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17. The targeting vector was constructed by modification of pMC1neo-polyA+ (Stratagene) with the use of segments of a C57BL/6 *Fgf4* genomic clone (*6*) and strategies based on published restriction maps and sequences (*18*): 5′ homology segment 4.25-kbp Sac I–Sac II genomic fragment including the transcriptional promoter and 105 bases of the 5′ untranslated region; Neo^r coding sequence, 1.0-kbp Mlu I–Bam HI segment lacking the polyoma virus promoter; and 3′ homology segment, 2.2-kbp Bss HII–Pvu II fragment (converted to Bam HI ends with linkers) extending from intron 1 into 3′ untranslated sequence.
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19. Nde I–linearized vector was electroporated (*2*) into CB1-4 ES cells [derived from C57BL/6 × Rb(11:16)2H/Rb(16:17)32Lub progeny], and standard procedures were used for G418 selection, DNA extraction, and Southern (DNA) blot hybridization (*27*).
20. Homologous recombinant ES cells were microinjected into MF1 (Harlan Sprague-Dawley) or C57BL/6 (Taconic Farms) blastocysts and fostered into avertin-anesthetized mice as described (*24*). Chimeras were crossed with C57BL/6 mice, and DNA was isolated from the tails of the F_1 progeny. Bgl II–digested DNA was hybridized with Neo^r and exon 1–specific probes generated by polymerase chain reaction (PCR) amplification of cloned wild-type or mutant sequences with oligos immediately upstream (CCACCGTTGCGTCCCTATT) and downstream (GGAGCTCGACTCTACTCAG) of the deleted exon. All animal work was done in accordance with federal and New York State guidelines.
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28. The χ^2 values for genotype distributions were derived with the use of a null hypothesis predicting 25% *Fgf4*$^{-/-}$ embryos. The significance of high-frequency abnormal E5.5 and E6.5 decidua from heterozygote intercrosses was assessed by χ^2, with the use of the combined average frequency of abnormal decidua seen in control (4 in 84 amongst *Fgf4*$^{+/-}$ × *Fgf4*$^{+/+}$ and wild-type progeny) and experimental matings to define null hypothesis abortive development rates of 7.7 and 11.6%, respectively.
29. Lysate (1 to 3 μl) was amplified in a 100-μl PCR reaction containing 2.5 U of native Pfu DNA polymerase (Stratagene), 20 mM tris-HCl (pH 8.2), 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 200 μM each deoxynucleoside triphosphate, and 50 pmol each of primers 1 (TCAAAAGGCTTCGGCGGCT), 2 (GAGAGCTCCAGAAGACCTG), and N (CGGGTGTTGGGTCGTTTGT) with 1 cycle at 97°C for 5 min, 55°C for 5 min, and 75°C for 2 min followed by 40 cycles at 97°C for 30 s, 55°C for 30 s, and 75°C for 2 min. Twenty microliters of each reaction was run on a 1.5% agarose gel, Southern blotted, and hybridized with a 438–base pair (bp) *Fgf4* probe generated by PCR with nested upstream (TACTGCAACGTGGGCATCGGA) and downstream (AAAGCCTAGGTGACCCTGGAC) primers. Control PCR experiments with limiting dilutions of *Fgf4*$^{+/-}$ genomic DNA template demonstrated that the sensitivities for detecting null or wild-type alleles are comparable.
30. We thank L. Schleifer for his support of this work. We also thank C. Epstein for providing CB1-4 ES cells; L. Breiman for statistical analyses; J. Griffith for providing oligonucleotides; and B. Galeano, C. Murphy, and D. Mahoney for graphics and photography. Supported in part by grants HD27198 and HD21988 from the National Institute of Child Health and Development.

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Inhibition of Ras-Induced Proliferation and Cellular Transformation by p16^{INK4}

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The cyclin-dependent kinase 4 (CDK4) regulates progression through the G_1 phase of the cell cycle. The activity of CDK4 is controlled by the opposing effects of the D-type cyclin, an activating subunit, and p16^{INK4}, an inhibitory subunit. Ectopic expression of p16^{INK4} blocked entry into S phase of the cell cycle induced by oncogenic Ha-Ras, and this block was relieved by coexpression of a catalytically inactive CDK4 mutant. Expression of p16^{INK4} suppressed cellular transformation of primary rat embryo fibroblasts by oncogenic Ha-Ras and Myc, but not by Ha-Ras and E1a. Together, these observations provide direct evidence that p16^{INK4} can inhibit cell growth.

