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REVIEW

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Inhibitors of cyclin-dependent kinase and cancer

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Abstract Recent research has yielded a dramatic increase in the number of connections between oncogenesis and the proteins which regulate the cell cycle. Three classes of protein which inhibit the activity of cyclin-dependent kinases (CDKs) have emerged as potential targets for oncogenic inactivation. p16 and related proteins inhibit the cyclin/CDK complexes which regulate the transition from G_1 to S phase; numerous studies have revealed that p16 is mutated in most tumor cell lines and in some types of primary tumor. p21/WAF1/Cip1 and the related p27Kip protein inhibit a broader range of cyclin/CDK complexes than p16. Although the absence of p21/WAF1/Cip1 from cyclin/CDK complexes is correlated with cellular transformation, no mutations in this gene have been found in tumors or tumor-derived cell lines. A third class of genes which are potential targets for oncogenic inactivation are the kinases and phosphatases which regulate the activity of cyclin/CDK complexes by phosphorylation and dephosphorylation of the CDK proteins. Disruption of any of these genes would result in loss of normal regulation of cell growth.

Abbreviations *ALL* Acute Lymphoblastic leukemias · *CAK* CDC2-activating kinase · *CDK* Cyclin-dependent kinase · *CDI* CDK inhibitor · *GM* Glioblastoma multiforme · *TGF-β* Transforming growth factor-β

Introduction

Progression through the cell cycle is regulated by proteins known as cyclins and their associated cyclin-dependent kinases (CDKs), as illustrated in Fig. 1. Mammalian cells contain at least 11 cyclins (A, B1, B2, C, D1, D2, D3, E, F, G, and H) and 5 CDKs (CDC2, CDK2, CDK4, CDK6, and CDK7); specific cyclin/CDK complexes regulate the different cell cycle checkpoints [1]. One of the most important checkpoints occurs in late G_1, just before the start of DNA replication. This checkpoint is known as START in yeast or as the restriction point in mammalian cells. The D-type cyclins associate with and activate the CDK4 protein, which allows the cell to pass the restriction point. After the cell has passed the restriction point the cyclinE/CDK2 complex forms, and this is believed to be necessary for initiation of DNA replication. CyclinA/CDK2 is required continuously for progression through S phase, and also for the G_2/M transition. Finally, entry into mitosis is signaled by activation of cyclin B/CDC2 complex.

Loss of regulation at cell cycle checkpoints has been linked to cancer, in many cases through changes in the cyclin/CDK complexes. In some cases this is due to disruption of the cyclin genes themselves [2]. Several D-type cyclin genes have been identified as proto-oncogenes, and cyclin E levels are altered in some tumors.

More recently a new type of cell cycle regulator, the CDK inhibitors (CDIs), have been identified. These are proteins which bind to and inhibit the activity of cyclin/CDK complexes. The CDIs are thought to inhibit cell growth in response to both external and internal signals; loss of the restraints imposed on the cell cycle by the CDIs are believed to result in continuous cell growth under conditions which would normally induce growth arrest. The potential tumor suppressor role of the CDIs is clear, and several types of CDI, which appear to have specific cell cycle inhibitory functions, have been cloned and characterized.

p16 and related genes

The closely related p15 and p16 genes are located adjacent to each other on human chromosome 9p21 [3]. The p18 gene product is also related to p16, although the homology to p16 (38% identity) is much less than that of p15 [4]. In the carboxy-terminal region, p15, p16, and p18 have 37%, 21%, and 24% amino acid sequence identity, respectively, to the TAN1 protein, the human homo-

