CANCER DRUG DISCOVERY AND DEVELOPMENT

Targets for Cancer Chemotherapy

Transcription Factors and Other Nuclear Proteins

Edited by

Nicholas B. La Thangue
Lasantha R. Bandara

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Cover illustration: DNA microarray analysis of human ovarium carcinoma cells treated with an HDAC inhibitor with the HDAC antagonist TSA superimposed on top (see Chapter 7 by Manfred Jung). Over-expressed genes light up in red, suppressed genes in green. Experiment carried out by Dr. Liming Wang at the Netherlands Cancer Institute.

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PREFACE

Nowhere has our favorite edict "Chance favors the prepared mind" been better illustrated than in the corridors of the pharmaceutical industry. From its conception as fungal spores contaminating a laboratory culture, to the rapid and sophisticated automated technologies of the present day, successful drug discovery continues to combine scientific breakthroughs with an element of luck and serendipity. We have witnessed huge technological developments in the automation and miniaturization of high throughput screening, as sources of chemical diversity in lead explosion, and genome-wide sequencing and proteomics programs in the supply of drug targets. Despite the indisputable benefits that these advances have brought to bear on improving efficiency and the probability of successful drug discovery, there remains unanimous recognition among drug discoverers that validated therapeutically relevant protein targets that contribute to the disease process provide an essential starting point for success.

For human health care, though we are witnessing considerable improvements in treating certain prevalent disease conditions, cancer remains a disease where there is a clearly unmet clinical need. Cancer is a devastating and incurable disease that affects all ages. Statistics tell us that, in the Western world, 1 in 3 people will suffer from cancer, and that 1 in 4 people will die from the disease. Its prevalence will soon outstrip cardiovascular disease. Indeed, though current cancer treatments may halt disease progression, the side effects are often severe and debilitating, and the therapeutic benefit to the patient frequently of limited value. Lack of tissue specificity, widespread nonspecific cytotoxicity and necrosis, and drug resistance are observed with many current clinical regimens. There is an undeniable need for better medicines and for redesigning cancer treatment.

It is becoming increasingly recognized that intracellular proteins provide an untapped source of therapeutic targets. Although most remain to be validated as drug targets, the large body of research evidence that has accumulated in recent years includes many examples of proteins that exhibit abnormalities in tumor cells. Elucidating the signal transduction pathways that govern the mitogenic response of cells to growth factors, together with the huge developments in understanding the cell cycle and gene expression control, are examples of research areas that have provided important insights into abnormalities that occur in cancers. However, cancer results from a multistep process in which the mutation of different genes culminates in the cell acquiring the capacity for continual growth, and thus numerous targets may be available for therapeutic intervention in a single tumor cell. Many of these genetic events occur in proto-

oncogenes, causing them to acquire increased activity and provide a permanent growth-promoting signal, or tumor suppressor genes, resulting in a loss of growth suppressing activity. The overriding consequence of these genetic abnormalities is a cancer cell that is liberated from its normal tightly regulated growth cycle.

We have seen important developments in understanding the mechanism of action of oncogenes and tumor suppressors, together with the pathways of control through which their effects on proliferation are mediated. For example, two of the most frequently inactivated tumor suppressor genes, the retinoblastoma gene Rb and p53, function as nuclear transcription factors that target genes involved in growth control. The mechanism of action indicates that pRb and p53 regulate transcription through chromatin-associated mechanisms. Oncogenes such as *myc* and *mdm2*, which frequently exhibit increased activity in tumor cells, act in a similar fashion.

The developments in understanding how oncoproteins and tumor suppressors exert effects provide a great resource that can be exploited in drug discovery. We know very well that aberrant control by these proteins provides the fundamental basis for a normal cell to become tumorigenic. Therapeutic approaches that target these proteins are therefore likely to offer new opportunities in the search for innovative, more specific, and efficacious medicines for treating the cancer patient.

Targets for Cancer Chemotherapy: Transcription Factors and Other Nuclear Proteins provides a series of authoritative and compelling accounts on selected examples of transcription factor oncoproteins and tumor suppressors, together with other nuclear and chromatin-associated proteins that are central to the phenotype of the tumor cell. By bringing together this group of expert commentaries, we aim to provide a detailed understanding of the latest research developments and the impact of this knowledge for cancer drug discovery.

Our book opens with a discussion from Kaelin on the E2F transcription factor, which plays an instrumental role in regulating progress into the S phase of the proliferative cycle, and clearly is of great relevance to the cancer cell owing to its frequent, if not universal, deregulation in human tumors. Berwanger and Eilers follow by describing Myc, a nuclear oncoprotein that functions as a transcription regulator and where recent research information has provided new mechanistic insights into growth control through the regulation of chromatin.

Angel and colleagues cover an equally important transcription factor in signal transduction, AP1, followed by Bhattacharya on the importance of the hypoxia-inducible transcription HIF factor that regulates angiogenesis required for tumor growth, and which offers great potential as a drug target. Trepel and colleagues overview recent developments in the β-catenin/TCF pathway, potentially of huge significance in cancer cells.

Research into chromatin control has shed light on the mechanisms that influence gene expression and accessibility to the transcription machinery. In turn, these studies have elucidated novel and interesting proteins that are endowed with enzyme activities required for chromatin modulation, and that play key roles in growth control. Thomson and Mahadevan describe the importance of histone acetylases, followed by Jung on the potential of deacetylases as cancer drug targets. Here, we know already of clinical trials underway with drugs that act as deacetylase antagonists.

Phosphorylation is known to have an important influence upon the activity of many transcription factors and other nuclear proteins, and several examples are discussed in which protein kinase regulation of transcription factors influences growth control. Rao and Patel overview cyclin-dependent kinases, frequently aberrantly regulated in tumor cells where they target and act through the pRb/E2F pathway, to drive early cell cycle progression. Mitogen-activated protein kinases, reviewed by Chiloeches and Marais, are an established group of drug targets that relay signals from growth factors to the nucleus, and regulate growth through the targeted phosphorylation of certain transcription factors.

Other nuclear mechanisms that influence growth could offer great value as cancer targets. The product of the tumor suppressor locus ink4/arf locus, the ARF protein, impedes the activity of the MDM2 oncoprotein to degrade p53, thereby facilitating the p53 response, representing an interaction that has attracted considerable interest in cancer drug discovery. Furthermore, we now understand that MDM2 regulates p53 activity by stimulating p53 breakdown through an ubiquitin-dependent pathway, and Klotzbücher and Kubbutat review recent progress in this area. In this respect, MDM2 mimics the action of certain viral oncoproteins, such as the oncogenic human papilloma virus E6 protein, a topic, and its application to new therapies, that is discussed in detail by Pim and colleagues.

Moreover, approaches that manipulate the mechanisms of DNA repair in response to DNA damage may alter the sensitivity of tumour cells to conventional chemotherapy, an exciting idea that is raised in the account from Gabriel and Ashworth on the role that the BRCA1/2 tumour suppressor proteins may play in DNA repair, and the opportunities for therapy that arise.

A review of an important series of developments surrounding the remarkable VP3 protein, known as apoptin, from the chicken anemia virus is provided by Noteborn. Apoptin causes apoptosis in cells that are malignant or transformed, but not in normal cells. Understanding the route through which apoptin stimulates apoptosis will likely open up new avenues for drug discovery in tumor cells.

In the final chapters, we conclude with reviews that move the emphasis from laboratory and pre-clinical-based anticancer drug discovery, to focus on the clinical disease, and address current knowledge of therapeutic applications for

E2F (Bertino) and HIF1 (Harris). Without doubt, it is pleasing and encouraging that application of these key targets has progressed to the clinical setting.

Our final review underscores the important contribution that drugs targeting transcription factors have made in current cancer treatments. Here, Oosterkamp and Bernards describe the mechanism of action of the nuclear hormone estrogen and androgen receptors and their value as anticancer targets.

Many of the proteins considered in this volume fulfill one of the most important and fundamental criteria in drug discovery, namely a validated target that contributes to the pathology of the disease. By providing this information in a single volume, together with the scientific and therapeutic rationale that justifies the approach and value of each target to cancer drug discovery, the cancer patient, and the pharmaceutical industry, we hope to have provided an authoritative account of an innovative scientific area that we believe could lead to a new class of specific target-based medicines for treating what remains an incurable disease. In completing the volume, we have tried at the very least to have prepared the mind of the cancer drug discoverer. After all, as all scientists know, chance does indeed favor the prepared mind.

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CONTENTS

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1 Cancer Chemotherapy Based on E2F and the Retinoblastoma Pathway

William G. Kaelin, Jr.

Contents

1. INTRODUCTION

Abnormal cellular proliferation is a hallmark of cancer. Specifically, normal cells do not undergo cellular division when faced with the absence of specific mitogenic signals or the presence of certain growth inhibitory signals. Cancer cells, in contrast, are relatively inured to such signals. Studies performed over the past decade or so suggest that this property of cancer cells is due, at least in part, to functional inactivation of the retinoblastoma protein (pRB). One consequence of pRB inactivation is deregulation of a cell-cycle regulatory transcription factor family called E2F. This chapter will outline therapeutic opportunities based on this knowledge.

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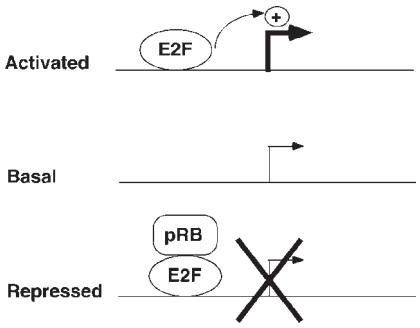

Fig. 1. Control of E2F-responsive promoters. E2F is a heterodimeric sequence-specific, DNA-binding, transcription factor. Free E2F is a transcriptional activator, whereas pRB converts E2F from a transcriptional activator to a repressor. Basal promoter activity is due to binding of non-E2F transcription factors (not shown for simplicity).

2. CONTROL OF E2F BY THE RETINOBLASTOMA PROTEIN

pRB is a nuclear protein that binds to members of a cell-cycle regulatory transcription factor family generically referred to as E2F [for reviews, see *(1–4)*]. There are six human E2F genes (*E2F1, E2F2,..., E2F6*). The products of these genes bind to specific DNA sequences as heterodimers with either DP1 or DP2. Both the E2F and DP subunit contact DNA via winged-helix DNA binding motifs. pRB binds to a collinear motif present in the C-terminal transactivation domains of E2F1, E2F2, E2F3, and E2F4 but does not recognize E2F5 and E2F6. E2F5, like E2F4, can bind to the pRB paralogs p107 and p130. It is now clear that complex formation with pRB (or its paralogs) converts E2F from a transcriptional activator to a potent transcriptional repressor (Fig. 1). E2F6, unlike the other E2F family members, is an intrinsic transcriptional repressor and does not interact with pRB family members.

E2F binding sites have been identified in a number of genes that play roles in DNA replication and cell-cycle progression. In normal cells, the transcription of these genes is repressed in G0 and G1 by pRB (or its paralogs) bound to DNA via E2F. In the presence of mitogenic signals, pRB becomes hyperphosphorylated, leading to a loss of pRB transcriptional repressor activity and ultimately the dissolution of pRB/E2F complexes. Neutralization of p107 and p130 may involve cytoplasmic sequestration in addition to changes in phosphorylation. E2F, unfettered by pRB family members, can then activate transcription (*see* Fig. 1). In model systems, transcriptional activation of E2F target genes is sufficient to induce quiescent or resting cells to enter S-phase.

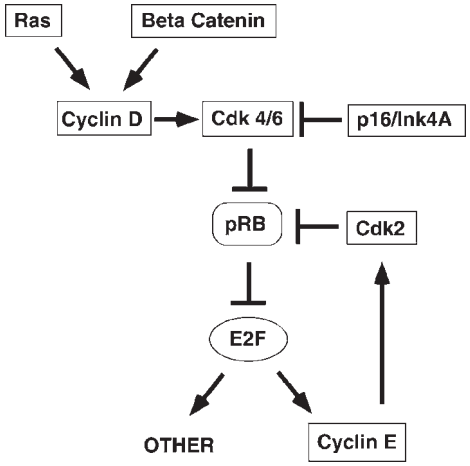

Fig. 2. The retinoblastoma pathway. pRB is negatively regulated by phosphorylation mediated by Cdk4/6 and Cdk2. These Cdks are positively regulated by D-type cyclins and cyclin E, respectively. D-type cyclins are negatively regulated by the Cdk inhibitor p16/Ink4A. Cyclin D1 levels are increased by Ras and beta catenin. Most human cancers harbor mutations affecting this pathway leading to functional inactivation of pRB and deregulation of E2F.

3. THE pRB PATHWAY

Phosphorylation of pRB is carried out initially by D-type cyclins (in conjunction with cdk4 or cdk6) followed later by cyclin E/cdk2 [for reviews, see *(3,5,6)*]. Cyclin A/cdk2 may serve to sustain pRB phosphorylation in S-phase. Most human cancers harbor mutations that directly or indirectly compromise the function of pRB [for reviews, see *(7–10)*] (*see also* Fig. 2). For example, *RB-1* mutations have been described in a number of cancers including retinoblastomas, sarcomas, small cell lung carcinomas, and breast carcinomas. All tumor-derived *pRB* mutants tested to date have lost the ability to repress E2F-responsive genes. Conversely, reintroduction of wild-type pRB into *RB-1 –/–* tumor cells leads to restoration of E2F control and suppression of proliferation.

Cancers without *RB-1* mutations frequently harbor mutations that lead to the untimely phosphorylation of pRB. Examples of such mutations include homozygous deletion of the *p16/INK4A* cdk inhibitor, amplification of *CYCLIN D1*, or gain-of-function mutations of *CDK4*. Reintroduction of non-phosphorylated pRB into such cells suppresses tumor cell growth. Inactivation of the *APC* tumor

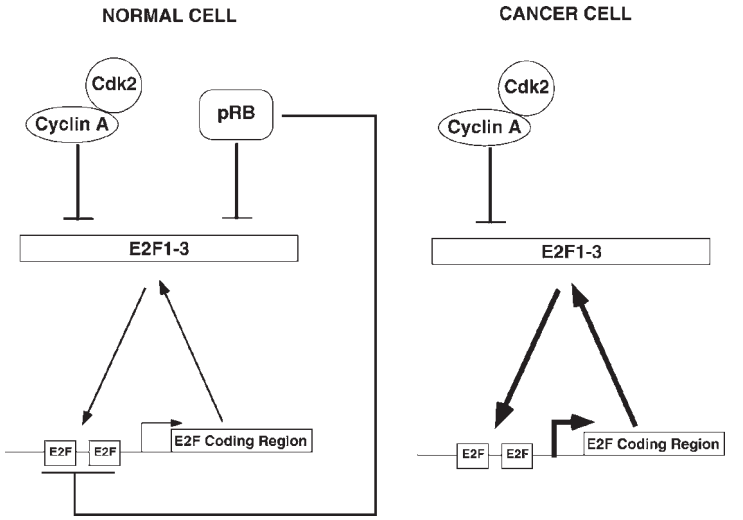

Fig. 3. Control of E2F in normal cells and cancer cells. E2F1, E2F2, and E2F3 contain N-ter-minal Cyclin A/Cdk2-binding motifs and C-terminal pRB-binding motifs. Cyclin A/Cdk2 inhibits E2F DNA-binding activity. pRB silences the E2F transcriptional activation domain and actively represses transcription when bound to DNA. E2F1, E2F2, and E2F3 are all encoded by E2F-responsive genes. As a result, functional inactivation of pRB in cancer cells might allow the establishment of a positive feedback loop.

suppressor gene in colorectal cancer leading to beta catenin stabilization *(11)*, as well as activating *Ras* mutations *(12)*, also lead to enhanced cyclin D activity and hence pRB phosphorylation. Thus, inactivation of pRB is a common, and possibly requisite, step in human carcinogenesis.

Interestingly, the *E2F1, E2F2,* and *E2F3* genes all contain E2F-responsive promoters. Thus, inactivation of the pRB pathway in cancer cells would potentially allow the establishment of a positive feedback loop wherein free E2F would drive the excessive transcription of *E2F1, E2F2*, and *E2F3* (Fig. 3). The studies perform to date clearly implicate E2F1 and E2F3 as playing critical roles in the control of cellular proliferation.

4. E2F-INDUCED APOPTOSIS

The untimely activation of *E2F* target genes can induce programmed cell death or apotosis. E2F is capable of transcriptionally activating the *ARF* gene.

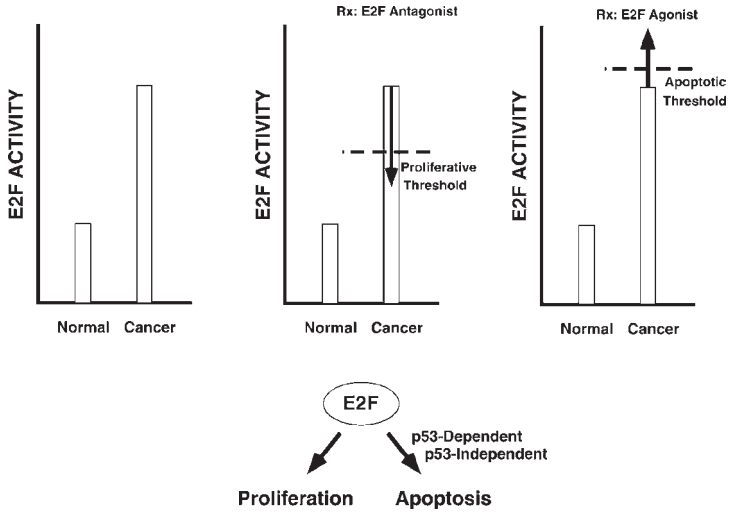

Fig. 4. Rationale for development of E2F agonists and antagonists. E2F can induce both cellular proliferation and apoptosis. The latter can occur via both p53-dependent and p53-independent pathways. Cancer cells are characterized by high levels of E2F transcriptional activity. An E2F antagonist might inhibit cell proliferation; whereas an agonist might induce apoptosis.

The Arf protein prevents the Mdm2 oncoprotein from targeting p53 for ubiquitin-dependent proteolysis [for review, see *(13–15)*]. Thus, high levels of E2F can stabilize p53, leading to death, which may be a contributing factor with respect to the frequent mutation of *p53* in human cancer. Moreover, some tumors that retain a wild-type *p53* allele fail to accumulate high levels of p53 protein due to loss of Arf or overproduction of Mdm2. In summary, mutations affecting the "pRB pathway" would be predicted to deregulate E2F and consequently provide a selection pressure to mutate a "p53 pathway" involving Arf, Mdm2, and p53. It is now clear, however, that E2F can also utilize p53-independent pathways to induce death under certain conditions and in certain tissues. For example, E2F can transcriptionally activate *p73*, a paralog of *p53*, as well as inhibit survival signals mediated by NFκB *(16–19)*. Cancer cells therefore appear to "walk a tightrope" with respect to E2F transcriptional activity. Too much E2F activity can induce apoptosis, despite the absence of p53, whereas too little can lead to cell-cycle arrest (Fig. 4).

5. INDUCTION OF E2F BY DNA DAMAGE

A number of recent studies have shown that E2F1 is induced by DNA damage such as following UV irradiation, gamma irradiation, or treatment with various chemotherapeutic agents *(20–24)*. In one study the degree of E2F1 induction correlated well with chemotherapy-induced apoptosis *(24)*. Although it is tempting to speculate that the increased E2F1 levels observed in these settings contributes to apoptosis, this has not been proven. Furthermore, the studies performed to date suggest E2F1 that accumulates following DNA damage is not transcriptionally active *(23,24)*.

6. MEASURMENT OF E2F ACTIVITY IN VIVO

A prediction of the above considerations would be that E2F-responsive genes would be more transcriptionally active in cancer cells than in their normal counterparts. Parr and coworkers made replication-defective adenoviral vectors in which a reporter gene was placed under the control of a constituitively active promoter or an E2F-responsive promoter *(25)*. Following stereotactic injection into an orthotopically growing rat glioblastoma, the E2F-responsive viral vector gave rise to reporter activity only within the tumor, whereas the control vector gave rise to reporter activity in both the tumor and surrounding normal brain tissue. Importantly, the E2F-responsive vector did not give rise to measurable reporter following infection of rapidly dividing normal liver cells. Replacement of the reporter gene used in these studies with a suicide gene led to selective killing of brain tumor cells with relative sparing of surrounding normal brain parenchyma by the E2F-responsive vector. These results suggest that normal cell cycles and cancer cell cycles differ, at least with respect to E2F control. Furthermore, they suggest that derepression of E2F in tumor cells is both measurable and potentially exploitable therapeutically.

7. E2F AS A DETERMINANT OF CHEMOSENSITIVITY

In theory, activation of E2F-responsive genes can have either positive or negative effects on chemosensitivity depending upon the nature of the chemotherapeutic agent [for a recent review, *see (26)*]. For example, increased E2F activity would be expected to increase the sensitivity of cells to agents that preferentially kill cycling cells. This would be especially true for S-phase–specific agents. In addition, E2F might prevent the cell-cycle arrest that would typically occur following DNA damage. Overriding such checkpoint controls might prevent the timely repair of DNA damage and thus lead to death. Furthermore, it is possible that proapoptotic signal(s) due to increased E2F might act additively or synergistically with proapoptotic signals resulting from the actions of chemotherapeutic agents. On the other hand, high levels of E2F might decrease sensitivity

to drugs which inhibit enzymes that are encoded by E2F-responsive genes. The exception to this rule would be instances where the drug/enzyme complex is, itself, responsible for killing (such as is the case with topoisomerase inhibitors) or where the enzyme converts a prodrug to a drug.

Experimental evidence is emerging in support of the above considerations. Wahl and coworkers showed that *RB-1 –/–* fibroblasts underwent apoptosis following certain forms of chemotherapy, whereas wild-type fibroblasts underwent a cell-cycle arrest *(27)*. Thus, in this model, the absence of a pRB-dependent cell-cycle checkpoint translated into a qualitatively different response to DNA damage. Similarly, pRB has been reported to suppress apoptosis following gamma irradiation *(28)*.

Bertino and coworkers reported that pRB loss in sarcomas was associated with increased levels of DHFR and thymidylate synthase, which translated into decreased sensitivity to methotrexate and 5-fluorouracil, respectively *(29)*. This same laboratory showed that forced expression of E2F1 in an osteosarcoma line led to decreased sensitivity to 5-fluorouracil but increased sensitivity to topoisomerase inhibitors *(30)*. Several other laboratories have also found that high levels of E2F1 increase sensitivity to topoisomerase inhibitors. Hiebert and coworkers showed that induction of E2F1 in 32D.3 myeloid cells increased sensitivity to the the topoisomerase II inhibitor etoposide *(31)*. El-Diery and coworkers showed that overexpression of E2F1 sensitized glioblastoma and bladder carcinoma cells to etoposide as well as to the DNA intercalating agent adriamycin *(24)*. Conversely, E2F1–/– fibroblasts were less sensitive than their wild-type counterparts to these two agents. Sehested and coworkers found that induction of E2F1 in osteosarcoma cells led to increased killing of sarcoma cells following treatment with topoisomerase I or II inhibitors *(32)*. The effect of E2F1 on chemosenstivity may also be cell-type dependent. Thus, in the 32D.3 system Hiebert and coworkers did not observe an effect of E2F1 on sensitivity to 5-fluorouracil or adriamycin.

8. DEVELOPMENT OF E2F ANTAGONISTS

The knowledge that transcriptional activation of E2F target genes drives cellular proliferation has generated interest in developing E2F antagonists for the treatment of proliferative disorders such as cancer, neointimal hyperplasia, and proliferative glomerulopathies. In principle, one might target E2F DNA-binding capability, E2F/DP heterodimerization, or E2F transcriptional activation activity. A number of concept validation experiments have been carried out in this area. Wu and coworkers showed that overproduction of a dominant-negative version of DP1 (capable of binding to E2F but not DNA) led to a G1 block in pRB-defective tumor cells *(33)*. Ishizaki and coworkers selected for synthetic RNAs capable of inhibiting E2F DNA binding activity *(34)*. Introduction of such an RNA into quie-

scent human fibroblasts prevented S-phase entry following serum readdition. Fabbrizio et al. selected for peptide aptamers that interact with E2F's DNA binding and dimerization domains *(35)*. One such aptmer was found to block E2F DNA binding activity in vitro, probably by preventing binding to DP. Like the Ishizaki synthetic RNA, this aptmer prevented quiescent fibroblasts from entering S-phase following serum stimulation. Bandara designed peptides capable of blocking E2F/DP heterodimerization based on conserved dimerization motifs present in both the E2F and DP family members *(36)*. Introduction of such peptides into E2F4/Ras-transformed fibroblasts led to apoptosis. Lesser degrees of apoptosis were observed in cervical carcinoma and osteosarcoma cells. Dzau and coworkers, as well as others, have developed synthetic oligonucleotides that act as "decoys" for E2F *(37–41)*. Such decoys have been used to inhibit vascular smooth muscle and mesangial cell proliferation in vitro and in mouse models following vascular injury. These agents have also been used to inhibit neointimal hyperplasia in vascular bypass grafts in both mouse models and in humans.

Collectively, these studies suggest that blockade of E2F DNA binding activity can inhibit cellular proliferation. To date there has not been a systematic evaluation of this approach with respect to differential effects on transformed versus nontransformed cells. Nor is it clear whether E2F inhibition will kill cells or merely induce a cell-cycle block. The studies of Bandara and colleagues raise the possibility that blockade of E2F DNA binding activity in tumor cells will induce apoptosis rather than cytostasis. On the other hand, apoptosis was not noted in the study of Wu and coworkers.

Although blockade of E2F DNA binding activity affects cell proliferation, and possibly cell viability, several questions remain surrounding the development of E2F antagonists. For example, to date there are no studies that show that blocking E2F transcriptional activation function, per se, will lead to inhibition of cell growth or apoptosis. For example, overproduction of dominant-negative E2F mutants that can bind to DNA, and yet not transactivate transcription, have generally not affected cellular proliferation *(42–45)*. Another concern relates to the fact that inhibition of E2F DNA binding activity should, in theory, prevent transcriptional repression by pRB family members (Fig. 1). In keeping with this idea, dominant-negative E2F mutants, as well as multimerized E2F DNA binding sites, can block transcriptional repression by pRB and p130 *(42–47)*. Thus, blockade of E2F DNA binding activity might, paradoxically, functionally inactivate the pRB tumor suppressor protein leading to undesirable effects in normal tissues.

9. DEVELOPMENT OF E2F AGONISTS

The knowledge that cancer cells are characterized by high levels of transcriptionally active E2F, coupled with the ability of E2F to induce apoptosis, has led to the somewhat heretical idea of treating cancer cells with E2F agonists (Fig. 4).

Several groups have pioneered the treatment of tumor cells with adenoviral vectors encoding E2F1. Such vectors have been used to treat melanomas *(48)*, breast cancers *(49)*, ovarian cancers *(49)*, esophageal cancers *(50)*, head and neck cancers *(51)*, glioblastomas *(52)*, and sarcomas *(53)* in mouse xenograft models. Superinduction of E2F1 in such tumor cells has led to apoptosis and tumor regression with little apparent toxicity to surrounding normal tissue.

Earlier studies showed that the physical interaction of cyclin A/cdk2 with E2F1 was necessary for the timely phosphorylation of the E2F1 heterodimeric partner DP1 in S-phase, which, in turn, neutralized E2F/DP DNA binding activity *(54–57)*. Chen and coworkers identified a short E2F1-derived peptide capable of blocking the interaction of cyclin A/cdk2 with E2F1 *(58)*. Similar peptidic motifs are present in a variety of cyclin/cdk2 substrates (including E2F2 and E2F3), and physically interact with a hydrophobic substrate recognition pocket formed by the cyclin *(59–65)*. Cell-membrane-permeable forms of such cyclin/cdk2 inhibitory peptides induced apoptosis in transformed cells *(58)*. In contrast, non-transformed cells did not die. Rather, these cells underwent a G1/S block but only if first induced to exit the cell cycle by serum withdrawal and then restimulated with growth factors in the presence of the cyclin/cdk2 inhibitory peptides. Deregulation of E2F1, using an inducible promoter, was sufficient to sensitize non-transformed cells to the killing effects of the cyclin/cdk2 inhibitory peptides. Interestingly, such cells appeared to arrest in S-phase prior to undergoing apoptosis. A similar phenotype was observed earlier following overproduction of an E2F1 mutant in which the cyclin A binding site was deleted *(54,66)*. Collectively, these results suggest that cyclin/cdk2 antagonists might selectively kill cancer cells, and are consistent with the view that this selectivity is at least partially related to differences in E2F activity between transformed cells and non-transformed cells.

Preclinical studies suggest that blocking E2F phosphorylation by cyclin/cdk2 would also enhance the effectiveness of conventional chemotherapy and radiotherapy. Hall and coworkers showed that overexpression of an E2F1 mutant lacking its cyclin A/cdk2 binding site led to increased accumulation of cells in S-phase, as expected, as well as enhanced sensitivity to campothecin *(66)*. Krek and coworkers showed that overproduction of a similar E2F1 mutant enhanced the killing of cancer cells by gamma irradiation irrespective of p53 *(67)*. Bertino and coworkers showed that overexpression of the cdk inhibitor p21 in pRB-defective tumor cells led to decreased phosphorylation of E2F, a S-G2 delay/arrest, and enhanced senstivity to chemotherapeutic agents such as doxorubicin, tomudex, and methotrexate *(68)*.

Kinases utilize ATP as a phosphate donor, and it is now well established that ATP-like molecules can be made into inhibitors of specific kinases. Accordingly, a number of ATP-like molecules are in various stages of development as cyclin/cdk2 inhibitors *(69)*. It remains to be determined whether the biological conse-

quences of blocking the interaction of cyclin/cdk2 with ATP will be the same as blocking the interaction of cyclin/cdk2 with substrates that utilize the peptidic motif described above.

10. SUMMARY

Studies carried out over the past decade have led to an emerging view that functional inactivation of pRB, and consequent derepression of E2F-responsive genes, is a driving force in cancer cell proliferation. Paradoxically, high levels of E2F can also lead to apoptosis. The proteins that regulate pRB and E2F are coming into view and have led to the notion of a "pRB Pathway." A number of these proteins are enzymes, and are thus potentially drugable. Inhibition of E2F would be expected to inhibit cellular proliferation, although it is not yet clear whether such inhibitors would preferentially affect cancer cells relative to normal cells. High levels of E2F in cancer cells might potentially be exploited in the design of cancer-selective gene therapy vectors. In addition, high levels of E2F might sensitize cancer cells to the proapototic affects of E2F agonists.

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2 Myc Oncoproteins as Targets for Therapeutic Intervention in Tumorigenesis

Bernd Berwanger and Martin Eilers

CONTENTS

1. INTRODUCTION

Myc genes form a small gene family that comprises four members: *c-MYC*, the founding member, *NMYC*, *LMYC*, and *SMYC*. A fifth member, *BMYC*, has high homology but lacks domains that are critical for the function of the other members; it may therefore be a dominant negative allele. The four canonical members of the gene family encode highly related proteins; indeed, there is clear genetic evidence demonstrating that at least N-Myc and c-Myc are functionally interchangeable (e.g., *1*). Thus, the main difference between the four proteins is their relative patterns of expression.

Myc proteins are overexpressed in a large percentage of human tumors. This is either due to mutations in the *MYC* genes; for example, a fraction of childhood neuroblastomas carry amplifications of the *NMYC* gene *(2)*; in many lymphomas,

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translocations are found that fuse the *c-MYC* coding sequences to promoters and enhancers derived from immunoglobulin or T-cell receptor genes (e.g., *3*). Alternatively, mutations in upstream signaling pathways that control the expression of *Myc* genes lead to a tumor-specific deregulation of expression of mRNA. For example, enhanced expression of *c-myc* in colon carcinoma may be due to mutations in the APC tumor-suppressor pathway *(4)*. Mutations affecting the TGF-beta pathway relieve expression of *c-MYC* from the repressive effects of TGF-beta *(5)*. Finally, some point mutations found in lymphomas in the amino-terminus of Myc affect protein stability and this may further enhance protein levels of Myc protein in these tumors *(6)*. As a result, there is often a significant difference between normal and neoplastic tissue in expression levels of Myc proteins. Because these proteins are powerful regulators of both cell proliferation and apoptosis, this difference alters the physiology of the cell and opens therapeutic windows that can be used for tumor-specific chemotherapy.

The biochemistry and biology of Myc proteins has been extensively reviewed and several in-depth reviews have appeared. In particular, numerous reviews have exhaustively summarized evidence supporting the underlying assumption of this chapter: that Myc proteins, owing to their widespread deregulated expression in human tumors and their well-established tumorigenic potential, are well-validated targets in tumorigenesis. This chapter does not attempt to present another general coverage of the field. Instead, we will focus on some key aspects of Myc biology that may be useful for designing both biochemical and cell-based assays aimed at interfering with Myc function in tumorigenesis.

2. BIOCHEMISTRY OF MYC PROTEINS

2.1 Transcriptional Activation

Myc proteins are transcription factors that belong to the helix-loop-helix/leucine zipper family of proteins (for recent reviews, *see 7,8*). All Myc proteins have the capacity to activate transcription; they do so as part of a heterodimeric complex with a partner protein, Max. (Fig. 1 summarizes currently known protein/protein interactions of Myc proteins.) The heterodimeric complex binds to specific sequences on DNA termed E-boxes with a core consensus sequence CACGTG. Several array projects and directed searches have identified multiple target genes that are upregulated by the Myc/Max heterodimer (*see*, for example: *9–11*). Both the helix-loop-helix domain and the leucine zipper of Myc interact with Max, generating a large surface area of interaction. All mutations in Myc that impair complex formation with Max abolish transformation (e.g., *12*). Indeed, the transformation deficiency caused by some specific mutations in the leucine zipper of Myc is rescued by compensating mutations in the leucine zipper of Max, providing unequivocal evidence that transformation by Myc requires Max as partner *(13,14)*. Because of the size of the interaction surface, it may be difficult to directly

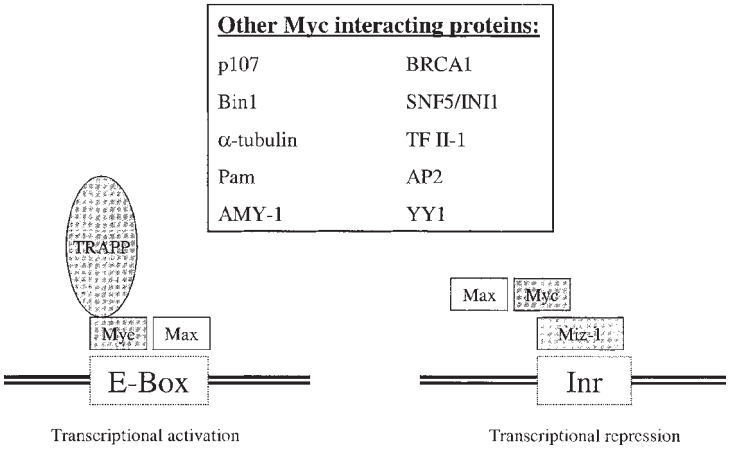

Fig. 1. Protein/Protein interactions of Myc. Heterodimers of Myc and Max bind to CACGTG motifs (E-Boxes) on DNA and activate transcription by recruiting histone acetyltransferase via interaction with TRRAP. Transcriptional repression is mediated by formation of complexes with Miz-1, which binds DNA at initiator elements (Inr).

interfere with heterodimerization of the Myc/Max complex; however, it is fair to assume that transcriptional activation per se by the Myc/Max heterodimer is a validated target for therapeutic intervention.

Strikingly, quite a number of transcription factors exist that have the same target sequence on DNA and can activate transcription, but are by no means oncogenic; some of them even have antiproliferative functions. Chromatin-immunoprecipitation assays provide clear evidence that one of these factors, USF, is bound to at least two Myc target genes in vivo, but fails to activate them. Thus, Myc/Max heterodimers must differ from USF at a step after DNA binding to provide transcriptional activation *(15,16)*. While the precise mechanisms of activation by Myc remain to be resolved for any gene, some components involved appear to emerge.

An initial clue came from sequence comparisons between different Myc proteins. Such comparisons pointed to the presence of two highly conserved elements in the amino-terminus of Myc proteins, termed Myc boxes. Mutations in MycboxI have varying effects in biological assays, defying a clear description of its function. MycboxI has been suggested to interact with Bin1 (a putative tumor-suppressor gene) and p107. However, clear evidence for interaction of endogenous proteins is lacking for both interactions *(17,18)*. The clearest evidence so far appears to be the suggestion that MycboxI has a regulatory role in the ubiquitin-mediated proteolysis of Myc, and this will be discussed later in this chapter *(19)*.

In contrast, mutations in MycboxII abolish all biological functions of Myc, clearly demonstrating that MycboxII is a central effector domain of the protein *(12)*. Recent work defines a large protein, TRRAP, that is a catalytically inactive member of the ATM/PI-3-kinase superfamily, as a MycboxII-interacting protein *(20,21)*. Evidence from dominant-negative alleles of TRRAP suggests that indeed Myc/TRRAP interactions are required for transformation. TRRAP is a member of two histone acetylase complexes, PCAF/GCN5 and TIP60 *(22,23)*. The fact that two other components of the TIP60 complex have similarly been found as proteins interacting with MycboxII suggests that at least the TIP60 complex is associated with Myc in vivo, although formal proof of this suggestion is lacking *(24)*.

These interactions provide for a simple working hypothesis, which would suggest that Myc recruits histone acetylase complexes to targets of activation via interaction with TRRAP. Curiously, this has not been formally demonstrated in the literature, and the field has been somewhat reluctant to embrace this hypothesis. Clearly, one reason for this reluctance is the observation that deletions of MycboxII have no deleterious effects on transactivation in transient reporter assays. However, this finding may be trivial since no proper chromatin is formed in transient transfection experiments.

Recent studies from my own laboratory in cooperation with Bernhard Lüscher address this question for one well-validated target gene of *Myc*, *cyclin D2* (C. Bouchard et al., unpublished). Using chromatin immunoprecipitation assays, we find that Myc proteins are bound to one of the E-boxes in the *cyclin D2* promoter in vivo. Activation of conditional alleles of Myc (so-called MycER chimeras, in which Myc activity is rendered regulatable by fusion with the hormone-binding domain of the estrogen receptor) leads to histone H4 hyperacetylation at a single nuclesosome in the promoter. Wild-type *Myc* recruits TRRAP to the promoter upon addition of hormone. In contrast, a MycboxII deletion mutant fails to upregulate cyclin D2, recruit TRRAP, and induce histone acetylation at the *cyclin D2* promoter. Taken together, the data strongly suggest that a model proposing that MycboxII directs histone acetylation via recruitment of histone-acetyl-transferase (HAT) activity at a Myc target gene in vivo applies at least to *cyclin D2*. The identity of the HAT involved remains unclear at present, although the preference for histone H4 points to Tip60 rather than PCAF. Clearly, targeting the HAT involved in the activation process may provide a key possibility to inhibit transformation by Myc.

2.2. Transcriptional Repression

Myc proteins have at least one additional mechanism to regulate transcription in addition to Max-dependent gene activation. Consistently, Myc proteins have been shown to be able to repress transcription of specific genes; in particular, the ability to repress transcription has been linked to the presence of so-called "initiator" elements of transcription in the promoter *(25)*. Such elements direct a pre-

cise start of transcription at a given promoter in the absence of a consensus TATA-box, strongly suggesting that sequence-specific transcription factors are at work here. Thus, the suggestion has been repeatedly put forward that Myc can interact with such factors to repress transcription.

The identity of the genes repressed by Myc makes repression an attractive target for interfering with Myc function. Two examples illustrate this point:

1. Consistently, expression of the DNA damage inducible gene, *gadd45*, has been identified as a target of repression by Myc *(26)*. *Gadd45* is a target for transcriptional activation by both the *p53* and the *BRCA1* tumor-suppressor genes *(27,28)*; its absence leads to genomic instability and the inability to mount checkpoint responses in the G2 phase of the cell cycle *(29)*. Indeed, Myc-transformed cells at least in culture show clear evidence of genomic instability when challenged with microtubule-disrupting drugs, which target a G2/M-checkpoint *(30)*. Restoration of Gadd45 expression may therefore re-establish the presence of such checkpoints and may therefore induce stable arrest and apoptosis in cells that have undergone events disrupting genomic integrity.
2. Similarly, the gene encoding p15ink4b has been identified as a target for repression by Myc *(31)*. Since p15ink4b is upregulated by the TGF-beta tumor-suppressor pathway, its repression by Myc is firmly linked to the resistance of Myc-transformed cells to the antimitogenic action of TGF-beta. Restoration of p15ink4b expression can be expected to sensitize transformed cells to TGF-beta, thus reestablishing normal control of cell proliferation.

The mechanistic basis of gene repression by Myc is not fully established and several suggestions have been made over time. For example, YY-1 and TFII-I have been suggested to be partner proteins of Myc that may mediate its repressive properties, but little follow up has been seen after the initial reports *(32,33)*. Work from my own laboratory in collaboration with Joan Massague's laboratory now suggests that repression of p15ink4b expression by Myc is mediated by interaction with the zinc finger protein, Miz-1, which was cloned several years ago in a two-hybrid screen using the carboxyl-terminus of Myc as bait *(34–36)*. Miz-1 binds to the start site of the promoter (which lacks a TATA-box but starts at a specific nucleotide and thus, by definition, is an initiator element) and transactivates expression. Myc binds to Miz-1 at this element and prevents recruitment of the co-activator p300, thus leading to repression.

Recent unpublished work now identifies individual key residues in Myc that are required for interaction with Miz-1; thus, the critical interaction interface may be small indeed, rendering it amenable for chemical disruption. However, it will be equally important to identify the enzymatic co-factors involved in gene repression (histone deacetylases, chromatin-remodeling enzymes) to be able to specifically target repression by Myc proteins. Such experiments are under way in several laboratories. One potential candidate is the tumor-suppressor *Ini1*,

which has been suggested to interact with the carboxyl-terminus of the c-Myc protein *(37)*.

3. TARGETING PROLIFERATION

A large body of evidence shows that Myc proteins act as key regulators of mammalian proliferation, both in tissue culture and in mouse models (for review, *see 38*). Furthermore, analysis of tumor biopsies strongly suggests that human neuroblastomas carrying an amplification of *NMYC* show a much higher proportion of proliferating cells relative to tumors lacking an amplification, supporting the notion that *Myc* genes deregulate cell proliferation in vivo. This function of *Myc* is evolutionary conserved, as *Drosophila Myc* clearly regulates cell proliferation *(40)*.

The precise mechanisms and identity of target genes involved in the control of cell proliferation by Myc are still a matter of intense debate. Well-validated direct target genes of Myc include *Cyclin D2 (10)*, *Cdk4 (41)*, *Cul-1 (42)*, and *Cks-2 (9)*. From these studies, several principles have emerged, which can be summarized as follows:

- The number of genes directly regulated by Myc proteins is large and several pathways are involved. Importantly, Myc appears to independently regulate both cell cycle progression per se and cell growth (increase in cell mass), which may in turn affect cell proliferation *(40)*.
- During the G1 phase of the cell cycle, Myc independently regulates E2F activity and cyclin E/Cdk2 kinase activity *(43)*. The pathway leading to active E2F-dependent transcription is not fully resolved; more is known about the link between Myc and cyclin E/Cdk2 kinase activity. Myc primarily regulates cyclin E-dependent kinase through affecting the metabolism of the inhibitor, p27. Deregulation of p27 metabolism is important for Myc to be mitogenic. Published data about relationship between Myc and p27 are summarized in Fig. 2.
- For most target genes, it has not been reported that they are dramatically overexpressed in Myc-induced tumors, altering the normal pathway of G1 progression. Thus, it is not immediately obvious whether interfering, for example, with Cdk2 activity opens a therapeutic window. The possible exception is degradation of p27, since a large body of data suggests that altered metabolism of p27 is a hallmark of advanced tumor stages (e.g., *44*).
- Deregulation of cell proliferation by Myc almost certainly contributes to checkpoint failure in tumors carrying *Myc* mutations *(45,46)*. For example, several human leukemias express both p53 and high levels of c-MYC; yet activation of p53 fails to induce cell cycle arrest because Myc overrides the action of physiological levels of p21. Chemically interfering with Cdk2 activity would therefore be expected to activate checkpoint responses. Because Cdk2 activity and differentiation are tightly linked in several systems, inhibition of Cdk2 activity can also be expected to promote differentiation of tumors that express deregulated *Myc* genes.

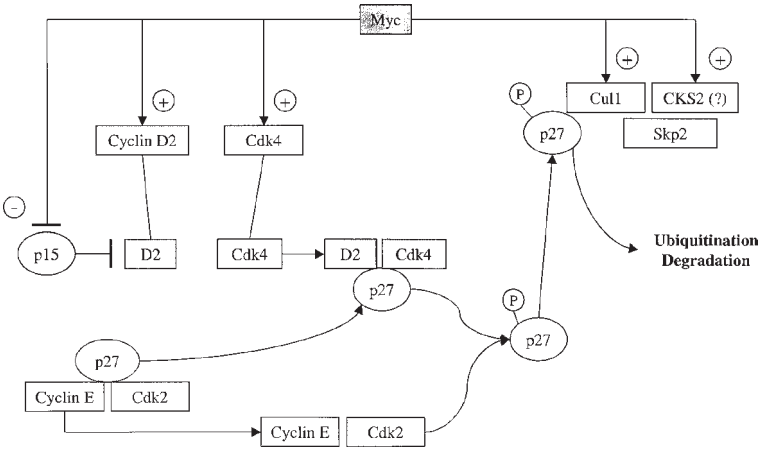

Fig. 2. Summary of interactions between Myc and p27. Following activation of Myc, p27 dissociates form Cyclin E-Cdk2 complexes and is sequestered by cyclin D2-Cdk4 complexes. Myc also activates Cul1 and CKS2, components of the SCF ubiquitin ligase complex, which ubiquitinates phosphorylated p27 targeting it for subsequent degradation.

In addition to growth factor signaling and Cdk2 activity, proliferation of human cells is regulated by telomere length; expression of the catalytic subunit of telomerase, *htert*, is rate limiting for telomere length and cell survival (e.g., *47*). There is clear evidence that tumor cells need to activate telomerase expression in order to proliferate. Myc activates htert expression via binding to several sites in the *htert* promoter *(48–50)*. As a consequence, Myc extends the life span of human primary cells in culture. It is likely, therefore, that inhibition of Myc function will limit proliferation of human tumors via a telomere-dependent mechanism. Formally, however, the contribution of Myc to telomerase promoter activity in human tumor cells has not been evaluated.

4. WHAT TO EXPECT IF SUCCESSFUL?

One key assumption underlying any attempt to target Myc proteins is that transformation is reversible. Clearly, if Myc were a runaway oncogene triggering irreversible changes when activated even briefly, then any attempt to interfere with its function at later stages in tumorigenesis would be futile.

From experiments in tissue culture, it is not entirely clear what to expect. In established cell lines, induction of proliferation and transformation are reversible if analyzed using a *MycER* gene *(51)*. However, when Ras and Myc are used

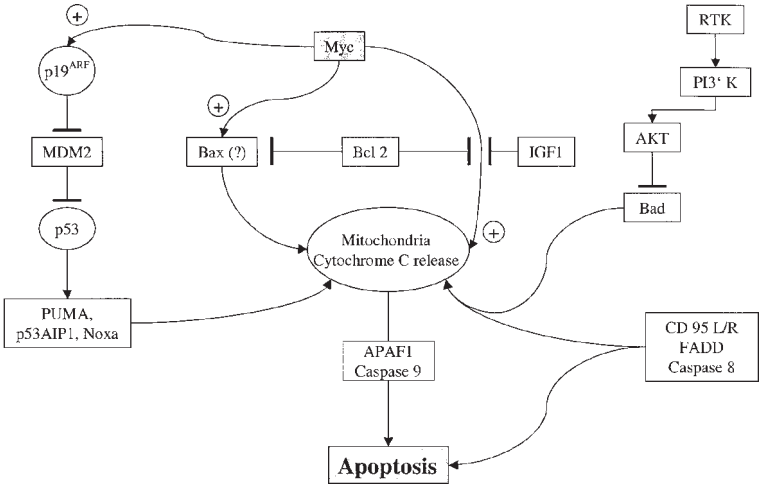

Fig. 3. Induction of apoptosis by deregulated Myc. While it is clear that Myc acts upstream of the cytochrome c release to induce apoptosis, the precise pathways leading to the release of cytochrome c are not fully resolved. Myc activates ARF and leads to a p53-dependent apoptosis, but p53-independent apoptosis by Myc has also been demonstrated.

to transform primary cells, the resulting transformed colonies have mutations in either *ARF* or *p53*, simply because both Ras and Myc induce expression of ARF, thus enhancing expression of p53 to a degree that is incompatible with cell viability *(52,53)* (*see* Fig. 3). At the same time, genetic studies show that lack of either p53 or ARF is sufficient to render cell susceptible for transformation by oncogenic Ras alone *(53)*. Therefore, Myc's only function in this context may be to facilitate the emergence of cells that carry *ARF* or *p53* mutations; once they emerged, Myc may be dispensable. For example, suppression of p15ink4b by Myc may simply extend the time that cells can accumulate *ARF* mutations before becoming senescent *(54)*. Thus, potentially, mutations in *Ras* genes bypass the need for Myc in tumorigenesis in vivo; indeed, results from transgenic systems suggest that this idea may indeed be valid.

Reversibility of tumor formation has been analyzed in transgenic animals in which the function of Myc has been made conditional, either using MycER chimeras or a tetracycline-inducible system. Several systems targeting different organs have been reported and the available results have been reviewed recently *(55)*. Experiments targeting Myc to the lymphoid compartment clearly demon-

strate reversibility of tumorigenesis in B-cells *(45)*. Similarly, hyperplasia of keratinocytes induced by conditional Myc in the dermis is reversible, although the emergence of tumors was not analyzed *(56)*. Thus, there is reason to believe that interference with Myc will revert the tumorigenic phenotype at least in some organs.

In contrast, activation of Myc in breast epithelium yielded tumors only some of which regressed after turning-off Myc. All non-regressing tumors carried activating mutations in *Ras* genes, suggesting that activation of Ras can bypass the need for Myc under these circumstances *(57)*. Clearly, then, the results of inhibiting Myc function in tumorigenesis may not be universal regression and may depend on the genetic circuitry of the specific tumor.

One should point out, however, that these experiments are done in mice, where telomere length is not an issue. If Myc is indeed a central player in maintaining telomerase activity in human tumors, inhibiting of Myc function may yield a universal cessation of tumor cell proliferation in humans. Inhibition of telomerase function by expression of a dominant-negative allele inhibits proliferation of most established tumor cell lines that were tested; the exception was a tumor cell line that lacked telomerase activity and may have activated an alternate mechanism of telomere lengthening *(58)*. Thus, inhibition of Myc function might limit tumor cell growth indirectly via its effect on *htert* expression.

There is reason to believe that, at least for some tumors, the effects of inhibiting Myc function would go beyond a reduction in tumor mass. The clearest evidence comes from a study using human astrocytoma cells *(59)*; in these experiments, the Myc antagonist Mad-1 was expressed using an adenoviral vector leading to an almost complete loss of tumorigenic potential of the cells. In particular, the remaining tumors that formed in xenografts displayed a high degree of differentiation, suggesting that interference with Myc function can induce cellular differentiation in a tumor environment. Thus, inhibition of Myc may lead to permanent changes in cell phenotype rather than a transient cessation of cellular proliferation. Similarly, there is some evidence to suggest that the deregulation of cell proliferation by amplified NMYC in human neuroblastoma is due to inhibition of an intrinsic differentiation program.

Recent work also suggests one possible mechanism as to how Myc may interfere with cellular differentiation, since the gene encoding Id-2 has been identified as a target for transcriptional upregulation by Myc *(60)*. Id-2 is a member of a class of four related proteins that bind to and inactivate HLH proteins involved in cellular differentiation. Ectopic expression of Id proteins can delay differentiation in a number of cellular systems. Whether specific Id proteins are consistently up-regulated in Myc-induced tumors remains to be fully resolved; if so, even transient interference with Myc function may induce an irreversible pathway of differentiation.

5. TARGETING APOPTOSIS

Deregulated expression of Myc proteins strongly induces active cell death—apoptosis—in addition to proliferation *(61,62)*. Initially, this was observed in tissue culture experiments using both fibroblast and lymphoid cell lines. Here, either constitutive or regulated activation of Myc (using a MycER chimera) led to an inability of cells to survive in the absence of survival factors; survival factors were defined operationally as a class of growth factors that can provide (an initially unknown) survival signal. As a consequence, cells expressing active Myc die in the absence of survival signals, whereas normal cells simply become arrested. Therefore, inhibition of defined survival signals may provide a large window of opportunity for tumor-specific therapeutic intervention.

The precise mechanism(s) by which Myc stimulates apoptosis are not fully resolved (*see* Fig. 3); therefore, it is not fully clear whether these pathways are mutated in tumors or whether they can be re-activated by therapeutic strategies *(63)*. Myc acts upstream of cytochrome c release to stimulate apoptosis *(64)*; one likely possibility is that it activates, like p53, known or unknown pro-apoptotic members of the bcl-2 family. One characterized pathway by which Myc induces apoptosis involves upregulation of the *ARF* promoter. However, the dependence of Myc-induced apoptosis on p53 is by no means absolute and—from tissue culture experiments—there is reason to believe that deregulated Myc can effectively kill cells even in the absence of p53 given appropriate circumstances.

Clear evidence exists that the pro-apoptotic function of Myc is not limited to tissue culture experiments but extends into transgenic tumor models; indeed, induction of apoptosis clearly limits the tumorigenic potential of Myc genes. Several examples exist in the literature. For example, comparison of the rate of apoptosis in tumors arising in MMTV-myc, MMTV-Ras, and MMTV-myc/Ras mice showed that the rate of apoptosis is surprisingly high in tumors from MMTV-myc mice *(65)*. Even more surprisingly, apoptosis was only moderatedly reduced in MMTV-myc/Ras mice despite the strong synergy that is observed in tumor formation. Furthermore, when conditional MycER proteins were activated in keratinocytes, little apoptosis was observed unless rare migrant cells occurred, which crossed the basal membrane *(55,56)*. Almost invariably, such cells underwent apoptosis and died. Inhibition of apoptosis promoted survival of such cells, allowing invasive malignancies to form. In this setting, therefore, targeting the signals that allow survival of keratinocytes in this foreign environment can be expected to significantly inhibit tumor progression.

A critical survival signal in fibroblasts expressing Myc is provided by the activation of the AKT kinase, in part because this kinase in turn phosphorylates and inactivates the pro-apoptotic Bad and pro-caspase 9 proteins (*66*; for review, *see 67*). The precise identity of survival signals may be cell-type-specific, however, since in lymphoid cells activation of Raf rather than Akt provides a survival

stimulus. For many human tumors, autocrine secretion of growth factors may provide a similar survival signal.

Clearly, however, in many tumor types inhibition from apoptosis is provided by deregulated expression of members of the *bcl-2* family. This situation can easily be visualized in tissue culture experiments, in which bcl-2 protects from apoptosis induced in cells expressing deregulated Myc by growth-factor withdrawal *(68)*; in transgenic mice, a potent synergy in tumor induction between Myc and bcl-2 is observed. Clearly, cell-based assays can be envisaged in which compounds are sought that re-activate the apoptotic function of Myc proteins in the presence of bcl-2 or bcl-xl. Specifically, if MycER proteins are used, compounds can be sought that kill cells expressing MycER and bcl-2 in the presence of estrogens, but have little or no effect on the same cells in the absence of estrogens. In this manner, cellular assays can be specifically targeted to the tumorigenic situation. Similar screens can be envisaged for situations, in which the AKT pathway has been activated.

6. SUPERACTIVATION OF MYC AS A THERAPEUTIC MEANS?

The potent ability of Myc to induce apoptosis opens the possibility of envisaging alternate strategies to kill tumors overexpressing Myc. Such strategies would make use of the observation that deregulation of Myc expression in tumors usually occurs at the level of mRNA and that further enhancing levels of active Myc protein can be expected to raise levels of apoptosis to a degree to inhibit tumor growth. Two such strategies can be envisaged:

1. Targeting degradation of Myc proteins: All Myc proteins known are extremely short-lived nuclear proteins that are rapidly degraded by the ubiquitine/proteasome-dependent pathway *(70)*. Degrons have been mapped in the amino-terminus of the protein, which overlap MycBoxI. Indeed, degradation of Myc is regulated by phosphorylation of specific amino acids close to MycboxI; mutation of these residues moderately stabilizes the protein *(19)*. The E3-ligases involved in degradation are currently unknown, but screens to identify them are under way in several laboratories. Potentially, therefore, transiently stabilizing *Myc* might push the levels of *Myc* proteins in cells with high levels of mRNA beyond a threshold that can be tolerated for survival, while leaving normal cells with low levels of Myc mRNA largely unaffected. The beauty of such an attempt would be the transient nature of the treatment, since cells would be eliminated from apoptosis.
2. Targeting Mad-dependent histone acetylation. Transcriptional activation by Myc/Max heterodimers is antagonised by heterodimers between Max and one of five different Mad (Mxi/Mnt) proteins (for review, *see 8*). These proteins recruit histone deacetylase complexes indirectly via a short motif in the amino-terminus, which binds to sin3A. Thus, the level of histone acetylation is a balance between

Myc-dependent acetylation and Mad-dependent deacetylation. Indeed, inhibition of deacetylation activates at least some target genes of Myc in vivo *(10)*. Since such compounds have clear antineoplastic function in vivo, it remains a clear possibility that they would deregulate the function of the Myc network sufficiently to kill tumor cells by apoptosis *(71,72)*.

7. SUMMARY

The biology of Myc proteins can be used to design a number of assays to specifically interfere with the role of Myc proteins in tumorigenesis; the extremely well-validated biology makes these proteins an attractive target.

Given that specific interference with protein/protein interactions of nuclear proteins may be difficult to achieve, two approaches hold the promise for success in the short time frame. One, cell based assays aimed at re-activating apoptotic strategies in human tumors, and two, interfering with the enzymatic activities required for transcriptional regulation by Myc proteins.

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3 The AP-1 Family of Transcription Factors

Structure, Regulation, and Functional Analysis in Mice

Marina Schorpp-Kistner, Peter Herrlich, and Peter Angel

CONTENTS

1. INTRODUCTION

The history of the exploration of transcriptional regulation can be told as a tale of increasing complexity, from single regulatory proteins selecting genes and obeying linear arrays of signals, to huge multiprotein complexes that are embedded in a network of circuitry—involving so many and even seemingly redundant components that it has become difficult to recognize a pattern of rationale. The factor AP-1 can serve as a prime example for this development.

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The term AP-1 stands for a family of proteins that bind as homo- or heterodimers to similar DNA motifs. Although the subunits forming these dimers are structurally related, they exhibit distinct properties. The number of possible subunit combinations creates an enormous functional diversity. Individual members of the AP-1 family can be engaged in totally different cell fates, from proliferation to apoptosis, from differentiation to carcinogenesis and tumor progression, and in survival and defense reactions. In addition to the combinatorial diversity, the AP-1 dimers integrate numerous signaling pathways stimulated by a great variety of extracellular cues. The synthesis of AP-1 subunits, their turnover, and their activation state are controlled by these signaling pathways. Through rapid and diverse responsiveness, the AP-1 family of transcription factors serves key roles in regulation of physiological and pathological processes.

The literature on AP-1 is large and cannot be reviewed in all details here. We like to refer to previous reviews *(1–4)* and we summarize here only basic features. Our emphasis will be on the advances made over the last five years when new organismic technologies have made possible to dissect the roles of individual AP-1 subunits and of their regulation in embryonic mouse development and in organ function.

2. THE AP-1 SUBUNIT FAMILY, DNA ELEMENTS, INTERACTION RULES

The core of the AP-1 family of transcription factors is formed by heterodimeric associations of members of the Jun (c-Jun, JunB, and JunD), Fos (with c-Fos, FosB, Fra-1, and Fra-2), and ATF (identified more recently mostly by yeast-two-hybrid screening; ATFa, ATF-2, ATF-3) proteins. Fos proteins heterodimerize exclusively with Jun proteins. Also ATF proteins are found predominantly in association with Jun. The AP-1 subunits share an evolutionary conserved domain that mediates DNA binding and dimerization, the so-called "bZip" region. bZip stands for the amino acid composition of two independently acting subregions: the "basic domain" ("b") is rich in basic amino acids and is responsible for contacting the DNA; the "leucine-zipper" ("Zip") region is characterized by heptad repeats of leucines forming a coiled-coil structure that is responsible for the dimerization. The structure of the Zip region in a hydrophilic environment demands immediate association with a partner and only the dimers bind to DNA. In addition to the leucines, other hydrophobic and charged amino acid residues within the leucine zipper region are responsible for specificity and stability of the dimers formed. The structure of the Zip region determines that Fos proteins cannot form stable homodimers nor heterodimers within the Fos group of proteins. The proteins heterodimerize efficiently with the Jun proteins. Homodimers of Jun proteins have been described. Their stability appears to be lower than that of Jun:Fos or Jun:ATF heterodimers. Although bzip regions interact in solution, stability

and probably specificity are influenced by the DNA element *(5)*. Jun:Jun and Jun:Fos dimers preferentially bind to the 7-bp motif 5'-TGAG/CTCA-3', whereas Jun-ATF dimers or ATF homodimers prefer a related 8-bp sequence with the consensus structure 5'-TTACCTCA-3'. Binding affinities and stabilities of Jun:Fos proteins are strongly affected by the individual Fos partner *(6)*. Since base pairs flanking the 7- and 8-bp motifs also influence DNA binding (for review *see* ref. *7* and references therein), in vitro binding studies using the DNA core elements can only give coarse hints. Certainly, the characteristics of the AP-1 DNA binding sites in promoters as well as the abundance of the individual AP-1 subunits are decisive for the selection of target genes. The existence of related DNA binding AP-1 molecules as well as of related DNA elements adds to the diversity of target genes that can be addressed. One could even imagine that different affinities could provide cells with a mechanism of fine-tuning.

In addition to the "classical" AP-1 members (Jun, Fos, ATFs), numerous other bZip proteins have been discovered, some of which can heterodimerize with the core AP-1 subunits, e.g., Maf, Maf-related proteins, Nrl, Smads, and Jun-dimerizing partners (JDPs). The function of these proteins in AP-1-regulated processes remains to be determined. Binding of AP-1 to DNA may also support binding of other transcription factors to adjacent or overlapping binding sites (composite elements) to allow the formation of "quaternary" complexes. The interaction of NF-AT and Ets proteins with the IL-2 and collagenase promoters, respectively, may serve as paradigms for this type of protein–protein interaction (for review *8, 9*). Several laboratories have reported an AP-1 association with NFκB *(10–13)*.

In contrast to the well-defined DNA binding domains of AP-1 bZip proteins, the structural properties of the domains mediating transcriptional activation of target genes (transactivation domain, TAD) are yet ill defined. The TAD (and its function) can be transferred to heterologous DNA binding domains that do not require heterodimerization, e.g., to that of the yeast transcription factor GAL4 or of the mammalian transcription factor GHF1 (Pit-1). Such chimeric proteins permit the identification of critical amino acids in TADs and the recognization that the TADs of individual Jun, Fos, and ATF proteins greatly differ in their transactivation potential. For example, extrapolating to the complete proteins, c-Fos, FosB, and c-Jun are strong transactivators, whereas JunB, JunD, Fra-1, and Fra-2 transactivate weakly. Under specific circumstances, these latter subunits may even act as repressors of AP-1 activity by competitive binding to AP-1 sites, or by forming inactive heterodimers with c-Fos, FosB, or c-Jun (for review *1,4*).

3. REGULATION BY ABUNDANCE

The abundance of individual AP-1 subunits varies during the cell cycle *(14)*. For instance, JunD levels are high in G0 and downregulated at the onset of the

G1 phase of the cell cycle, while c-Jun follows the reverse pattern, upregulated early in G1. Although AP-1 proteins appear to be present in all tissues, there are slight differences of expression, which is the major target of current organismic studies (below). For instance, basal layer keratinocytes of mouse skin express c-Jun, JunD, junB, c-Fos, and Fra-1 but not FosB *(15)*. In the granular layer of the epidermis only JunB and Fra-2 are found *(15,16)* (for review *17*). Also during embryonic development, AP-1 subunits are differentially essential as will be described below.

The abundance of AP-1 subunits is regulated on several levels: transcription, mRNA turnover, protein turnover by the ubiquitination/proteasome pathway *(4)*.

4. TRANSCRIPTIONAL AND POST-TRANSLATIONAL CONTROL OF AP-1 ACTIVITY

4.1. Promoter Regulation of c-jun and c-fos

Given the aspects of subunit heterogeneity, their dimerization and DNA binding rules, the control of transcription of AP-1 subunit genes, the time course of subunit synthesis, and the regulation of their function become important issues for an evaluation of the effect on target gene expression and on dependent phenotypes. *c-jun* and *c-fos* are so-called "early-response" or "immediate-early" genes, which are characterized by rapid and transient increases of transcription and translation in response to changes of environmental conditions. The promoters respond to growth factors, cytokines, and tumor promoters, to carcinogens, or to expression/activation of certain oncogenes. The upregulation is by and large transcriptional, while the transient nature is due to (regulated) high RNA and protein turnover. Promoter activation of *c-jun* or *c-fos* occurs in the absence of ongoing protein synthesis indicating that preexisting factors, whose activity is altered by post-translational modification (see subsequent section), are responsible for the regulation of promoter activity.

Most of our current knowledge on transcriptional activation of immediate early genes is derived from studies on deletion and point mutations of the *c-fos* and *c-jun* promoters, combined with in vitro and in vivo footprinting analyses. The serum-response element (SRE) occupied by and forming ternary complexes with the transcription factors p67-SRF and p62-TCF (which stands for a class of Ets-related proteins described as Elk or SAP) is required for the majority of extracellular stimuli including growth factors and phorbol esters. Changes in the phosphorylation pattern of SRF and, above all, of TCF regulate *c-fos* promoter activity by these stimuli. Other extracellular cues address the c-AMP response element (CRE) recognized by CREB (member of the ATFs), and the Sis-inducible enhancer (SIE) recognized by the STAT group of transcription factors at the receiving end of the Jak/Stat signaling pathway. CREB is activated by phosphorylation in response to increased levels of cyclic AMP or to growth factors, the

Jak/Stat pathway is activated by certain cytokines. The very transient induction of promoter activity by extracellular stimuli can be explained by increased phosphatase activity counteracting the activity of upstream protein kinases that address promoter-associated transcription factors described above. On the other hand, negative autoregulation (feedback) by newly synthesized c-Fos may also play an important role. The mechanism of this negative feedback loop is not fully understood *(18,19)*.

Analysis of deletion mutants of the *c-jun* promoter identified two AP-1-like binding sites (Jun1, Jun2), which are recognized by Jun:ATF heterodimers or ATF homodimers and which are involved in transcriptional regulation in response to the majority of extracellular stimuli affecting *c-jun* transcription. In response to G-protein-coupled receptors (e.g., the muscarinic acetylcholine receptor), EGF, and other growth factors, the AP-1-like binding sites and an additional element (recognized by MEF2 proteins) in the *c-jun* promoter cooperate in the transcriptional control of *c-jun*. Similarly to the factors binding to the *c-fos* promoter, the factors acting on the *c-jun* promoter are regulated by their phosphorylation status *(20,21)*.

Both *c-jun* and *c-fos* RNA levels are subjected to rapid turnover, which appears to be regulated by signaling pathways *(22)*.

4.2. Protein Kinases Acting on Preexisting Transcription Factors

The predominant modification relevant for protein activity is represented by phosphorylation at serine/threonine residues. Numerous protein kinases can act directly on transcription factors. Interesting for regulation are of course those that themselves are incorporated into signaling pathways. Mitogen-activated protein kinases (proline-directed kinases; MAPKs) are probably most critical for AP-1 activity regulated in response to extracellular stimuli. Depending on the type of stimuli MAPKs can be dissected into three subgroups: the extracellular signal-regulated kinases (ERK-1, ERK-2), which are robustly activated by growth factors and phorbol esters, but only weakly activated by cytokines and cellular stress-inducing stimuli (UV irradiation, chemical carcinogens). In contrast, Jun-N-terminal kinases (JNK-1, -2, -3), also known as stress-activated kinases (SAPK), and a structurally related class, p38 MAP kinases (p38α, -β, -γ), are strongly activated by cytokines and environmental stress, but are poorly activated by growth factors and phorbol esters.

These kinases themselves are under strict control of upstream kinases and phosphatases, which are part of individual signaling pathways initiated by specific classes of extra- and intracellular stimuli (growth factors, DNA damaging agents, oncoproteins). This network exhibits a high degree of evolutionary conservation from yeast to *drosophila* and mammals. This issue is, however, too complex to be discussed in greater detail in this chapter. (For in-depth information on this subject *see 23,24*.)

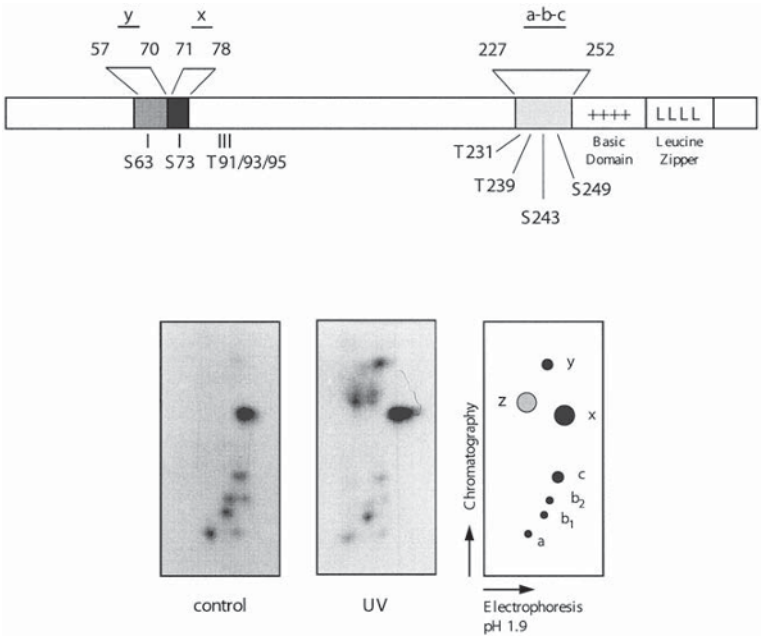

Fig. 1. Top: schematic diagram of the human c-Jun protein. Amino acids are numbered. The numbers on top refer to the trypsin cleavage sites that lead to the appearance of phosphopeptides after in vivo labelling of cells with ^{32}P-orthophosphate. The location of the tryptic peptides "a–c" in the DNA binding domain and peptides "x" and "y" in the transactivation domain are indicated. Bottom: Autoradiogram of in vivo labeled c-Jun protein, isolated by immunoprecipitation from untreated and UV-treated cells, digested with trypsin and separated by gel electrophoresis into two dimensions. On the right the positions of the tryptic peptides are schematically illustrated. Peptide "z," which is usually found in labeled Jun protein from cells treated with genotoxic agents, most likely represents a peptide containing residual phosphorylation at threonine-91, thr-93 and/or thr-95 of c-Jun.

ERK-1 and ERK-2 carry out mitogen-stimulated phosphorylation of Elk/SAP proteins in vivo. The sites phosphorylated by ERKs reside in the TADs of TCF proteins. Their phosphorylation has a positive regulatory role in transactivation *(25,26)*. The JNK/SAPKs were originally identified by their ability to specifically phosphorylate c-Jun at two positive-regulatory serine (Ser-63, Ser-73) within the transactivation domain *(27)* (Fig. 1). Hyperphosphorylation of both sites identified by 2D-phospho-peptide mapping (peptides x, y; Fig. 1), occurs in response to stress stimuli and oncoproteins and is responsible for transcriptional activa-

tion of c-Jun and its target genes. The JNKs can also phosphorylate and stimulate the transcriptional activity of ATF-2 and, to a lesser extent, of TCF proteins. The same serines in ATF2 serve as phospho-acceptor sites for p38, while Ser-63 and -73 of c-Jun are not affected by p38 *(28–31)*. Most likely, hyperphosphorylation of Jun, ATF, and TCF proteins results in a conformational change of the TADs allowing more efficient interaction with co-factors, such as CBP, which facilitate and stabilize the RNA polymerase II/initiation complex at target genes *(32)*. Interestingly, in addition to enhanced transactivation, hyperphosphorylation of the TAD of c-Jun also regulates the stability of c-Jun protein by reducing ubiquitin-dependent degradation *(33,34)*. Similarly, phosphorylation-dependent changes in the half-life of c-Fos have been observed *(35)*.

The DNA binding domain of c-Jun is phosphorylated at multiple sites by GSK-3 and/or casein kinase II (CK-II) (Fig. 1), which results in reduced DNA binding. In response to extracellular stimuli, such as UV, phosphorylation is reduced, thus enhancing DNA binding *(36,37)*. The mechanism (reduced activity of the kinase or enhanced activity of a phosphatase) has not yet been definitively clarified.

In addition to phosphorylation, other post-translational modifications regulate AP-1 activity. Oxidation of a cysteine in the basic region inhibits DNA binding *(38–40)*. There is pronounced regulation of nuclear localization *(41)*. The functional consequence of glycosylation *(42)* has not been revealed. Moreover, positive and negative interference of other cellular proteins with AP-1 activity (in addition to the protein kinases and coactivators described above) have been identified, for example, Maf, MyoD, YY1, STAT, SMADs, and Menin *(7)*. The mutual interference between AP-1 and nuclear receptors, particularly the glucocorticoid receptor (GR) represents the most extensively analyzed example for this type of crosstalk. There is evidence that the anti-inflammatory and immunosuppressive activities of glucocorticoids are mediated, at least in part, by GR-mediated repression of AP-1 activity *(43,44*; see below).

