CMLS, Cell. Mol. Life Sci. 58 (2001) 1907–1922
1420-682X/01/131907-16 $ 1.50 + 0.20/0
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CMLS Cellular and Molecular Life Sciences

Contributions in the domain of cancer research: Review

Negative regulators of cyclin-dependent kinases and their roles in cancers

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Received 25 May 2001; received after revision 29 June 2001; accepted 3 July 2001

Abstract. In the past decade, the discovery and characterization of cyclin-dependent kinases (CDKs), the engine cores of the cell cycle machinery, have advanced our understanding of the cell cycle. Both positive and negative regulators of CDKs have been characterized, accelerating the important research to unravel the mechanisms of the cell cycle disease – cancer. Cancer can originate from overexpression of positive regulators, such as cyclins, or from underexpression of negative regulators, such as CDK inhibitors (CKIs). CKIs are the focus of much cancer research because they are capable of controlling cell cycle proliferation – the Holy Grail for cancer treatment. CDKs can be inactivated by several mechanisms:, (i) by association with CKIs including p16 (INK4a), p15 (INK4b), p21 (Cip1), p27 (Kip1), and p57 (Kip2), (ii) by disassociation from their cyclin regulatory unit, (iii) by dephosphorylation of a conserved threonine residue in the T-loop, and (iv) by adding inhibitory phosphate. Here we discuss what is known about each mechanism with a hope that these insights will become useful in developing strategies to eliminate cancer in the future.

Key words. Cell cycle; INK4; CIP; KIP; 14-3-3; HER2; tumor suppressor; CDK.

Introduction

A major challenge faced by many cancer researchers is determining how oncogenic signals accumulate genetic changes during the evolution of cancer from normal cells. Cancer is indeed a proliferative disease of the cell cycle. In the past decade, advances in understanding the cell cycle regulatory machinery have demonstrated that cell cycle deregulation is one of the most frequent alterations during tumor development. Cyclin-dependent kinases (CDKs) and their cyclin partners are positive regulators, or accelerators, that induce cell cycle progression, whereas important negative regulators, such as cyclin-dependent kinase inhibitors (CKIs), act as brakes to stop cell cycle progression in response to regulatory signals [1]. For cells to grow normally, the positive and negative regulators of the cell cycle machinery must be very carefully controlled. Any deregulation of this machinery will result in abnormal cell growth. Therefore, the cell cycle machinery is like a complicated car system, containing accelerators and brakes to control the CDK engine. CDKs may be the most highly regulated enzymes characterized, and multiple mechanisms exist to regulate their activity. CDK activity is positively regulated by association with cyclins, by phosphorylation of the T-loop threonine by the CDK-activating kinase (CAK), and by dephosphorylation of threonine 14/tyrosine 15 residues mediated by the CDC25 phosphatase. On the other hand, CDK activity can be negatively regulated by association with CKIs, by dephosphorylation on a conserved T-loop threonine residue (T161 in human CDC2 and T160 in human CDK2) by the CDK-associated protein phosphatase (KAP), by negative phosphorylation of threonine 14/ty-

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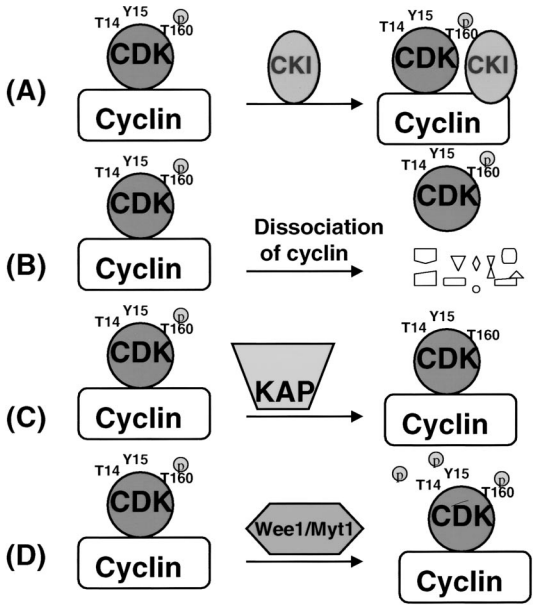

Figure 1. Mechanisms that negatively regulate CDK: binding to CKI (*A*), dissociation of cyclin (*B*), dephosphorylation of a conserved threonine residue in the T-loop (T160 in human CDK2) by KAP (*C*), and phosphorylation of T14, Y15 by WEE1/Myt1 kinase (*D*).

rosine 15 residues mediated by WEE1-like kinases, and by dissociation from cyclin [2] (fig. 1). In this review, we particularly focus on discussing the negative regulators of CDK, showing that their dysregulation can lead to uncontrolled cell growth and tumorigenesis. We hope that this review will generate further insights into the molecular mechanisms underlying unlimited proliferation during oncogenesis.

CKIs and cancers

INK4 family of CKIs

Two families of CKIs have been characterized based on their specificity of interaction with CDKs and on their sequence homology. The first is the INK4 family, which includes p15 (INK4b), p16 (INK4a), p18 (INK4c), and p19 (INK4d) [1]. These proteins are structurally related, each containing four ankrin repeats. The INK4 family recognizes CDK4 and CDK6, but not CDK2, and causes G1 arrest of the cell cycle by competing with cyclin D for binding with CDK4. p15 and p16 are frequently deleted in a variety of tumors and cancer cell lines, while no clear tumor suppressor function has been assigned to p18 [3, 4] or p19 [4, 5].

p16

The p16 protein, which was originally identified as a CDK4 interacting protein that inhibits CDK4-associated kinase activity [6], is located on chromosome 9p21 [7]. Due to this hot spot of genomic alterations in cancers, intense studies have focused on the role of p16 in tumorigenesis. Mutations of the p16 gene are frequently found in a variety of human malignancies and transformed cells [8, 9]. Furthermore, numerous studies have revealed a high frequency of p16 deletion in melanoma, mesothelioma, acute lymphocytic leukemia, osteosarcoma, renal cell carcinoma, and cancers of the esophagus, lung, pancreas, bladder, head and neck, breast, brain, and ovaries [10, 11]. The frequent inactivation of p16 in these tumors suggested that loss of p16 provides a selective cellular growth advantage. The tumor suppressive activity of p16 is attributed to its biochemical ability to bind both CDK4 and 6 and to inhibit the catalytic activity of the cyclin D/CDK enzyme complex required for phosphorylation of the retinoblastoma protein (pRb) for cell cycle progression. These observations provide strong genetic evidence of a tumor-suppressive function for p16.