The CDK4–cyclin D kinase complex promotes progression through the G_1 phase of the cell cycle (*1*). In normal cells, the retinoblastoma tumor suppressor protein (Rb) regulates cell proliferation by binding and sequestering transcription factors essential for S phase (*2*). These transcription factors are released at late G_1 by phosphorylation of Rb, thereby allowing cells to enter S phase (*2*). The main function of the CDK4–cyclin D kinase complexes may be to phosphorylate Rb at late G_1 (*3*). Indeed, transformed cell lines lacking functional Rb do not require the activity of the CDK4–cyclin D kinase to proliferate, and these cell lines are devoid of CDK4–cyclin D complexes (*4*). The p16^{INK4} protein has been biochemically characterized as a protein that specifically binds to and inhibits CDK4, and thus p16^{INK4} may regulate Rb phosphorylation (*5*). The p16^{INK4} protein appears to act as a tumor suppressor protein because the p16^{INK4} gene (also called *MTS1*, *CDK4I*, or *CDKN2*) is frequently deleted in tumor cell lines and shows a high frequency of point mutations and small deletions in some tumor cell lines and primary tumors (*6*).

To examine the effect of p16^{INK4} on entry into S phase, we microinjected cultured rat embryo fibroblasts (REF-52) arrested in G_0 by serum starvation with a DNA plasmid encoding activated Ha-Ras (V12Ras) together with a plasmid encoding human p16^{INK4} in either sense (p16^{INK4}-s) or antisense (p16^{INK4}-αs) orientation relative to the promoter (*7*). DNA synthesis was monitored 30 hours after injection by immunostaining of 5-bromodeoxyuridine (BrdU) incorporated into newly synthesized DNA (*8*). Microinjection of a V12Ras expression plasmid either alone or together with the p16^{INK4}-αs plasmid stimulated the incorporation of BrdU in ~25% of the injected cells, whereas only 2% of the cells injected with the vector plasmid stained positive for BrdU (Fig. 1, A and B). These results are consistent with the values previously reported for V12Ras-induced mitogenesis in this microinjection assay (*9*). V12Ras-induced stimulation of DNA synthesis was reduced by 80% upon coinjection of p16^{INK4}-s (Fig. 1, A and B). The expression of V12Ras and p16^{INK4} in the injected cells was confirmed by double immunofluorescence staining (Fig. 1C) (*10*). These results indicate that expression of p16^{INK4} can prevent V12Ras-induced entry into S phase.

To test the specificity of the inhibition of V12Ras-induced mitogenesis by p16^{INK4}, we asked whether the effect of p16^{INK4} could be counteracted by coexpression of a catalytically inactive CDK4 mutant. We expected that the exogenous CDK4 mutant might bind p16^{INK4} and relieve the p16^{INK4}-mediated inhibition of cell growth. The catalytically inactive CDK4 mutant, CDK4-K35M, has a methionine in place of the conserved lysine at position 35 that is probably required for binding to adenosine triphosphate (*11*). We first analyzed the ability of CDK4-K35M to bind to p16^{INK4}. In vitro–translated Cdc2, CDK2, CDK4, and CDK4-K35M proteins were incubated

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with purified glutathione-S-transferase (GST)–p16^{INK4} fusion protein, and the resulting complexes were recovered on glutathione-Sepharose beads. Cdc2 and CDK2 did not bind to GST-p16^{INK4}, whereas wild-type CDK4 did bind (Fig. 2A) (*5*). The catalytically inactive mutant CDK4-K35M also bound to GST-p16^{INK4} at least as efficiently as the wild-type protein (Fig. 2A). Injection of V12Ras and CDK4-K35M plasmids resulted in a moderate inhibition (29%) of V12Ras-induced DNA synthesis (Fig. 2B). This inhibition could result from the sequestration of D-type cyclins by CDK4-K35M. Injection of V12Ras and p16^{INK4}-s expression plasmids resulted in a 63% inhibition of V12Ras-induced DNA synthesis (Fig. 2B). This inhibitory effect was attenuated compared with that described in Fig. 1B because the amount of p16^{INK4}-s plasmid used in this experiment was reduced to half the amount used in the previous experiment in order to maintain the total amount of injected DNA constant among experiments. When cells were microinjected with a mixture of three plasmids expressing V12Ras, CDK4-K35M, and p16^{INK4}-s, the inhibitory effect of p16^{INK4} was relieved (Fig. 2B). This effect of the CDK4-K35M mutant could result from sequestering p16^{INK4} such that it no longer interacts with wild-type CDK4 or other presumptive targets of p16^{INK4}.

