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Growth suppression by $p16^{ink4}$ requires functional retinoblastoma protein

(cell cycle/cyclin D/cyclin-dependent kinases)

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ABSTRACT **$p16^{ink4}$ has been implicated as a tumor suppressor that is lost from a variety of human tumors and human cell lines. $p16^{ink4}$ specifically binds and inhibits the cyclin-dependent kinases 4 and 6. *In vitro*, these kinases can phosphorylate the product of the retinoblastoma tumor suppressor gene. Thus, $p16^{ink4}$ could exert its function as tumor suppressor through inhibition of phosphorylation and functional inactivation of the retinoblastoma protein. Here we show that overexpression of $p16^{ink4}$ in certain cell types will lead to an arrest in the G_1 phase of the cell cycle. In addition, we show that $p16^{ink4}$ can only suppress the growth of human cells that contain functional pRB. Moreover, we have compared the effect of $p16^{ink4}$ expression on embryo fibroblasts from wild-type and RB homozygous mutant mice. Wild-type embryo fibroblasts are inhibited by $p16^{ink4}$, whereas the RB nullizygous fibroblasts are not. These data not only show that the presence of pRB is crucial for growth suppression by $p16^{ink4}$ but also indicate that the pRB is the critical target acted upon by cyclin D-dependent kinases in the G_1 phase of the cell cycle.**

$p16^{ink4}$ was originally identified as a polypeptide bound to cyclin-dependent kinase 4 (cdk4) in human diploid fibroblasts transformed with the DNA tumor virus simian virus 40 (SV40) (1). The $p16^{ink4}$ gene was subsequently cloned using a two-hybrid protein interaction screen to identify proteins that can associate with human cdk4 (2). Interestingly, the same gene was identified as a putative tumor suppressor through an extensive analysis of deletions and rearrangements of chromosome 9p21 present in human tumor lines (3, 4). The $p16^{ink4}$ gene has since been found to be deleted or rearranged in a large number of human primary tumors (5). Binding of $p16^{ink4}$ to cdk4 prevents association of cdk4 with the D-type cyclins and results in an inhibition of the catalytic activity of the cyclin D/cdk4 enzymes (2). More recently, it was demonstrated that $p16^{ink4}$ can also bind and inhibit cdk6, the alternative kinase partner of the D-type cyclins (6). Hence, $p16^{ink4}$ seems to target specifically the cyclin D-dependent kinase activity by competing with D cyclins for binding to their kinase partners.

Complexes formed by cdk4/cdk6 and cyclin D have been strongly implicated in the control of cell proliferation during the G_1 phase of the cell cycle (7, 8). Three different human D-type cyclins have been identified, D1, D2, and D3 (9, 10). *In vitro*, complexes of cyclin D1, D2, or D3 and cdk4 or cdk6 can phosphorylate the product of the retinoblastoma tumor suppressor gene, pRB (11, 12). The phosphorylation of pRB, occurring in mid/late G_1, reverses its growth-inhibitory effect and enables cells to proceed from G_1 to S phase. The timing of cyclin D-dependent kinase activity and the onset of pRB hyperphosphorylation appear to coincide in the cell cycle. Taken together, these findings suggest that cyclin D/cdk complexes play a critical role in pRB hyperphosphorylation *in vivo*. If so, $p16^{ink4}$ could negatively regulate cell proliferation by suppressing hyperphosphorylation and functional inactivation of pRB.

Here we show that overexpression of $p16^{ink4}$ in certain cells will lead to an arrest in the G_1 phase of the cell cycle. In contrast, cells that lack functional pRB appear not to be affected by high levels of $p16^{ink4}$, demonstrating that pRB mediates the growth suppression by $p16^{ink4}$. Since $p16^{ink4}$ has been shown to specifically inhibit cyclin D/cdk4/cdk6 complexes, these findings indicate that pRB is the critical target of these complexes in order to promote progression through the G_1 phase of the cell cycle.