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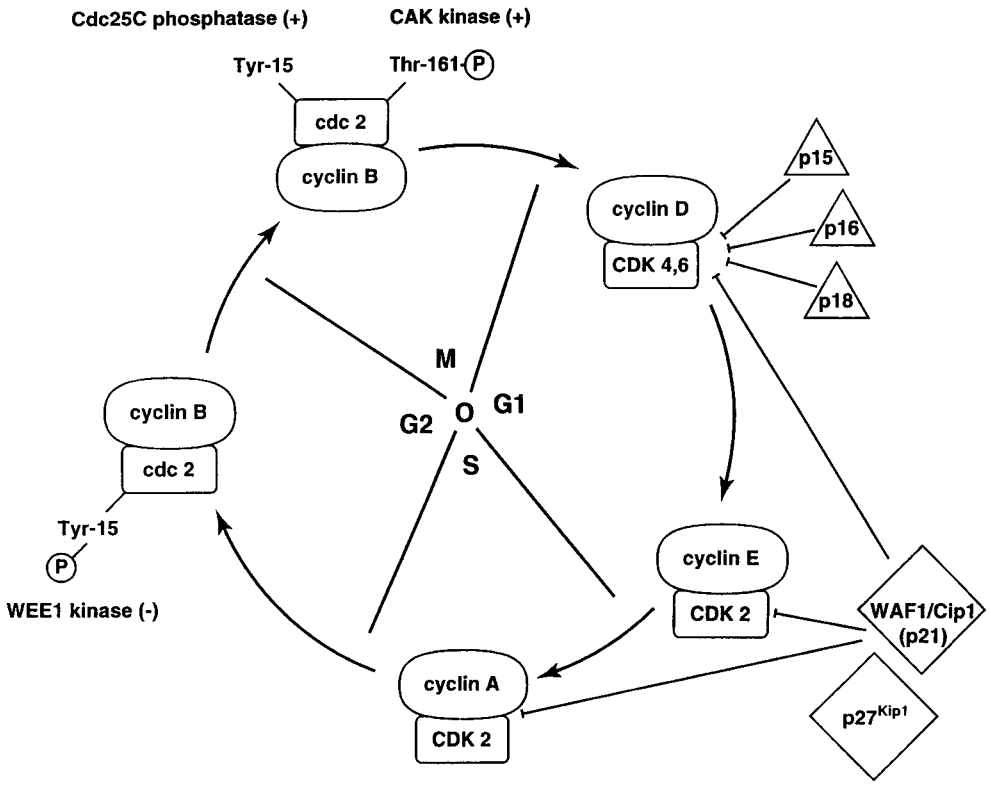

Fig. 1 Inhibitors of cyclin-dependent kinases. A schematic figure shows the cyclin/CDK complexes which are active at various points of the cell cycle. *Right*, inhibitory proteins which form complexes with CDKs; +, -, kinases or phosphatases which activate or inactivate CDC2, respectively

log of the *Drosophila* Notch protein [5]. The region of TAN1 homologous to p15 comprises a motif shared with the yeast cell cycle proteins CDC10 and SW16, with the human erythrocyte protein ankyrin, with the product of the human BCL3 gene, and with the host range proteins of the vaccinia and cowpox viruses. Since this motif is found in genes that regulate the cell cycle and differentiation, it has been inferred that this protein domain has some role in cell growth and differentiation, and that it may be involved generally in protein-protein interactions.

p15, p16, and p18 interact only with CDK4 and CDK6, forming a complex that forces dissociation of cyclins from CDKs. CDK4 and CDK6 are the CDKs which regulate passage through the START checkpoint at G1/S [3, 6]. This strongly suggests that p16 and related CDIs form a family of G_1 CDI proteins which can inhibit the passage of cells through START. Accumulation of p15 mRNA, but not that of p16 mRNA, is highly stimulated by treating HaCaT cells with transforming growth factor-β (TGF-β), and the amount of p15 protein associated with both CDK4 and CDK6 is increased. This suggests that p16 and related CDIs arrest cell growth at START in response to external signals. In lung cancer cells and urothelial cells mutations in the retinoblastoma gene (Rb) result in increased levels of p16 protein [7, 8], suggesting that p16 levels are regulated through a pathway involving Rb. The fact that the cyclin/CDK complexes which are inhibited by p16 also phosphorylate and inactivate Rb suggests the existence of a regulatory loop involving Rb and p16.