The frequent inactivation of p16 in cancer suggests that tight regulation of p16 is necessary to maintain normal cell growth. To further understand the biological roles of p16, we will review some of the important mechanisms by which p16 is regulated (fig. 2A). In addition to homozygous deletion and inactivating mutations, recent studies have identified inappropriate methylation of the p16 promoter as a major mechanism of gene silencing. Methylation of 5′ CpG islands is an important mechanism of transcriptional repression in general, and evidence suggests that methylation of the p16 promoter can play an important role in tumorigenesis [12]. Methylation of the 5′CpG island of p16 has been identified and associated with a complete transcriptional block in cancers of the head and neck, breast, prostate, brain, lung, colon, esophagus, and bladder [13]. Treatment of methylated cell lines with 5-deoxyazacytidine, a methylation inhibitor, results in a significant decrease in promoter methylation, leading to the reexpression of p16 and to G1 cell cycle arrest. Therefore, methylation is one of the mechanisms for silencing p16 expression. Recently, a novel gene SEI-1, cloned by interaction with p16, was shown to antagonize the function of p16 [14]. p34 SEI-1 renders the cyclin D1/CDK4 complex resistant to the inhibitory activity of p16. Interestingly, expression of SEI-1 is rapidly induced when quiescent fibroblasts are treated with serum. Furthermore, overexpression of p34 SEI-1 enables fibroblasts to proliferate under low-serum conditions. These observations suggest that p34 SEI-1 is a growth factor sensor that can counteract the inhibitory activities of p16. Importantly, SEI-1 has been mapped to chromosome 19q13.1–q13.2, where amplification is seen in several human cancers [15]. Amplification and overexpression of genes in the 19q13.1–q13.2 region has been proposed to contribute to cancer pathogenesis or progression. However, the roles of SEI-1 in tumorigenesis remain to be characterized.

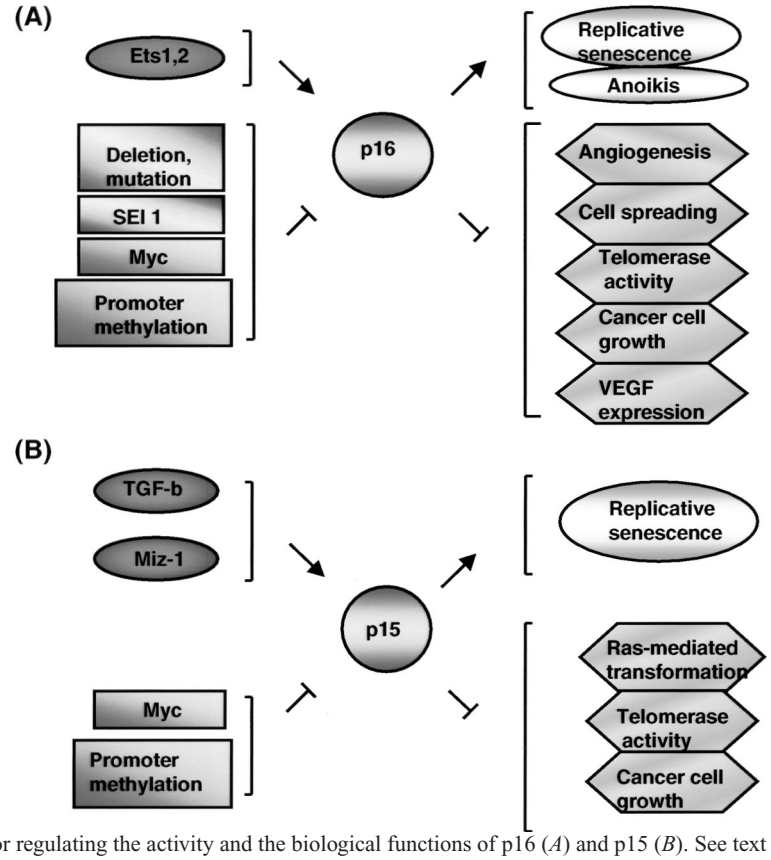

Figure 2. Mechanisms for regulating the activity and the biological functions of p16 (*A*) and p15 (*B*). See text for details.

Elevated levels of p16 are observed in senescent cells, suggesting that p16 is responsible for this state of permanent growth arrest. Overexpression of p16 can induce senescence-associated β-galactosidase (SA-β-gal) in cells, a characteristic of replicative senescence. In addition, p16 expression is lost when cells escape senescence checkpoints and achieve immortalization [16, 17]. On the other hand, reintroduction of exogenous p16 induces senescence in immortalized cell lines [18]. Recent research showed that p16 can be activated by the ETS1 and ETS2 transcription factors, which activate the p16 promoter through an ETS-binding site and induce SA-β-gal activity [19]. In senescent cells, increased expression of ETS1 can cause the upregulation of p16. Taken together, these observations demonstrate an important role for p16 in senescence and tumor immortalization. Telomere length is shortened as cells approach replicative senescence, and senescence can be overcome in telomerase-negative cells by expression of the catalytic subunit of telomerase. Such cells show a dramatically extended life span. Exogenous expression of p16 is associated with a decrease in telomerase activity [20]. Although the mechanism by which p16 causes downregulation of telomerase is unclear, a link certainly exists between p16 expression, telomerase activity, and cell immortalization.

Recent research has also shown that p16 plays a role in loss of cell adhesion (anoikis), in cell spreading, and in angiogenesis. Induction of apoptosis upon loss of anchorage is a characteristic of anoikis and has been shown to occur in epithelial and endothelial cells [21]. During transformation, most malignant cells become resistant to anoikis, allowing for possible tumor spreading, invasion, and metastasis. Reintroduction of p16 reconstitutes the apoptotic response to the loss of anchorage (anokis) in a variety of human cancer cells [22]. Anoikis induced by p16 is due to the specific transcriptional upregulation of the α_5 β_3 fibronectin receptor [22], which is frequently lost in transformation cells. It has also been shown that p16 expression can block α_V β_3 integrin-dependent cell spreading on vitronectin [23]. Cell attachment to vitronectin is mediated mainly through integrin receptors containing the α_V subunit, and the α_V β_3 integrin receptor is tightly associated with tumor growth and spreading [24]. Although transient expression of wild-type p16 results in loss of spreading on vitronectin, this effect can be reversed by expression of CDK6. These findings suggest a novel function for the p16 tumor suppressor protein in regulating matrix-dependent cell migration.

Vascular endothelial growth factor (VEGF) is a pivotal mediator of angiogenesis and plays an important role in the growth and metastasis of many types of human can-

cer. An additional study has shown that overexpression of p16 significantly reduces the expression of VEGF to inhibit tumor angiogenesis in p16-deleted glioma cells [25]. In addition, wild-type p16 inhibits neovascularization more potently than does wild-type p53 in vivo [25]. These findings indicate that p16 also plays an important role in the regulation of angiogenesis. However, how VEGF transcription is downregulated by p16 remains to be characterized.

