p16\textsuperscript{\textit{Ink4a}} Tumor Suppresser Function in Lung Cancer Cells Involves Cyclin-dependent Kinase 2 Inhibition by Cip/Kip Protein Redistribution\textsuperscript{1}

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Abstract

As cell cycle regulators whose activity is frequently altered in human cancers, cyclin-dependent kinases (cdks) are novel targets for therapeutic intervention. cdk inhibition is an emerging strategy for the treatment of non-small cell lung carcinomas (NSCLCs) because most derived cell lines express functional retinoblastoma protein (Rb) but appear to bypass its function with inappropriate cdk activity. Elevated cdk4/cdk6 activity in NSCLC cells is often due to inactivation of the p16\textit{Ink4a} cdk inhibitor. To model the effects of cdk4/cdk6 inhibition, we have expressed p16\textit{Ink4a} in a Rb-positive NSCLC cell line that lacks endogenous p16\textit{Ink4a} expression. Whereas cdk4/cdk6 inhibition and Rb dephosphorylation are expected on p16\textit{Ink4a} expression, we have also observed indirect cdk2 inhibition. cdk2 inactivation by the redistribution of other cdk inhibitors may be required for p16\textit{Ink4a}-mediated growth suppression of Rb-positive cells. The implications of such a requirement on the use of chemical cdk inhibitors to treat human cancers will be discussed.

Introduction

cdk\textsuperscript{2} activity is essential for eukaryotic cell proliferation and, when deregulated, can contribute to oncogenic transformation (1–3). Consisting of a catalytic subunit (cdk1 to cdk8) and a regulatory subunit (cyclin A to cyclin H), specific cyclin-cdk complexes are believed to promote cell cycle progression at discrete “execution points” of the cell cycle. cdk activation is regulated at multiple levels, including: (a) transient synthesis and degradation of regulatory subunits; (b) cyclin-cdk complex assembly; (c) subunit phosphorylation and dephosphorylation; (d) subcellular localization; and (e) binding of various protein inhibitors (4).

Because cells that commit to chromosome duplication in S phase will generally complete a cell cycle, proliferative decisions are made in G\textsubscript{1} phase, when cdk activity is determined by a balance of growth-stimulatory and growth-inhibitory signals. Above a critical threshold, G\textsubscript{1}-phase cdk activity promotes Rb phosphorylation and G\textsubscript{1}-S-phase transition. cdk activity in transformed cells is often decoupled from regulatory signals, allowing Rb phosphorylation and cell proliferation despite serum deprivation, cell-cell contact, loss of anchorage, and the presence of inhibitory cytokines.

Rb regulation of the G\textsubscript{1}-S-phase transition is frequently targeted for inactivation in human cancers (reviewed in Ref. 5). Whereas the Rb-1 gene is frequently deleted or mutated during the development of small cell lung cancers (6), most NSCLC cells express functional Rb but show altered regulation of cdk4 and the closely related enzyme, cdk6 (7–9). Common mutational events in NSCLC that are thought to contribute to unregulated cdk activity include K-ras activation, D-type cyclin overexpression, and inactivation of p16\textit{Ink4a}, whose expression is lost in approximately one-third of NSCLC tumors and in the majority of NSCLC-derived cell lines (7–11). Additionally, point mutations in either p16\textit{Ink4a} or cdk4 which prevent their association appear to compromise cdk4 inhibition and Rb function in other human cancers (12, 13).

Mammalian cells normally require both cdk4/cdk6 and cdk2 activity for S-phase entry (14–17). Whereas cdk2 inhibition prevents G\textsubscript{1}-S-phase transition in all known mammalian cells, cdk4/cdk6 inhibition only affects cells expressing functional Rb, implying that Rb is the key substrate of cyclin D-cdk4/cdk6 enzymes (18, 19). In an active, hypophosphorylated state, Rb can bind histone deacetylase and members of the E2F family of transcription factors, repressing the transcription of E2F-regulated genes and preventing S-phase entry (20–22). cdk phosphorylation of Rb during G\textsubscript{1} phase releases bound proteins, switching E2F-responsive genes from a state of transcriptional repression to one of activation as cells approach S phase (23–25). Genes activated by E2F release encode proteins required for G\textsubscript{1}-S-phase transition and DNA synthesis, including cyclin A, components of replication origins, ribonucleotide reductase, and DNA polymerase \(\alpha\) (26–28). After G\textsubscript{1}-S-phase transition, Rb phosphorylation is maintained until mitosis.