The p16 gene has been found to be mutated in the majority of tumor cell lines examined [9, 10]; however, p16 is mutated in a much smaller fraction of primary tumors [11, 12, 13], suggesting that p16 mutations may be linked to survival of cells in culture. Some specific tumor types show a higher proportion of p16 mutations. Analysis of p16 in 27 xenografts and ten cell lines derived from pancreatic adenocarcinoma showed homozygous deletions or sequence changes in 29/37 samples [15]. Unlike primary tumors, xenografts of human tumors in nude mice contain almost no nonneoplastic cells, yet provide an accurate representation of the genetic abnormalities found in the original tumor [14]. Six of the primary tumors from which the xenografts were derived were cryostat-dissected to enrich for neoplastic cells. All six DNAs from primary tumors contained p16 mutations identical to those found in the corresponding xenografts.

p16 mutations were also found in 14 of 27 primary esophageal squamous cell carcinomas examined [16], and all 14 cell lines derived from head and neck squamous cell carcinomas carried p16 mutations [17]. In two cases of head and neck squamous cell carcinomas the mutations in the p16 gene were identical in cell lines derived independently from metastatic tumors and the corresponding primary tumor. This identity is good evidence that the inactivation of p16 was an in vivo event. Similar results were obtained after analysis of lung cancers. Six of 20 non-small-cell lung cancers were found to contain deletions of p16 and/or p15, although no small cell lung cancers were found to contain such deletions [18]. It was suggested that these were in vivo events be-

cause one primary tumor contained the same deletion as the corresponding cell line, and because two cell lines originating from distinct metastatic sites in a single patient both contained deletions of p16.

p16 and p15 are often homozygously deleted in glioblastoma multiforme (GM) but not in other types of brain tumor, such as medulloblastomas and ependymomas [19]. Using a polymerase chain reaction based assay, it was determined that exon 1 of the p16 gene was deleted in 26 of 38 GM xenografts, but in none of 19 other brain tumor xenografts (14 medulloblastomas, 5 ependymomas). It was subsequently determined that the deleted regions were large, and 27 of 38 deletions in GM xenografts included both p16 and the adjacent p15 gene. However, when the 12 tumors without p16 deletions and the 11 tumors without p15 deletions were examined for point mutations in p16 and p15, none was found. This leaves open the possibility that the actual target gene in GM deletions is not p16 or p15, but a third gene, located between p16 and p15 on chromosome 9p21. However, recent experiments using fluorescence in situ hybridization suggest that the p16 region is the target of 9p deletions in gliomas [20]. A p16 probe was hybridized to chromosomes from primary GM tumors, and homozygous deletions of p16 were observed in six of nine tumors; only four of the nine tumors contained deletions of the nearby interferon type 1 gene cluster.

There is a substantial incidence of homozygous p16 deletions in acute lymphoblastic leukemias (ALL), but fewer deletions in other types of lymphoid malignancies. Analysis by DNA blots consistantly reveal deletions or rearrangements of p16 in 10–20% of B-precursor ALL samples [21 22, 23]. Results from T-precursor ALL are more variable, with the observed rate of p16 deletion ranging from 0% [21] to 25% [23] to 80% [22]. A lower incidence of p16 deletions has been observed in Burkitt's lymphoma and non-Hodgkin's lymphoma.

It is possible that p16 is identical to a gene for familial predisposition to melanoma located at 9p21, and known as the *MLM* gene, but studies of p16 mutations in MLM families have not been absolutely conclusive [24, 25]. One study found disease-associated mutations of p16 in 4 of 6 families with a high probability of linkage to 9p21, but the other found potential predisposing mutations in only 2 of 13 9p families screened. It is possible that there were undetected mutations in the 5' untranslated or promoter regions of p16 in some families that had no mutations in the p16 coding region. It is also possible that p16 cosegregates with melanoma because of its proximity to the genuine familial melanoma gene.