The mitogenic stimulation of quiescent cells by Ras is initiated by the rapid transcriptional activation of many cellular immediate-early genes including the c-*fos* gene (*12*). To determine whether the expression of p16^{INK4} interferes with early signaling events initiated by Ras, we examined the effect of p16^{INK4} on the expression of a V12Ras-induced reporter construct in which the transcription of the chloramphenicol acetyltransferase gene (CAT) is under the control of five copies of the serum response element (SRE) derived from the human c-*fos* promoter (5XSRE-CAT) (*13*). The V12Ras-induced transcriptional activation of the SRE is dependent, among other factors, on the p62^{TCF} transcription factor, which is phosphorylated and activated by mitogen-activated protein (MAP) kinase (*14*). REF-52 cells were microinjected with the 5XSRE-CAT reporter plasmid and CAT induction was monitored by immunofluorescence staining with antibodies to CAT. In this system, CAT induction can be detected as early as 3 hours after injection and is dependent on MAP kinase activation (9). REF-52 cells injected with a mixture of plasmids containing V12Ras, 5XSRE-CAT, and p16^{INK4}-αs or p16^{INK4}-s exhibited similar amounts of CAT staining indicating that the expression of p16^{INK4} does not interfere with Ras-regulated gene expression during early G_1 (Fig. 3). This result suggests that the inhibitory effect of p16^{INK4} is exerted during late G_1 phase, and it is consistent with the role of p16^{INK4} as an inhibitor of CDK4 because the activity of the CDK4–cyclin D complexes is required for cell cycle progression at late G_1 phase.

The p16^{INK4} protein may function as a tumor suppressor (6), and therefore we tested whether p16^{INK4} suppressed cellular transformation. The rat embryo fibroblast (REF) cotransformation assay (*15*) was used