Significantly, adenovirus-mediated gene transfer of p16 can suppress tumor growth, including that in tumor xenograft models of breast cancer [26], prostate cancer [27], esophageal cancer [28], and mesothelioma [29], suggesting that p16 could be considered for cancer gene therapy in clinical trials. Despite the large amount of data supporting the role of p16 as a tumor suppressor, its many biological roles remain to be elucidated. Future directions will need to address how transcriptional activators, such as ETS1and ETS2, can regulate p16 and their possible roles in tumorigenesis, and whether oncogenic signals can activate SEI-1 to antagonize the ability of p16 to facilitate tumor growth.

p15

The p15 gene is located at the Ink4 locus, 9p21, and its expression is induced in response to transforming growth factor beta (TGF-β) treatment [30]. Although evidence for a tumor suppressor role of p16 is abundant, the role of p15 in tumor suppression is unclear. Point mutations rarely occur in p15 [31]. In most tumors, homozygous deletions affect either both the p16 and p15 loci or the p16 locus alone [31]. Specific deletions of p15 sequences have been found in only a few cases of leukemia and lymphomas [31, 32]. In contrast, inactivation of p15 by hypermethylation seems to be frequent in leukemia and lymphomas [33–36], suggesting that silencing of the p15 promoter by methylation plays an important role in hematopoietic malignancies. Furthermore, aberrant p15 methylation seems to have important prognostic implications for risk assessment because patients with p15 methylation have overall shortened survival [34].

The c-Myc protooncogene has been found to be involved in the progression of a wide range of neoplasia [37]. The c-Myc protein acts as a transcription factor in conjunction with its transcriptional activation partner Max. Recently, Miz1, Myc-interacting zinc finger protein 1, was found to upregulate the expression of p15 by binding the initiator element of the p15 promoter [38]; however, this process can be antagonized by Myc. c-Myc and Max form a complex with Miz-1 at the p15 initiator and inhibit Miz-1-mediated transcriptional activation of p15. Interestingly, overexpression of Miz-1 causes G1 cell cycle arrest and inhibits cyclin D-associated kinase activity. Furthermore, TGF-β is known to downregulate Myc expression [39, 40]. These observations provide a potential model that explains how p15 could be regulated in response to TGF-β growth inhibitory signals: TGF-β may upregulate p15 mRNA expression by transcriptionally activating p15 promoter as well as by downregulating the promoter suppressor c-Myc, thereby releasing endogenous Miz-1 from inhibition by c-Myc. Indeed, evidence has shown that TGF-β signalling prevents recruitment of Myc to Miz-1, thereby enabling transcriptional activation of p15 [41]. Future directions will need to address whether other oncogenic signals can regulate Miz-1 and to elucidate roles of Miz-1 in tumorigenesis.

Despite the confusion in determining a role for p15 in cancer, cancer cells appear to have evolved an efficient mechanism to inactivate not only p16 but also nearby potentially important tumor suppressor genes, such as p15 (fig. 2B). In addition to growth inhibition, p15 has also been shown to be a strong inhibitor of cellular transformation by Ras [42]. Embryonic fibroblasts isolated from p15 knockout mice are susceptible to transformation by Ras, whereas wild-type embryonic fibroblasts are not. These studies indicate that p15 is involved in the tumor suppressor activity after inappropriate activation of oncogenic Ras in the cell. We should point out that the function of p15 is almost indistinguishable from that of p16 in terms of inducing replicative senescence, inhibiting telomerase activity, and inhibiting cell growth [43]. Therefore, p15 may become as useful as p16 in blocking cancer growth, thus fulfilling a role as a gene therapy agent.

CIP/KIP family of CKIs

The second family of CKIs is the CIP/KIP family, which shares homology at the N-terminal CDK inhibitory domain, and includes p21 (CIP1/WAF1/SDI), p27 (KIP1), and p57 (KIP2). The CIP/KIP family members interact with the cyclin-CDK complexes and inhibit kinase activity of cyclin A/CDK2 and cyclin E/CDK2. Overexpression of CIP/KIP inhibitors causes G1 cell cycle arrest, suggesting that they preferentially target the G1 cyclin-CDK complexes. Surprisingly, p21 and p27 have an important function in regulating cyclin D/CDK4 activity. Indeed, p21 and p27 are inhibitors of CDK2 and activators of CDK4 [44, 45]. Evidence has been provided that p21 and p27 positively promote assembly of cyclinD/CDK4 [44]. In addition, they promote cyclin D stability and nuclear localization [44]. Thus, a more accurate picture of the effect of CKIs on the cell cycle is that they are negative regulators of cyclin A/CDK2 and cyclin E/CDK2 complexes although they are also the positive regulators of cyclin D/CDK4 complexes.

p21

p21 [46–50] is a transcriptional target of p53 [48]. p21 protein is a critical determinant of the G 1 arrest in response to DNA damage [48] and also plays an important

role at the G2/M phase transition [48, 51–53]. So far, its role in tumorigenesis is not clear. Recently, in p53-negative patients, the survival rate of those with p21 expression was significantly better than that of those without p21 expression [54, 55]. Multivariate analysis revealed that p21 expression in the p53-dependent pathway was an independent prognostic factor. However, other observations have shown that the patients with p21 expression have a poorer prognosis than do those without p21 expression [56]. Further studies will be required to establish the role of p21 in cancer development.

To further understand the role of p21 in cancer requires reviewing several different signals that regulate p21 activity (fig. 3A). First, in addition to a response to p53 transcriptional activation, p21 responds to p53-independent signals [57] and has been implicated in terminal differentiation and senescence [58, 59]. p202 is an interferon (IFN)-inducible phosphoprotein (52 kDa) [60] that regulates the expression of p21 protein independent of p53 protein [61], contributing to the cell growth inhibitory activity of IFNs. How p202 activates p21 expression is not yet clear. Second, studies have demonstrated that p21 expression can be induced through activation of the signal transducers and activators of transcription (STAT) signal transduction pathway [62]. STATs are a family of latent cytoplasmic proteins that are activated to enter the nucleus and work with other transcription factors to transcriptionally activate downstream genes when cells are treated by various cytokines and growth factors [63]. The p21 promoter region was shown to bind STAT1, an essential component mediating cell growth suppression of the IFN-γ signal transduction pathway [62]. Importantly, increased DNA methylation in the p21 promoter region can prevent STAT1 from binding to the p21 transcription start site in rhabdomyosarcomas (RMSs), thereby inhibiting p21 induction and cell growth regulation through the IFN-γ/STAT signalling pathway [64]. Complete methylation within the STAT1-binding site correlates with decreased p21 mRNA expression in RMSs. In contrast normal tissues show a partial methylation pattern at the STAT-binding site. These studies suggest that methylation is one of the mechanisms for silencing p21 expression. The third way that p21 can be regulated is through Myc signalling. Overexpression of c-Myc reduces the levels of endogenous p21 mRNA and represses p21 promoter-luciferase reporter gene expression [65]. This suggests that the downregulation of p21 contributes to c-Myc-dependent entry into S phase. Currently, the mechanism of this repression is unclear. Whether the c-Myc interaction protein Miz-1 is involved in this repression remains an interesting topic of study. Of note is that TGF-β treatment