The WAF1/Cip1 and p27Kip genes

Another type of CDI gene, variously known as WAF1, Cip1, p21, CAP20, PIC1, and SDI1, was isolated as a p53-inducible gene [26], as an inhibitor of a wide variety of cyclin/CDK complexes [27, 28], and as a senescence-induced gene which inhibits DNA synthesis [29]. Transcription from the WAF1/Cip1 promoter is induced by p53 protein; signals such as DNA damage which activate p53 lead to accumulation of WAF1/Cip1 mRNA and protein. WAF1/Cip1 is believed to arrest cells at G_1/S by inhibiton of cyclin/CDK complexes necessary for progression through G1/S and by binding to proliferating cell nuclear antigen, the processivity subunit of DNA polymerase [30].

In normal cells this p53-induced growth arrest allows time for either repair of DNA damage before resumption of DNA replication or induction of apoptosis. Cells containing mutant p53 do not induce WAF1/Cip1, undergo growth arrest, or undergo apoptosis in response to DNA damage [31, 32]. The relationship between p53-dependent growth arrest and apoptosis is unclear at present, but evidence suggests that these functions are carried out by two independent pathways. Radiation causes normal human fibroblasts to undergo a prolonged arrest state resembling senesence, with long-term elevations of p53 protein, WAF1/Cip1 mRNA, and WAF1/Cip1 protein [33]. In contrast to certain leukemic cells, the irradiated fibroblasts are viable and do not undergo apoptosis, suggesting that the critical function of p53 is to induce growth arrest in cells with DNA damage. On the other hand, evidence from mice in which both p53 and the retinoblasoma genes (Rb) have been inactivated in the developing ocular lens suggests that it is the apoptotic function of p53 that eliminates cells undergoing uncontrolled proliferation [34, 35]. One model has proposed that the p53 pathway preserves genetic integrity by mediating either premanent growth arrest or apoptosis in response to DNA damage, depending on cell type. There is evidence that p53 mediates growth arrest and apoptosis by two distinct mechanisms. p53 can induce growth arrest by induction of the WAF1/Cip1 gene (and probably other genes), but p53-dependent apoptosis can occur in the absence of transcriptional activation of p53-target genes [36]. This result suggests that p53 is a component of the enzymatic machinery of apoptosis; it is possible that the extent of DNA damage determines whether p53 is utilized by the cell as a transcription factor or as an enzyme. It is also possible that cell type determines this choice.

Alterations in the level of WAF1/Cip1 expression have clear effects on cell growth. Overexpression of WAF1/Cip1 inhibits the growth of several tumor cell lines [26], and WAF1/Cip1 is associated with cyclin/CDK complexes in normal fibroblasts, but not in fibroblasts transformed with SV40 tumor virus [37]. However, there is no direct evidence that mutation of WAF1/Cip1 itself is involved in oncogenesis. Few mutations in WAF1/Cip1 have been found in the tumors examined so far [38]. This may be due to the fact that WAF1/Cip1 is essential for cell viability or to overlapping function with other CDIs.

WAF1/Cip1 is clearly involved not only in the p53 pathway but also in other pathways leading to cell differentiation. WAF1/Cip1 is induced in p53-mutant fibroblasts by treatment with various growth factors, phorbol ester, or okadaic acid [39]; phorbol esters also induce

WAF1/Cip1 in p53 mutant hematopoietic cells, and TGF-β induces WAF1/Cip1 in p53 mutant ovarian cancer cells [40, 41]. Recent work demonstrates that WAF1/Cip1 is induced by myogenin and MyoD in a p53-independent manner in myocytes and other types of cell [42, 43], which strongly suggests a role for WAF1/Cip1 in the process of growth arrest preceding differentiation.

Another CDI, p27Kip7, is 44% identical to WAF1/Cip1 in a 60 amino acid segment in the N-terminal half of the protein. Like WAF1/Cip1, p27 inhibits a large number of different CDK/cyclin complexes. In Mv1Lu mink epithelial cells, growth arrest in response to TGF-β or contact inhibition is mediated by p27 [44]. Treatment of quiescent T cells with interleukin-2 causes a decrease in p27 mRNA and loss of p27 protein associated with CDK2/cyclinE, while WAF1/Cip1 mRNA and protein levels increase [45]. This led to the suggestion that p27 may be the primary regulator of CDK activity as cells enter or leave quiescense, while WAF1/Cip1 may regulate CDK activity in cycling cells. This is clearly different from the model suggested for myocytes, where it was suggested that WAF1/Cip1 induction is associated with differentiation. Possibly CDIs have different functions depending on cell type.