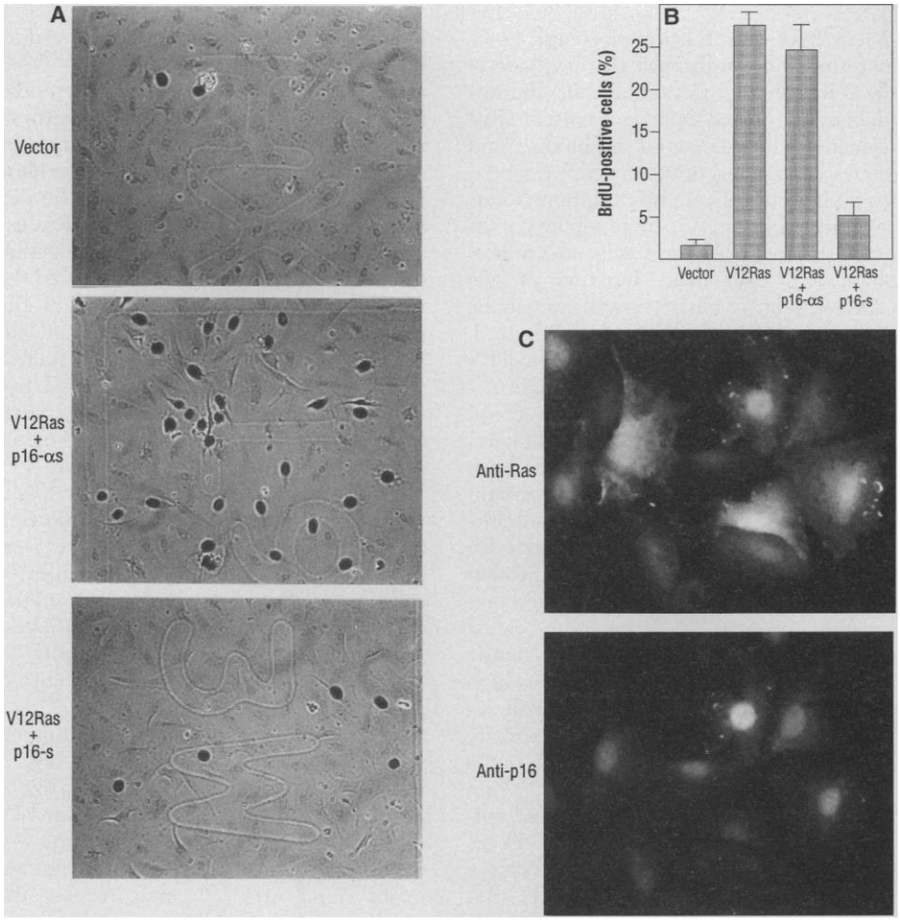

Fig. 1. Inhibition of V12Ras-induced DNA synthesis by p16^{INK4}. (**A**) Immunocytochemical staining of REF-52 cells with antibody to BrdU 30 hours after microinjection of the indicated expression plasmids (*7, 8*). (Top) pDCR (5 ng/ml) and pRc/CMV (40 ng/ml) vectors. (Middle) V12Ras (5 ng/ml) and p16^{INK4}-αs (40 ng/ml). (Bottom) V12Ras (5 ng/ml) and p16^{INK4}-s (40 ng/ml). (**B**) Percentage of BrdU-positive REF-52 cells 30 hours after microinjection of the indicated plasmids. Values correspond to the average of three independent assays in which at least 200 injected cells were scored per condition in each assay. Error bars correspond to the standard deviation. (**C**) Double immunofluorescence staining of REF-52 cells with antibody to Ras (Anti-Ras, top) or antibody to p16^{INK4} (Anti-p16, bottom) 15 hours after injection of both V12Ras (5 ng/ml) and p16^{INK4}-s (40 ng/ml) expression plasmids (*7, 10*).

Table 1. Effect of p16^{INK4} on cellular transformation induced by either Myc with V12Ras or E1a with V12Ras (*16*). Values shown are from two independent experiments.

Transfected DNA	Exp. 1		Exp. 2	
	No. of foci	%*	No. of foci	%
myc + V12*ras*	35	100	123	100
myc + V12*ras* + p16^{INK4}	7	20	18	15
E1a + V12*ras*	62	100	220	100
E1a + V12*ras* + p16^{INK4}	78	126	317	144

*Foci obtained in the presence of p16^{INK4} as a percentage of the number of foci obtained in its absence.

Table 2. Flow cytometry analysis of Saos-2 cells transfected with a plasmid encoding p16^{INK4} (*22*). Values are shown for a representative assay.

Transfected DNA	Percent of cells in		
	G_0/G_1	S	G_2/M
pRc-CMV	57	12	31
pRc-p16	55	12	33
pRc-p21	77	6	17
pRc-Rb	89	3	6