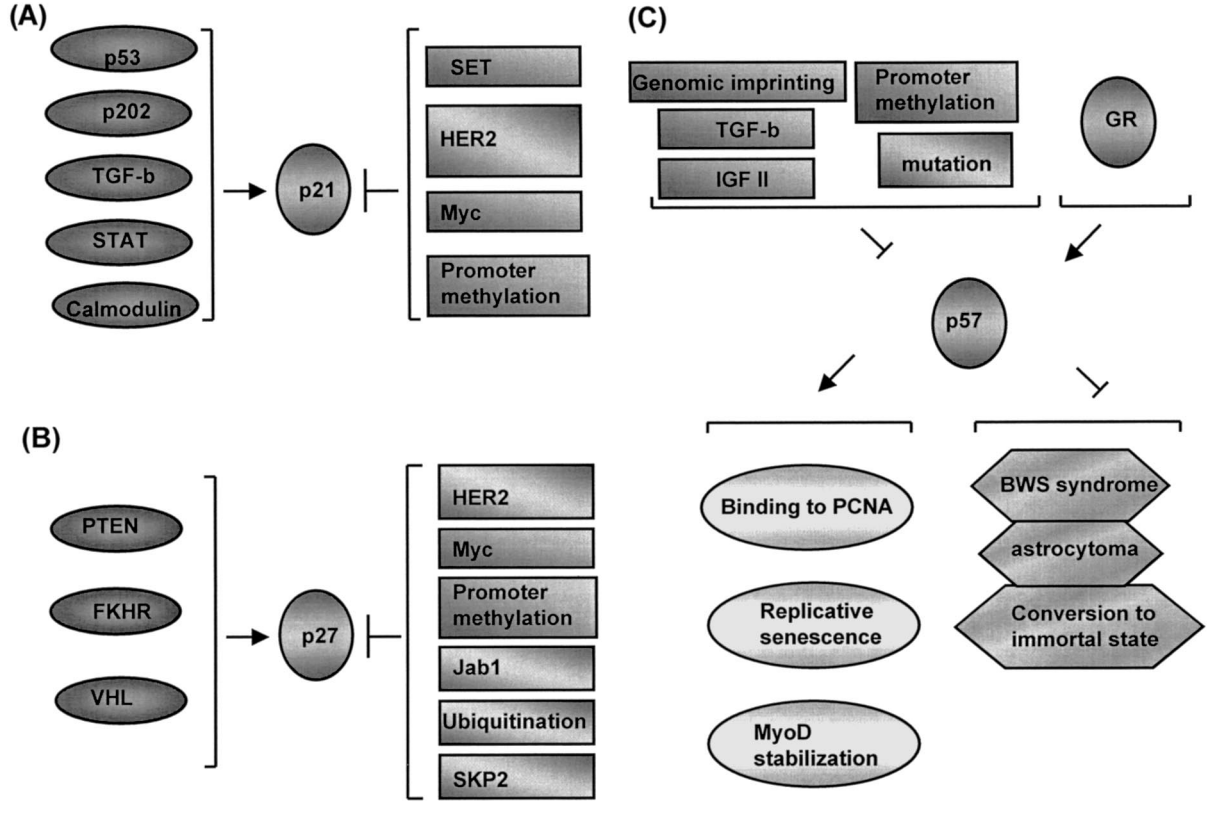

Figure 3. Positive and negative mechanisms to regulate p21 activity (*A*), p27 activity (*B*), and p57 activity and the biological functions of p57 (*C*). See text for details.

of immortalized human keratinocytes can upregulate p21 to inhibit cell cycle progression [66]. In addition, TGF-β also downregualtes c-Myc expression [40]. Therefore, TGF-β transcriptionally activates p21 by downregulating the expression of c-Myc. Another negative regulator of p21 is a protein called SET, a 39-kDa oncoprotein which binds to p21 at the carboxyl-terminal region to abrogate the inhibitory activity of p21 toward cyclin E-CDK2 [67]. Interestingly, SET does not bind to p27 to block its activity, suggesting that p21 is a specific target. Thus, SET appears to be a modulator of p21 in the G1/S transition by modulating the activity of cyclin E/CDK2. Whether SET is involved in tumorigenesis needs to be determined. Finally, the oncogene HER2/neu (HER2) has also been shown to negatively regulate p21 activity [68]. HER2-mediated cell growth requires the activation of Akt, which associates with p21 and phosphorylates it at threonine 145, resulting in cytoplasmic localization of p21 [68] (fig. 4). The mislocation of p21 prevents its growth inhibitory activity. In keeping with this observation, nuclear p21 (T145A) can preferentially suppress the growth of transforming cells. In addition to HER2, calmodulin binds to p21 and is involved in the regulation of its nuclear translocation [69]. Cells treated with anti-calmodulin drugs did not have the nuclear accumulation of p21, suggesting that calmodulin is involved in the regulation of p21 nuclear localization.

Taken together, the role of p21 in cancer is as unclear as that of p15, but p21 certainly serves as a target for different signals during tumorigenesis. Cancer cells appear to have evolved an efficient mechanism for first inactivating p53, the inducer of p21, so that the roles of p21 in cancer remain obscure. Recent studies have shown that overexpression of p21 suppresses the growth of mammary carcinoma [70], lung cancer [71], and prostate cancer [72], suggesting its potential use as a therapeutic agent for cancer [73].

p27

The p27 protein is a CKI that causes G1 arrest by inhibiting G1 cyclin-CDK activities [74, 75]. As a negative regulator of the cell cycle, p27 is a member of a new class of haplo-insufficient tumor suppressors [76]. In animal studies, the number of p27 gene copies decides the rate of tumor formation since p27 haplo-insufficient mice are hypersensitive to carcinogens [76]. Recently, p27 expression has been studied intensely in various tumors to define its role in tumorigenesis. Reduced expression of p27 is frequently detected in human cancers, including breast [77, 78], prostate [79], gastric [80], lung [81], skin [82], colon [83], and ovarian cancers [84]. Decreased expression of the p27 protein is correlated with cancer development and poor survival and is an important marker of cancer progression. Since p27 inhibits cyclin/CDK in a dose-dependent manner to control cell cycle progression [74, 85], decreased expression of p27 may result in abnormal cell proliferation in these cancers. The exact mechanism for decreased p27 expression at certain stages of cancer has been the focus of studies. Because p27 is posttranscriptionally regulated through the ubiquitin-mediated proteasome degradation pathway [86], low levels of p27 in many types of cancer may be caused by the enhancement of ubiquitin-mediated p27 degradation. For example, high-grade colon cancer samples contain high p27 degradation activity compared with that of low-grade samples [83].