MATERIALS AND METHODS

Plasmid Constructs. A human $p16^{ink4}$ cDNA was obtained by PCR amplification of a HeLa cell cDNA library (a kind gift of C. Sardet, Whitehead Institute) using two oligonucleotide primers, a 5′ primer (5′-GGAATTCACCACCATGGAGCCTTCGGCTGAC-3′) and a 3′ primer (5′-GGAATTCTCGAGTCAATCGGGGATATCTGAGGGACC-3′). A 472-bp fragment was amplified, purified on a low-melting agarose gel, and cloned directly into pGEM-T (Promega). The resulting plasmid was then used to isolate an *Eco*RI/*Xho* I fragment containing the $p16^{ink4}$ coding region, which was cloned into pcDNA3 (Invitrogen) to construct pCMV.$p16^{ink4}$. pCMV.CD20, pCMV.cdk4, and pCMV.cdk2 were all provided by S. van den Heuvel (Massachusetts Institute of Technology, Cambridge).

Recombinant Viruses. The *Eco*RI/*Xho* I fragment from pGEM.p16ink4 was cloned into the *Eco*RI/*Sal* I site of pBabe.puro (13). The resulting construct was introduced into ψ-CRE cells by electroporation. Two days after electroporation the medium was changed to Dulbecco's modified Eagle medium (DMEM) supplemented with 10% calf serum (CS) and 2 μg of puromycin per ml. Puromycin-resistant colonies were pooled and used as producer lines. The pBabe.puro vector was used to obtain a control virus-producing ψ-CRE line.

Cell Culture, Transfections, and Flow Cytometry. U2OS, SAOS-2, and C33A cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS). U2OS and SAOS-2 cells have been described previously (14); C33A cells (15) were provided by L. Zhu (Massachusetts General Hospital, Charlestown). ψ-CRE cells (16) were cultured in DMEM supplemented with 10% CS. Cells were transfected using the standard calcium phosphate technique (17). Two days after transfection, cells were prepared for and analyzed by flow cytometry analysis as described (15).

Infections. Cultures of virus-producing ψ-CRE cells were incubated overnight with 10 ml of DMEM containing 10% CS per 10-cm tissue culture dish. The medium was filtered through

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Abbreviations: cdk, cyclin-dependent kinase; RB, retinoblastoma; SV40, simian virus 40; large T, large tumor antigen.

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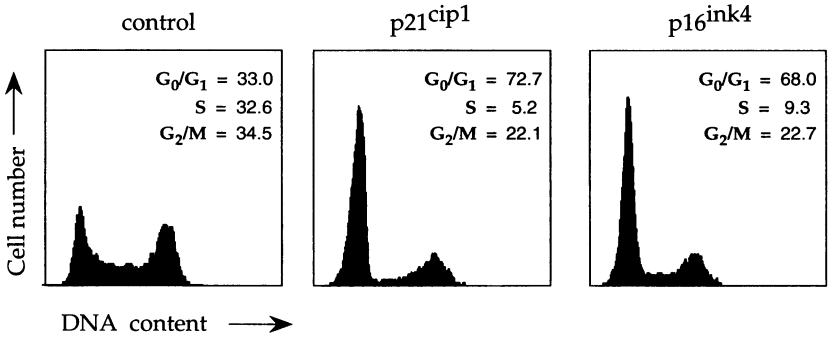

FIG. 1. G_1 arrest by $p16^{ink4}$. U2OS osteosarcoma cells were cotransfected with 20 μg of the indicated plasmids and 2 μg of pCMV.CD20 encoding a cell surface marker. Two days after transfection, cells were analyzed using flow cytometry. Histograms were obtained by collecting data from 5000 CD20-positive cells. The cell number is plotted on the *y* axis, and the relative DNA content is on the *x* axis. The percentage of cells in a particular compartment of the cell cycle is indicated. One representative experiment of at least five independent experiments is shown.

0.22-μm filters and supplemented with 8 μg of Polybrene per ml. Only freshly prepared virus stocks were used for infections. Cultures of mouse embryo fibroblasts were infected by replacing the medium with 5 ml of virus stock per 10-cm dish. This procedure was repeated once, after which cells were cultured in DMEM with 10% FCS for 2 days. Cells were subsequently placed under puromycin selection by changing the medium to DMEM with 10% FCS and 2 μg of puromycin per ml.