Although Rb is phosphorylated \textit{in vivo} by several cyclin-cdk complexes, including D-type cyclins (cyclins D1, D2, and...
D3) bound to cdk4 or cdk6, cyclin E bound to cdk2, and cyclin A bound to either cdk2 or cdc2, its initial phosphorylation and inactivation in G1 phase is a point of controversy. Rb contains at least 16 consensus cdk phosphorylation sites; although the functional significance of multiple, sequential phosphorylations is not completely understood, several phosphorylation sites are known targets of cyclin D-cdk4/cdk6 and cyclin E-cdk2 kinases (29, 30). Initial Rb phosphorylation by cyclin D-cdk4/cdk6 appears to enable cyclin E-cdk2 activation, leading to further Rb phosphorylation and E2F release (24, 31–33).

G1 phase ckds can be regulated by the binding of inhibitory proteins of two structurally distinct classes. Both the Ink4 (p16INK4a, p15INK4b, p18INK4c, and p19INK4d) and Cip/Kip (p21Cip1/Waf1, p27Kip1, and p57Kip2) families of proteins can inhibit ckds, but in response to different antiproliferative stimuli and with different specificities and mechanisms (3, 34). The Ink4 proteins are characterized by ankyrin-like repeats and by their exclusive binding to cdk4 and cdk6 (35). Cip/Kip proteins share a homologous NH2-terminal domain with both cyclin and cdk-binding regions, and bind a wide range of cyclin-cdk complexes including cyclin D-cdk4/cdk6, cyclin E-cdk2, and cyclin A-cdk2 (36). Individual Ink4 and Cip/Kip proteins can be permanently induced during differentiation or senescence or transiently expressed in response to antiproliferative signals (34, 37, 38). p16INK4a and possibly p15INK4b are tumor suppressors inactivated in various human cancers, whereas other cdk inhibitors do not appear to be targeted for inactivation at such a high frequency (39). Although no inactivating mutations have been detected in any Cip/Kip protein, reduced or absent p27Kip1 expression has been reported in various human cancers and is correlated with a poor patient prognosis (40–43).

NSCLC cell lines that retain Rb expression show p16INK4a inactivation by point mutation, transcriptional silencing, or homozygous gene deletion from chromosome 9 (10, 11, 44). The loss of p16INK4a protein expression in NSCLC cells is important to their proliferation because its reintroduction dramatically slows cell growth (10, 45). During cell transformation, p16INK4a inactivation is thought to undermine cdk4/cdk6 inhibition and cell cycle arrest in response to growth-inhibitory signals. p16INK4a expression in NHBE cells can be induced by either constitutive Ras signaling or senescence.

Because most normal cells are quiescent and lack cdk activity, chemical cdk inhibition may slow the proliferation of tumor cells with minimal effects on normal tissue. cdk inhibition as a therapy for lung cancer, frequently in combination with genotoxic agents such as cisplatin and Taxol (46–48), is currently under clinical evaluation. Chemical cdk inhibitors currently available include the selective compounds olomoucine, butyrolactone, and flavopiridol and the nonselective inhibitors 7-hydroxystaurosporine (UCN-01) and suramin (49–51). Cell cycle studies using these compounds in various mammalian cell culture models have confirmed their effect on cell cycle progression at both the G1-S- and G2-M-phase transitions (49, 50, 52). cdk inhibitors can also either induce or inhibit apoptosis, depending on whether cells are actively proliferating or synchronized to specific cell cycle positions with chemotherapeutics (46, 48, 49, 53, 54).

The identification of p16INK4a as a tumor suppressor inactivated in various human malignancies has prompted a search for specific cdk4/cdk6 chemical inhibitors. Although no cdk4/cdk6-selective compounds have been described to date, high-throughput screening and structural refinement will likely lead to their development (55–57). Currently, the only available cdk4/cdk6 inhibitor is flavopiridol, which is highly specific for ckds but is not selective for the cdk4/cdk6 subfamily (51, 58). Because the loss of p16INK4a expression in NSCLC is important for maintenance of the transformed phenotype, cdk4/cdk6-specific inhibitors may be of use in the treatment of Rb-positive lung cancers. To model the biochemical effects of cdk4/cdk6 inhibition by a selective cdk inhibitor, we have overexpressed the cdk4/cdk6-specific inhibitor p16INK4a in a Rb-positive NSCLC cell line that lacks endogenous p16INK4a protein expression (NCI-H460).