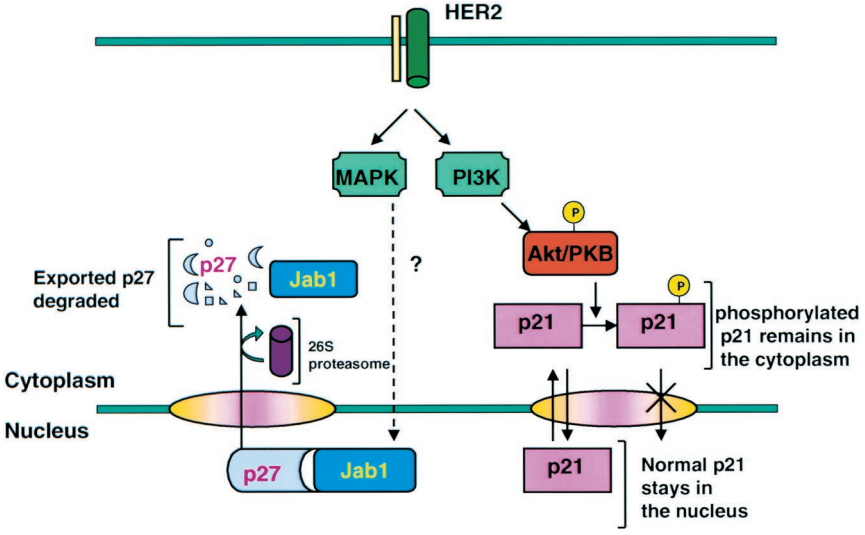

Figure 4. Regulation of p21 and p27 by HER2 oncogenic signals. Activation of PI3K and Akt leads to phosphorylation of p21. Phosphorylated p21 then remains in the cytoplasm. Activation of MAPK leads to the export of the Jab1/p27 complex from the nucleus to the cytoplasm through uncharacterized regulation (?). p27 is then degraded by the 26 S proteasome.

The cellular response to mitogenic stimuli requires tight regulation of p27 levels. Several signals contribute to this regulation (fig. 3B). First, the tumor suppressor gene PTEN (phosphatase and tensin homolog) [87], one of the most commonly inactivated genes in human cancer, is associated with increased expression of p27 [88, 89]. PTEN can reduce CDK2 activity, causing cell cycle arrest. Actually, the PTEN pathway regulates p27 protein stability through the reduction of SKP2, an important component of p27 ubiquitinase [90]. Antisense p27 oligonucleotides can abrogate the growth arrest mediated by PTEN, suggesting that p27 is a critical mediator of PTEN-induced G1 arrest and that p27 plays a role in the PTEN-regulatory cascade. Mutations in the PTEN gene are seen in brain, breast, prostate, endometrial, and skin cancers [87]. Determining whether p27 is deregulated in these tumors will be interesting. Second, transcriptional upregulation of p27 by the Forkhead transcription factors has been reported recently [91, 92]. The Forkhead transcription factors AFX, FKHR, and FKHR-L1 are involved in regulating longevity in *Caenorhabditis elegans*. Interestingly, overexpression of these Forkhead transcription factors causes growth inhibition in cell lines, suggesting that AFX-like proteins are also involved in cell cycle regulation. Importantly, AFX blocks G1 cell cycle progression by transcriptionally activating p27 [91]. Indeed, p27-deficient cells are significantly less inhibited by AFX activity than are their p27 wild-type counterparts, revealing that p27 is a major target of AFX-like Forkhead proteins. In light of this important link, defining whether inactivation of these proteins is involved in tumorigenesis is important. The third signal that can regulate p27 involves Von Hippel-Lindau (VHL) protein. VHL syndrome is an autosomal dominant familial cancer syndrome associated with several abnormalities, including renal cell carcinoma, cerebral hemangioblastomas, pheochromocytomas, and retinal angiomas [93, 94]. The VHL protein can negatively regulate cell cycle progression, which is associated with increased expression of p27 [95, 96]. p27 usually accumulates upon serum withdrawal. In a study using VHL-negative renal cell carcinoma cells, p27 accumulated only in the presence of the VHL upon serum withdrawal, suggesting that p27 is a downstream mediator of the VHL pathway [95, 96]. How VHL mediates increased expression of p27 remains to be determined.

In addition to signals that enhance p27 activity, there are signals that inhibit or silence p27 activity. First, dense methylation of a CpG island in the promoter region of p27 is observed in malignant melanomas [97], and is associated with transcriptional silencing of p27. Whether methylation of the p27 promoter region also occurs in other types of cancer remains to be shown. Second, c-Myc antagonizes the function of p27 [98]. c-Myc prevents p27 from binding cyclin E/CDK2 through sequestration. Sequestration of p27 is mediated via c-Myc-mediated induction of cyclin D1 and D2 [99, 100]. In a recent study, c-Myc transcriptionally repressed the gene expression of p27 [101]. The c-Myc repression region is located at the bp –20 to +20 region of the p27 gene. These data suggest that Myc controls the function of p27 in multiple ways. Interestingly, many cancers that overexpress HER2, including colon, breast, gastric, and lung cancers, also underexpress p27, leading to the hypothesis that a link exists between HER2 oncogenic signals and p27 regulation. HER2, a receptor tyrosine kinase oncogene, promotes mitogenic growth and transformation of cancer cells. Strong expression of HER2 in breast cancer has been associated with poor prognosis [102]. Reduced expression of p27 correlates well with poor clinical outcome in breast cancer. Interestingly, downregulation of p27 correlates with HER2 overexpression in primary breast carcinomas [103]. To address the molecular mechanism of this inverse correlation, evidence has demonstrated that reduced p27 levels are caused by enhanced ubiquitin-mediated degradation and that the HER-2/Grb2/MAPK pathway is involved in the decrease in p27 stability [104]. Furthermore, HER2 activity causes mislocation of p27 and JAB1 (an exporter of p27) [105] into the cytoplasm, thereby facilitating p27 degradation [104] (fig. 4). Thus, these observations show that various oncogenic signals downregulate p27.