Detection of $p16^{ink4}$ Expression. Spontaneously immortalized mouse embryo fibroblasts infected with control or $p16^{ink4}$-producing virus were metabolically labeled at 48 hr postinfection with 0.1 mCi of [^{35}S]methionine per ml (1 Ci = 37 GBq) for 4 hr. Cells were subsequently lysed in E1A lysis buffer (150 mM NaCl/50 mM Hepes, pH 7.5/5 mM EDTA/20 mM NaF/1 mM phenylmethylsulfonyl fluoride/10 μg of leupeptin per ml/10 μg of trypsin inhibitor per ml/0.1% Nonidet P-40). $p16^{ink4}$ was immunoprecipitated with an anti-human $p16^{ink4}$ polyclonal antiserum (PharMingen), and immunoprecipitates were separated on a 15% polyacrylamide gel. For Western blotting total cell extracts were prepared by adding 0.5 ml of Laemmli sample buffer to a confluent 10-cm dish. Extracted proteins were separated on a 15% polyacrylamide gel and blotted to nitrocellulose. p16 was detected with anti-$p16^{ink4}$ (PharMingen) diluted 1:1000 in phosphate-buffered saline (PBS) to which 5% dry non-fat milk was added. Bound antibody was detected with enhanced chemiluminescence (ECL) (Amersham) according to the manufacturer's directions.

[^{3}H]Thymidine Incorporation. Primary cultures (passage 3) were plated on 24-well plates 1 day prior to infection. Cells were infected with control or $p16^{ink4}$-encoding virus by three subsequent infections of 3 hr with 2 ml of conditioned medium from the ψ-CRE producer cells per well, supplemented with 8 μg of Polybrene per ml. Twenty-four hours postinfection cells were washed twice with DMEM. To each well 0.5 ml of DMEM containing 1 μCi of [^{3}H]thymidine per ml was added and cells were labeled for 4 hr. Cells were washed with PBS and fixed with methanol. Cells were washed three times with PBS and lysed in 0.5 ml of 0.2 M NaOH. [^{3}H]Thymidine incorporation was determined by scintillation counting.

RESULTS

Overexpression of $p16^{ink4}$ Causes a G_1 Arrest in U2OS Ostoesarcoma Cells. A common characteristic of tumor sup-

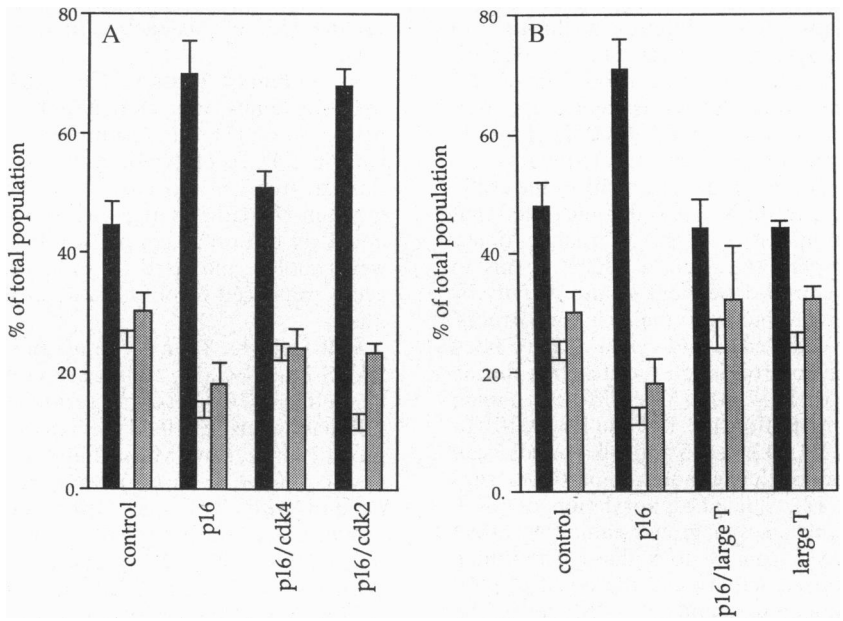

FIG. 2. Reversion of $p16^{ink4}$-mediated growth suppression by cdk4 and SV40 large T. (*A*) U2OS cells were transfected with 2 μg of pCMV.CD20 and 5 μg of the $p16^{ink4}$ expression vectors as indicated, and 15 μg of pCMV.cdk4 or pCMV.cdk2 was cotransfected as shown. (*B*) U2OS cells were transfected with 2 μg of pCMV.CD20, 10 μg of pCMV.$p16^{ink4}$, and 10 μg of pCMV.LT (large T) as indicated. The total amount of DNA in each transfection was normalized to 22 μg by addition of pcDNA3. Two days after transfection, cells were analyzed using flow cytometry. Black bars represent the percentage of G_0/G_1-phase cells, white bars represent S-phase cells, and gray bars represent G_2/M-phase cells. Standard errors calculated from percentages obtained from three independent experiments are indicated.