4.3. Transcriptional Regulation of junB, junD, fosB, fra-1, fra-2 Genes and Post-Translational Regulation of Their Gene Products

Similar to *c-jun* and *c-fos*, *junB* is a typical immediate-early gene. Its transcriptional regulation is complex with regulatory sequences dispersed across the complete *junB* locus including 5' and 3' flanking sequences. The organization of the *junB* locus is unique in that it contains nine distinct regions of non-coding DNA (so-called FECS, flanking evolutionary conserved sequences) that share 72% to 91% sequence identity between mouse and man *(45)*. So far, all reported *cis*-regulatory elements are located within these FECS. Numerous studies have contributed to the complex picture of *junB* regulation outlined in Fig. 2, which

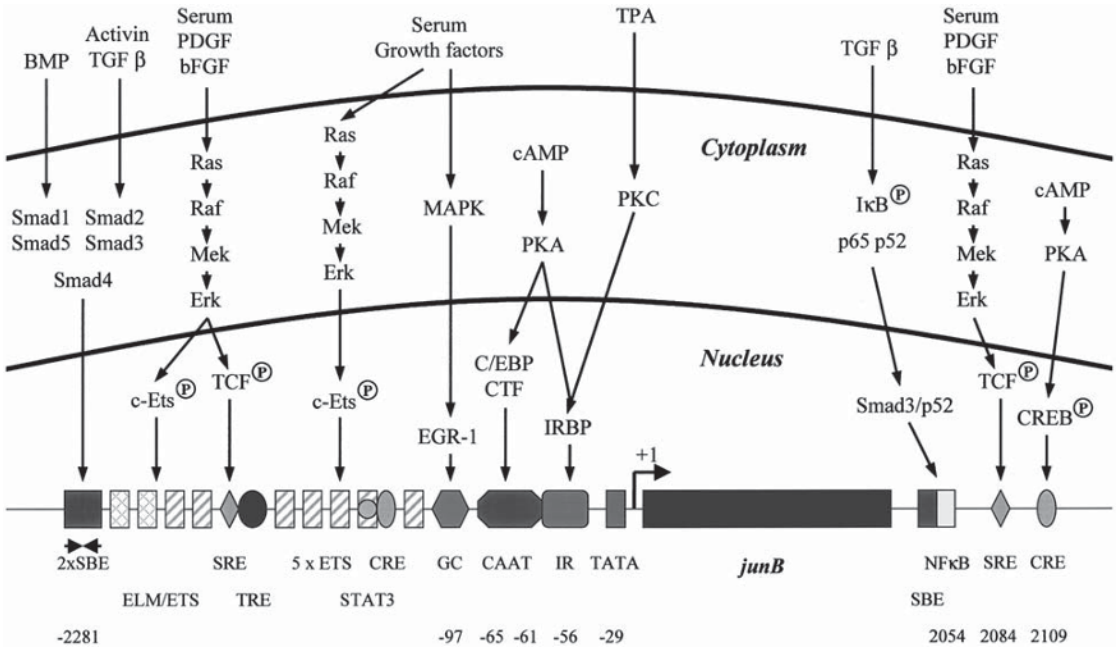

Fig. 2. Schematic representation of the mouse *junB* locus. Important *cis*-regulatory elements and their relative position with respect to the transcriptional start site are depicted at the bottom. Multiple pathways elicited by various signals leading to the transcriptional activation of *junB* are shown. For abbreviations of the signaling components, cis-regulatory elements and the individual references see text.

summarizes the location of described major *cis*-regulatory elements in the mouse *junB* promoter as well as the inducing signals and targeted pathways. Induction of *junB* in response to various mitogens is mediated by multiple Ets sites *(46)*, an IL-6 response element containing a STAT3 binding site and a CRE-like site *(47)*, a GC box with the potential to bind Egr-1/Zif 286 *(48)* or SP-1, an inverted repeat element *(49)*, and a novel myeloid-specific IL-6 response element (IL-6RE *(50)*, in the proximal promoter region (FECSI). Regulation of *junB* by v-src involves the CAAT and TATA box region *(51)*. Upstream of these elements two serum response elements SREs (at –1451/–1425 and –3100/–2500) have been found in the rat promoter *(52)*, whereas a TRE, a SRE, two Ets sites and two Ets-linked motifs (ELMs) that are specifically targeted by growth factor signaling pathways are located within FECSII and III of the mouse promoter (at about –1000 to –2000 *[53]*). The SRE and Ets sites exhibit enhancer-like activity because mutation of any of these elements including the ELMs significantly impairs *junB* induction. Moreover, mutations in the SRE or ELM appear to cause changes in the chromatin architecture, which are in turn associated with significant reduction of *junB* induction *(53)*. An SRE and a CRE site in the 3'-flanking sequences (FECS IX) contribute to *junB* induction by serum, PDGF, bFGF, phorbol ester, and forskolin, respectively *(54)*. However, this SRE is not conserved in the human *junB* promoter. Consistent with the general opinion that one major task of JunB is to act as a negative regulator of AP-1 function, of all AP-1 subunits studied only *junB* is highly induced at early times by negative regulators of cell growth, such as TGF-β and BMP-2 *(55)*. Two Smad binding sites (SBEs) arranged as an inverted repeat between –2813 and –2792 of the mouse *junB* promoter are required for transactivation by Smad3 and Smad4 and the repeat acts as a TGF-β, activin, and BMP-inducible enhancer *(56)*. Recently, an additional TGF-β responsive region, a NFκB site, could be identified within the 3'-regulatory sequences of *junB (57)*. The activation requires an intact NFκB pathway and depends on ligand-induced nuclear translocation of Smads. As part of the process, Smad3 associates with the NF-κB/rel member p52 and may act as a transcriptional coactivator *(57)*.

Despite this great body of information on the transcriptional regulation of JunB, there are only a few reports concerning its post-translational control. JunB contains a JNK docking site but lacks serine residues analogous to sites phosphorylated in c-Jun. This fact and initial studies suggested that JunB may not be a substrate addressed by JNKs *(58,59)*. On the other hand, a more recent study has identified threonines 102 and 104 of JunB as phosphoacceptor sites for JNK *(60)*. During G2-M transition of the cell cycle, JunB phosphorylation by the $p34^{cdc2}$-cyclinB kinase was observed *(61)*. Because this event can be associated with markedly decreased JunB levels in mitotic and early G1 cells, it has been proposed that this modification may target JunB for degradation *(61)*.

In contrast to *c-jun* and *junB*, expression of *junD* is barely affected by growth factor and phorbol ester treatment of cells. In fact, it is constitutively expressed

at high levels in many cell types *(62)*. The murine *junD* promoter harbors several conserved *cis*-acting elements, including a SP-1 binding site, a CAAT box, a Zif268/Krox24 motif, and a TRE-like sequence. However, the presence of an octamer motif recognized by the ubiquitous Oct-1 protein is mainly responsible for the high basal level of *junD* transcription and the non-responsiveness of the TRE *(63)*. Similarly to JunB, only little is known about changes in the phosphorylation pattern of JunD modulating its activity. Although JunD lacks a JNK docking site, it possesses a phosphoacceptor peptide identical to c-Jun and can be phosphorylated by JNK through heterodimerization with docking-competent partners *(59)*. Glycogen-synthase kinase 3 can phosphorylate JunD in a region proximal to its DNA binding domain and can attenuate its DNA binding capacity *(64)*. In contrast, there is evidence that serum-induced phosphorylation of JunD does not affect its DNA binding ability but rather may influence its transactivation potential *(65)*. This is supported by a recent report that ERK1/2-mediated phosphorylation of JunD and FosB is required for okadaic acid-induced transcription *(66)*.

Not much is known about the transcriptional regulation of *fosB* except that the promoter contains several consensus sequences, including an SRE and an AP-1 binding site whose relative positions are identical to those found in the 5'-upstream region of the *c-fos* gene *(67)*. The AP-1 site may be the target for the downregulation of the promoter by FosB and c-Fos. *Fra-1* has been identified as a positively regulated Fos target gene. Basal and AP-1-regulated expression of *fra-1* depends on regulatory sequences in the first intron that contains one consensus AP-1 element and two AP-1- like sites *(68)*. Fra-1 and also *c-jun* expression is increased upon overexpession of the β-catenin-T cell-factor/lymphoid-enhancer factor complex that is able to interact directly with the promoter regions of both *fra-1* and *c-jun (69)*. So far, a detailed analysis on Fra-1 regulation by posttranslational modification is still lacking, although there is evidence that Fra-1 phosphorylation by ERK1/2 may occur in response to insulin stimulation *(70)*.

Little is known about Fra-2, whose function and biology remain poorly understood. Expression of *fra-2* can be turned on by second messengers including cyclic AMP *(71)* and Ca^{2+} *(72)*. The Fra-2 protein is modified extensively through MAP kinase phosphorylation that triggers a positive auto-regulatory loop *(73)*.

Very little is known about transcriptional regulation of *atf* (*ATFa, ATF-2* and *ATF3*) genes, while ATF proteins were extensively analysed as substrates of the p38 and JNK protein kinases in response to genotoxic stress *(28–31)*.

5. JUN DIMERIZATION MUTANTS REVEAL DISTINCT FUNCTIONS OF SPECIFIC DIMERS

The seven AP-1 subunits can, in principle, form 18 different homo- and heterodimers. Current efforts are directed toward understanding the programs of gene expression controlled by each of these dimers. Several experiments to be described

below address this problem: the generation of dimerization mutants, and the total and conditional removal of individual subunit genes from mice.

As discussed above, the selectivity of dimer formation and their stability is dictated by the leucine zipper according to rules that have been worked out in solution in vitro *(74,75)*. In the presence of an AP-1 DNA element, however, subunits associate in a manner not exactly identical to that predicted *(5)*. By empirical site-directed mutagenesis, Jun mutants in the leucine zipper have been generated that prefer either Fos-related proteins or ATF2. These mutants were tested within the background of other AP-1 subunits in the nucleus by making use of the transforming ability of Jun in embryonic chick *(5)*. Retroviral introduction of the Jun mutants led to an interesting proof of dimer specificity: the Fos-seeking Jun mutants caused anchorage independent growth but not serum independence, while Jun mutants with preference for ATF2 conferred serum independence but no growth ability in soft agar. Thus, these two types of AP-1 dimers induced defined and different programs of gene expression.

6. AP-1 IN THE MOUSE ORGANISM

6.1. Lessons from Loss-of-Function Approaches: Disruptions of Subunit Genes Indicate Both Overlapping and Unique Functions

Despite being in the middle of the genomics era, a systematic classification of AP-1 dimer-specific target genes is still missing. Nevertheless, for almost all AP-1 members, mouse models involving genetic disruption and/or transgenic overexpression have contributed to an understanding of their normal physiological roles, as well as of their relation to disease. The distinct phenotypes emerging support the notion that AP-1 dimers exhibit specific and independent functions in vivo. As a general rule derived from all studies, the AP-1 subunits must be present in a complementary and coordinated manner in order to ensure proper development or physiology of the organism.

6.1.1. AP-1 Members Required for Embryonic Development

Loss of c-Jun, JunB, or Fra-1 is fatal for embryonic development. c-Jun null embryos die at midgestation (E12.5 to 13.5 [*76,77]*) owing to dysregulation in liver and heart development *(78)*. Mutant fetal livers are characterized by extensive apoptosis of both hematopoetic cells and hepatoblasts. The finding that c-Jun-deficient embryonic stem (ES) cells are not able to contribute efficiently to hepatic tissue in chimeric mice *(76)* supports an essential role of c-Jun in liver development. Nevertheless, *c-jun*$^{-/-}$ fetal liver cells are able to reconstitute all hematopoietic compartments of lethally irradiated recipient mice, excluding a strictly cell-autonomous defect *(78)*. This finding also suggests that c-Jun is not needed for the differentiation of mature B and T lymphocytes, albeit its strong induction

upon T cell activation *(79)*. Beside liver organogenesis, c-Jun is essential for the development of a normal heart outflow tract. c-Jun null fetuses show cardiac malformations that resemble the human disease of truncus arteriosus persistence *(78)*. Interestingly, mice carrying a conditional (floxed) allele of *c-jun*, survive the specific post-natal gene inactivation in hepatocytes. The mice display no overt histological or biochemical abnormalities, but show an impaired liver regeneration in response to partial hepatectomy *(80)* (Behrens A. and Wagner E.F., personal communication). A severe scoliosis phenotype was generated when the conditional *c-jun* allele was inactivated by the use of a Coll2a-Cre transgene suggesting that c-Jun regulates sklerotomal differentiation *(80)* (Behrens A. and Wagner E.F., personal communication).

Knowing the mechanism of protein activation by serine phosphorylation, one would expect that Jun mutant mice whose Jun cannot be phosphorylated should show a severe phenotype. However, knock-in mice expressing a mutated c-Jun protein in which the N-terminal phosphorylation sites at serines 63 and 73 were changed into alanines (JunAA mice) developed normally and were viable and fertile as adults *(81)*. However, these JunAA mice are resistant to epileptic seizures and neuronal apoptosis induced by the excitatory amino acid kainate, and JunAA fibroblasts show proliferation defects as well as apoptotic defects upon stress induction *(81)*. By contrast, T-cell proliferation and differentiation appear to be independent of c-Jun N-terminal phosphorylation, whereas efficient T cell receptor-induced thymocyte apoptosis is affected *(82)*. These findings suggest that N-terminal phosphorylation of c-Jun is redundant in many instances, presumably because partner subunits supply sufficient transactivation function. Nevertheless, Jun TAD phosphorylation may be required for stress-induced apoptosis. Interestingly, embryos deficient in both JNK1 and JNK2 do not close their neural tubes as a result of deregulated neuronal apoptosis *(83)*. Reduced apoptosis was observed only in certain areas of the developing brain, while increased apoptosis was observed in other regions *(83,84)*.

An interesting question is whether other members of the Jun family can substitute for c-Jun. To date this has been examined for JunB. To investigate whether the homologous JunB protein is able to execute c-Jun functions, knock-in mice having the *c-jun* allele replaced by *junB* were generated. Such mice were born with open eyes and normal livers. They died, however, a few hours after birth due to a malformed cardiac outflow tract *(80)* (E. Passague and E.F. Wagner, personal communication). Obviously, JunB is able to substitute for c-Jun in hepatic but not in cardiac development.

Targeted inactivation of JunB also resulted in embryonic lethality, at a stage earlier than the c-Jun knock-out. JunB null embryos died between d 8.5 and 10.0 of embryonic development owing to multiple defects in extraembryonic tissues *(85)*. Affected cell types/organs comprise the trophoblast giant cells, yolk sac

mesenthelium, and placental labyrinth. The observed phenotypes in JunB null embryos appear to result from severe impairment of general vasculogenic and angiogenic processes resulting in a failure of establishing proper vascular interactions with the maternal circulation. This interpretation is further supported by changes in the expression levels of proliferin, MMP-9, uPA, or VEGFR-1 genes that are directly or indirectly associated with vasculogenesis/angiogenesis. In tetraploid chimeras with extraembryonic wild-type tissues, the survival of the *junB*$^{-/-}$ embryos can be extended up to E12.5 demonstrating that the placentation defect is the cause for the early lethality. The rescued fetuses exhibited no overt phenotype. Their death at E12.5 is most likely due of a failure of *junB*$^{-/-}$ extraembryonic mesoderm to sustain and promote the growth of the umbilical vessels (*85* and M. Schorpp-Kistner, unpublished results). The lethal phenotype can be overcome by the expression of an Ubiquitin C-promoter-driven *junB* transgene *(85)*. Among the offspring of such *junB*$^{-/-}$/Ubiquitin C-transgene-positive mice, a strain lacking JunB in the myeloid lineage was obtained. These mice developed a transplantable myeloproliferative disease resembling human chronic myeloid leukemia *(86)*. A similar disease appeared in mice reconstituted with *junB*$^{-/-}$ES cells. In both cases, the loss of JunB resulted in increased numbers of granulocyte progenitors showing enhanced GM-CSF-mediated proliferation and extended survival associated with changes in the expression levels of the GM-CSFα receptor, of the antiapoptotic proteins Bcl2 and Bclx, and of the cell cycle regulators p16 and c-Jun.

Fra-1 is so far the only Fos member essential for embryonic development. Fra-1 null embryos died between E10.0 and 10.5 *(87)*. Similarly to JunB null embryos, the vascularization of the placental labyrinth was impaired. In contrast, the life span of Fra-1 null fetuses supported by wild-type extraembryonic tissues could be extended up to birth. These findings suggest that JunB and Fra-1, probably as heterodimers, address the same set of target genes responsible for the generation of a functional placental labyrinth. Fra-1-deficient embryos can be rescued from embryonal death by ubiquitin C-driven JunB overexpression *(87)*.

6.1.2. AP-1 Members that are Dispensable for Embryonic Development, but Exhibit Distinct Features

Mice lacking JunD, c-Fos, FosB, or ATF-2 are viable and fertile, although the adults show specific defects in distinct tissues, implying that only a subset of AP-1 target genes is affected in these mutants.

JunD-null mice develop normally. However, post-natal growth of homozygous *junD*$^{-/-}$ animals is reduced. JunD null males develop age-dependent defects in reproduction, hormone imbalance and impaired spermatogenesis *(88)*.

Fos-null mice are viable and fertile but suffer from severe osteopetrosis caused by the lack of mature osteoclasts *(89,90)*. The osteopetrosis causes extramedullary

hematopoiesis and lymphopenia *(91)*. Despite the rapid upregulation of Fos upon activation of T-lymphocytes and its regulatory role in cytokine gene expression, Fos is dispensable for normal thymocyte development and for the differentiation and functional activity of peripheral T cells *(92)*. Importantly, c-Fos-dependent functions in mice can be substituted by Fra-1, when the *fos* locus is deleted and replaced by the *fosl1* gene encoding Fra-1. *Fosl1* is a transcriptional target of c-Fos during osteoclast differentiation *(93)*, and can fully complement for the lack of c-Fos in bone development (in a gene-dosage-dependent manner) and in light-induced photoreceptor apoptosis (independently of the dosage). However, Fra-1 is not able to induce expression of c-Fos target genes in fibroblasts derived from the knock-in mice *(94)* suggesting the need for additional tissue-specific factors.

The embryonic development of FosB-deficient mice is normal *(95)*. Adult *fosB*$^{-/-}$ females, however, nurture insufficiently *(96)* which is interpreted to be due to the absence of FosB in a behavior-relevant hypothalamic region.

Data on targeted ablation of Fra-2 are still missing. Because of the broad expression pattern of Fra-2 during late embryonic *(16,97)* numerous tissues will likely be affected by a knock-out. A tissue-specific transgenic knock-down of Fra-2 expression mediated by a dominant-negative mutant of Fra-2 in the pineal gland led to the identification of two genes (encoding type II iodothyronine deionidase and nectadrin or CD24) specifically expressed in the pineal gland, whose expression is closely linked to *fra-2* expression *(98)*.

Of the ATF family, only ATF-2 has yet been inactivated. ATF-2 mutant mice that express small amounts of a mutant ATF-2 protein (ATF-2$^{m/m}$ *[99]*) are chondrodysplastic and neurologically abnormal *(100)*. Recently, cyclin D1 has been identified as a target gene affected in chondrocytes *(99)*. Analysis of immune system activation in these mice revealed a decreased immediate inflammatory gene induction affecting genes encoding transcription factors, adhesion molecules, cytokines and chemokines, in the early phase of an immune response. After one to two days, however, an overexuberant response was observed *(101)*. ATF-2-null mutant mice die shortly after birth and suffer from a disease resembling a severe type of human meconium aspiration syndrome *(102)*. An increased expression of hypoxia-inducible genes suggests that hypoxic distress may lead to strong gasping respiration, and consequently, to aspiration of the amniotic fluid containing meconium. A placental anomaly is probably responsible for insufficient oxygen supply prior to birth *(102)*.

6.2. Lessons from Overexpression Studies

c-Jun is the cellular homolog of *v-jun*, the transforming oncogene of the Avian sarcoma virus 17. Although c-jun is capable of transforming mammalian cells upon co-expression with Ras or Src *(103)*, *c-jun* overexpression in transgenic mice is not sufficient to induce tumor development *(104)*. Nevertheless, in coopera-

tion with c-Fos, c-Jun contributes to the formation of skeletal osteosarcomas *(105)* in the course of which the transformation depends on c-Jun N-terminal phosphorylation *(106)*. Overexpression of the *v-jun* oncogene in transgenic mice causes initially no phenotype, but after wounding, these animals show abnormal wound repair characterized by hyperplastic granulation tissue *(107)*.

Transgenic mice that ectopically express JunB in a broad range of tissues are phenotypically normal. By contrast, T-cell-specific JunB transgenic animals express cytokines in Th1 cells, which normally are restricted to Th2 cells *(60)*. Unfortunately, no gain of function approach has yet been described for JunD.

Overexpression of c-Fos in transgenic and chimeric mice causes osteosarcomas *(108)* and chondrosarcomas *(104,109)*. So far, c-Fos is the best studied AP-1 member, whose function is perfectly described by the complementing data of gain- and loss-of-function approaches. These data have identified c-Fos as a key regulator of the osteoclast–macrophage lineage determination and of bone remodeling *(110)*. Transgenic mice expressing v-Fos under the control of the human keratin-1 promoter exhibit hyperplasia, hyperkeratosis, and squamous papillomas in wounded ears *(111)* suggesting that both overexpression and loss of Fos activity interferes with skin homeostasis. Ectopic overexpression of a naturally occurring form of the FosB protein, ΔFosB, results in increased bone formation throughout the skeleton and in continuous post-embryonic increase in bone mass, leading to osteosclerosis. On the other hand, ΔFosB downregulates the expression of early adipogenesis markers and inhibits adipogenesis *(112)*. Because osteoblasts and adipocytes are thought to originate from a common precursor, ΔFosB can be considered a transcriptional regulator of osteoblastogenesis. Fra-1 is still another AP-1 member implicated in bone formation. Similarly to ΔFosB, Fra-1 overexpression in transgenic mice causes a progressive increase in bone mass leading to osteosclerosis of the entire skeleton, most likely due to enhanced osteoblast differentiation rather than proliferation *(113)*. However, an effect of Fra-1 overexpression on adipogenesis was not observed.

Mice overexpressing the related Fra-2 protein show ocular malformations due to a disrupted development of anterior eye structures *(114)*. There have been no reports on ATF-a or ATF-2 overexpression studies in transgenic mice.

In summary, major in vivo functions of all Fos family members, possibly with the exception of the yet poorly studied Fra-2 protein, are closely linked to osteogenesis (c-Fos to osteoclast-macrophage and ΔFosB and Fra-1 to osteoblast differentiation). A common biological process unifying the Jun members has not yet been identified. c-Jun can be associated with hepatogenesis and heart development, JunB with placentation (very likely endothelial cell differentiation/function), T cell differentiation and granulopoiesis, whereas JunD is implicated in spermatogenesis. Three AP-1 members appear to support placenta formation and/or function: JunB and Fra-1 are absolutely required for proper placentation, while in the absence of ATF-2 the number of cytotrophoblast cells is reduced.

6.3. AP-1 Target Genes

Although numerous promoters carry AP-1 binding DNA elements, only a few directly regulated and verified AP-1 target genes have yet been characterized. The recent explosion in gene targeting and "genomics" technologies has significantly increased the number of identified and physiologically relevant target genes. Well-studied systems to identify critical target genes are phenotypes of mouse mutants and cell lines derived thereof with major emphasis on cell cycle control and skin homeostasis (*see 4* and references therein). Despite this "new wealth," efforts are required to identify sets of target genes and biological processes that are addressed by individual AP-1 dimers. Classification studies for preferred DNA elements recognized by the individual dimers as well as the overexpression studies of individual subunits or pairs (including the Jun dimerization mutants) in tissue culture suggest that dimer-specific promoters are selected (e.g., *5,115,116)*. In the near future, a much more persuasive evidence for defined functions will come from the analysis of double or triple gene disruption studies in the mouse.

7. DOWN-MODULATION BY NUCLEAR RECEPTORS

AP-1 is not only an essential transcription factor in several genetic programs but also a mediator of emergency responses, e.g., stress response, acute phase response, septic shock, response to antigens, and induced apoptosis. The "immediate" nature of upregulated AP-1 synthesis and function matches these functional demands. Many emergency reactions are, however, detrimental for the organism and, therefore, need to be limited and counteracted. This control is achieved by negatively regulating components on many levels of the signal transduction chains, often organized in autoregulatory loops (e.g., TGFβ/Smad activates AP-1, which in turn inhibits TGFβ target genes; IL-6 released from macrophages in the development of septic shock acts on the HPA axis releasing cortisol, which in turn inhibits cytokine synthesis and prevents death from septic shock). Negative regulation is established, e.g., by inhibitory ligands of transmembrane receptors or by protein phosphatases that are associated with the protein kinases of the signal transduction chains and whose action is upregulated after stimulation of signaling. As a last rescue step, interesting brakes are built into the nucleus. One of these brakes will be described in this section: the modulating activity of the nuclear receptors.

The inhibitory action has been best characterized for the glucocorticoid receptor (GR). Glucocorticoids are the most widely used drugs prescribed. Their wanted action is based on this modulating activity. The glucocorticoid receptor is a ligand-activated transcription factor, which, as a homodimer, itself addresses promoters carrying palindromic so-called glucocorticoid-responsive elements (GREs) *(see 17)*. Upon binding the specific ligand hormone cortisol (cortico-

sterone in rodents), the cytoplasmic nuclear receptor dissociates from a complex of chaperones and is taken up into the nucleus. This process and the subsequent activation of GR-dependent genes depends on the hormone concentration with half maximal responses achieved by 10^{-7} *M* cortisol (or 10^{-8} *M* of the nondegradable analog dexamethasone; *118*). The inhibitory action of hormone on the emergency responses occurs, interestingly, at a 10-fold lower concentration of hormone *(118)*, which initially caused skepticism in the endocrinology community. The hormone dose dependence has since been reproduced by other laboratories (e.g., *119*) and hormone responses of different sensitivity have been described for other systems: in neural tissues, glucocorticoid hormone activates two receptors, the mineralocorticoid receptor MR and the GR. With increasing doses the MR responds first *(120)*. These dose dependencies may make physiological sense: in the hypothalamus–pituitary–adrenal axis, the autoregulatory down-regulation of cortisol release should occur only at excess concentrations. In the emergency response, the brake function is required irrespective of a GR-dependent gene program. This program may not even be wanted. The different hormone dose responses suggest the existence of two different GR-dependent molecular mechanisms.

Emergency responses are triggered by the activation of a limited number of transcription factors. AP-1, NF-AT, and NFκB are responsible for most of the responses. For example, the synthesis and release of numerous cytokines is regulated by AP-1 and NFκB. The anti-inflammatory action of cortisol, the rescue from septic shock by cortisol, and the inhibition of other emergency responses may therefore be based on a GR-dependent block of signaling to these transcription factors or a direct inhibition of their function. Interference with signaling, as well as with AP-1 and NFκB function, have indeed been well documented (*see 44*, for references).

Mutations introduced into the GR have produced convincing evidence for two separate functions exerted by the hormone receptor. To act as a transcription factor selecting GRE-containing promoters, the GR needs to be able to form homodimers on DNA and to assemble coactivators through its transactivation domains. Deletion or point mutation of the transactivation domains as well as mutations in the D-loop required for dimerization, abolish transcriptional activity. These mutations do not, however, destroy the ability of GR to inhibit AP-1 activation and activity *(121–124)*. GR appears to interfere with members of the AP-1 family on two levels: inhibition of JNK activity and inhibition of AP-1 after binding to appropriate target promoters. Mice carrying a D-loop mutation are viable *(123)*—quite in contrast to GR-null mice *(125)*—suggesting that the properties retained in the D-loop mutant are important for survival. The D-loop mutant-mice cannot activate GRE-promoters in response to hormone, while they are fully competent in the modulation of AP-1 and NFκB, and in the inhibition of JNK *(123, 124)*. This competence is reflected by the efficient induction of anti-inflammatory

mechanisms in vivo. For instance, the LPS-triggered release of TNFα and of IL-6 causes a rise of circulating corticosterone and the subsequent block of further TNFα release in both wild-type and D-loop mutant mice *(126)*. A phorbol-ester-induced inflammation of the skin is prevented by cortisol in both genotypes.

The interference with AP-1 and NFκB function after their activation is exerted by GR in a tethering reaction. Both transcription factors are inhibited while bound to the target gene promoters *(44,127,128)*. By chromatin-immunoprecipitation experiments, no obvious change in the preinitiation complex upon GR activation was found *(128)*. Transcriptional start was however prevented. Phosphorylation of a serine in the C-terminal tail of RNA polymerase II did not occur. The mechanism seems to differ from repressive actions of thyroid and retinoid receptors in that it was not trichostatin-sensitive (cited in *128*; and our own unpublished data). It is yet unknown which cofactors participate in the modulating action of the GR. The existence of additional factors must be postulated for several reasons *(44)*. The tethering mechanism "pioneered" by the GR-AP-1 interaction appears to occur also in other transcription factors, thus representing a novel and widely used property of transcription factors.

8. CONCLUSIONS

This chapter focused mainly on transcription factor data obtained with modern mouse technology. By comparison with results derived from cell culture and in vitro experiments, the organismic data not only exhibited enormous power, but also disproved previously developed hypotheses. With more refined recombinant mouse methodology, an even better insight is expected. Mutant mice will serve as disease models and will help to verify actions of drugs that had been developed in high-throughput procedures. The discrimination of two separate functions of GR will induce efforts to find ligands that selectively activate only one of the two functions, transcriptional activation or cross talk with AP-1. Available and new mouse mutants will ultimately be decisive in verifying such ligand actions.

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4 Hypoxia-Induced Factor-1 as a Target for Anticancer Therapy

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CONTENTS

1. INTRODUCTION

One of the most powerful recent advances in drug discovery has been the development of high-throughput screens that rapidly identify small molecules having specific biochemical, biological, and ultimately therapeutic effects. A candidate high-throughput screening target is ultimately validated only by the successful development of a pharmaceutical agent. For a putative target, there are three major points that need to be addressed to evaluate its anticancer potential. First, is the target in question important for the proliferation, survival, or metastasis of tumor cells? Second, is the target tractable for lead identification by high-throughput screening? Third, are facile methods available for determining if a candidate lead molecule effectively inhibits target function in vitro and in vivo? The purpose of this chapter is to address these issues with regard to the transcription factor HIF-1 (hypoxia-induced factor-1), and assess its suitability as a target for anticancer drug development.

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Table 1
Hypoxia and Hypoglycaemia Induced HIF-1 Target Genes *(10,98,99)*

Function	*Gene*
Glucose uptake and glycolysis	Glucose transporters (Glut-1*, Glut-3); Hexokinase 1,2; Phosphofructokinase L, and C; Aldolase-A* and C; Triosephosphate isomerase; Glyceraldehyde 3-phosphate dehydrogenase; Phosphoglycerate kinase 1*; Enolase 1; Pyruvate kinase M; Lactate dehydrogenase A*
Angiogenesis and vasodilation	Vascular endothelial growth factor (VEGF)*; Platelet derived growth factor B (PDGF-B); VEGF receptor (FLT-1); Nitric oxide synthase II; Haeme oxygenase 1; Endothelin-1; PAI-1
Neutralisation of extracellular acid	Transmembrane carbonic anhydrase 9 ,12 *(100)*
Growth factors & cell proliferation	Insulin-like growth factor 2; Insulin-like growth factor binding protein 1,2,3; P21; c-fos; c-jun; p35srj
Systemic functions	Erythropoietin; Transferrin; Adrenomedullin; α_1-adrenergic receptor; ceruloplasmin

*Indicates hypoglycemia induced HIF-1 target genes *(73,75)*.

2. BIOLOGY OF HIF-1 AND THE TISSUE ISCHEMIA RESPONSE

The function of blood, among other things, is to deliver oxygen and nutrients to tissues (and tumors), and to remove metabolites. Hypoxia refers to the reduction of oxygen supply despite adequate perfusion. Ischemia refers to a state of oxygen and nutrient deprivation accompanied by metabolite accumulation resulting from reduced perfusion.

2.1. Physiological Responses to Ischemia

The physiological responses to ischemia are geared to maintain cellular nutrient, oxygen, and metabolite homeostasis. These responses result from a coordinated response that leads to increased cellular glucose uptake *(1,2)*, a switch in ATP generation from oxidative phosphorylation to anaerobic glycolysis resulting in reduced oxygen consumption *(3,4)*, and the growth of new blood vessels, which increases the delivery of oxygen, nutrients, and the removal of cellular metabolites *(5–7)*. At a molecular level, the specific activation of hypoxia and hypoglycemia target genes (Table 1) is required for this fundamental metabolic adaptation. In broad terms, these genes encode glucose transporters, key glycolytic enzymes, vasodilatory molecules, angiogenic factors, and acid neutralizing enzymes.

2.2. Oxygen-Sensing and the Hypoxia Response Element

Enhanced expression of hypoxia target genes results from increased transcription and stabilization of target mRNAs in response to hypoxia. The response is also mimicked by desferrioxamine and cobalt chloride, and is inhibited by carbon monoxide, suggesting that the oxygen-sensing mechanism involves a heme-protein *(8,9)*. The mechanisms of oxygen sensing are controversial, and the reader is referred to a more comprehensive recent review on the subject *(10)*. The transcriptional component of the hypoxia response depends on hypoxia-inducible enhancers in the target genes, the first of which was identified in the *Epo* gene *(11–13)*. These elements are activated by hypoxia in diverse cell types indicating a common, widespread oxygen-sensing mechanism *(14)*. Analysis of other hypoxia-response genes established that these hypoxia-response elements (HREs) have the core consensus sequence 5'-A/GCGTG *(15)*.

2.3. Structure of Hypoxia-Induced Factor-1 (HIF-1)

HIF-1 was first identified as a ubiquitous hypoxia-induced HRE-binding factor in electrophoretic mobility shift assays *(16,17)*. Purification and cloning of HIF-1 showed that it consisted of two subunits, HIF-1α and HIF-1β or ARNT (aryl-hydrocarbon receptor nuclear translocator) *(18,19)*. Both peptides are members of the basic helix-loop-helix–PER-ARNT-SIM (bHLH-PAS) family of proteins. ARNT had previously been identified as a heterodimeric partner of the aryl-hydrocarbon receptor (Ahr), and is involved in the response to xenobiotics *(20)*. HIF-1α also heterodimerizes with the ARNT homologs ARNT2 and ARNT3 *(21,22)*. A HIF-1α homolog, HIF-2α (also known as EPAS1, HLF, HRF, and MOP2), is also induced by hypoxia, heterodimerizes with ARNT and ARNT homologs, and can activate the HRE *(21,23–26)*.

2.4. Regulation of HIF-1 Function by Hypoxia and VHL

HIF-1α (and its homolog HIF-2α) is the oxygen-sensitive component of HIF-1, and the protein is rapidly degraded under normoxic conditions (see Fig. 1A) *(19, 26,27)*. This occurs via the ubiquitin–proteasome pathway *(28–30)*, and is mediated by the tumor suppressor protein VHL *(31)*. Loss of VHL in tumors, such as renal cell carcinoma, leads to the constitutive activation of HIF-1 and up-regulation of hypoxia target genes *(31–34)*. VHL directly binds HIF-1α under normoxic conditions *(31)*, and together with other proteins (elongin C, Cul-2, and Rbx1), functions as an E3 ligase *(35–38)*, ubiquitinating HIF-1α, and targeting it for degradation *(39–42)*. Oxygen tension does not affect HIF-1α or ARNT mRNA levels *(27,43,44)*. The mechanism by which hypoxia inhibits VHL-mediated ubiquitination of HIF-1α is currently unknown, but is under intense scrutiny.

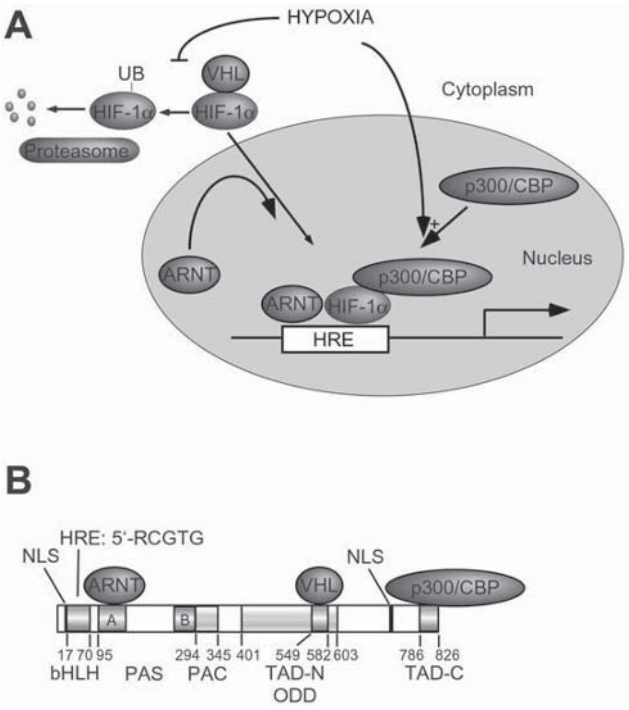

Fig. 1. (**A**) Hypoxia signalling and HIF-1 activation. HIF-1α is ubiquitinated by a VHL (von Hippel-Lindau gene product) containing complex, and targeted for degradation by the proteasome machinery. VHL-mediated ubiquitination is inhibited by hypoxia, leading to stabilisation of HIF-1α. HIF-1α enters the nucleus, heterodimerizes with ARNT, and binds the hypoxia-response element *(HRE)* in the promoter of hypoxia-target genes. Hypoxia enhances the interaction between HIF-1α and the transcriptional co-activator and histone-acetyl transferase p300/CBP. Recruitment of p300/CBP to the HIF-1 DNA complex leads to activation of gene transcription. (**B**) HIF-1α-domain structure and protein interactions. HIF-1α is a 826 residue peptide that contains a basic helix-loop-helix—PER-ARNT-SIM (bHLH-PAS) domain at its N-terminus *(19)*. This domain is required for heterodimerisation with ARNT, and for binding to the hypoxia-response element *(HRE) (56)*. The PAC domain occurs at the C-terminus of a subset of PAS domains, and may be important for PAS domain folding. Residues 401–603 contain a oxygen-dependent degradation *(ODD) (29,107)* domain which also binds VHL *(39)*, and has transactivation properties (TAD-N) *(57,108)*. The C-terminus of HIF-1α contains a separate hypoxia-inducible transactivation domain (TAD-C) *(57,108)*, that directly binds to the CH-1 domain of p300/CBP *(60,70)*.

2.5. Activation of HIF-1 Function by Cellular Growth Control Mechanisms

HIF-1α is also activated by a number of growth factors. These factors include insulin *(45,46)*, IGF-1 and 2 *(45,46)*, angiotensin II *(47)*, thrombin *(47)*, and PDGF *(47)*. In keeping with these observations, the activation of a number of downstream protooncogenes and protein kinases also induces HIF-1α protein expression. They include H-Ras *(48)*, v-src *(49)*, AKT *(50,51)*, and the p42/p44 MAP kinase, which has also been shown to directly phosphorylate HIF-1α *(52, 53)*. In prostate cancer cells, growth factors function through the PI3-kinase/ PTEN/AKT/FRAP pathway to activate HIF-1α *(51)*. In addition to activation by the loss of VHL, the loss of the tumor suppressors PTEN *(51,54)*, and p53 *(55)*, also result in HIF-1α activation. p53 promotes the ubiquitination and degradation of HIF-1α by MDM2 *(55)*. Thus, the regulation of HIF-1 appears to be intimately coordinated with cellular growth control mechanisms, implying that it may have a role in controlling nutrient, oxygen, and metabolite homeostasis in tumors, as well as in normal tissues.

2.6. Domain Structure of HIF-1α and Protein Interactions

HIF-1α is an 826 residue peptide that contains a basic helix-loop-helix–PER-ARNT-SIM (bHLH-PAS) domain at its N-terminus *(19)*. This domain is required for DNA binding and heterodimerization with ARNT (*see* Fig. 1B) *(56)*. HIF-1α has two transactivation domains *(56–58)*. The DNA-binding and transactivation domains are conserved in HIF-2α *(59)*. The N-terminal transactivation domain of HIF-1α, or TAD-N, overlaps with an oxygen-sensitive degradation domain or ODD *(29)*, which contains a binding site for VHL *(39)*. The C-terminus transactivation domain, TAD-C, functions to recruit the transcriptional co-activator p300/ CBP, via a direct physical interaction with the CH1-domain of p300/CBP *(60–63)*. The interaction is induced by hypoxia *(62)*, through a mechanism that is independent of HIF-1α stabilization. A leucine-rich interface in TAD-C, regulated by Cys800 in HIF-1α, is thought to play an important role in this interaction *(64)*. The structure of the HIF-1α C-terminus is not currently known. NMR evidence indicates that p300 CH-1 has two novel zinc-coordinating structures called "zinc bundles"—with the sequence C-X_4-C-X_8-H-X_3-C. The Zn-chelating residues are necessary for interaction with HIF-1α *(65)*. HIF-1α also apparently interacts with p53 and may play a role in the stabilization of p53 by hypoxia *(66)*. This result however appears to be controversial *(67)*.

2.7. Function of p300/CBP and Other Co-Activators

p300 and CBP (CREB-binding protein) are ubiquitous, evolutionarily conserved nuclear proteins. They function to connect several different signal-activated DNA-bound transcription factors (e.g., CREB) to RNA polymerase II,

basal transcription factors, and histone acetyl transferase (HAT) activities. These functions activate gene transcription, and result in the synthesis of mRNA *(68,69)*. The HIF-1α p300/CBP interaction is critical for the hypoxia response. Inhibition of p300/CBP function by the adenoviral oncoprotein E1A abolishes HIF-1 transactivation and cellular hypoxia responses *(60)*. Disruption of the HIF-1α–p300/CBP interaction by over expression of the high-affinity p300-CH1 interactor p35srj also blocks HIF-1 transactivation and hypoxia responses *(62)*. Similarly, the expression of dominant-negative HIF-1α molecules consisting of the TAD-C region also block HIF-1 transactivation and hypoxia signaling *(70)*. Other co-activators such as SRC-1 and TIF2 have also been implicated in HIF-1α co-activation, but it is not clear if they work through direct or indirect mechanisms *(71)*.

2.8. HIF-1 Is Essential for Hypoxic and Hypoglycemic Activation of Target Genes

Mice deficient in HIF-1α die *in utero* with severe neurological and cardiovascular developmental abnormalities, severe hypoxia, and extensive cell death *(72–74)*. Embryonic stem cells and embryoid bodies lacking HIF-1α have low levels of hypoxia and glucose-induced VEGF, glucose transporters, and glycolytic enzymes *(73)*. Similar effects have been noted in ARNT-deficient mice and cells *(75,76)*, which also show abnormal responses to glucose and oxygen deprivation. HIF-1 is therefore a key factor regulating oxygen and glucose homeostasis, and the response to ischemia. Unlike hypoxia, the stabilization of HIF-1α by glucose deprivation has not yet been demonstrated, and the mechanism by which it activates HIF-1 is unknown. HIF-2α is apparently not essential for oxygen homeostasis, but instead, is required for catecholamine homeostasis *(77)*.

3. ROLE OF HIF-1 IN TUMOR SURVIVAL AND ANGIOGENESIS

Blood vessels supplying solid tumors are frequently abnormal *(78)*, resulting in inefficient metabolite removal, and inadequate nutrient and oxygen delivery *(79)*. The majority of tumors are therefore hypoxic *(79)* and acidotic *(80)*. HIF-1 is activated in a broad spectrum of tumors, and appears to be important for tumor survival and growth.

3.1. HIF-1 is Activated in a Broad Spectrum of Tumors

HIF-1 activation has been detected in the perinecrotic areas of experimental tumors using a HRE reporter *(81)*, and by HIF-1 target gene expression *(76)*. Up-regulated HIF-1α has been detected by immunohistochemistry in naturally occurring colon, breast, gastric, lung, skin, ovarian, pancreatic, prostate, and renal carcinoma, bladder, kidney, in metastatic breast cancer, and in the perinecrotic

areas of glioblastomas *(82–84)*. HIF-2α is also strongly activated in tumor-associated macrophages *(84)*. The mechanism of HIF-1 activation in tumors likely involves a combination of tumor hypoxia, oncogene activation, and loss of tumor suppressors. Non-hypoxic activation of HIF-1, and the consequent induction of glucose transporters and glycolytic enzymes may play a major role in the development of aerobic glycolysis in tumors, known as the Warburg effect *(85)*. Other genetic alterations that likely contribute to this effect include the activation of the protooncogene c-myc *(86)*.

3.2. Genetic Evidence Indicates that HIF-1 Is Necessary for Tumor Growth

Three approaches have been used to genetically inactivate HIF-1 function. These approaches include mutagenesis of the murine hepatoma cell line Hepa1 to achieve a functional loss of ARNT *(76,87)*; the targeted disruption of ARNT *(88)* and HIF-1α *(72–74,89)* genes by homologous recombination; and overexpression of dominant-negative HIF-1α *(70)* or p35srj *(62)* molecules, which bind the CH1 domain of p300/CBP and block HIF-1 transactivation. Cell proliferation and tumor formation studies have, in the main, indicated that loss of HIF-1 function results in slower xenograft tumor growth with reduced vascularity *(49, 70,74,76)*. The one exception to this has been the study from Carmeliet et al. *(89)*. Here the authors targeted the HIF-1α gene by homologous recombination, and compared the growth of HIF-1α –/– embryonic stem (ES) cells with that of a ES cell clone carrying a "random" integration of the targeting vector. It was found that loss of HIF-1α was associated with *reduced* apoptosis, and *enhanced* tumor growth. The possibility that "random" integration of the targeting vector in the control cells may have inadvertently disrupted a cell survival gene, or have activated a proapoptotic gene, was not addressed in this study. In another study, Maltepe et al. found that ARNT –/– ES cells grew as well as wild-type cells in a xenograft model *(88)*. These tumors expressed ARNT2, which can associate with HIF-1α to form a functional HIF-1 molecule. As a consequence, these tumors were not defective in VEGF expression, and grew normally *(88)*. Somewhat surprisingly, the growth of HIF-1-deficient cells in monolayer cultures is not significantly impaired, even under hypoxic or hypoglycemic conditions *(72,73,76)*. This observation could be explained by the fact that in monolayer cultures, tumor cells undergo hypoxia-induced apoptosis only under acidotic conditions *(90)*. With regard to HIF-1-deficient cells, there are no data on monolayer cell growth under conditions that mimic ischemia, i.e., combined hypoxia–hypoglycemia with acidosis

In summary, HIF-1 is activated in most tumors, either by hypoxia or by a tumor mutation. The genetic evidence, with one notable exception, indicates that HIF-1 is required for tumor growth and vascularization.

4. TRACTABILITY OF HIF-1 FOR HIGH-THROUGHPUT SCREENING

Current understanding of HIF-1 function allows us to configure both biochemical and cell-based high-throughput screening assays designed to identify inhibitory small molecules. The biochemical assays could use protein–protein, or protein–DNA interactions critical for HIF-1 function as a screening target. The cell-based screen could use the hypoxia-response element itself as the target.

4.1 Protein Interaction Based Screens

Key protein interactions required for normal HIF-1 function include the HIF-1α–ARNT–DNA interaction, and the HIF-1α–p300 interaction. These interactions are well defined, and can be demonstrated easily in vitro, and in vivo. Disruption of the HIF-1α–ARNT interaction by dominant-negative HIF-1α molecules, which bind ARNT but lack the transactivation domain, inhibits hypoxia responses *(56)*. Disruption of the HIF-1α–p300CH1 interaction either by the high affinity p300-CH1 binder, p35srj *(62)*, or by dominant negative HIF-1α peptides that bind p300-CH1 inhibits HIF-1 transactivation, and hypoxia responses *(70)*. Dominant negative HIF-1α peptides also inhibit xenograft tumor growth *(70)*. These results provide the proof-of-principle that the HIF-1α–p300CH1 interaction is a valid target for the development of anti-cancer therapy.

High-throughput assays to identify small molecules that disrupt the HIF-1α–p300CH1 interaction could use recombinant, epitope-tagged p300 and HIF-1α TAD-C molecules. The interaction between these two molecules could then be assayed using techniques such as DELFIA (Perkin-Elmer), where one protein partner is attached to the solid phase, and formation of a complex is detected by a europium-labeled antibody directed against the second protein partner, which is added in solution. Other techniques include Homogeneous Time-Resolved Fluorescence (HTRF), and Scintillation Proximity Assay (SPA, Amersham Pharmacia Biotech). A 32-mer peptide from p35srj efficiently disrupts the HIF-1α–p300 CH1 interaction in vitro, and can be useful as a positive control when developing such assays *(62)*.

Ideally one would select compounds that work by binding HIF-1α rather than p300CH1, as binding to CH1 might produce unexpected effects. Because the p300-CH1 binding domains of HIF-1α and HIF-2α (i.e., the last 40 amino acids) are highly conserved, these screens would be expected to identify some molecules capable of interfering with HIF-2α transactivation as well. Similar approaches could also be taken for the HIF-1α–ARNT interaction, and the interaction of HIF-1α + ARNT with a hypoxia response element. The main advantage of a biochemical screening approach is that the mechanism of action of the compound is well understood. This knowledge aids in developing novel molecules with improved structure–activity profiles. Detailed information regarding the struc-

ture of the relevant interaction surfaces is likely to aid in rational drug design and modification of initial "hits" from a high-throughput screen. The main disadvantage is that, with a few exceptions, e.g., GPIIb/IIIa antagonists, it has been difficult to develop molecules that efficiently disrupt protein–protein interactions in cells.

4.2. Cell-Based Screens

In principle, a HRE upstream of a reporter gene such as luciferase could be stably transfected into a cell line, such as Hep3B, that is known to have a robust response to hypoxia. These cells could then be directly screened in high-throughput format for inhibition of hypoxia-induced luciferase activity by individual compounds. A major problem here is the nonspecific inhibition of luciferase transcription by cytotoxic compounds, or compounds that nonspecifically inhibit transcription. This could be overcome by using a modification of the reporter system *(91)*, where the HRE drives a tetR–KRAB repressor fusion, and under hypoxic conditions, the tetR–KRAB represses transcription from a tetO–luciferase reporter. Inhibition of hypoxia signaling or HIF-1 function by a compound would activate rather than repress the luciferase reporter, thus excluding nonspecific compounds. The advantage of a cell-based system is that the entire pathway, i.e., hypoxia sensing–HIF-1 activation–co-activator recruitment is being screened. Compounds detected in such assays are less likely to have problems with cellular uptake and nonspecific cytotoxicity. The disadvantage is that the biochemical target is not well defined, making structure–activity analysis and rational drug design more difficult.

5. MONITORING THE EFFICACY OF COMPOUNDS THAT INHIBIT HIF-1

What changes would be expected if we blocked hypoxia-induced glycolysis and angiogenesis in tumor cells? How can we assess these changes first in cell-culture, in tumor xenografts and animal models, and ultimately in the clinical situation?

5.1 Cell Culture

A logical first place to start would be the transcriptional response to hypoxia, which includes the induction of glycolytic enzymes and angiogenetic factors. Induction of these genes is disrupted in cells with genetic defects in hypoxia signaling, and should also be blocked by successful pharmacological inhibition of the HIF-1 pathway. DNA microarrays that include a spectrum of known hypoxia and hypoglycemia target genes and other genes as controls are likely to prove useful here. Assays of VEGF secretion and assays of glycolytic enzymes could be used to back up the transcriptional assays. The next step would be to determine

if the candidate lead compounds affect glucose uptake, glycolytic flux, and ATP/high-energy phosphate generation under conditions resembling tissue ischaemia —i.e., combined deprivation of oxygen and glucose. Under these conditions a potentially effective compound would also result in inhibition of cell growth, and increased apoptosis as a result of ATP depletion.

5.2. Animal Models and Clinical Trials

Small molecules that inhibit tumor hypoxia and hypoglycemic responses would be expected to inhibit tumor angiogenesis, resulting in reduced blood flow, increased hypoxia, and reduced microvessel density. These molecules are also likely to inhibit glucose uptake, glycolytic flux, and, importantly, ATP and high-energy phosphate generation. These changes should ultimately lead to apoptosis, and a reduction in tumor size. As compounds in early developmental stages are unlikely to be highly potent, it is important to have sensitive methods to determine if the compound in question will produce the desired changes in tumor metabolism in vivo. Ideally these methods should also be non-invasive, and allow for serial measurements, both in animal models and in the clinical setting.

Magnetic resonance spectroscopy (MRS) and proton magnetic resonance imaging (MRI) of tumors are likely to prove highly valuable, as they are noninvasive and can be performed repeatedly. These methods have been reviewed in depth elsewhere, and are summarized in Table 2. 31P MRS is likely to be the most informative single indicator of tumor status, as it can provide information about the levels of cellular high-energy phosphates (phosphocreatine and ATP), intracellular pH, and cellular biomembrane turnover *(92–94)*. Another method, called gradient recalled echo MRI (GRE-MRI) can be used to monitor tumor oxygenation and blood flow *(94,95)*. As these parameters are likely to be affected by changes in tumor metabolism and angiogenesis, they would be ideal for the investigation of small molecules that block HIF-1 function.

6. POTENTIAL ADVERSE EFFECTS OF TARGETING HIF-1

What could go wrong as a result of pharmacologically targeting HIF-1? The potential adverse effects can only be speculated upon, but animal studies and clinical trials would need to incorporate mechanisms to detect them. HIF-1 is required for embryonic development, and anatagonists will very likely have teratogenic effects. Clearly a drug that targets HIF-1 in tumors will also target it in other ischemic tissues, with potentially deleterious effect. For instance, in patients with ischemic heart disease, HIF-1 very likely plays an important role in the survival of poorly perfused myocardium *(96)*, and inhibition may have serious consequences. With regard to antineoplastic therapy, HIF-1 inhibition is likely to have complex effects. Hypoxia is known to enhance tumor radio-resistance *(79)*. Blocking HIF-1 function and consequent exacerbation of tumor hypoxia may

Table 2
Predicted Effects of Interfering with the Hypoxia Pathway in Tumors, and Possible Methods of Measurement

Cellular response to hypoxia and hypoglycemia	
Reduced target gene activation	DNA microarrays; VEGF-ELISA in supernatants *(32)*; LDH–enzyme assay *(101)*
Reduced glycolysis, glucose uptake	Glycolytic flux measurements (e.g., 3H-glucose) Lactate generation–(culture supernatant) *(101,102)*
ATP depletion	Luciferase assay for ATP in cell extracts.
Inhibition of cell growth	Growth curves; 3H-thymidine uptake; Growth in soft agar *(101)*
Increased apoptosis	TUNEL, DNA fragmentation, other
Inhibition of tumor angiogenesis	
Reduced blood flow	GRE MRI imaging of deoxyhaemoglobin *(94,95)*; GdDTPA uptake and washout *(93)*; 2H MRI *(92)*
Increased hypoxia	GRE MRI imaging of deoxyhaemoglobin. *(94,95)*; SR-4554 fluorinated 2-nitroimidazole retention *(103)*
Reduced microvessel density	Intravital microscopy in subcutaneous models *(104,105)*
Reduced VEGF expression	*In situ* hybridization *(76)*
Inhibition of tumor glucose uptake, glycolysis, and ATP generation	
Reduced glycolytic flux	31P-MRS *(92)*
ATP depletion	31P-MRS (NTP/Pi ratio) *(92–94)*
Inhibition of tumor growth	
Apoptosis	PUFA accumulation 1H MRS *(106)*
Size	MRI *(94)*
Pathological assays	
Tumor dimensions	Size and weight *(102)*
Tumor vascularization	Histological scoring
Tumor necrosis and apoptosis	Histopathological scoring

Abbreviations: GdDTPA, gadolinium DTPA; MRI, magnetic resonance imaging; MRS, magnetic resonance spectroscopy; PUFA, polyunsaturated fatty acids; GRE, gradient recalled echo.

aggravate this effect. Reduction of tumor blood flow may result in reduced antineoplastic drug delivery to the tumor *(79)*. However, HIF-1 anatagonists may be useful in combination with drugs such as tirapazamine, which are preferentially toxic to hypoxic cells *(79)*. Hypoxia may also result in clonal selection of tumor cells with reduced propensity to apoptosis and loss of p53 *(97)*, resulting in a more malignant phenotype.

7. CONCLUSIONS

The evidence to date suggests that HIF-1 is important for the proliferation, and survival of tumor cells in experimental models, and that it is up-regulated in diverse naturally occurring tumors by hypoxia or tumor mutation. A considerable amount is known about the biology of the HIF-1 system, allowing the design of biochemical or cell-based lead identification by high-throughput screens. Non-invasive methods are available for determining the efficacy of candidate lead molecules in animal models, and in the clinical situation. HIF-1 therefore appears to fulfill the criteria required for an anti-cancer target. HIF-1 antagonists are likely to have complex, but potentially useful effects in cancer therapy.

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5 Nuclear β-Catenin Signaling as a Target for Anticancer Drug Development

Eun Joo Chung, Paolo Bonvini, Ho Jung Oh, Len Neckers, and Jane Trepel

Contents

1. INTRODUCTION

β-Catenin is a multifunctional protein that acts as a component of the adherens junction regulating homotypic cell–cell adhesion and as a co-activator of the transcription of lymphoid enhancer binding factor/T cell-specific factor (LEF/TCF) target genes *(1–3)*. β-Catenin was first identified as a 92–94 kDa protein associated with the cytoplasmic tail of the E-cadherin adhesion protein *(4,5)*. At that time, two other members of the catenin family, α and γ catenin, were also isolated in association with E-cadherin *(6)*. The name catenin is derived from the Latin *catena* or chain, because the catenins were thought to link E-cadherin and the cytoskeleton *(7)*. This hypothesis was substantiated when it was shown that the ability of E-cadherin to function as a mediator of homotypic cell–cell interactions is dependent on catenins *(8–11)*.

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An additional function of β-catenin was suggested when the mammalian gene was cloned and found to be a homolog of the *Drosophila* gene *armadillo*, a segment polarity gene involved in the wingless (Wg) signaling pathway*(12)*. The Wg signaling pathway of *Drosophila* is homologous to the mammalian Wnt pathway *(1,13,14)*. In the Wg or the Wnt pathway, signaling starts with interaction of Wg or Wnt, which are secreted ligands, with their cell surface receptor frizzled. Frizzled activates disheveled, leading to down-regulation of the activity of a serine/threonine kinase known as Zeste white-3 in *Drosophila* or GSK-3β in mammalian cells. This kinase phosphorylates armadillo or β-catenin, leading to β-catenin ubiquitination and degradation by the proteasome *(1,15–18)*. The signaling function of β-catenin is mediated through its interaction with members of the LEF/TCF family of transcription factors in the Wg and Wnt pathway. These HMG (high mobility group) proteins bind DNA in a sequence-specific manner, but utilize the transactivation domain of β-catenin to induce gene expression *(1–3,19)*. β-Catenin has also been identified in *Xenopus*, where it was demonstrated that injection of β-catenin into the ventral embryonic region induces secondary axis formation *(20)*. Further experiments confirmed a role for β-catenin in early *Xenopus* development *(14,21)*.

β-Catenin clearly plays a critical role as a transcriptional regulator in early development and as a component of the epithelial adherens junction. Recently, however, aberrant regulation of β-catenin has been detected in familial and sporadic cancer, and considerable attention has been focused on the role of β-catenin in malignant transformation *(13,19,22–27)*. In this chapter, we will review the function of β-catenin with an emphasis on its role as a transcriptional co-activator, summarize how β-catenin is regulated, and discuss deregulation of β-catenin in cancer. The data suggest that the β-catenin pathway is an important new target for anticancer drug development. We end the chapter with a brief overview of potential avenues for therapeutic intervention in β-catenin signaling pathways.

2. STRUCTURE OF β-CATENIN

β-Catenin is composed of three regions. The N-terminal region, which consists of approximately 130 amino acids, contains the consensus phosphorylation sites for GSK-3β that are critical in targeting β-catenin to the proteasome. Mutations in the β-catenin N-terminal region make it resistant to targeted degradation by the proteasome, leading to stabilization of cytoplasmic β-catenin, translocation to the nucleus, and enhanced nuclear signaling. Consistent with a role for β-catenin in malignant transformation, mutations in this region have been identified in a wide variety of human cancers. The N-terminal region also contains a transactivation domain. The central region of β-catenin consists of 12 imperfect repeats of 42 amino acids known as armadillo repeats. This region is required for interaction of β-catenin with various proteins including LEF/TCF family members,

Axin, APC, and E-cadherin. The C-terminal region of β-catenin consists of approximately 100 amino acids. This region contains a second, independent transactivation domain *(1,19)*. The histone acetylase transcriptional co-activators p300 and CBP have been reported to bind to both the N-terminal and the C-terminal transactivation domains of β-catenin *(28–30)*.

3. REGULATION OF β-CATENIN STABILITY AND SUBCELLULAR LOCALIZATION

Posttraslational serine/threonine and tyrosine phosphorylation of β-catenin and its associating proteins regulate β-catenin's stability and subcellular localization. Whereas serine/threonine phosphorylation is widely recognized to target β-catenin for ubiquitination and proteasomal degradation, tyrosine phosphorylation of β-catenin negatively regulates E-cadherin/β-catenin association, resulting in profound effects on the subcellular distribution of β-catenin. Additionally, phosphorylation of multiple β-catenin-associating proteins both positively and negatively regulate their activities.

3.1. Components of the Destruction Complex: GSK-3β, Axin, APC, and SCF$^{\beta TrCP}$

Degradation of β-catenin requires the scaffold protein Axin, the APC tumor suppressor protein, and the serine/threonine kinase GSK-3β, the so-called "negative regulatory complex" *(31–35)*. Phosphorylation at N-terminal sites is a prerequisite for β-catenin ubiquitination by the E3 ubiquitin ligase complex SCF$^{\beta TrCP}$ *(36–39)*. In vivo, aberrant expression of β-catenin by inactivation of APC, as well as by mutations of β-catenin, has been found in an increasing number of human cancers, and is correlated with the failure of tumor cells to degrade β-catenin *(40–42)*. Sequence analysis of the APC gene has identified frequent C-terminal truncations that lead to abrogation of the APC/Axin complex, without perturbing APC affinity for β-catenin *(43–45)*. Likewise, sequence analysis of the β-catenin gene has revealed frequent mutation of the four consensus motifs for GSK-3β phosphorylation *(44,46,47)*.

Hart et al. have identified β-TrCP as a novel β-catenin interacting protein by yeast two-hybrid screening analysis *(39)*. β-TrCP functions as a receptor for targeting phosphorylated proteins to the proteasome, and simultaneously recruits both β-catenin and the Skp1/Cul1/E2 ubiquitination apparatus *(48–50)*. In vitro, binding to β-TrCP is restricted to wild-type β-catenin, and binding is greatly enhanced by prior GSK-3β-dependent phosphorylation *(39)*. Full-length β-TrCP inhibits the activation of β-catenin-driven reporter plasmids when transfected in 293 cells, whereas expression of a dominant negative mutant β-TrCP that lacks the F-box domain (ΔF-β-TrCP) down-regulates β-catenin ubiquitination and potentiates its transactivation capacity by inducing β-catenin nuclear translocation *(39,51)*.

Although β-catenin recognition by the ubiquitination machinery totally depends on GSK-3β, which in normal cells is always found in β-catenin immunocomplexes, it is important to note that β-catenin does not contain GSK-3β binding sites, as evidenced by the failure of immobilized β-catenin to bind directly to purified GSK-3β, and by the low level of phosphorylation of β-catenin in vitro in the presence of GSK-3β *(32,52,53)*. In contrast, β-catenin associates directly with APC and Axin, suggesting that the β-catenin/GSK-3β interaction is a consequence of a reciprocal association with Axin *(31,33,52,54)*. APC, in fact, contains three 15 amino acid repeats and seven 20 amino acid repeats that allow the formation of the APC/β-catenin/Axin ternary complex *(33,55)*. However, like β-catenin, APC does not bind directly to GSK-3β, even though phosphorylation of Axin-bound APC by GSK-3β has been shown to greatly enhance its binding affinity for β-catenin *(56,57)*.

A key step in regulating β-catenin proteolysis is the control of Axin stability *(58)*. Like APC and β-catenin, Axin is phosphorylated by GSK-3β *(52,53)*. GSK-3β phosphorylation has been found to stabilize Axin, and it can up-regulate phosphorylation of β-catenin in vitro, even in the absence of APC *(59,60)*. Ectopic expression of Axin in tumor cells containing inactive APC is sufficient to functionally reconstitute the destruction complex, indicating that there is no absolute dependence on APC for the degradation of β-catenin, while Axin may represent a limiting factor *(56,58,61)*.

Recently, the catalytic subunits of the serine/threonine protein phosphatases 2A (PP2A) and 2Cα (PP2Cα) were show, to interact with Axin *(62,63)*. Both PP2A and PP2Cα act as negative regulators of axin. Coexpression of PP2Cα and LEF-1 in COS-1 cells was found to synergistically up-regulate the luciferase activity of a LEF-1-luciferase reporter plasmid, due to axin destabilization caused by its interaction with PP2Cα and its subsequent dephosphorylation *(63)*. Willert and colleagues have demonstrated that, similar to Wnt stimulation or GSK-3β inhibition by lithium chloride, PP2A stimulates Axin dephosphorylation, thus reversing the high affinity of phosphorylated Axin for β-catenin, and resulting in β-catenin/axin dissociation *(64,65)*. Recently, several downstream effector proteins in the Wnt signaling pathway have been found to bind directly to Axin and GSK-3β, causing the accumulation of cytoplasmic β-catenin secondary to either Axin down-regulation or inhibition of GSK-3β kinase activity *(66–71)*.

3.2. The Role of Protein Tyrosine Kinases and Phosphatases in the Subcellular Distribution of β-Catenin

Posttranslational regulation of β-catenin level also depends on tyrosine phosphorylation. Unlike serine phosphorylation, tyrosine phosphorylation does not influence the association of β-catenin with the APC/Axin/GSK-3β ternary complex, but instead modulates β-catenin interaction with E-cadherin and with nuclear

co-factors. Deregulation of membrane-bound and receptor tyrosine kinases (RTKs) has frequently been observed in human tumors and tumor cell lines, often associated with loss of epithelial differentiation and gain of invasiveness *(72–78)*. Several protein tyrosine kinases, including v-src, $pp60^{c\text{-}src}$, Ras, Fyn/Fer, Met, EGFR, and erbB2, have been found to phosphorylate both catenins and cadherins *(73,79–85)*. Expression of EGFR correlates with a more invasive phenotype, lymph node metastasis, and poor prognosis in a variety of human malignancies *(86–95)*. In human quiescent epithelial cells at confluence, β-catenin mainly associates with both E-cadherin and α-catenin, but not with EGFR. In contrast, stimulation of cell growth by tryptic digestion of quiescent cells facilitates β-catenin binding to EGFR and induces its tyrosine phosphorylation, and this is further potentiated by EGF treatment *(96–99)*. Similarly, the transmembrane tyrosine kinase c-Met appears to regulate cell growth and motility, as a consequence of down-regulating E-cadherin-catenin-dependent cell–cell adhesion *(100–102)*.

Mutational analysis has identified an EGFR-binding site in the last three armadillo repeats of β-catenin, while the N- and C-terminal regions have been shown to contain EGFR/erbB2 phosphoacceptor sites *(103)*. Recently, Roura and colleagues found that β-catenin possesses two tyrosine residues,Tyr-86 and Tyr-654, capable of being phosphorylated by $pp60^{c\text{-}src}$. Although Tyr-86 showed a higher stoichiometry of phosphorylation, only $pp60^{c\text{-}src}$ phosphorylation of Tyr-654 significantly decreased β-catenin affinity for E-cadherin *(104)*. Treatment of src-transformed cells with tyrosine kinase inhibitors, or expression of dominant inhibitory c-src proteins, has been shown to reduce β-catenin phosphorylation and to restore E-cadherin/β-catenin association *(105,106)*.

In a more recent publication, Roura et al. have demonstrated that introduction of a negative charge at position 654 has a dual effect on β-catenin. First, by dissociating the carboxy tail of β-catenin from its armadillo domain, phosphorylation of Tyr-654 alters β-catenin's conformation, thereby facilitating the binding of the nuclear factor TATA-binding protein (TBP). At the same time, introducing a negative charge in this position hampers the binding of E-cadherin, which is regulated by charge complementarity *(107)*. Our laboratory recently demonstrated that, in melanoma cells, β-catenin is phosphorylated by erbB2, and erbB2 down-regulation results in both the dephosphorylation of β-catenin and the inhibition of its transcriptional activity. At the same time, the amount of E-cadherin-associated β-catenin dramatically increases. Replacement of Tyr-654 with a neutral residue abrogated the ability of erbB2 to disrupt β-catenin/E-cadherin association, while replacement of Tyr-654 with a charged amino acid negated the effects of erbB2 inhibition and resulted in β-catenin that was constitutively dissociated from E-cadherin *(108)*. These data strongly suggest that Tyr-654 is an erbB2-dependent phosphoacceptor site, and they further demonstrate the importance of β-catenin tyrosine phosphorylation in regulating β-catenin's activity and localization, even in the absence of other mutations or deletions in β-catenin's regulatory components.

β-Catenin and E-cadherin have been shown to directly interact with protein tyrosine phosphatases. Several independent investigations have observed that ectopic expression of protein phosphatases results in a significant reduction of growth factor-dependent tyrosine phosphorylation and cytoplasmic accumulation of β-catenin, while also inhibiting cell motility in vitro and tumor growth in vivo *(99,109–113)*. In contrast, treatment with the phosphatase inhibitor pervanadate increased tyrosine phosphorylation of both E-cadherin and β-catenin *(99)*.

3.3. The Serine/Threonine Kinases PKB/Akt and ILK Indirectly Regulate β-Catenin's Stability and Subcellular Localization

Another signaling pathway that can regulate β-catenin stability involves protein kinase B (PKB/Akt). When activated, this serine-threonine kinase phosphorylates GSK-3β on Ser-9, thereby inhibiting its activity *(114,115)*. Akt is downstream of several growth factor receptors, including the insulin receptor *(116–119)*. Previous reports have shown that insulin/IGF-1 stimulation of intact cells leads to stabilization of β-catenin *(120)*. As the EGF receptor and erbB2 also promote activation of Akt, it would not be surprising to find that these kinases, while disrupting β-catenin/E-cadherin association, on the one hand, resulting in increased levels of monomeric β-catenin, simultaneously favor stabilization of monomeric β-catenin by stimulating the Akt-dependent inhibition of GSK-3β.

The serine/threonine kinase, integrin-linked kinase (ILK) also can phosphorylate β-catenin, disrupting its association with E-cadherin and increasing its nuclear translocation, consistent with the anchorage-independence and invasive phenotype of ILK-overexpressing epithelial cells *(121)*. Interestingly, ILK also stimulates the repression of transcription of the E-cadherin gene, thus potentiating the stability and transcriptional activity of β-catenin via independent mechanisms *(122,123)*. As expected, inhibition of ILK results in suppression of β-catenin-LEF/TCF-dependent transcription, as monitored by cyclin D1 down-regulation, as well as in stimulation of transcription of E-cadherin *(124)*. ILK activation has recently been shown to occur following insulin treatment. Insulin treatment of quiescent IEC-18 intestinal epithelial cells resulted in rapid up-regulation of ILK and activation of Akt, with concomitant inhibition of GSK-3β kinase activity *(125)*. Thus, insulin, and perhaps other growth factors, utilize overlapping protein kinase cascades to simultaneously activate multiple signaling pathways that combine to i) dissociate β-catenin from E-cadherin, ii) protect monomeric β-catenin from degradation, and iii) potentiate the transcriptional activity of β-catenin.

3.4. APC and E-Cadherin Regulate the Intracellular Trafficking of β-Catenin

A third level of regulation of β-catenin is represented by the opposing actions of E-cadherin and APC. E-cadherin and APC form similar but non-overlapping

complexes with β-catenin both in vitro and in vivo *(126–128)*. Displacement of β-catenin from APC immunocomplexes by increasing the expression of full-length E-cadherin has revealed that, at least in vivo, these complexes are mutually exclusive *(127,129)*. Orsulic and colleagues have demonstrated that the association of LEF-1 with the uncomplexed pool of β-catenin in vitro is negatively regulated by increasing amounts of either APC or E-cadherin *(130)*, supporting the hypothesis that both E-cadherin and APC are tumor suppressor proteins. These data are also consistent with the fact that overexpression of ectopic full-length APC reverts the transformed phenotype of APC–/– neoplastic cells *(40,131)* and with the observation that metastatic carcinomas often down-regulate either the expression or function of E-cadherin. Re-establishing the function of E-cadherin reverts tumor cell lines to a benign epithelial phenotype and arrests tumor development *(24,132–135)*.

For many years the nuclear import/export of β-catenin has been elusive and intriguing, since sequence analysis of the β-catenin gene failed to detect either nuclear localization signal (NLSs) or nuclear export signal (NESs) sequences. Furthermore, the finding that β-catenin could localize within the nucleus independent of its interaction with LEF-1 or importin α/β suggested the existence of alternative partners mediating its nuclear transport. Neufeld and colleagues have identified two NLSs that are required for the nuclear import of APC *(136)*. Introduction of a negative charge on Ser-2054 of APC, adjacent to the second NLS domain, or its substitution by an aspartic acid residue (Asp-2054), potentiated the nuclear translocation of full-length APC *(137)*. These data are consistent with earlier findings that the phosphatase activity of APC-bound PP2A enhanced APC-mediated β-catenin degradation, presumably occurring in the cytoplasm *(138)*. In addition, these data are consistent with what had been previously observed by Rosin-Arbersfeld and Henderson, namely, that APC contains several NES sequences that are responsible for shuttling APC and APC-bound β-catenin to the cytoplasm in a CRM1-dependent manner *(139,140)*. Alanine substitutions of conserved residues within the NES of APC strongly compromised APC's nuclear export, thereby accumulating β-catenin within the nucleus *(137)*. Thus, APC would appear to be one of the long-sought-after partner proteins that shuttles β-catenin in and out of the nucleus. Intringuingly, the APC mutation cluster region found in multiple cancers overlaps the NES, explaining why such APC mutants can affect the export function of APC, but not its association with β-catenin *(139)*.