Taken together, p27 is clearly a tumor suppressor deregulated in many types of cancer and an important target of different signals during oncogenesis. Future directions will need to address how tumor suppressors such as VHL and PTEN can induce p27 to control cell cycle progression and how oncogenic signals from HER2 or c-Myc can antagonize the function of p27 to facilitate tumor growth.

p57

The p57 protein is a maternally expressed, paternally imprinted CKI located on chromosome 11p15.5 [85, 106]. p57 is a candidate tumor suppressor gene because of its location, biochemical activities, and imprinting expression. So far, many studies in signal regulation have been focused on p21 or p27, whereas little is known about p57. Animal studies have shown that p57 knockout mice have altered cell proliferation and differentiation, and have a variety of other abnormalities, including muscle defects, bone defects, a cleft palate, adrenal cortical hyperplasia and cytomegaly, and gastrointestinal tract defects [107, 108]. Many of these defective phenotypes are shared with patients with Beckwith-Wiedemann syndrome (BWS), a childhood overgrowth syndrome, suggesting that loss of p57 plays a role in BWS [107, 108]. Indeed, patients with BWS have p57-specific gene mutations [109]: a mutation in the CDK inhibitory domain causes loss of most of the protein, and a frameshift mutation results in disruption of a carboxy-terminal domain that is conserved in both p27 and p57 (QT domain). Thus, characterizing the regula-

tory signals of p57 to further understand its functional roles in BWS and in cancer cells is important.

Other than those in BWS, p57 mutations in the other human cancers are rare [110, 111], suggesting that other mechanisms of transcriptional or posttranslational silencing are involved in the loss of p57 function in other types of cancer. Decreased expression of p57 has been found in bladder carcinoma [112] and gastric cancer [113]. In most cases, p57-associated mutations are not found, but p57 mRNA levels are significantly diminished in cancer cells compared to normal cells. These results suggest that inactivation of p57 gene expression, rather than mutations in the p57 gene, accounts for the involvement of p57 in other types of cancer. Transcriptional repression could be a result of DNA methylation of the promoter. Indeed, hypermethylation of the p57 promoter is an alternative signal for inactivating the p57 gene [114]. Again, the p57 gene is activated in the cell lines with silenced p57 after treatment with a methylation inhibitor, suggesting hypermethylation of the promoter. In addition to genomic imprinting and methylation, several other signals contribute to p57 regulation (fig. 3C). First, insulin-like growth factor-II (IGF-II) plays an important role in embryonic growth, and aberrant IGF-II expression has been documented in several human disorders, including BWS and a wide variety of tumors. The expression of p57 is reduced in primary embryo fibroblasts upon IGF-II treatment and in mice with high levels of serum IGF-II [115]. In addition, in a mouse study for BWS, IGF-II and p57 indeed acted in an antagonistic manner [116]. Therefore, IGF-II and p57 play antagonistic roles in controlling cell proliferation during normal development. TGF-β is a third signal that regulates p57. TGF-β plays a variety of signalling roles in numerous cells and sometimes exerts contradictory effects, depending on the cell type. Although TGF-β mainly suppresses cell growth in epithelial cells, it can promote cell growth in other cells, such as osteoblasts. A recent study showed that TGF-β causes p57 degradation in osteoblasts, suggesting a new system for regulating p57 [117]. Because TGF-β plays an important role in tumorigenesis, determining whether regulation of p57 by TGF-β also plays a role will be interesting.

p57 has important functions such as binding to proliferating-cell nuclear antigen to prevent DNA replication and inhibit cell transformation [118]. It can also be induced transcriptionally after glucocorticoid treatment and is involved in glucocorticoid-induced antiproliferation [119]. It also stabilizes MyoD during muscle differentiation [120, 121]. Recently, overexpression of p57 has been shown to cause a cell growth arrest and senescent phenotype in astrocytoma cells, as demonstrated by the accumulation of the SA-β-gal marker [122]. Furthermore, expression of p57 is involved in inhibiting the conversion of conditionally immortal human mammary epithelial cells to the fully immortal phenotype, suggesting that p57 provides an important barrier against indefinite proliferation [123]. These studies suggest that p57 can be used as a tumor suppressor agent to induce senescence and block immortalization in cancers.

Overall, little is known about the biological function of p57, and the relationship between p57 expression and cell proliferation under physiological circumstances therefore requires further exploration. The p57 promoter remains to be characterized, as do the many regulatory signals for p57 [85]. Once completed, these studies will certainly help us understand more about the role of p57 in cancer.

Ubiquitination and cancer

Recently, the most important progress made concerning cell cycle deregulation in cancer was the discovery of SKP2, a regulator involved in p27 degradation [124]. SKP2 is a subunit of SKP1-Cul1-F-box (SCF) protein-ubiquitin ligase complexes and has been characterized as being required for the ubiquitination and consequent degradation of p27 [124]. SKP2 binds p27 phosphorylated on Thr187 by CDK2, to facilitate the ubiquitination of p27. In animal studies, SKP2 knockout mice grow more slowly and have smaller organs than controls [125]. Furthermore, SKP2-deficient cells show high levels of p27 and have larger nuclei with abnormal DNA content ranges from 2 to 16C, suggesting polyploidy. Abnormal amplification of the centrosome is also apparent in SKP2–/– mouse embryonic fibroblasts due to the retention of a high level of cyclin E [125]. These observations highlight the importance of SKP2 in positively regulating cell proliferation. Indeed, SKP2 was originally identified as one of two proteins that interact with the cyclin A/CDK2 complex [126]. SKP2 is required for the G1/S transition in both transformed cells and diploid fibroblasts [126], and SKP2 overexpression induces quiescent fibroblasts to replicate their DNA in the presence of low serum [127]. Because the abundance of SKP2 is greatly increased in many transformed cells, SKP2 has also been hypothesized as being involved in cancer.

Since p27 degradation is enhanced in many aggressive human tumors, the role for SKP2 in p27 degradation may be related to oncogenesis and tumor progression. Recent studies have shown that SKP2 expression correlates positively with the grade of malignancy and inversely with p27 levels in human lymphomas [128]. In addition, increased levels of SKP2 protein are associated with reduced p27 in a subset of oral cancer [129]. SKP2 can also cooperate with H-Ras G12V to transform primary rodent fibroblasts, resulting in anchorage-independent growth and tumor formation in nude mice [129]. These observations suggest that SKP2 has oncogenic potential and may therefore be a protooncogene. Since SKP2 mediates the ubiquitin-dependent proteolysis of p27, its overexpres-

sion may lead to the aggressive phenotype and poor prognosis in human tumors with loss of p27. Thus, examining whether SKP2 is deregulated in cancers with low levels of p27 is important. Future directions will need to address which oncogenic signals regulate SKP2 activity and how they do so to degrade p27.

KAP and cancer

The CDK-associated protein phosphatase KAP is a human dual-specificity protein phosphatase that dephosphorylates CDK2 on threonine 160 in a cyclin-dependent manner. KAP is expressed at the G1/S transition of the cell cycle and forms stable complexes with CDK2. Overexpression of wild-type KAP delays cell cycle progression in cells, and the delay is dependent on the phosphatase activity of KAP [130]. KAP always dephosphorylates Thr160 in native monomeric CDK2. The binding of cyclin A to CDK2 inhibits the dephosphorylation process but does not prevent the binding of KAP to the cyclin A/CDK2 complex. Because the dephosphorylation of CDK2 at T160 by KAP inhibits CDK2 activity, KAP might function like a tumor suppressor.