pressor genes is their ability to inhibit cell proliferation when overexpressed in sensitive cell lines (14, 15, 18, 19). To determine whether $p16^{ink4}$ can inhibit cell proliferation, we employed a modified flow cytometry technique (15). Control vectors or expression vectors coding for $p16^{ink4}$ were cotransfected with a plasmid expressing the CD20 cell surface marker (20). Cotransfection of this plasmid enabled us to identify cells that contain transfected DNA by staining with an anti-CD20 monoclonal antibody conjugated to fluorescein isothiocyanate, and the DNA content of the transfected cells could be measured independently by propidium iodide staining.

Transfection of an expression plasmid encoding $p16^{ink4}$ in the human osteosarcoma cell line U2OS resulted in a very significant increase (≈35%) in the fraction of CD20-positive cells in the G_0/G_1 phases of the cell cycle, consistent with its putative role as tumor suppressor. Similarly, transfection of another inhibitor of cyclin/cdks, $p21^{cip1}$, also caused an accumulation of cells in the G_0/G_1 phases of the cell cycle (Fig. 1). This latter inhibitor is known to antagonize several cyclin/cdk complexes, including cdks 2, 4, and 6 (21–23), and our own work has confirmed that overexpression of $p21^{cip1}$ in a variety of cell lines causes a G_1 arrest (R.H.M. and R.A.W., unpublished results).

Growth Suppression by $p16^{ink4}$ Is Abolished by cdk4 and SV40 Large Tumor Antigen (Large T). Since $p16^{ink4}$ competes with cyclin D for binding to cdk4 and cdk6, we reasoned that substantial overexpression of cdk4 should partially or completely relieve the $p16^{ink4}$-imposed cell cycle arrest. Indeed, the increase in the G_0/G_1 population established by $p16^{ink4}$ in U2OS cells was greatly reduced when a plasmid coding for human cdk4 was cotransfected (Fig. 2*A*). In contrast, cotransfection of an expression vector encoding human cdk2 did not significantly affect the arrest mediated by $p16^{ink4}$ (Fig. 2*A*). This reinforces the notion that the inhibition of cell proliferation by $p16^{ink4}$ is mediated through its ability to bind cdk4/6 and indicates that $p16^{ink4}$ acts as an inhibitor of cyclin D/cdk complexes not only *in vitro* but also *in vivo*.

Upon transformation with a variety of viral oncoproteins, cdk4 is no longer found in complex with D cyclins but associates exclusively with $p16^{ink4}$ (1). This finding suggests that the critical substrates of cyclin D/cdk complexes themselves have been inactivated by viral transformation, thereby obviating cyclin D/cdk activity. To confirm that cyclin D/cdk4/cdk6 function is no longer needed in the presence of DNA tumor virus oncoproteins, we cotransfected a SV40 large T expression vector together with a $p16^{ink4}$ vector. Fig. 2*B* shows that growth inhibition induced by $p16^{ink4}$ was completely reversed by cotransfection of SV40 large T. This demonstrates that SV40 large T can indeed drive cell proliferation in cells that lack cyclin D-dependent kinase activity. Similarly, it was recently demonstrated that suppression of cellular transformation by $p16^{ink4}$ can be overcome by the E1A oncoprotein (24). Since SV40 large T and E1A are known to bind and inactivate pRB, these findings seem consistent with the hypothesis that $p16^{ink4}$ inhibits cell proliferation through its effects on pRB. However, based on these observations one cannot rule out that growth suppression by $p16^{ink4}$ might be mediated through other viral oncoprotein targets—for example, p107 or p130, both of which are also bound and inactivated by SV40 large T and E1A.