The role of E-cadherin in regulating β-catenin expression is also becoming more complex *(22,141)*. The number of different extracellular stimuli that modulate cadherin localization and its ability to form protein–protein interactions is constantly expanding *(142,143)*. Also, there is a growing body of evidence suggesting that cadherins are not only involved in the maintenance of functional epithelial tissues, but also participate in the transduction of extracellular signals *(144,145)*. Structurally, the cytoplasmic tail of the cadherin molecule binds to different

members of the catenin family, including β-catenin, γ-catenin, or plaklogoblin, and $p120^{ctn}$, strongly anchoring them to the actin cytoskeleton *(4,146–150)*. E-cadherin induces β-catenin translocation to the plasma membrane and prolongs β-catenin half-life by impeding β-catenin interaction with the components of the cytosolic negative regulatory complex *(37)*. Engagement of β-catenin also benefits E-cadherin stability. In the absence of β-catenin, the cytoplasmic domain of E-cadherin is unstructured and subject to proteasome-dependent proteolysis due to the presence of a PEST sequence that overlaps the β-catenin binding site *(151)*.

Transfection of full-length N-cadherin or E-cadherin in CHO cells has been shown to stabilize β-catenin and to inhibit β-catenin-driven transcription *(152)*. Under these circumstances, endogenous β-catenin is localized to the plasma membrane. Consistent with these data, even in nontransformed cells the subcellular localization of β-catenin is predominantly nuclear when E-cadherin expression is down-regulated. Likewise, E-cadherin–/– embryonic stem (ES) cells were shown to contain most of their β-catenin within the nucleus, in contrast to their wild-type counterparts in which β-catenin was found primarily at the plasma membrane *(130)*. Transfection with degradation-resistant (S33A) β-catenin dramatically increased transcription in E-cadherin–/– ES cells, while transfection of full-length E-cadherin strongly repressed such β-catenin-driven transactivation. Therefore, the proper localization of significant amounts of cadherins at the plasma membrane, or stabilization of membrane-bound cadherin should be able to inhibit β-catenin-mediated oncogenesis *(153)*. In support of this hypothesis, it has been shown that exogenous calveolin-1, which acts as a scaffolding protein within membrane calveolae, co-localizes with and recruits E-cadherin and β-catenin to the plasma membrane of MDCK cells *(154)*. As expected, calveolin-1 overexpression inhibits LEF-directed transcription, mediated by both Wnt stimulation and wild-type or mutant (S33Y) β-catenin ectopic expression.

4. TRANSCRIPTIONAL ACTIVITY OF β-CATENIN

4.1. Nuclear Localization of β-Catenin

The ability of β-catenin to function as a transcriptional activator is dependent on its nuclear localization and association in the nucleus with LEF/TCF family members. The mechanism of translocation of β-catenin into the nucleus is not clear. However, recent studies have demonstrated that APC acts as a CRM-1-dependent chaperone of β-catenin, and that APC induces export of nuclear β-catenin (see above). Thus, APC interdicts β-catenin signaling both by promoting degradation of β-catenin in the cytoplasm and by facilitating export of β-catenin from the nucleus *(13,139,155)*.

4.2. Regulation of Gene Expression by LEF/TCF

LEF and TCF bind to specific consensus sequences in the promoters of target genes via their HMG domains. When tested against a promoter-reporter consist-

ing of concatenated LEF/TCF-responsive sites, LEF and TCF were unable to independently activate transcription. In this system, TCF-dependent activation is dependent on co-expression of β-catenin *(14,156,157)*. Mapping experiments using armadillo demonstrated that the armadillo C-terminal domain is sufficient for co-activation of TCF-responsive transcription *(158)*.

A second mode of regulation of gene expression has been described for LEF/TCF. In some systems the data suggest that TCF can repress transcription of target genes in the absence of an activating stimulus. Upon activation, cytoplasmic β-catenin is stabilized, translocates to the nucleus, binds TCF, and relieves TCF-mediated repression *(1,14,15)*.

4.3. β-Catenin-LEF/TCF Target Genes

The best-studied function of Wnt signaling is the regulation of cell fate, which is achieved by altering the transcriptional program of target cells. Consistent with that function, many of the genes regulated by the Wnt pathway are transcription factors or secreted signaling molecules (see Table 1). Analysis of two target genes, *Ultrabithorax* and *siamois*, demonstrated that Wnt signaling activates transcription via an armadillo/HMG protein complex bound to specific sites in the promoters of target genes. However, in both studies, Wnt signaling alone did not determine normal patterns of gene expression, and the promoter appeared to integrate other signaling inputs for determination of the transcriptional response *(158–162)*. Wodarz and Nusse have suggested that such a combinatorial mechanism may explain how Wnt signaling can differentially regulate gene expression depending on the cellular context. For example, Wg activates expression of achaete in the wing imaginal disc, and represses achaete expression in the eye imaginal disc. The authors suggest that cooperation with other signaling pathways in a cell type-specific manner may explain the observation that ubiquitous Wg expression is not accompanied by ubiquitous activation of Wg target genes *(163)*.

LEF/TCF family members were originally studied as regulators of the expression of the *Xenopus* genes *siamois* and *twin*, which are required for activation of the Spemann organizer and development of the embryonic body axis *(159–161,164)*. In mammalian development, the *Cdx* homeobox gene is a Wnt/β-catenin target. *Cdx* is expressed first in association with establishment of the embryonic axis, and later in embryogenesis when expression is limited to the intestinal endoderm. In adulthood, *Cdx* is expressed in the proliferating cells of the intestinal crypt *(165)*.

The first β-catenin target gene clearly involved in human cancer was *c-myc (166)*. Vogelstein and colleagues have shown that mutation of APC is an early event in transformation of normal colonic epithelial cells. The subsequent accumulation of cytoplasmic β-catenin leads to increased nuclear β-catenin and enhanced expression of β-catenin-LEF/TCF target genes in colon cancer cells. As shown by Vogelstein et al., the *c-myc* gene, which is frequently overexpressed in colon cancer, is under the control of APC and β-catenin-LEF/TCF. McCormick et al.

Table 1
Target Genes for β-Catenin-LEF/TCF Complex

Target gene	*Species*	*Cell type*	*Response*	*Function*	*Ref.*
c-myc	human	colorectal cancer cell line	activation	oncogene	*(166)*
cyclin D1	human	colon cancer cell line	activation	regulator of the progression of	*(167–169)*
	human	colon cancer cell line	activation	cells into the proliferation stage	
	human	breast cancer cell line/ breast cancer	activation	of the cell cycle	
peroxisome proliferator-activated receptor δ (PPARδ)	human	colorectal cancer cell line	activation	one of the nuclear receptor superfamily which includes the steroid hormone, thyroid hormone, and retinoid	*(170)*
c-jun	human	colonic adenocarcinoma cell line	activation	member of activator protein-1 (AP-1) transcription factor	*(171)*
fra-1	human	colonic adenocarcinoma cell line	activation	member of activator protein-1 (AP-1) transcription factor	*(171)*
urokinase-type plasminogen activator receptor (uPAR)	human	colonic adenocarcinoma cell line	activation	tumor invasion and metastasis	*(171)*
zonula occludens-1 (ZO-1)	human	colonic adenocarcinoma cell line	suppression	tight junction of associated protein	*(171)*
multidrug resistance 1 (MDR1)	human	colorectal carcinoma cell line	activation	major causes of drug resistance	*(179)*
novel band 4.1-like protein 4 (NBL4)	human	colon cancer cell line	activation	membrane skeleton protein	*(218)*
matrix metalloproteinase-7	mouse	colon and small intestine tumor	activation	factor for early tumor growth, later progression steps	*(172,173)*
(MMP-7, matrilysin)	human	colon cancer cell line	activation		
	human	colon cancer cell line	activation		*(178)*
monocyte chemotactic protein-3 (MCP-3)	mouse	fibroblast cell line	suppression	activates monocytes, lymphocytes	
	human	colon cancer cell line	suppression	dendritic cells, NK cells, and granulocytes	
Wnt-1 induced secreted protein (WISP)	mouse	mammary epithelial cell line	activation	CCN family of growth factor	*(219)*
	mouse	mammary tumor	activation		
	human	colon cancer cell line	activation		

WISP-1	rat	kidney fibroblast cell line	activation	CCN family of growth factor	*(174)*
connexin 43 (Cx43)	mouse	mammary epithelial cell line	activation	gap-junctional protein	*(220)*
	rat	pheochromocytoma cell line	activation		
cyclooxygenase-2 (COX-2)	mouse	mammary epithelial cell line	activation	inducible isoform of prostaglandin synthase	*(177,221)*
	rat	colon tumor	activation		
Cdx1	mouse	embryonic stem cell	activation	homeobox gene involved in axis formation	*(165)*
	rat	embryonic endoderm	activation		
engrailed-1	mouse	embryo	activation	midbrain development in vertebrate	*(222)*
Brachyury (T)	mouse	embryo	activation	regulation of morphogenetic cell movements	*(223,224)*
	mouse	embryonic stem cell	activation		
gastrin	mouse	small and large intestine	activation	gastrointestinal hormone and growth factor	*(175)*
TCF1	mouse	epithelial cells of intestine and mammary gland	repression	prevention of neoplasm	*(176)*
E-cadherin	mouse	in vitro	activation	one of the cell adhesion molecules	*(225)*
chordin	*Xenopus*	embryo	activation	dorsalizing factor	*(226)*
twin	*Xenopus*	embryo	activation	homeobox gene involved in axis formation	*(164)*
goosecoid	*Xenopus*	embryo	activation	homeobox gene involved in axis formation	*(164)*
Xenopus nodal-related 3 (Xnr3)	*Xenopus*	embryo	activation	dorsal axis factor	*(227)*
siamois	*Xenopus*	embryo	activation	homeobox gene involved in dorsal axial patterning	*(159–161)*
fibronectin	*Xenopus*	fibroblast	activation	cell-substrate adhesion molecule, extracellular matrix glycoprotein	*(180)*
engrailed-2	*Xenopus*	embryo	activation	homeobox gene involved in axis formation	*(228)*
engrailed	*Drosophila*	embryo	activation	homeobox gene involved in axis formation	*(158)*
Ultrabithorax (Ubx)	*Drosophila*	embryo	activation	homeobox gene involved in axis formation	*(158,162)*
egl-20	*C. elegans*	migratory neuroblast QL cell	activation	Hox gene involved in development of the posterior body region	*(229)*
mab-5	*C. elegans*	migratory neuroblast QL cell	activation	Hox gene involved in development of the posterior body region	*(230)*

(167) and Shtutman et al. *(168)* demonstrated that the cell cycle-regulatory gene *cyclin D1* is a β-catenin-LEF/TCF target in colon cancer. A variety of evidence has demonstrated that, when overexpressed, *cyclin D1* can act as an oncogene by promoting deregulated cell growth. As noted by Lin et al. *(169)*, *cyclin D1* is a target for β-catenin in breast cancer, where there is a high correlation between β-catenin expression and cyclin D1 expression.

Through an analysis of global gene expression profiles in human colorectal cancer cells, Kinzler et al. *(170)* demonstrated that *PPARδ* is an APC target gene. Furthermore, the authors demonstrate that the nonsteroidal anti-inflammatory (NSAID) drug sulindac inhibits *PPARδ*-driven gene expression. Thus, the chemopreventive activity of the NSAIDs in suppressing colorectal tumorigenesis might be related to their ability to inhibit PPARδ-activity, which is deregulated in response to APC inactivation.

To identify β-catenin-LEF/TCF target genes, Mann et al. *(171)* transfected β-catenin in colorectal cell lines with low β-catenin expression and analyzed differential expression in response to β-catenin by cDNA microarray. The authors identified increases in two transcription factors, *fra-1* and *c-jun*, both members of the AP-1 complex. Urokinase-type plasminogen activator, which is known to be activated by AP-1, was also induced. There was decreased expression of zonula occludens-1 (ZO-1), which plays a role in epithelial polarization. The authors concluded that β-catenin may facilitate loss of epithelial polarization and enhance proteolytic activity in colorectal carcinomas.

Brabletz et al. *(172)* demonstrated that the matrix metalloproteinase MMP-7 is a β-catenin target gene. MMP-7, which is overexpressed in 80% of human colorectal cancers, plays a role in early tumor growth and may function in invasion and metastasis. The authors conclude that these data explain the high level of MMP-7 expression in colon cancer, i.e., as a consequence of APC or β-catenin mutations, and suggest that via MMP-7 overexpression, loss of wild-type APC may influence later stages of colon cancer progression. Crawford and colleagues demonstrated that in intestinal tumors the metalloproteinase matrilysin is a target of β-catenin transactivation, suggesting a potential connection between β-catenin nuclear signaling and increased cell invasive potential *(173)*.

Suppression subtractive hybridization was used by Xu et al. *(174)* for identification of WISP-1 (Wnt-1 induced secreted protein1) as a β-catenin target. The mouse mammary epithelial cell line C57MG and *Wnt-1*-expressing C57MG cells were compared. WISP-1 is in the CCN family of growth factors. The promoter of *WISP-1* is activated by Wnt-1 and β-catenin expression, and overexpression of *WISP-1* induced morphologic transformation, accelerated growth, and higher saturation cell density in normal rat kidney fibroblasts. Interestingly, the LEF/TCF sites on the *WISP-1* promoter played a minor role in activation by Wnt-1 or β-catenin, but a CREB site was identified as critical in the response.

Koh et al. *(175)* identified the hormone gastrin as a target of β-catenin in colon cancer and used a genetic approach to directly test its functional significance. β-Catenin activated the gastrin promoter when transiently expressed in colon cancer cells. Consistent with a role for this gene as a β-catenin target, gastrin expression is induced early in colon cancer progression, and less processed forms of gastrin are potent colonic trophic factors. Overexpression of the glycine-extended form of gastrin in mice heterozygous for the *APC* gene ($APC^{min+/-}$) induced a significant increase in polyp formation. In contrast, gastrin-deficient $APC^{min+/-}$ mice showed a significant decrease in polyp formation, a decrease in the proliferation rate of polyp cells, and an increased survival time. These data provide genetic evidence that activation of gastrin by β-catenin may play a role in progressive malignant transformation.

Inactivating mutations in APC or activating mutations in β-catenin lead to inappropriate activation of TCF4. Clevers and colleagues demonstrated that the TCF family member *TCF1* is a target of the β-catenin–*TCF4* complex *(176)*. In contrast to other β-catenin targets, however, *TCF1* does not enhance the malignant phenotype. The most abundant *TCF1* isoforms lack the region for β-catenin interaction. *TCF1* null mice develop adenomas at an increased rate. Thus, *TCF1* appears to act as a tumor suppressor, and may function as a feedback repressor of β-catenin–TCF signaling.

Expression of COX-2, an inducible form of prostaglandin synthase, has been detected in intestinal tumors in which APC is mutated. Howe et al. *(177)* demonstrated that COX-2 is a transcriptional target of Wnt-1. Genetic ablation of the COX-2 gene or pharmacologic inhibition of COX-2 dramatically reduced the incidence of colonic tumors in the APC min mouse.

Fluorescence differential display of changes in gene expression in response to overexpression of activated β-catenin in murine fibroblast L cells identified monocyte chemotactic protein-3 (MCP-3) as a target of β-catenin. As reported by Fujita et al. *(178)*, MCP-3 is down-regulated in association with activated β-catenin binding to a LEF/TCF consensus sequence in the MCP-3 promoter. Expression of MCP-3 in colon cancer cells induces expression of several differentiation markers. The authors suggest that inappropriate activation of β-catenin may inhibit MCP-3-induced differentiation, and thus contribute to transformation of colonic epithelial cells.

Yamada et al. *(179)* used cDNA microarray to analyze gene expression in the colorectal carcinoma cell line DLD-1 engineered with a tetracycline-inducible truncated dominant-negative TCF4. The authors found that the *multidrug resistance 1* (*MDR1*) gene was down-regulated after inactivation of TCF4, and that there are TCF4-β-catenin-responsive sites in the *MDR1* promoter. The authors noted that MDR1 levels are up-regulated in adenomas and adenocarcinomas of patients with familial adenomatous polyposis (FAP), which is characterized by germ-line

APC mutations and accumulation of cytoplasmic β-catenin. Furthermore, P-glycoprotein, the *MDR1* gene product, has been shown to protect cells from apoptotic stimuli including TNF, fas ligand, and UV irradiation, and thus may provide another mechanism by which overexpression of β-catenin promotes transformation.

In a study by Gradl et al. *(180)*, β-catenin was shown to control transcription of the cell-substrate adhesion molecule fibronectin. The Wnt/Wg responsive region was mapped to a LEF/TCF binding site in the fibronectin promoter. Overexpression of cadherins shifted β-catenin to the plasma membrane and down-regulated fibronectin expression. The ability of β-catenin to transactivate the fibronectin promoter was dependent on the subtype of HMG family member expressed in the cell. The authors propose that induction of fibronectin and other mesenchymal adhesion proteins by β-catenin may drive the epithelial-mesenchymal transition, which is characteristic of the malignant phenotype.

4.4. Coordination of TGF-β and Wnt Signaling

Depending on the setting, TGF-β and Wnt/β-catenin signaling can be cooperative or antagonistic. In certain events of embryonic development, such as spatial restriction of early gene transcription to the Spemann's organizer, TGF-β and Wnt/Wg pathways cooperate. In *Drosophila*, the TGF-β family member Dpp and Wg cooperate to regulate the expression of *Ultrabithorax* in the endoderm, through a LEF site and a Dpp-responsive element. In the *Drosophila* leg disc compartment, however, Dpp and Wg are antagonistic. Labbe et al. *(181)* reported a potential molecular mechanism for this cooperation. They demonstrated that Smads, the downstream effectors of the TGF-β pathway, physically associate with LEF/TCFs. Additionally, they show that for the *Xenopus twin* promoter, which is cooperatively activated by the Wnt and TGF-β pathways, both Smad and LEF/TCF binding sites are required. They propose that Smad regulation of LEF target genes depends on the physical association of LEF and Smad and on the presence of a Smad-binding element (SBE) adjacent to the LEF/TCF binding site. Conversely, they propose that LEF binding sites that are not in close proximity to an SBE will not be regulated by the Smad pathway.

Nishita and colleagues demonstrate in *Xenopus* that β-catenin and LEF1/TCF form a complex with Smad4, an essential mediator of TGF-β family signaling, and this complex synergistically affects expression of the Wnt target gene *twin (182)*. They suggest that a limited pool of Smad4 could affect the efficacy of Wnt signaling or the availability of Smad 4 for the TGF-β signaling pathway. Furthermore, the authors suggest that the cooperation of TGF-β and Wnt signaling may also play an important role in carcinogenesis, citing the work of Takaku et al. *(183)*. These investigators introduced the Smad4 mutation into the $Apc^{\Delta 716}$ knockout mouse. These mice are a model for FAP, and normally develop large numbers of benign adenomatous polyps. Inactivation of Smad4 in these animals induced progression of adenomatous polyps to malignant, invasive adenocarcinomas,

thus demonstrating how inactivation of TGF-β signaling can cooperate with activated β-catenin in promoting malignant transformation.

5. ONCOGENEIC ACTIVITY OF β-CATENIN

5.1. Deregulation of β-Catenin Adhesion Function in Cancer

Because the cadherin/catenin complex can bind adjacent epithelial cells together, it can be hypothesized to play a role in regulation of invasion and metastasis. Reduced, absent, or disorganized expression of E-cadherin has been described in carcinoma, including gastric, head and neck, bladder, prostate, colon, and breast cancer *(184)*. As re-introduction of the E-cadherin gene into invasive cancer cells that had suppressed E-cadherin reduced their invasive activity, E-cadherin appears to act as an invasion and metastasis suppressor gene *(132)*. Furthermore, suppressed E-cadherin not only negatively impacted adhesion, but also resulted in an increase of cytosolic β-catenin and accumulation of nuclear β-catenin in breast cancer cell lines *(185)*. In colon cancer, decreased cadherin/β-catenin association is causally linked to increased β-catenin-regulated gene expression and increased cell division *(186)*.

5.2. Evidence that β-Catenin Causes Cancer by Activation of LEF/TCF Target Genes

β-Catenin was recently shown to efficiently induce neoplastic transformation of the SV40-transformed rat kidney epithelial cell line RK3E. Transformation by β-catenin was associated with activation of TCF-responsive genes. Furthermore, overexpression of TCF lacking the β-catenin binding domain blocked β-catenin-induced neoplastic transformation, emphasizing the importance of β-catenin-dependent activation of TCF-responsive genes in this process *(15)*. Further evidence that β-catenin can induce malignant transformation by activation of LEF target genes was provided by experiments showing that expression of a chimeric protein of the LEF DNA binding domain fused to the transactivation domain of VP16 or the estrogen receptor induced malignant transformation of chick embryo fibroblasts.

6. β-CATENIN AS A TARGET FOR ANTICANCER DRUG DEVELOPMENT

Recent epidemiological studies have revealed that the nuclear localization of β-catenin is an important prognostic factor related to unfavorable outcome in several malignancies *(186–189)*. These data, together with overwhelming experimental evidence that nuclear β-catenin functions as an oncogene, predict that antagonists of β-catenin signaling and/or nuclear localization will be of significant clinical benefit.

Although small molecule screening programs with this goal in mind are still in their infancy, the technology exists to achieve rapid progress. For example, existing β-catenin/TCF and β-catenin/LEF reporters, whose readout is luciferase activity, are ideally suited as initial screens for use in microtiter plate-based high-throughput-screening regimens. Likewise, the crystal structure of a β-catenin/TCF complex has recently been reported *(190)*, allowing investigators to define critical motifs on β-catenin that are uniquely required for interaction with TCF and other associating proteins. With such information in hand, molecular modeling can be used to design potential small-molecule inhibitors that are specific for the catenin/TCF interaction (while not affecting catenin association with E-cadherin, for example).

Small-molecule inhibitors of β-catenin signaling, arrived at serendipitously, already exist. This has been possible because of a more complete understanding of the multiple signaling pathways that impact on β-catenin.

6.1. Inhibition of β-Catenin Nuclear Signaling

Retinoids are potent regulators of embryogenesis, cell proliferation, epithelial cell differentiation, and carcinogenesis *(191)*. Retinoids have two major receptor proteins, the retinoic acid (RA) receptor (RAR) and the retinoid X receptor (RXR), that can mediate regulation of gene expression either directly, by binding to RA-responsive elements (RAREs), or indirectly, by inhibiting activity of other transcriptional regulators such as AP-1.

Many downstream pathways are regulated by retinoids. According to recent reports, retinoids are associated with β-catenin signaling and cadherin-β-catenin adhesion function *(192,193)*. In breast cancer cells, retinoid-activated RAR, but not RXR, competes with TCF for binding to β-catenin. Although retinoids have not been shown to regulate expression or degradation of β-catenin, they can reduce β-catenin nuclear signaling by inhibiting complex formation of β-catenin and TCF. Retinoids can also increase cadherin expression and function in breast cancer cells. As increased cadherin can tether β-catenin to the plasma membrane, nuclear signaling of β-catenin may be reduced by depleting the β-catenin cytoplasmic pool, which has certainly been shown experimentally *(127,130,185,194)*. Therefore, retinoids should be considered as a therapeutic agent for cancers in which β-catenin nuclear signaling contributes to transformation.

Nonsteroidal anti-inflamatory drugs (NSAIDs), such as aspirin and indomethacin, have been shown to attenuate β-catenin/TCF signaling *(195)*. In four colon carcinoma cell lines, NSAIDs effectively inhibited the transcriptional activity of a β-catenin/TCF reporter gene, as well as that of *cyclin D1*, an endogenous β-catenin/TCF sensitive gene. Interestingly, these drugs most likely functioned to modulate TCF activity without directly affecting β-catenin or its association with TCF.

NSAIDs also inhibit COX-2 *(196,197)*, a proinflamatory and prooncogenic enzyme in the prostaglandin pathway that has been implicated in colorectal and

other cancers *(198–204)*. Specific COX-2 inhibitors such as rofecoxib (Vioxx) have also been shown to be effective in inhibiting polyposis in a mouse model harboring mutated APC *(205)*. Although the mechanism of action of COX-2-specific agents is not known, the nonspecific COX inhibitor indomethacin seems to reduce nuclear β-catenin level while augmenting localization of both β-catenin and E-cadherin to the plasma membrane *(206)*.

6.2. Regulation of Upstream Signals

Growth factors and other soluble ligands, including epidermal growth factor (EGF), insulin, insulin-like growth factor-1 (IGF-1), hepatocyte growth factor, and intestinal trefoil factor, can increase and stabilize the levels of free cytosolic β-catenin as described earlier in this chapter *(96,98,120,122,193,207)*. Mechanistically, this activity is generally mediated by either serine–threonine or tyrosine phosphorylation of β-catenin itself, E-cadherin, GSK-3β, or other components of the β-catenin regulatory network. As an example of the complexity of these effects, EGF and IGF-1 stimulate receptor tyrosine kinases, which i) directly phosphorylate β-catenin on tyrosine, thereby reducing its affinity for E-cadherin, while ii) simultaneously activating Akt kinase which then phosphorylates GSK-3β, thus inhibiting its ability to phosphorylate β-catenin and preventing β-catenin's recognition by the ubiquitination machinery.

Integrin-linked kinase (ILK) is downstream of both growth factor receptors and integrins, transmembrane proteins that are regulated by attachment to extracellular matrix. As such, it can be activated by diverse extracellular signals and, as we have described earlier, plays multiple roles in the β-catenin pathway. ILK directly phosphorylates and inhibits GSK-3β, down-regulates E-cadherin expression, up-regulates LEF-1 expression, and stimulates Akt. The net result of all of these activities is increased β-catenin-driven transcription *(121,208,209)*.

Based on these data, kinase inhibitors would seem to be useful agents to inhibit or reverse the transcriptional activity of β-catenin. Because multiple kinases, with both serine/threonine and tyrosine specificity, affect the β-catenin pathway, it is not clear whether specific inhibitors of a particular kinase will have clinical benefit. Certainly, in defined experimental settings, small-molecule inhibitors of ILK are effective in reversing β-catenin-driven transcription *(124,210,211)*. One would expect that inhibitors of EGF and insulin/IGF-1 receptors, and of c-met receptor tyrosine kinase *(212)*, might be similarly beneficial. Small-molecule Akt inhibitors have been shown to block β-catenin stabilization and GSK-3β inhibition in response to Akt activation *(213)*. We recently reported that inhibition of erbB2 by a small molecule in melanoma cells reversed the tyrosine phosphorylation of β-catenin, inhibited β-catenin-driven transcription, and markedly elevated the percentage of total cellular β-catenin associated with E-cadherin *(108)*.

Given the multiplicity of overlapping kinases that impact the β-catenin pathway, a broad-acting inhibitor, capable of down-regulating both tyrosine and

serine/threonine kinases might be of particular benefit. The efficacy of one such agent, the heat shock protein 90 (Hsp90) inhibitor geldanamycin, has been demonstrated in vitro *(108)*. Hsp90 inhibition is uniquely appealing in the context of β-catenin inhibition since EGF receptor, insulin/IGF-1 receptor, c-met, erbB2, and Akt all are client proteins of Hsp90 and thus depend on this chaperone for stability and activity *(214–216)*.

7. CONCLUSION

Although disregulation of β-catenin signaling is considered to be one of the earliest steps in tumor progression, it is clear that abrogation of β-catenin nuclear signaling with concomitant enhancement of its plasma membrane association with E-cadherin can reverse the strongly tumorigenic phenotype of even advanced malignancies. In tissue culture, down-regulation of the β-catenin/LEF/TCF pathway in established undifferentiated tumor cells is associated with the establishment of a more differentiated epithelial phenotype *(217)*. Since most, if not all, β-catenin target genes positively regulate cell growth and survival, interdiction of β-catenin nuclear signaling should have benefit in both the prevention and treatment of cancer.

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6 Histone Acetyltransferases as Potential Targets for Cancer Therapies

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Contents

1. INTRODUCTION

Tumors develop through aberrant cell proliferation caused by defects in regulatory mechanisms governing the cell cycle or, conversely, in mechanisms controlling programmed cell death (apoptosis). One way in which both these mechanisms can be disrupted is through unregulated expression of key genes. It is therefore important to understand the control of genes, such as proto-oncogenes and anti-oncogenes that are potentially involved in cancer, to gain insights into why they are expressed aberrantly and how this can be rectified. This chapter will focus on one class of nuclear proteins, the histone acetyltransferases, which are involved in the control of gene expression and have been implicated in certain types of cancer.

The steady-state levels of histone acetylation in the cell are maintained by a delicate balance between the action of histone acetyltransferases (HATs) and histone deacetylases (HDACs). Discussion of the role of HATs in cancer began with the discovery of histone acetylation by Vincent Allfrey, who first postulated its involvement in gene regulation *(1)*. Allfrey and colleagues were the first to demonstrate a correlation between enhanced acetylation of nucleosomes and the activation of two oncogenes, c-*fos* and c-*myc (2,3)*. These early studies remained correlative until three recent advances reignited interest in the role histone acetylation

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might play in cancer and which are discussed in this chapter. The first of these advances was the discovery that certain coactivators, such as p300, CBP, and pCAF, already implicated in the transcriptional activation of protooncogenes as well as in the action of DNA tumor viral products, were HATs (Section 3.). Second, the development of chromatin immunoprecipitation (ChIP) assays allowed the use of antibodies against specific modifications on histone tails to isolate only that chromatin carrying these modifications, a powerful methodology that allowed proof of enhanced histone acetylation on active proto-oncogenes (Section 5.1.). Finally, the discovery that mutations and chromosomal abberrations in certain human cancers involved either the inactivation or misregulation of human HATs showed that this class of enzymes has direct pathological potential in the development of cancer (Sections 5.2.–5.4.).

Interestingly, it would seem that aberrant HATs can function as either oncogenes or tumor-suppressor genes. The apparent contradiction between oncogenic and antioncogenic modes of causality may find explanation in the way these enzymes function within complexes and how they are targeted to specific genes. These considerations impinge on derivation of bioactive compounds acting on HATs to treat cancer.

2. CHROMATIN AND TRANSCRIPTION

Over the last 20 or so years, there has been extensive research into the basic components of the eukaryotic transcription complex (*4,5,* and references therein). Based largely on the reconstitution of the basal transcription machinery in vitro, this work allowed elucidation of the sequence of events that occurs during the initation of transcription. Although revealing much of the detail and the complex nature of the transcription process, these experiments were carried out on naked DNA templates, and do not address issues related to the chromatinized state of DNA.

In vivo, DNA is compacted over 1000-fold, first by wrapping around an octamer of core histone proteins, consisting of two copies of H2A, H2B, H3, and H4, forming a structure called the nucleosome (6). The string of nucleosomes, the 10 nm filament, is further folded into higher orders of compaction forming the 30 nm fiber, and further to form chromosomes. A major current challenge is to determine how the transcriptional apparatus accesses this template and which protein factors are required to allow this.

In studies of chromatin, there have been persistent indications that post-translational modifications of histone tails, most notably acetylation of lysine residues, were associated with actively transcribed genes *(7–9)*. Allis and co-workers identified the yeast protein Gcn5 as a histone acetyltransferase *(10)*. Notably, this protein had also previously been identified as a transcriptional coactivator, linking the process of histone acetylation to transcriptional activation for the first time. There has since been an explosion in the number of HATs identified, and their involvement in the regulation of gene expression is now unquestionable.

Table 1
Properties of Known Histone Acetyltransferases

HAT Subunit	*Complex*[a]	*HAT Histone Specificity*[b]	*Proposed Cellular Function*
Gcn5	yADA	**H3/**H2B	Transcriptional coactivator
	ySAGA	**H3**	Transcriptional coactivator
	hGcn5	**H3**	Transcriptional coactivator
PCAF	hPCAF	**H3**	Transcriptional coactivator
Sas3	yNuA3	**H3/**H4	Silencing/transcriptional elongation
Esa1	yNuA4	**H4/**H2A	Transcriptional coactivator
Tip60	hTip60	**H4/H2A**	DNA repair/apotosis
MOF	dmMSL	**H4**	Dosage compensation
MOZ			Unknown
MORF		**H4/**H3	Unknown
Elp3	yElongator	**H3/H4/H2A/H2B**[c]	Transcriptional elongation
HBO1	yORC complex	**H3**[d]	DNA replication
p300/CBP		**H3/H4/H2A/H2B**	Transcriptional coactivator
SRC-1	Nuclear receptor	**H3/**H4	Transcriptional coactivator
ACTR (PCIP)	Nuclear receptor	**H3/**H4	Transcriptional coactivator
TIF2	Nuclear receptor	nd	Transcriptional coactivator
$TAF_{II}250$	hTFIID	**H3/**H4[c]	General transcription factor
ATF2		**H4/H2B**	Transcription factor

[a]The best well-characterized complexes from different species are indicated: y, yeast; h, human; dm, *Drosophila melanogastor*.

[b]Histone specificity is against nucleosome substrates. Main histone acetylated is shown in bold, with secondary substrates in normal type.

[c]HAT activity has only been shown against free histones.

[d]HBO1 activity was very weak against nucleosomes.

3. DIFFERENT CLASSES OF HATs AND THEIR COMPLEXES

There are now at least 16 known HATs from a variety of organisms (Table 1). HATs are classed overall as type A, the nuclear HATs, or type B, the cytoplasmic HATs *(11)*. Here, we will concentrate on the type A HATs that are mainly involved in the regulation of transcription. The type B HATs are involved in deposition-linked acetylation, which occurs on histones prior to their incorporation into chromatin. This complexity is further enhanced by the existence of HAT proteins in large multi-subunit complexes and their apparent involvement in a number of different cellular processes (summarized in Table 1). The main families of type A HAT enzymes are summarized briefly in the following section. A more extensive discussion of these proteins can be found in other reviews *(11,12)*.

3.1. GCN5-Related N-Acetyltransferase (GNAT) Family

The two main members of this family, Gcn5 and PCAF, are closely related proteins. Gcn5 has homologs in yeast and human, whereas PCAF appears unique to higher eukaryotes.

GCN5 was first identified as a transcriptional activator and was subsequently shown to be the yeast homolog of the *Tetrahymena* p55 protein, the first nuclear HAT to be identified *(10)*. Gcn5 is the catalytic HAT in two distinct high-molecular-weight complexes, ADA and SAGA *(13)*. The ADA complex contains the adaptor proteins ADA2 and ADA3 in addition to Gcn5, whereas the SAGA complex contains these three proteins plus an additional 13 subunits. The precise role for these distinct complexes in the cell remains unclear, although it is possible that they are involved in the regulation of different subsets of yeast promoters. Gcn5 was first shown to acetylate histone H3 and H4 peptide substrates on distinct sites *(14)*. Notably it was only able to acetylate nucleosomal substrates when incorporated into either ADA or SAGA complexes. These complexes showed a preference for H3, but were also able to acetylate an expanded repertoire of lysine residues on the H3 tails *(15)*.

A second, well-studied, member of this family is PCAF (reviewed in *16*). This protein was first identified as a protein whose interaction with p300/CBP (see below) was disrupted by the presence of the viral protein E1A *(17)*. The HAT activity of PCAF was shown to be directed primarily against H3 and to a lesser extent H4 *(17)*. Subsequently, PCAF was shown to be present in a larger complex, containing up to 20 additional subunits *(18)*. Interestingly, this complex contains a subset of TAFs, proteins previously identified as components of the general transcription factor TFIID (reviewed in 19). Similarly, the yeast SAGA complex also contains TAF subunits *(20)*, suggesting that they function in a more ubiquitous fashion than was previously thought. In addition, the human homolog of Gcn5 is present within an almost identical complex to PCAF, the only discernible difference being the identity of the catalytic HAT *(18)*, suggesting similar functions for these complexes. However, gene knockout experiments showed that while the hPCAF knockout was viable, the hGcn5 knockout was lethal. The survival of the hPCAF knockout was probably the result of an up-regulation of hGCN5 within these mice *(21)*. These data suggest that these two closely related complexes are involved in distinct but partially overlapping functions within the cell.

3.2. MYST Family

This family takes its name from four of the originating members, MOZ, Ybf2/Sas3, Sas2, and Tip60. These HAT complexes have various roles that include involvement in dosage compensation, silencing, DNA repair and apoptosis (Table 1).

The yeast proteins Sas3 and Esa1 are the catalytic subunits of the NuA3 and NuA4 complexes, respectively *(22,23)*. These complexes primarily acetylate histone H3 (NuA3) or H4 (NuA4). Unlike other yeast HATs, Esa1 is an essential gene, with mutants showing defects in cell cycle progression *(24,25)*. However, a point mutation in the acetyl-CoA binding site of Esa1 resulting in defective HAT activity showed no obvious effect on growth *(26)*. This difference between the point mutation and the deletion of the gene suggests that the complex may have a role dis-

tinct from the acetylation of histones. More recently, the NuA4 complex has been shown to have a role in the regulation of ribosomal protein gene expression *(27)*.

Another member of the MYST family of HATs, Elp3 has been identified in yeast *(28,29)*. This protein was identified as a subunit of the yeast elongator complex and implicates histone acetylation in transcriptional elongation. Similarly, the NuA3 complex has been genetically linked to Spt16, a component of the elongation factor FACT *(23)*.

Human Tip60 was originally identified in a yeast two-hybrid screen to identify proteins interacting with the HIV protein Tat *(30)*. Its putative HAT domain was later shown to be active against free histones, although it had poor activity toward nucleosomal substrates *(31)*. The recent characterization of the human Tip60 complex has revealed potential roles for this HAT complex in double-stranded DNA repair and apoptosis *(32)*.

HBO1 was identified in a complex with ORC1, a protein that binds to replication origins *(33)*. HBO1 was subsequently shown to possess HAT activity, primarily against histones H3 and H4 *(33)*, implying a role for histone acetylation in DNA replication. Interestingly, another MYST family member, Sas2, has also been genetically linked to ORC function in yeast *(34)*.

MOF has been implicated as a HAT involved in the process of dosage compensation in *Drosophila*, whereby the X chromosomes of the male flies are transcriptionally activated two-fold to ensure parity with the female *(35)*. MOF is part of a larger complex called MSL, which contains four other subunits plus an RNA species roX2 *(36)*. This complex has been shown to acetylate histone H4 specifically on Lys16, a characteristic of male X-chromosomes *(36,37)*.

MOZ, a human protein that has homology to the MYST family of HATs, has recently been shown to be a functional HAT *(38)*. This protein is of interest because its genetic locus is unstable and a number of chromosome translocations are associated with this gene (*see* Section 5.4.).

3.3. p300/CBP Family

CBP was originally identified as a transcriptional coactivator of CREB *(39)* and p300 as a protein interacting with the viral E1A protein *(40,41)*. These proteins are implicated in a wide range of cellular functions, including cell proliferation and differentiation (reviewed in 42). A number of functional domains have been identified in these proteins, such as HAT and bromodomains, allowing interaction with a wide variety of transcription factors, including c-jun, Elk1, and p53 *(43)*, viral oncogenes including E1A, SV40 large T antigen *(44)*, and other HATs, including PCAF *(17)*, suggesting that they play an important role in transcriptional regulation *(45)*.

The striking similarities in sequence and function of these proteins suggest that they may perform redundant functions within the cell. However, a number of studies have revealed functional differences. For example, retinoic acid-induced

F9 cell differentiation was shown to require p300 but not CBP *(46)*. Additionally, a homozygous p300 knockout was embryonic lethal, suggesting that CBP was unable to compensate for its loss *(47)*. It is therefore unclear as to the extent of overlap in function between these proteins.

Identification of HAT activity within these proteins *(48,49)* revealed an additional functional aspect. These proteins are uniquely able to acetylate all four core histones within a nucleosomal context, but the true in vivo acetylation sites have yet to be identified. However, the HAT activity has been shown to be important for their transcriptional activation function in vivo *(50)*, although an additional role as a noncatalytic transcriptional adaptor cannot be ruled out. It is noteworthy that p300/CBP is able to acetylate a large number of transcription factors in vitro (*see* Section 4.2.), raising the possibility of non-histone substrates for these enzymes in vivo. In addition chromosome translocations and mutations associated with p300 and CBP are implicated in human disease (*see* Section 5.4.).

3.4. Nuclear Receptor Coactivator Family

A number of nuclear receptor coactivators have been shown to possess HAT activity, linking this modification with transcriptional activation in response to hormones *(51)*. There are three known nuclear receptor coactivators that possess HAT domains: SRC1 *(52)*, ACTR *(53)*, and TIF2 *(54)*. These coactivators are recruited to promoters by nuclear receptors, such as the estrogen and progesterone receptors, displacing repressor complexes and can themselves interact with additional HAT activities such as p300/CBP (reviewed in 55). ACTR and SRC1 have been shown to be acetylated by p300/CBP, and this has the effect of attenuating ligand-dependent transcriptional activation (*56, see* below). ACTR and SRC-1 can acetylate nucleosomes primarily on H3 *(52,53)*; however, no functional HAT activity has yet been demonstrated for TIF2. Interestingly, as discussed for MOZ, TIF2 is involved in chromosome translocations implicated in leukemia (*57,58*; *see* Section 5.4.).

3.5. Additional HATs

Identification of HAT activity in the human TFIID subunit $TAF_{II}250$, as well as its *Drosophila* and yeast homologs, places these enzymes right at the core of the basal transcription machinery *(59)*. $TAF_{II}250$ does not show any homology with other known HATs, and so may represent the first member of a new family of HATs. $TAF_{II}250$ also possesses kinase activity *(60)* and histone H1 ubiquitination activity *(61)*, suggesting that this subunit can regulate transcription in a number of different ways. A temperature sensitive (ts) mutant cell line with a mutation in the HAT domain of $TAF_{II}250$ underwent G1 arrest and apoptosis at the restrictive temperature, suggesting a functional role for the HAT domain in $TAF_{II}250$ regulated gene expression. The genes affected were mainly involved in cell cycle control and DNA synthesis *(62)*.

The transcription factor ATF2 has recently been demonstrated to have HAT activity, and exhibits a unique substrate specificity (Table 1; *63*). This is the first demonstration of a HAT with site-specific DNA binding activity, and so suggests a mechanism for targeting the HAT activity to specific promoters in vivo. Interestingly, phosphorylation of ATF2 increased its HAT activity and CRE-dependent gene transcription in vivo, implying a control of HAT activity via cellular signaling pathways.

4. THE FUNCTION OF HATs

Although it is clear from the preceding section that HATs are involved in a wide range of cellular functions, their precise molecular function is only beginning to be unraveled. Histones are the most well-studied substrate, but it is becoming apparent that HATs can acetylate other proteins, giving rise to speculation that acetylation may have wider functional implications *(64)*. In this section, we briefly discuss the role of HATs in vivo with regard to acetylation of histones and non-histone proteins.

4.1. Histone Acetylation

The acetylation states of histones are maintained by a delicate equilibrium between the action of HATs and histone deacetylases; however, its molecular function is still contentious. One long-held view is that acetylation neutralizes the positive charge on lysine residues within histone tails, thus disrupting the interaction with the negatively charged DNA wrapped around the protein core. However, the crystal structure of the nucleosome reveals interesting aspects about the location and possible role of histone tails *(65)*. The histone tails were observed to be unstructured and flexible, but rather than contacting DNA wrapped around the histone octamer as previously supposed, the tails protrude through the gap between the two wraps of DNA, and may well contact adjacent nucleosomes via interactions with the histone octamer rather than the DNA. For example, the H4 tail was observed to make contacts with the H2A/B dimer of an adjacent nucleosome *(65)*. This finding would seem to suggest that the function of the tails may be to stabilize higher-order structure of the chromatin, rather than involvement in the structure of a single nucleosome *(6,65)*. As an extension of this model, acetylation of the tails may be involved in destabilizing higher-order chromatin structure, thus allowing easier access of transcription factors and coactivators.

The most well-studied process affected by acetylation is transcription. More recently, a role in processes such as DNA replication, recombination and repair, and apoptosis has emerged *(12)*. The importance of acetylation to transcription has been shown genetically in yeast, where mutation of the HAT domain of Gcn5 resulted in defective expression from several Gcn5 regulated promoters *(66,67)*. In contrast, whole genome analysis of temperature-sensitive mutations in Gcn5 indicated that expression of only approx 5% of yeast genes were altered at the

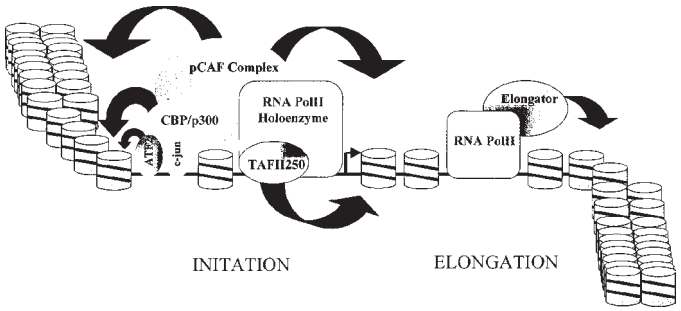

Fig. 1. Schematic representation of potential HAT complexes present during transcription of a proto-oncogene. During transcriptional activation of a proto-oncogene a number of HAT complexes are present at the promoter of the gene. These include the site-specific DNA binding protein ATF-2, coactivator complexes such as p300/CBP and PCAF and the TAFII250 subunit of the TFIID general transcription factor. These enzymes may acetylate both histones and non-histone proteins. Subsequent elongation of the RNA polymerase II may also require the action of HAT complexes, represented here by the Elongator complex identified in yeast. Note that this diagram is not to scale.

non-permissive temperature *(68)*. This number may be an underestimate, as Gcn5 may be more involved in gene regulation at specific stages of the cell cycle or under different environmental conditions. For example, a recent study suggests that the SAGA complex may be critical for expression of genes during mitosis *(69)*. Interestingly, at non-permissive temperatures, some genes were overexpressed and others repressed, indicating a potential role for Gcn5 in both activation and repression of genes in yeast. However, it remains a possibility that not all of these effects arise directly from altered histone acetylation, but rather from modified transcriptional coactivator function or acetylation of transcription factors (*see* Section 4.2.).

The favored model for targeting of HAT function in gene expression is the recruitment of complexes by site-specific DNA-binding transcription factors to specific genetic loci. Promoter-proximal acetylation of histones then permits a transcriptionally competent chromatin structure to be formed (Fig. 1; *70*). Transcription-factor-dependent recruitment of HATs has been demonstrated in yeast for the SAGA complex *(66,71–73)*. Additionally, the interaction of CBP/p300, PCAF, and nuclear receptor coactivators with different site-specific transcription factors suggests a mechanism for their targeted recruitment.

Recent elegant studies have revealed sequential recruitment of HAT and ATP-dependent chromatin remodeling factors to promoters prior to gene expression. Studies on expression of the yeast *HO* gene revealed a requirement for prior recruitment of the Swi/Snf remodeling factor by the transcription factor Swi5p before the SAGA complex is recruited *(71,72)*. Subsequently, sustained association of the Swi/Snf complex may depend upon acetylation of histones by the SAGA

complex *(74)*. In contrast, the reverse is true at the human β-*IFN* promoter. Upon virus infection, a multisubunit complex called the enhancesome is assembled at the promoter, requiring recruitment of the human Gcn5 (hGcn5) complex followed by recruitment of the hSwi/Snf complex and nucleosome remodeling *(75)*. However, in each of these two cases, there may be a requirement for histone acetylation to allow remodeling factors to be maintained at the promoter *(74)*.

Histone tails undergo a variety of other post-translational modifications in addition to acetylation *(8,9)*. It is therefore possible that acetylation in combination with other modifications can act as a mark for a particular chromatin state. These modified tails also have the potential to act as binding platforms for the recruitment of regulatory factors to specific regions of chromatin. Recently, examples of this type of interaction have been reported. Proteins such as the HATs, PCAF, and $TAF_{II}250$ have recently been shown to bind to acetylated histone tails via their bromodomains *(76,77)*. Furthermore, the heterochromatin protein HP1 was shown to bind, via its chromodomain, to histone H3 methylated on Lysine 9 *(78)*.

In addition to highly targeted modes of histone acetylation discussed above, there also appears to be another more global mode of histone acetylation within chromatin. It has been shown that the yeast genome as a whole is highly acetylated, with an average of 13 acetyl groups per nucleosome reported in yeast *(79)*. Further, genetic studies in yeast have suggested that dynamic acetylation/deacetylation occurs over entire loci and that discrete regions of hyperacetylation correlate with gene promoters *(80,81)*. Similar conclusions have been reached from studies of the chicken β-globin locus, where high levels of acetylation correlate with increased DNase I hypersensitivity over the entire locus *(82)*. Additionally, regions of hyperacetylation map to the locus control region, which controls transcription throughout the locus *(83)*.

These studies suggest that acetylation may be associated with transcriptionally competent regions of chromatin and that the targeted recruitment of HATs and hyperacetylation of promoter nucleosomes is involved in regulating gene expression within that region. Whether the same HAT complexes and sites of modification are involved in both modes of acetylation remains to be determined.

4.2. Non-Histone Protein Acetylation

There is much less information available as to the function of non-histone protein acetylation. The majority of the protein targets are transcription factors (reviewed in *11,12*), although chromatin-associated proteins such as HMG14 and 17 *(84,85)*, and nuclear import factors *(86)* have also been reported to be acetylated.

The functional role of acetylation of these proteins appears to be context-dependent. Acetylation can modulate the DNA-binding efficiencies of transcription factors, either increasing [p53 *(87)*, MyoD *(88)*, EKLF *(89)*] or decreasing [CDP/cut (*90)*] DNA binding. Acetylation has also been reported to increase protein half life [E2F1 *(91)*], and modulate binding to nucleosomes [HMG14 and 17

(84,85)]. The role of acetylation in controlling p53 function is of interest because p53 is one of the most highly mutated proteins detected in human cancers, and it implies a level of p53 regulation additional to its phosphorylation *(92)*.

The acetylation of the nuclear receptor coactivator ACTR provided a striking example of an acetylation-regulated process in vivo *(56)*. These authors found that ACTR was acetylated by CBP/p300 in an estrogen-dependent manner, causing a disruption of the interaction between the receptor and coactivator, and so attenuating the transcriptional response. Therefore, in contrast to histone acetylation, coactivator acetylation can repress transcription.

Acetylation of these proteins has mainly been shown in vitro, with a few notable exceptions [p53 *(87)*], using p300/CBP or PCAF as HATs. It will be of interest to determine how physiologically significant acetylation of these factors is and which of the many HAT complexes are involved. Furthermore, it is notable that the majority of protein factors reported to be acetylated are also phosphorylated, as are histones. It will be of interest to determine whether acetylation and phosphorylation of these factors contribute in a combinatorial manner to their function.

5. POTENTIAL ROLE OF HATs IN CANCER

Given that HATs and acetylation play an important role in the control of oncogene expression and other cellular processes, it is no surprise that they have been implicated in human disease, particularly cancers. Interestingly, they appear to be able to act as both oncogenes and tumor suppressors, depending upon the genetic context. In this section, we discuss the main areas of evidence linking HAT activity to aberrant cell proliferation and disease states.

5.1. HATs and the Transcriptional Activation of Oncogenes

Histone acetylation was first implicated in the activation of proto-oncogenes by the work of Allfrey and colleagues. It was found that nucleosomes that assumed a more "open" conformation, making it possible to isolate them on organomercurial columns by virtue of the increased accessibility of a cysteine residue on histone H3, contained higher levels of histone acetylation *(2,3)*. Using this assay, it was demonstrated that nucleosomes associated with proto-oncogenes, such as c-*fos* and c-*myc*, were retained when the gene was activated but not when it was inactive. This finding was the first link between more "open" nucleosomes, histone acetylation, and active proto-oncogenes. With the development of ChIP assays that allow specific immunoselection of acetylated nucleosomes, a number of labs applied this technique to proto-oncogenes with somewhat conflicting results *(93,94)*. However, our own recent work, along with that of others, has established that increased acetylation of H3 and H4 is intimately linked to c-*fos* and c-*jun* expression *(95–97)*.

Over the last decade, it has emerged that histone H3 phosphorylation is also involved in proto-oncogene induction (*98–100*; reviewed in *7,101*), and, most important, in 1994, a potential link between H3 phosphorylation and acetylation

was described *(102)*. This link led to the recent demonstration that both modifications of H3—phosphorylation and acetylation (called phosphoacetylation hereafter)—were specifically associated with active c-*fos* and c-*jun* nucleosomes *(95,103)*, implicating two enzyme systems—protein kinases and HATs—in this double-modification associated with proto-oncogene activation. Over this period, it was concurrently demonstrated that certain coactivators are involved in the transcriptional activation of proto-oncogenes. The final piece in the chain of evidence that links histone acetylation to proto-oncogene induction was the demonstration that these coactivators, such as CBP, p300, and pCAF possess intrinsic histone acetyltransferase activity (*see* Section 3.), suggesting a high level of HAT activity is present at such promoters (Fig. 1). Recently it has emerged that histone acetylation/deacetylation is a very dynamic process at proto-oncogene promoters, even in resting cells. Interestingly, although increases in acetylation of H3 and H4 are observed upon transcription of these genes, the modification is not uniformly distributed along the gene *(96)*. These studies provide clear insight into the remarkable complexity of nucleosome modification associated with proto-oncogene induction, and identify HATs and histone acetylation as a key factor in this process.

The precise functional significance of histone acetylation to proto-oncogene induction remains unclear, and the potential modes of function discussed above (Section 4.) could apply. However, if HATs and histone acetylation alone are sufficient for proto-oncogene induction, it may be argued that constitutively active HATs may themselves be oncogenes. Present evidence indicates that this is unlikely for two reasons. First, there is abundant evidence that phosphorylation of transcription factors, proteins of the general transcriptional apparatus, and nucleosomal proteins are essential for proto-oncogene activation (reviewed in *101,104*); HATs alone might be insufficient. Second, experiments using inhibitors of deacetylases that produce clear enhancement of histone acetylation do not generally lead to proto-oncogene activation, although there are exceptions *(97)*. Nevertheless, there are circumstances (discussed further below) where an unregulated HAT may be capable of acting as a dominant oncogene.

5.2. HATs as Targets for Viral Oncogenes

Viral infection leads to a reprogramming of gene expression that drives the cell through the cell cycle, aiding viral replication. This reprogramming requires that the virus modulates genes involved in cell proliferation, and inhibits those required for differentiation pathways. In extreme cases, this can lead to tumor formation. Two of the best-studied examples of viral proteins that can manipulate the cell in this way are the adenovirus E1A and the SV40 large T antigen proteins.

Studies have shown that these proteins predominantly target the retinoblastoma (Rb) protein or p300/CBP *(44)*. The interaction of E1A with the tumor-suppressor Rb has extensively been studied, and has been shown to relieve the repression

of the E2F family of transcription factors by Rb, driving the cell through G1 and into S phase (reviewed in *105*). One of the main results of E1A binding to p300/CBP appears to be disruption of its interaction with PCAF, which has the result of increasing cellular proliferation *(17)*. Indeed, the overexpression of PCAF can inhibit the E1A-induced increase in cell proliferation. In addition, conflicting reports suggest that E1A binding to p300 can either increase *(106)* or decrease *(107)* its intrinsic HAT activity, and p300-dependent transcriptional activity.

The modulation of HAT activity by E1A may lead to changes in expression of cellular proto-oncogenes, such as c-*fos* and c-*myc* (discussed above), or may result in inhibition of differentiation pathways. In support of this latter possibility, E1A binding to PCAF has been shown to disrupt myogenesis *(108)*. Therefore, regulation of HAT activity by E1A is one mechanism, in addition to Rb interaction, whereby viral infection can lead to cellular transformation.

Interaction of viral oncoproteins with CBP/p300 and PCAF may also mediate gene expression via more indirect routes. As mentioned above, p53 is acetylated in vivo *(87),* which increases transcriptional activation of p53-dependent genes mediating cell cycle arrest *(92)*. Modulation of CBP/p300 and PCAF HAT activity by viral oncogene binding can lead to decreases in p53 acetylation *(109)* and so transcriptional activity. Therefore, one component of E1A-mediated cellular transformation may be a reduction in the acetylation of transcription factors that mediate cell cycle arrest and apoptosis.

5.3. HATs as Potential Tumor-Suppressor Genes

Recent work shows that HATs can function as tumor-suppressor genes, i.e., loss of function and/or loss of heterozygosity (LOH) causes tumorigenicity. Experimental evidence for this hypothesis has been generated from gene knockout experiments in mice. Homozygous deletions for either *p300* or *CBP* were embryonic lethal *(47)*, suggesting an important role for these proteins during early development and indicating that these proteins were not functionally redundant. Notably, the $p300^{+/-}$ heterozygote also showed a high level of embryonic lethality, but those mice that did survive to adulthood showed no discernible phenotype. In addition, a double $p300^{+/-}$ $CBP^{+/-}$ heterozygote was also embryonic lethal. These studies imply that the overall levels of the p300 and CBP proteins are critically important during early development *(47)*.

In addition, cells derived from the $p300^{+/-}$ heterozygote were deficient in proliferation, implying a direct role for p300 in cell proliferation. Therefore, the inhibition of p300 activity by E1A, as discussed above, should lead to growth retardation, which is clearly not the case. This paradox may be explained by a differential effect of p300/CBP on gene transcription depending upon the transcription factors that bind to the promoters. Interestingly, $p300^{+/-}$ cells exhibited defects in retinoic-acid-dependent gene expression, but not in CREB-dependent gene expression *(47)*.

CBP$^{+/-}$ heterozygotes, while not being embryonic lethal, exhibited defects in hematopoiesis, and, with increasing age, an increased occurrence of hematologic malignancies *(110,111)*. These malignancies are characterized in some cases by loss of heterozygosity (LOH) in the *CBP* allele, a classical hallmark of a tumor-suppressor gene *(111)*. Notably, the loss of one allele of *CBP* is the genetic basis of Rubinstein–Taybi syndrome *(112)*. One of the characteristics of this disease is an increased risk of tumor formation *(113)*, further supporting a tumor-suppressor function for CBP.

Additionally, there have been numerous reports of mutations in *p300* or *CBP* being associated with cancers. Missense mutations in *p300*, coupled to deletion of the second allele, have been reported in colorectal and gastric carcinomas *(114)*. In addition, mutations in numerous epithelial cancers have been mapped to *p300 (115)*. These mutations included a truncation of the protein that removed the HAT domain. Furthermore, these mutations were linked to the loss of the second allele of *p300* in five out of the six cases examined. Finally, a novel homozygous mutation in *p300* that removes the bromodomain has been reported in a cervical carcinoma cell line *(116)*. Therefore, mutation of a protein–protein interaction domain of p300 may be a mechanism for disrupting HAT activity in the cell. Notably, this mutated form of p300 was severely inhibited in its ability to activate the Cdk kinase inhibitor p21 gene promoter, suggesting a mechanism for increased cell proliferation.

5.4. Chromosomal Aberrations Involving HATs in Cancer

Some of the clearest evidence for the role of HATs in cancer comes from the identification of chromosome aberrations associated with leukemias (Table 2; *117,118*). These translocations may result in unregulated HAT activity, either by the loss of critical regulatory domains or targeting of HAT activity to regions of the genome with which they are not normally associated. Notably, the amplification of the steroid receptor coactivator *ACTR* (*AIB*) locus has been linked to breast and ovarian cancers, supporting a role for unregulated HAT activity and the onset of cancer *(119)*.

The mixed lineage leukemia (*MLL*) locus has a number of chromosome translocations associated with it that result in cancer *(120)*. These translocations mainly arise as a side effect of cancer therapies, most commonly after treatment with DNA topoisomerase II inhibitors. *MLL* is present on chromosome 11q23 and has homology to the *Drosophila* trithorax protein, and so potentially regulates genes controlling development, the misregulation of which could produce cancer *(121)*. Two translocations involving the *MLL* locus that result in leukemia are caused by a fusion between *MLL* and *p300*/*CBP (122–125)*. These fusions maintain the majority of the CBP/p300 proteins, including the HAT domain.

A recent study characterized the transforming ability of an MLL-CBP fusion protein both in vitro and in vivo *(126)*. These authors found that a fusion protein containing MLL and the HAT and bromodomains of CBP was sufficient for

Table 2
Chromosome Alterations Involving HATs

HAT	*Fusion*	*Number of HAT Domains*	*Karyotype*	*Key References*
CBP	MLL-CBP	1	t(11;16)(q23;p13)	*123,124,125,126*
CBP	MOZ-CBP	2	t(8;16)(q11;p13)	*127*
CBP	MORF-CBP	2	t(10;16)(q22;p13)	*129*
p300	MLL-p300	1	t(11;23)(q23;q13)	*122*
p300	MOZ-p300	2	t(8;22)(p11;q13)	*128*
TIF2	MOZ-TIF2	2	inv(8)(p11q13)	*57,58*

in vitro and in vivo transformation. This observation suggests that the chromatin associating and modifying activities of CBP are sufficient to induce transformation *(126)*. It is also notable that 100% of mice infected with the MLL-CBP fusion protein develop leukemias very early, suggesting that this fusion protein is highly oncogenic, even though both endogenous alleles of *CBP* are intact.

A second translocation event involving CBP/p300 generates a fusion between *MOZ* and *CBP (127)* or *p300 (128)*. This translocation is associated with acute myeloid leukemia (AML). MOZ (monocytic leukemia zinc finger protein) is a protein of unknown function, but has recently been shown to possess HAT activity *(38)*. The *MOZ-CBP* fusion protein results in the fusion of the Zn-finger and HAT domains of MOZ with virtually the entire CBP or p300 protein, and therefore generates proteins containing two functional HAT activities *(126,127)*. It remains unclear if one or both these HAT domains contribute to the onset of leukemia.

Additionally, *MOZ* has been identified as being involved in a chromosome translocation in which it is fused to the nuclear receptor coactivator HAT *TIF2* (Table 2; *57,58*). This fusion results in the same type of leukemia phenotype as the *MOZ-CBP* fusion. The resulting fusion proteins contain both the HAT domains as well as the CBP interacting domain of TIF2. It is not clear whether the observed leukemia phenotype results from the fusion of TIF2 to MOZ or the ability of CBP to now interact with MOZ via interaction with TIF2.

Finally, a further translocation involving *CBP* has recently been reported. This translocation results in a fusion of *CBP* with *MORF (129)*, a member of the MYST family of HATs, again creating a fusion protein with two functional HAT domains.

Although these studies show that a role for mutations in HATs in various leukemias, it remains unclear whether the creation of these fusion proteins leads to inactivation of CBP/p300 or to enhanced or re-directed activity. The fact that the HAT domains remain intact in the majority of mutants suggests that HAT activity is retained. Furthermore, it is interesting that in a number of cases the fusion protein gains two HAT domains, possibly upregulating HAT activity at particu-

lar targets. These proteins may therefore be acting as oncogenes by increasing HAT activity and driving cell proliferation. It will be interesting to determine which, if any, genes are upregulated in such leukemias. An alternative explanation, in keeping with a potential tumor-suppressor activity, may be that the fusion protein loses its ability to interact with critical cell cycle regulators, such as p53, or transcription complexes important for maintaining the differentiated state of the cell, and that this contributes to increases in proliferation.

6. HATs AS TARGETS FOR THE DERIVATION OF CHEMOTHERAPEUTIC DRUGS

There has been some contention as to whether chromosomal aberrations observed in human cancers indicate that HATs function as oncogenes or as anti-oncogenes, an important factor in considering HATs as potential targets for pharmaceutical intervention. For example, Kung et al. *(111)* and Gayther et al. *(115)* found that loss of heterozygozity at *CBP* and *p300* loci, respectively, are implicated in specific leukemias. This finding suggests a clear tumor-suppressor function for these genes in normal cells. However, Lavau et al. *(126)* show that a fusion protein comprising MLL fused to CBP was very highly and specifically oncogenic, producing myelomonocytic leukemias in 100% of infected mice, even though they expect both host *CBP* alleles to be normal. These considerations would feature prominently in choosing a strategy to develop HATs as targets for cancer chemotherapy.

Clearly, it is inappropriate to suppose that a general HAT inhibitor or activator may be of use in treating all cancers. The administration of HAT inhibitors may enact pharmacologically what LOH at the *CBP* and *p300* loci achieve genetically and may cause or worsen the prognosis of certain types of cancers. On the other hand, if a specific HAT or HAT fusion acts in an oncogenic mode, it may be logical to try to derive specific inhibitors of that HAT, or possibly, inhibitors of fusion protein function. This derivation could conceivably be achieved not just by inhibiting HAT activity, but by interfering with fusion protein interactions, because all present indications are that HATs must be highly targeted to specific genes, and this targeting is brought about by several motifs such as bromodomains and PHD fingers that mediate these interactions. For example, the MLL-CBP fusion protein may be highly oncogenic as result of the specific targeting of the fusion protein to certain genes, and interfering with these interactions may be a potential way forward. However, high-throughput screens for compounds affecting enzyme activity are much more convenient and better established than those for altering protein–protein interactions. Despite these difficulties, the prevalence of cancer and the increasing evidence for a role for HATs in the onset and/or progression of this disease renders it an interesting target for pharmaceutical intervention.

Note added in proof: Since the writing of this chapter, a number of reports have been published that further implicate a functional role for HAT activity in the onset of cancers.

Vries et al. report that the transcription factor c-jun is acetylated in vivo, possibly by p300/CBP, and that this is essential for the repression of the collagenase promoter by EIA *(130)*. c-jun is a proto-oncogene that controls the expression of many cellular genes. The fact that E1A can repress genes through interaction with acetylated c-jun implies that inhibition of HAT activity directed toward c-jun may be one possible mechanism for preventing E1A-mediated cell proliferation.

Kitabayahi et al. have recently shown that the MYST family member MOZ is part of a complex with AML and CBFβ transcription factors. Together these factors regulate the expression of certain hematopoietic cell-specific genes and are required for differentiation of a mouse myeloid cell line into monocytes. Interestingly they also show that the fusion between MOZ and CBP, associated with the onset of acute myeloid leukemia *(see* Section 5.4), inhibits the differentiation of these cells and may indicate a mechanism for increased cell proliferation associated with cancer *(131)*.

A role for CBP/p300 in muscle cell differentiation has recently been described. Using small molecule inhibitors of CBP/p300, first described by Lau et al. *(132)*, Harel-Bellan and collegues show that the HAT activity of CBP/p300 is required for the differentiation of mouse myoblasts into myotubes *(133)*. This approach opens the way for a detailed analysis of the role of CBP/p300 in different biological processes and may lead to the development of effective clinical therapies.

Furthermore, recent reports have identified a novel HAT enzyme and also described a new yeast HAT complex. The transcriptional coactivator CIITA, which is essential for transcription of MHC class II genes, was shown to possess intrinsic HAT activity directed against H3 and H4 *(134)*. This coactivator was shown to bypass the requirement for TAFII250 in the transcription of specific genes. Additionally the yeast Sas2 protein, a MYST family member, has recently been purified in a high molecular weight complex and been demonstrated to play a role in silencing at the *HML* locus and telomeres *(135)*.

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7 Histone Deacetylases

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CONTENTS

1. INTRODUCTION

1.1. The Impact of Histone Acetylation and Deacetylation on Chromatin Structure and Gene Activity

The nucleosome is the repeating structural unit in the chromatin. It consists of the DNA and the protein part, the histones. The protein component is assembled by the core histones H2A, H2B, H3, and H4 *(1)*. The amino-terminal ends of the histone proteins are subject to post-translational modifications, namely, acetylation, methylation *(2)*, phosphorylation, and ADP-ribosylation. The reversible acetylation is an important regulator of chromatin higher-order structure and its interactions with nuclear non-histone proteins *(3)*. The level of acetylation is maintained by histone acetyltransferases (HATs) and histone deacetylases (HDACs). This chapter reviews the literature concerning the impact of histone deacetylase on chromatin structure and gene activity especially in the context of the pathogenesis and potential treatment of cancer.

The phenomenon of histone acetylation and a link to RNA synthesis was discovered by Allfrey and coworkers over 30 years ago *(4)*, but its significance remained elusive for a long time. The histones H3 and H4 are important regulators of chromatin fiber folding and interactions in between fibers *(5,6)*. Acetylation of the ε-amino group of the side chains of lysine residues in the histone proteins disrupts

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chromatin fiber folding and interactions with other proteins *(7)*. The increased DNase sensitivity of acetylated chromatin regions suggests a decondensed structure *(8)* and a high level of acetylation is also associated with high levels of transcriptional activity *(1)*.

At first it was thought the acetylation of the lysine side chain would eliminate the interaction of the positively charged lysines to the negatively charged phosphates in the DNA backbone, but after the X-ray analysis of a nucleosome particle was achieved, such a simple model could not be upheld anymore. The histone tails that are acetylated are sticking out from the core and are rather involved in the contact to neighboring particles *(9)*. Thus, it is suggested that acetylated lysines serve as flags for enzymes and cofactors involved in transcription and have an impact on chromatin superstructure.

The significance of the influence of histone deacetylase on transcription was conceived when the first mammalian HDAC was isolated, and its amino acid sequence revealed a homology to the yeast transcriptional regulator rpd3 *(10)*. Since then, an enormous amount of information has been accumulated regarding different HDAC subtypes, their interactions with other regulatory proteins, and their significance for gene regulation and possibly pharmacotherapy.

1.2. Histone Deacetylases and Histone Acetyltransferases as Mediators of Reversible Histone Acetylation and Methods for the Identification of HDAC Activity

Histone acetyltransferases and deacetylases establish and maintain the reversible acetylation of the lysine side chain of histone proteins. The acetyltransferases utilize acetyl-CoA to transfer an acetyl group to the lysine side chain. The deacetylases cleave the acetamide, which results in a protonated free amino group in the lysine side chain and the liberation of acetate.

To determine whether a newly isolated protein has histone deacetylase activity, biochemical in vitro assays are used. Traditionally, radiolabeled histones are used as the substrate and the liberation of [^{3}H]acetic acid is measured by scintillation counting. The histones can be obtained from cell cultures or from chicken reticulocytes *(11)*. Similarly, labeled oligopeptides with 8 *(12)* or 24 *(10)* amino acids that are derived from N-terminal histone sequences have also been used. For high-throughput inhibitor screening, a scintillation proximity assay using a [^{3}H]acetylated and N-terminally biotinylated peptide and streptavidin-coated beads has been established *(13)*. Alternatively, fluorescent substrates of histone deacetylase have recently been identified. A fluorescein-labeled octapeptide with the same sequence as the previously described radioactive analog was shown to be converted by rat liver histone deacetylase and could be used for the in vitro determination of site-selectivity among multiple lysine sites in a substrate *(14)*. A simple acetyl-lysine derivative that in the meantime has become commercially available was identified as a convenient nonradioactive substrate for histone deace-

tylase activity *(15)*. This derivative can be used in the purification and characterization of histone deacetylases and for the determination of inhibitors from the nanomolar to the millimolar range *(16)*. The assay is run by HPLC and allows a moderate throughput.

Histone deacetylase activity leads to histone hypoacetylation, whereas its inhibition results in chromatin hyperacetylation. Acitivity can be monitored by the isolation of histones and gel electrophoresis *(11)*, and further substantiated by immunoblotting *(17)*. There is also a high-throughput version of the antibody-mediated analysis of histone hyperacetylation *(18)*.

2. HISTONE DEACETYLASES

This chapter is intended to provide an overview of the known histone deacetylases and the mechanism of action. It also describes proteins interacting with HDAC activity and effects of that recruitment on transcriptional regulation.

2.1. Structural Features of Human HDACs

Nine human proteins with histone deacetylase activity have been described in the literature. HDAC1 *(10)*, 2 *(19)*, 3 *(20,21)*, and 8 *(22)* belong to the human class I that displays homology to the yeast protein rpd3. The class II is composed of four representatives so far [HDAC 4–6 *(23)* and 7 *(24)*], which show homology to the yeast enzyme HDA1. Additionally, another group of proteins with deacetylase activity was discovered with the protein Sir2 and similar enzymes [called group III histone deacetylases *(25)*].These group III histone deacetylases have been linked to the process of aging, as an increased expression of Sir2 in *Caenorhabditis elegans* leads to a prolonged lifespan *(26)*. Their difference from other discovered deacetylases is shown by the dependence of their activity on NAD^+ *(27)*. Additionally, histone deacetylases from other organisms, e.g., mice *(28)*, maize *(29)*, and *Plasmodium (30)* have been characterized. Some HDACs show a high degree of similarity to rpd3-type enzymes, while others are quite different, e.g., the nucleosomal acidic phosphoprotein maize HD-2 *(31)* that has been attributed a class of its own [also called class III deacetylase *(32)*]. HD-2 is so far the sole member of this class and does not show significant homology to the other classes but rather to other phosphoproteins such as B23 or FKBPs *(32)*. The class I members share about 50 % sequence identity among each other, and the sequence identity of class I toward class II is about 25%.

So far, no information on the three-dimensional structure of a mammalian or plant HDAC has been published. But a homolog was crystallized from the bacterium *A. aeolicus* termed HDLP, and crystal structure data are available *(33)*. Although this bacterium does not have histones, the enzyme is able to deacetylate histones in vitro, showing that HDACs belong to an "old" protein family, and showing similarity with archaeal and eubacterial enzymes, such as acetoin-utilization protein and acetylpolyamine amidohydrolase.*(34)* Comparison of active sites revealed

complete conservation in human HDACs. The enzyme was also co-crystallized with the inhibitors SAHA and trichostatin A (see below). The X-ray analysis revealed that the enzymatic catalysis is zinc-dependent. The metal ion is responsible for weakening the acetamide bond making it susceptible for hydrolytic attack by a water molecule. The active site is built by two charge-relay systems and bears resemblance to both metalloproteases and serine proteases. The zinc is complexed by two aspartates and a histidine, with a water molecule serving as a fourth ligand in the free enzyme. Upon binding to hydroxamate-based inhibitors, the water is displaced, resulting in a pentacoordinated zinc complex with the hydroxamic acid occupying two binding sites *(33)*.