KAP mutations recently identified in hepatocellular carcinoma cause defective binding of KAP to CDK2, suggesting a possible role for KAP mutations in hepatocarcinogenesis [131]. In contrast, other studies have shown that the KAP gene is overexpressed in breast and prostate cancers, thereby acting as an oncogene [132] (fig. 5A). The mechanism behind KAP overexpression is not clear, but the KAP gene is mapped to the 14q22 locus [133], where chromosome abnormalities are linked to several types of cancer. In primary cancer studies, the normal prostate glands show no detectable expression of the KAP protein, whereas invasive prostate carcinomas have high levels of KAP in epithelial cells. Furhermore, normal mammary gland epithelium stains weakly for KAP, whereas invasive carcinoma cells of the breast show significant expression. These findings suggest that overexpression of KAP is frequently found in both breast and prostate cancers. Consistent with these observations, abrogation of KAP expression by antisense oligonucleotides results in reduced populations of S phase cells and reduced CDK activity [132]. Furthermore, blocking KAP expression leads to inhibition of the transformed phenotype and tumorigenic potential in a mouse cancer model [132]. In addition to showing that suppression of KAP expression reduces the S phase population, an earlier study also showed that selected KAP transfectants had a reduced S phase fraction [130]. How both exogenous overexpression and decreased KAP expression might contribute to blocking cell cycle progression remains to be resolved. However, abnormal expression of KAP clearly plays an important role in tumor development. Further investigation is warranted.

WEE1/Myt1 and cancers

Entry into mitosis requires the activity of CDC2 coupled with cyclin B. Phosphorylation of the CDC2 on threonine 14 and tyrosine 15 residues inhibits CDC2 activity. CDC2 is kept inactive by this inhibitory phosphorylation until the completion of DNA replication, and the inhibitory phosphorylation is maintained in cells arrested in the G2 phase following DNA damage. Human WEE1 (HWEE1) phosphorylates Tyr-15 on CDC2, whereas human Myt1 (HMyt1), a membrane-anchored protein kinase, can phosphorylate CDC2 on threonine 14 and tyro-

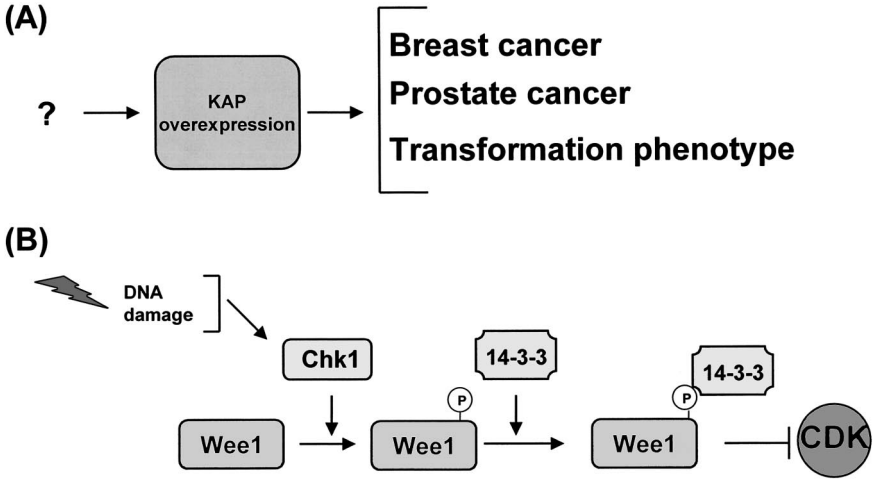

Figure 5. Regulation of KAP and WEE1. (*A*) An uncharacterized mechanism (?) leads to KAP overexpression, and KAP may then act as a potential oncogene to be involved in tumorigenesis. (*B*) DNA damage activates Chk1, and Chk1 and 14-3-3 act as positive regulators of WEE1 to block CDK activity.

sine 15 residues [134–137]. At the G2/M transition, the CDC2-inhibitory phosphorylation is removed by a dual-specificity phosphatase called Cdc25C [138]. Unlike HWEE1, HMyt1 is capable of phosphorylating cyclin-associated CDC2 but not CDK2, indicating that the substrate specificity of HMyt1 is more restricted than that of HWEE1 [139]. Because of their roles as negative regulators of CDK, characterizing their participation in tumorigenesis is of great importance. The WEE1 gene was recently found to be significantly suppressed in colon carcinoma cells, suggesting its role in tumorigenesis and its potential role as a tumor suppressor [140]. This finding suggests that genetic defects targeting cell cycle regulation occur not only at the G1/S transition but also at the G2/M transition during tumor development. The defects in WEE1 expression may additionally enhance the ability of tumor cells to proliferate. The suppression of HWEE1 in colon cancer and its potential role in tumor growth warrant further investigation in other types of cancer.

To further understand the role of WEE1/Myt1 in cancer, a review of HWEE1 regulation is necessary. In yeast, a homolog of HWEE1 called Mik1 is positively regulated by the DNA damage checkpoint control [142]. DNA damage increases Mik1 abundance in a checkpoint kinase 1 (Chk1)-dependent manner [142]. Interestingly, Mik1 is ubiquitinated, suggesting that the DNA damage checkpoint prevents Mik1 degradation [142]. In *Xenopus* egg extracts, Chk1 can phosphorylate WEE1 on Ser549, mediating binding of WEE1 to 14-3-3 proteins [141]. Interestingly, binding of 14-3-3 can stimulate the kinase activity of WEE1 against CDC2 [141] (fig. 5B). Taken together, these results suggest that Chk1 and 14-3-3 proteins act together as positive regulators of WEE1. With our quickly expanding knowledge of cell checkpoint control, we can expect more insight into how WEE1 is regulated in mammalian cells. Future directions will need to address how checkpoint controls are involved in regulating WEE1 stability and how oncogenic signals cause the downregulation of WEE1 to facilitate tumor growth.

14-3-3 sigma: a new class of CKI and cancer

The 14-3-3 sigma gene is one of the seven members of the 14-3-3 family. It was originally characterized as the human mammary epithelial-specific marker, HME1 [143], and has been shown to be induced by p53 for involvement in G2 cell cycle checkpoint control after DNA damage [144]. 14-3-3 sigma has also been shown to sequester cyclin B1/CDC2 complexes in the cytoplasm during G2 arrest and its absence allows cyclin B1/CDC2 complexes to enter the nucleus, causing mitotic catastrophe [145, 146] (fig. 6). During this process, the nuclear exporting sequence (NES) of 14-3-3 sigma is required to sequester CDC2 in the cytoplasm [146]. Recently, 14-3-3 sigma was also cloned by expression cloning and characterized as a negative regulator of CDK [146]. 14-3-3 sigma has also been shown to specifically interact with CDK2, CDC2, and CDK4 and can inhibit CDK activities to block cell cycle progression, thus defining it as a new class of CKI [146].