Overexpression of $p16^{ink4}$ Does Not Arrest Human Cell Lines That Lack Functional pRB. To resolve among these possibilities, we studied the effects of overexpressing $p16^{ink4}$ in two human tumor cell lines known to lack functional pRB, specifically the SAOS-2 osteosarcoma cell line and a cervical carcinoma cell line, C33A. Expression of $p16^{ink4}$ in these cell lines did not give rise to any increase in the G_1 population, in sharp contrast to the G_1 arrest previously observed in U2OS cells (Table 1). However, expression of $p21^{cip1}$ blocked SAOS-2 and C33A cells in G_1, similar to the effect it had in U2OS cells (Table 1). This is in agreement with the observation that $p21^{cip1}$ inhibits a larger array of cyclin/cdk complexes than does $p16^{ink4}$ and inhibits DNA replication by a mechanism independent of cyclin/cdks (25). A similar lack of growth suppression by $p16^{ink4}$ when expressed in human cell lines that do not contain functional pRB was recently observed by others (24, 26). However, while consistent with the role of pRB as the critical mediator of cyclin D/cdk4/6 control, these observations are hardly definitive, since many distinct genetic lesions are likely to exist in these cell lines. Moreover, SAOS-2 cells have been reported to have very low levels of cyclin D1 (14, 27, 28), and complexes of D1 and cdk4 or cdk6 could not be detected in C33A cells (27, 28), further confounding the interpretation of these data.

Effects of $p16^{ink4}$ on Wild-Type and RB Mutant Mouse Embryo Fibroblasts. We reasoned that unequivocal evidence for a role for pRB in $p16^{ink4}$-mediated growth arrest could only be obtained through a comparison of two cell types that are genetically identical, except for the presence or absence of pRB. For this reason, we decided to study the effects of overexpression of $p16^{ink4}$ in primary mouse embryo fibroblasts derived from wild-type embryos and from RB nullizygous embryos (29). Another advantage of using these cells is that the endogenous levels of cyclin D1 and cdk4 are very similar in both cell types (R.E.H., unpublished observations). To express $p16^{ink4}$ in these cells, we generated a retrovirus carrying the $p16^{ink4}$ coding region and the puromycin-resistance gene, the latter being used to select for successfully infected cells. As a control, we infected cells with a similar virus that lacked the $p16^{ink4}$ coding region.

Infection of RB nullizygous fibroblasts as well as the wild-type fibroblasts with the control virus gave rise to a large, similar number of puromycin-resistant cells, confirming the comparable infectability of the two cell types (Fig. 3). However, infection of these two cell types with the much lower-titered $p16^{ink4}$ virus gave a very different outcome: 10–20 colonies per 10-cm culture dish were obtained upon infection of the pRB homozygous mutant cells, while no colonies arose from the infected wild-type cultures (Fig. 3). This provided clear evidence that the presence of pRB is critical to $p16^{ink4}$-mediated growth arrest.

To substantiate further these observations, we determined the levels of $p16^{ink4}$ following infection with the $p16^{ink4}$ retro-

Table 1. Absence of a $p16^{ink4}$-induced growth arrest in human tumor lines that lack functional pRB

	Transfected plasmid								
	Control			pCMV.$p21^{cip1}$			pCMV.$p16^{ink4}$		
Cell line	G_0/G_1	S	G_2/M	G_0/G_1	S	G_2/M	G_0/G_1	S	G_2/M
U2OS	44 ± 4	19 ± 4	37 ± 5	73 ± 4	5 ± 4	22 ± 5	68 ± 6	9 ± 5	23 ± 2
SAOS-2	47 ± 2	22 ± 2	31 ± 4	64 ± 1	9 ± 2	27 ± 1	36 ± 1	21 ± 1	43 ± 1
C33A	48 ± 4	24 ± 4	28 ± 5	63 ± 4	14 ± 4	23 ± 5	44 ± 6	22 ± 5	34 ± 2

Cells were transfected with 20 μg of the indicated plasmid and 2 μg of pCMV.CD20. Two days after transfection the cells were prepared for flow cytometry. Indicated is the percentage of CD20-positive cells present in the respective phases of the cell cycle. The percentages and standard errors shown here are obtained by combining the data of three independent experiments.