2.2. Interaction of Histone Deacetylase with Other Nuclear Proteins and Targeted Deacetylase Activity

The second key finding after the discovery of the homology of HDAC to the yeast transcriptional regulator rpd3 *(10)* was that histone deacetylase activity is recruited by DNA-sequence-specific proteins *(35)*. These proteins are transcriptional repressors that direct the HDAC activity to certain promoters. Below, the interaction of various transcriptional repressors and nuclear receptors with histone deacetylase is discussed.

2.2.1. Transcriptional Corepressors

2.2.1.1. YY1. The transcription factor YY1 (Yin Yang 1) can act either as an activator or repressor of transcription. Numerous promoters and enhancers contain YY1-binding sites *(36)*. It was shown that binding of HDAC2 *(19)* and also HDACs 1 and 3 *(21)* by YY1 is necessary for transcriptional repression at YY1-dependent promoters. It is interesting that, on the other hand, YY1 is also able to bind the histone acetyltransferases CBP and p300 *(37)*, which are transcriptional coactivators. Therefore, the effect on transcription mediated by YY1 may be dependent on the nature of the bound chromatin-modifying enzyme.

2.2.1.2. The Sin3-Complex and Interacting Proteins. The Sin3-complex is one of the main multiprotein-complexes that have been found to be associated with HDACs. The members of the complex are Sin3, the nuclear corepressor NCoR, RbAp46, RbAp 48, SAP18, and SAP30. Sin3 is a corepressor protein that may be active in the absence of HDAC, but is necessary to mediate the repressive functions of other regulatory proteins such as Ume6 *(38)* or the Mad/Max heterodimer *(39)*. Ume6 is a DNA-binding protein that regulates genes involved in meiosis and, in that case, deacetylation of a specific lysine in histone H4 has been linked to its activity *(40)*.

Mad displaces Myc from the Myc-Max heterodimer that is responsible for the transcription of growth-stimulating genes such as the *E2F* gene *(39)*. A histone deacetylase inhibitor will relieve the Mad/Max-mediated repression occurring

throughout differentiation directly, and also by inhibiting the activity of the B-lymphocyte-induced maturation protein (Blimp-1). Transcription of the c-*myc* gene is repressed by Blimp-1 during B-cell differentiation, and thus an indirect effect on Mad/Max affected repression can be observed as well *(42)*. Therefore, HDAC inhibition may lead to an increased activity of *myc*-dependent pathways, which seems counterproductive for cancer therapy. On the other hand, *myc* works in part by suppressing the expression of the tumor-suppressor protein p21 and the expression of the latter is increased after histone deacetylase inhibition *(43)*.

The RbAp proteins are thought to influence the interaction of enzymes involved with histone acetylation with their substrates *(44)*, and mediate the interaction of HDAC to the retinoblastoma protein pRB (see below) *(45)*. Another histone deacetylase complex called CoREST does not contain RbAp proteins, but the CoREST protein showing homology to polyamine oxidase has been described recently *(46)*. SAP30 is required for NCoR-mediated repression by antagonist-bound estrogen receptor, but not for NCoR-mediated repression by unliganded retinoic acid receptor, suggesting that SAP30 is involved in the recruitment of the Sin3–HDAC complex to a certain subset of NCoR corepressor complexes *(47)*.

2.2.1.3. The Mi2- or NuRD-Complex. The Mi2 complex of HDACs 1 and 2 also contains RbAp proteins, and additionally MTA2 and MBD3. Mi2 is a polypeptide related to the metastasis-associated protein 1 (MTA1) that occurs in patients with dermatomyositis who have a high rate of malignancy *(17)*. This complex is also associated with ATP-dependent chromatin remodeling activity *(48,49)*, and has thus also been termed NuRD (nuclear-remodeling histone deacetylase complex).

2.2.1.4. Retinoblastoma Protein (pRb). The retinoblastoma protein is a tumor-suppressor protein that exerts its beneficial activity by binding the transcriptional activator E2F *(50)*. This binding is critical for downregulating the activity of E2F-dependent genes such as thymidine kinase or dihydrofolate reductase. It has been shown that this effect of pRb is mediated by the association with histone deacetylase *(51–53)*. In light of this information, again an inhibition of histone deacetylase seems counterproductive for the treatment of cancer. However, the net effect of histone deacetylase inhibitors on cancer cell growth is proving otherwise and possible explanations are given in Section 3.1.3.

2.2.1.5. ATM. The *ATM* gene is mutated in patients with ataxia telangiectasia, a disease characterized by high sensitivity to radiation. The ATM protein acts as a sensor of radiation-induced cellular damage, and contributes to cell cycle regulation, signal transduction, and DNA repair. It has been shown that ATM associates with HDAC, that the extent of association is increased by ionizing radiation *(54)*.

2.2.1.6. Others. For numerous other proteins an interaction with one or several of the HDACs has been described *(32)*. While some interact via the two major protein complexes (Sin3 or NuRD), there are other interactions where such a link has yet to be established. One example with particular interest to cancer is *BRCA1*.

BRCA1 was the first breast cancer susceptibility gene to be identified. Mutations in *BRCA1* increase the risk of developing breast cancer *(55)*, and are associated with HDAC activity *(56)*. An association of HDAC4 with MAP kinases and its phosphorylation also links chromatin remodeling with the Ras pathway *(57)*. HDAC also associates with DNA-topoisomerase II that is involved in DNA replication and repair *(58)*. Interactions of HDACs 4 and 5 with the cytoplasmatic 14-3-3 proteins regulate availability of those deacetylases in the nucleus *(59)*. Another extracellular association has recently been described for HDAC7 and the endothelin receptor *(60)*.

2.2.2. Nuclear Hormone Receptors

The thyroid receptor interacts in its unliganded state with a corepressor complex containing histone deacetylase. Upon binding of the natural ligand triiodothyronine (T3), the corepressor activity is exchanged with coactivator complexes containing HAT activity *(61)*. T3 and retinoic acid (RA) receptors both regulate transcription of certain genes through binding to a common hormone response element (HRE) in the promoter *(62)*.

The retinoic receptors RAR and RXR also bind HDAC in their unliganded state and repress transcription. Binding of the natural retinoids all-*trans* retinoic acid (at RAR) and 9-*cis* retinoic acid (at RXR) leads to displacement of the corepressor complex with concomitant binding of activator proteins and transcriptional activation *(63)*. Agonistic activity at retinoid receptors leads to terminal cellular differentiation and inhibition of cancer cell proliferation *(64)*.

Similar findings have been made for the estrogen receptor *(65)*, and repression by estrogen antagonists relies on the NCoR-dependent recruitment of HDAC, thus casting doubt on the use of HDAC inhibitor therapy in sexual-hormone-dependent cancers *(47)*. Nevertheless, estrogen-dependent MCF-7 breast cancer cells are suppressed in their growth by HDAC inhibitors *(66)*. Additionally, HDAC inhibition leads to estrogen receptor re-expression in non-estrogen responsive breast cancer cells, which may open new opportunities for certain estrogen receptor negative cells *(67)*.

2.2.3. Crosstalk with DNA Methylation

The methylation of cytosines in DNA is another pathway of transcriptional repression *(68)*. In the last two years, experimental evidence has shown that there is a connection between DNA methylation and histone acetylation. Methylated DNA in the chromatin binds to the transcriptional repressor MeCP2, which has been shown to associate with the Sin3/HDAC complex. The repression mediated by MeCP2 and methylated DNA can be reversed by inhibition of histone deacetylase *(69,70)*. Another protein that is able to recognize methylated DNA is the demethylase MBD2, which is a member of the Mi2–HDAC complex *(71)*.

2.3. Deacetylation of Non-Histone Substrates

As of now, there are only two examples of proteins other than histones that can be substrates for histone deacetylases. There are already more examples of proteins that are acetylated by histone acetyltransferases and, thus, reversible protein acetylation might be a general mechanism of controlling protein activity such as phosphorylation.

One example is the important tumor-suppressor protein p53, which can be acetylated by the HAT CBP *(72)*. While acetylation leads to activation, deacetylation results in a decreased activity of the p53-dependent BAX-promoter *(73)*. The interaction of p53 with HDACs is mediated by the MTA2 (also termed PID) protein present in the NuRD complex *(74)*. PID expression leads to a diminished response of p53 pathways and is able to modulate p53-conferred growth arrest and apoptosis. Therefore, HDAC inhibitors may also exert their anticancer activities in part by restoring otherwise suppressed p53 function.

The other example for non-histone deacetylation substrates is the E2F-class of transcription factors. Acetylation by p300 and CREBP increases the affinity of E2F to its binding site on DNA, while HDAC is able to mediate the deacetylation *(75)*. Again, this seems to be a potential counterproductive effect of histone deacetylase inhibitors because it should contribute to the relief of pRb-controlled transcriptional repression.

3. HISTONE DEACETYLASE AND CANCER

While section 2 contains some general information on regulation of transcription via HDAC and also possible links to cancer, here specific findings that concern certain types of malignancies are presented. We describe the role of HDACs in the activity of certain oncogenes in the pathogenesis of cancer, and present inhibitors of histone deacetylase along with data concerning their existing or potential application in cancer therapy.

3.1. Examples of Deacetylase–Cancer Links

3.1.1. Fusion Proteins and Cancer

The involvement of HDACs in the pathogenesis of malignant disease on a molecular level was first proven in acute promyelocytic leukemia (APL). Mutant oncogenic receptor fusion proteins PML-RARα and PLZF-RARα that are formed by chromosomal translocations recruit HDAC to suppress the differentiation process in the leukemic cells *(76,77)*. The histone deacetylase inhibitor trichostatin A is able to increase retinoid activity in PML-RARα cells and overcome retinoid resistance in the PLZF-RARα cells. This resistance is based on an additional HDAC-binding site in the PLZF-fusion protein that is not retinoid sensitive. There is a case study where a patient with a retinoid resistant APL was treated with a combination

of a retinoid and the HDAC inhibitor phenylbutyrate. A complete remission of disease was achieved. This study shows that histone hyperacetylation can be achieved relatively safely in humans, and that the link between HDAC activity and cancer pathogenesis can be used for causal treatment *(78)*.

In acute myeloid leukemia (AML), the *AML1* gene is disrupted by a translocation that results in a fusion to the ETO (eight, twenty-one) protein. This and similar fusions inhibit *AML1*-dependent transcription *(79)*. It has been shown that inhibition is mediated by NCoR and Sin3-dependent recruitment of histone deacetylase. A HDAC inhibitor impairs the ability of the fusion protein to repress transcription *(80,81)*. Retinoic signaling can be restored by HDAC inhibition in primary blasts from AML patients *(82)*. PML-RAR and AML1-ETO were shown to exist in oligomeric forms that recruit NcoR, and oligomerization of transcription factors was recognized as new way of oncogenic activation *(83)*.

Another example is the *LAZ3/BCL6* (lymphoma-associated zinc finger 3/B cell lymphomas 6) gene that is frequently altered in non-Hodgkin lymphomas. This gene encodes a transcriptional repressor that contains a conserved domain called BTB/POZ (bric-a-brac tramtrack broad complex/pox viruses and zinc fingers). This domain interacts with the SMRT protein, and thus the Sin3–HDAC complex *(84,85)*. Thus, increasing evidence accumulates that links aberrant HDAC recruitment to the pathogenesis of certain cancer types *(86)*.

3.1.2. Disruption of Normal Processes by Oncogenes

There are a number of examples that link oncogenesis and HDAC recruitment. V-ErbA, the mutated form of the thyroid receptor, exerts its transforming activity by NCoR-dependent recruitment of HDAC *(87,88)*. V-ski, a viral form of the protein c-ski, contributes to cancer pathogenesis by disturbing HDAC recruitment. V-ski lacks a Sin3-binding domain and abrogates Mad- and pRb-mediated repression *(89,90)*. E7 is the main transforming protein of human papilloma virus type 16 (HPV16), which disturbs the pRb/E2F interaction and is involved in the formation of cervical cancer. Mi2, a component of the NuRD histone deacetylase complex, binds directly to the E7 zinc finger. The binding to a histone deacetylase complex is an important parameter for the growth-promoting activity of the E7 protein *(91)*. Thus, HDAC inhibitors may have potential for cancer chemoprevention in papilloma virus-positive patients.

3.1.3. Links on the Protein Expression Level

Inhibitors of histone deacetylase may lead to induction of differentiation, growth arrest, and/or induction of apoptosis in cancer cells. The main mediator of the anticancer activity of HDAC inhibitors is thought to be p21/WAF1/CIP1 *(92,93)*. p21 is an endogenous inhibitor of cyclin-dependent kinases (cdks) and leads to pRb hypophosphorylation, which, in turn, activates its repressive functions that are not all involving HDAC recruitment *(32)*. An increase in the accumulation

of acetylated histones was monitored at the p21(WAF1) promoter proving a direct effect on p21 expression *(94)*. Thus, this finding may explain why HDAC inhibitors lead to growth arrest despite the potential of hampering some of the pRb-mediated pathways.

Other examples of the effects on the protein level are down-regulation of c-Myc *(95)*, cyclin D1 *(96)*, surface adhesion molecules *(97)*, or the antimetastatic protein Drg-1. Drg-1 induces changes consistent with differentiation in colon cancer cells and in turn up-regulates the expression of several colonic epithelial cell differentiation markers *(98)*. The mechanism of induction of apoptosis is largely unclear, but an up-regulation of the mediator of apoptosis, caspase-3 *(99)*, seems to be an important event. A pan-caspase inhibitor prevents apoptosis after histone hyperacetylation in breast cancer cells. Interestingly, in the same study, it was shown that normal breast cells are far less sensitive to HDAC inhibitor treatment *(100)*. An increased expression of TGFβ type II receptor was observed following HDAC inhibition, and the potentiation of TGF signaling pathways is suggested as a possible mechanism of the anticancer activity of HDAC inhibitors *(101)*.

3.2. Histone Deacetylase Inhibition and Cancer

This section is intended to give a complete review on the HDAC inhibitors available today with an emphasis on reported anticancer activities. A more-detailed report on structure-activity data can be found in a review article *(102)*. So far there are little data on subtype selective inhibition of HDACs and the outcome of such an inhibition is unclear. Selective inhibition of HDAC subtypes by antisense oligonucleotides has been patented *(103)*.

3.2.1. Butyrate, Phenylbutyrate and Related Short Chain Fatty Acids

The inhibition of histone deacetylase by butyrate has been known for almost 20 years, and its differentiating effect on cancer cells has been demonstrated *(104)*. While there was some efficacy in a case of AML *(105)*, the clinical results were usually disappointing *(106)*, owing to rapid metabolism and weak inhibitory potency requiring extremely high doses. The odor developed by the patients due to the appearance of the acid in the sweat and breath further limits its use *(107)*. Phenylacetate and phenylbutyrate display HDAC inhibitory and anticancer properties similar to butyrate *(108)*. Structure-activity relationships show that the fatty acids are inhibitors in the high-micromolar or low-millimolar range *(109)*. The most promising clinical results stem from the use of phenylbutyrate together with retinoic acid as mentioned above *(78)*. Phenylbutyrate is known for its encouraging safety profile from the treatment of urea-cycle disorders *(110)* or thalassemia *(111)*. Generally, multigram doses have to be applied. The use of prodrugs such as tributyrin *(98)* or pivaloyloxymethylbutyrate *(112)* may lead to an improvement.

3.2.2. Trichostatin A and Analogs

(*R*)-Trichostatin A (TSA) **1** (*see* Fig. 1) from *Streptomyces hygroscopicus (113)* was initially discovered as an antifungal agent, but later identified to be an inducer of terminal cell differentiation that effects histone hyperacetylation *(114)*. TSA is an inhibitor in the low-nanomolar range, but the corresponding carboxylic acid is devoid of activity *(115)*. The enantiomer of **1** does not show inhibitory activity *(116)*. The anticancer effects of trichostatin A have been demonstrated on a multitude of cell lines. TSA has been studied in numerous models of leukemia (see above) as well as solid tumors. Induction of apoptosis in colorectal carcinoma cells has been shown *(99)*, as well as induction of differentiation in prostate cancer cells *(117)*. Trichostatin A was also effective in a mouse model of breast cancer, where it displayed low toxicity *(118)*. Amide analogs **2** of trichostatin A are inhibitors of HDACs and inducers of terminal cell differentiation in Friend mouse erythroleukemia cells (MELC) in concentrations down to 600 n*M (119,120)*. The enyne oxamflatin **3** is also structurally related to trichostatin, with an IC_{50} value of less than 1 μ*M (121)*. Another compound containing a 6-aminocaproic acid spacer is a compound termed Scriptaid **4** that induces histone hyperacetylation and leads to a facilitation of transcriptional events *(122)*.

3.2.3. Cyclic Tetrapeptides

A set of hydrophobic cyclotetrapeptides from natural sources that all have in common the presence of the unusual amino acid (*S*)-2-Amino-9,10-epoxy-8-oxodecanoic acid (L-Aoe) were also identified as inhibitors of histone deacetylase *(123,124)*. The epoxyketone was first thought to be essential for activity, as reduction or nucleophilic attack resulted in inactive compounds *(123,124)*. While trapoxin A **5a** was shown to be an irreversible inhibitor in the low nanomolar range *(123)*, contrary findings were made for the related HC toxin **5b**, which inhibits maize enzyme reversibly *(124)*. An analog of **5a** called K-trap was prepared *(125)*, coupled to an agarose matrix and used for the first isolation of HDAC-1 as mentioned above. The discovery of apicidin **5c** and apicidin A, which only possess a ketone function but still are active in the low-nanomolar range, has shown that the presence of the epoxy group is not a prerequisite for activity. **5c** was isolated in an antiparasital screening and inhibits the histone deacetylase of pathogens such as *E. tenella* but also suppresses leukemia cell growth *(12)*. A number of derivatives such as the 9-hydroxy- or 9-acyloxyapicidins have been patented *(126)*. Recently, FR225497 (the corresponding ketone analog of trapoxin A) has been isolated from *Helicoma sp.* and patented as an agent with potent inhibitory action on histone deacetylase and anticancer activities *(127)*. There are also trapoxin analogues that combine cyclotetrapeptide and hydroxamic acid moieties *(128)*. While D-L-L-(A_1-A_3),L-L-D-, and D-L-D isomers in the latter series are highly active, the L-D-L isomer was inactive *(129)*. The epoxyketone function cannot generally be replaced by simple ketones as the corresponding analog of HC

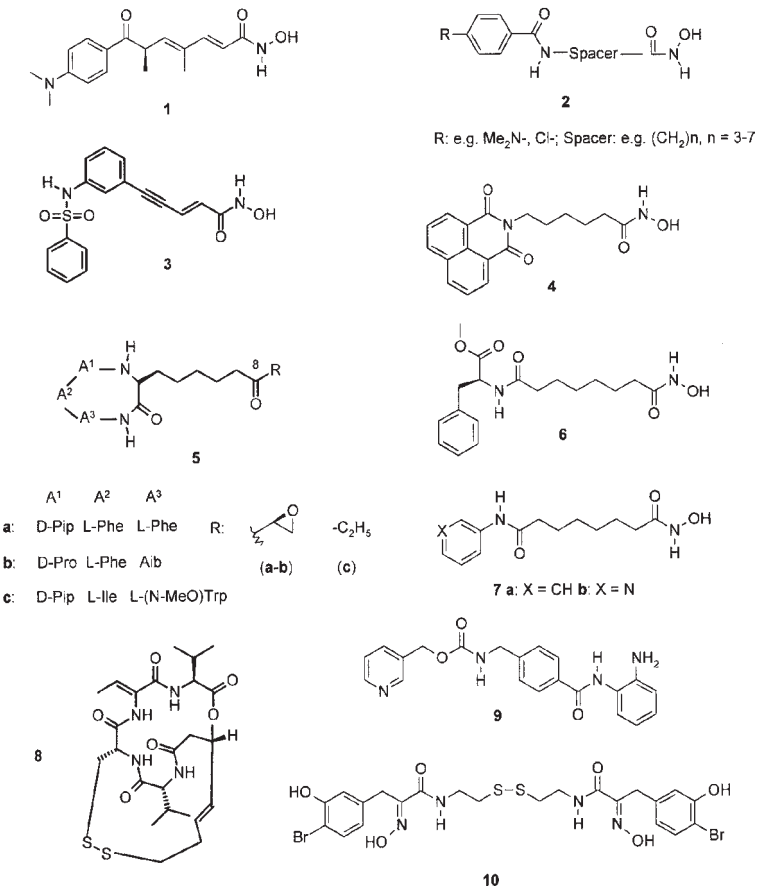

Fig. 1. Inihibitors of histone deacetylase.

toxin is inactive *(130)*. The importance of an aromatic amino acid, especially tryptophan or derivatives thereof, has been highlighted *(130)*. Exceptionally potent inhibitors are some quinolone analogs *(131)*, and the hydroxamic acid analog of apicidin *(132)*. The most potent HDAC inhibitor described so far is an epoxyketone analog of apicidin (IC_{50} < 100 p*M*) *(132)*. Apicidin also shows an increase in p21 expression associated with its anticancer activity *(133)*, and its anti-invasive and detransforming activities in H-ras-transformed breast cancer cells, MCF10A cells, shows potential for the prevention of metastases as well

(134). Another recently discovered tetrapeptide without an epoxy group is the diol diheteropeptin, but its activity is only observed at 25 μ*M (135)*. A natural product inhibitor also containing epoxide functions is depudecin that was isolated from the fungus *A. brassicicola (136)*.

3.2.4. Analogs of Tetrapeptides

Simple analogs of cyclic tetrapeptides that also contain suberic acid linkers but hydroxamate instead of epoxyketone or ketone functions such as M232 **6** *(119)* are inhibitors of HDAC in the low-micromolar range *(16)*. Together with some of the trichostatin amide analogs **2**, they were the first synthetic non-tetrapeptide compounds that were discovered as inhibitors of histone deacetylase activity with potency in the submicromolar range.

The structurally related hybrid polar compounds (HPC), also termed hybrid polar drugs in the earlier literature, form a class of compounds that were known as inducers of cell differentiation for some time *(137)*. The first representative was hexamethylenebisacetamide (HMBA), which is active against cancer cells in the millimolar range *(138)*. So-called second-generation HPCs such as SAHA (suberoylanilide hydroxamic acid) **7a**, SBHA (suberic bishydroxamic acid), or CBHA (m-carboxycinnamic acid bishydroxamide) exert their differentiating activity in the low-micromolar range, and were later identified as inhibitors of HDAC as well, while this is not the case for HMBA *(139)*. Also, a similar urea analog is a HDAC inhibitor *(139)*. SAHA **7a** has been proven to be active in the chemoprevention of chemically induced mammary tumors in the rat *(140)*, as well as in the regression of established tumors in the same model. SAHA has entered a phase I clinical trial for the treatment of cancer *(141)*, and such a trial is intended for an analog called pyroxamide **7b** active in a rat mammary cancer model *(142)*. N-alkylation in hydroxamic-acid-based HDAC inhibitors leads to a loss in activity demonstrated on the N-methyl derivative of SAHA *(16)*. The related azelaic bishydroxamate has been reported to trigger a G2 phase cell cycle checkpoint, which is defective in several tumor cell lines. This leads to aberrant mitosis and eventually cell death with a selectivity for tumor cells versus normal cells *(143)*.

3.2.5. Depsipeptide and Thiols

The depsipeptide FR901228 **8** (now termed FK228) had already entered a clinical trial for the therapy of cancer due to its preclinical activity when it was discovered to be a HDAC inhibitor in the low-nanomolar range *(144)*. Its pro-apoptotic features again are paralleled by an increased p21 expression, a phosphorylation of Bcl-2 *(145)*, down-regulation of cyclin D1, and up-regulation of cyclin E *(146)*. The phase I trial showed that reversible thrombocytopenia was the dose-limiting side effect *(147)*. A total synthesis has been achieved *(148)*, but, so far, the mode of enzyme inhibition is only subject to speculation. Opening of the disulfide bridge leads to a thiol that may be able to enter the active site and

complex the zinc ion. In that regard it is noteworthy that garlic constituents and their metabolites such as diallyldisulfide (up to 40 % inhibition between 40 and 100 μ*M*) and allylmercaptan (IC_{50} = 40 μ*M*) also are inhibitors of HDAC *(149)*.

3.2.6. Benzamides (MS275)

A series of benzamides is described to have HDAC inhibitory properties in the low micromolar range. A 2'-hydroxy or amino function seems to be essential for the activity and the IC_{50} values are in the low-micromolar range *(150)*. The 2'-amino compound MS-275 **9** from that series (called MS-27-275 earlier) is the first HDAC inhibitor with oral anticancer activity in an animal model. No severe side effects in the mice were registered *(151)*. The structurally related acetyldinaline is currently in clinical trials for the treatment of cancer *(152)*, but no information on HDAC inhibition has been published.

3.2.7. Psammaplins

A new inhibitor from natural sources is psammaplin A **10**, which contains an α-oximatoamide function as a novel group responsible for inhibition at the catalytic side of HDAC. The disulfide group is also an essential feature for HDAC inhibition by **10** and a series of synthetic analogs is presented as well *(153)*.

3.2.8. Synergistic Actions with Other Anticancer Agents

The most data available concern the interaction with HDAC inhibitors and retinoids in different models of leukemia as discussed above, but other types of carcinoma show a potentiation of retinoid activity as well *(154)*. A synergy of retinoic acid and the hybrid polar drug CBHA was also shown in a mouse model of neuroblastoma. CBHA displayed activity as a single agent and was well-tolerated *(155)*. Another very promising synergy has been demonstrated with agents leading to DNA hypomethylation, thus attacking two different but interrelated modes of transcriptional silencing. A combination of trichostatin with azacytidine leads to a dramatic potentiation in the activation of silenced genes *(156,157)*, and the DNA methylation status has an important impact on the effectiveness of induction of apoptosis by depsispeptide *(158)*. Also, classic cytostatic agents have been combined with HDAC inhibitors. For example, doxorubicin enhanced cytotoxicity of depsipeptide *(159)*. On the other hand, trichostatin also increased the expression of a protein responsible for efflux of cytostatic agents from cancer cells *(160)*. The topoisomerase II interaction with HDAC seems to be necessary for the activity of etoposide, and inactivation by trichostatin A occurred. On the other hand, no effect was observed on the topoisomerase-I-dependent camptothecin *(161)*.

4. OUTLOOK

An increasing wealth of information is linking histone deacetylase activity to the pathogenesis of cancer. This chapter reviewed some of those connections

with regard to protein–protein interactions and consequences on the protein expression level. Further information can be gained from numerous reviews *(32,63, 162–173)* in the field. Those findings and the data from the anticancer activity of HDAC inhibitors show highly promising potential for histone deacetylase as a target for anticancer therapy. One major concern is of course the safety when attacking such a fundamental mechanism, but the few animal studies available (see above) are encouraging. The gene expression during mouse embryogenesis was affected, but without apparent toxicity *(174)*. Many open questions await further research in the field. Such issues are the significance of the HDAC subtypes respectively, a potential benefit of selective inhibition of those, the question of tissue distribution of various subtypes, the role of accessory proteins in HDAC multiprotein complexes, the significance of extranuclear deacetylase activity, or the relevance of non-histone deacetylation. The data from the ongoing clinical trials are awaited with great interest to see whether the promise may be fulfilled in the future.

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8 Cyclin-Dependent Kinases and Their Small-Molecule Inhibitors in Cancer Therapy

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Contents

1. INTRODUCTION

Cell cycle, the process by which cells reproduce, plays a central role in the growth and development of all life *(1–4)*. Deregulation of cell cycle control, leading to a net increase in the total number of cells, is one of the initial events in the development of all cancers *(5)*. Most drugs that are used to treat cancer rely on this differential cell proliferation to achieve selective toxicity. Considerable progress has been made in understanding cell cycle progression in normal and neoplastic situations, the challenge is to translate this understanding into useful cancer therapies *(6–18)*. In this chapter, we discuss the role of cyclin-dependent kinases (CDKs) in cell cycle transitions and how their activity is controlled in normal and cancerous states. We will discuss the modulation of CDK activity by small-molecule inhibitors, and how some of these drugs act in preclinical and clinical models of cancer.

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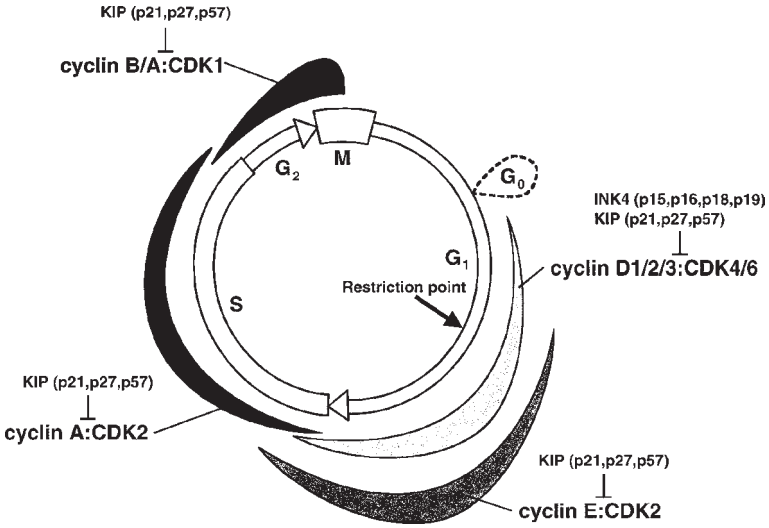

Fig. 1. The four phases of cell cycle progression: Early G_1 phase where mitogenic and antimitogenic signals are integrated. Prior to commitment to passage through restriction point, the cells can withdraw from cell cycle and become quiescent (G_0). Once cells pass through the restriction point, they are irreversibly committed to cell cycle progression. Passage through restriction point is marked by phosphorylation of retinoblastoma (Rb) protein by G_1 CDKs (CDK4/6 and CDK2). The appearance and disappearance of different CDKs is controlled by interaction with INK4 (inhibitor of CDK4) or KIP (kinase inhibitory proteins) families of CDK inhibitory proteins. DNA synthesis (S) and preparation (G_2) for mitosis (M) are three other phases of the cell cycle.

Cell division is a highly regulated and organized process that can be divided into four phases (Fig. 1). After reaching a sufficient size, cells make a commitment to divide (early G_1 phase) by integrating environmental cues *(19,20)*. Cells committed for division are said to have passed a restriction point *(21)*. Committed cells prepare for division (late G_1 phase, preparation for DNA synthesis), duplicate their DNA (S phase), duplicate centrosomes to facilitate chromosome segregation (G_2 phase), and then separate the duplicated chromosomes into two daughter nuclei (M phase). The daughter cells can opt for differentiation or for additional rounds of division (additional cycles of G_1, S, G_2, and M phases) or for quiescence, where the cells are alive but not dividing (G_0 phase). One of the major conceptual advances in the cell cycle research is the recognition of checkpoints that establish the biochemical dependency of cell cycle transitions unrelated to substrate–product relationship *(22,23)*.

Cell cycle transitions are mediated by cyclin-dependent kinases (CDKs, catalytic partner) *(19,24)*, heterodimeric enzymes that require activation by associated cyclins (regulatory partner). CDK2, CDK3, CDK4, and CDK6 mediate passage through restriction point *(21)*. Dominant-negative mutants of CDK1 arrest cells in the G_2/M phase transition, whereas mutants of CDK2 and CDK3 cause a G_1 block *(25)*. Although there have been some studies on CDK3 *(26,27)*, the precise role of CDK3 in G_1/S phase transition is not well understood, and will not be considered in this chapter. Three different cyclin Ds (D1, D2, and D3) are known to interact with CDK4 or CDK6 in a combinatorial fashion to activate their kinase activity. D-Cyclins are synthesized in early/late G_1 phase of the cell cycle and their synthesis is considered as a delayed early response. Cyclin E activates CDK2, and probably CDK3, and is expressed in late G_1 and early S phase. Cyclin A can also activate CDK2, and begins to appear during G_1/S transition and stays throughout S phase. As cells enter G_2 phase, cyclin A:CDK2 complexes are replaced by cyclin B/A:CDK1 (cdc2) complexes, which persist into M phase (Fig. 1). Activity and specificity of CDKs are controlled by the nature of the associated cyclins. Cyclins are less abundant than CDKs; cyclin A2 and B1 are 30-fold less abundant than CDK1 and cyclin A2 and E are 8-fold less abundant than CDK2 *(28)*, and their levels vary during different phases of the cell cycle *(29)*. These variations are one of the means by which CDK activity is controlled during cell cycle. Another way by which CDK activity is controlled is by interaction with CDK inhibitory proteins (CKIs). There are two families of CKIs: INK4 (inhibitor of CDK4) family—p15, p16, p18, and p19—and KIP (kinase inhibitory proteins) family—p21, p27, and p57 *(19,24)*. The INK4 family of proteins interacts exclusively with CDK4/6, whereas the KIP family of proteins interacts with CDK2 and CDK4/6. The sequential appearance and disappearance of these inhibitors are important in ensuring that initiation of subsequent steps is delayed until the completion of earlier steps *(19,24)*.

One of the major functions of CDK4/6 is to inactivate the growth-inhibitory effects of retinoblastoma protein (pRb) by phosphorylation *(30,31)*. pRb binds to E2F and modulates the transcriptional activity of E2F. Many of the genes, including cyclin E, whose products are necessary for DNA replication are transcriptionally activated by E2F *(32)*. pRb inhibits E2Fs transcriptional ability. In addition, pRb can mediate active repression by recruiting histone deacetylases (HDACs), and chromosome remodeling BRG1, SWI/SNF complexes *(30)*. Transcriptional activity of E2F can be enhanced by interactions with coactivators CBP/p300. Functional inactivation of pRb by phosphorylation is complex *(33,34)*, and is mediated by multiple phosphorylation events mediated by CDK4/6 and CDK2 *(30, 35–37)*. One of the downstream effectors of pRb is cyclin E, whose expression can lead to an autocatalytic inactivation of pRb and increased E2F activity. Unlike CDK4 and CDK6, which have narrow substrate specificity, CDK2 has very broad substrate specificity. Cyclin E:CDK2 can phosphorylate p27, targeting p27 for

proteolysis *(38)*, thus establishing a positive feedback loop and promoting G_1 to S transition *(39)*. Cyclin E:CDK2 can establish a similar positive feedback loop phosphorylating cdc25A and increasing its activity to dephosphorylate inhibitory phosphorylation of CDK2 *(40)*. Cyclin E:CDK2 phosphorylation is coupled to histone biosynthesis through the phosphorylation of NPAT *(41,42)*. Cyclin A:CDK2 is involved in DNA replication, probably by phosphorylating CDC6 and ORC1 *(43)*, and in centrosome duplication by cooperating with E2F and additional unknown CDK2 substrates *(44)*. Cyclin A:CDK2 can inactivate E2F by phosphorylating DP1 *(45)* whereas cyclin E:CDK2 can activate E2F by phosphorylating E2F5 *(46)*. Multiple activities of cyclin E may be contributing to its ability to overcome the need for pRb inactivation during G_1 to S phase transition *(47,48)*, and to the suppression of many defects associated with a cyclin D1-null by a cyclin E→D1 "knock-in" *(49)*.

The importance of deregulated CDK activity in proliferative disorders, in particular cancer, is widely recognized and there are several excellent reviews covering various aspects of cell cycle research *(4,14,15,19,20,24,50–54)*. In this chapter we concentrate on those CDKs that participate in G_1 to S transition, with a particular emphasis on the recent progress made in discovering small molecule CDK inhibitors.

2. CONTROL OF CDK ACTIVITY

Figure 2 presents some of the ways of controlling CDK activity in cells. In this section, we will discuss some of the pharmacological approaches for manipulating CDK activity.

2.1. Cyclin Levels (Fig. 2, 1 and 2)

Cyclins are regulatory partners of CDKs and their association increases the enzymatic activity of CDK by about 100-fold by moving PSTAIRE helix into the catalytic cleft and rotating it by 90º. Cyclin binding also moves the T loop out of the catalytic cleft of CDK relieving the blockade of the ATP binding site *(55)*. As the name cyclin implies, levels of cyclins vary during different phases of cell cycle (cyclin D peaks in early to mid-G_1, cyclin E peaks in late-G_1 and S, cyclin A peaks in S, and cyclin B peaks in G_2/M phases). Mitogens signal synthesis of cyclin D (Fig. 2, 1). One could inhibit mitogen signaling to control cyclin D1 level, thus inhibiting cell cycle progression. The abrupt transitions in cyclin levels are largely brought about by ubiquitin-mediated proteolysis (Fig. 2, 2) *(29,56–60)*. One could visualize enhancing this proteolysis as a way to modulate cyclin levels, thus decreasing CDK activity. However, it is important to recognize that increased proteolysis contributes to a decrease in p27 level, which can increase CDK activity *(61,62)*. It will be necessary to selectively degrade cyclins without affecting p27 levels if this approach for drug discovery is to be useful. Selectively decreasing the synthesis of cyclins is a difficult target from a drug discovery perspective.

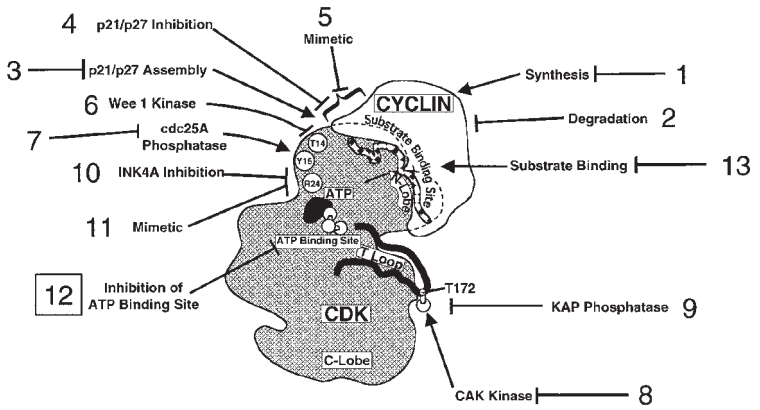

Fig. 2. Different ways of modulating CDK activity are indicated by the numbers in the figure. Most of the currently available small molecule inhibitors compete with ATP-binding site *(12)*, although some of the peptide inhibitors mimic the action of INK4 or KIP inhibitors *(5,11)*. Activating phosphorylation occurs on T160 (CDK2) or on T172 (CDK4). Inhibitory phosphorylation occurs on T14 and Y15 (CDK2) or on Y17 (CDK4). CDK4 interaction with p16 is dependent on a critical R24 residue in CDK4.

2.2. Assembly (Fig. 2, 3)

Recombinant cyclin E and CDK2 can associate with each other forming functional cyclin E:CDK2 complexes. However, a similar situation does not hold for the formation of functional cyclin D1 and CDK4 complexes *(63)*. This dichotomy has raised the question of additional proteins participating in the assembly process. Several lines of investigations have led to the idea that heat shock protein Hsp90 and CDC37 play an important role in the assembly of functional cyclin D1:CDK4 complexes *(64,65)*. Although p21 and p27 were initially identified on the basis of their ability to inhibit CDK activity *(24)*, they also function to enhance the assembly of cyclin D1:CDK4 complexes (Fig. 2, 3). Cells nullizygous for both p21 and p27 are defective in their ability to form functional cyclin D1:CDK4 complex *(66)*. However, recent work raises questions about the generality and the significance of p21/p27 mediated assembly of cyclin D3:CDK4 *(67)*. Maturation of human cyclin E requires eukaryotic chaperonin CCT protein *(68)*. Chaperonins are known to be associated with several cell cycle regulatory molecules, viz., CDK4, wee1, pRb, and p27 *(69)*. It is difficult to visualize how one can build selectivity with chaperonins as a drug discovery target.

2.3. CDK Phosphorylation (Fig. 2, 6–9)

CDK activity is inhibited by threonine and tyrosine phosphorylation (Fig. 2, 6) carried out by wee1 kinase *(70)*, and is enhanced by threonine phosphorylation (Fig. 2, 8) carried out by CDK activating kinase (CAK) *(56,71,72)*. Cdc25 reverses the inhibitory phosphorylation of CDKs by dephosphorylation (Fig. 2, 7) *(73,74)*. Cdc25A is considered to be a drugable target, which when inhibited could lead to a decrease in CDK activity *(73–75)*. A discussion of cdc25A as a drug target is outside the scope of this review.

Optimal phosphorylation of CDKs results in a 100,000-fold increase in catalytic efficiency and an approximate 1,000-fold increase in the overall turnover rate by reorganizing the substrate-binding site *(55,76)*. Activation by threonine phosphorylation is mediated by CAK, a complex containing cyclin H:CDK7 (Fig. 2, 8), and this phosphorylation is reversed by a phosphatase (Fig. 2, 9). There does not seem to be any change in the level/activity of this enzyme that is related to the phase of cell cycle or to the neoplastic state of the cell *(77)*, making it difficult to discover selective inhibitors to this target. This situation is made more uncertain with the recent suggestion that a new gene may code for the actual enzyme mediating this phosphorylation *(71,72)*.

2.4. CDK Inhibitory (CKI) Proteins (Fig. 2, 4, 5, 10, and 11)

Of the two families of CKI proteins, INK4 family of proteins (p15, p16, p18, and p19) selectively bind to CDK4 or CDK6, but not to CDK2, and KIP family of proteins (p21, p27, and p57) bind to heterodimeric cyclin:CDK complexes (Fig. 2, 4). INK4 family of proteins binds to either monomeric CDKs or to heterodimeric CDKs resulting in an inhibition of CDK activity. KIP family of proteins binds only to heterodimeric cyclin:CDK complexes. The consequence of this binding depends on the particular CDK present in the complex. Binding of KIP family to cyclin D:CDK4 does not result in the inhibition of CDK activity. However, cyclin E:CDK2 activity can be completely shut down by the binding of p27 *(24)*. CDK4 and CDK6 can act as a sink to titrate KIP proteins away from CDK2, increasing CDK2 activity *(24,78)*. In the trimeric p27:cyclin A:CDK2 complex, p27 helix mimics the ATP substrate, and the insertion of p27 helix into the catalytic cleft directly blocks ATP binding *(55)*. In contrast to KIP family, INK4 family of proteins (p15, p16, p18, p19) bind only to CDK4/6 (Fig. 2, 10), preferentially to monomeric CDKs, although they are capable of binding to heterodimeric cyclin: CDK4/6 complex. In p16:CDK6 structure, p16 binds next to the catalytic cleft, opposite to the cyclin-binding region. Because the binding sites do not overlap, p16 is also able to bind to the heterodimeric cyclin:CDK6 complex. p16 binding makes N and C lobes of CDK rotate 15º through a vertical axis, leading to misalignment of PSTAIRE helix with cyclin. In addition, p16 binding leads to a distortion in the ATP binding site *(55)*. In p18:K-cyclin: CDK6 structure there is a

misalignment of ATP binding site, catalytic residues, and distortion of cyclin-binding site *(79)*.

Independent of the structural information, there have been several experiments to identify peptides derived from p21/p27 (Fig. 2, 5) and from p16 (Fig. 2, 11) that inhibit CDK activity *(80)*. These studies have led to the identification of minimal domains with inhibitory activity in vitro and in vivo: p21—FYHSKRRLIFSK; GSKACRRLFGPV; p27—KPSACRNLFGPV; p16—84–103 [90–97] *(81–83)*. Constrained peptide libraries (aptamers) have been used to identify peptides that inhibit CDK2 with distinct substrate specificity and when expressed in cells can lead to a retardation of progression through G_1 *(84)*. Peptides derived from Nck5a, a neuronal-specific activator of CDK5, have been shown to inhibit CDK2 activity *(85)*. Peptides derived from p16, coupled to TAT protein transduction domain, have been used in cells to achieve G_1 cell cycle arrest *(86)*. However, this knowledge has yet to be converted into the development of small-molecule CDK inhibitors, and so is not further discussed in this chapter.

2.5. Inhibition of ATP-Binding Site (Fig. 2, 12)

Cells use cyclin-dependent kinase inhibitors (CKIs) to inhibit ATP binding to the CDKs and to inhibit CDK activity by changing the ATP-binding pocket. From the initial discovery of staurosporines as kinase inhibitors *(87)* and characterization of staurosporine:kinase crystal structures *(88)*, there has been an explosion in the discovery of a large number of small-molecule CDK inhibitors *(89–91)*. This approach is preferred by chemists, because the inhibitory molecules to be tested will be mimicking a well-established natural ligand, ATP, and because of the possibility of using structural information in SAR efforts *(92–95)*. A detailed discussion of CDK inhibitors with ATP antagonistic activity discovered so far is presented in Section 4.

There are at least nine CDKs in the human genome, of which four (CDK2, 3, 4, and 6) are involved in G_1 to S transition *(96)*. CDKs form a small part of the larger family of protein serine/threonine kinases, estimated at about 950, out of an estimated total number of 1,100 protein kinases, in the human genome *(97)*. This multiplicity of kinases raises a number of questions about selectivity—(a) definition of the desired selectivity, (b) measurement of the desired selectivity, and (c) clinical evaluation of the consequences of achieving the desired selectivity. Structural differences in the ATP-binding pocket of different kinases have been translated to the discovery of selective inhibitors. Cyclins determine the substrate selectivity of CDKs, illustrated by the changes in substrate selectivity seen between cyclin A:CDK2 and cyclin E:CDK2, and by how viral cyclins alter selectivity of CDK6 *(43)*. Cyclin A has a hydrophobic patch, 35 Å away from the active site, containing the MRAIL sequence that contacts the RNLFG sequence common to a number of substrates and inhibitors of CDKs. This docking site is critical for

phosphorylation of substrates containing RXL motifs (pRb) but not for substrates lacking this motif (histones). This hydrophobic patch is necessary for cyclin A function in cells *(98)*. Mutational analysis has identified a substrate-targeting domain in cyclin E necessary for phosphorylation of pRb *(99)*. We believe that further increases in selectivity are likely to come by incorporating substrate-binding sites in drug discovery approaches. Selectivity in action must ultimately be judged from the perspective of the substrate modification and activity.

2.6. Substrate Binding (Fig. 2, 13)

Cyclins play an important role in CDK substrate selection *(43,98,99)*. Structural studies have focused on how RXL (cyclin-binding motif) and LXCXE (part of cyclin structure) contribute to substrate selection. RXL motif in p27 (CRNLFGP) is thought to bind to a hydrophobic surface on cyclin A, which is conserved between cyclins A, B, D, and E, that lies opposite to the CDK2-binding site *(100,101)*. LXCXE motif is present in cyclin D and a number of Rb-associated proteins. The "A" and "B" domains of Rb pocket together encode a tightly associated tandem duplication cyclin-binding motif. The LXCXE motif of HPV E7 peptide binds in an extended conformation to the cyclin-binding motif encoded by the "B" domain Rb *(102)*. Cyclin E has VXCXE sequence that has been proposed to target substrates *(99)*. Cyclin A has an equivalent sequence that could be playing a role in targeting substrate *(100)*.

The number of substrates known to be phosphorylated by cyclin D:CDK4/6 is small (Rb, p107, p130) compared to cyclin A/E:CDK2 (Rb, p107, p130, p27, cdc25A, DP1, E2F5, CDC6, ORC1,and NPAT) *(3,41,42,46)*. Some substrates like Rb are thought to play an important role in regulating the progression of cell cycle *(30,31)*. Other substrates like E2F, CDC6, ORC1, and NPAT play an important role in the initiation of DNA synthesis *(3,30)*. The cyclin-binding motif, RXL, is present in E2F and CDC6 (RRLVF) *(35,103)* and is absolutely required for the association of cyclin:CDK complexes with these proteins. Systematic investigation of peptide substrates has demonstrated the importance of the RXL motif and its relative location from the site of phosphorylation (SPXK) in CDC6 *(103)*. This study has pointed out that RXL motif could be increasing the local concentration of substrate as well as orienting the phosphorylation site with respect to CDK2 active site for efficient phosphorylation. This study has raised the possibility of mimicking the RXL motif in developing specific small-molecule inhibitors of substrate recognition by CDKs. In fact, early studies show that such inhibitory peptides lead to the selective killing of transformed cells in which E2F pathway has been deregulated *(104)*. These studies highlight the possible usefulness of substrate-selective small-molecule CDK inhibitors, discovered either by rational design of peptidomimetic drugs or by high throughput screening with appropriate substrates.

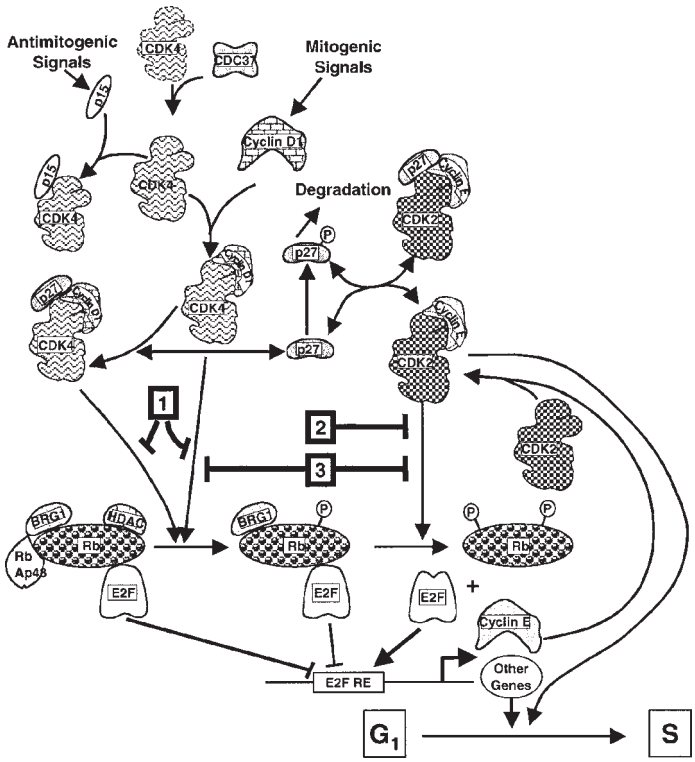

Fig. 3. CDK (or CDK6) and CDK2 control progression through G_1 phase by interacting with proteins (CDC37, p27) that facilitate assembly with cyclins (D1 or E) forming functional complexes or with proteins (p15, p27) that inhibit kinase activity. Rb is initially phosphorylated by CDK4 kinase and subsequently by CDK2 kinase. Activity of Rb is controlled by the nature of the interacting proteins, which is influenced by the Rb phosphorylation state. Rb controls the transcriptional activity of E2F, which is responsible for transcription of genes necessary for G_1 to S phase transition. Three different ways of inhibiting G_1 CDKs are suggested—selective inhibition of CDK4 kinase activity (**1**), or selective inhibition of CDK2 kinase activity (**2**) or selective inhibition of both CDK4 and CDK2 kinase activity (**3**).

2.7. Selectivity

Both cyclin E:CDK2 and cyclin D:CDK4/6 are involved in the cumulative and complex phosphorylation of pRb leading to its functional inactivation *(33)*. Activities of CDK2 and CDK4 are modulated by p27 protein function (Fig. 3).

Cyclin D:CDK4 complexed with p27 remains enzymatically active, whereas cyclin E:CDK2 complexed with p27 is enzymatically inactive. Increased expression of CDK4 could act as a sink for p27 and relieve inhibition of CDK2 by p27 *(24)*. Defects in the growth of CDK4-null fibroblasts are associated with increased binding of p27 to cyclin E:CDK2 and was partially relieved in a double CDK4-null and p27-null fibroblasts *(105)*. Cyclin D1 and p27 double-null mice corrected cyclin D1-null mice defects in body weight, early lethality, retinal hypoplasia, and male aggressiveness, and p27-null mice defects in body weight, retinal hyperplasia, and embryo implantation *(106,107)*. These results demonstrate that p27 negatively regulates cyclin E:CDK2 activity and that p27 can be sequestered by cyclin D:CDK4. Cyclin E:CDK2 is downstream of cyclin D:CDK4 and can largely bypass pRb in regulating the cell cycle *(107)*. In addition, cyclin E:CDK2 modulates the activities of many proteins necessary for proper execution of cell cycle through phosphorylation *(3)*, raising the question of the type of selectivity one is looking for in a CDK inhibitor. In developing inhibitors of G_1 CDKs, one can think of developing inhibitors that are selective for CDK4/6 (Fig. 3, 1), for CDK2 (Fig. 3, 2), or for both CDK4/6 and CDK2 (Fig. 3, 3). It is likely that inhibitors equally selective for CDK2 and CDK4, but not for CDK1, would allow better evaluation of the role G_1 CDK inhibitors in cancer therapy.

3. CDK ACTIVITY IS DEREGULATED IN CANCER

CDK activity is positively controlled by cyclins and CDKs, and negatively controlled by CKIs (p16, p21, and p27). A linear Rb pathway can represent these relationships, p16 $\dashv$ cyclin:CDK $\dashv$ Rb $\dashv$ E2F $\rightarrow$ G_1 $\rightarrow$ S *(31,108,109)*. Increased activity of positive controllers or decreased activity of negative controllers lead to the same end point of increased CDK activity and increased cell proliferation. Viewed this way, possibly 100% of human cancers have alterations leading to increased CDK activity *(19)*. Many of these alterations are in cell cycle control genes themselves *(14,19,20,50,53,110,111)*. Protein levels of p27 are dramatically decreased in a large number of breast cancer and colon cancer cases and this decrease is a prognostic marker for the disease. Lack of p27 is expected to contribute to increased cell proliferation *(61,62)*. Loss of function of tumor suppressor genes p15, p16, and Rb by epigenetic alterations e.g., hypermethylation is very frequent in a large variety of cancers *(112,113)*. Cdc25A is overexpressed in primary breast cancers, leading to an increased CDK2 activity *(114)*. Negative regulators of cell cycle progression, viz., p16, p27, and Rb, are frequently mutated in a large variety of cancers *(61,62,115–117)*.

CDK activity is increased in a large proportion of cancers due to alterations in cytoplasmic signal transduction pathways. These include alterations in Ras *(118)*, in transmembrane (EGFR, PDGFR) and cytoplasmic (Src, Abl) tyrosine kinases, and cytoplasmic serine/threonine kinases (Raf and AKT) *(119)*. Many of the onco-

genes enhance proliferation by increasing cyclin D1 levels *(118)*. Rac or CDC42 induced S-phase entry in Rat-1 R12 cells is correlated with cyclin D1 accumulation and enhanced E2F-mediated transcription *(120)*. *neu* (*c-erbB-2*, *HER-2*) codes for a receptor tyrosine kinase that is amplified in about 25% of human breast tumors *(121)*. Transformation by activated *neu* requires cyclin D1 signaling pathway *(122)*. Inactivating mutations in TGF-β receptor are common in many colorectal and gastric cancers. Inactivation of TGF-β decreases the expression of growth-suppressing genes (p15 and p21), and increases the expression of growth-promoting genes (*cdc25A* and *c-myc*) resulting in uncontrolled cell proliferation *(123)*. Wnt signaling pathway alterations are quite common in colorectal cancer *(124, 125)* resulting in increased β-catenin activity. Increased β-catenin activity leads to increased cyclin D1 transcription *(126)*. Wnt1 and MEK1 cooperate to transform cells in culture. Dominant-negative cyclin D1 mutant inhibited Wnt-activated MEK1-dependent S-phase entry suggesting that cyclin D1 is a critical downstream target of Wnt1- and MEK1- dependent cell proliferation *(127)*. Alterations in PTEN-PI3-kinase-AKT pathway are common among a large number of cancers *(128–132)*. PTEN modulates cell cycle progression and cell survival by regulating phosphatidylinositol-3,4,5-trisphosphate and AKT/protein kinase B signaling pathway *(133)*. PIK3A, which codes for catalytic subunit of phosphatidylinositol-3-kinase, is an oncogene that is amplified in ~40% of ovarian cancers *(134)*. PTEN is unable to suppress growth in Rb-null cells and cyclin D and CDK4 co-expression can overcome PTEN-mediated growth suppression. PI3K signaling activates E2F by inducing cyclin D3 *(135,136)*. Loss of PTEN increases PI3-K and increases AKT activity. One of the consequences of this increased AKT activity is a decrease in p27 levels, leading to increased CDK activity *(133,137)*. N-myc amplification is common in neuroblastoma, but there are no reported changes in Rb pathway. However, N-myc expression increases Id2 expression, which in turn overrides the tumor-suppressive activity of Rb *(138)*. Genetic or epigenetic alteration of genes directly or indirectly involved in cell cycle control ultimately leads to changes in cell cycle control genes and is responsible for the vast majority of human cancer.

4. CDK INHIBITORS WITH ATP ANTAGONISTIC ACTIVITY

Given the importance of CDKs in the regulation of cell cycle and its altered expression/activity pattern in most of the human cancers, considerable effort has been focused on the development of small-molecule CDK inhibitors for the past decade. While targeting regulators of CDK activity offers a promising approach to modulating CDK activity, most efforts have focused on targeting enzymatic activity of the complex. X-ray crystallographic analysis of a number of CDK complexes and computational chemistry combined with combinatorial chemistry and high-throughput screening have led to the development of a number of small-

molecule inhibitors that are ATP-competitive. These inhibitory molecules are derived from a large number of chemical classes. Activity (expressed as IC_{50} in μ*M*) of several CDK inhibitors toward a number of kinases is summarized in Table 1. Flavopiridol, UCN-01, and Ro 31-7453 are the most advanced molecules that are currently in clinical trials. Chemical structures for some of these molecules are shown in Fig. 4. In this chapter, we will discuss a few molecules from these classes in a greater detail.

4.1. Purine and Pyrimidine Analogs

6-Dimethylaminopurine (Fig. 4, **1**), originally identified as a mitotic inhibitor of sea urchin embryos, was the first CDK inhibitor to be identified, but is relatively nonspecific and only weakly inhibits (IC_{50} = 120 μ*M*) CDK1 *(139)*. Structural homology searches led to the identification of several purines and pyrimidine analogs that are more potent with slightly increased selectivity. These include isopentenyladenine (for cdc2, IC_{50} = 55 μ*M*), alkynylated purines (for CDK1, IC_{50} = 0.18–1.2 μ*M*), bohemine, suramine, olomucine (for CDK1/CDK2, IC_{50} = 7 μ*M*), roscovitine (for CDK1/CDK2, IC_{50} = 0.7 μ*M*), toyocamycin (for CDK1, IC_{50} = 0.5 μ*M*), hymenialdisine (for CDK1, IC_{50} = 0.022 μ*M*), CVT-313 (for cyclin A:CDK2, IC_{50} = 0.5 μ*M*, Ki = 0.095 μ*M*), MDL-44 (for cyclin E:CDK2 and cyclin D1:CDK4, IC_{50} = 0.01 and 0.25 μ*M* respectively), CGP-60474 (for CDK1 and CDK2, IC_{50} = 0.02 and 0.05 μ*M* respectively), NU-6027 (for CDK1 and CDK2, IC_{50} = 2.6 and 2.2 μ*M*, respectively), NU-2058 (for CDK1, IC_{50} = 6 μ*M*), and many others that have been extensively reviewed in recent years *(8,12,16,140–142)*.

More recently, combinatorial chemistry approaches were used to modify the purine scaffold to generate tri-substituted purine combinatorial libraries designed specifically to inhibit CDK2 *(143)*. These libraries were used in a solution phase phosphorylation screen to identify a group of novel compounds (Purvalanol-A, Purvalanol-B, Compound 52, and Compound 52Me). Among these, the most potent inhibitor was Purvalanol-B (for cyclin B:CDK1, cyclin A:CDK2, cyclin E:CDK2, and p35:CDK5, IC_{50} = 0.006, 0.006, 0.009, and 0.006 μ*M*, respectively), corresponding to a >1000-fold increase in potency over olomucine. Purvalanol (Fig. 4, **2**) showed remarkable selectivity toward CDKs (except cyclin D1:CDK4, IC_{50} = 0.893 and >10.0 μ*M* for purvalanol-A and purvalanol-B, respectively) with a little or no inhibitory activity against a battery of serine/threonine and tyrosine kinases *(143)*. X-ray crystallographic analysis of the human CDK2:purvalanol-B complex showed that purvalanol-B fits into the ATP-binding pocket just like other ATP-competitive inhibitors. Purvalanol-A was a more cell-permeable compound with an average IC_{50} of 2.0 μ*M* in inhibiting the growth of NCI tumor panel of 60-cell lines *(143)*. Consistent with its in vitro activity against CDK1, treatment with Purvalanol-A caused U937 human histocytic lymphoma cells to arrest in the G_2/M phase of the cell cycle.

Table 1
Inhibitory Activity of Small Molecules Against Enzymatic Activity of Kinases

	Cyclin B:CDK1 μM	*Cyclin A:CDK2* μM	*Cyclin E:CDK2* μM	*Cyclin D:CDK4* μM	*p35:CDK5* μM	*GSK-3β* μM	*ERK1* μM	*PKC-α* μM	*PKA* μM
6-Dimethylaminopurine	120								
Isopentenyladenine	55	50		200	90	60	90	43	
Olomucine	7	7	7	>1000	3	100	30	>1000	
Roscovitine	0.7	0.7	0.7	>100	0.16	130	34	>100	
Hymenialdisine	0.022	0.007	0.04	0.6	0.028	0.015	2		8
Purvalanol A	0.004	0.07	0.035	0.85	0.075	13	9	>10	
Purvalanol B	0.006	0.006	0.009	>10	0.006	>10	3.333	>100	
Butyrolactone I	0.68	1.5			0.15	100	94	160	260
Staurosporine	0.006	0.007		>10	0.004	0.015	0.02	0.0027	0.0082
UCN-01	0.031	0.03		0.032	0.15	0.07	0.91	0.007	
Flavopiridol	0.4	0.1		0.4	0.17	0.45		6	145
Kenpaullone	0.4	0.68	7.5	>100	0.85	0.023	20	>100	
10-Bromo-Paullone	1.3	3	4		2.7		88	>100	
Alsterpaullone	0.035	0.015	0.2	>10		0.004	22	>100	
Indigo	>1000	70	>1000	>100	>100	550	>100	>100	
Indirubin	10	2.2	7.5	12	5.5	0.6	>100	>100	
5-Chloro-Indirubin	0.4	0.75	0.55	6.5	0.8	0.05	>100	>100	
Indirubin-3'-monoxime	0.18	0.44	0.25	3.33	0.1	0.022	>100	27	
Indirubin-5'-Sulphonic acid	0.055	0.035	0.15	0.3	0.065	0.28	38	>100	
PD0183812	>40	0.2095	0.165	0.008				>50	
PD171851	0.06	0.01		0.0042					
SU9516	0.04	0.022		0.2				>10	
SU9675	0.36	0.025		0.28				7.8	
SU9678	0.009	0.003		0.51				>10	
SU11533	0.005	0.002		>10				>10	
GW-8510	0.107	0.01		0.129					
GW-9499		0.0035							
AG12286 (Ki)	0.0022	0.0057		0.013				11.5	>20
Ro 31-7453	3.8	1.3	1.9	2.5				10.8	>10

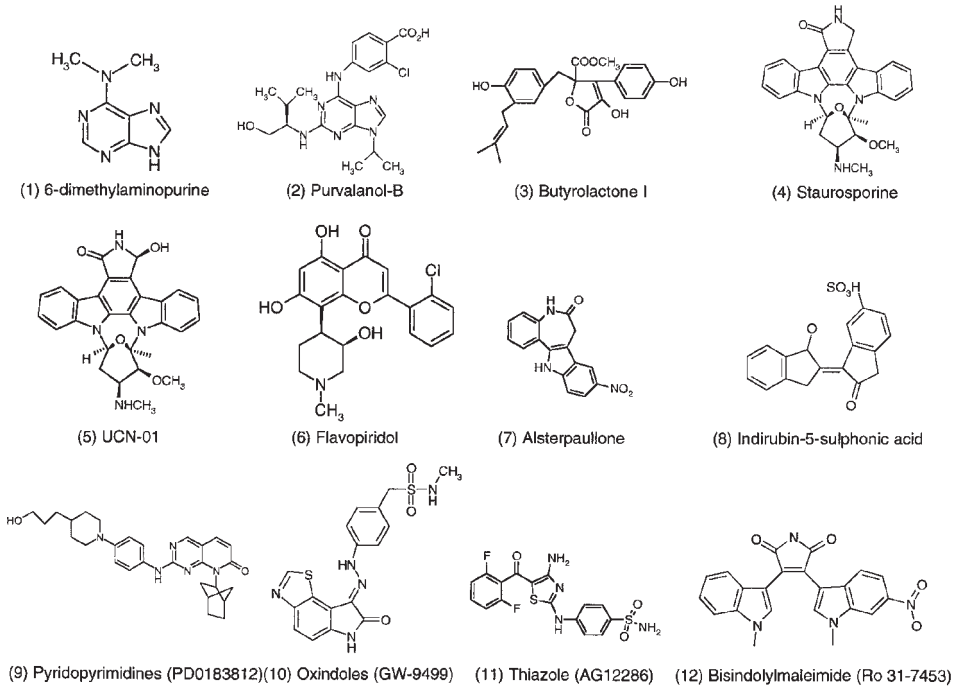

Fig. 4. Chemical structure of selected CDK inhibitors.

4.2. Butyrolactone I

Butyrolactone I (Fig. 4, **3**) was originally isolated as a metabolite from *Aspergillus terreus* var. *africanus* IF08835 and later identified as a selective inhibitor of CDK1 (IC_{50} = 0.68 μ*M*) and CDK2 (IC_{50} = 0.82 μ*M*) with little or no inhibitory activity against MAP kinase, PKC, PKA, and tyrosine kinases *(144)*. Butyrolactone I is active in inhibiting growth of various non-small-cell lung cancer (IC_{50} = 50–76 μg/mL) and small-cell-lung cancer cell lines (IC_{50} = 21–88 μg/mL). Treatment of PC-14 cells with 20 μg/mL butyrolactone I inhibited CDK1 kinase activity by 40% of control and arrested non-synchronized cells in G_2/M phase of the cell cycle suggesting that butyrolactone I acts predominantly on CDK1 kinase *(145)*.

4.3. Staurosporines

Staurosporine (Fig. 4, **4**) has an indolocarbazole moiety and was first isolated from *Streptomyces staurosporeus* during a search for new alkaloids present in actinomycetes *(87)*. It is one of the most potent general inhibitors of protein kinases. It inhibits kinases that belong to different groups (PKC, PKA, CDKs, p60v-src kinase, Calmodulin kinase II, and EGF-receptor-encoded protein kinase) with remarkably high potency (in p*M*–n*M* range), but with very low specificity. Staurosporine is equally potent in inhibiting the growth of wide variety of cancer cell lines and causes cell cycle arrest at growth inhibitory concentrations.

UCN-01 (7-hydroxystaurosporine) (Fig. 4, **5**), an analog of staurosporine, has potent activity against protein kinases (IC_{50} for PKC, CDK1, CDK2, CDK4, CDK6 = 7, 31, 42, 32, and 58 n*M*, respectively), similar to staurosporine *(146)*. UCN-01 has antiproliferative activity and induces G_1-phase accumulation in several human tumor cell lines. Treatment of human tumor cells with UCN-01 resulted in dephosphorylation of Rb and CDK2 proteins and increased the amount of CDK inhibitors, p21 and p27, *(146,147)* leading to arrest in G_1 phase and contributing to antitumor activity of the compound. UCN-01 enhanced ubiquitin–proteaosome-pathway-mediated degradation of E2F1 protein in the human gastric cancer cell line SK-GT5 *(148)*. Additionally, UCN-01 enhanced the anticancer effects of mitomycin C *(149)*, cisplatin *(150)*, and 5-fluorouracil *(151)* in vitro and in vivo. Because of the unique mechanisms of tumor growth inhibition in preclinical models, UCN-01 is anticipated to be effective in the clinic and is currently undergoing clinical trials as an anticancer agent in the United States and Japan.

In humans, UCN-01 has an extremely long half life (>24 d), a low systemic clearance (mean 8.7 ± 6.4 mL/h/m^2) and distribution volume due to specific binding of UCN-01 to human α-$_1$ acid glycoprotein with high affinity *(152)*. The peak plasma concentrations (C_{max}) ranged from 1 to 65 μg/mL. Dose-limiting toxicity (DLT) with UCN-01 was observed at 42.5 mg/m^2/d on a 72 h continuous infusion schedule *(153)*. Observed toxicities were nausea/vomiting, symptomatic hyperglycemia, and pulmonary toxicity characterized by substantial hypoxemia. Other

reversible toxicities include myalgias, hypotension, and headaches. There was a suggestion of activity with partial response for 8 mo in a patient with melanoma, and disease stabilization in a few patients with non-Hodgkin's lymphoma or leiomyosarcoma *(153)*.

4.4. Flavopiridol

Flavopiridol (Fig. 4, **6**) is a synthetic analog of a natural alkaloid rohitukine isolated from the stem bark of *Dysoxylum binectariferum*, a plant indigenous to India *(154)*. Flavopiridol is ATP-competitive and a nonselective kinase inhibitor with potent activity against cyclin B:CDK1 (IC_{50} = 0.4 μ*M*), cyclin A:CDK2 (IC_{50} = 0.1 μ*M*), cyclin D1:CDK4 (IC_{50} = 0.4 μ*M*), CDK7 (IC_{50} = 0.3 μ*M*), and moderate activity against EGF-receptor tyrosine kinase (IC_{50} = 21 μ*M*) and PKA (IC_{50} = 122 μ*M*) *(154–157)*. Flavopiridol has a very potent (mean IC_{50} = 66 n*M*) antiproliferative activity against NCI's 60-cell line panel and human tumor xenografts in nude mice *(158–160)*. Flavopiridol caused cell cycle arrest in both G_1 and G_2/M phases *(154,155,161)* and apoptosis in several human cancer cell lines *(158,162–164)*. Flavopiridol induced G_1 arrest in MCF7 cells was associated with a reduced level of phosphorylated form of pRb *(155)*. When A549 non-small-cell lung carcinoma cells were exposed to flavopiridol in combination with paclitaxel, cytarabine, topotecan, doxorubicin, or cisplatin, in vitro cytotoxicity increased *(165)* in a schedule-dependent manner.

Flavopiridol is the first CDK inhibitor to enter clinical trials as an anticancer agent. In phase I clinical trial, flavopiridol was administered as a 72 h continuous infusion every 2 wk *(166–168)*. Secretory diarrhea was observed at the DLT. Maximally tolerated dose (MTD) of 50 mg/m^2/d × 3 d was observed at the NCI clinical study *(166)* and 40 mg/m^2/d × 3 d was observed at the University of Wisconsin clinical study *(167)*. For patients receiving diarrhea prophylaxis regimen, the MTD was 78 mg/m^2/d × 3 d. The DLT at the higher MTD consisted of reversible hypotension, local tumor pain, and flu-like symptoms consisting of anorexia, fatigue, fever, and malaise. Interestingly, in beagle dogs, the DLT was gastrointestinal and the MTD was 26 mg/m^2/d given as a 72 h continuous infusion with the steady-state plasma concentration of 91 n*M* *(168)*. The pharmacokinetic parameter estimates of flavopiridol from the phase I study were as follows: total clearance, 17.23 L/h/m^2; terminal half-life, 11.6 h; and apparent volume of distribution, 13.16 L/m^2. A median C_{max} of 425 n*M* was seen at 78 mg/m^2/d dose *(166)*. Approximately 30% of the patients were found to have a post-infusional increase in drug concentration at 3–24 h after the end of the infusion, possibly due to resorption of the drug from the gastrointestinal tract *(169)*. Flavopiridol has been shown to undergo glucuronidation in hepatic microsomal preparations and in hepatic perfusion studies conducted in rats *(169,170)*. The glucuronidation rate was found to vary up to six-fold in microsomal preparations prepared from 48 patients *(171)*. NCI trial employing daily bolus infusion established an MTD

of 37.5 mg/m^2/d for five consecutive days every three weeks schedule with DLT consisted of nausea, vomiting, neutropenia, fatigue, and diarrhea at 53 mg/m^2/d *(168)*. Another trial at NCI employing sequential infusion of paclitaxel and flavopiridol established 135 mg/m^2 of 3 h paclitaxel and 80 mg/m^2 of 24 h flavopiridol as MTD for this schedule *(172)*. In this trial, clinical responses were observed in paclitaxel refractory esophagus and prostate cancer patients.

In a recently completed phase II consortium study at the University of Chicago, flavopiridol (administered by continuous infusion in 35 patients at 50 mg/m^2/d $\times$ 3 d every two weeks) was ineffective in metastatic renal cancer patients *(173)*. In addition to the diarrhea (77%) observed in phase I studies, asthenia (83%) and serious vascular thrombotic events (26%) were observed in these patients *(173)*. With the same treatment schedule, no response to flavopiridol was seen in two other phase II clinical trials for stage IV non-small, cell lung cancer (18 patients), or for metastatic colorectal cancer (14 patients) *(174,175)*. However, in another phase II study, flavopiridol was active against metastatic gastric cancer using the same schedule *(176)*. One patient with liver metastases showed PR, 3/13 had stable disease, and 8/13 progressed on therapy. Thrombosis (42%) was the main toxicity in addition to toxicities observed in the phase I trial *(176)*.

Flavopiridol is being tested in phase II studies in lymphoma (relapsed mantle cell and intermediate/high-grade non-Hodgkin's lymphoma), chronic lymphocytic leukemia, and prostate cancer patients as a 72 h continuous infusion. Flavopiridol is also undergoing investigation in combination with either cisplatin or paclitaxel as a 24 h infusion *(8,177)*. It remains to be seen if flavopiridol will ultimately be found to have clinical activity as a 72 h continuous infusion or with some other route of administration.