Several lines of evidence suggest that loss of 14-3-3 sigma function correlates with cell transformation. 14-3-3 sigma suppresses the anchorage-independent growth of

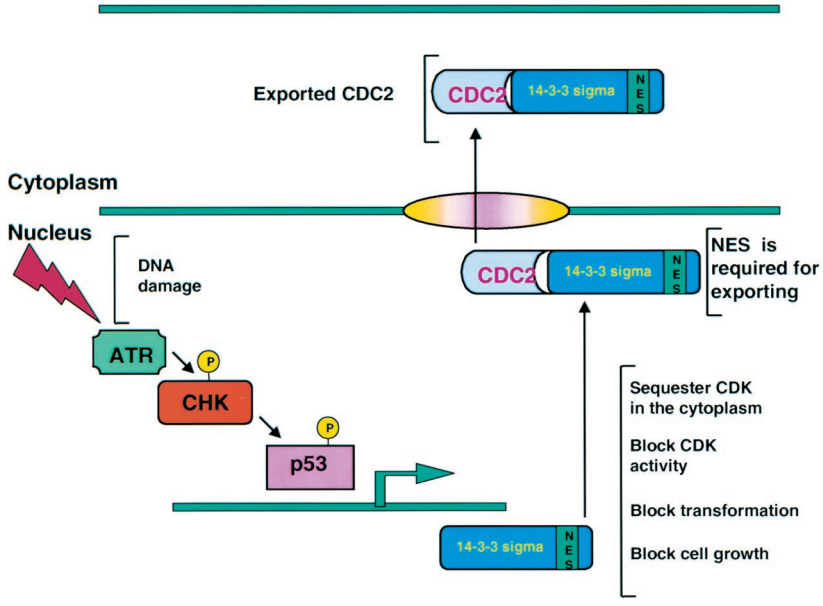

Figure 6. 14-3-3 sigma and CDC2 translocation. DNA damage activates Chk and p53, and p53 transcriptionally activates 14-3-3 sigma. 14-3-3 sigma then inhibits CDK activity, blocking cell growth and cell transformation. 14-3-3 sigma contains a nuclear exporting signal (NES) required to sequester CDC2 in the cytoplasm.

several breast cancer cell lines [146]. In addition, 14-3-3 sigma expression levels are diminished in v-Ha-ras-transformed mammary epithelial cells, mammary carcinoma cells [143], SV40-transformed human keratinocytes [147], head and neck squamous cell carcinoma lines [148], primary bladder tumors [149], and colonic polyp specimens [150]. These observations demonstrate that loss of 14-3-3 sigma function contributes to malignant transformation. The implication of loss of 14-3-3 sigma function in tumorigenesis was further supported by recent studies showing hypermethylation of CpG islands and transcriptional silencing of the 14-3-3 sigma gene in breast cancer [151], gastric cancer [152], and hepatocellular carcinoma [153]. These findings suggest that loss of 14-3-3 sigma function plays an important role in the development of cancer. Therefore, 14-3-3 sigma is also a candidate tumor suppressor.

Loss of control of genomic stability is crucial in the development of cancer, and p53 is important in regulating responses to DNA damage to maintain this stability. p53 function is lost in almost half of all human cancers, yet the defects in the other half remain elusive. p53-independent regulatory mechanisms are possibly lost in these cases, but another possibility is that different points of p53-dependent pathways may be inactivated. Since 14-3-3 sigma is regulated by p53 to guard genomic stability [144], dysregulation of 14-3-3 sigma may account for some cancers that do not have mutations in p53. Therefore, p53 possibly acts as the center of a complex network of signaling pathways and other components of these pathways, such as 14-3-3 sigma, may pose alternative targets for inactivation. 14-3-3 sigma is induced by p53 and is involved in the regulation of DNA damage [144]. Fluorescence in situ hybridization analysis of metaphase chromosomes has shown that 14-3-3 sigma is localized to chromosome 1p35 [144], where a high percentage of loss of heterozygosity (LOH) is seen in different solid tumors including breast tumors [154]. On the basis of LOH, 1p35 has been proposed to harbor a tumor suppressor gene [155]. The role of 14-3-3 sigma in negatively regulating cell cycle progression and cell transformation makes it a prime candidate for this tumor suppressor. Still remaining to be determined are whether expression of 14-3-3 sigma is dysregulated in many other types of cancer, how 14-3-3 sigma distinguishes itself from other isoforms that are also involved in the cell cycle, and what other signals regulate the activities of 14-3-3 sigma to facilitate the development of cancer.

Conclusion

In this review, we have discussed the negative regulators of CDK. As we learn more about the mechanisms underlying the cell cycle regulatory machinery, the importance of exploiting these mechanisms to control cancer will become clear. Translating our knowledge of these basic cell cycle regulatory mechanisms into understanding the significance of cancer etiology and treatment is important. Future basic studies need to be directed at identifying the critical substrates for cyclin/CDK complexes, at learning about the detailed regulation of the members of the INK4 and CIP/KIP inhibitors, and at understanding the role of the ubiquitination process in cancer. With regard to clinical applications, we expect the discovery of more comprehensive assessment methods for evaluating diverse functional parameters of the cell cycle machinery in cancer patients, especially parameters involving negative regulators. Furthermore, we hope that diverse and efficient drugs will be designed to target CDKs and the ubiquitination process. Indeed, natural CKIs including p16, p21, and p27 have been an inspiration for designing mechanism-based CKIs. The design of pharmaceutically useful molecules has been greatly boosted by the recent determination of CKI/CDK structures. To date, some of these pharmacologic CKIs have been used in treatment strategies for many types of cancer. Proteasome inhibitors have also become novel, potential anticancer agents. Some of these proteasome inhibitors cause accumulation of CKIs such as p21 and p27, and inhibit tumor cell growth, induce apoptosis, and even help cells overcomes drug resistance [156, 157]. Some are in the process of clinical trials [158]. Furthermore, potential gene therapeutic strategies are also being established based on these negative regulators, to inhibit transformation and cancer growth. Gene therapy is an exciting new approach for cancer therapy and holds great promise. Although most cancers are treatable by surgery, radiation therapy, and chemotherapy, many patients are appropriately considered as candidates for gene therapy clinical trials designed to improve therapeutic results and decrease the morbidity of treatment. In the future, we know that new drug compounds, treatment strategies, and gene therapy protocols will be established to help fight cancer on the basis of our understanding of the cell cycle and cancer. Complete control of this proliferative disease may not be too far in the future.

Acknowledgements. We would like to thank the William McGowan Charitable Foundation and the Susan Komen Breast Cancer Foundation for their kind support. Special thanks to Ms. Kerry Wright for editing. We apologize to our many colleagues whose work we were unable to cite due to space constraints.

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