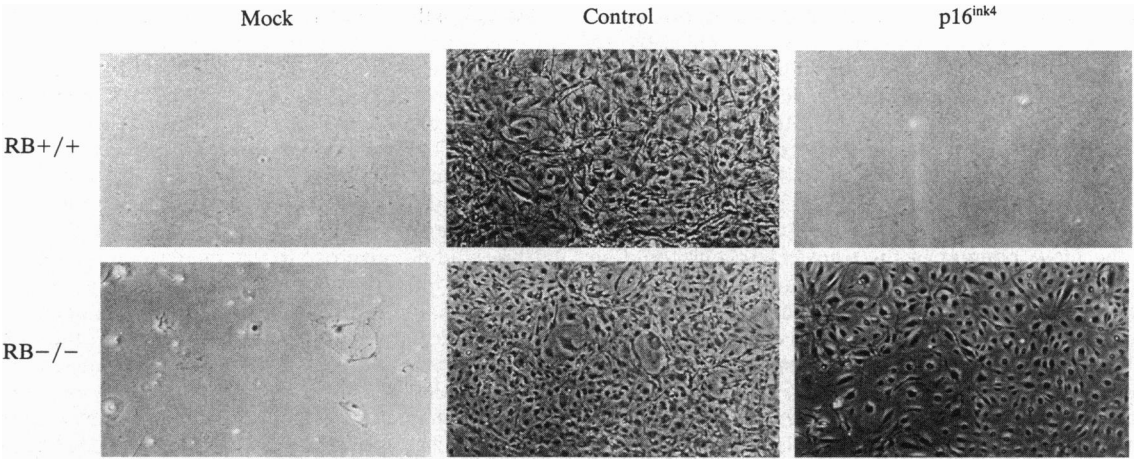

FIG. 3. Effect of p16^{ink4} on wild-type and RB nullizygous mouse embryo fibroblasts. RB homozygous mutant (*Lower*) and wild-type (*Upper*) embryo fibroblasts were infected with p16^{ink4}-encoding virus or control virus as indicated. Mock-infected cells were maintained in DMEM containing 10% CS for the same period of time. Twenty-four hours postinfection the growth medium was switched to medium containing 2 μg of puromycin per ml. Pictures were taken 2 weeks after initiation of puromycin selection. At this time the uninfected dishes no longer contained any cells. (×40.)

virus vector. Since the number of successfully infected primary cells was rather low, we infected spontaneously immortalized RB nullizygous and wild-type embryo fibroblasts with the p16^{ink4}-encoding virus. As shown in Fig. 4*A*, greatly increased amounts of p16^{ink4} could indeed be immunoprecipitated after infection with this virus (in this case from the mutant cells) (lane 2). In addition, a clear coimmunoprecipitating band is seen (Fig. 4*A*) that comigrates with murine cdk4 (not shown). Upon puromycin selection of these immortalized cultures infected with the p16^{ink4} virus, a large fraction of the RB nullizygous fibroblasts continued to grow (>10^5 colonies per 10-cm dish), but only a very small number of wild-type cells survived (2–5 colonies per 10-cm dish). We compared the level

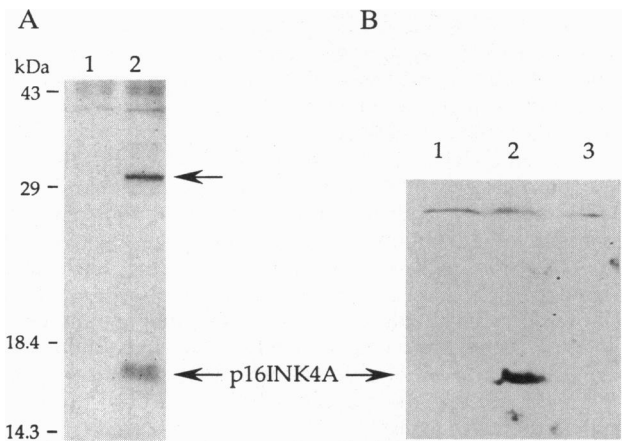

FIG. 4. Expression of p16^{ink4} in spontaneously immortalized mouse embryo fibroblasts. (*A*) Spontaneously immortalized fibroblasts derived from RB nullizygous mouse embryos were infected with control (lane 1) or p16^{ink4} encoding virus (lane 2). Two days postinfection cells were metabolically labeled, p16^{ink4} was immunoprecipitated, and immunoprecipitates were separated on a 15% polyacrylamide gel. Molecular size markers are shown in kDa. The coimmunoprecipitating band of ≈32 kDa is indicated with an arrow. (*B*) Spontaneously immortalized fibroblasts derived from RB nullizygous (lanes 1 and 2) or wild-type (lane 3) mouse embryos were infected with control (lane 1) or p16^{ink4}-encoding virus (lanes 2 and 3). Two days postinfection the culture medium was changed to medium containing 2 μg of puromycin per ml. Cells were grown on this selective medium for 2 months, after which the amount of p16^{ink4} in each population was determined by Western blotting. Equal amounts of protein were loaded in each lane.

of p16^{ink4} in these immortalized, puromycin-selected wild-type and RB mutant populations by immunoblot analysis. p16^{ink4} was readily detected in the RB nullizygous population (Fig. 4*B*, lane 2), while no p16^{ink4} was detectable in the wild-type cells that survived infection with the p16^{ink4} virus followed by puromycin selection (Fig. 4*B*, lane 3). This clearly indicates that the RB nullizygous cells can tolerate high levels of p16^{ink4}, whereas the wild-type cells cannot.