Our results demonstrate that in addition to inhibiting cdk4 and cdk6, p16INK4a expression in NCI-H460 cells indirectly inhibits cdk2. Whereas cdk4 and cdk6 inhibition can be attributed to direct p16INK4a binding, cdk2 inactivation is mediated by the redistribution of p21Cip1/Waf1 and p27Kip1 proteins from cyclin D-cdk4/cdk6 complexes to cyclin E-cdk2 complexes. cdk2 inhibition may be required for p16INK4a-mediated cell cycle arrest of Rb-positive NSCLC cells, as has been shown for Rb-positive human osteosarcoma (U2OS) cells (59). If small molecule cdk inhibitors do not function similarly to p16INK4a in displacing p21Cip1/Waf1 and p27Kip1, cdk4/cdk6 inhibition by chemotherapy will not be equivalent to cdk4/cdk6 inhibition by gene-based therapy and may therefore prove a less effective therapy.

Results

Cyclin Expression and Rb Phosphorylation in NCI-H460 Cells Is Consistent with a Normal Cell Cycle. Before introducing a cell cycle regulator such as p16INK4a to cells lacking endogenous expression, we wished to characterize their pattern of cyclin expression and Rb phosphorylation. Isolation of synchronous populations of cells demonstrated that despite their rapid proliferation and transformed phenotype, NCI-H460 NSCLC cells have an apparently normal profile of cyclin expression and Rb phosphorylation. FACS profiles of cells enriched for early G1 phase, late G1 phase, S phase, and G2-M phase are presented in Fig. 1A. In Fig. 1B, increasing elutriator fraction numbers correspond to samples of increasing cell size and cell cycle transit, beginning with the smallest, early G1 phase cells. Beginning with G1 phase cells, cyclin D1 is the first cyclin whose expression reaches a maximum level before declining, followed closely by cyclin E as cells approach the G1-S-phase boundary, then by cyclin A as cells transit S phase, and finally by cyclin B1 as cells enter mitosis (Fig. 1B). Although its levels do not change appreciably in synchronous cells of some lineages, cyclin D1 protein cycles in proliferating NCI-H460 cells. The pattern of Rb phosphorylation is also characteristic of a normal cell cycle because early G1 phase cells have Rb in a faster-migrating, hypophosphorylated form, which gradually

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4 B. Grimison, unpublished results.
Fig. 1. **A**, cell cycle distribution of control (con) and elutriated NCI-H460 cells as measured by propidium iodide staining of cellular DNA. FACS profiles for elutriator fractions enriched for early G1-phase cells (fraction 2), late G1-phase cells (fraction 5), S-phase cells (fraction 8), and G2-M-phase cells (fraction 11) are presented. Successive elutriator fractions contain cells of increasing size and cell cycle transit, beginning with small, early G1-phase cells. **B**, expression of cell cycle-regulatory proteins in asynchronous (con) and synchronous populations of NCI-H460 cells isolated by centrifugal elutriation (fractions 2–13). Protein extracts were prepared from each fraction, and equal amounts of protein were analyzed by Western blotting with antibodies to the indicated cell cycle regulators, using actin as a loading control.

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No cellular apoptosis was apparent during p16\(\text{INK4a}\) expression because cell numbers did not decrease during a proliferation assay, and no cells with a subdiploid DNA content were detected by FACS analysis (Fig. 2, A and B). Importantly, p16\(\text{INK4a}\) expression did not increase the levels of the cdks inhibitors p21\(\text{CIP1/WAF1}\) and p27\(\text{KIP1}\) during suppression of NCI-H460 cell growth (Fig. 2C).