4.5. Paullones

Paullones were recently identified as potent, ATP-competitive, inhibitors of CDKs from a National Cancer Institute's computer-based algorithm COMPARE search to find molecules with flavopiridol-like activities. Paullones constitute a new family of benzazepinones with promising antitumoral properties. Kenpaullone (NSC-664704) is the most potent inhibitor of cyclin B1:cdc2 (IC_{50} = 0.4 μM). It inhibited cyclin A:CDK2 (IC_{50} = 0.68 μM) and p35:CDK5 (IC_{50} = 0.85 μM) moderately, inhibited cyclin E:CDK2 (IC_{50} = 7.5 μM), ERK1 (IC_{50} = 20 μM), ERK2 (IC_{50} = 9 μM), c-src (IC_{50} = 15 μM), c-raf (IC_{50} = 38 μM), casein kinase 2 (IC_{50} = 20 μM) at higher concentration and was inactive (IC_{50} > 100 μM) against cyclin D1:CDK4, MAPKK, c-jun kinase, all PKC isoforms, cAMP- and cGMP-dependent protein kinases, casein kinase 1, and insulin-receptor tyrosine kinase *(178)*. Kenpaullone inhibited the growth of tumor cells in culture (mean GI_{50} = 42 μM) and caused serum-starved human breast cancer MCF10-A cells to remain in the G_1 phase of the cell cycle *(178,179)*.

Recently, paullones were also shown to be potent inhibitors of glycogen synthase kinase-3β (GSK-3β) (IC_{50} = 4–80 n*M*) and the neuronal p25:CDK5 (IC_{50} = 20–200 n*M*) *(180)*. Importantly, alsterpaullone (Fig. 4, **7**) inhibited the in vivo phosphorylation of tau, at sites normally phosphorylated by GSK-3β in Alzheimer's disease. Clearly, paullones represent a novel structural class with a potential to result in molecules with potent activity to treat proliferative and neurodegenerative diseases.

4.6. Indigoids

Indirubin, an isomer of indigo, is the active ingredient of Danggui Longhui Wan, a complex herbal mixture that is used in traditional Chinese medicine to treat chronic myelocytic leukemia. Indirubin-5-sulfonic acid (Fig. 4, **8**) is the most potent and selective CDK inhibitor in this class, and is active against cyclin B:CDK1 (IC_{50} = 0.055 μ*M*), cyclin A:CDK2 (IC_{50} = 0.035 μ*M*), cyclin E:CDK2 (IC_{50} = 0.15 μ*M*), cyclin D1:CDK4 (IC_{50} = 0.3 μ*M*), and p35:CDK5 (IC_{50} = 0.065 μ*M*), with little or no activity against MAPKK, c-raf, c-jun kinase c-src, c-abl, all PKC isoforms, cAMP- and cGMP-dependent protein kinases, casein kinase 1 and 2, and insulin-receptor tyrosine kinase *(181)*. Although, other indigo analogs are also ATP-competitive and selective inhibitors of CDKs, they are less potent. For example, IC_{50} for indirubin, 5-chloroindirubin and indirubin-3'-monoxime against cyclin B:CDK1 is 10, 0.4, and 0.18 μ*M*, respectively *(181)*. The most potent indigoid in an in vitro kinase assay, indirubin-5-sulfonate, has marginal effects on cell proliferation, probably because of its limited cell permeability. However, indirubin-3'-monoxime inhibited the proliferation of all cancer cell lines tested in a dose-dependent manner. Analysis of the cell cycle distribution showed a marked G_1 arrest in Jurkat and MCF7 cells at low indirubin-3'-monoxime concentrations, whereas at higher concentrations cells accumulated in G_2/M phase in most of the cell lines (HBL-100, MCF7, Jurkat, CCL-39, PC12, L1210, K562, and HL-60) tested. In Jurkat cells, G_1 arrest was accompanied by a decrease in phosphorylated form of pRb *(181)*.

4.7. Pyridopyrimidines

PD0183812 (8-ethyl-2-phenylamino-8*H*-pyrido[2,3-*d*]pyrimidine-7-one) is a potent and selective cyclin D1:CDK4 inhibitor that belongs to a series of [2,3-*d*] pyridopyrimidines under evaluation by Parke-Davis & Co, as potential treatments for Rb-positive tumors *(182–184)*. PD0183812 (Fig. 4, **9**) inhibited purified cyclin D1:CDK4 with an IC_{50} value of 0.008 μ*M* but showed 26-, 20-, >5000-, 1078-, and 6250-fold less activity against cyclin A:CDK2 (IC_{50} = 0.209 μ*M*), cyclin E:CDK2 (IC_{50} = 0.165 μ*M*), cyclin B:CDK1 (IC_{50} = >40 μ*M*), FGF receptor (IC_{50} = 8062 μ*M*), and PKC (IC_{50} = >50 μ*M*), respectively *(182–184)*. Interestingly, the parent structure (6-aryl-substituted pyrido[2,3-*d*]pyrimidine-7-one) of this molecule is a potent inhibitor of tyrosine kinases (EGFR, FGFR, PDGFR,

and c-src), but is inactive against CDKs *(185)*. Based on computer modeling, it was hypothesized that pyrido[2,3-*d*]pyrimidine-7-one lacking 6-aryl-substitutent would be able to bind in the ATP-binding pocket of CDKs. Indeed, X-ray crystallographic analysis of a related compound with CDK2, revealed that this molecule occupied the ATP-binding pocket *(183)*. PD0183812 inhibited proliferation of HCT116 human colon carcinoma cell line with an $IC_{50} = 0.213$ μ*M*. Cell cycle distribution analysis showed that Rb-positive tumor cells (MDA MB-453) were arrested in G_1 after treatment with PD0183812, whereas Rb-negative (MDA MB-468) cells continued to cycle *(183,184)*. Earlier compounds in this series, PD171851 and PD172803, showed remarkably similar biological activity profile *(186,187)*. PD171851 was active against cyclin D1:CDK4, cyclin A:CDK2, and cyclin B:CDK1 with IC_{50} of 0.0042, 0.01, and 0.06 μ*M*, respectively, and arrested Rb-positive human breast carcinoma cell line, MCF7, in G_1 phase of the cell cycle at growth inhibitor concentration ($IC_{50} = 0.8$ μ*M*). PD172803 (for cyclin D1:CDK4, $IC_{50} = 0.209$ μ*M*) reduced phosphorylation of S780 on pRb, a site selectively phosphorylated by CDK4, in a time-dependent manner in MCF7 cells *(186)*.

4.8. Oxindoles

Oxindole is a novel structural class recently disclosed by several pharmaceutical companies as an inhibitor of CDKs. Berlex Bioscience disclosed oxindole-I as a CDK4 inhibitor ($IC_{50} = 4.9$ μ*M*). Oxindole-I potently inhibited the growth of Rb-positive MCF7 ($IC_{50} = 0.42$ μ*M*) and ZR-75-1 ($IC_{50} = 0.1$ μ*M*) cell lines, while it was not active against Rb-negative BT-549 cell line *(188)*. SUGEN has developed SU-9516, a 3-substituted, oxindole-based inhibitor of CDK activity, which is currently under evaluation for pharmacokinetic parameters, chemical and metabolic stability, and in vivo efficacy. The compound has an IC_{50} of 0.04, 0.022, and 0.2 μ*M* for CDK1, CDK2, and CDK4, respectively, without much activity against PKCα ($IC_{50} = >10$ μ*M*), p38 kinase ($IC_{50} = >10$ μ*M*), EGFR tyrosine kinase ($IC_{50} = >10$ μ*M*), and PDGFR tyrosine kinase ($IC_{50} = 18$ μ*M*) *(189)*. SU-9516 has an antiproliferative effect in A431 ($IC_{50} = 2.2$ μ*M*), NCI-H460 ($IC_{50} = 3.6$ μ*M*), RKO human colon carcinoma cells with wild-type p53 ($EC_{50} = 6.5$ μ*M*) and SW480 human colon carcinoma cells with mutant p53 ($EC_{50} = 6.0$ μ*M*). Additionally, SU-9516 is also able to decrease phosphorylation of pRb by CDK2 and CDK4 *(189)*. Glaxo Wellcome has developed oxindole analogs based on the X-ray crystallographic structure of the CDK2-ATP binding site. These are potent cyclin:CDK2 inhibitors with IC_{50} value of 0.0023, 0.0062, 0.01, and 0.0035 μ*M* for GW-2059, GW-5181, GW-8510, and GW-9499 (Fig. 4, **10**), respectively *(190, 191)*. GW-9499 inhibited the growth of Rb-positive RKO cells ($IC_{50} = 0.86$ μ*M*) at lower doses than Rb-negative human breast carcinoma MDA MB-468 cells ($IC_{50} = 3.4$ μ*M*) and normal human diploid fibroblasts ($IC_{50} = 8.0$ μ*M*), required higher doses.

4.9. Thiazoles

AG12275 and AG12286 (Fig. 4, **11**) belong to a novel 2,4-diaminothiazole class of CDK inhibitors recently disclosed by Agouron Pharmaceuticals *(192, 193)*. AG12275 is the most potent and highly selective CDK4 inhibitor reported to date. AG12275 is a potent inhibitor of cyclin D3:CDK4 (k_i = 0.0033 μ*M*) but is 70- and 100-fold less active against cyclin A:CDK2 (k_i= 0.22 μ*M*) and cyclin B: CDK1 (k_i= 0.32 μ*M*), respectively. AG12286 is a potent inhibitor of cyclin B: CDK1 (k_i= 0.0033 μ*M*), cyclin A:CDK2 (k_i= 0.0033 μ*M*), and cyclin D3:CDK4 (k_i= 0.0033 μ*M*), but was not selective. However, both of these molecules are competitive inhibitors with respect to ATP and do not have activity against PKC, cAMP-dependent kinase, ERK2, VEGFR tyrosine kinase, and FGFR tyrosine kinase *(192,193)*. These compounds are active in inhibiting the growth of human colon carcinoma (HCT116, COLO205, and SW480), human osteosarcoma (U2OS and Saos-2), and human breast carcinoma (MCF7, MDA MB-435, and MDA MB-468) cell lines at submicromolar concentrations. Interestingly, AG12275, a potent CDK4 inhibitor, induced G_1 arrest in Rb-positive (HCT116 and MDA MB-453) cell lines but produced no cell cycle effect in Rb-negative (MDA MB-468) cell line, while AG12286, a potent broad spectrum CDK inhibitor, induced G_2/M arrest in all three cell lines *(193)*. Both of these compounds were able to reduce the level of S780 phosphorylation on pRb. In HCT116 xenograft nude mice model, AG12275 dosed at 100 mg/kg caused 70% inhibition of tumor growth (p = 0.003) versus vehicle treated control animals at d 41 *(194)*.

4.10. Bisindolylmalaemides

Roche has identified Ro 31-7453 (Fig. 4, **12**), a novel bisindolylmaleimide, as a new class of cell cycle inhibitors and apoptosis inducers. Ro 31-7453 is moderately active against cyclin B:CDK (IC_{50}= 3.8 μ*M*), cyclin A:CDK2 (IC_{50} = 1.3 μ*M*), cyclin E:CDK2 (IC_{50} = 1.9 μ*M*), and cyclin D1:CDK4 (IC_{50} = 3.8 μ*M*), but was not very active against PKA (IC_{50} = >10 μ*M*), PKCα (IC_{50} = 10.8 μ*M*), casein kinase 2 (IC_{50} = >10 μ*M*), Raf kinase (IC_{50} = >10 μ*M*), and topoisomerase (IC_{50} = >5 μ*M*) *(195–198)*. However, it is very active (IC_{50} = 20–300 n*M* range) in inhibiting growth of more than 30 human tumor cell lines, including multidrug resistant (MDR) cell lines and normal WI38 human lung cell line, prostate (PrEc) and smooth muscle (UASMC) cell lines *(195–198)*. Ro 31-7453 has moderate but broad-spectrum activity against more than 30 different targets screened by Roche so far, and the true molecular target for this molecule is not clear. At antiproliferative concentration, Ro 31-7453 caused a significant accumulation of MDA MB-435 cells in M-phase of the cell cycle followed by an induction of apoptosis in a dose-dependent manner *(195–198)*. In preclinical in vivo models, this compound showed significant antitumor effects including growth suppression, shrinkage, and cure, depending on the model and the therapeutic regimen used. Administration of Ro

31-7453, by continuous infusion, intraperitoneally and/or orally, showed potent antitumor activity against human breast (MDA MB-435), colorectal (RKO, HT-29, HCT116), NSCLC (A549), prostate (DU-145), paclitaxel-resistant colorectal (SW480), MDR-resistant colorectal (LS1034), and MDR-resistant uterine (MES-SA/DX-5) xenograft models. It was also active against syngeneic MTLn3 rat mammary adenocarcinoma and in the transgenic *MIN* (Multiple Intestinal Neoplasia) mouse model. Preclinical toxicities at high doses consisted of weight loss, leukopenia, mild anemia, degenerative changes in the small intestines and bone marrow, and unscheduled deaths *(195–198)*. In a preclinical dose escalation study, oral administration of Ro 31-7453 at lower dose (100 mg/kg/dose) spread over longer treatment duration produced maximum efficacy with minimum toxicity *(195–198)*. Ro 31-7453 is extensively metabolized in the body following intravenous and/or oral administration. Four metabolites of Ro 31-7453 with similar in vitro potency have been detected in the plasma of mice, rats, dogs, and monkeys *(195–198)*.

Ro 31-7453 is currently being evaluated in phase I clinical trials with an oral dose schedule. DLT consisted of myelosuppression, stomatitis nausea, vomiting, diarrhea, fatigue, alopecia, and one death was observed at 800 mg/m^2/d *(199)*. The area under curve (AUC) and trough concentrations of Ro 31-7453 and its metabolites appeared dose proportional with $t_{1/2}$ of about 12 h and a t_{max} of about 4 h. There were indications of antitumor activity in two patients with NSCLC. The MTD is expected to be 560 or 660 mg/m^2/kg/d for 4 d *(199)*.

5. CONCLUSIONS AND PROSPECTS

Since the discovery of cyclins and CDKs about 10 years ago *(200–203)*, there has been considerable progress made in understanding cell cycle *(1,3,30)*. This understanding is leading to a discovery of novel drugs for cancer therapy *(7,9,10, 12,16,80,89,90,94,204)*. The next few years will see the clinical evaluation of many drugs, targeted to CDKs, allowing an evaluation of the usefulness of CDK inhibitors in cancer therapy.

An ideal cancer drug is potent and selective in killing tumor cells, while leaving normal cells unharmed. Many of the drugs discussed in this review are expected to benefit from target-driven therapeutic window *(18)*. It is to be anticipated that more drugs exploiting context-driven therapeutic driven window will be discovered in the future.

Although G_1 CDKs are important in regulating Rb function and are highly deregulated in human cancers, many of the drugs that have been characterized so far (Staurosporines, Flavones, Paullones, Indirubin, and Hymenialdisine) appear to selectively inhibit CDKs other than G_1 CDKs (CDK1 involved in G_2–M transition and CDK5 involved in tau-phosphorylation). Flavopiridol, an inhibitor of CDKs, inhibited transcription by RNA polymerase II by inhibiting pTEFb's ability

to phosphorylate RNA polymerase II *(205)*. Flavopiridol is also reported to bind DNA, providing a potential explanation for its ability to kill non-cycling cells *(206)*. Indirubin and Paulones inhibit GSK-3β and p25:CDK5, besides inhibiting CDK1 *(204)*. UCN-01, initially thought to act through its inhibitory effects on CDK1, inhibits the ability of chk1 to phosphorylate cdc25C, thus keeping cdc25C nonfunctional *(207)*. Although hymenialdisine is capable of inhibiting CDKs, it is also a potent inhibitor of transcription initiated by NF-κB without inhibiting AP1, CCAAT/enhancer binding protein and Sp1 initiated transcription. Inhibition of NF-κB is not through an inhibition of protein kinase C activity *(208)*.

More clinical information on drugs inhibiting G_1 CDKs is necessary before we can adequately discuss their clinical usefulness. However, the data generated with CDK inhibitors highlight the need to redefine our approaches to drug discovery efforts and the concept of selectivity. It would be important to incorporate features that will allow discovery of substrate-selective CDK inhibitors *(103, 209–212)*. Physiologically relevant cellular targets for the current CDK inhibitors need to be identified *(213)*. We need to increase our current understanding of the selectivity of a number of CDK inhibitors from pregenomic investigational approaches (one gene/protein at a time) to genomic investigational approaches *(214–216)*. Our understanding of drug selectivity is likely to become more comprehensive. The challenge would be to relate this information with the clinical outcomes to gain insight into where the future drug discovery efforts should go. Progress in microarray technology (mRNA and/or protein) holds the promise of increased recognition of the underlying genetic diversity that exists in the seemingly uniform disease classes *(217)* by correlating this diversity with clinical outcomes. This understanding could lead to more effective therapies for smaller and more homogeneous disease subclasses.

This chapter has focused on the uses of CDK inhibitors in cancer therapy. However, CDKs play an important role in other proliferative disorders, viz., chemotherapy-induced alopecia *(218)*, neuronal death after reperfusion injury *(219)*, β-amyloid evoked cell death *(220)*, cytokine responsiveness of T cells *(221)*, and diabetic nephropathy *(222)*. Some of the apoptosis in β-amyloid evoked cell death is mediated through Rb pathway deregulation of E2F1 activity *(223)*. In addition, CDK5 and GSK-3β phosphorylate tau protein and hyperphosphorylated tau aggregates into paired helical filaments, a characterstic feature of Alzheimer's disease. The observation that a number of CDK2/4 inhibitors decrease CDK1, CDK5, and GSK-3β activities raise the possibility of these inhibitors being used not only in cancer therapy but also in Alzheimer's disease *(204,224)*. CDK inhibitors may also be useful in other proliferative disorders.

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9 Mitogen-Activated Protein Kinase Cascades as Therapeutic Targets in Cancer

Antonio Chiloeches and Richard Marais

Contents

1. INTRODUCTION

Mammalian cell growth is regulated by a large number of environmental cues in the form of extracellular signals. These signals stimulate changes in cell metabolism and gene expression, and induce complex cellular responses such as proliferation, differentiation, or death. However, the genetic mutations that accumulate in cancer allow cells to grow with apparent disregard for their environment, so that, even in the absence of appropriate signals, cells continue to proliferate, or they fail to differentiate or die when instructed to do so. In the past, cancer therapeutic agents were developed in the absence of a clear understanding of the mechanisms that regulate cell growth. The agents that were produced were generally developed to target rapidly dividing cells and are, on the whole, extremely toxic and associated with barely tolerable side effects, because they also target healthy dividing cells. A great deal of research has therefore been directed at understanding the molecular mechanisms that regulate cell growth and to determine why cancer cells grow with such apparent disregard for their environment. It was anticipated that this would provide new molecular targets that were associated with only the rapid division associated with cancer cells, but not with the rapid division associated with normal cells. The hope was that agents that blocked the activity of these targets would be specific for cancer cells over normal cells,

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leading to fewer side effects and, consequently, offering improved treatments for cancer patients.

These studies have led to the discovery of a number of intracellular signaling pathways that are responsible for transmitting signals from the plasma membrane to the nucleus. The activity of these pathways maintains normal cellular homeostasis in response to the environment and inappropriate elevation of the activity of these pathways has been linked to the pathogenesis of cancer. These pathways therefore represent the sought after new therapeutic targets for cancer. In this chapter, we discuss one such family of signaling pathways, the mitogen-activated protein kinase (MAPK) cascades and consider the prospects for targeting these cascades in the treatment of cancer.

1.1. The Mitogen-Activated Protein Kinase Cascades

The MAPKs are a large family of serine/threonine-specific protein kinases that were originally described as proteins that became phosphorylated following mitogen stimulation of eukaryotic cells. It was subsequently shown that these proteins were in fact protein kinases that were activated following stimulation of cells by a number of environmental stimuli (for reviews, *see 18,56,67,88)*. Activation occurs when the proteins are phosphorylated on a threonine and tyrosine in a region called the T-loop, which is located within the kinase domain. MAPKs are conserved from yeast to mammals and are the last components of three-tiered, protein kinase cascades (Fig. 1). These cascades consist of a serine/threonine-specific MAPK kinase kinase (MAPKKK), which phosphorylates a MAPK kinase (MAPKK) in its T-loop, stimulating the activity of the MAPKK. The MAPKKs are dual specificity protein kinases that phosphorylate and activate the MAPKs. The MAPKs regulate complex cellular events, because they phosphorylate and regulate the activity of a large number of cellular proteins, including transcription factors, proteins that regulate chromatin structure, metabolic enzymes, and proteins that regulate the cytoskeleton (Fig. 1).

In mammals, at least five families of MAPKs have been described (Fig. 2). These are the Extracellular signal Regulated protein Kinases (ERK1 and ERK2), the c-Jun N-terminal Kinases (JNK1, 2, 3), the p38MAPKs (p38MAPKα, β, γ, δ), and two additional families that contain only one member each, ERK3 and ERK5 (Fig. 2). Although all of the MAPKs are activated by threonine and tyrosine phosphorylation within their T-loops, the different families respond to distinct extracellular signals. Thus, ERK1 and ERK2 are responsive mostly to anabolic signals provided by peptide growth factors and hormones and are associated with cell growth and differentiation. The JNK and p38MAPK families, by contrast, are mostly responsive to physical cellular stress and cytokines and are associated with cell death through apoptosis. However, as we shall see, these distinctions may be too rigid. ERK5 appears to be activated both by anabolic signals and by cellular stress.

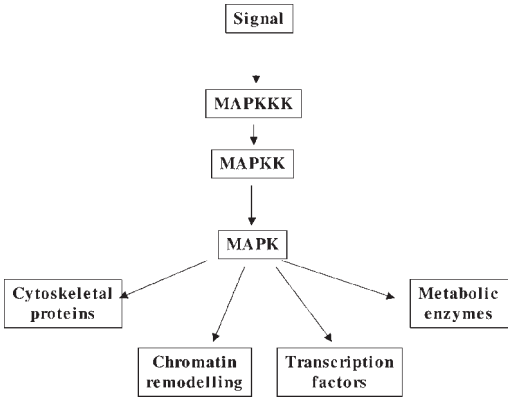

Fig. 1. Schematic overview of the MAPK cascades. The MAPK cascades consist of a three-tiered protein kinase cascade, in which an extracellular signal stimulates the activity of a MAPKKK, which then activates a MAPKK, which activates a MAPK. The MAPK is able to phosphorylate a number of proteins, that regulate metabolism, cytoskeletal proteins, chromatin remodeling and transcription factors.

Like the MAPKs, a number of MAPKKs have also been identified. These are called MEK1, MEK2, MKK3, MKK4, MEK5, MKK6, and MKK7 (*see 18,91)*. Similarly, several MAPKKKs have been described, including the Raf proteins, Mos, TPL-2, the mixed lineage kinases (MLKs), the MEK kinases (MEKK1–4), TAK1, and the apoptosis signal-stimulating kinase (ASK) (*see 18,27,44,73,74, 82)*. There is a great deal of specificity within these pathways and the components are organized into distinct cascades or modules (Fig. 2). In part, the specificity within these modules occurs because the different MAPKKs are restricted in the MAPKs that they can phosphorylate and activate. Thus, ERK1 and ERK2 are likely to be the only physiological substrates of MEK1 and MEK2 and the JNKs are likely the only physiological substrates for MKK4 and MKK7, whereas the p38MAPKs are the preferred substrates for MKK3 and MKK6 (Fig. 2; *see 18,56)*. Furthermore, despite being most similar to MEK1/2, MEK5 only activates ERK5 and MEK1/2 cannot activate ERK5 *(25,50,125)*; the MAPKK for ERK3 has yet to be characterized.

At the level of the MAPKKKs, the picture is less clear and the direct upstream activators of many of the MAPKKs have not been identified or fully characterized. However, our understanding of the regulation of the ERK1/2 pathway is reasonably complete. The regulation of this pathway has been determined by a

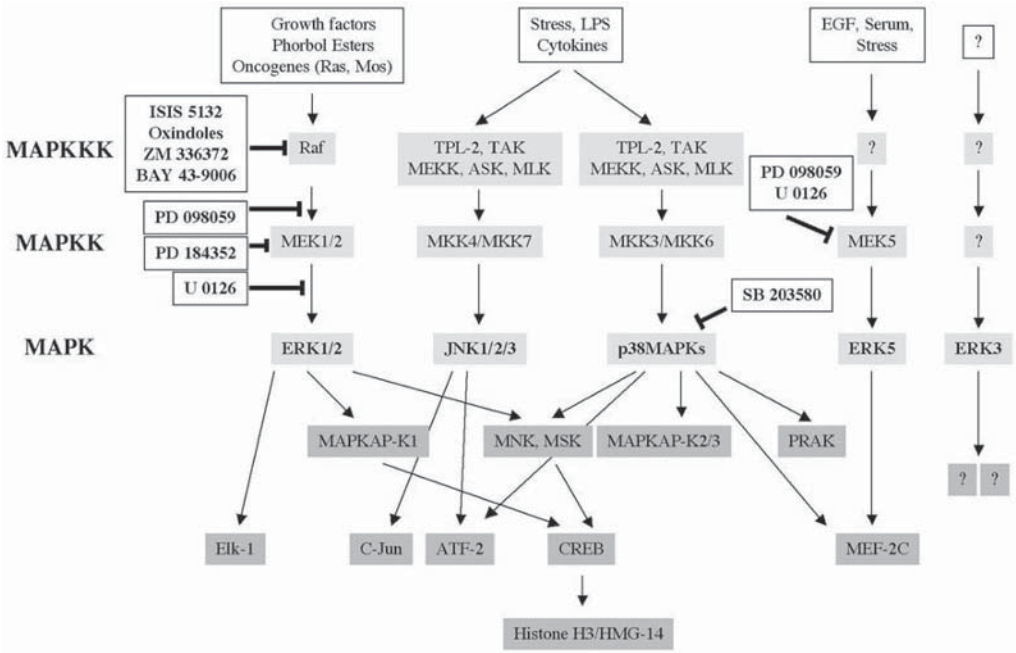

Fig. 2. The MAPK cascades in mammalian cells. There are five MAPK cascades, the signals known to stimulate the activity of each cascade are in the white boxes shown at the top. The MAPKKKs, the MAPKKs, and the MAPKs are shown in the light grey boxes. The kinases and nuclear proteins downstream of the MAPKs are in dark grey boxes. Chemical inhibitors of the different cascades are shown in bold text in white boxes. See text for details.

combination of genetic studies in worms and flies with biochemical studies and cell biology studies performed in tissue culture systems. These studies led to the discovery of a linear pathway from receptors at the cell surface to the regulation of gene expression in the nucleus (*see 64,88)*. In this pathway, growth factors stimulate receptor tyrosine kinases (RTKs), which are embedded in the plasma membrane and this leads, through adaptor proteins and exchange factors, to activation of the Ras proteins *(92)*. These are small guanine nucleotide binding proteins that are bound to the inner surface of the plasma membrane and are activated by exchange of GDP for GTP, which induces a conformational change in Ras proteins and allows them to bind to downstream effector proteins (*see 65,107)*. One family of Ras effector proteins are the Raf proteins, of which there are three isoforms in mammals (Raf-1, A-Raf, B-Raf) (*see 62,73)*. In the presence of inactive Ras, Raf proteins are cytosolic, but they are recruited to the plasma membrane in the presence of active Ras.GTP. The Raf proteins are the MAPKKKs of the ERK signaling cascade and Raf-1 is the most highly studied isoform. Raf-1 is activated at the plasma membrane in a Ras-dependent fashion, but its binding to Ras. GTP is not sufficient to stimulate its activation, and other events, including Raf-1 phosphorylation, oligomerization, association with other proteins, interactions with membrane lipids, and, possibly, a Ras-induced conformation change are required *(28,60,73)*. Active Rafs phosphorylate and activate MEK1 and MEK2, which in turn activate ERK1 and ERK2. The Raf proteins are therefore responsible for coupling growth-factor mediated RTK stimulation at the plasma membrane to cytosolic activation of the ERKs.

The MAPKKKs responsible for activating the JNKs and p38MAPKs are less well defined. The MLKs, MEKK1–4, TAK1, the ASKs, and TPL-2 can all activate both the JNKs and the p38MAPKs (*see 18,27,56)*, but the mechanism of activation of these MAPKKKs is less well characterized than Raf-1. MEKK2/3 can also activate the MEK5/ERK5 pathway *(10,102)*, but since many of these studies were performed with overexpressed proteins, the specificity inherent in these pathways may have broken down and the physiological interactions between the different MAPKKKs and MAPKKs require further analysis. For example, under some cicumstances MKK4 can activate p38MAPKs *(113)*. Finally, some of the MAPKKKs have only been able to stimulate the activity of the MAPKs in cell based studies, so it is possible that they are not direct MAPKKKs, but that additional kinases are required.

1.2. Protein–Protein Interactions Determine Cascade Specificity

Protein–protein interactions clearly play an important role in the regulation of the specificity of the different MAPK cascades. Direct interactions between the different components of each pathway have been described and these interactions may be facilitated or stabilized by specialized scaffold proteins. Thus, for

example, the protein MP1 (MEK Partner 1) interacts with MEK1 and ERK1 to enhance ERK1 activation *(90)*. Another protein, the kinase suppressor of Ras (ksr) binds to Raf-1, MEK, and ERK suggesting a scaffold function, but its physiological role remains enigmatic because it can mediate activation and suppression of ERK signaling in different systems *(20,46,105,118)*. Similarly, the Raf kinase inhibitor protein (RKIP) binds to Raf-1, MEK, and ERK *(121)*. MEK and ERK can bind to RKIP simultaneously, but the binding of Raf-1 and MEK is mutually exclusive. RKIP appears to be a negative regulator of ERK signaling, because it acts as a suppressor when over-expressed and ERK signaling is enhanced when RKIP is down-regulated *(120)*.

Scaffold proteins have also been described for the JNK cascade. Two cytoplasmic JNK-interacting proteins (JIP-1 and JIP-2) bind selectively to the JNKs but not to the other MAP kinases *(18,21,110,119)*. JIP-1 and JIP-2 also bind to MKK7 and members of MLK family and overexpression of JIP1 causes increased JNK activation by MKK7 and the MLKs, presumably by facilitating the formation of JNK-activating complexes *(109)*. Recently, an unrelated protein, called JIP3, has been identified that appears to act as a scaffold for the MLKs, MKK7, and JNKs *(51)*. Finally, the proteins MEKK1 and MEKK2 are MAPKKKs that also appear to act as a scaffold *(11,117)*; they can bind simultaneously to the MAPKK, MKK7, and to the JNK MAPKs. Indeed, MEKK1 may also play a more general scaffolding role, as it appears also to bind to all members of the Raf/MEK/ERK cascade *(49)*.

1.3. Nuclear Translocation of the MAPKs

The MAPKs are all activated in the cytosol and yet (with the exception of ERK3) all have been shown to be able to phosphorylate a large number of nuclear proteins, because (with the exception of ERK3) all the MAPKs can translocate to the nucleus when activated. Although the translocation typically only involves a small fraction of the activated kinase, it occurs rapidly and can persist for many hours. The mechanism of translocation has been extensively studied in the ERKs, but remains elusive as they do not contain either a nuclear localization signal or a nuclear export sequence, and both passive and active uptake have been described *(1,9)*. Since MEK1 and MEK2 do possess nuclear export signals, one model is that they anchor ERK1/2 in the cytoplasm through direct association *(33)*. Intriguingly, translocation of the ERKs to the nucleus does not require their phosphorylation or activation, because versions of the ERKs that cannot be phosphorylated still translocate to the nucleus, suggesting that activation of MEK1/2 may release the ERKs for passive or active uptake into the nucleus. It has also been suggested that the phosphatases that dephosphorylate and inactivate the ERKs act to anchor the ERKs in the cytosol, or shuttle them from the nucleus to cytoplasm upon deactivation *(9)*.

1.4. MAPK Substrates and the Regulation of Cellular Responses

With the exception of ERK3, all of the MAPKs have been shown to phosphorylate transcription factors and are presumed thereby to regulate directly the transcription of immediate-early genes such as *c-fos* and *c-jun* (for review, *see 106*). Furthermore, activation of the ERK cascade can induce transcription of proteins such as cyclin D, $p16^{INK4}$, $p21^{Waf1/Cip}$, and $p27^{Kip}$, which regulate the cell cycle *(63,89)*. It is also thought that the MAPKs are important mediators of chromatin remodeling through the phosphorylation of nucleosomal proteins such as HMG-14 and histone H3 and through induction of histone acetylation. Since so many proteins are MAPK substrates, the importance of any individual substrate to the response that is elicited is difficult to determine. Furthermore, some stimuli can activate multiple MAPKs cascades simultaneously, although with different time-courses and to different extents and different MAPKs can target the same substrate, making it difficult to assign specific effects to particular substrates.

While some of the phosphorylation events that are mediated by the MAPKs are direct, others are performed by downstream kinases that are themselves activated by the MAPKs. The ERKs activate three closely related protein kinases known as MAPK-activated protein kinases-1a, 1b, and 1c (MAPKAP-K1a/b/c; also known as RSK1/2/3; Fig. 2) *(100,124)*. Similarly, the p38MAPKs activate two closely related kinases called MAPKAP-K2 *(98)* and MAPKAP-K3 (also called 3pK) *(68)* and the protein kinase PRAK (p38-regulated activated kinase) *(76)*. In some cells, MAPKAP-K2 and -K3 are only activated by stimuli that activate the p38MAPK cascade *(12,15)*, but in others, all three MAPK cascades can activate MAPKAP-K3 *(95)*. In vivo, the MAPKAP-K1 family is only activated by agents that activate the ERK cascade *(95)*. Both the MAPKAPK-1s and MAPKAP-K2 can phosphorylate transcription factors, providing an indirect mechanism by which MAPKs regulate gene expression (Fig. 2) *(31,99,103)*. In vivo activation of ERK and p38MAPK also leads to activation of the mitogen- and stress-activated kinase (Msk) *(19)*, the MAPK integrating kinase (Mnk) *(34, 108)*, and the protein kinase RSK-B *(81)*. Thus, in addition to the complexity of the different cascades themselves, additional complexity is associated with downstream kinases that are directly activated by the MAPKs.

Another important aspect of MAPK signaling is the concept that both signal duration and strength are important mediators of the biological responses that they regulate (*see 66,89*). For example, in PC12 cells, transient ERK signaling stimulated by EGF leads to cell proliferation, whereas sustained signaling induced by NGF leads to differentiation. The importance of signal strength is exemplified by the observation that low levels of Raf-mediated ERK signaling leads to cell proliferation, whereas strong signaling leads to cell cycle arrest, which appears to be mediated by the induction of the cell cycle inhibitors $p21^{Waf1/Cip1}$ *(42,58, 94,116)* or $p16^{INK4}$ *(126)*, or through direct inhibition of the cyclin-associated

kinases CDK2 and CDK4 *(86)*. Similarly, constitutively active forms of MEK or ERK can induce increased levels of $p21^{Waf1/Cip1}$ in response to some agonists *(42)*, but through unknown mechanisms. In some cells, the induction of $p21^{Waf1/Cip1}$ and $p16^{INK4}$ is p53 independent *(94,116,126)*, whereas in others, ablation of p53 abrogates Raf-induced $p21^{Waf1/Cip1}$ expression *(58)*. The ability of MAPKs to regulate gene expression clearly explains, in part, why they are able to mediate complex cellular responses.

2. THE ROLE OF MAPK SIGNALING IN CANCER

2.1. ERK Signaling and Cancer

Although oncogenic versions of the ERKs have not been identified in human cancer and it has been difficult to mutate these proteins to create oncogenic proteins, several observations have implied that ERK signaling is an important pathogenic factor, at least in some cancers. Early studies had identified a number of powerful transforming agents of tissue culture cells, which were subsequently shown to be upstream of ERK. Consequently, it was shown that ERK activity was elevated in cell lines transformed by expression or oncogenes such as *v-ras*, *v-raf*, *v-src*, and *v-mos* (*see 56*). Interfering mutants of ERK (in which the T-loop sites were mutated to prevent their phosphorylation) or suppression of ERK expression using antisense constructs were used to block growth factor-mediated DNA synthesis and cell proliferation *(77,79,80)*. Similarly, interfering mutants of MEK1 were used to block growth factor-mediated proliferation and caused reversion of *v-src*- and *v-ras*-mediated transformation *(14)*. Constitutively active versions of MEK1 were shown to stimulate growth-factor-independent proliferation of fibroblasts in tissue culture and cells expressing these constructs and able to grow both in soft agar and as tumors in nude mice *(14,61)*.

These studies established that ERK was an important mediator of cell growth in model systems, but what of its role in human cancer? The first evidence of this came from the observation that mutant, activated Ras was found in ~30% of human cancers and is often associated with elevated ERK kinase activity *(7,114)*. The incidence of these mutations varies greatly among different cancers. For example, Ras mutations are found in more than 90% of adenocarcinomas of the pancreas, in ~50% of colon carcinomas, and only in ~30% of lung cancers. Hyperactivation of proto-oncogenes upstream of Ras has also been described in clinical samples of human cancer. This hyperactivation is often due to elevated activation of both RTKs and non-receptor tyrosine kinases (NRTKs) and has been associated with elevated Ras and ERK activity (for review, *see 13,52*). Tyrosine kinase hyperactivation can be caused by overexpression of the RTKs and NRTKs proteins as is seen with the platelet-derived growth factor receptor (PDGFR), which is often overexpressed in cancer of mesenchymal and glial origin. Similarly, overexpres-

sion of both the RTK c-erbB-2 and the NRTK Src is associated with breast cancer and in the case of c-erbB-2 is associated, at least in node-positive disease, with a poorer prognosis. Point mutations and fusion proteins involving both RTKs and NRTKs have also been documented (for review, *see 87*). Examples are the mutations described in the RTKs c-fms, c-Kit, and RET, that have been isolated from clinical samples of hematological cancers. Chromosomal translocations, leading to gene fusions that express proteins with elevated tyrosine kinase activity, have also been documented. Examples are the Tel-PDGFRb fusion, formed between the kinase domain of the PDGFR and the transcription factor Tel, a similar fusion between Tel and the NRTK Abl (Tel-Abl) and a fusion between the Bcr gene and Abl (Bcr-Abl). The elevated activity of these tyrosine kinases is often linked to hyper-activity in the Ras-ERK signaling pathway.

2.2. JNK Signaling and Cancer

It is generally accepted that the JNKs play a role in mediating cell death through apoptosis, rather than playing a role in cell growth and, like the ERKs, oncogenic versions of the JNKs have not been described. It is presumed that JNK-mediated, stress-induced apoptosis does not occur in tumor cells and that the JNK cascade is a "tumor suppressor" in normal cells. This view is supported by the identification of MKK4 as a candidate tumor-suppressor gene that may also play a role in suppressing metastasis *(101,104,122)*. JNKs may therefore seem unlikely candidates as therapeutic targets in cancer treatment, but indirect evidence for a role for these kinases in cancer pathology is available. Ras can induce c-jun phosphorylation, which is mainly mediated by JNK *(85,96)* and mutation of the Jun phosphorylation sites can suppress Ras-induced tumorigenicity *(4)*. Cells lacking c-jun lack many of the transformation characteristics normally seen in cells expressing oncogenic Ras and c-jun phosphorylation also appears to be required for *v-fos* transformation, but not for transformation mediated by *v-src (47)*.

More direct evidence that the JNKs play a direct role in cancer pathogenesis come from the observation that JNK kinase activity is elevated in several tumor cell lines *(18,45)*, and there is evidence to suggest that JNK is required to maintain tumor cell growth. Anti-JNK antisense oligonucleotides suppress EGF-stimulated growth of A549 human lung carcinoma cells *(8)* and JNK inhibition causes cell cycle arrest and apoptosis of MCF-7 and HCT116 cells *(83,84)*. These effects may be p53-dependent, because JNK inhibition only induces high levels of $p21^{Waf1}$ in parental but not in p53-defficient cell lines *(84)* and it appears that JNK2 may play a more important role in maintaining cell growth than does JNK1. These data suggest that JNK inhibition may be particularly effective in tumors lacking functional p53, but this may be of limited utility, as not all p53- deficient cells undergo apoptosis in response to JNK inhibition. In T89G cells (which have mutant p53), for example, JNK inhibition induces high levels of $p21^{Waf1}$ and cell cycle arrest, but not apoptosis *(83)*.

3. THERAPEUTIC AGENTS THAT BLOCK MAPK SIGNALING

Among the first compounds developed to target signaling molecules were agents that antagonized the activity of tyrosine kinases shown to have elevated activity in human cancer. Many of these compounds have now entered clinical trials (for review, *see 22*), some with spectacular results. The compound STI 571 (signal transduction inhibitor 571), an Abl kinase antagonist indicated for treatment of chronic myelogenous leukemia (CML) deserves special mention. CML is a hematological stem cell disorder, the hallmark of which is the Philadelphia chromosome. This reciprocal translocation generates the Bcr-Abl fusion protein mentioned above and is found in 95% of CML patients and approx 5% of adults with acute leukemia. In the phase I trial, approx 50% of patients responded to STI 571 treatment, showing clear signs of clinical improvement (*see 35*). Incredibly, although STI 571 also antagonizes the activity of c-Kit and the PDGF receptor, it proved to be particularly nontoxic and was very well tolerated by patients.

The unexpected success of STI 571 in the clinic demonstrates the potential for compounds that target hyperactive tyrosine kinases. However, these compounds are generally developed to target the upstream components of the signaling pathways and cannot therefore be considered to be pure ERK cascade antagonists, as they will block the activity of other signaling pathways. These compounds also suffer the disadvantage that they are only indicated for cancers where the tyrosine kinase they target is hyperactivated, so they have limited use. The development of compounds that target the downstream signaling pathways that are hyperactivated in a larger number of cancers from different tissues may therefore be of greater utility. The understanding that we have of the ERK cascade has enabled the development of screens to identify compounds that target its components. A convenient feature of this cascade in screening protocols is its three-tiered nature. Thus, high throughput screens that measure the activity of the cascade allow three enzymes to be screened simultaneously. Using these approaches, a number of compounds have been developed that target this cascade, although, as we shall see, other compounds have been developed using assays that did not specifically target ERK signaling.

3.1. Inhibitors of the Raf Protein Kinases

Clearly, a concern associated with inhibitors of the Raf proteins is that they may be very toxic, as these proteins are involved in maintaining normal cellular homeostasis. This view is supported by the observations that mice that are null for Raf-1 die *in utero* at an early age *(43,69,115)*. However, we have recently created a "knock-in" mouse in which the genomic copy of the Raf-1 gene was replaced with a mutant Raf-1 gene that expresses a protein with undetectable kinase activity *(43)*. The animals that express two copies of this mutant protein appear

to be normal, are fertile, and live a normal life span. Thus, Raf-1 protein expression is required during development, but its kinase activity is dispensable during development and in adult life, and so inhibitors of these kinases may not be as toxic as may have been predicted. To date, four classes of compounds that target Raf have been described.

3.1.1. Raf-1 Antisense Oligonucleotides

The compound ISIS 5132 is a phosphorothioate oligodeoxynucleotide antisense compound that targets the 3' untranslated region (UTR) of the human *Raf-1* gene *(72)*. ISIS 5132 appears to mediate degradation of *Raf-1* mRNA and thereby suppresses protein expression. It appears to be highly specific, because in tissue culture studies it blocks expression of Raf-1, without affecting A-Raf expression *(71)*. ISIS 5132 has been tested against human tumor xenografts in nude mice, and, although there is some concern regarding the original published data, it has been shown to be active against tumors that have elevated Ras signaling *(70,71)*.

Two concurrent clinical trials have been initiated using ISIS 5132 in patients with a range of solid tumors *(16,78,97)*. Interestingly, it was well tolerated, producing only mild side effects, and these were attributed to the antisense technology, rather than sequence specific effects. This raises interesting issues relating to the safety of this approach. In the mouse studies that were performed with ISIS 5132, the toxicity could not be fully tested, because the sequence that was targeted is not conserved in the mouse gene. Therefore, it is likely that the mouse *Raf-1* gene was not targeted. In one of the clinical trials, the levels of Raf-1 mRNA were found to be suppressed in peripheral blood mononuclear cells from the treated patients *(78)*. This demonstrates that these compounds do not only target tumor cells, but also target the Raf-1 in normal tissues. With reference to the mouse knock-out studies, this may have been expected to be lethal, but these studies show that some reduction in Raf-1 protein in normal tissues can be tolerated. Furthermore, some potentially encouraging antitumor responses have been observed in the clinical trials, and ISIS 5132 is being progressed into a phase II trial and also into another Phase I trial in combination with conventional chemotherapeutic agents *(16,78)*. The results from these studies will prove very interesting, as they will both test the use of antisense technologies in the clinic and determine whether general targeting of Raf-1 protein is a feasible approach.

3.1.2. Oxindoles

The oxindoles are a series of benzylidene-1H-indole-2-one derivatives that were identified using a single step, scintilation-proximity assay with Raf-1 phosphorylating MEK *(53)*. A structure-activity-relationship (SAR) analysis of over 2000 compounds was undertaken and a number of compounds were identified, examples of which are shown in Figs. 3A and B and which inhibit Raf-1 with potencies in the low n*M* range. Some of these compounds were also shown to be

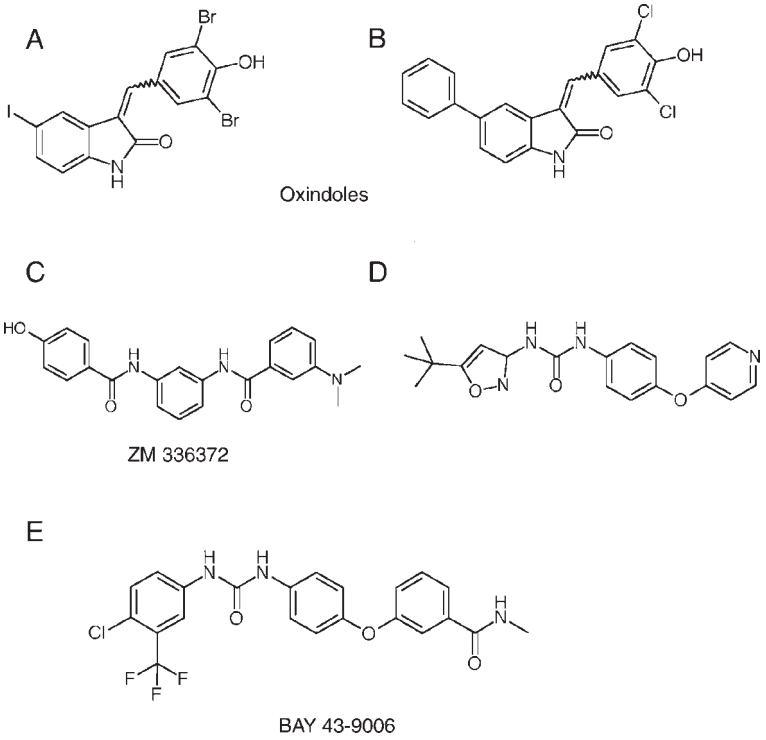

Fig. 3. Raf protein kinase inhibitors. The chemical structures of the Raf protein inhibitors are shown. (**A,B**) Oxindoles. Two examples of oxindoles that are specific inhibitors of Raf-1 are shown. (**C**) ZM 336372. (**D**) The lead compound, *N*-(5-*tert*-butyl-3-isoxazolyl)-*N'*-(4-phenoxy-phenyl)urea is shown. (**E**) The clinical derivative of this compound, BAY 43-9006 is also shown.

potent inhibitors of EGF-mediated MAPK activation in vivo *(53)*. Since the first description of these compounds, there have been no follow-up studies and so the clinical progression of these compounds is unclear.

3.1.3. ZM 336372

The compound ZM 336372 (*N*-[5-(3-dimethyl-aminobenzamido)-2-methyphenyl]-4-hydroxybenzamide; Fig. 3C) was identified in a high-throughput screen using a single-step coupled assay with Raf-1, MEK1, ERK2, and myelin basic protein (MBP) as sequential substrates *(38)*. Upon deconvolution, ZM336372

was found to be ineffective against MEK1 or ERK2, but to be a potent Raf-1 inhibitor at low ATP concentrations. ZM 336372 did not inhibit a range of other kinases, but it did inhibit B-Raf somewhat less efficiently than Raf-1 and, curiously, it inhibited the activity of p38MAPKα and β, a point to which we shall return subsequently. Despite being an effective inhibitor of Raf-1 in vitro, the results with ZM 336372 in tissue culture cells were both disappointing and curious. Pretreatment with this compound did not block growth factor-mediated MEK and ERK activation and did not suppress the proliferation of cells stimulated with a variety of mitogens *(38)*. Instead, and rather curiously, treatment of cells with ZM 336372 resulted in a hyperactivation of Raf-1, but without stimulating the activity of MEK or ERK. The mechanism of this paradoxical activation is unknown, but is Ras independent and did not appear to be due to the inhibition on the p38MAPKs. These data suggest that Raf-1 is feedback-inhibited by its own basal kinase activity and that agents that suppress this basal activity consequently stimulate to its own activation. This could be (and has been, *see 38*) interpreted to suggest that the inhibition of Raf-1 may not be a viable approach for cancer treatment. However, it is not known how ZM 336372 inhibits Raf-1 in vitro, nor is it known how it activates Raf-1 in vivo. It should be noted that the inhibition in vitro was tested at low ATP concentrations and that at the higher concentrations present in cells, the effects of this compound may be different. The mechanism of action of this compound surely hides interesting aspects of Raf-1 regulation that are not yet understood.

3.1.4. Compounds Based on *N*-(5-*tert*-butyl-3-isoxazolyl)-*N'*-(4-phenoxy-phenyl)urea

The value of Raf-1 as a therapeutic target was recently demonstrated in a series of exciting posters presented at the 11th NCI-EORTC-AACR Symposium on New Drugs in Cancer Chemotherapy (Amsterdam, 7–11 November 2000). Using a high-throughput screen in which MEK was phosphorylated by Raf-1, a urea was identified that was able to suppress the activity of the Raf-1 protein. This compound was matured through SAR analysis to produce the compound *N*-(5-*tert*-butyl-3-isoxazolyl)-*N'*-(4-phenoxy-phenyl)urea (Fig. 3D) *(59)*. This compound is the preclinical candidate and inhibits Raf-1 with an IC_{50} of approx 230 n*M*, but was inactive against a range of other protein kinases *(111)*. It also blocks B-Raf activity in vitro and was able to suppress the activation of MEK stimulated by an inducible version of B-Raf. The compound also suppressed EGF-stimulated ERK activation in A431 cells and blocked both the anchorage-dependent and -independent growth of HCT116 cells in culture *(111)*. Typical dosing schedules of 10–300 mg/kg daily for 14 d were shown to give regressions of human tumor xenografts in nude mice and increased survival of animals bearing tumors with activated Ras *(24)*. Two further very important features of these compounds is that they are orally available (bioavailability approx 70%) and are quite nontoxic and appear

to be well tolerated *(40)*. Together with favorable pharmacokinetics *(40)*, these compounds are the most promising to be developed that target the Raf proteins. The clinical candidate (BAY 43-9006, Fig. 3E) has an in vitro IC_{50} value against Raf-1 of approx 6 n*M* and inhibits cell proliferation with an IC_{50} of approx 2–3 μ*M* (J. Lyons, personal communication). The growth of human tumor cell lines in soft agar is significantly more sensitive to this compound, with an IC_{50} of approx 6–7 n*M*. This compound has now entered clinical trials and the results are eagerly awaited.

3.2. MEK Inhibitors

A number of inhibitors of the MEK MAPKKs have been developed. Two of these compounds (PD 98059, U0126) have been used extensively to study the ERK signaling pathway in tissue culture systems and a number of studies have been performed on the mechanisms by which these inhibitors function, as will be described below. A third compound (PD 184352) has recently been shown to be active against human tumor xenografts in nude mice, suggesting that these compounds may be useful in the clinic.

3.2.1. PD 098059

The flavione, PD 098059 [2-(2'-amino-3'-methoxyphenyl)-oxanaphthalen-4-one] (Fig. 4A), was identified in a high-throughput screen in which MEK1, ERK, and MBP were used as sequential substrates *(23)*. The MEK1 that was used in this screen was produced in bacteria and had not been activated, so the assay depended on basal kinase activity. PD 098059 suppressed basal MEK1 kinase activity with an IC_{50} of approx 2–10 μ*M* and also suppressed the activity of constitutively activated MEK1, in which the T-loop phosphorylation sites were substituted for glutamates (MEK1-2E) at similar levels *(2,23)*. In tissue culture, PD 098059 suppressed PDGF stimulated ERK activation and DNA synthesis in Swiss 3T3 cells, with an IC_{50} of approx 7 μ*M* *(23)* and also prevented ligand stimulated ERK activation in a number of cell systems *(2)*. PD 098059 is more potent against MEK1 (IC_{50} approx 7 μ*M*) than against MEK2 (IC_{50} approx 50 μ*M*) and also suppressed MEK1-activation-stimulated by MEKKs in vitro. It suppressed the basal activity of ERK in *ras*-transformed cells, reverted their transformed morphology and suppressed the ability of *ras*- and *raf*-transformed cells to grow in soft agar *(23)*. Finally, PD 098059 proved to be highly specific for MEK, failing to inhibit the activity of a number of other protein kinases (including other MAPKKs) *(2,17,23)*, although recently it was shown to suppress signaling through the MEK5/ERK5 cascade *(48)*.

The exquisite specificity displayed by PD 098059 is most likely explained by its mechanism of action. PD 098059 is not competitive with ATP and therefore did not block the activity of MEK that had been preactivated by Raf-1 in vitro or the in vitro activity of MEK that had been activated in vivo *(2)*. It did, however,

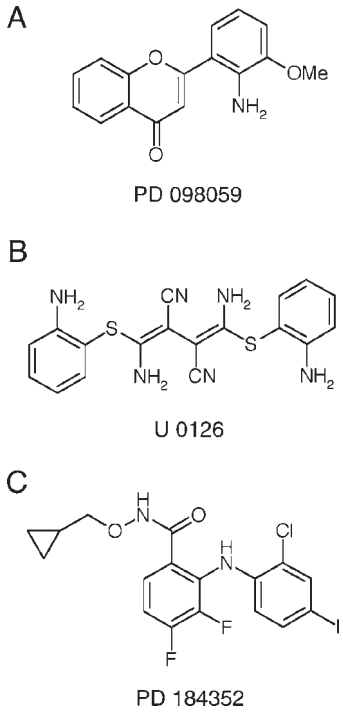

Fig. 4. MEK protein kinase inhibitors. The chemical inhibitors of the MEK proteins are shown. (**A**) PD 098059. (**B**) U0126. (**C**) PD 184352.

block Raf-1-mediated MEK1 phosphorylation and activation in vitro and thus PD 098059 appears to work, not by blocking MEK kinase activity, but by uncoupling the Raf-MEK activation step, presumably by binding to MEK and preventing its binding to Raf-1 *(2)*. PD 098059 is therefore a compound that blocks MEK activation, rather than one that inhibits its activity.

3.2.2. U0126

Another MEK inhibitor, the compound U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene) (Fig. 4B) was not identified in a biochemical screen, but in a cellular-based screen that measured AP-1-mediated gene expression *(29)*. U0126 was found to block AP-1-dependent gene expression but without blocking the DNA binding activity of AP-1 and, subsequently, it was shown

that it targeted the Ras signaling pathway. In TPA-treated cells, U0126 blocks ERK, but not Raf or MEK activation and U0126 inhibits the in vitro activity of MEK, but not Raf or ERK, demonstrating that it targets MEK *(29)*. U0126 inhibits both MEK1 and MEK2 with similar potencies (approx 70 n*M* and approx 60 n*M*, respectively) and like PD 098059 it is not competitive with ATP and also is not competitive with ERK. U0126 appears to bind to the same or an overlapping site on MEK that is bound by PD 098059, because their binding is competitive, but U0126 binds with higher affinity *(29)*. Despite this sharing of a common binding site, these compounds appear to have different mechanisms of action. Thus, whereas PD 098059 appears to prevent MEK from being activated by Raf proteins, U0126 does not block MEK activation, but does prevents it from activating ERK. This also appears to be true of their activity in vivo. Whereas PD 098059 suppresses COX1 and COX2 gene expression, U0126 does not *(6)*. Never the less, like PD 098059, U0126 is very specific for MEK and does not block the activity of a variety of other kinases *(17,29)*, but does block MEK5/ERK5 signaling *(48)*.

Both PD 098059 and U0126 have proven to be extremely useful tools for tissue culture based studies. Both block the growth of Ras-transformed cells in vitro *(2,23,29)* and have proven extremely useful for studying the activity of the Ras-ERK pathway in tissue culture systems. Because these compounds are not ATP competitive, their activity will not depend on the levels of ATP in the cells. However, there is no information on the use of these compounds in animals, presumably due to unfavorable pharmacokinetics or unacceptable toxicity, and clinical trials using these compounds have not been reported.

3.2.3. PD 184352

PD 184352 2-(2-chloro-4-iodo-phenylamino)-*N*-cyclopropylmethoxy-3,4-difluorobenzamide (Fig. 4C) was also developed using a single-step high-throughput screen with GSTMEK, GSTERK, and MBP as substrates, followed by chemical maturation of the lead compound *(93)*. PD 184352 inhibits MEK activity in vitro with an IC_{50} of 17 n*M*, but does not inhibit ERK and does not inhibit a number of other kinases *(93)*. Like the other MEK inhibitors, PD 184352 is not competitive for either ATP or ERK binding, and although it is not known to compete with PD 098059 or U0126 for binding to MEK, it is possible that all three compounds bind to the same site. In tissue culture, PD 184352 inhibited PDGF-mediated ERK phosphorylation and suppressed the elevated ERK activity seen at steady state in a number of tumor cell lines. The anchorage-dependent growth of cells with elevated ERK activity was considerably more sensitive to PD 184352 (IC_{50} approx 0.15 μ*M*) than the growth of cells which did not have elevated ERK activity (IC_{50} >10 μ*M*) and PD 184352 reversed the morphological transformation of cells with elevated ERK activity *(93)*. One important point about PD 184352 is that it is a cytostatic rather than a cytotoxic and, when removed from the growth medium, cell growth is restored and their transformed morphology reappeared

(93). PD 184352 also suppressed the invasion of colon 26 cells through matrigel, suggesting that it may possess antimetastatic properties.

The most exciting data with PD 184352, however, comes from the in vivo studies performed in nude mice. Although a full toxicological study has not yet been published, the compound was extremely well tolerated by mice that received up to 6 g/kg over a 14 d period *(93)*. The levels of ERK phosphorylation in human tumor xenografts excised from the mice that were treated with PD 184352 were significantly reduced and remained suppressed for up to 9 h following drug administration *(93)*. PD 184352 administration suppressed the rate at which human tumor xenografts with elevated ERK phosphorylation grew in nude mice, but did not provide any survival advantage to mice inoculated with tumors that did not have elevated ERK phosphorylation *(93)*. Although two different tumor models were used for these studies, this result suggests that PD 184352 is targeting the ERK cascade in vivo and providing a therapeutic advantage.

3.3. Inhibitors of the MAPKs

It is intriguing that despite the large number of high-throughput screens that have been conducted, no direct inhibitors of the ERKs have been described. There could be many reasons for this. It is possible that these compounds simply do not exist in the compound libraries that have been examined, or that they have been discovered, but not made public. Alternatively, it is possible that the structure of these MAPKs do not lend themselves to inhibition and that potential compounds that are produced are simply screened out when cross-examined against other kinases to determine specificity. Antisense approaches have been suggested for both of these proteins, but these have only been used for tissue culture studies and, as yet, there are no clinical trials reported. However, the situation with the p38MAPKs is different. A number of compounds have been described that are exquisitely selective for the p38MAPKs. The interest in these compounds lies in their use in the treatment of rheumatoid arthritis and one compound, SB 203580, has been shown to reduce joint edema in models of induced arthritis in mice and rats *(3)*. These compounds are unlikely to be used for the treatment of cancer, however, because the role of p38MAPKs in cancer is unclear. Nevertheless, they offer interesting insights into specificity and why these compounds inhibit the p38MAPKs and not the ERKs or JNKs.

The pyridinyl imidazole compounds were originally identified due to their ability to inhibit LPS-induced IL-1 and TNFα synthesis in monocytes *(55)*. The compounds SB 203580 (Fig. 5) and SB 202190 were among the most potent and are competitive with ATP. Thus, SB 203580 does not block the activation of p38MAPK by MEK3/MEK6, but it does block both the basal and stimulated activity of p38MAPK (*see 5,54*). Despite the high conservation between the four p38MAPKs, SB 203580 inhibited p38MAPKα and p38MAPKβ in vitro with

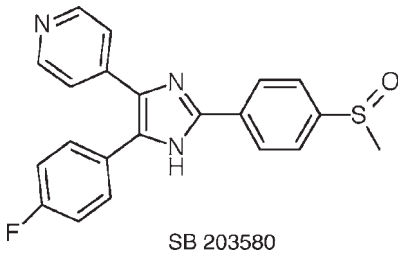

Fig. 5. SB 203580, a p38MAPKα and p38MAPKβ inhibitor. The chemical structure of SB 203580, a p38MAPKα and β inhibitor is shown.

IC_{50} values of approx 50 n*M*, but did not inhibit p38MAPKγ or δ, or a number of other protein kinases *(13,15,17,36,123)*.

The reason for this extraordinary specificity can now be explained, because the X-ray crystal structures of p38MAPKα bound to SB 203580 have been resolved *(112)*. SB 203580 binds to the ATP binding pocket in a region that is overlapping with, but distinct from, ATP. The crystal structure reveals that SB 203580 does not inhibit other protein kinases, because the 4-fluorophenyl moiety cannot be accommodated in their ATP-binding pockets. In p38MAPKα and p38MAPKβ, there is the relatively small amino acid threonine at position 106 (Fig. 6), which creates a cavity in the wall of the ATP-binding pocket into which the 4-fluorophenyl moiety neatly fits. In the majority of other kinases, including p38MAPKγ and δ, the position equivalent to position 106 contains a relatively large amino acid, such as a methionine or glutamine (Fig. 6) so there is no cavity for 4-fluorophenyl moiety to fit into. Indeed, when a methionine is introduced into position 106 of p38MAPKα or p38MAPKβ they become insensitive to SB 203580 *(26)* and insertion of a threonine into this position in ERK2 or JNKs renders these proteins sensitive to inhibition by SB 203580 *(30,37)*. Ironically, one of the kinases that naturally possesses a threonine at the position equivalent to 106 of p38MAPK is Raf-1 (Fig. 6). This lead to the observation that the in vitro kinase activity of Raf-1 can be blocked by SB 203580, with an IC_{50} of approx 360 n*M* and that like ZM 336372, Raf-1 is activated in a paradoxical manner in SB 203580 treated cells *(39)*. Thus it appears that the compounds ZM 336372 and SB 203580 are structurally related. They appear to regulate Raf-1 through common mechanisms and this presumably explains why, as mentioned above, ZM 336372 also suppresses the activity of the p38 MAPKs *(38)*.

4. CONCLUSIONS

It is clear that the ERK signaling cascade plays an important role in cancer pathogenesis and it has been validated as a *bona fide* target for novel therapies.

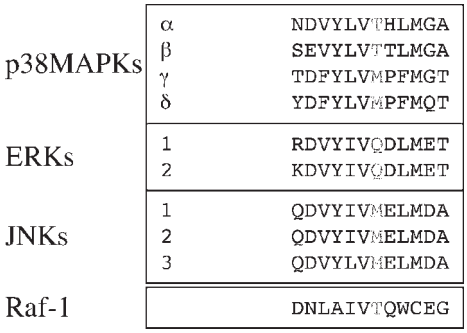

Fig. 6. Comparison of the amino-sequences flanking threonine 106 of p38MAPKα, the residue that confers sensitivity to SB 203580 and which is highlighted in grey. The following amino acids are shown: p38MAPKα: 100–111; p38MAPKβ: 100–111; p38MAPKγ: 103–114; p38MAPKδ: 101–112; ERK1: 116–127; ERK2: 98–110; JNK1: 102–113; JNK2: 102–113; JNK3: 140–151; and Raf-1: 396–407. The single amino acid code is used and the position equivalent to Thr106 of p38MAPKα is highlighted in bold in all of the other kinases.

The concept of this cascade as a linear pathway from plasma membrane to nucleus has proven to be somewhat naive and it is now clear that there is a significant amount of cross-talk with other pathways (*see 32,41*). We do not fully understand the regulation of this cascade, but we have sufficient knowledge to develop therapeutic compounds that target this pathway and some of these are now entering the clinic. It is also true that other agents that have entered the clinic also target this pathway. The compound 17-AAG, an inhibitor of the Hsp90 proteins, also targets (among other proteins) the Raf family members, which become degraded when cells are treated with this compound *(75)*. It is unclear what proportion of the antitumor activity of Hsp90 is due to the targeting to the Raf proteins, but either in combination with other pathways, or if targeted alone, blocking the ERK pathway is clearly a feasible approach for the treatment of some cancers. It is possible that the JNK pathway may also represent a good cancer therapy target, but as yet, no inhibitors of this pathway have been described.

It is curious that the compounds that have been developed appear to be particularly nontoxic. This is true of the compound STI 571, but appears also to be true of the compounds directed toward the ERK signaling pathway. The original goal was to identify targets that were tumor specific, so that compounds could be developed that were tumor selective. However, we now know that the so-called cancer targets are active in the majority of normal cells as well, where they regulate cellular responses to the environment. It would therefore be reasonable to expect some toxicity from compounds that target the processes that maintain normal

cell homeostasis and yet, in preclinical studies and the phase I trials, toxicity does not appear to be the problem that was feared. It may be that these compounds target only the elevated, constitutive signaling that is required to maintain high rates of tumor cell growth and do not suppress the signaling that occurs in normal cells. An important area of study for the future will be to determine how this distinction is made. However, perhaps one of the prices to pay for this lack of toxicity is the fact that these compounds are cytostatic and not cytotoxic. Therefore, although they may stabilize disease, they may fail to effect any cures. It is possible that they will be most effective if combined with conventional, cytotoxic therapeutic agents. Alternatively, in solid tumors, they may affect angiogenesis, a process that requires ERK signaling *(57)* and this may starve the tumor of nutrients and mediate an effective cure. It should be remembered that STI 571 is also a cytostatic drug and yet it clearly has excellent effects in the clinic; perhaps cytostatic drugs that are free of toxic side-effects are a preferable way to treat cancer.

A final point about specificity. The mechanisms of specificity that are achieved by drugs that target the Raf-MEK-ERK signaling pathway are certainly interesting when we consider that the majority of tyrosine kinase inhibitors are ATP competitive *(22,87)*. ZM 336372 is an intriguing compound, and it is not known how BAY 43-9006 suppresses Raf-1 activity, but the MEK inhibitors appear to function by decoupling the components of the cascade and thereby preventing signaling. Targeting the protein–protein interactions may therefore be the key to specificity in these sorts of compounds. Elucidating the mechanism of action of ZM 336372 is likely to reveal interesting information about how Raf-1 is regulated and may hint as to what will be required from the next generation of ERK cascade inhibitors. The Ras-Raf-MEK-ERK signaling pathway is now established as an important target in cancer therapy and the results of the clinical trials should prove both interesting and exciting.

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10 Mdm2 and ARF

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Contents

1. INTRODUCTION

One of the major defense mechanisms against the development of cancer is governed by the tumor-suppressor protein p53. The p53 protein can induce growth arrest and apoptosis, and, owing to these important functions in controlling cell proliferation, p53 itself has to be tightly regulated. The identification of Mdm2 as a major player that effects p53 protein stability, and the discovery that the tumor-suppressor protein p14ARF is an important regulator of Mdm2, initiated a series of discoveries that gave new insight into hitherto unknown molecular mechanisms that govern p53 functions. Although most of the research about Mdm2 and ARF concentrate on their roles in regulating p53, there is growing evidence that Mdm2 and ARF also possess p53-independent functions. Our increasing understanding of all functions of Mdm2 and ARF will further consolidate our knowledge about the interplay of key players of cell cycle regulation and will surely contribute to the development of new therapeutical approaches in cancer treatment.

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2. MDM2

Mdm2 was originally identified from amplified DNA sequences associated with mouse double minute chromosomes in a spontaneous transformed mouse 3T3 cell line *(1)*. Subsequently, Mdm2 was shown to possess oncogenic potential, because NIH 3T3 and rat cells overexpressing Mdm2 induced tumors in nude mice *(2)*. Mdm2 was also identified independently as a 90 kD protein that can interact with the p53 tumor-suppressor protein *(3,4)*, and the corresponding human gene has been mapped to chromosome 12q13-14 *(5)*.

Mdm2 is overexpressed in a variety of different tumors either by gene amplification, increased transcription, or enhanced translation. Using Southern blotting and quantitative PCR, it was found that *mdm2* gene amplification ranges between 2- and 10-fold *(6)*. Based on examination of a wide variety of different tumors, an overall *mdm2* gene amplification frequency of 7% has been calculated *(6)*. The highest frequency of 20% was observed in soft tissue tumors, osteosarcomas showed 16%, and esophageal carcinomas 13%. Overexpressed Mdm2 was also frequently found in human breast carcinomas *(7,8)*. Overexpression, however, is not the only mechanism resulting in deregulation of Mdm2 expression that can be found in tumors. Frame shift, mis-sense, or non-sense mutations within the zinc-binding regions were also described for *mdm2* analyzed in a variety of tumors *(9)*.

2.1. Structure of Mdm2

The human *mdm2* gene encodes a protein of 491 amino acids, whereas the murine homolog consists of 489 amino acids *(5)*. Protein sequence alignment of Mdm2 from different species revealed four major conserved protein domains (*10* and unpublished results) (Fig. 1A). Conserved region I includes about 90 amino acids at the N-terminus, and several proteins have been identified that bind to this region of Mdm2, among them p53 (Fig. 1b). Interaction of Mdm2 with p53 can result in inhibition of the transactivation function *(11–15)*, and mediates the proteolytic degradation of p53 *(16–18)*. Based on sequence comparison with the amino acid stretch of p53 that interacts with Mdm2, the p53 homolog p73 *(19–22)* and the transcription factor E2F-1 *(23)* have also been identified as Mdm2 binding proteins. Further N-terminal binding proteins are the cell fate protein Numb *(24)*, DNA polymerase ε *(25)*, the E2F-1 binding protein DP-1 *(23)*, and the co-transactivator p300 *(26)*. The conserved regions II and III represent a highly acidic region and a zinc-finger domain, respectively. To these regions, the tumor-suppressor proteins ARF *(27,28)* and Rb *(29)*, the ribosomal protein L5 *(30)*, and the very recently identified MTBP (*M*dm *t*wo *b*inding *p*rotein) *(31)* can bind. The C-terminal homologous region includes a RING-finger domain *(32,33)* that is responsible for the interaction with RNA *(30)*. In principle, RING-finger domains are known to interact with DNA, RNA, and proteins, and, recently, it has been shown that these motives are directly involved in targeting proteins for degradation by helping to transfer

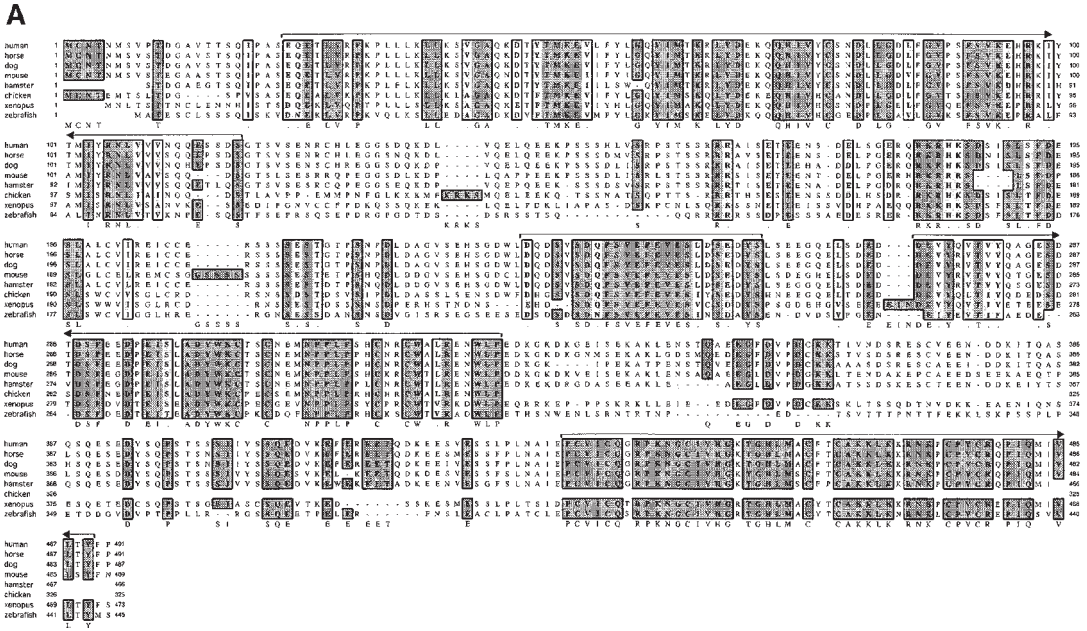

Fig. 1. (**A**) Comparison of amino acid sequences of Mdm2 homologs from different species. Sequence alignment was performed using MacVector software package version 6.5 (Oxford Molecular Ltd., UK). Conserved region one to four are marked with lines above the sequences. Identical amino acids are dark shaded; similar amino acids are light shaded. The corresponding nucleic acid sequences were obtained from the following references and from Genbank, respectively: *H. sapiens (5)*, accession number Z12020; *Equus caballus (196)*, acc. no. AF121140; *Canis familiaris (197)*, acc. no. AB031276/AF322416; *Mus musculus (2)*, acc. no. X58876; *Mesocricetus auratus (198)*, acc. no. U10982 (partial cds); *Gallus gallus*, acc. no. AF005045 (partial cds); *Xenopus laevis (199)*; *Danio rerio*, acc. no. AF10255.