Finally, we investigated whether infection with the p16^{ink4} virus would cause a significant inhibition of S-phase entry. To do so, we infected primary cultures of wild-type and RB homozygous fibroblasts with high multiplicities of the control and p16^{ink4} viruses and measured levels of [^{3}H]thymidine incorporation 24 hr after infection (Fig. 5). A very significant inhibition (44%) of thymidine incorporation was observed in wild-type embryo fibroblasts infected with the p16^{ink4} virus compared to the control virus. In contrast, the fibroblasts from the RB nullizygous embryos were unaffected. From this experiment we cannot distinguish whether the observed reduction in thymidine incorporation in the wild-type cells is the

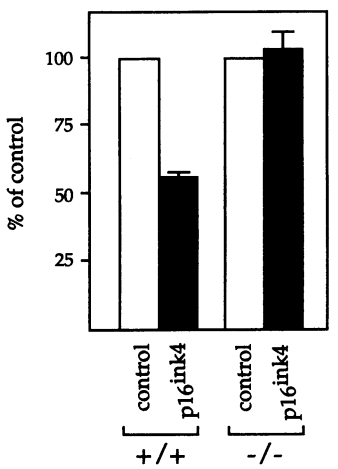

FIG. 5. p16^{ink4} blocks S-phase entry in wild-type but not RB nullizygous fibroblasts. Primary cultures of wild-type and RB nullizygous mouse embryo fibroblasts were seeded on 24-well plates and infected with control or p16^{ink4}-producing virus. Twenty-four hours after infection [^{3}H]thymidine incorporation was determined. Values of thymidine incorporation from the cultures infected with the control virus were set at 100%.

result of an arrest in G_1 or in another phase of the cell cycle. However, it clearly demonstrates that the effect of $p16^{ink4}$ on cell cycle progression is mediated by pRB and seems most consistent with an arrest in G_1.

DISCUSSION

Here we show that the growth-suppressive effect of $p16^{ink4}$ is dependent on the presence of functional pRB. Since the actions of $p16^{ink4}$ are likely mediated largely, if not entirely, through inhibition of cyclin D/cdk4/cdk6 complexes, these findings indicate that pRB is the critical target of these complexes. These findings are consistent with the observation that microinjection of anti-cyclin D1 antibodies or cyclin D1 antisense plasmids, which causes a G_1 arrest in normal fibroblasts, has no effect in cells lacking functional pRB (30). In contrast, microinjection of anti-cyclin E antibodies will cause a G_1 arrest in wild-type as well as RB mutant cells, indicating that this latter cyclin has distinct substrates that are critical for cell cycle progression (31).

The evidence presented here connects two tumor suppressors, $p16^{ink4}$ and pRB, in one pathway controlling cell growth. In this pathway cyclin D/cdk complexes would mediate the inactivation of pRB, either directly or indirectly. This inactivation causes the release of several proteins, including the E2F transcription factors, which allows entry into S phase. Interestingly, a number of distinct genetic aberrations have been observed in human tumors, which could all possibly result in loss of growth regulation through this pathway. On the one hand, loss of pRB or $p16^{ink4}$ by point mutation or deletion has been demonstrated in a variety of human tumors (3, 4, 32). On the other hand, translocation and amplification of cyclin D1 or cdk4 have also been observed in a number of tumors (33–36). Finally, although no examples are known at present of amplification of one of the E2F family members in human tumors, overexpression of E2F-1 was shown to cause neoplastic transformation of cells in culture (37).

Several lines of evidence suggest that inactivation of this growth-regulatory pathway might be an absolute requirement for the progression of certain tumors. A recent report demonstrated that 85% of the human astrocytomas analyzed showed either homozygous deletion of $p16^{ink4}$ or, alternatively, amplification of the cdk4 gene (38). Also, a number of groups recently observed an inverse correlation between the expression of wild-type pRB and $p16^{ink4}$ in many human tumor cell lines (39–41). Now that it is becoming evident what the components of this pathway are, it will be of interest to check the status of each of these components within one type of tumor.

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