**Ectopically** **Expressed** p16\(\text{INK4a}\)** Inhibits** **cdk4, cdk6,** and **cdk2** **without ** **Binding** **cdk2.** Because ectopic p16\(\text{INK4a}\) expression in NSCLC cells induced a cell cycle arrest with reduced Rb phosphorylation, we measured the in vitro activity of cdk4, cdk6, and cdk2 before and after p16\(\text{INK4a}\) introduction (Fig. 3A). As expected, cdk4, cdk6, and cyclin D1- and D3-associated kinase activity on GST-Rb was reduced on p16\(\text{INK4a}\) expression (cyclin D2 protein expression was not detectable in NCI-H460 cells). Additionally, in the absence of induction of the cdk2 inhibitors p21\(\text{CIP1/WAF1}\) and p27\(\text{KIP1}\), cdk2 activity was eliminated after p16\(\text{INK4a}\) introduction. The complexity of cdk regulation offers several possible mechanisms by which cdk2 can be inhibited. Coimmunoprecipitation experiments demonstrated that whereas direct cdk binding can account for the effects of p16\(\text{INK4a}\) on the activity of cdk4 and cdk6, it cannot explain the associated cdk2 phosphorylation form, consistent with G1, cdks inhibition and cellular G1- -phase arrest. Detection of cyclin E protein during a p16\(\text{INK4a}\)-mediated arrest was not expected because its expression is largely regulated by E2F, and Rb hypophosphorylation (Fig. 2C) represses E2F activity. Previous studies involving inducible p16\(\text{INK4a}\) expression in osteosarcoma cells have also reported sustained cyclin E protein levels during p16\(\text{INK4a}\) expression (59, 63), and we have observed the same pattern of cyclin expression in primary lung epithelial (NHBE) cells after Ad-p16\(\text{INK4a}\) infection. Cyclin E transcription and protein stability during p16\(\text{INK4a}\) expression are currently under evaluation because E2F-independent mechanisms may contribute to the regulation of cyclin E levels.
inhibition because no binding was detectable (Fig. 3B). The lack of cdk2 binding is consistent with the specificity of p16Ink4a because no association of p16Ink4a with either cdk2 or cdc2 in any cell type has been reported in the literature.

**p16Ink4a Expression Redistributes p21Cip1/Waf1 and p27Kip1 from cdk4/cdk6 to cdk2.** Whereas ectopic p16Ink4a expression in NSCLC cells did not affect the levels of p21Cip1/Waf1 or p27Kip1, the ability of Cip/Kip proteins to bind various cyclin-cdk complexes allows for their redistribution. Ink4 and Kip proteins are known to collaborate in arresting the growth of mink lung epithelial (Mv1Lu) cells exposed to the inhibitory cytokine TGF-β (64). In this case, TGF-β induces p15Ink4b expression, promoting the formation of inactive p15Ink4b-cdk4 and p15Ink4b-cdk6 assemblies. Before TGF-β treatment, p27Kip1 is bound to cdk2, cdk4, and cdk6 complexes. Because Ink4 and Cip/Kip proteins share overlapping binding sites on cdk4 and cdk6, p15Ink4b induction redistributes p27Kip1 from cdk4 and cdk6 complexes to cdk2 complexes (64, 65). Immunoprecipitation of cdk complexes before and after ectopic p16Ink4a expression demonstrated that Ink4 protein expression in NCI-H460 cells similarly redistributes Cip/Kip proteins from cdk4/cdk6 complexes to cdk2 complexes (Fig. 4A). Control virus-infected cells show p21Cip1/Waf1 and p27Kip1 associated with cdk2, cdk4, and cdk6 complexes. When ectopically expressed, p16Ink4a binds cdk4 and cdk6 at the expense of p21Cip1/Waf1 and p27Kip1. Without an observable change in the absolute Cip/Kip protein levels in the cell (Fig. 2C), the amount of p21Cip1/Waf1 and p27Kip1 bound to cdk2 increases after p16Ink4a expression, at a time point when kinase assays indicate that all cdks are inhibited (Figs. 3A and 4A).

Although p21Cip1/Waf1 and p27Kip1 redistribution to cdk2 complexes may be sufficient for cdk2 inhibition, other mechanisms may also contribute. Cyclin absence may explain the lack of cdk2 activity; however, coimmunoprecipitation demonstrates that cyclin E remains associated with cdk2 and even appears to be up-regulated during p16Ink4a expression (Fig. 4A). We have not investigated p57Kip2 induction or cdk2 phosphorylation status, and we cannot exclude their involvement in the observed inhibition of cdk2 activity.