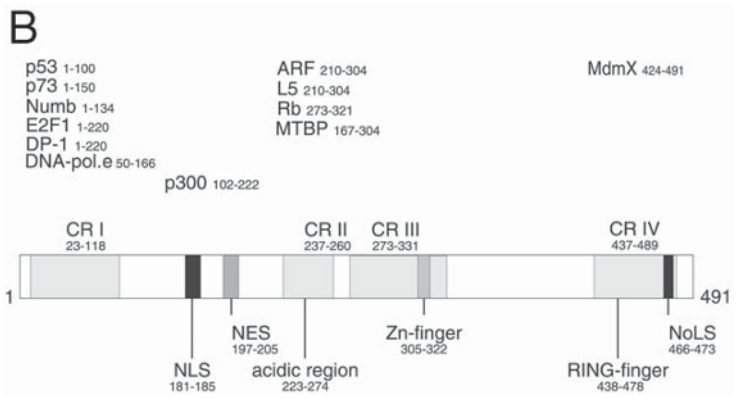

Fig. 1. (**B**) Primary structure and interacting proteins of Mdm2. (CR I-IV, conserved regions I-IV; NLS, nuclear localization signal; NES, nuclear export signal; NoLS, nucleolar localization signal). Numbers indicate amino acids stretches within Mdm2 to which the appropriate interacting protein binds.

ubiquitin *(34–36)*. The only homolog of Mdm2 *(37,38)* identified so far, termed MdmX, can form heterodimers with Mdm2 via RING-finger domains *(39)*. Mdm2 contains several potential phosphorylation sites, and phosphorylation might count in part for the discrepancies between the predicted and the apparent molecular weight (55 kD and 90 kD, respectively) *(5)*. Noteworthy, several splice variants of the Mdm2 mRNA and Mdm2 proteins with different apparent molecular weights ranging from 57 to 85 kD have been detected in several tumor cells *(14,40)*. The alternative Mdm2 mRNA and protein species, however, have not been observed in normal cells, and it is unclear whether they contribute to tumorigenesis *(41)*.

Shorter regions of homology include a nuclear *(5)* and nucleolar *(42)* localization signal as well as a nuclear export signal *(43)*, all of which are involved in regulation of the subcellular localization of Mdm2.

2.2. Function of Mdm2

2.2.1. Regulation of Tumor-Suppressor Protein p53 and p53 Family Members

Although identified as p53 binding protein, expression of or interaction with Mdm2 is not a prerequisite for any of the known functions of p53 *(44,45)*. The pivotal role of Mdm2 in regulating p53, however, is evident in *mdm2*-deficient mice that die very early in embryonic development, probably due to deregulation of p53-mediated apoptosis *(46–48)*. This phenotype is rescued by simultaneous deletion of the *p53* gene. $p53^{-/-}$ and $p53^{-/-}/mdm2^{-/-}$ mice show no differences in incidence and spectrum of spontaneous tumor formation *(49)*, and cells derived

from these mice showed the same growth characteristics in vitro, suggesting that regulation of p53 is the sole function of Mdm2. p53 is a transcription factor and binding of Mdm2 occurs within the transactivation domain of p53 *(11)* resulting in the inhibition of transcriptional transactivation of *p53* target genes such as cyclin-dependent kinase inhibitor p21. *mdm2* by itself is a p53 target gene *(50)*, and it is now well established that both proteins form a regulatory negative feedback *(12)*. p53 functions mainly as a safeguard mechanism in response to different types of cellular stress *(51)*. In unstressed cells, p53 protein is maintained at a low level owing to constant degradation of the protein, resulting in a very short half-life. Degradation of p53 occurs via the ubiquitin-dependent pathway *(52)*, in which proteins are targeted for degradation by covalent attachment of several ubiquitin molecules to specific lysine residues. For this, ubiquitin molecules are first activated by an ubiquitin-activating enzyme E1, and subsequently transferred to the target protein via ubiquitin conjugating enzyme E2 and ubiquitin ligase E3 *(53)*. Recently, Mdm2 has been identified as a protein that can effect p53 protein stability *(16–18)*, and it is now established that Mdm2 can function as an E3 ubiquitin ligase for p53 *(54)*. A conserved cysteine residue within the RING-finger domain of Mdm2 (cysteine 464) forms an activated thioester bond with ubiquitin, from which the ubiquitin molecule is subsequently transferred to p53. Deletion or mutation of the RING-finger domain results in loss of ubiquitin ligase activity of Mdm2 *(54–57)*. Interestingly, several other proteins containing RING-finger domains such as BRCA and CBL have been found to interact with E2 ubiquitin-conjugating enzymes *(34,35)*. Furthermore, a variety of known E3 ubiquitin ligases were found to contain such RING-finger domains as Ubr1p, the human homolog of the yeast N-recognin that targets proteins for degradation via recognition of specific N-terminal amino acid residues of target protein *(58)*, and Apc 11, a component of the anaphase promoting complex *(59)* that regulates cell cycle progression by degradation of mitotic cyclins. A general concept is now emerging in which target specificity in ubiquitin-dependent protein degradation is achieved by RING-finger domain containing proteins like Mdm2 that interact specifically with the appropriate target proteins (like p53), and simultaneously with E2 ubiquitin conjugating enzymes, thereby acting as bridging proteins allowing transfer of ubiquitin molecules from E2 ubiquitin-conjugating enzymes to the target protein *(60)*. RING-finger domains, however, are not essential for the transfer of ubiquitin from E2 enzymes onto target proteins. Another class of E3 ubiquitin ligases, the so-called HECT (*h*omologous to the *E*6AP *c*arboxyl *t*erminus) domain containing proteins, do not contain RING-finger domains *(61,62)*, and the E3 ubiquitin ligase function of Mdm2 has actually been identified by sequence alignment with the HECT domain protein E6AP *(54)*, an E3 ubiquitin ligase that is involved in p53 degradation caused by viral proteins *(63)*. Whether Mdm2 belongs functionally to the HECT domain or the RING-finger domain E3 ligases therefore remains to be elucidated.

Mdm2 as well as p53 predominantly localize to the nucleus but both are constantly shuffled between nucleus and cytoplasm *(43,64)*, and nuclear import and export of Mdm2 has been shown to be required for Mdm2-mediated degradation of p53 *(65–67)*. There are, however, conflicting results in the ability of both proteins to exit the nucleus independently. Whereas a p53 mutant that is unable to bind mdm2 behaves like wild-type p53 with regard to subcellular localization *(68)*, other reports demonstrated that the RING-finger domain but not the NES of Mdm2 is required for nuclear export of p53 *(69–71)*. These results suggest that Mdm2-mediated ubiquitination of p53 might be a prerequisite of p53 nuclear export, although Mdm2 can ubiquitinate and degrade cytoplasmic p53 mutants *(72)*. It has been shown, however, that the RING-finger domain of Mdm2 can interact with other cellular proteins, e.g., MdmX *(39)*, or can be modified by attachment of the ubiquitin homolog SUMO-1 *(73)*, and the contribution of these modifications to the regulation of subcellular localization of Mdm2 and p53 are still unknown.

Mdm2 can interact with the co-transactivator protein CBP/p300 promoting Mdm2-mediated degradation of p53 *(26)*. p300, however, can also stabilize p53 in response to DNA damage *(74)*, and enhance p53-dependent transactivation by mediating acetylation of p53 *(75)*. Kobet et al. reported that Mdm2 can form a ternary complex with p300 and p53 resulting in suppression of p300-mediated p53 acetylation and activation *(76)*. A model has been suggested in which interaction between Mdm2 and p300 can either result in activation or inhibition of p53 function *(26)*, but the molecular mechanism awaits further clarification.

Mdm2 has been shown to interact with the recently identified p53 homologous protein p73 *(77)*, resulting in inhibition of its ability to transactivate p53/p73 target genes such as *p21*. In contrast to p53, interaction with Mdm2 does not result in proteolytic degradation of p73 *(20–22)*. One possible reason might be that p73 cannot interact simultaneously with Mdm2 and p300 *(22)*. Mutational analysis of p53 and p73 revealed a unique amino acid stretch within the p53 sequence that confers sensitivity to Mdm2-mediated degradation, indicating that an additional yet unknown protein might be involved in degradation of p53 by Mdm2 *(78)*.

2.2.2. p53 Independent Functions

Controlling p53 activity and stability is the best-characterized function of Mdm2. Interaction of Mdm2 with several other key players in cell cycle regulation (e.g., Rb, E2F-1, DP-1), however, suggests that Mdm2 might posses additional, possibly p53-independent functions, and data from several studies support this notion. Overexpression of Mdm2 in *p53* null cells induces anchorage-independent growth *(79)*, and tissue-specific overexpression of Mdm2 in mice induces S-phase independently of p53 *(80)*. Furthermore, transgenic mice that overexpress Mdm2 from its native promoter show spontaneous tumor formation even in the absence of p53, and the frequency of tumor formation correlated with the expression level of Mdm2 *(81)*. Interestingly, mice overexpressing Mdm2 from a CMV promoter did

not develop more spontaneous tumors than the control mice, but show skin abnormalities possibly due to increased DNA synthesis, regardless of their *p53* status *(82)*. All p53-independent functions of Mdm2 described are so far based on studies in which Mdm2 has been overexpressed, and it is still unclear whether there is a p53-independent function of Mdm2 under physiological conditions. Human tumors, however, that contain *p53* mutation and *mdm2* gene amplification are associated with a worse prognosis than those containing just one of the two alterations *(83)*, indicating that at least under pathological conditions, additional functions of mdm2 might be of significance.

Mdm2 has been shown to interact with the transcription factor E2F-1 *(23)*. E2F-1 belongs to a family of heterodimeric transcription factors each consisting of one of six E2F subunits bound to a DP family member *(84)*. E2F transcription factors can promote entry into S-phase by transactivating several genes essential for DNA synthesis. Interestingly, some members of the E2F family such as E2F-1 can also promote apoptosis. Interaction of Mdm2 with E2F-1 results in increased transactivation of E2F responsive genes but can also lead to destabilization of E2F-1 and DP-1 and a reduction in their ability to promote apoptosis independently of p53 *(85)*. This result raises the interesting possibility that some of the oncogenic properties of Mdm2 are due to the modulation of the activity of E2F by blocking the apoptotic pathway while enhancing S-phase promoting activities of E2F.

In a screen for proteins that can overcome TGF-β induced growth arrest, Sun et al. identified Mdm2 as a protein that confers TGF-β resistance independently of p53 *(86)*. TGF-β mediated growth arrest depends on members of the family of SMAD transcription factors that translocate to the nucleus in response to TGF-β and co-expression with Mdm2 blocks nuclear accumulation of SMAD proteins. This effect is also mediated by the Mdm2 homolog MdmX, but does not involve direct interaction between SMAD and either Mdm2 or MdmX *(87)*. Noteworthy, using Mdm2 inducible cell lines, Blain and Massagues could not observe an effect of Mdm2 on TGF-β signaling *(88)*.

Mdm2 has also been shown to interact with the human homolog of the *Drosophila numb* gene product. During cell division Numb is asymmetrically expressed within the cell resulting in two daughter cells that develop differently. Mdm2 can destabilize numb and co-expression of Mdm2 and Numb leads to translocation of cytoplasmic Numb into the nucleus *(24)*. The physiological significance of this observation, however, remains to be clarified.

Recently, Boyd et al. identified a novel Mdm2 interacting protein, MTBP *(31, 89)*. MTBP shows some homology to the yeast BOI proteins that are involved in negatively regulating cell growth. Overexpression of MTBP induces growth arrest, even in the absence of p53, and this function of MTBP can be suppressed by Mdm2. Interestingly, Mdm2 does not effect the stability of MTBP and further studies will be, therefore, necessary to elucidate the exact function of MTBP and its regulation by Mdm2.

2.3. Regulation of Mdm2

Inhibition of p53 activity is by far the best-characterized function of Mdm2 and most of our knowledge about regulation of Mdm2 based on studies that investigated regulation of p53 functions. p53 is activated in response to several forms of cellular stress, and as a prerequisite, its degradation and the inhibition of its transactivation activity by Mdm2 has to be blocked. Although the p53 protein alone is subject to different forms of post-translational modifications that contribute to a release from Mdm2-mediated inhibition *(90)*, Mdm2 itself is also modulated by many different mechanisms, such as transcriptional control, posttranslational modification, regulation of protein stability, subcellular localization, and protein–protein interaction. It is important to bear in mind that all mechanisms that regulate Mdm2-mediated inhibition of p53 might also be relevant in the regulation of other functions of Mdm2.

2.3.1. Transcriptional Regulation

The mouse *mdm2* gene consists of 12 exons *(41)*, and two promoters were identified for the *mdm2* gene. The first promoter is located upstream of the coding sequence and is probably responsible for constitutive expression. The second promoter lies within the first intron and is flanked by p53 binding sites. It is well established that p53 can transactivate Mdm2 expression *(12,50,91,92)*. In response to stress signals such as UV irradiation, treatment with topoisomerase inhibitors, camptothecin, and hypoxia, a decrease in Mdm2 mRNA level has been observed *(90,93–97)* resulting in the reduction of Mdm2 protein level that would allow accumulation of p53. Recent evidence suggests that the transcriptional regulation of Mdm2 expression by p53 is limited to condition of stress, and in unstressed cells Mdm2 expression does not depend on functional p53 *(98)*. Mdm2 and p53 expression can be regulated independently *(99–101)*, supporting the idea that in addition to p53, other factors such as basic fibroblast growth factor *(102,103)*, insulin growth factor *(104)*, thyroid hormones *(105)* and Ras *(106)* can effect Mdm2 mRNA expression.

2.3.2. Regulation by Post-Translational Modification

Although initial downregulation of Mdm2 mRNA expression in response to some form of stress signals could be observed, additional mechanisms exist that regulate Mdm2 function. DNA damage in response to ionizing radiation induces phosphorylation of Mdm2 dependent of the *ataxia telangiectasia* gene product (ATM) and ATM can directly phosphorylate Mdm2 in vitro at two different sites within the C- and N-termini, respectively *(107)*. Phosphorylation of Mdm2 at the N-terminus could directly affect the Mdm2/p53 interaction, whereas C-terminal phosphorylation raises the interesting possibility that phosphorylation of Mdm2 at the C-terminus might modulate its degradation activity without affecting binding to p53. The double-stranded DNA-dependent protein kinase (DNA-PK) *(108)*

can phosphorylate Mdm2 on serine 17, resulting in a decrease of p53/*Mdm2* complexes in vitro and inhibition of p53 transactivation by a Mdm2 mutant that cannot be phosphorylated at this amino acid was more effective compared to wt Mdm2. This indicates that phosphorylation of Mdm2 might weaken the interaction with p53 allowing activation of p53 target genes required for cell cycle arrest even in the presence of increased Mdm2 protein levels.

2.3.3. Regulation by Degradation

Like p53 Mdm2 is constantly degraded via the ubiquitin-dependent degradation pathway *(109)*. The Mdm2 protein has a very short half-life which increases upon deletion or mutation of the C-terminal RING-finger domain, indicating that this region confers protein lability *(57)*. Interestingly, attachment of ubiquitin molecules to lysine residues of Mdm2 depends on the ability of Mdm2 to function as E3 ubiquitin ligase and Fang et al. identified Mdm2 as its own E3 ubiquitin ligase *(55)* by demonstrating that in vitro, for efficient ubiquitination of Mdm2, only the E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme, and Mdm2 itself are necessary and sufficient.

Recently, the ubiquitin homolog SUMO1 (small ubiquitin-related modifier) has been identified as another protein that can be covalentely, but reversibly, attached to Mdm2 *(73)*. SUMO1 transfer onto target proteins depends on an enzyme cascade similar to that required for ubiquitin transfer *(110)*. However, SUMO1 is incapable of self-conjugation, and therefore does not form long chains like ubiquitin, a prerequisite required by the 26S proteasome and subsequent degradation *(111,112)*. The effect of attachment of SUMO1 molecules onto proteins appears to be much more diverse than ubiquitin attachment, and ranges from changes in activity, subcellular localization, protein-binding capacity, and protein stability *(113)*. Attachment of SUMO1 to *Mdm2* enhances its protein stability and in response to UV treatment, the amount of Mdm2–SUMO1 conjugates decreases. A single-point mutation within the RING-finger domain of Mdm2 prevented not only the attachment of SUMO1 but also the transfer of ubiquitin and its E3 ubiquitin ligase activity in vitro *(73)*. These data suggest an attractive model in which stress signals induce the replacement of stabilizing SUMO1 with ubiquitin within the RING-finger domain of Mdm2 resulting in enhanced degradation of Mdm2, and by that in up regulation of p53. In contrast to the in vitro data, in vivo experiments showed that attachment of SUMO1 to Mdm2 actually resulted in an inhibition of the ability to mediate the degradation of p53 *(73)*. Since the RING-finger domain of *Mdm2* carries the nucleolar localization signal, and can interact with several proteins, transfer of SUMO1 onto Mdm2 might regulate Mdm2 function by effecting its subcellular localization, or its ability to bind to other proteins. Interestingly, SUMO1 is also attached to p53 in response to UV treatment, resulting in activation of p53-dependent transactivation *(114,115)*. SUMO1 modifications, therefore, appear to play a dual role in the launch of a p53

response, by directly activating p53, and simultaneously blocking the p53 inhibitor Mdm2.

Mdm2 has also been identified as a substrate for members of the caspase family *(116,117)*. Caspases are proteases that are activated during apoptosis *(118)*, and cleavage of Mdm2 into two fragments *p60* and *p30*, respectively, can be observed in primary cells that undergo apoptosis *(116)*. A highly conserved caspase cleavage site (DVPD) can be found within the Mdm2 amino acid sequence at amino acid position 358 (human Mdm2). In vitro cleavage by caspase 3 and caspase 8 gives two fragments of the identical size as observed in vivo. Interestingly, a 60-kD fragment, which can bind but not degrade p53, is often constitutively expressed to high levels in several tumor cell lines *(119)*. This caspase appears to be distinct from known caspases, because it does not cleave poly(ADP-ribose) polymerase, a typical substrate of apoptotic caspases, and it is active even in nonapoptotic tumor cells *(120)*. Furthermore, a cell line expressing a temperature-sensitive *p53* mutant showed increase of Mdm2 cleavage at the permissive temperature, even in the absence of activation of apoptotic caspases suggesting that p53 could modulate its own inhibition by Mdm2 through mediation of Mdm2 cleavage. Whether cleavage of Mdm2, however, represents a mechanism that results in complete inactivation of Mdm2, or a mechanism to regulate the outcome of a p53 response is still unclear.

2.3.4. Regulation by Protein–Protein Interaction

Several Mdm2 interacting proteins have been identified and some of them have been shown to modulate Mdm2 function. In addition to p14ARF, which is described below, two additional proteins have been shown to regulate Mdm2, the Mdm2 homolog MdmX and the tumor-suppressor protein Rb. MdmX was initially isolated in an expression screen designed to identify p53 binding proteins *(37)*. Similar to Mdm2, MdmX can bind to p53 within its N-terminal transactivation domain, and inhibits the transactivation activity of p53. *mdmX* expression is not induced in response to DNA damage *(37,38)*, and does not seem to be a p53 target gene. During early development, it cannot substitute for *mdm2* since deletion of *mdm2* in mice results in a lethal phenotype even in the *mdmX* wild-type background. Recently, MdmX was found to interact with Mdm2 in vitro and in vivo via their homologous RING-finger domains *(39,121)*. Although MdmX binds to p53, it cannot mediate its degradation in the absence of Mdm2 *(122)*. Interestingly, wt MdmX or mutants that are unable to bind p53 or Mdm2 can inhibit Mdm2-mediated degradation of p53. This inhibitory effect is abolished by simultaneous deletion of the p53 and the Mdm2 binding sites within the MdmX protein, suggesting that MdmX can block Mdm2-mediated degradation of p53 by competing for p53 binding and by blocking Mdm2 ubiquitin ligase activity, respectively. MdmX is a nuclear protein, but is unable to shuttle out of the nucleus *(122)*, indicating that preventing p53 shuttling might be yet another mechanism by which

MdmX can reverse Mdm2 function. Clearly, further studies are necessary to elucidate the mechanism by which MdmX regulates Mdm2, and it will be of great interest to learn more about the mechanisms that regulate MdmX function, and whether MdmX can modify all functions of Mdm2.

Another protein that has been found to bind to Mdm2 is the retinoblastoma gene product Rb. Rb belongs to a family of pocket proteins that can bind and negatively regulate the transcription factor E2F. Interaction between Mdm2 and Rb is independent of their ability to bind to E2F, and requires the C-terminal region of Rb necessary for growth-suppression function *(29)*. Rb mediates growth suppression by binding to E2F/DP hetero dimers and interaction with Mdm2 results in the release of free E2F/DP leading to S-phase entry. Recently, it has been found that interaction of Rb with Mdm2 inhibits Mdm2-mediated degradation of p53 resulting in accumulation of p53, and an increase of p53-dependent apoptosis *(123)*. Rb does not compete for p53 binding of Mdm2, and the three proteins can form a trimeric complex. Although interaction of Rb with Mdm2 can stabilize p53, it does not reverse the inhibitory effect of Mdm2 on p53-dependent transactivation and the ability of p53 to induce G_1 arrest. Therefore, Rb/Mdm2 interaction promotes the selective activation of p53-dependent apoptosis, possibly due to a release of Mdm2-mediated inhibition of the transrepression function of p53 *(124)*. The exact mechanisms and the physiological significance of the ability of Rb to modulate p53 functions by interacting with Mdm2 remain to be elucidated.

3. ARF

ARF (murine $p14^{Arf}$, human $p19^{Arf}$) has been found as an *a*lternative *r*eading *f*rame of the *INK4a* gene locus (now called *INK4a/ARF* locus) already known to encode the tumor-suppressor protein $p16^{INK4a}$ and located on human chromosome 9p21 and murine chromosome 4, respectively *(125–128)*. $p16^{INK4a}$ was originally identified as an inhibitor of the cyclin D dependent kinases (Cdk 4/6) that regulate cell cycle progression by phosphorylating and inactivating the tumor-suppressor protein Rb *(125)*. In human tumors, inactivation of $p16^{INK4a}$ and Rb are mutually exclusive alterations, and deregulation of the p16INK4a/CDK4/cyclin D/Rb pathway is found in the vast majority of human tumors *(129)*. Surprisingly, ARF was found to mediate growth suppression similar to $p16^{INK4a}$, suggesting that ARF, too, might be a tumor suppressor protein. ARF, however, can induce growth arrest only in the presence of wild-type p53, while $p16^{INK4a}$ functions independently of p53 indicating that both proteins act via different pathways *(130–133)*. The contribution of both proteins to tumor development in humans has been studied by thorough analysis of the genetic alteration of the *INK4a/ARF* gene locus. Homozygous deletion of the locus, resulting in inactivation of both proteins, is a common alteration and has been found in about 14%

of all human tumors analyzed *(129)*. Point mutations could be found in 5% of the tumors, most often in exon 2 shared by both proteins, while a subset of tumors carried point mutation within exon 1α exclusively effecting $p16^{INK4a}$ function. Expression of ARF and $p16^{INK4a}$, respectively, are controlled by two independent promoters *(134–136)*, and silencing the $p16^{INK4a}$ promoter by methylation was detected in 19% of all human tumors analysed. Recently, inactivation of the ARF promoter by methylation has been identified in colorectal and gastric cancers *(137,138)*. The critical role of ARF in tumorigenesis is further supported by the identification of a germline deletion of *ARF* that does not effect expression of p16INK4a, in a family of which members are predisposed to melanomas and neurally derived tumors *(139)*.

The contribution of both proteins to tumor suppression has also been analyzed using different knock-out mice. Homozygous inactivation of *ARF* by disruption of exon 1β in mice leads to a high frequency of spontaneous tumor development in a $p16^{INK4a}$ wild-type background. Fibroblasts derived from these mice are unable to undergo replicative senescence and, similar to $p53^{-/-}$ fibroblasts, do not require a cooperative oncogene for transformation by activated ras *(130,140)*, supporting the concept of ARF as an independent tumor-suppressor protein.

3.1. Structure of ARF

The *INK4a/ARF* gene locus encompasses four exons termed exon 1α, 1β, 2, and 3 (Fig. 2A). The α transcript encoding $p16^{INK4a}$ is composed of exon 1α, 2, and 3, while the ARF transcript (or β transcript) is formed by a different 5' sequence (exon 1β) spliced to exons 2 and 3 *(141–143)*. Owing to a frame shift at the exon 1β/exon 2 boundary, the ARF transcript encodes a protein with a completely unrelated amino acid sequence to $p16^{INK4a}$ *(132)*.

Human ARF protein consists of 132 amino acids and murine ARF of 169 amino acids, resulting in proteins of 14 kD and 19 kD. Sequence analysis revealed that the shorter human ARF results from a premature stop codon within human exon 2 *(129)* suggesting that the additional C-terminal portion found in mice and rat might not contribute to ARF functions. ARF is a highly basic nuclear protein that predominantly localizes to the nucleolus *(27,132,133)*, and a single nucleolar localization signal (NoLS) at the N-terminus of murine ARF has initially been identified between amino acids 26 and 37 *(144,145)*. Regulation of subnuclear distribution of ARF, however, appears to be much more complex because several regions within the N- and C-termini of the protein have been identified as contributors to its nucleolar localization *(146–148)* (Fig. 2B). A N-terminal NoLS resides within amino acid residues 1–14 *(146,148)* and shows a high degree of homology to the corresponding region in the murine counterpart. Deletion of this region disrupts nucleolar localization of human ARF, and fusion to an unrelated protein confers nuclear localization *(146)*. A second NoLS was found in the C-terminal half of the protein in the region between residues 80–90 and 83–101 *(146,147)*.

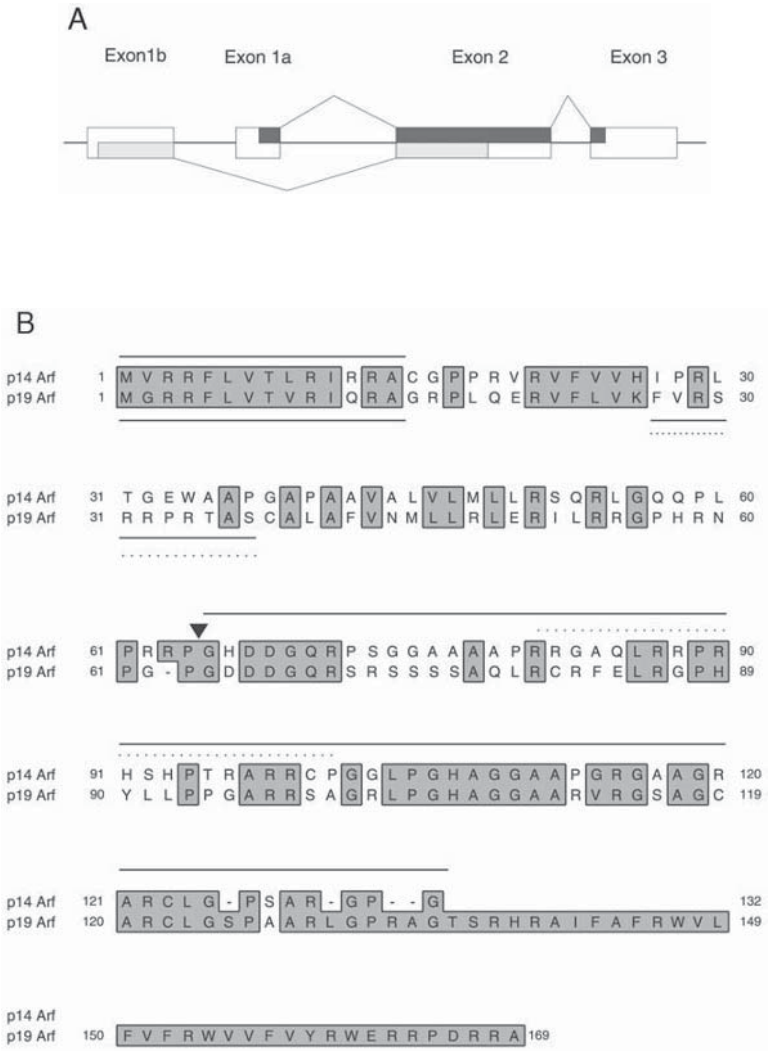

Fig. 2. (**A**) Structure of *INK4a/ARF* gene locus. Black bloxes indicate *p16INK4a* coding region, grey boxes mark *ARF* coding regions. (**B**) Amino acid comparison of human and murine ARF proteins. Solid lines mark Mdm2 binding regions, dotted lines mark NoLS, arrow indicates exon 1β/exon 2 boundary.

Deletions within this region of the *ARF* gene have been found in several human tumors. Owing to the resulting frame shift mutations, these tumors express chimeric proteins composed of the ARF N-terminus and the $p16^{INK4a}$ C-terminus *(129,149)*. Those mutant proteins showed a different localization pattern when compared with wild-type ARF pointing towards the possibility that alteration in the nucleolar localization of ARF might be one mechanism to alter ARF function.

ARF can interact with the Mdm2 protein (see below), and within murine and human ARF the far N-termini have been identified as one Mdm2 binding site (Fig. 2B). A second Mdm2 binding region (aa 26–37) is unique to murine ARF, because it could not be identified in the human homolog. Instead of a second N-terminal Mdm2 binding site, the C-terminal region of human ARF encoded by exon 2 can interact with Mdm2, albeit to a lesser extent than the N-terminal Mdm2 binding site *(145,146,148,150)*. It is striking that the regions that contribute to the nucleolar localization of ARF are also involved in MDM2 binding, indicating that interactions of both proteins might affect their intranuclear localization (*see* below). The exact physiological significance of the different NoLS and Mdm2 binding sites is still unclear. One has to bear in mind, however, that ARF is a highly basic protein (p*I* ~ 13) that interacts with the acidic domain of Mdm2 (ARF binding site p*I* ~ 3), to which other basic proteins such as the ribosomal protein L5 (see above) bind as well. NoLS are not defined by a common target signal, but consist of a stretch of several basic amino acids *(151)*. It therefore might be possible that some of the observations concerning interaction and localization of subfragments of ARF occur due to the experimental set up (overexpression of protein fragments). Future approaches, however, that aim to interfere with specific regions of the endogenous proteins will surely elucidate the functional significance of specific regions within the ARF protein.

3.2. Functions of ARF

The identification of Mdm2 as an ARF binding protein *(27,152)* provided the first direct link between ARF expression and p53-mediated growth arrest. Binding of ARF occurs within the central acidic region of Mdm2 that does not overlap with the p53-binding region, NLS, NES, NoLS, or the RING-finger domain carrying the ubiquitin ligase function of Mdm2. Introduction of ARF into p53 wild-type cells results in accumulation of p53, and this function depends on the ability of ARF to bind to Mdm2 *(150)*. Initial reports suggested that ARF promotes the degradation of Mdm2 *(152)*, but recent studies demonstrated that ARF expression actually leads to accumulation of Mdm2 *(133,148)*. These results are supported by the observation that binding of ARF to Mdm2 blocks the ubiquitin ligase activity of Mdm2 not only toward p53, but also toward Mdm2 itself *(153)*. Inhibition of the ubiquitin ligase activity of Mdm2 due to the interaction with ARF could account for ARF-dependent stabilization of p53. However, recent reports indicate that additional mechanisms exist by which ARF can alter Mdm2-

mediated degradation of p53, namely, by affecting the subcellular localization of Mdm2. As mentioned earlier, Mdm2-mediated degradation of p53 requires the relocalization of both proteins from the nucleus to the cytoplasm. Binding of ARF to Mdm2 interferes with this function of Mdm2 indicating that ARF might stabilize p53 by blocking nuclear export of Mdm2 *(147,154)*. Upon binding the ARF/Mdm2 complex relocalizes to the nucleolus, and there is a close correlation of ARF mutants between the ability to stabilize p53 and sequestering Mdm2 to the nucleolus, indicating that nucleolar localization is important for the inactivation of Mdm2 by ARF. In the absence of ARF, Mdm2 localizes predominantly to the nucleoplasm, but the identification of a mutant of Mdm2 not sequestered to the nucleolus despite being able to interact with ARF indicates that the NoLS of ARF are not sufficient to relocalize the ARF/Mdm2 complex to the nucleolus. Surprisingly, deletion of the ARF binding site results in a Mdm2 mutant that localize to the nucleolus even in the absence of ARF. Hence, binding of ARF to Mdm2 appears to induce a conformational change of Mdm2 leading to the unmasking of the otherwise cryptic NoLS of Mdm2 *(42,145)*. Although the occurrence of ternary complexes consisting of ARF, Mdm2, and p53 has been reported *(27,150, 152)* there is no evidence that p53 is drawn into the nucleolus by binary ARF/Mdm2 complexes, but resides and accumulates in the nucleoplasm indicating that ARF activates p53 by dislodging the negative regulator Mdm2, allowing induction of p53 dependent growth arrest *(150,155)* (Fig. 3A).

Several reports have demonstrated that growth arrest induced in tumor cell lines by ectopic expression of ARF depends on the presence of functional p53 *(130–133)*. Recent reports, however, demonstrated that re-activation of ARF in mouse embryo fibroblast (MEF) immortalized by ARF antisense constructs induces replicative senescence even in the absence of p53 *(156)*. Furthermore, p53-independent growth arrest induced by ARF depends, at least in some situations, on Mdm2, and could be overcome by either antisense-mediated inhibition of $p16^{INK4a}$ expression, overexpression of Mdm2, or overexpression of E2F-1. These data indicate that ARF might function by regulating both the Rb and the p53 pathways possibly via Mdm2. Weber et al. *(157)*, however, found that MEF lacking both ARF and p53 are resistant to ARF overexpression, whereas MEF lacking ARF, p53, and Mdm2 were not. In this setting, p53/Mdm2-independent function of ARF did not depend on Rb indicating that, in the absence of p53 and Mdm2, ARF can mediate growth suppression via other, as yet unknown pathways. The notion that ARF might function through additional p53-independent pathways is supported by the observation that inactivation of ARF frequently coexists with p53 mutations in non-small-cell lung cancer *(158)*.

3.3. Regulation of ARF

During murine development ARF as well as $p16^{INK4a}$ proteins are initially undetectable *(132,159)*, and whereas p16INK protein levels increase in spleen and

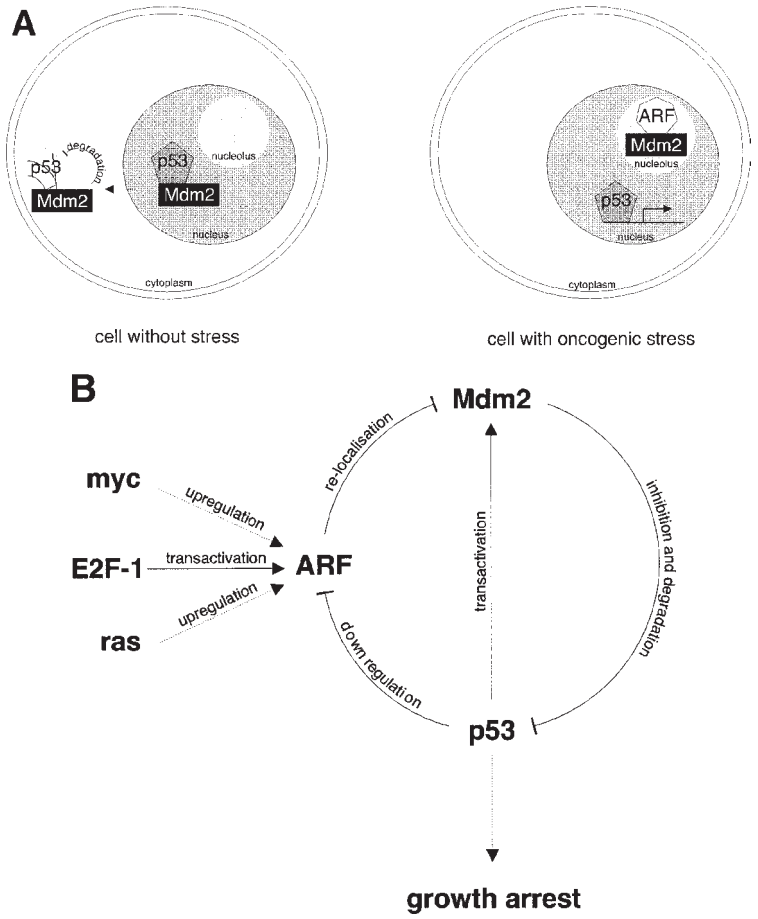

Fig. 3. (**A**) ARF mediated relocalizaton of Mdm2 prevents p53 degradation. (**B**) ARF links oncogenic proliferation signals to the p53/Mdm2/ARF regulatory circuit.

lung during aging, ARF protein remains undetectable in all tissue analyzed. In isolated MEF, both proteins accumulate during cellular senescence, indicating that they might counteract tumor development by limiting the replicative capacity of a cell. Although both proteins are encoded by a single genetic locus, expression

is controlled by different promoters. In tumor cell lines, p16INK4a expression inversely correlates with the Rb status of the cells *(160,161)*, whereas ARF expression was found to be independent of functional Rb *(142)*. However, inverse correlation of ARF and p53 expression has been observed *(132)*, and induction of exogenous p53 in a p53/Rb negative cell line that expresses high levels of ARF results in reduction of ARF protein levels *(133)*. Furthermore, p53 has been found to directly repress the ARF promoter *(135)*, indicating that ARF and p53 form a regulatory feedback loop similar to that observed between p53 and Mdm2 or $p16^{INK4a}$ and Rb, respectively.

As mentioned earlier, ARF can stabilize p53, and it is well known that p53 is activated by DNA damage. Initial studies indicated that *ARF* null cells arrest in response to γ-irradiation, indicating that ARF is a prerequisite for the activation of p53 after DNA damage *(130,133)*. Recent data, however, demonstrate that ARF might participate in the maintenance of the cell cycle arrest in response to DNA-damage *(162)*. Further studies will be necessary to clarify the exact contribution of ARF in the activation of a p53 response after DNA-damage.

In the absence of a cooperative oncogene or inactivation of a tumor-suppressor gene oncogenic Ras-induced senescence is accompanied by the accumulation of p53 and $p16^{INK4a}$ *(163,164)*. It has been demonstrated that Ras not only induces ARF mRNA and protein expression, but that ARF is essential for p53-dependent growth arrest induced by Ras *(165)*. The S-phase promoting transcription factor E2F-1 can induce apoptosis *(166)* and mice lacking E2F-1 develop spontaneous tumors *(167,168)* indicating that E2F-1 can act as a tumor-suppressor gene. E2F-1 induced apoptosis is, at least in part, mediated by p53, and *ARF* has been identified as a gene that is directly transactivated by E2F-1 *(135,166,169)*. The data suggest that ARF might function as a sensor for abnormal growth signals that can prevent hyperproliferation by activation of the p53 pathway (Fig. 3B). The concept of ARF/p53 as checkpoint for hyperproliferation gained further support by the finding that the oncogenes *myc*, adenoviral E1A, and *v-abl* can also induce apoptosis by activating ARF *(170–172)*. Apoptosis induced by oncogenes, however, does not always involve activation of ARF. Overexpression of the E7 protein of high-risk human papillomaviruses can result in p53 stabilization even in the absence of ARF *(173)* and senescence induced by oncogenic Raf did not depend on p53 *(174)*, indicating that ARF and p53-independent mechanisms exist that can prevent oncogene-induced hyperproliferation.

Recently, a binding site for the transcription factor DMP1 has been identified within the ARF promoter *(175)*. DMP1 has been identified due to its ability to bind to D-type cyclins *(176)* and induces growth arrest when overexpressed in mouse fibroblasts *(177)*. DMP1 induced growth arrest depends on the presence of ARF and p53 *(175)*, indicating that in addition to E2F, other transcription factors exists that can directly transactivate ARF expression. In contrast to *ARF* knockout mice,

animals lacking functional DMP1 rarely develop spontaneous tumors, but show increased sensitivity in response to ionizing radiation with regard to tumor development *(178)* indicating that ARF-mediated tumor suppression is not exclusively achieved through DMP1.

4. MDM2 AND ARF AS TARGETS IN CANCER THERAPY

The tumor-suppressor protein p53 is central part of a major defense pathway against neoplastic transformation, and several mechanisms have been identified in tumors that result in loss of p53 function, namely, mutation of the gene, cytoplasmic retention, degradation by viral proteins, Mdm2 overexpression, and inactivation of ARF. In many tumors, occurrence of one of these p53 inactivating mechanisms appears to bypass the need for a second alteration within this pathway, suggesting that inactivation of p53 by one or another mechanism might contribute to the development of all human malignancies. Hence, reactivation of p53 represents one of the most promising approaches to block tumor growth. In addition to attempts that aim to reintroduce wt p53, e.g., by gene therapy *(179,180)*, or to reactivate mutant p53 *(181)*, blocking the interaction of p53 and Mdm2 in tumor cells expressing wild-type p53 represents another promising therapeutic approach. The p53/Mdm2 interface has been studied extensively *(182–185)*, and small peptides or antibodies that release p53 bound to Mdm2 by competitive binding can restore p53 function *(18,186)*. Based on the observation that ARF can activate p53 by binding and blocking Mdm2-mediated ubiquitination, similar approaches were undertaken that aim to reactivate p53 using small peptides derived from the ARF amino acid sequence. A peptide spanning the first 20 amino acids of ARF was identified as able to block Mdm2-dependent ubiquitination of p53 in vitro and in vivo *(187)*. Interfering with Mdm2 function by expression of Mdm2 antisense oligonucleotides *(188–191)* and adenoviral-mediated ARF gene transfer in cancer cells also result in p53-dependent growth arrest and apoptosis *(192)*. In addition to the approaches that aim to block Mdm2 function, Mdm2 might be, when overexpressed in tumor cells, a suitable target in tumor immunotherapy. Autoreactive cytotoxic T lymphocytes (CTL) that have been stimulated using a synthetic Mdm2 peptide have been shown to selectively kill tumor cells in vitro and delay growth of lymphomas and melanomas in mice *(193–195)*. These data further support the value of the concepts that utilize Mdm2 and ARF as targets for the development of new cancer therapies.

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11 The Breast Cancer Susceptibility Genes *BRCA1* and *BRCA2*

Anastasia Gabriel and Alan Ashworth

Contents

1. INTRODUCTION

About one in eleven women in the Western world develop cancer of the breast, and at least 5% of these cases are thought to result from a hereditary predisposition to the disease *(1,2)*. Two breast cancer susceptibility (*BRCA*)[+] genes have been mapped and cloned, and mutations in these genes account for most families with four or more cases of breast cancer diagnosed before age 60. Women who inherit loss-of-function mutations in either of these genes have up to an 85% risk of breast cancer by age 70 *(1)*. Both *BRCA1* and *BRCA2* are thought to be tumor-suppressor genes, as the wild-type allele of the gene is observed to be lost in tumors of heterozygous carriers. As well as breast cancer, carriers of mutations in these genes are at elevated risk of cancer of the ovary, prostate, and pancreas.

[+]Upper case and italics ie *BRCA1* and *BRCA2* indicate the genes in humans whereas *Brca1* and *Brca2* denote the equivalent mouse genes. Plain text, eg BRCA1, is used for the corresponding proteins.

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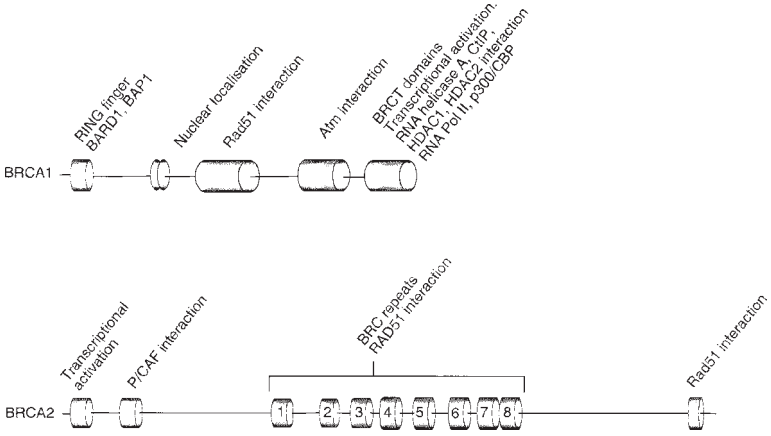

Fig. 1. Features of the BRCA1 and BRCA2 proteins. Structural features and regions of interaction with other proteins are indicated. More information can be found in references 1–3.

Surprisingly, despite the association with inherited predisposition, somatic disease-causing mutations in *BRCA1* or *BRCA2* are extremely rare in sporadic breast cancers *(1,2)*. Functions for the BRCA proteins in both transcriptional regulation and DNA repair/recombination have been suggested *(2,3)*. However, it is still unclear how loss of *BRCA* gene function leads to tumorigenesis.

2. *BRCA1* AND *BRCA2* GENES AND THEIR ENCODED PROTEINS

The *BRCA1* gene, which maps to human chromosome 17q21, consists of 22 coding exons and encodes a protein of 1863 amino acids (Fig. 1). Most of the BRCA1 protein shows no sequence similarity to previously described proteins apart from the presence of a RING zinc finger domain at the N-terminus of the protein, and two "BRCT" repeats at the C-terminus. RING-finger domains may be responsible for protein/protein interaction, and the BRCA1 RING finger, like some others of this family, may be involved in facilitating protein degradation *(4)*. Furthermore, the BRCA1 RING finger interacts with the RING-finger domain of another protein, BARD1, also implicated in protein degradation. The BRCT repeat is a poorly conserved domain found in a range of proteins many of which

are involved in either DNA repair or metabolism, such as RAD9 and XRCC1. Although there has been some controversy regarding the location of the BRCA1 protein in the cell, most authors now believe that the protein is present within the cell nucleus *(2)*; within S phase of the cell cycle, BRCA1 localizes to discrete foci within the nucleus *(5)*.

The *BRCA2* gene, which maps to human chromosome 13q12, has 26 coding exons and encodes a protein of 3418 amino acids with a molecular weight of 384 kDa, and localizes to the nucleus (Fig. 1). The only obvious feature of the BRCA2 protein is the presence of eight copies of a 30–80 amino acid repeat (the BRC repeat) in the portion of the protein encoded by exon 11; these repeats are able to bind the RAD51 protein implicated in DNA repair and recombination *(3)*.

3. GENETICS OF *BRCA1* AND *BRCA2*

Breast cancer exhibits familial association in that the disease is about twice as common in the mothers, sisters, and daughters of carriers as in the general population. This familial risk rises to about five-fold where the cancer occurs before 40 years. Mutations in *BRCA1* and *BRCA2* account for most inherited susceptibility to breast cancer in families with several (>6) affected individuals. However, it has been estimated that, overall, *BRCA1* and *BRCA2* mutations might account for only 20–25% of familial risk *(6)*. None of these other putative *BRCA* genes (*BRCA3*, *-4 -5*, etc.) have yet been mapped or cloned.

Carriers of mutations in *BRCA1* or *BRCA2* have an up to 85% chance of developing breast cancer by age 70, but this might differ among different populations *(1)*. Hundreds of different mutations in *BRCA1* and *BRCA2* have been described [see the Breast Cancer Information Core (BIC) database on the World Wide Web at http://www.nhgri.gov/Intramural_research/Lab_transfer/Bic/]. Some mutations are found more commonly than others, usually due to founder effects in certain populations. For example, in the Ashkenazi populations, two *BRCA1* mutations (185delAG and 5382insC) and one *BRCA2* mutation (6174delT) are common, and are detected in a significant proportion of early onset breast cancer cases *(1)*. A few disease-causing missense changes, most notably in the RING finger region of *BRCA1*, have been noted, but the majority are truncating nonsense or frameshift mutations spread throughout the genes. Some evidence for a genotype/phenotype correlation for an elevated risk of ovarian cancer has been presented for both *BRCA* genes, but this remains to be definitively proven.

Evidence is accumulating for the effect of modifying genes on the penetrance of certain mutations in the *BRCA1* and *BRCA2* genes. For example, the 999del5 mutation in *BRCA2*, prevalent in the Icelandic population, appears to be associated with male breast cancer in some families but not others. No modifying genes have yet been identified, but rare alleles at a VNTR (variable number of tandem repeats) linked to *HRAS1*, the Harvey-Ras proto-oncogene, have been suggested

to increase the risk of ovarian cancer modestly in individuals carrying a *BRCA1* mutation. This area is certain to receive much more attention in the next few years.

4. CLINICAL ASPECTS

Analysis of the pathology of breast tumors that arise in carriers of mutations in *BRCA1* or *BRCA2* revealed that their properties differ from each other and from sporadic cases *(7,8)*. Tumors in both *BRCA1* and *BRCA2* carriers tend to be of higher grade than sporadic cases, and *BRCA1* tumors are much more likely to be negative for the estrogen receptor and to have P53 mutations than sporadics. In addition, *BRCA* tumors differ morphologically from each other and from sporadic tumors. The major differences manifest in tubule formation, mitotic count, and lymphocytic infiltration. Independent features that could be attributed to *BRCA1* mutation were a higher mitotic count, "pushing" tumor margins, and higher lymphocytic infiltration. *BRCA2* tumors did not show significant tubule formation and lacked pushing tumor margins. These differences and the fact that *BRCA2* mutation is also associated with a higher incidence of male breast cancer and a decreased frequency of ovarian cancer suggests that the two genes may have some independent functions.

Whether the survival rates of women with breast cancers who carry *BRCA1* or *BRCA2* mutations are different from sporadics is controversial. Early reports suggested that the prognosis was better than for matched individuals with sporadic tumors. However, other studies have suggested that the survival is worse in carriers. Larger, longer-term studies are required to resolve this issue.

The high rates and early onset of breast (up to 85% by age 70) and ovarian (up to 40% lifetime for *BRCA1* carriers) cancers in mutation carriers have important clinical management implications. Regular mammographic screening is indicated but is of unknown effectiveness in younger women. Bilateral prophylactic mastectomy has been shown to be effective in considerably reducing the risk of breast cancer in women with a family history *(9)*. However, this procedure can carry with it psychological and physical morbidity. Prophylactic oophorectomy has also been shown have some effect in reducing breast cancer risk in *BRCA1* mutation carriers *(10)*. This finding may indicate that hormone intervention therapies, such as tamoxifen, might be effective in reducing the risk of breast cancer.

5. MOUSE MODELS FOR LOSS OF *BRCA1* AND *BRCA2*

Germline manipulation has been used to create mice carrying several different putative null alleles of both *Brca1* and *Brca2*. Mice heterozygous for these mutations have not shown elevated susceptibility to cancer of the mammary gland or indeed of any other tissue. It is possible that the rate of loss of the wild-type allele is insufficient to lead to a population of null cells large enough for tumorigenesis.

This finding might relate to differences in breast physiology or development between mice and humans *(2)*.

In contrast to heterozygotes, mice that are homozygous for null alleles of the *Brca* genes are very severely affected. *Brca1* and *Brca2* have indispensable roles during mouse development and null mutations for both genes result in embryonic lethality between d 5.5 and 9.5 of embryogenesis, the phenotype of *Brca1–/–* embryos being more severe than of *Brca2–/–* embryos. A lack of cell proliferation has been suggested as the explanation for the failure of *Brca1* and *Brca2* null embryos to develop. Two hypomorphic (partial loss-of-function) alleles of *Brca2* have been described that result in some homozygous mutant mice surviving. These develop thymic lymphomas with high frequency *(11,12)*. The lethality of homozygosity for a *Brca1* null allele has been circumvented by mammary gland-specific deletion of the gene *(13)*. Mammary gland tumors that had many of the morphological features of human breast cancers occurred in these animals. Tumor cells showed extensive aneuploidy, chromosomal rearrangements, and alterations in p53 expression. This result suggests that aneuploidy results in changes that are selected for during tumor growth and development. These mice should be useful in the development of novel preventive or therapeutic approaches.

6. POSSIBLE ROLES OF BRCA1 AND BRCA2 IN TRANSCRIPTIONAL REGULATION

There is accumulating evidence for a role in transcriptional regulation for BRCA1, and to a lesser extent for BRCA2. Disregulation of target genes consequent to the loss of *BRCA* genes is a plausible mechanism to explain, at least in part, tumorigenic progression. However, the exact function of the BRCA proteins in transcriptional regulation is not yet understood. Various genes, such as *GADD45* and the cell cycle regulator $p21^{WAF1}$, have been suggested to be regulated by BRCA1 *(3)*. Interestingly, *GADD45* has been implicated in the regulation of genetic stability, the G_2-M checkpoint and centrosome duplication *(14)*. These processes are also disrupted in *Brca1* knock-out mice *(13)*.

However, there are no reports, as yet, of BRCA1 binding DNA and acting directly as a transcription factor. Rather, BRCA1 appears to exert its influence on transcription as a cofactor or adaptor because it can interact with both DNA-binding transcription factors and the RNA Pol II holoenzyme. The C-terminal domain of BRCA1 consists of two BRCT domains that interact with multiple transcriptional activators and repressors such as p300/CBP *(15)*. BRCA1 may also function as a coactivator of p53-mediated transcription *(16)*. Transcriptional regulation has also been proposed as a function of BRCA2 because sequences encoded by exon three, when fused to the GAL4 DNA-binding domain, can activate transcription of a reporter gene. However, it is not clear whether these sequences can function similarly in the context of the whole protein *(3)*.

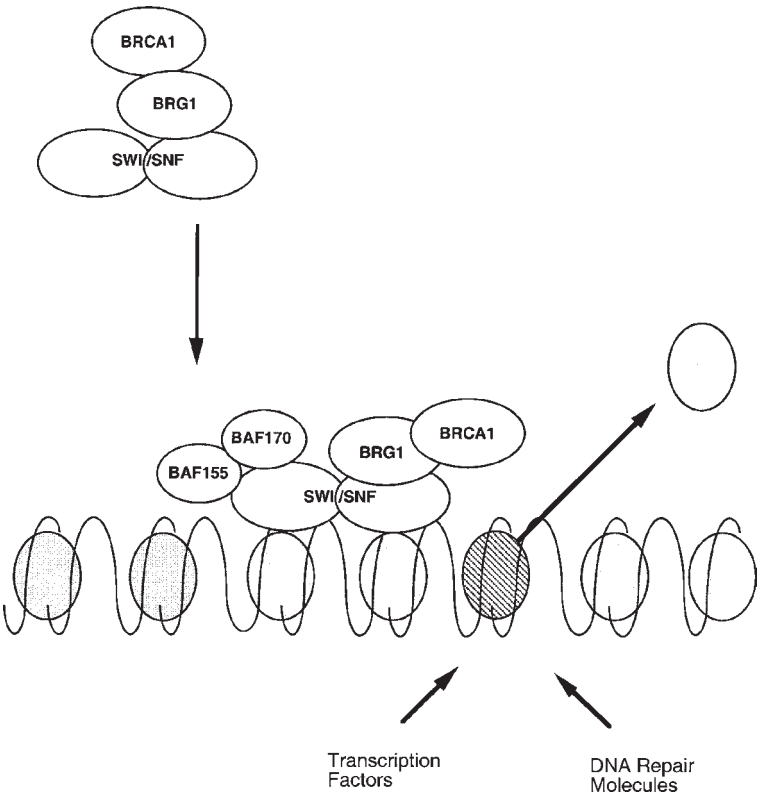

Fig. 2. SWI/SNF chromatin remodeling nucleosomes: BRCA1 binds the BRG1 subunit of the SWI/SNF-related complex. This interaction could allow the remodeling of the nucleosomes to allow the chromatin access to transcription factors, which bind BRCA1, or DNA repair molecules, which are associated with BRCA1 or BRCA2.

An alternative route for transcriptional activation by BRCA1 may be by chromatin remodeling (Fig. 2; *17*). The SWI/SNF-related complex is involved in the remodeling of chromatin to mobilize nucleosomes, permitting the access of transcription factors for their binding sites. The association of BRCA1 with the SWI/SNF-related complex could, partially, explain the role of BRCA1 in transcription. Of the multiple proteins in the SWI/SNF-related complex, BRCA1 interacts specifically with the BRG1 subunit *(17)*. Transient expression of BRCA1 results

in a three-fold stimulation of the p53 promoter. Furthermore, expression of a dominant negative BRG1 abrogates the BRCA1-mediated stimulation of the p53 gene promoter. This potential role of BRCA1 in chromatin remodeling suggests a possible mechanism for the transcriptional regulation of a number of genes.

Chromatin remodeling has also been proposed to be an important pathway used by transcription factors to activate DNA replication. The acidic activation domain of BRCA1, implicated in transcriptional regulation, may also stimulate DNA replication by chromatin remodeling *(18)*. Tethering this domain of BRCA1 to a yeast replication origin by fusion with a DNA binding domain results in the modification of the local chromatin structure and the stimulation of replication. Cancer-predisposing mutations in this region of BRCA1 affect chromatin modeling and abolish activation of transcription and replication *(18)*. Whether the role of BRCA1 in DNA replication is pertinent to the pathogenesis of breast cancer is not yet clear.

BRCA1 may also function as a transcriptional co-repressor. Recently, a protein that interacts with BRCA1, ZBRK1, has been identified that may be involved in BRCA1-mediated regulation of *GADD45* transcription *(19)*. ZBRK1 encodes a 60-kD protein that has a N-terminal KRAB, a transcriptional repression domain, and eight central zinc-finger domains, which can bind a specific consensus sequence in DNA; a sequence similar to this consensus has been identified in the *GADD45* gene. Interaction of BRCA1 with ZBRK1 resulted in the transcriptional repression of *GADD45* suggesting a functional role for BRCA1 as a co-repressor. It is suggested that the main function of BRCA1 in the regulation of *GADD45* was to silence the gene in the normal uninduced state. Upon DNA damage, this repression is released, allowing the transcriptional activation of *GADD45*. Indeed, it has been demonstrated that UV treatment of cells results in the down-regulation of *BRCA1* mRNA *(20)* and is consistent with induction of *GADD45* upon DNA damage *(21)*. Alternatively phosphorylation of BRCA1, by either ATM or hCds1/Chk2 could be the driving factor that promotes dissociation from ZBRK1, and thus the induction of *GADD45*.

7. BRCA1 AND BRCA2 AND DNA REPAIR

Mouse cells with *Brca1* or *Brca2* mutations are hypersensitive to ionizing radiation, a genotoxic treatment that causes primarily double strand breaks in DNA *(11,12)*. This finding and the association of both BRCA1 and BRCA2 with RAD51, a protein that plays a key role in the repair of double strand breaks (DSBs) by homologous recombination, suggests that BRCA1 and BRCA2 play a part in the cellular response to DNA double strand breaks (Fig. 3). Furthermore, BRCA1 also associates with the RAD50/MRE11/nibrin complex, which is thought to process DNA double strand breaks for repair by the processes of both nonhomologous end joining and homologous recombination *(22)*. BRCA1 and BRCA2 can coexist

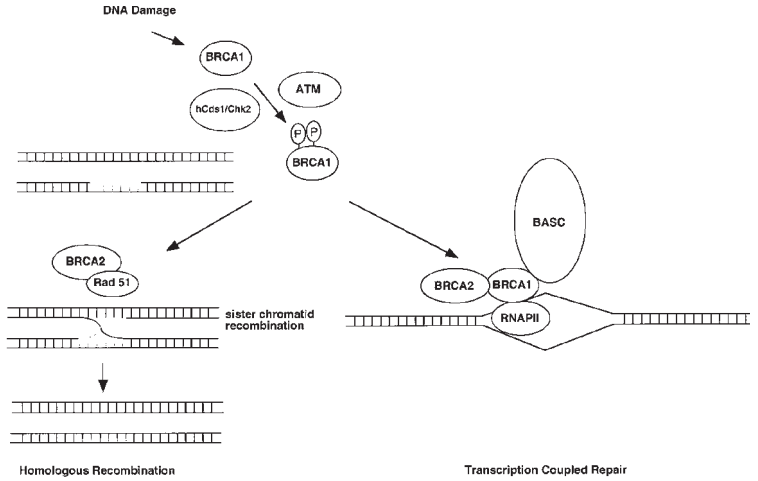

Fig. 3. BRCA1 and BRCA2 are thought to be involved in two forms of repair following DNA damage. DNA damage, by γ-irradiation, results in the phosphorylation of BRCA1 by ATM or hCDS1/Chk2. If a double strand break occurs and homologous recombination is initiated, BRCA1 may bind BRCA2 and Rad51 to allow invasion by the sister chromatid and use of this chromatid as a template for repair. This would lead to the faithful repair of the DNA damage incurred. Transcription coupled repair also relies on BRCA1 and BRCA2. DNA damage at the sites of transcription could recruit BRCA2 and the BRCA1 associated BASC complex. This complex contains mismatch repair enzymes which are also involved in transcription coupled repair (see text).

in a biochemical complex and co-localize in subnuclear foci and relocate to PCNA+ replication sites following DNA damage *(3,23)*. Together, these data suggest that BRCA1 and BRCA2 are involved in homologous recombination-mediated repair of double strand DNA breaks. There is also some evidence that BRCA1 and BRCA2 may have a role in the mechanistically independent process of the transcription-coupled repair of oxidative DNA damage *(24)*. Spontaneous chromosomal abnormalities are observed at high frequency in untreated *Brca1* and *Brca2* mutant cells, implying that these genes act to repair DNA damage, which occurs as a consequence of normal cell division, as well as that caused by genotoxic agents *(25,26)*.

Aneuploidy is observed in many breast tumors as well as in the tumors that arise in the *Brca1* and *Brca2* knockout mice *(25,26)*. There are many ways in which a cell can become aneuploid, including chromosomal rearrangement or aberrant DNA repair. However, other routes to aneuploidy do exist. At the end of mitosis, each daughter cell inherits one of the two centrosomes, and begins duplication

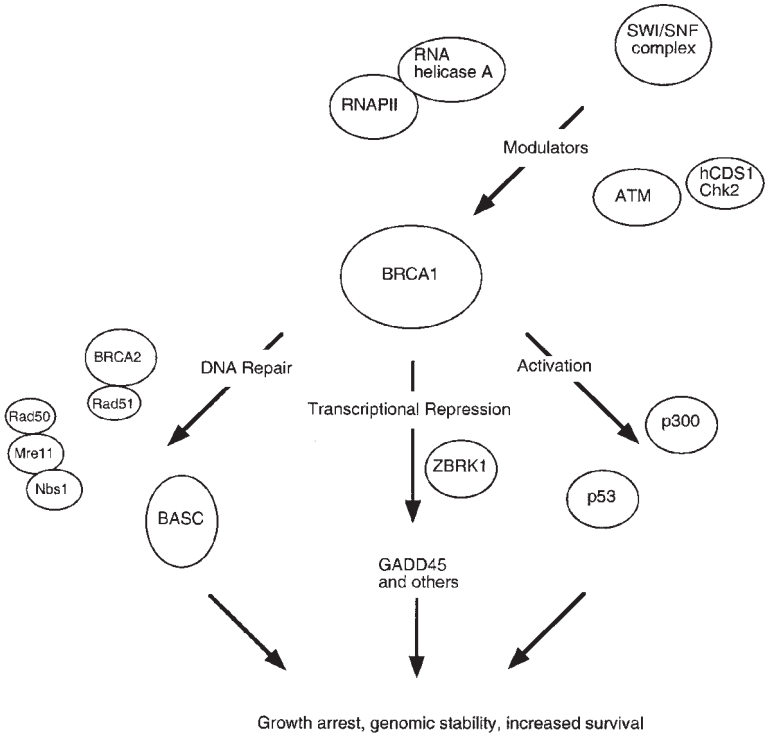

Fig. 4. The tumor-suppressor BRCA1 and its activity in DNA repair and transcription. BRCA1 can be regulated by phosphorylation after DNA damage by ATM or hCds1/Chk2. This can lead to the initiation of DNA repair by recruitment of such molecules as BRCA2/Rad51 or Rad50/Nbs1/Mre11. Transcription coupled repair can also be promoted by the interaction of BRCA1 with the BASC complex. BRCA1 activity is modulated by its interaction with the BRG1 subunit of the SWI/SNF-related complex, resulting in the remodeling of local chromatin structure for access to DNA repair molecules or transcription factors. Transcription can also be activated through interactions with p53 and p300 or BRCA1 can repress transcription by its association with ZBRK1 (figure modified from ref. *38*).

at the G_1/S transition so that two centrosomes are present during mitosis. BRCA1 protein has been reported to be located at the centrosome *(27)*, and recent studies have found that a high proportion of *Brca1* and *Brca2* mutant cells contain supernumerary centrosomes *(26,28)*. Centrosome amplification or possible centrosome fragmentation has also been seen in cells that lack the RAD51-like recombination

proteins XRCC2 and XRCC3 *(29)*. This finding might explain the high degree of aneuploidy seen in *BRCA* mutant breast tumors *(3)*. Recently, a serine/threonine kinase, STK15, has been identified that is amplified in many breast tumors, and is localized to the centrosome. Interestingly, centrosome amplification is seen in approx 12% of breast tumors. Overexpression of STK15/BTAK in a near diploid breast cancer cell line resulted in an abnormal centrosome number and an induction of aneuploidy *(30)*. It is not yet clear whether there is any relationship between STK15/BTAK amplification and *BRCA* mutation. If this relationship exists, one potential route of therapeutics could be to identify inhibitors of STK15/BTAK kinase activity, which could used to regulate centrosome amplification and allow appropriate chromosome segregation.

BRCA1 is hyperphosphorylated in response to ionizing radiation, but the outcome of this remains unclear. It has recently been demonstrated that BRCA1 is phosphorylated by ATM and in ATM–/– cell lines BRCA1 could not be hyperphosphorylated in response to γ-irradiation. The importance of the phosphorylation of BRCA1 in response to ionizing radiation was demonstrated using a BRCA1-deficient cell line (HCC 1937), which is radiation hypersensitive. Expression of wild-type BRCA1 in this cell line reduced the radiation sensitivity, but the introduction of BRCA1 with alanine substitutions at the potential ATM phosphorylation sites (S1423A and S1524A) fails to rescue this hypersensitivity, suggesting the functional importance of these two sites *(31)*.

BRCA1 is also phosphorylated upon γ-irradiation at residue S988 by the checkpoint protein kinase hCds1/Chk2. BRCA1 and hCds1/Chk2 normally co-localize in discrete nuclear foci, but following phosphorylation at S988 of BRCA1 they disperse. This release of BRCA1 from hCds1/Chk2 is required for restoring survival in a BRCA1-deficient cell line following γ-irradiation *(32)*. It is suggested that phosphorylation of BRCA1 upon DNA damage and the resultant dispersal of these foci allows BRCA1 to perform its putative DNA damage repair function.

A role for BRCA1 in DNA damage repair is further supported by the discovery that BRCA1 resides in a multisubunit protein complex, the so-called BASC complex (BRCA1-associated genome surveillance complex) (Fig. 4). Purification of proteins associated with BRCA1 revealed a complex that contained many proteins associated with the repair of damaged DNA, including ATM, BLM, and DNA replication factor C. Two mismatch repair proteins, MSH2 and MHS6, were also found to reside in this complex. These proteins are also necessary for transcription coupled repair (TCR). BRCA1 mutant cells are deficient in TCR and the identification of these mismatch repair proteins in association with BRCA1 could provide a possible explanation for this defect. Mismatch repair proteins also have a role in recognizing abnormal DNA structures, for example, at sites of replication fork collapse, suggesting that this complex may also be involved in genome surveillance. It seems possible that BRCA1 acts a scaffold organizing different types of DNA damage sensors and thereby coordinating repair *(33)*.

The association of BRCA1 in the SWI/SNF-related complex, described above, may also link chromatin remodeling to DNA repair via the activation of sets of genes that are required for responses to DNA damage, including *GADD45* and $p21^{WAF1}$. Furthermore this BRCA1-associated complex could play a direct role in DNA repair by allowing the DNA repair machinery access to damaged DNA by mobilization of nucleosomes.

In mammalian cells two major pathways exist to repair chromosomal DSBs: nonhomologous end joining (NHEJ) and homologous recombination (HR). HR can maintain genome integrity by the faithful repair of the DSB using the sister chromatid as a template. In an elegant set of experiments *(34)*, the group of Maria Jasin introduced a direct repeat recombination substrate containing the cleavage site for a rare-cutting endonuclease, allowing the creation of double strand breaks into a *Brca1* mutant embryonic stem cell line. Using this system, they have demonstrated that mutation in *Brca1* can result in a decrease in repair via HR and the elevation of NHEJ. A reduction in the ability to repair by HR and an elevation in repair by NHEJ suggests a possible route of mutagenesis through aberrant DNA repair *(34)*. This result correlates with the chromosomal abnormalities observed in *Brca1*-deficient mice and, in conjunction with the centrosome abnormalities described, could lead to the aneuploidy that is so frequently seen in breast tumors. A direct role for BRCA2 in homology directed repair has yet to be established, but seems likely given its interaction with RAD 51.

8. THERAPEUTIC EXPLOITATION OF BRCA DEFICIENCY

Gene therapy has been long mooted as an approach to correcting genetic defects, and has been used in an attempt to correct the defect in *BRCA1* patients. Using a retrovirus containing a splice variant of *BRCA1*, clinical trials have been set up to investigate its efficacy *(35)*. Apart from the general problems associated with gene therapy (vector delivery and immune response), one problem that is apparent with this type of approach is that tumors have already lost both copies of the relevant *BRCA* gene. In animal models, inactivation of either *Brca1* or *Brca2* results in the induction of aneuploidy, a phenomenon that is also observed in many breast cancers. This aneuploidy is thought to result in mutations in a number of key genes, allowing uncontrolled growth. Thus, replacing *BRCA* genes in tumors that are already aneuploid would not necessarily overcome the growth advantage they possess.

Because many BRCA tumors exhibit a prevalence of mutations in *p53* and subsequent loss of p53 function, the exploitation of this defect provides a basis for therapy. The loss of p53 function has been cleverly exploited by using a selectively replicating adenovirus (ONYX-015) to design therapeutic strategies. The E1B gene product of this virus will normally bind to wild-type p53. However, inactivation of the *E1B* gene via mutation allows the virus to replicate only in cells that do not have functional p53 *(36)*. Success of this approach with concomitant

chemotherapy in the killing of tumor cells in animal models has been demonstrated *(37)*, and could be a basis of targeting specifically to breast tumors in conjunction with conventional therapies. Indeed, phase II trials of ONYX-015 with cisplatin treatment was able to enhance the efficacy of cisplatin alone in squamous cell carcinomas of the head and neck *(38)*.

Identifying the key genes regulated by the BRCA proteins would be an another important step in devising therapeutic strategies specific to *BRCA* tumors. For example, some of the genes that have been identified downstream of BRCA1 are *GADD45* and $p21^{WAF1}$. The identification of other genes controlled by the BRCA proteins could also be achieved by using isogenic cell lines and subjecting these to microarray analysis. This approach would more faithfully represent the situation that occurs in breast cancers and could yield clues as to the genes that are regulated by BRCA1 or BRCA2. Furthermore, this approach might identify pathways that are involved in the loss of the BRCA genes that could also be targeted for therapeutics.

One of the major defects in the BRCA1 mutant cells is the inability of BRCA to repair DSBs faithfully by HR, which leads to genetic instability. The consequent increase in NHEJ could also result in a mutagenic phenotype *(34)*. One possible route of therapy would be to also inactivate the NHEJ pathway. This inactivation in conjunction with the inability to repair DSBs by HR could render *BRCA*-deficient cells more sensitive to genotoxic assaults such as γ-irradiation (which induces DSBs) or chemotherapy, and thereby more efficiently kill the cells. Molecules involved in NHEJ such as DNA-PK could be specifically targeted for the rational design of inhibitors, and could lend selective toxicity toward BRCA mutations.

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12 Human Papillomavirus

Targets for Therapeutic Intervention

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CONTENTS

1. INTRODUCTION

Human papillomaviruses (HPVs) are the causative agents of a number of important human diseases. The best characterized of these is cervical cancer, where the virus is found in over 99% of cases. This cancer is the third major cause of cancer-related death in women worldwide, with over 370,000 new cases per annum *(1)*. HPVs are also associated with the development of cutaneous squamous cell carcinoma (SCC), particularly in immunocompromised individuals and renal transplant recipients *(2,3)*. HPV infection also produces a number of nonmalignant, but nonetheless cosmetically unpleasant, lesions. Therefore, any effective therapeutic for HPV-induced diseases would be extremely beneficial on humanitarian grounds as well as on an economic level.

Of 100 different HPV types so far identified, only a small subset are associated with the development of cancer *(4)* and they are accordingly classified as either ''high-'' or ''low-'' risk types. The major high-risk types associated with the

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development of cervical cancer are HPV-16 and HPV-18 *(5)*. A key element in the identification of possible targets for therapeutic intervention is the fact that the virus continues to be retained and expressed in cell lines derived from cervical tumors, many years after the initial transforming events *(6–9)*. This finding argues strongly for a role of the virus in the continued proliferation of the tumor and the derived cell lines. Indeed, more recent studies have shown that if the oncoprotein expression can be inhibited (see below), then proliferation ceases, and cell growth arrest and/or apoptosis ensues.

An important feature during the development of HPV associated malignancy is the loss of replicative competence of the virus and integration of the viral DNA into the host genome. During this time, large regions of the viral genome are often deleted; however, there is invariable retention of two viral genes, *E6* and *E7 (6,10)*. We now know that these are the two major viral transforming genes that are responsible for the induction of cell immortalization and the subsequent continued proliferation that ultimately gives rise to malignancy *(11,12)*. A great deal of work has now been done in attempting to elucidate the mechanisms of action of these two viral proteins, and a concomitant plethora of interacting cellular target proteins has been identified. In this chapter, we shall attempt to highlight the activities of these two viral proteins that offer the best potential as targets for therapeutic intervention. However, prior to discussing the viral oncoproteins, we shall first consider the potential of the viral E2 protein as a therapeutic target, since regulation of viral replication and transcription is still considered to be an aspect of the viral life cycle that offers good prospects for the development of useful therapeutics.

2. HPV E2

The HPV E2 protein is the major regulator of viral gene expression, and plays an intimate role in the initiation of viral DNA synthesis. As such, it would appear to be an ideal target for therapeutic intervention during the viral life cycle. E2 binds to its cognate DNA recognition sequence, ACCGN4CGGT, which is repeated several times within the upstream regulatory region (URR) of all papillomaviruses *(13)*. The E2 protein itself is structurally conserved among different papillomaviruses, and can be divided into three structural domains *(14)*: an amino terminal transactivation domain, a flexible hinge region, and a conserved carboxy terminal DNA binding and dimerization domain. Early studies first demonstrated that E2 was a potent transcriptional activator *(15)*. However, later work suggested that in the context of the natural viral promoter the HPV E2 protein would function as a transcriptional repressor *(16)*. It is now clear that E2 binds to its different consensus recognition sites within the viral URR with different affinities. At low concentrations, sites distal to the promoter are occupied and E2 functions as a transcriptional activator, while at high concentrations, it binds to sites close to the promoter and functions as a transcriptional repressor *(17,18)*.