Regulation of cyclin/CDK activity by phosphatases and kinases

In addition to regulation through association with proteins such as p16 or WAF1/Cip1, the activity of CKDs is regulated by phosphorylation/dephosphorylation of the CDK protein. Cyclin B/CDC2, the complex which must be activated for entry into mitosis, is inactivated by phosphorylation of CDC2 on tyrosine 15 and threonine 14. Tyrosine 15 is phosphorylated by the WEE1 tyrosine kinase; threonine 14 by a distinct kinase that has not yet been identified [46, 47]. After completion of DNA synthesis tyrosine 15 and threonine 14 are dephosphorylated by CDC25 phosphatases, allowing entry into mitosis. In normal cells DNA damage prevents Tyr 15/Thr-14 dephosphorylation, but many tumor cell lines activate cyclin B/CDC2 and enter mitosis reguardless of the state of the DNA [48, 49]. This suggests that defects in either the phosphorylation or dephosphorylation of CDC2 might have a role in oncogenesis.

Mammalian cells contain three CDC25 genes; *Cdc25A, Cdc25B,* and *Cdc25C*. Both CDC25B and CDC25C dephosphorylate Tyr-15 and Thr-14 of CDC2 CDK, but CDC25C undergoes phosphorylation and activation by the CDC2 kinase, forming a positive feedback loop [50]. CDC25A is activated by cyclinE/CDK2-dependent phosphorylation at the G_1/S transition, suggesting that CDC25A regulates the activity of CDK complexes [51, 52]. Supporting this idea is the fact that CDK2 complexes are negatively regulated by Tyr-15/Thr-14 phosphorylation [53, 54].

CDC2 is also phosphorylated on threonine 161 by CDC2-activating kinase (CAK); this phosphorylation is necessary for binding of cyclin and full activity [55, 56]. CDK2 is also phosphorylated on the equivalent residue [52], and CAK is capable of activating CDK2 in the presence of either cyclinA or cyclin B [57]. The phosphatase which dephosphorylates threonine 161 would clearly be an important regulator of cell growth, but little is known about this phosphatase at present.

The product of the CDI1 gene is a phosphatase which interacts with CDC2 and CDK2 (but not CDK4) and removes phosphates from tyrosine residues [58]. When overexpressed in HeLa cells, Cdi1 delays progression through the cell cycle. CDI1 shows only a weak homolgy to other phospatases, such as the CDC25 proteins, indicating that CDI1 represents a novel class of phosphatases that function in cell cycle progression.

The kinases and phosphatases which regulate CDK/cyclin activity are all possible targets for oncogenic inactivation. In addition, the proteins such as CDC25, CAK, and CDI1, which interact directly with the cyclin/CDK complexes, there are undoubtedly other proteins forming signal transduction pathways which regulate the activity of the CDK kinases and phosphatases. A perturbation anywhere along these pathways might lead to uncontrolled growth and contribute to oncogenesis.

Conclusions

The number of known proteins involved in the regulation of cyclin/CDK activity, and by extension regulation of movement through the cell cycle, has grown rapidly in recent years. Because disruption of normal cell cycle regulation is a key event in oncogenesis, all the cyclin/CDK regulators are potential targets for oncogenic inactivation. To date two genes encoding cyclin/CDK regulators, p16 and WAF1/Cip1, have been analyzed for mutations in primary tumors and tumor cell lines. Although no such mutations have been observed in the WAF1/Cip1 gene, the results with p16 seem to confirm the hypothesis that mutation of cyclin/CDK regulatory proteins can contribute to oncogenesis. In certain types of tumor, such as pancreatic adenocarcinoma and squamous cell carcinoma, mutation of p16 seems to be an in vivo event contributing to tumorogenesis. Mutational analysis of other genes encoding cyclin/CDK regulatory proteins will almost certainly identify additional targets of oncogenic inactivation.

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