To determine whether Cip/Kip redistribution is unique to NCI-H460 cells, we performed similar coimmunoprecipitation experiments with a second Rb-positive NSCLC cell line (NCI-H661), and NHBE cells. Although cdk2 activity was not assayed, in both cell types adenovirus-mediated p16Ink4a expression led to G1-phase cell cycle arrest (data not shown), a decrease in p21Cip1/Waf1 and p27Kip1 association with cdk4 and cdk6, and a simultaneous increase in association with cdk2. In control virus-infected cells, Cip/Kip proteins are bound to cdk2, cdk4, and cdk6; p16Ink4a expression disrupts p21Cip1/Waf1 and p27Kip1 interactions with cdk4 and cdk6, promoting their relocation to cdk2 (Fig. 4, B and C). As was observed for NCI-H460 cells, p16Ink4a expression had no effect on the total levels of p21Cip1/Waf1 and p27Kip1 protein (data not shown), and cyclin E remained associated with cdk2 during Cip/Kip protein redistribution (Fig. 4, B and C).
Discussion

The redistribution of p21 Cip1/Waf1 and p27Kip1 cdk inhibitors that we observed during the cell cycle arrest of primary lung and RB-positive NSCLC cells ectopically expressing p16Ink4a is reminiscent of the collaboration of p15Ink4b and p27Kip1 in the growth arrest of mink lung epithelial cells exposed to TGF-β (64, 65).

Overexpression of p16Ink4a and induction of p15Ink4b therefore appear to be equivalent in displacing Cip/Kip proteins from cyclin D-cdk4/cdk6 complexes, leading to the increased association of p21Cip1/Waf1 and p27Kip1 with cyclin E-cdk2 complexes and the subsequent inhibition of cdk2 kinase activity, as shown in the present study. Physiological induction of p16Ink4a in primary cells can occur during replicative senescence or in response to constitutive mitogenic signaling as part of a proposed tumor surveillance mechanism (66–69). Consistent with these findings, primary lung epithelial (NHBE) cells show elevated p16Ink4a protein after extended cultivation or during ectopic expression of activated H-Ras and are sensitive to adenovirus-mediated p16Ink4a overexpression.4 Our demonstration of p21Cip1/Waf1 and p27Kip1 redistribution during p16Ink4a expression in primary (NHBE) cells argues that this response to Ink4 protein accumulation is not limited to established cell lines. Whether physiological p16Ink4a accumulation, as seen during primary cell senescence, has the same effect on Cip/Kip protein interactions with cdks is under investigation.