As well as regulation of viral gene expression, as a consequence of different levels of E2 expression, a number of alternatively spliced forms of the protein have also been described. These forms consist of the carboxy terminal DNA binding and dimerization domain of the protein, but lack the amino terminal transactivation domain *(19)*. This sE2, or repressor form, has been shown to negatively regulate the activity of the full-length E2 protein, either through competition for the DNA recognition site or by forming heterodimers with the full-length protein *(17,20)*. Although mutant viruses that fail to produce this protein exhibit increased levels of gene expression and DNA replication activity, consistent with a role in negatively regulating full-length E2, these viruses fail to be maintained as episomes *(21)*. This result suggests that negative regulation by the sE2 protein is essential for the normal life cycle of the virus, and hence may also represent an aspect of E2 function that could be targeted for blocking viral replication.

In addition to regulating viral gene expression, E2 stimulates viral DNA replication. DNA replication initiates at the viral *ori* located within the viral URR, which encompasses an E1 recognition site and two E2 binding sites. Viral DNA replication itself is driven by the E1 protein. This is the origin binding protein, which possesses both ATPase and helicase activities *(22–25)*, and unwinds the viral *ori* of DNA replication, as well as the DNA template ahead of the replication fork *(26)*. E1's binding to DNA and the initiation of viral DNA replication by E1 alone is weak *(22,27)*. However, E1 and E2 form a complex that greatly increases the binding of E1 to the *ori*, thereby stimulating the initiation of viral DNA replication *(28)*. Once replication begins, E1 remains part of the replication complex, but E2 is no longer required *(26,29)*.

Although the crystal structure of the E2 transactivation and DNA binding domains has been determined *(30,31)*, little is known about the precise mechanism of action of the E2 protein. A number of cellular partners of E2 have been identified, including TFIIB *(32)*, TBP *(33)*, p300/CREB binding protein (CBP) *(34,35)*, and AMF-1 *(36)*, all of which are intimately involved in the regulation of transcription. AMF-1 would appear to be required both for the ability of E2 to stimulate viral DNA replication and to activate viral gene expression. The latter activity is not surprising, because recent studies have reported association between AMF-1 and p300, thereby enhancing the association of p300 with E2 *(37)*. However, the role of AMF-1 in viral replication is still unknown. The interaction between E2 and p300 probably accounts for the major transcriptional activity of E2, where the presence of p300/CBP results in synergistic activation of E2 responsive promoters. Obviously, blocking the binding of E2 to its DNA target sequence, to the *ori* binding protein E1, or to its known cellular targets, all offer potential ways of inhibiting E2 function and hence blocking viral replication.

Certainly, inhibition of DNA binding activity or blocking association with E1 will seriously disrupt the virus life cycle. Mutating a number of the E2 recognition sites within the URR abolishes the late stages of the viral life cycle *(38)*. In addition,

the use of a 15mer amino acid peptide to block the E1–E2 association has been shown to effectively inhibit viral DNA replication *(39)*, demonstrating proof of principle that small-molecule inhibitors could be developed to target the E1–E2 *ori* binding complex. However, targeting the transactivation functions of E2 may be more complex. One report suggests that the transactivation activity of E2 is not required for completion of the viral life cycle *(40)*, implying that the major functions of E2 are related to the regulation of viral DNA replication. Even if transactivation by E2 should prove to be required for viral replication, this activity involves complex associations with cellular transcription factors, and blocking these associations might also alter normal cellular pathways of transcriptional regulation. This finding suggests that any strategies aimed at blocking E2 function should target its DNA binding activity or its ability to interact with E1. These aspects have the added value of being interactions that are restricted to the virus, and hence are likely to offer higher degrees of specificity.

There are, however, some caveats to E2 being a good candidate for therapeutic intervention in HPV-induced diseases. A major goal of the work on HPVs is blocking the processes of malignant transformation and progression. Unfortunately in over 70% of cervical cancers the viral DNA becomes integrated into the host genome. As a consequence, there are often deletions in large sections of the viral genome, and E2 is frequently lost. Therefore, in the majority of cancers, E2 is no longer present, and hence would not be a useful target for therapeutic intervention.

Blocking E2 activity earlier in viral infection could provide an effective therapy. This is certainly true for low-grade lesions and skin papillomas, which are cosmetically disfiguring. In this case, inhibiting E2 function, especially with respect to its interaction with E1 or its DNA recognition site, would inhibit the ability of the virus to replicate. It should be borne in mind, however, that inhibition of E2 function in a high-risk virus infection may have certain risks attached to it. A hallmark of virus-induced cancer is the loss of the ability of the cells to differentiate and the concomitant loss of replicative competence of the virus. Blocking E2 function in a high-risk virus infection could mean that the virus would remain longer within the infected epithelium, providing additional time for the events leading to transformation to take place. There are also recent reports suggesting that E2 has a variety of other activities including the induction of apoptosis or senescence, which may inhibit transformation *(41–44)*. How specific these activities of E2 are with respect to the levels of E2 expressed in a viral infection remain to be determined, but again, blocking these functions could have a negative impact on the outcome of the disease.

In summary, E2 represents a valid target for therapeutic intervention in a low risk viral infection, but its use as a target for treatment of high risk infections is somewhat questionable.

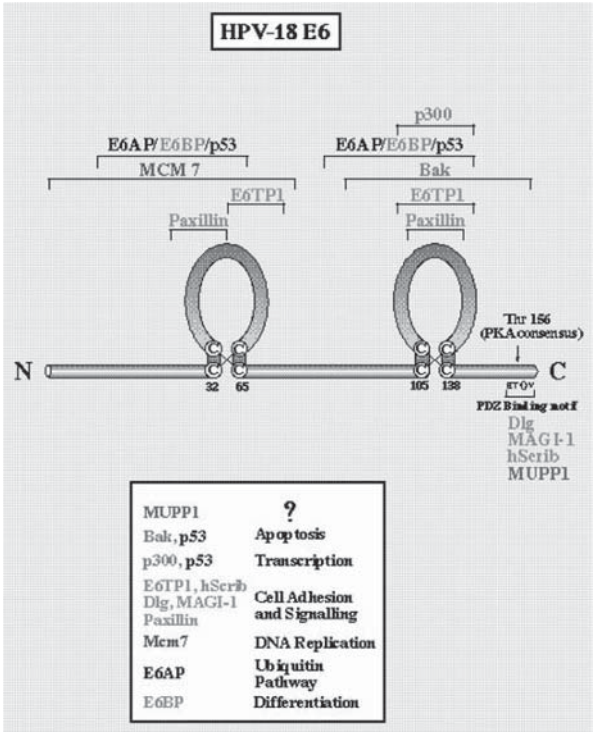

Fig. 1. Schematic diagram showing the HPV-18 E6 protein. The two zinc fingers are indicated, together with some of the known cellular targets and the regions to which sites of interaction have been mapped.

3. HPV E6

HPV E6 is one of two major oncoproteins encoded by the virus and, as such, represents a major target for therapeutic intervention in HPV-induced malignancy. The E6 protein is a small 150 amino acid polypeptide, and has four Cys-X-X-Cys motifs (*see* Fig. 1), which permit the formation of two zinc-binding fingers *(45, 46)*. These motifs are strictly conserved in all known E6 proteins, and their integrity appears to be essential for E6 function *(47,48)*. The E6 proteins have been shown to interact with a variety of important cellular proteins, including p53/E6AP *(49,50)*, E6BP *(51)*, E6TP-1 *(52)*, Bak *(53)*, Mcm7 *(54)*, paxillin *(55)*, p300/CBP *(56)*, and a number of PDZ domain-containing targets *(57)*. However, dissecting

the importance of these different interactions for E6 function has proved to be extremely difficult largely because many of the associations are frequently overlapping and span large regions of the E6 protein. Thus, strategies to eliminate one set of interactions are likely to also perturb others. Therefore, in this section we will focus on those interactions that are either the most well-defined and/or offer the most potential for the development of an effective therapeutic agent.

The E6 protein is found in very low abundance, and this has made its analysis in vivo difficult, although it appears to be present within nuclear, cytoplasmic, and membranous fractions of the cell *(47,48,51)*. Being in low abundance, E6 has evolved a system for inactivating cellular proteins that are present at much higher concentrations, by using the enzymatic, proteasome-mediated degradation of many of its target proteins.

4. CELLULAR TARGETS OF E6

4.1. p53

The best known and most studied interaction of HPV-16 or HPV-18 E6 is with the p53 tumor suppressor protein, which it targets for ubiquitin-mediated degradation *(49,58)*. E6 binds to E6AP, the prototype HECT domain protein, and forms an E3 ubiquitin–protein ligase that ubiquitinates p53 *(50,59,60)*, resulting in rapid p53 degradation by the 26S proteasome. Thus, p53 levels are extremely low in cervical tumor cell lines, and, consequently, they fail to undergo p53-induced growth arrest or apoptosis in response to DNA damage *(61,62)*. It is now widely assumed that this activity of E6 contributes to the accumulation of DNA damage within the immortalized keratinocyte, and thence contributes toward malignant progression. Therefore, blocking the E6/E6AP/p53 complex is a major therapeutic goal, and there is ample evidence that the p53-responsive pathways in cervical tumor-derived cell lines are fully functional *(63,64)*. The use of antisense oligonucleotides against E6AP resulted in the upregulation of functional p53 in certain HPV-positive tumor cells *(65)*. More recently, the use of small peptides to block the ability of E6 to degrade p53 also resulted in up-regulation of p53 and induction of apoptosis *(66)*, again suggesting that small-molecule inhibitors of the E6/E6AP/p53 complex might be a valid therapeutic option. However, it is unlikely that this approach will be effective in all HPV-induced tumors. Inhibition of E6-induced degradation of p53 in some tumor cells does not always result in up-regulation of p53, and additional genotoxic insults may be necessary to obtain a full p53 response *(64)*. There are also reports of mutations within p53 in metastatic cervical cancers, and these are likely to render useless any attempts to reactivate p53 function in these cases *(67)*.

In terms of the viral life cycle, blocking the E6–p53 association is also likely to prevent viral replication. A central aspect of the viral life cycle is the induction of S phase in differentiating suprabasal cells, via the action of E7 (*see* below),

and amplification of the viral genomes. However, the host cell responds to this unscheduled DNA replication by mounting a p53 response, which, in turn, is overcome by the action of E6. In addition, mutations within E6 that abolish the interaction with p53 also disrupt the viral life cycle *(68)* early in the process of infection. Hence, this interaction would also appear to offer excellent potential for treating the benign stages of viral disease.

4.2. Bak

It is now generally accepted that one of the major roles of E6 during viral infection is prevention of premature apoptosis. Obviously, one molecular basis for this is via the p53 association. However, recent studies also suggest that inactivation of Bak is an additional means by which E6 inhibits cellular apoptotic pathways *(53,69)*. Bak is a potent inducer of apoptosis *(70,71)*, and belongs to the Bcl-2 family of proteins *(72)*. Bak forms pores within mitochondrial membranes resulting in a cytochrome flux, caspase induction, and subsequent apoptosis *(73)*. During the normal processes of keratinocyte differentiation, Bak protein becomes highly expressed in the later stages of differentiation *(74)*. Recent studies also suggest that Bak can be up-regulated in response to UV irradiation, and thereby acts as a protection against UV-induced DNA damage in squamous epithelia *(69)*. It is now clear that E6 proteins from a wide range of papillomaviruses all share the ability to inhibit Bak-induced apoptosis *(69,75)*. As with p53, this involves targeting Bak for proteolytic degradation through a process that also involves E6AP. Thus, it is tempting to suggest that the E6–Bak interaction represents a good therapeutic target, especially since it would seem potentially efficacious against the E6 proteins from both mucosal and cutaneous HPV types: one of the few targets known to fulfill this criterion. The major drawback, however, appears to be that Bak is only encountered at a late stage in viral infection. Most of the early stages of the viral life cycle would likely be complete, and inhibition of this association would probably only reduce virus yield rather than abolish virus infection. The relevance of this association for viral-induced malignancy thus remains to be determined.

4.3. PDZ Domain-Containing Proteins

Recent studies, using a K14 promoter to express E6 or E7 in the basal keratinocytes of transgenic mice, showed that both proteins could induce tumor formation. An interesting finding from these studies was that the tumors in mice expressing E6 showed greatly increased malignant potential compared with those in the E7-expressing mice *(76)*. This finding indicates that the activities of E7 may promote tumor formation (*see* below), while those of E6 may accelerate the progression of benign tumors into malignancy. A great deal of effort has been expended in defining those activities of E6 that are unique to the high-risk types, and hence contribute directly to the processes of malignant progression. A striking feature, unique

to the E6 proteins from high-risk virus types, is an extended carboxy terminus containing a PDZ binding motif *(57,77)*. The HPV-18 E6 has a perfect consensus binding motif, while that of HPV-16 E6 is suboptimal. Together with a number of studies showing that HPV-18-containing tumors are the more aggressive and more prone to recurrence *(78–80)*, this result may suggest that E6's interaction with this class of target proteins might be responsible for its ability to promote malignancy. Hence, this class of interactions might represent ideal therapeutic targets for the later stages of virus-induced disease.

The E6 proteins of HPV-16 and HPV-18 bind to a number of PDZ domain-containing proteins, including MUPP1 *(81)*, hScrib *(82)*, and the MAGUK proteins, Discs Large (Dlg) *(57,77)* and MAGI-1 *(83)*. In each case the proteins are targeted for ubiquitin-mediated degradation *(81–84)*. Although the exact functions of all these cellular proteins are not yet clear, a number of insights have come from work in *Drosophila*. Knock-outs in Dlg are lethal, and mutations in this locus are characterized by uncontrolled cell proliferation and loss of cell polarity *(85, 86)*; as a consequence, Dlg is classified as a potential tumor suppressor. Recent studies also suggested that Scribble, the *Drosophila* equivalent of hScrib, cooperates with Dlg in the regulation of cell polarity and epithelial cell organization *(87)*. It is clear that should these proteins fulfill a similar role in higher eukaryotes, then their abolition by HPV E6 could contribute to metastatic progression. Interestingly, Dlg has also been shown to complex with the adenomatous polyposis coli gene product (APC) and to potentiate the binding of APC to β-catenin *(88, 89)*. This pathway is disrupted in the majority of colon tumors, and it is intriguing that E6 appears to perturb, at least in part, the same pathway during HPV-induced malignancy.

PDZ domains are 80–90 amino acid motifs that function as specific protein recognition domains *(90,91)* and act as molecular scaffolds in the formation of signaling complexes at specialized membrane sites *(92–94)*. Although all PDZ domains are homologous, there are certain differences in sequence that determine substrate specificity. Indeed, it has been shown that MAGI-1 interacts with β-catenin only through PDZ domain 5 *(95)*, and recent studies also suggest that the E6 interaction with these multiple PDZ domain containing proteins is highly specific. Thus, only single PDZ domains on Dlg and MAGI-1 *(164,165)* are recognized by the E6 protein. Because the structures of a number of PDZ domains have been solved *(96–98)*, and the binding motif on E6 is small and exposed, the rational design of relatively nontoxic chemotherapeutic agents, capable of specifically inhibiting the interaction between E6 and this class of targets should be possible.

4.4. Other Targets

The E6 protein has been shown to interact with a number of other cellular proteins, including E6TP-1, c-Myc, Mcm7, E6BP, paxillin, and p300/CBP, and

although it is entirely possible that these interactions may have chemotherapeutic potential, less is known about them than those discussed above. Therefore, we shall review them more briefly.

4.5. Interactions Involving E6-AP

The HPV-16 and HPV-18 E6 proteins, bind a putative GAP protein, E6-targeted protein 1, E6TP-1, and induce its degradation *(52)*. Interestingly, this protein has a PDZ domain but does not bind to E6 through it, thus providing further evidence for the specificity of PDZ domain binding discussed above. Little is known about the functions of E6TP-1, but it maps to a putative tumor-suppressor locus on chromosome 14 *(99)*, and it is thought to be involved in negative regulation of mitogenic pathways. E6AP can bind simultaneously, indicating that it may be involved in the degradation of E6TP-1.

HPV-16 E6 has been reported to enhance the degradation of the cellular oncoprotein, c-Myc, in an E6-AP-dependent fashion *(100)*. Unscheduled expression of c-Myc gives rise to high levels of apoptosis *(101)*, and its down-regulation appears to be required to promote keratinocyte differentiation *(102,103)*. Thus, E6's degradation of c-Myc seems logical for the virus and a potentially useful point for early antiviral chemotherapy.

Therefore, any strategies designed to interfere with E6/E6-AP functions with respect to p53 or Bak, may also be effective with respect to the E6 interactions with E6TP-1 and c-Myc.

4.6. Interactions Without Degradation

A number of cellular targets of E6 do not appear to be targeted for ubiquitin-mediated degradation. The E6 proteins of HPV-16 and of bovine papillomavirus type 1 (BPV-1) have also been shown to bind to paxillin *(55)*. Paxillin relays messages from the plasma membrane to focal adhesions and to the actin cytoskeleton, and is activated by tyrosine phosphorylation upon treatment with growth factors *(104)*. The BPV-1 E6 binding blocks paxillin's interactions with vinculin and with the focal adhesion kinase, disrupting the actin cytoskeleton *(105)*, thus interfering with cell signaling, and perhaps altering the architecture of the cell in a manner advantageous for the production of progeny virus. Interestingly, both polyomavirus middle T protein and the adenovirus E4 orf4 target the Src family of kinases, and thence affect the phosphorylation of paxillin *(106,107)*, again demonstrating that DNA tumor viruses target similar pathways. This result might suggest that this interaction is a useful target for chemotherapy. However, HPV-16 E6 is expressed at much lower levels than BPV E6, and as the effect of BPV E6 on paxillin appears to be that of competition with normal cellular partners *(105)*, the relevance of this interaction for HPV E6 proteins remains to be determined.

HPV-16 E6 has also been shown to bind and inhibit the activity of the CBP/p300 transcriptional co-activator *(56)*. However, the HPV-6 and HPV-18 E6 proteins have also been shown to bind to and induce the degradation of AMF-1/Gps2, a protein that enhances p300 activity *(37,108)*, and which, intriguingly, is also required for E2 function (*see* above). Therefore, it is possible that it is the degradation of AMF-1/Gps-2 by E6 which is responsible for the apparent effects of E6 upon p300/CBP activity, and this may also represent a feedback mechanism for regulating E2 function.

The CBP/p300 complex binds a number of important cellular proteins involved in the control of cell growth and differentiation, and is involved in NFκB activation. Dysregulation of the NFκB pathway can result in hyperproliferation of the stratum spinosum, the epithelial layer in which HPV DNA amplification occurs *(109,110)*. NFκB activation increases upon viral infection, and it activates transcription from a number of genes involved in local immune response *(111)*. The advantages to the virus in inhibiting these pathways are obvious, and clearly chemotherapeutic intervention at this point would probably abort the viral infection. However, because the CBP/p300 complex is involved in a large number of pathways, intervention at this point should be done with caution. In addition, until it becomes clear whether this effect of E6 is directly upon p300/CBP, or whether it is mediated by AMF-1/Gps2 thereby regulating E2 activity, no rational design of chemotherapeutic agents will be possible.

4.7. Control Mechanisms

Because HPV E6 is expressed at low levels but has a large number of cellular targets, there needs to be a system for controlling substrate specificity. One of the simplest controls is likely to be proximity; interactions only occur if E6 and its target are in the same cellular compartment. As yet, no localization controls for E6 have been described, but since different studies have found E6 in different cellular compartments, it is probable that they exist.

One of the major means of altering protein specificity is posttranslational modification, and E6 has now been reported to interact with two kinases. PKN, a serine-threonine kinase, has been shown to phosphorylate E6 at a site in the carboxy terminal half of the protein *(112)*. However, no alteration in E6 activity has yet been reported upon PKN phosphorylation. In contrast, the high-risk HPV E6 types have a consensus PKA site within the carboxy terminal region of the protein that overlaps the PDZ binding domain *(113)*. Phosphorylation of this site by PKA abrogates the ability of HPV-18 E6 to bind to Dlg and target it for degradation. Thus, a simple posttranslational modification of E6 completely alters its ability to recognize at least one substrate (and probably the whole range of its PDZ-containing targets). This finding provides a further demonstration of the potential of the PDZ binding activities of E6 as a compelling target for chemotherapeutic intervention.

5. HPV E7

HPV *E7*, like *E6*, continues to be retained and expressed in cells derived from cervical tumors *(7)*, and is essential for maintenance of the transformed phenotype *(114–116)*. Numerous studies have used antisense oligonucleotides to suppress both E7 expression and tumor cell growth *(117–119)*, confirming the efficacy of targeting E7 to block malignant progression. Recent studies also found that E7 was required for completion of the viral life cycle *(68)*, demonstrating that it is also a valid therapeutic target during the early stages of viral infection.

E7 is a small, multifunctional protein, whose number of identified cellular targets continues to grow. As was highlighted with E6, many of the same domains of E7 interact with multiple cellular targets; inhibition of one interaction may also inhibit several others, so that specific inhibition of any single interaction may prove impossible.

HPV E7's primary function is linked to perturbation of the normal cell cycle. HPVs commence their vegetative replication cycle in terminally differentiating keratinocytes, which have switched off their replication machinery and exited the cell cycle. To replicate their DNA, high-risk HPVs, in particular, must restart the cellular replication machinery at a point when their host cell differentiation is well advanced. An analysis of the cellular proteins with which E7 interacts, most of which are involved in the normal regulation of the cell cycle, shows how this is achieved.

The E7 protein is around 100 amino acids long, and is highly conserved in structure between different HPV types. The E7 protein can be divided into three conserved domains: CD1, CD2, and CD3 (*see* Fig. 2). CD1 and CD2 share homology with conserved regions 1 and 2 (CR1 and CR2) of Adenovirus E1a, and CD2 also shares homology with SV40 large T antigen. The CD3 domain of E7 consists of a cysteine loop structure in the C-terminal half of the protein, shown to bind zinc and to contribute toward homodimerization *(46,120)*.

5.1. Interactions with Pocket Protein Family Members

As mentioned above, expression of E7 from a K14 promoter in transgenic mouse epithelia results in benign tumor cell growth *(76)*, highlighting the potential of E7 to drive cell proliferation. It is not surprising, therefore, that a large number of the cellular targets of E7 are involved in the regulation of cell cycle progression. These targets include cyclins A and E, the AP-1 family of transcription factors, and, perhaps most important, members of the "pocket protein" family—pRb, p107, and p130 *(121–126)*. These pocket protein interactions are considered paramount to E7 function, since they are major regulators of the cell cycle, responsible for modulating the expression of genes required for cell cycle progression *(127–129)*. Indeed, mutations within E7 that block these associations greatly reduce E7 transforming potential *(130,131)*, as well as preventing its ability to support viral replication *(68)*.

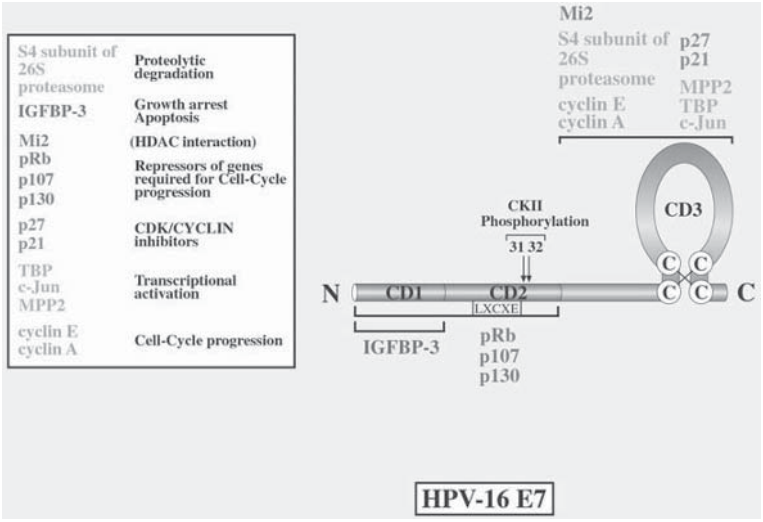

Fig. 2. Schematic diagram showing the HPV-16 E7 protein. The zinc-finger region and CD1, 2, and 3 are indicated. Also shown are some of the cellular targets and the regions to which sites of interaction have been mapped.

The E7 proteins from high-risk HPVs interact more strongly with pRb than those from low-risk HPVs, and do so via an LXCXE motif lying between residues 20 and 30 of CD2 *(132)*. During normal cell cycle progression, cyclin/CDK hyperphosphorylation of the pocket proteins causes release of E2F transcription factor family members and concomitant upregulation of genes required for cell cycle progression *(129)*. During HPV infection, interaction of HPV E7 with pocket proteins causes release of free E2F independently of cyclin/CDK activity. However, binding of E7 to pRb is, by itself, insufficient for both release of free E2F and inhibition of pRb DNA binding activity, and sequences within the C-terminal half of E7 are also required *(133,134)*. This protein-binding pocket of pRb, p107, and p130 also binds to range of cellular proteins that contain an LXCXE motif. These proteins include the D-type cyclins *(135,136)*, retinoblastoma binding proteins 1 and 2 (RBP1 and 2) *(137)*, and histone deacetylases 1 and 2 (HDAC1 and 2) *(138,139)*. This last set of interactions with HDACs allow the pocket proteins to actively repress genes whose expression is required for cell cycle progression during G_1 *(140)*. Interestingly, HPV-16 E7 has also been shown to be able to disrupt this complex *(138)*, and the finding that E7 can bind to HDACs 1 and 2 via an intermediate protein, Mi2, using residues in CD3 *(141)* suggests how this activity may occur.

As mentioned above, E7 binding to pRb alone is insufficient for complete inactivation of pRb function, and recent studies have shown that E7 can target pRb for ubiquitin-mediated degradation *(142,143)*. A model for how this targeting is achieved is suggested by the discovery that E7 interacts with the S4 subunit of the 26S proteasome *(144)*, thus increasing the ATPase activity of the S4 enzyme, which is involved in proteasome assembly. The region of E7 required for its interaction with S4 is also required for the release of free E2F from pRb *(134)*, suggesting that degradation of pRb is required for optimal E7 activity, and representing an additional target domain for small-molecule inhibitors. This result also raises the question of whether there are other targets of E7 that may also be degraded.

Thus, there are multiple levels at which a potential therapeutic could be aimed with respect to the E7–pocket protein interactions; however, the most appealing is still inhibition of the basic association. Unfortunately, the number of cellular proteins containing an LXCXE motif that bind to pocket protein family members obviously raises the spectre that attempts to block pocket protein interactions with E7 may encounter problems of specificity. However, a recent study showed that some proteins with an LXCXE motif, such as HDAC1, were still able to bind to a mutant pRb lacking the LXCXE-binding cleft. Furthermore, the mutant pRb used was still able both to induce cell cycle arrest and to repress E2F-mediated transcription, but in a manner resistant to inactivation by E7 *(145)*. This study has very important implications for the design of inhibitors to block the E7–pRb interaction, since it suggests inhibition may be achieved without drastically affecting normal pRb functions. Hence, this "pocket protein" binding region of E7, or the binding pocket on pRb itself, represent excellent potential target domains for small-molecule inhibitors.

Although inhibition of the E7–pocket protein interaction is likely to be an effective therapeutic option, a word of caution is required. A mutant of E7 defective in pRb binding could still cooperate with E6 in the immortalization of primary keratinocytes *(146)*, and E7 stimulation of proliferation does not necessarily correlate with pRb binding *(147)*. These observations imply that other interactions with cellular proteins play important roles in E7 function, and should be taken into account in the design of therapeutic strategies.

5.2. Interaction with p21 and p27

Another level of cell cycle control is performed by the cyclin-dependent-kinase (CDK) inhibitors p21 and p27. CDK inhibitors block cell cycle progression in response to a variety of antimitogenic and differentiation-related signals *(148)*. Both p21 and p27 are transiently upregulated during keratinocyte differentiation; the p21 induction being p53-independent *(149)*. HPV E7 antagonizes the p27 inhibition of cyclin E/CDK activity and cyclin A expression *(150)*, and this ability correlates with its interacting with p27 via a domain in the C-terminal half of E7

(150). A similar interaction occurs with p21, allowing E7 to block both the p21 inhibition of cyclinE/cdk2 activity and the p21 inhibition of PCNA-dependent DNA replication *(151,152)*. Therefore, as well as upregulating E2F responsive promoters, E7 also directly targets cyclin-dependent-kinase inhibitors, thereby providing an extra impetus to cell cycle progression. However, whether these interactions with CDK inhibitors are relevant targets for therapeutic intervention remains to be determined.

5.3. Transcriptional Activation of Cyclins and Interaction with Cyclins/cdks

E7 has been shown to sequentially activate the cyclin E and cyclin A promoters *(153)*. E7 induction of cyclin E expression was shown to require residues within CD2 of E7, probably as a consequence of its interaction with cellular pocket proteins and release of E2F. In contrast, for cyclin A upregulation, residues within both CD1 and CD2 were shown to be required. E7 has also been shown to become part of two cyclin-containing complexes; the S-phase-specific E2F/cyclin A/p107/cdk2 complex *(154)*, and the G_1-specific E2F/cyclin E/p107/cdk2 complex *(122)*. Such complexes undoubtedly assist in overriding normal cell cycle controls and are probably essential for viral DNA replication, but it is unclear whether these activities contribute to the malignant potential of E7.

5.4. Interaction with Elements of the Transcriptional Machinery

HPV E7 has also been shown to be able to activate transcription independently of E2F *(155)*. This activation appears to be related in part to its ability to interact with the core component of the TFIID transcription factor complex—the TATA box binding protein (TBP) *(156)*—and to a TBP-associated factor TAFII10 *(157)*. Interestingly, the association with TBP is regulated by casein kinase II (CKII) phosphorylation of E7 *(156)* on serine residues 31 and 32 in CD2 *(158)*. Blocking CKII phosphorylation of E7 should reduce the malignant potential of E7, because mutants within the CKII phosphorylation site are greatly reduced in their transforming potential *(158)*. This potential can be restored, however, by replacing the two serines with two acidic residues, suggesting that the negative charge at this site enhances E7 function *(159)*. CKII phosphorylation of E7 thus appears to be an effective target for cancer therapy. Its value as a target during viral replication is doubtful, however, because mutants within this region of E7 retain the ability to undergo a complete viral life cycle *(68)*.

As well as interacting with basal transcription elements, E7 has been shown to interact with the Ap-1 family of transcription factors, including c-Jun, JunB, JunD, and c-Fos. Residues within CD3 of E7 are required, and as a consequence of this interaction, c-Jun-responsive promoters are upregulated *(123)*. The rele-

vance of this interaction was demonstrated by a study showing that expression of dominant negative c-Jun in HPV-transformed keratinocytes can suppress anchorage-independent growth *(160)*, suggesting that up-regulation of c-Jun-responsive promoters by E7 may contribute to the malignant potential of this protein. Taken together, these studies suggest that targeting the interactions of E7 with the basal elements of the transcriptional machinery offers excellent possibilities for therapeutic intervention in the progression to malignancy.

5.5. Other Activities of HPV E7

At this stage, it is worth pointing out that the major contributor to the aggressive nature of a tumor, and hence the prognosis for a patient, comes not from the initial immortalization event, but from the metastasis of the tumor, and its ability to evade the host immune system. Recent studies have shed some light on these aspects of cervical cancer. HPV-16 E7 abrogates interferon-α signaling by inhibiting induction of interferon-α-inducible genes, probably by binding via CD2 to the p48 DNA-binding component of the interferon-stimulated gene factor 3 (ISGF3) transcription complex *(161)*. HPV-16 E7 expression was also shown to inhibit both interferon regulatory factor-1 (IRF-1) and NFκB function, thus potentially impairing the IFN response in HPV-infected cells *(162)*. Recent studies have also begun to focus on the vascularization of cervical tumors, and HPV-16 positive SiHa cells have been shown to release E6, E7, and vascular endothelium growth factor (VEGF) into the extracellular medium. This study also showed that E7 inhibited the cellular immune response to recall and alloantigens, and enhanced the release of angiogenic cytokines such as TNFα, IL-β, and IL-6 by macrophages *(163)*. Although the precise mechanism by which this occurs is not clear, this is an important observation, implying that, in addition to its cell cycle interactions and proliferative properties, E7 might have functions which aid virally infected cells or tumors to evade the immune system and enhance vascularization. Both of these steps have important implications for tumor progression, and open up new levels at which therapies might be applied to patients suffering from cervical cancer.

6. SUMMARY

It is clear from the above discussion that there are several options for blocking viral replication and/or HPV-induced malignancy. Some of these are highlighted in Fig. 3. While E2 would appear to be an ideal target during the early stages of viral infection, in the later stages of transformation, E7 would appear to offer good possibilities. However, as with E2, a note of caution with E7 is required. Inhibition of E7 function in a primary infection is likely to result in a loss of replicative competence of the virus, and there is evidence that this might give rise to viral integration *(68)*. If this is the case, it is essential that any therapy that targets E7 function is applied for the duration of time to clear the virally infected cell, anything less

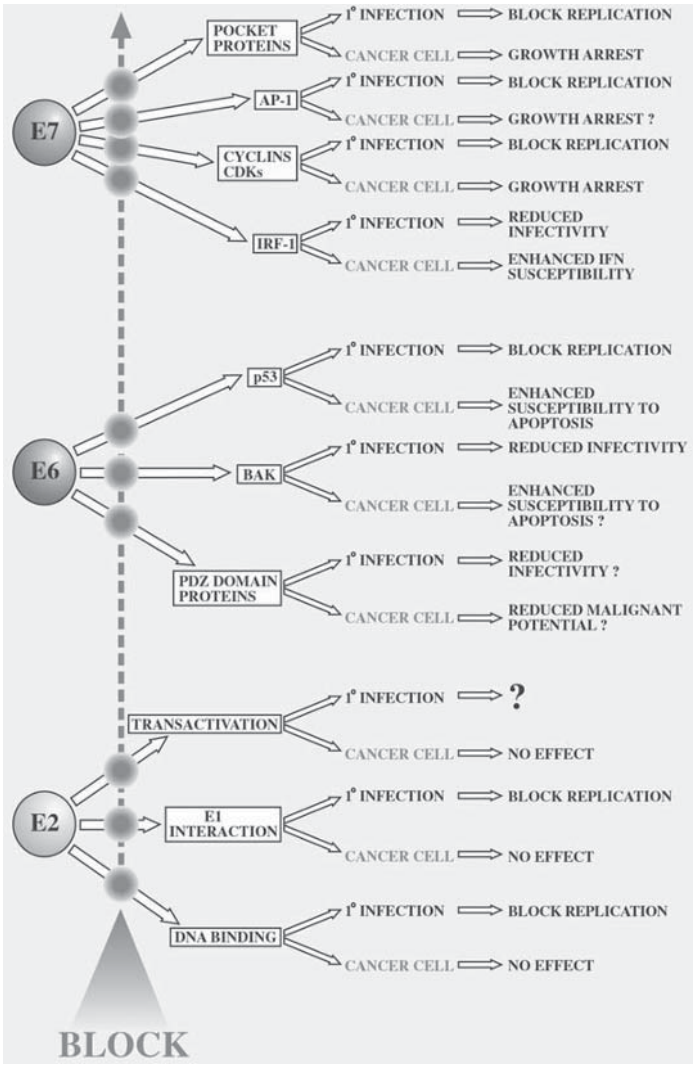

Fig. 3. Potential therapeutic targets for blocking E2, E6, and E7 functions. Also shown are the possible consequences of such inhibitors upon the normal viral life cycle, and also in the later stages of malignant progression.

may result in E7 being reactivated and the processes leading to immortalization initiated. The ideal viral protein for therapeutic intervention therefore appears to be E6, because a key element here is the induction of host apoptotic pathways, thereby eliminating a primary viral infection as well as the virus-transformed cancer cell. As with all therapeutic approaches, a combination of therapies, in particular, reactivation of p53 and MAGUK protein function, together with IFN treatment while blocking E7 inhibition of IRF-1 activation, would appear to offer one such means of treating HPV-induced diseases.

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13 Apoptin as an Anticancer Therapy

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Contents

1. INTRODUCTION

During the life of humans and animals, dysfunctional cells undergo apoptosis in an active and programmed fashion, a process that allows the organism to function normally. The program to enter apoptosis is present in each cell of the body and "waits" for an apoptosis signal. Such signals can be either extracellular or intracellular *(1)*. The process of apoptosis can be divided into two parts: the decision phase and the execution phase. During the decision phase, a cascade of gene-regulated processes is activated that leads to the actual execution of apoptosis. At each step of the decision cascade, the apoptotic signal can be either prevented/delayed or stimulated, which implies that cells have genes with pro- and antiapoptotic activities *(2)*. During the execution phase, cellular processes take place that will lead to a total metamorphosis of the cell. Specifically, the cellular membrane forms so-called blebbing structures, and the DNA in the nucleus

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strongly condenses. Somewhat later, the cell will disintegrate into several fragments, the so-called apoptotic bodies. These bodies are taken up and digested by neighboring cells and macrophages, which prevents the release of cellular debris that cause inflammatory reactions in the body *(3)*.

Impairment of apoptosis plays an important role in a large number of pathophysiological processes. Elevated apoptotic activity leads to syndromes like AIDS and Parkinson's disease, whereas decreased apoptosis may lead to autoimmunological diseases and cancer *(4)*. Several gene products have been implicated in controlling the apoptotic process. The tumor-suppressor protein p53 plays an essential role in the repair of DNA damage and will, if necessary, induce apoptosis. Without a functional p53, the cell will survive with DNA damage and might grow into tumors. Consistent with this idea, more than half of all human tumors harbor mutations in the *p53* gene *(5)*. In addition, chromosomal translocations can lead to a high synthesis of antiapoptotic Bcl-2 proteins. Overexpression of Bcl-2 and other antiapoptotic Bcl-2-like proteins have been connected to the occurrence of tumors such as lymphomas, breast, and prostate tumors *(6)*. Besides these p53 and Bcl-2 pathways, other distinct apoptotic decision pathways, like that downstram of Fas, have been described to play an important in the development of tumors *(7)*.

The apoptotic process plays a role not only in the development of tumors, but must also become active in a successful anti-cancer treatment. Tumor cells overwhelmingly contain genetic lesions in the apoptotic decision pathway, but not in the execution phase. This means that tumors can go into regression as soon as the right apoptotic signal is offered. Unfortunately, many conventional (chemo)-therapies fail because they induce apoptosis via p53 and/or are inhibited by antiapoptotic proteins such as Bcl-2 and Bcr-abl *(8)*.

In this chapter we discuss the tumor-specific characteristics of the apoptosis-inducing protein Apoptin. The applied and mechanistic studies performed to date reveal the potential of Apoptin for anti-tumor therapy strategies.

2. CAV INDUCES APOPTOSIS

2.1. History

Viruses are known that inhibit apoptosis and/or induce apoptosis *(9)*. An example of the former are certain DNA tumor viruses, while an example of an apoptosis-inducing virus is the chicken anemia virus (CAV), which belongs to the genus *Gyrovirus* of the family *Circoviridae (10)*. CAV causes clinical symptoms in newborn chickens and subclinical symptoms in older ones. Among the clinical signs are severe depletion of cortical thymocytes and erythroblastoid cells in the bone marrow, leading to immunodeficiency and anemia. The depletion of thymocytes observed after CAV infection is caused by apoptosis, as DNA isolated from the thymus of infected chickens shows an apoptosis-specific laddering pattern. More-

over, electron-microscopic analysis of the cortex of CAV-infected chickens reveals cells containing condensed chromatin next to the nuclear membrane and apoptotic bodies in the cytoplasm of epithelial cells *(11)*. A striking feature of CAV is that it can replicate and induce apoptosis in vitro only in transformed chicken cell lines, which suggests that at least a part of its life cycle is regulated by transforming events.

CAV is a small nonenveloped virus containing a single-stranded circular DNA genome of 2.3 kb. In 1990, our laboratory elucidated the sequence of the CAV replicative double-stranded DNA form. From its genome, a single polyadenylated polycistronic mRNA is transcribed containing three partially or completely overlapping genes. As three different frames are used, three distinct proteins (VP1 [51.6 kDa], VP2 [24.0 kDa], and VP3 [13.6 kDa]) are generated *(12)*. Thus far, no other cellular or viral protein has been found that resembles one of the CAV proteins.

2.2. A Single CAV Protein, Apoptin, Induces Apoptosis

We have analyzed which CAV protein is responsible for the induction of apoptosis. To this end, plasmids encoding VP1, VP2, or VP3 were transfected into cultured chicken transformed mononuclear cells. The cells were analyzed by indirect inmunofluorescence using specific antibodies, and the DNA was stained with propidium iodide. Early after transfection, VP3 is dispersed as fine-granular structures throughout the nucleus, and is also situated partially in the cytoplasm. Somewhat later, when the cells become apoptotic, VP3 becomes aggregated in larger, discrete nuclear structures. At this time, nucleosomal laddering can be seen in the DNA from VP3-expressing cells, but not in the DNA from cells transfected with a control plasmid *(13)*. Expression of the CAV gene *VP3* alone was sufficient for the induction of apoptosis as observed in CAV infection. Moreover, during the Apoptin-induced apoptotic process, characteristic Apoptin-positive "doughnut-like" structures similar to those observed in CAV-infected cells became visible. The fact that VP3 can induce CAV-specific apoptosis on its own prompted us to rename VP3, Apoptin®. The VP2 protein also has some apoptotic activity, although much weaker than that of Apoptin, whereas VP1 was shown not to harbor apoptotic activity (Noteborn et al., unpublished data).

The Apoptin protein is 121 amino acids long, contains many proline, serine, and threonine residues, and has a positively charged C-terminus. Apoptin contains both a putative nuclear export as well as a putative nuclear localization signal *(14)*. Apoptin was found to co-localize with the chromatin in morphologically nonapoptotic cells, and with the condensed DNA in apoptotic ones. A mutant Apoptin protein that lacks a part of the positively charged amino acids remained partially localized in the cytoplasm and had a reduced apoptotic activity *(15)*. The positively charged characteristic of Apoptin may allow interaction with nucleic acids, consistent with its nuclear localization. The presence of Apoptin in the

chromatin structure and its high proline content may cause disturbance of the supercoiled organization, which could then result in apoptosis. Another possibility is that Apoptin acts as a transcriptional regulator of genes influencing the apoptotic process.

3. APOPTIN KILLS TUMOR CELLS SPECIFICALLY

3.1. In Vitro Effects in Human Tumor Cells

We have shown that Apoptin is able to induce apoptosis in a large variety of human tumor cell lines. Among these are cells derived from breast tumor, hepatoma, cholangiocarcinoma, colon carcinoma, lymphoma, leukemia, lung tumor, neuroblastoma, osteosarcoma, ovarian cancer, prostate cancer, or squamous cell carcinoma. In addition, Apoptin was also proven to induce apoptosis in transformed cells from other species (*16*, unpublished results). While Apoptin induces apoptosis with variable kinetics in different tumor cell lines, it always reaches 90–100% of the Apoptin-positive cells 6 d after transfection.

Early after transfection, immunofluorescence and electron-microscopy studies revealed that in human osteosarcoma-derived Saos-2 cells, Apoptin is present both in the endoplasmic reticulum and in the nucleus. At later time point after transfection, a large amount of Apoptin protein accumulates in the heterochromatin. This results in the characteristic CAV-induced apoptotic nuclear structures described above. Somewhat later, the cellular DNA condenses and is fragmented (*13,16*; Mommaas, unpublished results). These data, combined with those described for the avian-transformed cells, led us to conclude that Apoptin can induce apoptosis in a great variety of tumor cells in a similar if not identical manner to that of CAV.

3.2. In Vitro Effects in Normal Human Cells

Under tissue culture conditions, Apoptin cannot induce apoptosis in normal healthy human cells such as keratinocytes, endothelial cells, phytohemagglutinin-stimulated T cells, fibroblasts, myoblasts, and smooth muscle cells. In addition, rodent embryo diploid fibroblasts and hepatocytes from adult rats were also shown not to undergo apoptosis. Limited long-term expression of Apoptin in normal human fibroblasts revealed that Apoptin has no toxic or transforming activity. Interestingly, the differential killing of tumor versus normal cells is paralleled by a difference in subcellular localization: in tumor cells, Apoptin accumulates in the nucleus, while in normal cells, it is predominantly present in cytoplasmic structures *(17)*.

Thus, one possible reason for the tumor specificity of Apoptin-induced apoptosis is that Apoptin can bind to chromatin structures only in tumor cells. Preliminary microinjection experiments of human tumor cells and normal fibroblasts with bacterially produced Apoptin–MBP fusion protein confirm the tumor-speci-

fic activity and localization of Apoptin (Zhang et al., unpublished results). To prove that Apoptin is harmless in healthy cells in vivo, we are testing transgenic Apoptin mice. These mice harbor the Apoptin gene under the regulation of the widely active MHC-1 promoter in all their cells, and express the transgene in a number of tissues and organs. Nevertheless, these Apoptin mice look healthy, breed normally, and do not seem to suffer any adverse effects from the constant production of Apoptin. Currently, experiments are in preparation to examine whether the Apoptin mice are more resistant to tumor-inducing agents in comparison to their wild-type littermates (Pietersen and Noteborn, unpublished results).

3.3. Effects in Cancer-Prone Cells

We have found evidence that transient transfection of human diploid cells with the SV40 transforming large T antigen will render these normal cells susceptible to Apoptin-induced apoptosis *(18)*. Upon transfection, the Apoptin protein becomes translocated from the cytoplasm into the nucleus. These results imply that expression of a transforming protein is sufficient to render normal cells susceptible to Apoptin, and the establishment of a stable transformed state is not required.

UV irradiation causes an aberrant SOS response in primary diploid cells from cancer-prone individuals. This response can be described as a transient transformation event and as expected, under these circumstances Apoptin can induce apoptosis. In cells derived from healthy individuals, UV irradiation does not result in Apoptin-induced apoptosis or nuclear localization *(19)*.

Taken together, these results clearly indicate that Apoptin induces apoptosis in a transformed and/or tumorigenic cellular background. Figure 1 shows a schematic representation of the Apoptin activity and its cellular localization in normal, cancer-prone and tumorigenic cells.

3.4. Tumor-Specific Events

The tumor-specific characteristics of Apoptin seem to focus on its tumor-specific cellular localization; namely, Apoptin is present predominantly in the nucleus of tumor cells, whereas it is mainly localized in cytoplasmic structures in nontransformed cells. To determine whether nuclear localization is important for Apoptin's tumor-specific activity, we have constructed an Apoptin protein containing the nuclear location signal of SV40 large T antigen (NLS-Apoptin). Transient transfections of normal human fibroblasts with plasmids encoding this NLS-Apoptin or wild-type Apoptin showed that NLS-Apoptin accumulated in the nucleus, whereas, as expected, Apoptin was mainly situated in the cytoplasm. Strikingly, although NLS-Apoptin was present in the nucleus of normal diploid fibroblasts, it was not able to induce apoptosis (Danen-van Oorschot, unpublished results).

These data suggest that Apoptin becomes modified in a differential way in tumor cells versus normal cells and/or encounters proteins with a tumor-specific

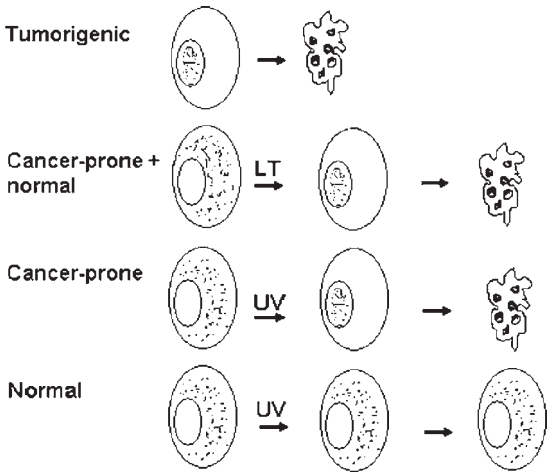

Fig. 1. Under transformed situations. Apoptin is located in the nucleus whereas in normal cells Apoptin is situated within cytoplasmic structures. All analyzed human tumor cells (tumorigenic) undergo Apoptin-induced apoptosis, while normal cells do not. Transient synthesis of a transforming agnet like SV40 large T antigen (LT), however, results in the nuclear localization of Apoptin and induction of apoptosis in cells derived from healthy individuals (normal) as well as in cells derived from cancer-prone individuals (cancer-prone). The latter cells also undergo Apoptin-induced apoptosis upon UV-treatment, whereas normal cells do not. See for further details also the text.

behavior absent in normal cells. Alternatively, normal cells might contain Apoptin-inhibiting proteins. Another possibility, the existence of cellular counterparts of Apoptin and, therefore, interaction of this pathway with Apoptin, have so far not been discovered. The isolation of Apoptin-associating proteins, by means of a yeast-two-hybrid screen, revealed novel proteins with tumor-specific characteristics (Danen-Van Oorschot and Rohn, unpublished results). It is hoped that these proteins will help to elucidate the tumor-specific mechanism of Apoptin-induced apoptosis. Finally, in vitro kinase assays that combined bacterially produced Apoptin with lysates derived from human tumor cells versus human nontransformed cells showed that Apoptin becomes phosphorylated in a tumor-specific manner. It seems plausible that a modified Apoptin protein can induce apoptosis, whereas an unmodified one cannot. These results are currently being confirmed in vivo (Rohn and Zhang, unpublished results).

All of these ongoing experiments should uncover the mechanism of tumor-specific Apoptin-induced apoptosis as well as provide better insight into steps

essential to the development of tumors. The following sections will discuss the relationship of Apoptin with known apoptosis factors.

4. P53-INDEPENDENT APOPTOSIS

The role of p53 in apoptosis and chemosensitivity provides a provocative link between factors that influence tumor development and those involved in drug toxicity. Current clinical studies suggest that one of the major causes of resistance to the conventional chemotherapy or radiation therapy are mutations within the tumor-suppressor gene *p53*. Hence, strategies that enhance p53 activity or that can circumvent p53 by appropriate manipulation of downstream targets are likely to have therapeutic benefit *(5)*.

Therefore, we have investigated whether Apoptin requires functional p53. To that end, we first transiently transfected a plasmid encoding Apoptin into human osteosarcoma-derived Saos-2 cells lacking *p53*, Saos-2 cells expressing a nonfunctional form, and U2OS cells expressing wild-type *p53*. In all three cell lines, Apoptin was able to induce apoptosis to the same extent, which suggests that it induces apoptosis in a p53-independent way *(15)*. Consistent with this result was the finding that inhibitors of p53, such as the adenovirus E1B-55K protein (Zhuang et al., unpublished data) or the SV40 large T antigen have no effect on Apoptin-induced apoptosis. Furthermore, in cell lines derived from human hepatoma, squamous cell carcinoma, and cholangiocarcinoma that do not express p53 at all, or express nonfunctional p53, we found that Apoptin induces apoptosis (*20*; unpublished results).

All of these results strongly suggest that Apoptin acts via a p53-independent apoptosis route or downstream from p53 and that, therefore, Apoptin is a potential anti-cancer agent.

5. BCL-2 STIMULATES APOPTIN-INDUCED APOPTOSIS

Bcl-2 is known to inhibit p53-mediated apoptosis *(6,21)*. In contrast, Apoptin was shown to induce apoptosis in human malignant blood cells expressing high levels of endogenous Bcl-2 due to a chromosomal translocation. In fact, Apoptin induced apoptosis even faster than in transformed blood cells with a normal level of Bcl-2 *(22)*. These results suggested that Bcl-2 has an enhancing effect on Apoptin-induced apoptosis. Consistent with this hypothesis, human Saos-2 cells were transiently transfected with plasmids encoding Apoptin alone or together with Bcl-2. Several days after transfection, Saos-2 cells transfected with both Apoptin and Bcl-2 underwent much faster apoptosis than cells expressing Apoptin alone *(21,23)*.

Apparently, Bcl-2 accelerates Apoptin-induced apoptosis in transformed mammalian cells, which is surprising, as Bcl-2 is known to inhibit apoptosis. One possible explanation could be that Apoptin induces cleavage of Bcl-2 by activated

caspases, resulting in a proapoptotic form of Bcl-2 *(24)*. However, a mutant form of Bcl-2 that can no longer be cleaved by caspases can accelerate Apoptin activity to the same extent as wild-type Bcl-2 (Danen-Van Oorschot, unpublished results). Because immunoprecipitation assays showed that Bcl-2 does not co-precipitate with Apoptin, it is likely that the effects of Bcl-2 on apoptosis are not due to direct interaction between the two proteins.

In view of the results obtained with co-expression of SV40 large T antigen and Apoptin, one might expect that overexpression of the proto-oncogene Bcl-2 in normal human cells might also force Apoptin to enter the nucleus, resulting in the induction of apoptosis. However, normal diploid cells expressing Apoptin alone or with Bcl-2 did not enter the apoptotic process. This means that an unknown transforming factor and/or event has an effect on Apoptin and/or Bcl-2 independent of Bcl-2's antiapoptotic activity.

6. ROLE OF CASPASES AND MITOCHONDRIA

Caspases are cellular proteases that play an essential role in the execution phase of the apoptotic process by cleaving a large number of proteins, which in turn leads to the typical morphology of apoptosis *(25,26)*. All caspases cleave after an aspartatic acid residue, and specificity is largely determined by the tetrapeptide directly N-terminal to the cleavage site. Functionally, caspases form a cascade of specific proteases and can be divided into initiator (upstream) and effector (downstream) caspases *(27)*.

Many apoptotic stimuli induce loss of mitochondrial integrity followed by a drop of the mitochondrial inner potential, opening of the permeability transition pores, and release of proapoptotic factors, such as cytochrome c, AIF, and Smac/DIABLO, some of which can activate caspases *(28–30)*. The antiapoptotic proteins Bcl-2 and Bcl-x_L can inhibit apoptosis by inhibiting opening of the permeability transition pore and the release of mitochondrial factors *(31)*.

We have examined the involvement of caspases and mitochondria in Apoptin-induced apoptosis in human tumor cells. Apoptin-induced apoptosis was not affected by the cow-pox-derived protein CrmA, an upstream-caspase inhibitor, whereas the baculovirus-derived p35 protein, a downstream-caspase inhibitor, did. In addition, immunofluorescence studies using a specific antibody showed that caspase-3 was present only in Apoptin-positive cells that already revealed apoptotic morphology. The fact that caspase-3 activation was never seen to precede late-stage apoptotic characteristics implies that it represents a late event in Apoptin-induced apoptosis; in other words, it may be a consequence, not a cause. Cells expressing both Apoptin and p35 showed only a slight change in nuclear morphology, indicating that an inhibitor of downstream caspases could at least partially suppress Apoptin-induced apoptosis. In most of these cells, however, cytochrome c is still released and the mitochondria are not stained by CMX-Ros,

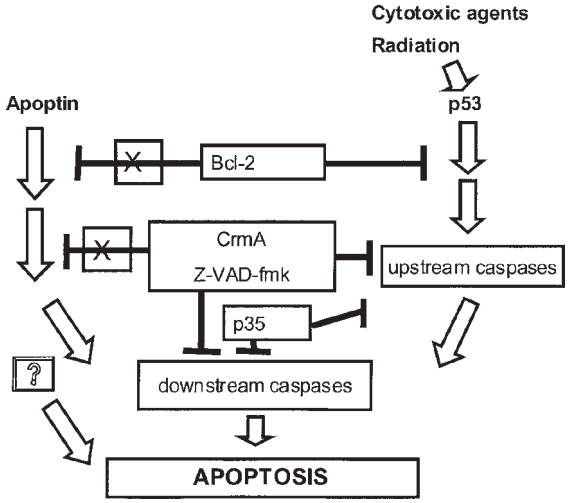

Fig. 2. Schematic representation of the apoptotic pathway induced by Apoptin versus the p53-regulated apoptotic pathway. See for further details also the text.

indicating a drop in mitochondrial membrane potential. Thus, although the final apoptotic execution events are blocked by p35, the apoptotic processes mediated through the mitochondria are not *(32)*.

Taken together, it cannot be concluded that mitochondria are essential for the early stages of Apoptin-induced apoptosis. If anything, mitochondrial involvement, which in the absence of p35 is almost only observed in late-stage apoptotic cells, thus seems to be an effect rather than a cause of apoptosis induction *(32)*. This hypothesis is strengthened by the fact that Bcl-2, which inhibits apoptosis by preventing cytochrome c release and the drop in mitochondrial membrane potential, does not inhibit Apoptin-induced apoptosis. On the contrary, as discussed above, overexpression of Bcl-2 even accelerates the apoptotic process induced by Apoptin *(20–23)*.

A schematic representation of the Apoptin apoptosis pathway showing its p53-independence, Bcl-2 stimulation, and involvement of downstream caspases and mitochondria is given in Fig. 2.

7. ANTITUMOR THERAPY

Because the Apoptin gene specifically induces apoptosis in tumor cells in a p53-independent way, is stimulated by Bcl-2, and is not completely blocked by downstream caspase inhibitors, Apoptin holds promise as the basis for an effective and

specific antitumor therapy. Currently, we are developing therapies using two strategies: exploiting the anti-cancer activity of Apoptin itself and finding drug targets in the Apoptin pathway.

A first approach for determining the potential of Apoptin itself for anticancer therapy is an efficient uptake of the Apoptin gene. One has to use either viral vectors expressing the Apoptin protein or a nonviral delivery system. In collaboration with others, systems have been developed or are under development that enable efficient delivery of Apoptin in tumors. One system relies on recombinant viruses such as retroviral-based plasmovirus- *(33)*, parvovirus- *(34)*, or adenovirus-vectors *(35)*, which can produce Apoptin in tumor cells, but are no longer virulent. Another strategy is to construct DNA plasmids that encode Apoptin. These plasmids can be transduced into a tumor cell by means of (bio-)chemical additives and produce Apoptin protein. Third, we are also developing methods based on transduction of Apoptin protein itself in tumor cells *(36)*.

The first animal studies have been carried out with a recombinant Ad5 adenovirus vector into whose genome the Apoptin gene has been integrated *(35)*. Toxicity tests in rats have shown that Apoptin has no significant detrimental effect. Short-time studies with a human hepatoma model in nude mice showed that Apoptin exerts an antitumor effect by inducing apoptosis. Specifically, a single intratumoral injection of the Ad5 vector encoding Apoptin into the xenogeneic hepatoma tumor resulted in a significant reduction of tumor growth. In the meantime, we have been able to show that Apoptin also has long-term antitumor effects and survival benefits in this hepatoma model (Van der Eb and Pietersen, unpublished results). The treated tumors revealed a significant reduction in tumor growth of the majority of the tumors, and 3 out of 11 tumors underwent complete regression. Studies with other tumor models such as melanoma, cholangio carcinoma, colon carcinoma, and breast tumors are underway or will be started soon.

In combination therapies, Apoptin may even decrease the effective dose of existing or novel (Apoptin-based) cytotoxic agents, resulting in a reduction of possible side effects of chemo- and related antitumor therapies.

The second strategy in developing an antitumor therapy based on Apoptin is the characterization of proteins involved in the Apoptin pathway. Presently, we are studying Apoptin-associating proteins and other processes that have tumor-specific activities (Danen-Van Oorschot and Rohn, unpublished results). All these studies should help to elucidate the tumor-specific mechanism of Apoptin-induced apoptosis and, will hopefully pinpoint specific drug targets enabling improved antitumor (chemo-)therapies.

8. CONCLUSIONS

The elucidation of a tumor-specific apoptosis pathway such as that induced by the viral protein Apoptin will result in an improved understanding of tumor

development as well as treatment of tumors. Resistance to induction of apoptosis plays an important role in failures in treatment of many tumors by chemotherapy and radiation therapy. Novel strategies for anticancer therapies focus on bypassing the tumor's resistance to this therapy. The protein Apoptin combines two essential properties: specificity and efficacy. It induces apoptosis in tumor cells, but not in normal healthy cells, and can exert its activity under conditions in which the most common anticancer drugs fail.

Animal studies prove that Apoptin might become a very promising anticancer drug. Furthermore, studies on the mechanism of Apoptin's tumor-specific apoptosis pathway should provide specific drug targets, resulting in the development of improved antitumor (chemo-)therapies.

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14 E2F and Cancer Chemotherapy

Debabrata Banerjee and Joseph R. Bertino

Contents

1. INTRODUCTION

Detailed studies on progression (governing the biochemical events) through the G_1/S boundary have indicated that a number of key cell cycle proteins exert their influence in forcing the cell through the G_1/S boundary. Among these are the cyclinD/CDK activities that phosphorylate the tumor suppressor pRb and release the transcription factor E2F (a family of proteins from E2F-1 through 6, discussed in detail in other chapters and reviewed in *1, 2*). The release of "free" E2Fs, in conjunction with other factors, leads to the increase in levels of proteins required for DNA synthesis, eventually allowing the cell to duplicate itself *(3)*. Among these factors, alterations in pRb have been observed in many if not a majority of human tumors, either as a result of mutations/deletions in the gene encoding pRb or qualitative abnormalities in the protein, such as hyperphosporylation induced by abnormal cyclinD/CDK activity. The result of a compromised pRb function is the release of free E2F, which accelerates premature entry of the cell into the S phase *(4,5)*. Under normal cellular circumstances, premature entry into the S phase would be dealt with severely by invoking programmed cell death or apoptosis *(6,7)*. In tumor cells, that have lost some or all apoptosis signaling mechanisms, such a premature entry into the S phase is tolerated and leads to continued growth *(8–10)*. The consequence of increased E2F-1 appears to be fatal in normal cells owing to activation of downstream executioners of cell death.

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However, in the face of a sustained high level of E2F-1, some cells may escape the death sentence by altering the apoptotic signaling. This escape has been demonstrated in an experimental transgenic mouse model system by Conner et al. *(11)*, where human E2F-1 under the control of an albumin enhancer promoter was overexpressed in the transgenic liver. The transgenic mice showed no signs of abnormal liver growth early, but as they aged they all developed adenocarcinomas of the liver. Continuous evaluation of the E2F-1 transgenic animals revealed that early on an increased rate of cellular proliferation was matched by increased apoptosis. However, in later stages of tumor development, the level of proliferation was always greater than apoptosis *(11)*. Several other studies have indicated that inappropriately high levels of E2F can have dual functions depending upon the stage of tumor development *(1,2)*.

2. E2F-1 OVEREXPRESSION IN HUMAN TUMORS AND EFFECTS ON CHEMOSENSITIVITY

2.1. Cell Lines Lacking pRb with Mutant p53 Have Increased Levels of Free E2F and Are More Resistant to Methotrexate (MTX) and 5-Fluorodeoxyuridine (FdUrd)

Growth inhibition studies indicate that cell lines that lack functional pRB such as HS-18, a human liposarcoma line, and *SaOS2*, a human osteosarcoma line, are intrinsically resistant to methotrexate and 5-fluorodeoxyuridine as compared to the HT-1080 Rb+/+ fibrosarcoma cell line (10–12-fold and 4–11-fold higher, respectively). These Rb–/– cell lines showed a 2–4-fold increase in DHFR levels and a 3–4-fold increase in TS levels, respectively, when compared to the HT-1080 Rb+/+ cell line. The increase in the DHFR and TS levels were not due to gene amplification, suggesting that transcriptional regulation of these genes may have been affected by absence of functional pRb *(5)*. Restoration of functional pRb by transfection of the Rb cDNA in *SaOS2* cells increased sensitivity to both MTX and FdUrd, with a concomitant decrease in levels of DHFR and TS. Sensitivity to other drugs such as VP-16, cisplatin, and doxorubicin were unchanged in pRb restored cells, although the Rb–/– cells were marginally more sensitive to doxorubicin, VP-16, and cisplatin. Electrophoretic gel mobility shift assays (EMSA) indicated that *SaOS2* cells had higher levels of free E2F as compared to the levels in the pRb restored cells. Results of these studies indicate that higher levels of free E2F may lead to intrinsic drug resistance, particularly antimetabolite resistance in tumor cells that have disrupted pRb function as a mutation, deletion, or truncation. High levels of free E2F, especially E2F-1, have been reported to cause apoptosis in cells containing an intact apoptotic signaling pathway, beginning with a wild-type p53 function. Since p53 function in tumors is frequently disrupted, it is possible that lack of functional pRb and higher levels of free E2F would lead to resistance rather than to apoptosis.

2.2. Forced Overexpression of E2F-1 and Drug Sensitivity

In order to understand the role of each of the individual E2F family members on drug sensitivity, we overexpressed E2F-1 in HT-1080 cells *(12)*. Cells overexpressing E2F-1 grew at a slightly faster rate in vitro, as well as in vivo. Increased expression of E2F-1 in these cells was associated with an increase in TS levels, and to a lesser extent DHFR and TK levels. Cells overexpressing E2F1 were more resistant to 5FU and serum deprivation, but were more sensitive to doxorubicin and SN38, an active metabolite of irinotecan. Sensitivity to taxol remained unchanged. These results indicated that transcription of TS in particular was regulated by E2F-1, either directly or indirectly. The increased sensitivity to topoisomerase poisons was of interest, as irinotecan has recently been approved for the treatment of colorectal cancer, a tumor that in some patients has high levels of E2F-1 (vide infra).

2.3. Dominant Negative E2F Blocks Cell Cycle Progression and Leads to Decreased Levels of DHFR and TS

Expression of a dominant negative mutant of E2F containing the DNA binding domain of E2F under the control of a tetracycline-regulated promoter was found to inhibit E2F transcriptional activity in *SaOS2* osteosarcoma cells as well as VA-13 cells (a SV-40 transformed human fibroblast cell line). Expression of the dominant negative E2F (E2F97) resulted in decreased levels of DHFR and TS besides cyclin D1, cyclin A, and p53 *(13)*. This finding supports the previous obser-vations from pRb restoration studies, as well as the E2F-1 overexpression studies, that levels of the chemotherapeutically important targets, such as DHFR and TS, are influenced by E2F activity. Expression of the dominant negative E2F possibly blocks all or some E2F transcriptional activity by binding to the consensus E2F binding regions on the DNA. Although a quantitative measurement of the effect of expressing the dominant negative E2F on the individual E2F family members has not been carried out, a general inhibition of the E2F activity appears to have the desired inhibitory effect on the cell cycle and related gene expression in tumor cells.

2.4. Studies in Human Tumors

Saito et.al. *(14)* first reported E2F-1 overexpression as a result of amplification in a human erythroleukemia cell line HEL. They proposed that E2F-1 overexpression in erythroid progenitors may stimulate abnormal cell proliferation by overcoming the negative effects of pRb. Studies from other laboratories have also suggested that deregulated or overexpressed E2F (especially E2F-1) leads to abnormal proliferation or blocks terminal differentiation and loss of leukemogenicity in hematopoietic cells, including myeloblastic leukemia cells *(15,16)*.

Recent work from several groups has shown that levels of TS in colon tumors predict not only for clinical outcome after fluoropyrimidine-based chemotherapy

Table 1
Differential Gene Expression Levels in Hepatic and Lung Metastasis of Colon Cancer—Q-RT-PCR Analysis

Tumors[a]	*Prior Rx*	*DHFR*	*TS*	*E2F-1*	*Cox2*	*Topo I*
H1	FU/LV/MTX	0.9	3.4	19.3	18.8	1.2
H2	none	5	7.5	25.8	137.1	8
H3	FU/LV	0.8	2.3	22	32.6	4
H4	FU/LV	1.9	2	5.6	20.2	3.5
H6	FU/LV	0.7	2.3	12.6	13.2	1.9
H7	none	0.3	4.1	12.5	15.4	2.5
H8	FU/LV	0.5	5.9	7.2	15	1.2
H9	none	0.3	13	5.5	5.7	6.6
H10	FU/LV	8.9	0.8		323.1	0.2
H11	none	0.5	4.3	18.5	19.9	1.8
H12	FU/LV/MTX	0.6	6.7	5.4	4.2	7.9
	median	0.7	4.7	13.5	18.8	3.5
L2	FU/LV	1.1	6.9	25.3	45.9	1.5
L3	none	3.1	22	127.8	30.2	4.1
L4	FU/LV	3.1	20.7	62.5	88.3	3.8
L5	FU/LV	0.2	37	60	10.2	3.5
L6	FU/LV	1.4	21	53.4	50.7	6.5
L7	FU/LV	1.1	21	77.7	49.5	7.2
L8	FU/LV	2.8	9.4	41.7	101.7	10.9
	median	1.4	19.7	64.1	49.5	5.4

[a]H = hepatic metastasis; L = lung metastasis.
Levels of TS > 4.0 have been associated with lack of response to 5FU.

but, more importantly, for overall survival *(17–18)*. As discussed, forced overexpression of E2F-1 in cell culture studies indicated a correlation between TS and E2F-1 *(12)*. In order to examine whether a similar relationship exists between E2F-1 and TS in human colon tumor samples, we evaluated colon tumor samples for expression levels of these genes. Pulmonary metastasis were found to express higher levels of TS mRNA and protein than hepatic metas-tasis *(19)*. Levels of E2F-1 expression in the pulmonary metastasis were higher than in the hepatic metastasis, and correlated with the higher levels of TS in the former *(20)*. Besides TS, we have also found that Cox-2 expression levels were significantly higher in lung metastasis (median values 49.5) over hepatic metastasis (median values 18.8) as shown in Table 1. Values presented are ratios of gene to β-actin mRNA.

Several reports indicate that Cox-2 levels are up-regulated in tumors, and more significantly, this up-regulation correlates with a more invasive phenotype *(21,22)*. An earlier study involving forced overexpression of E2F-1 in head and

neck squamous carcinoma cells demonstrated that clones which overexpressed E2F-1 were more invasive than clones that did not, and the invasive phenotype correlated with an invasive phenotype *(23)*.

Suzuki et al. *(24)* have reported that 40% of gastrointestinal cancers overexpress E2F-1, and in some of these tumors, an increase in gene copy number for E2F-1 was noted. Another report from Masunaga et. al. *(25)* indicated that liver metastasis of colorectal cancers have higher levels of TS than primary tumors, and this correlates with higher levels of E2F-1 mRNA expression.

As discussed above, higher levels of E2F-1 and TS were found in lung metastasis as compared to liver metastasis. Together with our data showing that forced expression of E2F-1 resulted in overexpression of TS, these findings raise the question as to why lung metastasis contain high levels of TS and its transcription factor E2F-1. We have preliminary evidence that high levels of E2F-1 may be a result of gene amplification. DNA isolated from 40 tumor samples from lung (L) and liver (H) metastasis, and the colon tumor cell line HCT-8 were analyzed by real-time quantitative PCR using primers specific for E2F-1 DNA. Relative to the HCT-8 DNA copy number, approximately half the samples had increased gene copies for E2F-1 (Iwamoto, Banerjee, and Bertino, unpublished results).

Mutations in E2F-1 have not been reported in human tumors, although several reports indicate overexpression and/or mutations in the E2F-4 gene in several human cancers, including hematologic malignancies and GI tumors *(26,27)*. The significance of these findings is not clear.

3. E2F-1 AS A TUMOR TARGET

The observation that E2F levels are elevated in tumors, including gastric and colon cancers, suggests that this family of transcription factors may be a target for therapy. The DNA sequence to which these proteins bind are well characterized, and several groups have devised strategies to interfere with the DNA binding function of the E2Fs. Some of these approaches are discussed below. The main approaches reported thus far to inhibit the function of E2Fs are:

1. Using oligonucleotides as decoys.
2. Using an antisense oligonucleotide to inhibit E2F synthesis.
3. Using decoy dominant inhibitory proteins or peptides that interfere with the E2F-DNA interaction.

3.1. Decoy Oligonucleotides to Block E2F-1 Function

Transcription factors usually bind to very specific consensus binding sites on relatively short stretches of DNA. Therefore, designing a specific oligonucleotide that can block the DNA binding domain of the protein may effectively block the transcription factor function. Advances in the field of modified oligonucleotide

(ODN) design, synthesis, and delivery have permitted investigators to adopt this strategy for inhibiting E2F function in the treatment of various proliferative disorders. Although the E2F family is made up of at least seven individual members, it has been a general finding that inhibiting the DNA binding ability of E2F-1 has the most profound effect on cell proliferation. The best example of this application comes from the work of Morishita et al. *(28)* and Mann et al. *(29,30)*, in which an E2F oligonucleotide decoy inhibited smooth muscle cell proliferation and thereby blocked neointimal hyperplastic response to acute injuries usually associated with grafting. Autologous vein grafts are widely used in patients who suffer from occlusive disease of the coronary or lower extremity arteries. Immediately after grafting, the thin-walled vein undergoes neointimal hyperplasia leading to a thickening of the vessel wall to withstand the higher pressure in the arterial circulation. This neointimal hyperplastic process also renders the vessel wall susceptible to atherosclerosis. Studies with the E2F ODNs have progressed from animal models to limited human trials and should prove to be valuable in controlling vein graft failures.

3.2. Strategies for Inhibiting E2F-1 Function Using Peptides

As with the decoy ODN approach, one can also envision blocking the transcriptional activity of E2F-1 by blocking the DNA sites with peptides that contain the DNA binding domain, but not the transactivation domains. Several investigators have utilized this approach to target E2F-1 activity for induction of cell cycle block, apoptosis, or down-regulation of E2F-1 target genes that are also targets for common chemotherapeutic agents such as dihydrofolate reductase (DHFR) and thymidylate synthase (TS). Short peptides of 10–18 amino acids in length have been identified and used to induce apoptosis in neoplastic cells that normally overexpress E2F-1 *(31)*. These peptides have been tagged with a sequence of peptides that facilitate intracellular entry and nuclear localization known as penetratin sequences (identified in proteins from *Drosophila sp.*), and used in in vitro experiments to demonstrate the induction of apoptosis. Control or scrambled peptides were unable to induce apoptosis in the same cells where E2F-1 specific peptides had been shown to be effective. The mechanism of this therapeutically important activity was attributed to the ability of these peptides to competitively block the DNA sites to which the native E2F-1 normally binds. These peptides appear to contain a net basic charge, and it has been speculated that these peptides are minor groove binders. A dominant negative mutant of E2F-1 can block S phase progression induced by E1A or E2F-1 by competing for the available E2F binding sites, thereby excluding the native E2F-1 from these sites. Affinity studies suggest that the dominant negative peptide has a higher affinity for the E2F-1 sites than the native E2F-1 protein and this may be a useful reagent in inhibiting E2F activity *(32)*. The side effects of reducing or inhibiting the "general" or total E2F activity of normal cells is unknown.