Ink4 protein induction and Cip/Kip protein relocation to cdk2 are proposed to be a general mechanism of cdk inhibition and mammalian cell growth arrest in response to various antiproliferative signals (70). Controversially, this model of inhibitor redistribution proposes that p21Cip1/Waf1 and p27Kip1 exist in active cyclin D-cdk4/cdk6 complexes before the elevation of Ink4 protein levels. Whether Cip/Kip proteins are universal cdk inhibitors is an unresolved question, with different experimental systems producing conflicting results (71–73). The isolation of cyclin-cdk complexes with kinase activity despite the presence of stoichiometric amounts of bound p21Cip1/Waf1 and p27Kip1 is difficult to reconcile with...
available structural information for a crystallized complex of cyclin A-cdk2-p27Kip1 (74). In that trimeric structure, extensive interactions between p27Kip1 and cyclin A-cdk2 dramatically distort the enzyme’s catalytic cleft and prevent ATP binding. Until the structure of a cyclin D-cdk4/cdk6-Cip/Kip protein complex is solved, it will be unclear how p21Cip1/Waf1 or p27Kip1 can exist in an active cyclin-ckd complex, although a growing body of evidence supports this (72, 75–77). Evolutionary distance between cdk subfamilies may explain differing sensitivities to inhibitory proteins and drugs. For example, despite their similarity to other cdk6, cdk2 does not bind Ink4 proteins, and cdc2 (cdk1) does not bind either Ink4 or Cip/Kip proteins. A recent phenotypic analysis of p21Cip1/Waf1 and p27Kip1 doubly null mice indicates that the role of Cip/Kip proteins is not limited to cdk inhibition. Despite their ability to inhibit cdk2 complexes, Cip/Kip proteins are essential for the assembly and nuclear localization of cyclin D-ckd4/ckd6 complexes (77). Inhibitor redistribution and the identification of p21Cip1/Waf1 and p27Kip1 as assembly factors suggest that Cip/Kip proteins and cyclin D-ckd4/ckd6 kinases are functionally interdependent. Whereas cyclin D-ckd4/ckd6 complexes require p21Cip1/Waf1 and p27Kip1 for assembly, in the absence of Ink4 protein expression, these complexes can act as reservoirs for Cip/Kip proteins, limiting their ability to inhibit cyclin E-ckd2 enzymes. This noncatalytic role for cdk4 and cdk6 complexes is important to the regulation of a normal cell cycle because mitogen-stimulated D-type cyclin expression in early G1 phase (Fig. 1) leads to assembly of cyclin D-ckd4/ckd6 complexes, sequestering Cip/Kip proteins and allowing the activation of cdk2 as cells approach S phase (78, 79). Redistribution of Cip/Kip proteins during Ink4 protein expression suggests that cdk2 inhibition is required for efficient growth arrest of mammalian cells (59, 63). Human osteosarcoma cells (U2OS) and rodent fibroblasts engineered to overexpress cyclin E and constitutively activate cdk2 are able to bypass a growth arrest imposed by hypophosphorylated Rb after expression of either p16ink4a or a phosphorylation site-deficient form of Rb (59, 80). Furthermore, endogenous cyclin E overexpression is seen in a subset of primary human breast tumors that express Rb but are resistant to p16ink4a overexpression (81). The ability of cyclin E to promote S-phase entry without Rb phosphorylation and E2F activation implies the existence of a cdk2-regulated pathway parallel to Rb phosphorylation which, under certain circumstances, can substitute for E2F activation (80). G1 phase acceleration by ectopic cyclin expression is additive when both cyclin D1 and cyclin E are expressed, arguing that two rate-limiting events are required for G1-S-phase transition, and that these events are independently controlled by cyclins D1 and E (23). A cdk2-regulated event parallel to Rb phosphorylation may explain why cdk2 inhibition by antibody microinjection or a dominant-negative cdk2 mutant arrests Rb-negative cells in G1 (14, 15). Whereas both p16ink4a and a phosphorylation site-deficient form of Rb can arrest Rb-positive cells in G1, only p16ink4a-expressing cells maintain a prolonged arrest correlated with the inhibition of cdk2 by Cip/Kip protein redistribution (82).

The observation of p16ink4a inactivation in various human cancers has prompted efforts to develop drugs that can mimic its cellular function. However, compounds currently under development as specific cdk4/ckd6 inhibitors may not fully substitute for p16ink4a protein during suppression of cell growth. If cdk2 inhibition is required for efficient cell cycle arrest, chemical inhibitors specific to cdk4/ckd6 may only have a lasting effect on cell growth if they redistribute Cip/Kip proteins to cdk2. Whereas small molecule inhibitors may effectively inhibit cdk4/ckd6 activity, they may be too small to displace associated Cip/Kip proteins. If so, cdk4/ckd6 inhibition by chemotherapy will not be mechanistically equivalent to cdk4/ckd6 inhibition by a gene-based therapy. Chemical inhibition of cdk4/ckd6 may be more similar to expression of a dominant-negative form of cdk4 or a phosphorylation site-deficient form of Rb, both of which do not affect cdk2 activity and can only temporarily arrest cell growth. When specific cdk4/ckd6 chemical inhibitors are developed, an important functional test in cultured cells will be whether p21Cip1/Waf1 and p27Kip1 are redistributed, and whether cdk2 activity is inhibited.

Materials and Methods

Cell Culture. NCI-H460 and NCI-H661 cells were originally established at the National Cancer Institute, Washington, DC (83) and obtained from the University of Colorado Cancer Center Tissue Culture Core Facility. Cells were cultured in RPMI 1640 with 10% fetal bovine serum in a humidified 37°C incubator. NHBE cells and serum-free bronchial epithelial cell growth medium were purchased from Clonetics. NHBE cells were cultured in a humidified 37°C incubator and expanded by approximately six population doublings prior to use.

Recombinant Adenoviruses. The recombinant adenovirus encoding p16ink4a protein was provided by Introgen Therapeutics (Houston, TX); its original construction is described in Jin et al. (45). The Ad-GFP virus used as a control was generously provided by Dr. Jerome Schaack (Department of Microbiology, University of Colorado Health Sciences Center, Denver, CO).

Western Blotting. Cells were washed with PBS and lysed with hot Laemmli sample buffer (84) at 2–5×10^6 cells/ml. Lysates were boiled, sheared through a syringe needle, and stored at −20°C. Volumes of cell extracts corresponding to approximately 75 µg were loaded on 7%, 10%, or 12% polyacrylamide gels and then electroblotted (Idea Scientific) at 15 V for 45 min onto nitrocellulose (Schleicher & Schuell). The following primary antibodies were used in this study: (a) anti-cyclin A (#06-138), anti-cyclin B1 (#05-158), anti-cyclin D1 (#06-137), anti-cyclin E (#06-459), anti-cdk2 (#06-505), and anti-cdk4 (#06-139) from Upstate Biotechnology; (b) anti-actin (I-19) and anti-cdk6 (C-21) from Santa Cruz Biotechnology; (c) anti-p21Cip1/Waf1 (Ab-3) and anti-p27Kip1 (Ab-1) from NeoMarkers; (d) anti-p16ink4a (Ab-2) from Oncogene Research; and (e) anti-Rb (#14001A) from Pharmingen. Horseradish peroxidase-conjugated goat antimouse IgG (Jackson ImmunoResearch) or goat antirabbit IgG (Bio-Rad) was used as secondary antibody, and immune complexes...
were detected using Western blot chemiluminescence reagent (New England Nuclear).

**DNA Staining of Cell Nuclei with Propidium Iodide and FACS Analysis.** Subconfluent cells were harvested using PBS with 1 mM EDTA, and approximately 1 × 10^6 cells were analyzed for cell cycle distribution. Cells were washed with PBS, fixed with ice-cold 70% ethanol, and then washed with PBS before resuspension in 0.5 ml of 30 mM sodium citrate and 70 μM propidium iodide. After adding 1 μl of 9.5 mg/ml DNase-free RNase A, samples were incubated at 37°C for 30 min in the dark and stored overnight at 4°C (85). FACS analysis was performed by the University of Colorado Cancer Center Flow Cytometry Core, and cell cycle distributions were estimated using ModFit software.

**Immunoprecipitation/kinase Assays.** Kinase assays were performed as described by Matsushime et al. (86), except that a GST fusion to the COOH-terminal 137 amino acids (amino acids 792–928) of human Rb was used as a substrate (87). Approximately 3 × 10^6 control and adenovirus-infected cells were washed with PBS and scraped into 1 ml of HEPES/Tween lysis buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, 0.1% Tween 20, and 10% glycerol] with freshly added protease and phosphatase inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 2 μg/ml aprotinin, 10 μg β-glycerophosphate, 1 mM NaF, and 0.1 mM sodium orthovanadate). Lysates were frozen in liquid nitrogen and stored at −80°C until use. After thawing on ice, lysates were clarified by centrifugation at 10,000 × g for 10 min at 4°C, and protein concentrations were determined using a Bradford protein assay.

Immunoprecipitations were performed with 500 μg of lysate and 0.5 μg of antibody bound to 10 μl of protein A- or protein G-Sepharose (Amersham Pharmacia Biotech) by rotating for 3 h at 4°C. The antibodies used to immunoprecipitate cdk2, cdk4, and cdk6 are the same as those used for Western blotting; antibodies to cyclins D1 and D3 were from NeoMarkers (clones DCS-11 and DCS-28.1). Immunocomplexes were washed three times with cold HEPES/Tween lysis buffer and twice with cold kinase buffer [50 mM HEPES (pH 7.5), 10 mM MgCl₂, 2.5 mM EGTA, 1 mM DTT, 10 mM β-glycerophosphate, 1 mM NaF, and 0.1 mM sodium orthovanadate, and 20 μM ATP]. Samples were resuspended in 30 μl of kinase buffer containing 2 μg of GST-Rb fusion protein (see below) and 10 μCi of [γ-32P]ATP (Amersham Pharmacia Biotech); after incubation at 30°C for 30 min with occasional mixing, reactions were stopped by adding 30 μl of hot 2× Laemmli sample buffer and boiling for 5 min. Samples were resolved by SDS-PAGE in 10% gels and Western blotted for immunoprecipitated and coimmunoprecipitated proteins.

**Acknowledgments**

We thank Maria C. Todd for critical reading of the manuscript and James V. DeGregori for assistance with adenovirus production and infections.

**References**