3.3. Utilizing High Levels of E2F-1 Expression in Tumors to Express Suicide Genes Downstream of E2F-1 Promoters

As tumors have higher levels of E2F-1 than normal tissue, several investigators have proposed using E2F promoters to express suicide genes such as HSVTK or E1A to kill tumor cells. Parr et al. *(33)* have reported on the use of E2F promoter driven HSVTK to target brain tumors without affecting the surrounding normal brain cells. In vitro studies have demonstrated the specificity of the principle using the *lacZ* reporter gene expressed downstream of a promoter element that contains the consensus E2F binding sequences. In order to determine whether the specific expression of the reporter gene/suicide gene in tumor cells was due to enhanced S phase, a partial hepatectomy model was chosen to examine the issue. It is well known that the liver regenerates to normal size after partial hepatectomy, and that a synchronous increase in S phase cells can be seen in the regenerating liver. Infection of the regenerating liver with an adenovirus construct Adv-E2F-βgal did not result in extensive staining of the regenerating liver, although the Ki67 (for PCNA) staining indicated that a majority of the liver cells were in the S phase. Tumors, on the other hand, were stained dark blue due to expression of the *lacZ* gene. Mutations in the factor binding regions of the E2F promoter (deletion mutants) resulted in loss of tumor specificity. Use of a strong nonspecific promoter such as CMV resulted in expression of the *lacZ* gene in all cells including normal cells. Treatment of the glioma model with an adenovirus vector containing the E2F promoter driven HSVTK and GCV resulted in remarkable tumor regression, without any toxicity to normal tissue. In contrast, the CMV promoter driven HSVTK/GCV construct resulted in normal tissue toxicity indicating that E2F-1 driven HSVTK is specifically expressed in tumors and not in normal tissues. Amin et al. *(34)* have taken a similar approach for selective expression of the *E1A* gene driven off the E2F promoter using a mutant oncolytic adenovirus. Their hypothesis is that ovarian cancer and non-small-cell lung cancer cells as well as tumors overexpress E2F-1, and therefore utilizing the E2F-1 driven *E1A* gene, will selectively kill these tumor cells. This approach adds further specificity by expressing the *E1A* gene off the tumor-specific E2F promoter.

3.4. Interfering with E2F–DP Interactions

E2F DNA binding activity requires heterodimer formation between E2F and DP family members. Thus far, seven E2F family members and three DP proteins have been described, so as many as 21 combinations are possible that can bind to pRb or related proteins to repress transcriptional activation. DP-1, and not DP-2 or 3, is present in most cells, decreasing the level of this complexity somewhat. As mentioned, high levels of E2F-1, in particular, can cause increased rates of cell proliferation and, in some cells, apoptosis. As E2F, in particular E2F-1, is dysregulated in many tumor cells as a consequence of pRB inactivation, interdicting its

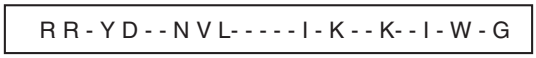

Fig. 1. DEF consensus sequence.

function is an attractive target for controlling cancer cell proliferation. Bandara et al. *(31)* generated peptides that prevent E2F–DP dimerization. A region within the DP-1 protein, referred to as the DEF box, is strikingly similar to a domain in the E2F protein (*see* Fig. 1), suggesting to these authors that it may have an important role in dimerization and/or DNA binding. In a series of elegant studies, they were able to show that a minimal inhibitory peptide, a 19 amino acid-residue sequence from the N-terminal half of the DEF box, retained activity of the DEF peptide, and disrupted binding of E2F–DP-1 to its DNA binding region. When these peptides were introduced into tumor cells using membrane-permeable forms, apoptosis was induced. Cell types differed in their susceptibility to these peptides, related to the level of endogenous E2F. It may be possible to find small organic molecules that mimic the action of these peptides, thus generating a compound that may be more useful in the clinic. However, the presence of the DEF region in E2F-2,-3,-4, and 5, may limit the specificity of this strategy, as the other E2F forms may have different, possibly opposing actions.

3.5. Inhibition of Cyclin/cdk2

The finding that the DNA-binding ability of E2F-DP heterodimers is negatively regulated by cyclin A/cdk2 *(1,2)* led Kaelin and co-workers to study peptides that were derived from the E2F1, E2F2, and E2F3 binding motif, or the cyclin/cdk2 binding motifs in p21-like cdk inhibitors and from the p27/cyclin A/cdk2 crystal structure as inhibitors of cyclinA/cdk2 activity *(35)*.

Peptides containing this motif not only inhibited the phosphorylation of substrates by cyclin A/cdk2 or cyclin E /cdk2, but remarkably, cell membrane permeable forms of these peptides induced transformed cells but not untransformed cells to undergo apoptosis. As many types of cancer have deregulated E2F because of pRB loss or mutation or upstream pRB regulators, e.g., overexpression of cyclin D, inhibitors that block cyclin/cyclin-dependent kinase interaction (Fig. 2) may be selective for these tumors. Based on these encouraging studies, it is likely that small non-peptide molecules will be developed that target these kinases. Whether these agents will be specific for tumor cells, and lack host toxicity remains to be seen. The development of STI571, a BCR/ABL kinase inhibitor, as a minimally toxic and effective drug to treat chronic myelocytic leukemia *(36)* pro-

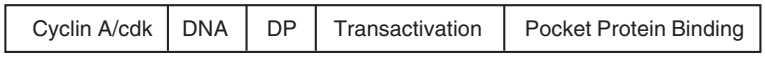

Fig. 2. Domains in E2F-1 protein.

vides hope that this degree of specificity may be obtained in tumors with dysregulated E2F.

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15 Hypoxic Signaling Pathways as Targets for Anticancer Therapy

Heidi M. Sowter and Adrian L. Harris

Contents

1. HOW DOES HYPOXIA ARISE IN TUMORS?

Solid tumors, which account for more than 90% of all human cancers, are poorly oxygenated compared to normal tissues; needle oxygen probe measurements show a range of 1.3% to 3.9% median oxygen concentrations in tumors compared to 3.1% to 8.7% in normal tissues (for review *see 1*). In up to 82% of readings taken from tumors, oxygen concentrations are less than 0.3%, that is, they show regions of hypoxia (generally defined as oxygen concentrations less than 1%), a phenomenon rare in normal tissues. Hypoxia arises when tumor cells proliferate out of the diffusion zone of the local vascular supply by cells growing around the vascular core; cells in these areas are more acidic and nutrient-starved

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than those in well-vascularized areas. The relative distance from a vessel at which hypoxia develops is also dependent on the metabolic activity of the tumor. Some oncogenes, *myc* and *ras*, for example, will stimulate tumors to proliferate more rapidly than other transformation pathways and hence increase oxygen consumption.

Tumors react to hypoxia by stimulating growth of endothelial cells from surrounding blood vessels, a process known as angiogenesis. However, tumor blood vessels are different from those in normal tissue as they are tortuous with sluggish and irregular blood flow; this leads to less efficient oxygen delivery and propagates the hypoxic tendency of tumors (for review *see 2*). Hypoxia arising from diffusion limitation is regarded as chronic, while perfusion fluctuation resulting from faulty tumor blood vessels induces acute, reversible hypoxia.

Anemia commonly develops in cancer, either from blood loss (as in gastrointestinal malignancy) as a result of treatment with chemotherapy or radiotherapy, or through the mechanisms of anemia of chronic disorders. Anemia will therefore exacerbate both chronic and acute hypoxia (for review *see 3*).

2. WHY IS HYPOXIA IMPORTANT IN TUMOR THERAPY?

2.1. Treatment Resistance

It has been known for many years that solid tumors have regions that are resistant to cancer treatment. This heterogeneity is caused in part by the different oxygen tensions that extend throughout the tumor, and there are several reasons why hypoxic areas of tumors can escape radiation and chemotherapy. Hypoxic cells of solid tumors are further away from blood vessels, leading to poor drug delivery to these areas. More importantly, hypoxic cells are nonproliferating or slowly proliferating, and most chemotherapy drugs target rapidly dividing tumor cells only (*see* Fig. 1). Radiation treatment also fails to kill hypoxic cells, since oxygen radicals are required to make permanent the DNA damage that leads to cell death. One study has shown that hypoxia-induced radiation resistance of advanced cervical cancer contributes more to treatment failure than stage of local disease *(4)*.

2.2. Promotion of Metastasis

Not only is hypoxia one of the major causes of relapse after treatment, it can also lead to the evolution of more aggressive and resistant tumors. Cells respond to hypoxia by increasing the production of proangiogenic and prometastatic factors, such as vascular endothelial growth factor (VEGF). Acute and chronic hypoxia, however, activates cell death pathways, and hypoxic areas of tumors are subject to p53-dependent apoptosis. While it may seem as though natural cellular defenses are succeeding where chemotherapy and radiation treatment failed, this does not prove to be the case. Hypoxia increases the mutation rate of cells *(5)*, and cells mutated such that they are deficient in functional p53 survive to form a more

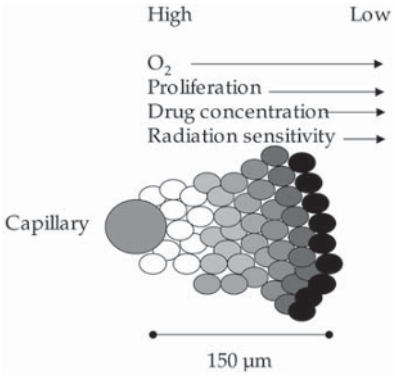

Fig. 1. A schematic representation of the tumor microenvironment. As the oxygen concentration decreases with increasing distance from the capillary, cell proliferation, radiation sensitivity and drug concentration also decrease.

resistant tumor less susceptible to cell death signals. This resistance has been demonstrated in mouse embryonic fibroblast cells, which showed a greater degree of p53-mediated apoptosis in hypoxic compared with aerobic culture *(6)*. Subsequent rounds of hypoxia and aerobic recovery revealed the ability of hypoxia to select for $p53^{-/-}$ (apoptosis resistant) over $p53^{WT}$ cells. It is now known that cells in an hypoxic environment have impaired cellular repair functions, causing hypermutability to DNA damage and increased genetic instability *(7)*.

2.3. Indication of Poor Prognosis

Not surprisingly, tumor hypoxia has emerged as a predictor of poor prognosis. Oxygen profiling of human squamous cell cancer of the uterine cervix has revealed that hypoxic cervical cancers are associated with diminished apoptotic potential and are more aggressive, and that tumor oxygenation is the strongest predictor of overall and disease-free survival *(8,9)*. However, hypoxia is a predictor of poor prognosis regardless of treatment modality, so therapy resistance is not the only explanation. The presence of hypoxia induces expression of many genes that promote a more aggressive tumor phenotype (*see* Table 1).

3. MEASUREMENT OF OXYGEN CONCENTRATIONS IN VIVO

Because the presence of a hypoxic microenvironment has many implications for treatment strategy and prognosis, many methods have been developed to enable in vivo monitoring of oxygen tension in human tumors. Polarographic needle electrodes are able to automatically sample oxygen levels in different areas of

Table 1
Gene Products Regulated by Hypoxia[a]

Oxygen Transport
Ceruloplasmin; **Erythropoietin**; Heme oxygenase 1; **Transferrin**; **Transferrin receptor**

Angiogenesis
α1β-Adrenergic receptor; alpha(v)beta(3) *(45)*; **Adrenomedullin**; Cyclooxygenase-2; **Endothelin-1**; **VEGFR1**; **Nitric oxide synthetase**; Placenta growth factor; **plasminogen activator inhibitor-1**; Transforming growth factor β; **VEGF**; Angiopoietin-2 *(46)*; Tie2 *(47)*

Glycolysis and Glucose Uptake
Aldolase A and **C**; **Enolase 1**; **Glucose transporter 1** and **3**; **Glyceraldehyde-3-phosphate dehydrogenase**; **Hexokinase 1** and **2**; **Lactate dehydrogenase A**; **Phosfructokinase L** and **C**; **Phosphoglycerate kinase 1**; **Pyruvate kinase M**

Transcription Factors
jun; *fos*; p53; Nuclear factor κB; HIF-2α (EPAS); Heat shock factor

Apoptotic/cell death genes
Nip3 NIX *(48)*; Fas/CD95/APO1 *(49)*

Others
Adenylate kinase 3; Aminopeptidase A; **Carbonic anhydrase 9** and **12** *(27)*; Collagen type V alpha 1; Cyclin G2; Epidermal growth factor; DEC1; GADD153; Glucose related protein 78; HAP-1; **Insulin-like growth factor (IGF) 2**; **IGF binding protein 1, 2** and **3**; Interleukin 6 and 8 *(50)*; low density lipoprotein receptor-related protein 1; MIC2/CD99; Monocyte chemotactic protein 1; Ornithine phosphorylase; **Platelet-derived growth factor B**; **p21**; **p27**; **p35sjr**; **Retrotransposon**; Telomerase components *(51)*; Tissue factor; Transglutaminase; Tyrosine hydrolase

[a]The data shown has mostly resulted from work on human tumor cells or endothelial cells. Genes known to be HIF-1 dependent are shown in bold. Unless stated, information was taken from *(40–44)*.

tumors, thereby revealing local gradients *(10)*. It is, however, an invasive technique, and the volume of tissue able to be sampled is limited. ^{31}P-Magnetic resonance spectroscopy (MRS) has been developed as a noninvasive technique, whereby tumor oxygen tension is measured as a correlate of cellular bioenergetic status; this method has been shown to be somewhat variable, however *(11)*. Chemical probes able to accumulate in hypoxic cells can be administered before tumor excision, and have the advantage of being only minimally invasive. Most are nitroimidazoles, and they can be visualized using autoradiography, immunohistochemistry, or MRS following appropriate tagging of the probe (for review *see 12*). Changes in the blood flow of human tumors have been measured using Doppler probes; this allows the measurement of acute hypoxia, which may not be detected over the short sample times required for other hypoxia probes *(13)*.

4. HOW DO CELLS SENSE THEY ARE HYPOXIC?

Because even slight reductions in the oxygen concentrations of virtually every cell type can result in the induction of specific genes involved in oxygen homeostasis, at least one molecular oxygen sensor must exist. As low oxygen tension can be mimicked by the addition of desferrioxamine and cobalt chloride to cells, it is thought by some that the main oxygen sensor in cells is a haem protein *(14)*. The cytochrome b protein NADPH oxidase has been proposed as a candidate for this haem protein, since it is capable of the generation of hydrogen peroxide *(15)*. Other data have also suggested the role of a redox mechanism in oxygen sensing, involving the production of hydrogen peroxide *(16)*. Hydrogen peroxide production is dependent on oxygen, since it is reduced during hypoxia, and may therefore act as a signal pathway intermediate. Although adaptive responses to hypoxia may occur via several different molecular mechanisms, one signal transcription factor family has emerged as playing a critical role in cellular and systemic oxygen homeostasis. These proteins belong to the hypoxia-inducible factor (HIF) family, and are heterodimers consisting of two subunits. The alpha subunits, HIF-1α, HIF-2α (also known as EPAS1) and HIF-α dimerize to ARNT (also known as HIF-1β) to form the functional transcription factors HIF-1, HIF-2, and HIF-3 *(17,18)*. In normoxia, the von Hippel-Lindau (VHL) protein, bound to elongin C, elongin B, Cul2, and Rbx1, degrades the alpha subunits in an oxygen-dependent manner. In hypoxia, this cannot occur, and the alpha subunit moves to the nucleus, dimerizes with ARNT, and is able to activate target genes (for review *see 17*).

The best characterized hypoxic transcription factor, HIF-1, has already been described extensively in Chapter 4. However, some gene products that are up-regulated by HIF-1 provide potential targets for therapy and markers of hypoxia will be discussed in detail below.

5. HIF-1 INDUCIBLE GENES AS POTENTIAL TARGETS FOR CANCER THERAPY

Genes that are transactivated by HIF-1 contain a sequence of 50 bp or less, known as hypoxia response elements (HREs), which are able to bind HIF-1. Many target genes that are transactivated by HIF-1 have been identified, and are shown in Table 1. The products of these genes either increase oxygen delivery or mediate adaptive responses to hypoxia, and many are being investigated as to their potential role in cancer therapy.

5.1. VEGF

VEGF is a powerful angiogenic agent, which acts through two receptors, VEGFR-1 (also known as Flt-1) and VEGFR-2 [also known as KDR/flk-1 (for review *see*

19). VEGF and VEGFR-1 are strongly upregulated by hypoxia *(20)*, and have been established as HIF-1 target genes *(21)*, although various oncogenes are also known to upregulate VEGF (for review *see 22*). The expression of VEGF and its receptors is crucial for tumor development, and is correlated with the incidence of tumor metastasis *(23)*. For this reason, VEGF is an attractive therapeutic target, and various strategies designed to inhibit VEGF have already been shown to supress the growth of xenografts in vivo and tumor metastases in vivo (for review *see 24*).

5.2. Carbonic Anhydrases

Carbonic anhydrases are important for pH regulation, because they increase or decrease acidity by catalyzing the reversible hydration of carbon dioxide to carbonic acid *(25)*. Because mRNA encoding carbonic anhydrase 9 (CA IX) and 12 (CA XII) are down-regulated by pVHL *(26)*, and hypoxia is associated with low pH, our group has investigated the possibility that these enzymes are HIF-1 responsive. We demonstrated that mRNA encoding CA IX and CA XII are strongly inducible by hypoxia in a variety of cell types, and that CA IX is tightly regulated by an HRE close to its transcriptional start site *(27)*. Moreover, CA IX protein is expressed in a perinecrotic manner in many types of human cancer, overlapping with expression of VEGF mRNA and the hypoxia marker pimonidazole *(27)*. Normal tissue surrounding tumors shows little or no CA IX expression, indicating that CA IX is tumor-specific. Because carbonic anhydrase inhibitors suppress the invasion of renal carcinoma cell lines in vitro *(28)*, and have synergistic effects with chemotherapeutic agents in animal models *(29)*, targeting of tumor-associated CA IX may reduce the aggressiveness of tumors. Also, CA IX staining may be used diagnostically to indicate localization and intensity of hypoxia.

6. HIF-1-INDEPENDENT PATHWAYS

6.1. Nuclear Factor-κB

Another transcription factor, nuclear factor-kappa B (NF-κB), is activated by hypoxia *(30)*, although unlike HIF-1, other stimuli, such as cytokines, oxidants, viral and bacterial infections, and ultraviolet light, are also able to induce the transactivation of genes downstream of NF-κB *(31)*. In its inactive state, NF-κB is bound to an inhibitor, IκB, which, upon activation, is degraded via the ubiquitin-proteosomal pathway to release NK-κB for translocation to the nucleus. However, the hypoxia-induced activation of NF-κB does not involve the degradation of IκB, suggesting that hypoxia induces a different regulatory mechanism to other stimuli. NF-κB could provide one mechanism by which hypoxia and inflammation often occur coincidentally, since genes bearing NF-κB binding motifs include inflammatory cytokines, chemokines, inflammatory enzymes, and leukocyte adhesion molecules *(30)*. NF-κB is activated during radiotherapy and che-

motherapy, and up-regulates antiapoptotic proteins that interfere with the efficiency of both treatments *(32)*. By inhibiting NF-κB with adenovirally delivered IκBα, Wang et al. have shown that chemoresistant tumors become sensitized to tumor necrosis factor alpha (TNFα) and the chemotherapeutic agent, CPT-11, resulting in tumor regression *(33)*.

6.2. c-AMP

A number of hypoxia-inducible genes contain neither NF-κB nor HIF-1 regulatory elements, implicating additional mechanisms of regulation. Hypoxia is known to down-regulate the activity of cyclic AMP (cAMP)-generating machinery, leading to diminished cAMP signaling and, depending on the gene, influence activation or repression of transcription *(34)*. One group of genes to undergo reduced transcription as a result of lowered cAMP levels are the cAMP response element binding (CREB) proteins, a family of transcription factors that bind to cAMP response elements (CREs) in DNA. CRE-bearing genes (such as TNFα, interleukin 8, E-selectin, and cyclooxygenase-2) normally under negative regulation are therefore activated as a result of hypoxia (for review *see 35*).

Recent evidence has emerged that indicates crosstalk between the hypoxia-inducible pathways. Some genes, TNFα, for example, contain binding sites for both CREB and NF-κB, and so can be activated by both hypoxia and inflammatory stimuli *(36)*. Additionally, CREB and associated proteins interact at the level of DNA binding with HIF-1 *(37)*.

6.3. ELK-1

Another pathway involved in hypoxia-induced transcription is the PKCβII pathway, which activates the transcription factor ELK-1 in response to low oxygen *(38)*. ELK-1 induces transcription of early growth response gene-1, which also binds DNA to induce other genes, such as tissue factor *(38)* and insulin-like growth factor II *(39)*.

7. THE EFFECT OF HYPOXIA ON MACROPHAGES

Cellular responses to hypoxia have mostly been elucidated using epithelial tumor cells and endothelial cells, although macrophages also respond to hypoxia in a number of ways. Macrophages form a significant proportion of solid tumor mass, and predominate in regions of necrosis in a hypoxia-driven manner [Fig. 2 (for review *see 52*)]. Hypoxia induces tumor cells to secrete monocyte chemotactic protein 1 (MCP-1), which attracts macrophages; however, as hypoxia inhibits the ability of macrophages to respond to MCP-1, once they arrive into necrotic areas they are unable to leave *(53)*. Once in necrotic tissue, macrophages respond to hypoxia by secreting more MCP-1, as well as producing angiogenic cytokines such as VEGF and TNFα *(52)*. Indeed, increased tumor macrophage index is

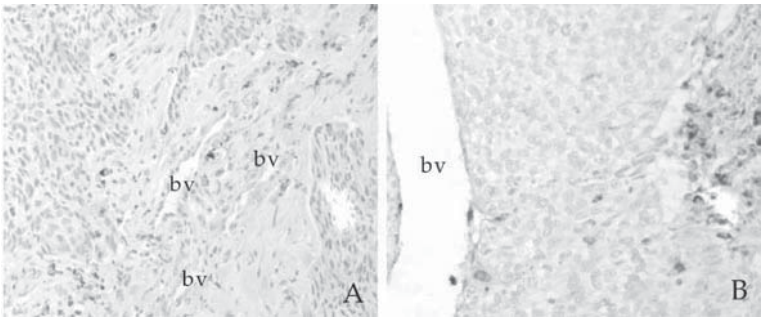

Fig. 2. Sections of a lung carcinoma from (**A**) a well-vascularized area and (**B**) a perinecrotic area. (**A**) Macrophages in oxygenated areas of the tumor are evenly distributed. (**B**) Macrophages are hypoxia-trophic, and migrate from blood vessels to areas of low oxygen tension, where they accumulate.

associated with the poor prognosis and angiogenesis of breast cancer *(54)*. Recent evidence from our group suggests that hypoxic signaling in macrophages is mediated mainly by HIF-2α, which is detected by immunohistochemistry and *in situ* hybridization in tumor macrophages and some bone marrow macrophages *(55)*.

8. STRATEGIES TO OVERCOME THE PROBLEM OF HYPOXIC CELLS IN TUMORS

8.1. Re-oxygenation

As explained above, hypoxia leads to the radiation and chemotherapy resistance of tumors. One strategy to overcome hypoxia-driven drug resistance is to re-oxygenate the targeted tissue, thereby improving radiation response and cell proliferation. This approach has been attempted by delivering a combination of nicotinomide and carbogen (95% oxygen plus 5% carbon dioxide; 56), or to have patients breath 100% oxygen at increasing pressures during radiotherapy *(57)*. These approaches have only shown marginal benefit, because hyperbaric chambers present technical difficulties and result in normal tissue toxicity, and tissues rapidly adapt or show heterogeneity in response *(57)*.

8.2. Hypoxia-Specific Cytotoxins

Another strategy to overcome therapy resistance has emerged with the development of toxins specific for hypoxic cells. One such drug is tirapazamine (TPZ), which shows a hypoxic cytotoxicity ratio (HCR) of 300 (a value of 1 indicates no differential between well-oxygenated and hypoxic cells; for review *see 58*). TPZ

acts as a substrate for various intracellular reductase enzymes, and the addition of an electron to TPZ by these reductases results in the formation of a free-radical intermediate. In well-oxygenated cells, the free radical is rapidly oxidized; however, without oxygen, the reactive radical can cause structural damage to other molecules by removing hydrogen atoms. If radicals are produced close to DNA, then single- and double-strand breaks occur, leading to cell death *(58)*. As TPZ does not kill non-hypoxic cells, used alone it has no effect on overall tumor growth, in either experimental animal models or clinical trials *(58)*. However, used in conjunction with radiation or the anticancer drug cisplatin, tumor cell kill is potentiated, and patient survival is significantly increased *(59,60)*.

Other drugs have been developed which are toxic to both hypoxic and normoxic cells, although these drugs show better results when used in combination with radiotherapy. One example is mitomycin C, a quinone antibiotic that requires reductive metabolism for activity. As mitomycin C also shows systemic toxicity to well-oxygenated cells, cure rates using a combination of mitomycin C and radiation therapy in head and neck cancers are higher than using radiation alone *(61)*. Experimental data have suggested that mitomycin C is not an ideal drug to use in conjunction with radiation. Mitomycin C has an HCR in the range of 1–5, indicating a much lower level of hypoxic toxicity than TPZ, and very low levels of oxygen are required to produce maximum differential *(62)*. If tumors were to be treated with a combination of radiotherapy and mitomycin C, therefore, a population of cells at intermediate oxygen concentrations would not be sensitive to cell death by either method.

8.3. Disrupting Hypoxia-Inducible Transcription

An attractive strategy to kill hypoxic tumor cells is to abrogate the activity of HIF-1, which mediates the induction of genes necessary for survival in regions of low oxygen concentrations. Since HIF-1 is expressed at negligible levels in normal tissues, therapy would be specific and safe. One group has engineered vectors to express high levels of transactivating domain C (TAD-C), a polypeptide that forms part of the p300/CBP interacting domain of Hif-1α *(63)*. Interaction of the HIF1 complex with p300/CBP is necessary for the transcription of HRE-containing target genes, and the TAD-C polypeptide was able to interfere with normal Hif-1α-mediated transcription. In vivo, tumor growth of cells that had been transfected with TAD-C vectors was significantly attenuated compared to wild-type xenografts. One fundamental problem of this approach is the feasibility of gene delivery to hypoxic regions of solid tumors, which, as described above, are further away from blood vessels, requiring the delivery vehicle to penetrate through multicell layers. Treatment of intermittent areas could be achieved if repeated injections were administered, although metastases further away from the primary site may be missed.

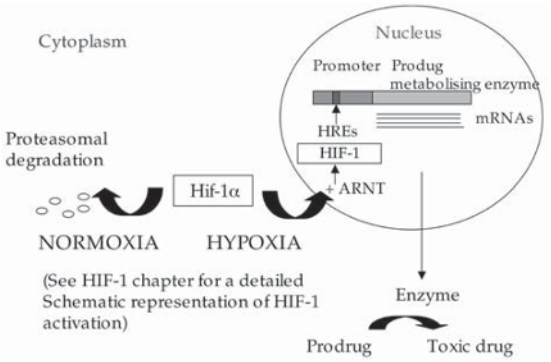

Fig. 3. How the microenvironment of solid tumors can be used in gene therapy. mRNA encoding an enzyme under the control of an HRE is transcribed in hypoxic cells, in a Hif-1α-dependent manner. When the tumor is subjected to a prodrug, these cells convert the drug to its toxic form and die. Normal cells, which do not express the enzyme, are not sensitive to the prodrug.

9. TARGETING HYPOXIC CELLS

While the above strategies deal with the problem of hypoxic cells in solid tumors, it has been proposed that tumor hypoxia may in fact be advantageous to cancer therapy. Hypoxia clearly distinguishes tumors from normal tissue, which is rarely subject to low oxygen concentrations. Toxic drugs could therefore be targeted to tumors, leading to a safer treatment with fewer side effects than conventional chemotherapy and radiotherapy. Additionally, hypoxia is a very powerful inducer of gene expression, and vectors containing HREs from the promoter regions of hypoxia-responsive genes such as VEGF *(64)* and Epo *(65)* can drive the HIF1-mediated transcription of transgenes within a vector. Our group has designed a vector in which the HRE from the mouse phosphoglycerate gene is used to drive expression of cytosine deaminase, a bacterial enzyme that converts the prodrug 5-fluorocytosine to 5-fluorocil (5-FU; 66). Tumor xenografts of cells transfected with this vector were more sensitive to treatment by the prodrug, compared to untransfected mammalian cells which do not express the enzyme (*66*; Fig. 3). Another group have used the HRE-containing promoter region of the VEGF gene to drive expression of the herpes simplex virus thymidine kinase gene selectively under hypoxic conditions. This gene sensitizes cells to ganciclovir, and accordingly, administration of glanciclovir to mice bearing tumors formed by cells containing this vector results in tumor regression *(67)*.

One advantage of this type of gene therapy is that it can be directed to endothelial cells, which are subject to acute periods of hypoxia in vivo *(68)*. Unlike gene

therapy directed at tumor cells, endothelial cell-specific gene therapy can be delivered systemically, and killing a small number of endothelial cells can result in the death of the large numbers of tumor cells that rely on them *(69)*.

Another method of targeting drugs to tumors is to exploit the natural hypoxic trophism of human macrophages. Our group, in collaboration with Griffiths et al., manipulated macrophages to contain human cytochrome P450, a prodrug converting enzyme, engineered to be under HRE control (*see* Fig. 3). We were able to deliver the macrophages to the hypoxic core of multicellular spheroids, which were then sensitized to the cytotoxic effects of cyclophosphamide *(70)*.

10. CONCLUSION

Many solid tumors are refractory to conventional anticancer therapies. A major part of this resistance is due to regions of low oxygen tension, which results in the presence of hypoxic, nonproliferating cells. Hypoxia is an abnormal physiology that exists in all solid tumors, regardless of their origin and location, and can be exploited as such to achieve very specific target delivery of anticancer drugs. Hypoxia also mediates the induction of factors that promote tumor angiogenesis and an aggressive tumor phenotype. Many of these factors are under the control of HIF-1, and one potential approach to overcome hypoxia-induced treatment resistance, therefore, is to inhibit the HIF-1α pathway or key downstream genes.

While strategies to exploit hypoxia as a therapeutical target for tumors are ongoing, it is also important to investigate the extent of hypoxia in individual tumors, so that the most appropriate treatment can be selected. Such studies involve correlating oxygen levels in tumors, either by biopsy or by scanning the whole tumor, with the response to anticancer drugs. The level of hypoxia in tumors could also give valuable information as to the likelihood of metastatic spread and the phenotype of the tumor. Clinical studies to this end, as well as hypoxia-based therapy strategies, are currently under investigation.

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16 Androgen Receptor and Estrogen Receptors

H.M. Oosterkamp and R. Bernards

Contents

1. GENERAL INTRODUCTION

The androgen receptor (AR) and the estrogen receptors (ER) are members of the nuclear receptor (NR) family. These NRs are distinguished from the other transcription factors by their ability to control gene expression upon ligand binding (steroids, retinoids, thyroid hormone, vitamin D, fatty acids, and other small hydrophobic molecules). Their combined effects are vast, influencing virtually every fundamental biological process, from development and homeostasis, to proliferation and differentiation.

All NRs display a modular structure, with five to six distinct regions, termed A–F *(1)*. (Fig. 1) The N-terminal A/B region contains the activation function (AF-1) that can activate transcription constitutively. Region C encompasses the DNA-binding domain (DBD). Region E consists of the ligand-binding domain (LBD), a dimerization surface, and the ligand-dependent transcriptional activation function 2 (AF-2).

The NR can be separated into two classes based on their association with other proteins in the absence of hormone *(2)*. Figure 2 shows the classic ligand-dependent activation of the steroid/thyroid hormone receptor family members that interact

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Fig. 1. Functional domains of nuclear receptors. A/B and F domains vary in size and primary sequence among the superfamily. CoR refers to corepressor binding site present in some nuclear receptors. AF-1 and AF-2 refer to two distinct activation functions.

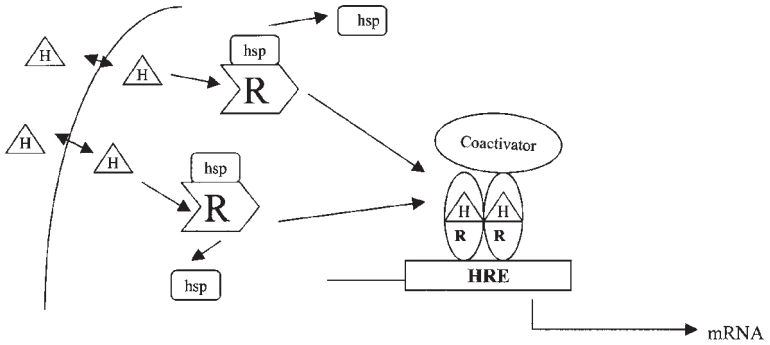

Fig. 2. Activation of steroid hormone receptors. In the absence of hormone, receptor monomers (R) are associated with heat shock proteins. Hormone (H) diffuses freely into the cell, binds to the receptor resulting in dissociation of the heatshock proteins, dimerization of the receptor and binding to target DNA sequences (HRE). Subsequently coactivators are recruited producing a transcriptionally active complex.

with heat shock proteins. Included in this group are the estrogen receptor (ER), progesterone receptor (PR), androgen receptor (AR), glucocorticoid receptor (GR), and mineralocorticoid receptor. In the absense of hormone, each receptor monomer is associated with a protein complex that contains heat shock protein 90 as well as a number of other proteins. This receptor complex is incapable of binding to DNA, and is either cytoplasmic or loosely bound in the nucleus. The steroid diffuses into the cell and binds to the ligand-binding domain of the receptor, inducing a conformational change that favors dissociation of the heat shock protein complex and tight binding to DNA. The receptors bind as homodimers or heterodimers to specific response elements that consist of inverted palindromes separated by three nucleotides *(3)*; they then interact with basal transcription factors, coactivators, and other transcription factors to induce and/or repress transcription of the target gene.

Receptors such as the thyroid hormone receptor, retinoic acid receptor, and vitamin D receptor do not interact with the heat shock proteins, and bind to DNA in the absence of ligand, repressing the transcription of the target gene. These receptors typically form heterodimers with retinoid X receptors. In the absence of ligand, these receptors interact with repressor proteins, blocking the activity of the dimer. Ligand binding induces dissociation of the corepressor, allowing interaction with other transcription factors and coactivators, which results in induction of target genes.

The activity of nuclear receptors is modulated by interactions with other proteins. Multiple coactivators have been identified that connect or bridge the DNA-bound NR to proteins in the preinitiation complex and thereby enhance transcription. Besides this bridging function, some coactivators can modify chromatin by histone acetylation and make promoters more accessible for the binding of other transcription factors. The opposites of coactivators are corepressors, which are recruited into the receptor-DNA-bound complex in the absence of ligand and actively inhibit transcription of the target gene through recruitment of histone deacetylases (HDACs) *(4)*.

It is becoming increasingly apparent that besides binding of their cognate ligands, non endocrine pathways, including those involving protein kinases and metabolic products, play a role in NR signaling. Whether a receptor can be activated in the absence of hormone appears to depend upon a number of factors, including the receptor type, the cell and promoter context, and the activation of the signalling-specific pathways.

2. THE ANDROGEN RECEPTOR

2.1. Introduction

Prostate cancer is the second leading cause of death among men in Western countries *(5,6)*. The prostate is an androgen-regulated organ. Prostate development from the urogenital sinus, as well as its growth, differentiation, and maintenance of function in adult life, depend on androgen activity *(7,8)*. The androgen testosterone (T) is mainly produced and secreted by Leydig cells in the testis and is converted into dihydrotestosterone (DHT) by the 5α-reductase enzymes *(9)*. The 5α-reductase enzyme type II is expressed in the male urogenital tract and is responsible for the conversion of testosterone to dihydrotestosterone in the prostate. DHT is the more potent androgen, with a higher binding affinity to the AR *(10)*. Besides the testis, the adrenals secrete large amounts of the inactive precursor steroids dehydroepiandrosterone (DHEA), its sulfate (DHEAS), and androstenedione. DHEA and androstenedione can be converted to T in most peripheral tissues, including the prostate.

As is the case with normal prostate development, prostate cancer is, at least initially, largely dependent on androgens for growth and survival. Most patients

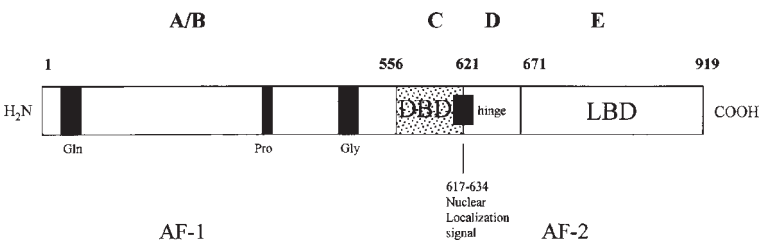

Fig. 3. Schematic illustration of the primary structure of the human androgen receptor. The letters A–E represent different functional domains. Within the N-terminal A/B region, variable glutamine(Gln) and glycine (Gly) are found.

respond favorably to androgen ablation therapy, which has become a standard treatment of metastatic disease. However, finally all patients with metastatic disease will relapse with clinically defined androgen-independent prostate cancer. In order to develop new therapies for this resistant disease, it is important to identify different molecular mechanisms that may be involved in prostate cancer development and progression. In this chapter, we will discuss the recent progress that have been made in the understanding of the mechanisms that play a role in androgen-(in)dependent prostate cancer.

2.2. Structure of the Androgen Receptor

The AR was cloned in 1988 *(6,11)*. The AR gene is located on the short arm of the X chromosome *(12)*. The entire gene encodes eight exons *(13)*. The large first exon encodes the entire N-terminal domain. Three repeat sequences exist in this first exon: a CAG (glutamine) repeat, a GGN (glycine) repeat, and a GCA (proline) repeat (Fig. 3). The CAG repeats are polymorphic in length, varying from 11 to 31 repeats in normal individuals. It is suspected that abnormal lengths of this polymorphic region may be associated with prostate cancer *(14)*. Because of the above mentioned variable repeats, different lengths of the AR have been reported (910, 917, 918, and 919 residues) with molecular weights between 100 and 110 kDa *(15)*. A schematic representation of the primary structure of the AR is given in Fig. 3. The AR has two major transactivation domains *(6)*: AF-1 in the A/B region of the N-terminal domain *(16,17)* and AF-2 in the C-terminal ligand-binding domain (LBD) *(18)*. The DNA-binding domain referred to as the C region consists of 68 amino acids that fold into two zinc fingers involved in the recognition of androgen response elements (AREs) on androgen-regulated genes. At the distal end of the C region and within the hinge region (D region), there is a bipartite nuclear targeting sequence (amino acids 617–633) responsible for androgen-regulated nuclear import of the androgen receptor *(19)*.

2.3. Androgen Receptor Function

2.3.1. Activation by its Natural Ligand and Role of Steroid Receptor Coactivators

In its inactive state, the unliganded AR is associated with heat shock proteins and is unable to perform its transactivating function. This inactive complex can be located both in the cytoplasm and nucleus. Testosterone or DHT binding to the AR induces an allosteric conformational change of the protein that results in hormone-dependent, DNA-independent phosphorylation of the receptor at several sites and dissociation from heat shock proteins. This process allows the AR to dimerize, which is essential for binding to AREs in the promoter of target genes *(1,20)*.The DNA-bound AR next recruits coactivators resulting in stable assembly of the preinitiation complex and transcription initiation by RNA polymerase II *(4,21)*. Several AR coactivators, such as ARA70 *(22)*, ARA55 *(23)*, ARA54 *(24)*, ARA160 *(25)*, and Rb *(26)*, have been isolated and characterized. Besides, CBP [CREB (cAMP response element)-binding protein] was revealed as a coactivator for the AR. Upon ligand binding, the AR can recruit CBP to communicate with the transcription initiation complex and activate transcription. CBP also plays a role in the cross-talk between AR and AP-1, because its recruitment by AR titrates CBP away from AP-1 resulting in inhibition of AP-1 transactivation *(27–29)*.

2.3.2. Androgen-Regulated Genes

Various genes have been identified that are regulated by androgens. These include the prostate-specific antigen (*PSA*) gene, which encodes a prostate-specific protease, now widely used as a tumor marker for prostate cancer. *PSA* contains an upstream ARE; thus it is believed that androgens directly regulate its transcription rate. Human glandular kallikrein (hK2) is another androgen-regulated protein. It is primarily expressed in the prostate and also contains an ARE. Furthermore, the AR induces stimulation of epidermal growth factor receptor (EGFR), keratinocyte growth factor (KGF), CDK2, CDK4, p21, and vascular endothelial growth factor (VEGF), and it represses transforming growth factor β (TGF-β), p16/INK4A, and bcl-2 *(30)*.

2.3.3. Ligand Independent Activation of AR

Aberrant activation of the AR may be one of the mechanisms that contributes to progression of prostatic carcinoma to an androgen-independent stage. Similarly to other steroid receptors, the AR is involved in cross-talk with the signaling pathways mediated by protein kinases. In transient transfection assays, insulin-like growth factor-I (IGF-I), KGF, and EGF activate the AR to different extents in the absence of androgen *(31)*. This activitiy can be blocked by antiandrogens. Ligand-independent activation of the AR was also reported for substances that directly activate the protein kinase A and C signaling pathways *(32–36)*. All these

substances were able to potentiate the effects of low concentrations of androgen, thus reducing a concentration of steroid needed for maximal activation of the AR. This reduction may be particularly important in patients with advanced prostate cancer in which serum levels of androgen are continuously suppressed. However, all of the above mentioned studies are performed in cell cultures, and the outcome of this nonsteroidal activation of the AR depends on a cellular and promoter context. Mechanisms responsible for AR activation by protein kinase activators are only partly understood. Altered phosphorylation, enhanced nuclear localization, or increased DNA binding of the AR by protein kinase activators may all play a role.

Two groups demonstrated a role for HER-2 in the development of hormone-independent prostate cancer. Craft et al. *(5)* showed that androgen-independent sublines of human prostate cancer xenografts expressed higher levels of HER-2 receptor tyrosine kinase than did androgen-dependent sublines. Additionally, overexpression of HER-2 in a LNCaP prostate cancer cell line caused the cell line to become androgen independent. Furthermore, it was demonstrated that overexpression of HER-2 increased the expression of PSA, especially at low androgen levels, and activation of PSA transcription by HER-2 was shown to require functional AR. Chang's group *(37)* found that HER-2 activates AR through MAP kinase. The clinical implication of this HER-2 → MAP kinase → AR → PSA pathway remains to be demonstrated. There are conflicting data about the overexpression of HER-2 in different stages of prostate cancer *(38*, and references therein). Signoretti et al. *(39)* analyzed HER-2 at DNA, RNA, and protein levels in prostate tumors representing different clinical stages. They found increasing mRNA and protein HER-2 expression levels with progression to androgen independence. However, unlike what is seen in breast cancer, in prostate tumors no concordance between *HER-2* gene amplification and overexpression was seen. Trastuzumab (Herceptin), a monoclonal antibody against HER-2, has been shown to prolong survival in advanced, refractory, HER-2-positive breast cancer *(40)*. Whether there is a role for trastuzumab in treating androgen-independent HER-2-positive prostate cancer is under current study *(41)*. Other investigational therapies against the HER-2 signaling cascade are ansamycins, which produce a rapid reduction in the level of HER-2 expression in cell lines that overexpress HER-2 *(42)*, antisense approaches *(43)*, and novel antibodies directed towards the dimerization of HER-2 *(44)*.

Knudsen et al. *(45)* have shown that cyclin D1, which was found to induce ER activity, can also complex with AR, but instead inhibits its transcriptional activity.

An Italian group reported recently that prostate cancer cell proliferation can be triggered by steroid-induced formation of a ternary complex constituted of the AR, ERβ, and the tyrosine kinase Src, leading to activation of the Src/Raf-1/Erks signal-transducing pathway *(46)*.

2.3.4. Androgen Resistance

Most androgen-independent prostate tumors continue to express AR as well as the androgen-dependent gene *PSA*, which indicates that these cells maintain a functional AR signaling pathway despite very low levels of testosterone. In the literature, four possible mechanisms that can lead to the emergence of androgen-independent prostate cancer are suggested: (1) 30% of androgen-independent prostate carcinomas show amplification and overexpression of the wild-type AR gene *(47)*. (2) AR gene mutations can lead to altered hormone specificity of the AR. The first mutation reported to lead to androgen-independence was a missense mutation that caused a substitution of alanine for threonine at amino acid 877 (T877A) in the ligand-binding domain of the AR *(48)*. The T877A mutation expands the ligand specificity of the androgen receptor allowing it to bind estrogens, progestagens, and adrenal androgens, as well as many antiandrogens. This mutation is frequently found in cases of prostate cancer resistant to endocrine therapy*(49,50)*. Taplin at al. found that mutated AR (His874Tyr or Thr877Ser) from two patients with metastatic androgen-independent prostate cancer could be stimulated by estrogen and progesterone *(51)*.The total number of reported AR mutations is increasing *(52)*. Recently, Zhao et al. discovered that the L701H mutation in the ligand-binding domain of the AR in the prostate cancer cell line MDA PCa 2b (derived from a bone metastasis of a hormonal-therapy-resistant prostate cancer patient) makes the AR highly sensitive to cortisol and cortisone, but less sensitive to androgen stimulation *(53)*. Little is known about the frequency of this L701H mutation in prostate cancer. (3) One could speculate that altered function of AR coactivators and corepressors may play a role in the emergence of androgen-independent prostate cancer *(30,54)*. (4) In addition, as already mentioned in the previous chapter, mitogenic signaling pathways, such as those activated by HER-2 overexpression, might lead to androgen independence. Further understanding how AR is activated at low androgen levels will be important for the development of new therapies to treat this otherwise incurable disease.

3. THE ESTROGEN RECEPTORS

3.1. Introduction

Breast cancer is the most common cancer in women in the Western world. Approximately 60–70% of all breast cancers are estrogen receptor (ER) positive *(55)*.Only about half of ER-positive patients will respond to the various hormonal therapies available. Of those who do initially respond, most will eventually develop hormonally unresponsive disease following a period of treatment, even though ER is often still present. Since estrogens and ER play a pivotal role in the development and progression of breast cancer as well as the treatment and outcome of

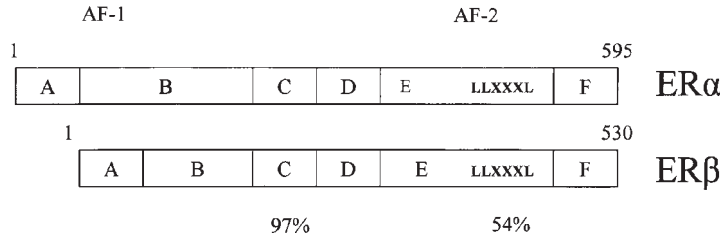

Fig. 4. Structure and functional domains of ERα and ERβ. **AB**: transcription activation; **C**: DNA binding and receptor dimerization; **D**: nuclear localization signal; **E**: ligand binding domain, coactivator binding, transcription activation, receptor dimerization; **F**: contributes to transactivation capacity, but function to a large extent unknown. The overall sequence identity between ERα and ERβ is 47%. There is little or no homology between their AF-1 domains. The DNA- and ligand-binding domains are well conserved.

breast cancer patients, mechanisms underlying regulation of ER gene expression and function are key areas of study.

3.2. Structure of the Estrogen Receptors

The ER gene was initially cloned from the ER-positive human breast cancer cell line MCF-7 in 1986 *(56,57)*. It is located on chromosome 6q25.1 and consists of eight exons *(58)*. The structure of the ER, like the other nuclear receptors, can be divided into six conserved, functional domains, A–F *(59)* (Fig. 4). The A/B domain of the ER protein contains a constitutive, estrogen-independent transcriptional activation function, AF-1 *(60)*. Deletion mapping and mutagenesis of human ERα revealed that phosphorylation at Ser^{118} is required for full AF-1 activity *(61)*. The C-domain possesses two zinc-finger DNA-binding motifs essential for binding to estrogen responsive elements (ERE) in the promoter regions of target genes *(59,62)*. The D or hinge region may be involved in estrogen-mediated transcriptional repression *(63)*, while the E domain contains the hormone binding site, the region required for stable dimerization of the receptor, and a second estrogen-inducible transcriptional activation function, AF-2 *(60)*. The function of the F-domain is presently unclear, but it may interact with cell-type-specific factors that regulate ER function *(64)*. AF-1 and AF-2 can act independently and synergistically to enhance transcription, and their activities have been shown to be influenced by promoter context and cell type *(60,65)*. Both AF-1 and AF-2 are presumed to interact with a complex array of coregulator proteins that mediate the interactions between receptors and the basal transcription machinery and remodeling of chromatin structure (*see* below).

It was previously assumed that ER was indispensable for maintenance of life, since no cases had been reported of humans or animals with an inactivated or

deleted receptor. However, in 1994 a case report was published describing a man with estrogen resistance *(66)*. This person lacks a functional ER due to a cytosine to thymine transition at codon 157 of both alleles, resulting in a premature stop codon. The patient was tall (204 cm [80.3 in.]) and had incomplete epiphyseal closure, with a history of continued linear growth into adulthood despite otherwise normal pubertal development. He also had severe osteoporosis and reduced fertility. This case demonstrated that deletion of ER is not lethal, and that a functional ER is necessary for bone maturation and mineralization in both women and men. Furthermore, in 1993, development of an ER knock-out mouse strain was reported *(67)*. In this mouse strain, specific estrogen binding could still be observed in some tissues, suggesting the existence of a second ER.

This second ER termed ERβ *(68,69)*, was discovered in 1996 with the previously defined ER now referred to as ERα. ERβ was initially cloned from rat prostate *(68)*, and the human clone was retrieved from testis *(69)*. ERβ is located on chromosome 14q22-24 *(70)*. ERβ is somewhat shorter than ERα, consisting of 530 amino acids *(71)* (*see* Fig. 4). Human ERα and ERβ share modest overall sequence identity (47%) *(69)*. The region of highest homology is in the DNA-binding domain (95%). The hormone-binding domain is also relatively well conserved (58% identity). The A/B domains are poorly conserved (only ~20%), suggesting that their AF-1 activities might be different, and possibly, that different coactivators interact with this region (*see* below).

3.3. Estrogen Receptor Function

3.3.1. Knock-out Studies and Tissue Distribution

Examination of the tissue distribution of ERα and ERβ and the results of knock-out studies suggest that the two receptors may have both distinct and redundant functions. Using commercial polyclonal antisera against peptides specific to human ERβ, Taylor et al. *(72)* have determined the sites of ERβ expression in archival and formalin-fixed human tissue and compared its expression to that of ERα. ERβ was localized to the cell nuclei of a wide range of normal adult human tissues including breast, ovary, Fallopian tube, uterus, lung, kidney, brain, heart, prostate, and testis. ERβ expression does not appear to be linked to ERα expression, raising the possibility that there are distinct ERα- and ERβ-dependent pathways. In the endometrium, both ERα and ERβ were observed in luminal epithelial cells and in the nuclei of stromal cells, but, significantly, ERβ was weak or absent from endometrial glandular epithelia. The prostate lacks ERα staining, but is immunopositive for ERβ. Increased ERβ immunoreactivity was noted in the glands of normal resting breast when compared with the glands of proliferating breast. In adult human bone, ERβ protein is expressed in cells of osteoblast lineage and in osteoclasts. In developing human bone ERα is predominantly expressed in cortical bone, whereas ERβ shows higher levels of expression in cancellous bone *(73)*.

Studies in mice lacking ERα, or ERβ, or both receptors reveal the distinct roles of each receptor in mammary gland development and reproduction. ERα knock-out mice (αERKO) show absence of breast development in females and infertility caused by reproductive tract and gonadal and behavioral abnormalities in both sexes *(67)*. In ERβ knock-out mice sexually mature females are fertile and exhibit normal sexual behavior, but have fewer and smaller litters than wild-type mice. Superovulation experiments indicate that this reduction in fertility is the result of reduced ovarian efficiency. The mutant females have normal breast development and lactate normally. Young, sexually mature male mice show no overt abnormalities and reproduce normally. Older mutant males display signs of prostate and bladder hyperplasia *(74)*. To further clarify the roles of each receptor in the physiology of estrogen target tissues, mice lacking both ERα and ERβ were generated *(75)*. Both sexes of αβ estrogen receptor knock-out (αβ ERKO) mutants exhibit normal reproductive tract development, but are infertile. Ovaries of adult αβ ERKO females exhibit follicle transdifferentiation to structures resembling seminiferous tubules of the testis, including Sertoli-like cells and expression of Müllerian-inhibiting substance, sulfated glycoprotein-2, and Sox9. Therefore, loss of both receptors leads to an ovarian phenotype that is distinct from that of the individual ERKO mutants, which indicates that both receptors are required for the maintenance of germ and somatic cells in the postnatal ovary.

We will discuss the possible role of ERβ in the development of breast cancer and the resistance to hormone therapy later.

3.3.2. Activation by its Natural Ligand and Role of Steroid Receptor Coactivators

In the absence of ligand, ERα is predominantly located in the nucleus *(76–78)* as part of a complex with heat shock proteins. Ligand binding to ER displaces the heat shock proteins and allows phosphorylation of the receptor at serine residues within the AF-1 domain *(61,79)*. This ligand binding also causes a conformational change in the protein accommodating a productive interaction and transcriptional synergism between AF-1 and AF-2 *(80–82)*. These changes allow for receptor dimerization, and two ERα molecules complexed with hormone bind specifically to a consensus 13-bp palindromic estrogen responsive element (ERE) located upstream of target genes *(1,62,83)*. (Formation of ERα and ERβ heterodimers has been demonstrated both in vitro and in vivo *(71,84)*, but the physiological role of the heterodimer is unclear.) By binding to DNA, the transcription activation function AF-2 of ERα is activated, the basal transcription machinery is contacted directly, and other nucleoproteins are recruited. These nucleoproteins interact with the ERα protein dimer to modify the expression of the estrogen-responsive genes (reviewed in Horwitz et al. *(85)*, Glass et al. *(86)*, and McKenna et al. *(87–89)*. Some of these receptor-interacting proteins function as transcriptional coativators, such as the p160 coactivators SRC-1/N-CoA1 *(90,91)*, TIF-2/GRIP-1 *(92,93)*,

and AIB1/RAC3/ACTR/P/CIP *(94–96,97)*. A distinctive structural feature of these p160 coactivators is the presence of multiple LXXLL signature motifs, which comprise determinants for direct interactions with the nuclear-receptor ligand-binding domain. The p160 coactivators modify local chromatin structure through histone acetylation, thereby facilitating RNA pol II recruitment, and are thought to recruit additional transcriptional cofactors, P/CAF and p300/CBP *(91,98,99)*. P/CAF and p300/CBP make independent contacts with the nuclear receptor, as well as with one another *(97,100)*. In addition, P/CAF and p300/CBP have HAT activity *(101–103)*. A surprising deviation from the p160/CBP paradigm, and perhaps from our traditional view of transactivation in general, is the identification of an RNA that acts as a coactivator for steroid receptors. This SRA (steroid-receptor RNA activator) is present in a SRC-1 complex *(104)*. DiRenzo et al. *(105)* demonstrated that ATP-dependent chromatin remodeling plays a role in the regulation of ER signaling. It was shown that transcriptional activation by ERα requires functional BRG-1, and that the coactivation of estrogen signaling by either SRC-1 or CBP is BRG-1 dependent. In addition, a distinct multiprotein complex, called DRIP or TRAP, first found to be involved in thyroid hormone receptor *(106)* and vitamin D receptor signalling *(107)*, has also been implicated recently in ERα and ERβ action through a ligand dependent interaction with its PBP/TRAP220/DRIP205 subunit and the AF-2 domain *(108)*.

Other nucleoproteins can function as corepressors *(87,89)*. Recently, it was shown that tamoxifen can recruit the corepressors N-CoR and SMRT to the promoter of the ER target genes cathepsin D and pS2 *(109)*.

3.3.3. ERα and ERβ Mediated Transcription via AP-1

Besides this classical mechanism of direct DNA binding, the two ER subtypes can also regulate transcription via an activator protein (AP)1 response element *(110)*. AP-1 response elements are regulated indirectly through interactions between ER and the AP-1 transcription factors c-Fos and c-Jun. These transcription factors regulate genes involved in many cellular processes, including proliferation, differentiation, cell motility, and apoptosis. Thus, the ER–AP-1 interaction could be important clinically. When signaling is mediated via AP1, ERα and ERβ signal in opposite ways *(111)*. When bound to ERα, estradiol activates transcription, whereas with ERβ transcription is inhibited. However, antiestrogens bound to ERβ are potent transcriptional activators at an AP1 site, acting as estrogen agonists rather than antagonists. This finding could have important implications for the differential effects of selective estrogen receptor modulators (SERMS) and antiestrogen resistance.

3.3.4. ER Target Genes

Knowledge of which genes are actually regulated by ERα is of interest, since the expression of ERα in breast cancer is an important predictor of response to

hormone treatment. ERα regulated genes that have lost this regulation during malignant transformation could potentially cause resistance to hormone therapy. Thus far, reports have been published on seven genes of which expression has been found to be regulated by ERα. The genes are the progesterone receptor *(112)*, *cathepsin D* (*CATD*) *(113)*, *pS2 (114,115)*, *c-Myc (116)*, *TGFα (117)*, *c-fos (110, 118)*, and *VEGF (119)*.

3.4. Crystal Structure

ER undergoes extensive conformational changes after ligand binding as revealed by recent crystal structures of ERα bound to various ligands *(120,121)*. The ER ligand binding EF domain (LBD) has been shown to be composed of 12 α-helices, forming a pocket to capture the ligand. Ligand binding only causes a shift in helix 12 at the C-terminal LBD without affecting the other regions. The conformation of the ERα LBD is determined by the nature of the particular ligand that is bound. In the estrogen or DES liganded complex, helix 12 containing the AF2 core is repositioned as a "lid" over the LBD cavity in a way that p160 coactivators can accommodate within a hydrophobic cleft of the LBD. This repositioning occurs through direct contacts with the LXXLL motif. Estrogen antagonists such as tamoxifen and raloxifene appear to alter the position of the AF2 core such that helix 12 itself occupies the hydrophobic cleft in the LBD, thereby precluding coactivator binding. In 1999, the crystal structure of the LBD of ERβ in the presence of the phytoestrogen genistein and the antagonist raloxifene was resolved showing similarity with the ERα-LBD three-dimensional structure *(122)*.

3.5. Ligand-Independent Activation of ER

Cross-talk with other growth-factor signaling pathways represents another way in which ER can affect important cellular processes. Phosphorylation at Ser^{118} of ERα is required for full AF-1 activity *(61)*. This residue is a direct substrate for mitogen-activated protein kinase (MAPK), providing a link between ER action and the Ras–MAPK signaling cascade *(79)*. EGF *(123)*, insulin or coexpression of Ras can activate this MAPK pathway leading to phosphorylation at Ser^{118}. Tremblay et al. recently showed that phosphorylation of Ser^{106} and Ser^{124} in AF-1 of ERβ by MAPK resulted in increased interaction of ERβ with SRC-1 *(124)*. There is also considerable cross-talk between ERα and insulin-like growth factor (IGF) signal transduction pathways. ER functions to increase levels of several of the key IGF signaling molecules, and IGFs, in turn, may activate ER *(125,126)*. Additionally, ERα is a target for tyrosine phosphorylation. Activation of the HER-2 receptor in breast cancer cells by the peptide growth factor heregulin leads to direct and rapid phosphorylation of ER on tyrosine residues. This phosphorylation is followed by interaction between ER and estrogen-response elements in the nucleus, and production of an estrogen-induced protein, progesterone receptor *(127)*. A single tyrosine residue located immediately adjacent to the AF-2

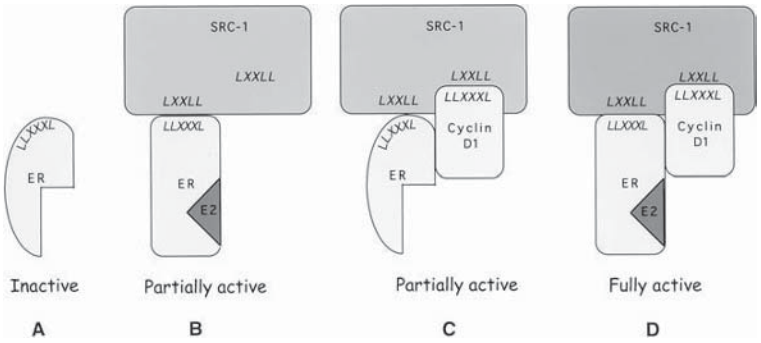

Fig. 5. Multiple ways to activate the estrogen receptor. In the absence of ligand, ER is unable to interact with steroid receptor coactivators (SRCs) directly as its leucine-rich coactivator interaction motif (AF-2), indicated as LLXXXL) is sterically unavailable for SRC interaction (**A**). Hormone binding by ER exposes AF-2 and allows recruitment of SRCs to ER, leading to activation of ER (**B**). Hormone-independent binding of cyclin D1 to ER provides a single leucine-rich interaction motif for SRCs on the cyclin D1/ER complex, which is present in the carboxyl terminus of cyclin D1. This results in partial activation of ER (**C**). Ligand binding of ER in the presence of cyclin D1 provides two independent interaction surfaces for SRCs: one is formed by the leucine-rich motif in AF-2 of ER and a second in the carboxyl terminus of cyclin D1 (**D**). The observed synergism between estrogen and cyclin D1 in ER activation may result from their cooperative recruitment of SRCs to the ER. The protein interaction motifs are shown in italics (L = leucine, X = any amino acid).

has been identified as a substrate for the src-family tyrosine kinases *(128)*. This cross-talk between signaling pathways could conceivably contribute to the development of estrogen independence and/or clinical resistance to hormone therapy.

Cyclin D1 can activate ERα in a ligand-independent and CDK-independent fashion *(129,130)*. By acting as a bridging factor between ERα and SRCs, cyclin D1 can recruit SRC-family coactivators to ERα in the absence of ligand, resulting in ligand-independent transcription *(131)* (Fig. 5). Cyclin D1 can also interact with P/CAF, facilitating the formation of a ternary complex in which P/CAF associates with ERα, leading to transcriptional activation *(132)*. Recently, it was reported that the functional interaction between cyclin D1 and ERα is regulated by a signal transduction pathway involving the second messenger, cyclic AMP *(133)*. The cyclin D1 gene is amplified in approx 20% of breast cancers, and the protein is overexpressed in 50% of cases *(134)*. Remarkably, cyclin D1 is overexpressed preferentially in ERα-positive breast cancers. Because ERα overexpressing breast cancers often occur in postmenopausal women–who have low levels of circulating estrogens–it is possible that the frequently overexpressed cyclin D1 in these tumors may, at least in part, be responsible for stimulating ER activity.

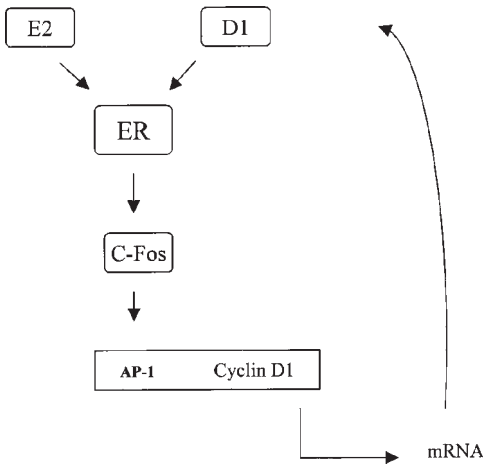

Fig. 6. Positive stimulatory loop of ER and cyclin D1. Estrogens bind to ER and stimulate the expression of cyclin D1 via AP-1. The increased cyclin D1 level leads to both CDK-dependent and CDK-independent cell cycle progression. The latter being the result of enhanced ER transcriptional activation mediated by cyclin D1.

The precise molecular mechanism by which estrogen and ER control cyclin D1 expression is at present poorly defined. Cyclin D1 does not represent a classical ER target gene, because the cyclin D1 promoter lacks an ERE. Altucci et al. *(135)* mapped the estrogen responsive region to a fragment between –944 and –136 of the cyclin D1 promoter. This region contains an AP-1 site, suggesting that estrogens can modulate cyclin D1 expression via AP-1. Combining the above-mentioned observations, we assume that ERα and cyclin D1 form a positive stimulatory loop in which estrogens stimulate the expression of cyclin D1 via AP-1, leading to both CDK-dependent and CDK-independent cell cycle progression. The latter being the result of enhanced ER transcriptional activation mediated by cyclin D1 (Fig. 6). Whether cyclin D1 has a role as a prognostic or predictive marker in breast cancer is still uncertain *(134)*.

In transient transfection assays, *BRCA1* was found to inhibit signaling by the ligand-activated ER-α through the estrogen-responsive enhancer element and to block the transcriptional activation function AF-2 of ERα. These results raise the possibility that wild-type *BRCA1* suppresses estrogen-dependent transcriptional pathways related to mammary epithelial cell proliferation, and that loss of this ability contributes to tumorigenesis *(136)*. However, the fact that *BRCA1*$^{-/-}$ tumors are mostly ERα negative argues against this model *(137–140)*.

3.3.5. Regulation by Antiestrogens

Inhibition of ER activity in cancer cells by antiestrogens such as 4-hydroxytamoxifen (OHT) has led to their use as therapeutic agents for the treatment of breast cancer. However, the mixed agonist/antagonist OHT inhibited only AF-2 function *(141)*. In fact, OHT functions as an agonist in uterine tissue and activates ERα in a cell-type and promoter-specific manner. The agonist activity of OHT at ERα requires an intact AF-1 domain *(142)*, and OHT cannot block the activation of ERα via the MAPK pathway *(79,123)*. The partial agonistic effect of OHT at ERα on a basal promoter linked to an ERE, was not seen in this setting with ERβ *(143,144)*.

Unfortunately, all patients eventually relapse on tamoxifen treatment. The mechanisms that lead to tamoxifen resistance are not completely understood, as a variety of mechanisms have been proposed but only limited evidence exists to substantiate them *(145)*. Mechanisms that can potentially give tamoxifen resistance in ERα positive tumors are altered metabolism of OHT; decreased intracellular drug concentrations; enhanced biological mechanisms for circumvention of tamoxifen cytotoxicity; stimulation of ERα positive cells by the agonistic component of OHT; altered levels of ERα in the tumor; variant or mutant ERs; and changes in expression of ER regulated genes.

In an effort to develop new and more effective antiestrogens, a new, pure antiestrogen was synthesized. ICI 182,780 (Faslodex) demonstrates a pure anti-estrogenic profile on all genes and in all tissues studied to date *(146)*. ICI 182,780 is also devoid of agonist activity in animal models and clinical trials, inhibiting growth of the breast and endometrium. In animal models, it does not cross the blood–brain barrier and appears to be neutral with respect to lipids and bone. ICI 182,780 down-regulates the estrogen receptor and is active in tamoxifen-resistant breast carcinoma. In a small, Phase II study, durable responses were seen. Phase III clinical trials are in progress comparing ICI 182,780 with anastrozole and tamoxifen in the treatment of patients with advanced breast carcinoma *(147)*.

3.3.6. The Role of ERα and ERβ in the Development of Breast Cancer

The number of normal breast epithelial cells expressing ERα is quite small, with estimates of between 7% and 17% ERα-positive ductal epithelial cells being reported in normal human breast tissue *(148,149)*. However, 60–80% of all human primary breast tumors express significant amounts of ERα *(150)*. This suggests that the expression of ERα signaling pathways is a selective advantage for breast cancer development.

The role of ERβ in carcinogenesis and progression of breast cancer is far from clear. Recent data considering the role of ERβ in the development of breast cancer show controversial results, probably due to small population samples, different techniques, and cut off points used. In Table 1, an overview of the results obtained in the different publications is given. Four studies *(151–154)* suggest that upon

Table 1

Author	*No pts*	*Technique*	*Tissue*	*Outcome/comment*
Leygue et al. *(151)*	18	RT-PCR	normal breast vs primary breast tumor same patient	ERα/ERβ in tumor greater than in normal tissue $p < 0.02$
Speirs et al. *(152)*	83	RT-PCR	normal breast tissue (n = 23) vs malignant breast tissue ($n = 60$)	In normal breast tissue ERβ predominates. Coexpressed ERα and ERβ associates with positive lymph nodes and higher tumor grade
Speirs et al. *(163)*	17	RT-PCR	primary breast tumor n = 8 tamoxifen S n = 9 tamoxifen R	ERβ ↑ in tamoxifen R. Comment: tam R group contains more pts with positive lymph nodes and high grade tumors
Jävinen et al. *(155)*	92	immunohistochemistry, confirmation by mRNA in situ hybridization. cut off ≥ 20% cells +	primary breast tumor	55/92 (59.8%) ERβ+. ERβ+ was statistically significant associated with: PR +, ERα +, lymph node –, slow proliferation, premeno pauzal state
Iwao et al. *(164)*	116	real-time PCR	primary breast tumor	ERβ↑ in ERα – and PR – tumors
Iwao et al. *(153)*	123	real-time PCR	normal breast tissue (n = 11) vs malignant breast tissue (n = 112)	ERβ↓ and ERα↑ during carcinogenesis of breast cancer
Mann et al. *(157)*	47	immunohistochemistry Cut off ≥ 10% cells +	primary breast tumors	33/47 (70%) ERβ+ 30/47 (64%) ERα+. How many for both receptors positive?
	118	same as above	primary breast tumors adjuvant treatment with tamoxifen	78/118 (66%) ERβ+ ERα+ correlates with better survival $p = 0.0077$
Roger et al. *(154)*	130	immunohistochemistry	71 BBD 59 CIS vs 118 normal breast tissue	ERβ↑ normal breast nonproliferating BBD ERβ↓ proliferating BBD and CIS
Omoto et al. *(156)*	88	immunohistochemistry	primary breast tumors	52/88 (59.1%) ERβ+. No correlation with node status, grade. Significant correlation with ERα+

BBD, benign breast disease; CIS, carcinoma *in situ*.

transition from normal to cancerous tissue, the expression of ERβ decreases together with an increase of the ERα expression. This marked and early decreased level of ERβ protein expression suggests a protective effect of ERβ against the mitogenic activity of estrogens in mammary premalignant lesions. Two other groups, however, reported exactly the opposite, showing a correlation between ERα and ERβ expression in breast cancer specimens *(155,156)*. One group claims that it has found a correlation between ERβ positivity of a tumor and better survival after adjuvant tamoxifen treatment *(157)*. However, these authors did not report on how many of the ERβ positive cases were also positive for ERα. The possible survival benefit could therefore be the result of unreported ERα positivity.

Using the current assay for ER status, which uses immunohistochemistry with ERα specific antibodies, a significant number of women with invasive breast cancer whose tumors are ERβ positive, but ERα negative, will be determined to have a negative ER status and may not receive adjuvant hormonal therapy. Although there are no studies to date showing that ERα negative but ERβ positive tumors respond to treatment with antiestrogens, this could be a possibility, based on studies in cell lines.

In summary, we can conclude that although one has the expectation based on preclinical data that there is a different role for each estrogen receptor during breast development and breast carcinogenesis, clinical studies performed so far are unable to prove this.

4. CONCLUDING REMARKS

ERα and its regulated genes play an important role in the development and progression of breast cancer. Overexpression of ERα is of important predictive value for the response to tamoxifen treatment *(158)*. The role of ERβ in the development of breast cancer is not clear yet. Unfortunately, not all ERα positive tumors respond to hormone therapy, and eventually, all initial responders will relapse. In order to optimize the treatment for breast cancer patients, we need better tools to predict the outcome of therapy. One step forward is the FASAY in which functionality of the ER is tested *(159)*. The recently developed DNA microarray technique will further clarify the pathways involved in breast cancer develoment, and will help us to identify expression profiles, predicting response to (hormone) therapy *(160–162)*. These new expression profiles probably will completely replace our current classification system of breast tumors.

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CANCER DRUG DISCOVERY AND DEVELOPMENT

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Targets for Cancer Chemotherapy

Transcription Factors and Other Nuclear Proteins

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Recent scientific discoveries concerning therapeutically relevant protein targets that contribute to cancer pathology have opened new possibilities in the search for effective cancer treatments. In *Targets for Cancer Chemotherapy: Transcription Factors and Other Nuclear Proteins,* a panel of leading basic researchers, pharmaceutical scientists, and clinical oncologists provide, in an easy-to-understand format, a detailed account of these latest research developments and spell out their implications for cancer drug discovery and clinical application. The authors identify and illuminate selected transcription factor oncoproteins and tumor suppressors, together with nuclear proteins that are central to the phenotype of the tumor cell and including recently elucidated enzymes involved with chromatin control. The emphasis is on new targets and approaches to cancer treatment derived from the cancer cell cycle, gene and chromatin control targets, and angiogenesis. Among the targets considered are E2F, Myc oncoproteins, hypoxic signaling pathways, Mdm2 and ARF, the AP-1 family of transcription factors, histone deacetylase, histone acetyl transferase, apoptin, and nuclear -catenin signaling.

Up-to-date and insightful, *Targets for Cancer Chemotherapy: Transcription Factors and Other Nuclear Proteins* shows clearly how our new understanding of critical molecular targets in cancer cells can be applied to cancer drug discovery to provide new drug discovery platforms both for future development and for new target-based medicines for cancer patients.

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- **Easy-to-understand digest of recent developments and their relevance to cancer patients**

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