to examine and compare the antioncogenic activity of $p16^{INK4}$ when cells were transformed with plasmids encoding either c-Myc and V12Ras or adenovirus E1a and V12Ras. In this assay, the oncogenic activity of cooperating oncogenes is measured by the number of foci that appear in the cell culture monolayer 7 to 10 days after transfection and by the malignant phenotype of these transformed foci as determined in part by their ability to be established as permanent cell lines (*16*, *17*). Under the conditions used, transfection with either V12Ras or c-Myc expression plasmids did not produce foci (*15*, *18*). Monolayer cultures of early-passage REF cells were transfected with pairs of plasmids encoding either c-Myc and V12Ras or E1a and V12Ras in the presence or absence of an equimolar amount of a plasmid encoding $p16^{INK4}$ (*16*). Transfection of REF cells with a mixture of plasmids encoding $p16^{INK4}$, Myc, and V12Ras resulted in an 80 to 85% reduction in the number of foci compared with that generated by the combination of Myc and V12Ras (Table 1). Moreover, the foci that arose in the presence of $p16^{INK4}$ exhibited an attenuated malignant phenotype as evidenced by a reduction in their subcloning efficiency (*17*) and by the slow growth rate of the subclones obtained (*18*). In contrast, $p16^{INK4}$ had no effect on focus formation induced by E1a with V12Ras (Table 1). The $p16^{INK4}$ protein was expressed in all the subclones derived from cells transfected with the mixture of plasmids encoding E1a, V12Ras, and $p16^{INK4}$, as determined by protein immunoblotting (*17*). These results suggest that $p16^{INK4}$ can suppress cellular transformation and that this suppression is overcome by the E1a oncoprotein. Because the induction of cellular transformation by E1a is probably mediated, at least in part, through its ability to bind and inactivate Rb (*19*), one possible interpretation of our results is that the $p16^{INK4}$-mediated suppression of cellular growth is dependent on the presence of Rb. To further investigate the functional relation between $p16^{INK4}$ and Rb, we tested whether cells lacking the Rb gene are insensitive to the growth-suppressor activity of $p16^{INK4}$. The human osteosarcoma Saos-2 cells are characterized by a nonfunctional deletion of the Rb gene and have been used extensively as an $Rb^{-/-}$ cell line model (*20*). Proliferating Saos-2 cells were transiently transfected with a plasmid encoding the cell-surface marker CD20 to detect the transfected cells, together with one of the plasmids encoding $p16^{INK4}$, Rb, or the general CDK inhibitor p21 (*21*). After transfection, cells were stained for the presence of the CD20 marker and their DNA content was analyzed by flow cytometry (*22*). Transient expression in Saos-2 cells of either the general CDK inhibitor p21 or Rb significantly decreased the number of cells in S and (G_2 + M) phases, suggesting the induction of G_1 arrest (Table 2). In contrast, no G_1 arrest was observed when Saos-2 cells were transiently transfected with $p16^{INK4}$ (Table 2). These results suggest that the absence of functional Rb protein renders the cells insensitive to the growth-suppressor activity of $p16^{INK4}$. In this context, it is of interest to note that in many tumor cell lines the presence of $p16^{INK4}$ is accompanied by the absence of functional Rb protein, and conversely the absence of $p16^{INK4}$ is accompanied by the presence of functional Rb (*23*, *24*). The inverse correlation between $p16^{INK4}$ and Rb expression further supports the hypothesis that $p16^{INK4}$ and Rb function within the same pathway. Together, our data indicate that $p16^{INK4}$ can inhibit cell proliferation, possibly as a result of the inhibition of CDK4 and hence the accumulation of Rb in its dephosphorylated growth-inhibitory state at G_1.

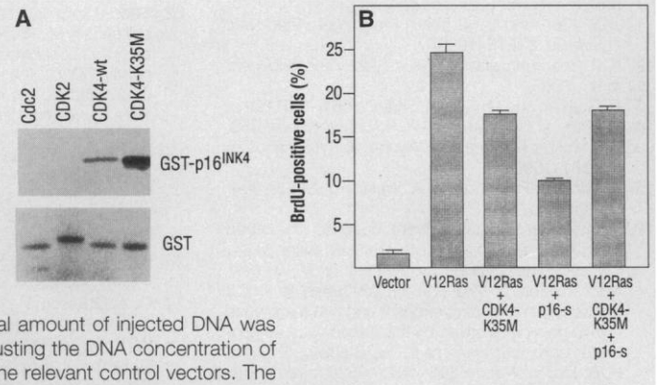

Fig. 2. Effect of a catalytically inactive CDK4 mutant on inhibition of V12Ras-induced DNA synthesis by $p16^{INK4}$. (**A**) Binding assay of ^{35}S-methionine–labeled in vitro-translated CDKs to GST-p16 (*5*). (**B**) Percentage of BrdU-positive cells 30 hours after injection of the indicated expression plasmids into REF-52 cells (*7*, *8*). The total amount of injected DNA was maintained constant by adjusting the DNA concentration of the injected solutions with the relevant control vectors. The concentrations of the injected expression plasmids or the corresponding control vectors were as follows: V12Ras or pDCR vector, 5 ng/ml; $p16^{INK4}$-s or pRc/CMV vector, 20 ng/ml; and CDK4-K35M or pCEP4 vector, 20 ng/ml. Values correspond to the average of two independent assays scoring at least 200 injected cells per condition in each assay. Bars indicate the range of values obtained in the two assays.

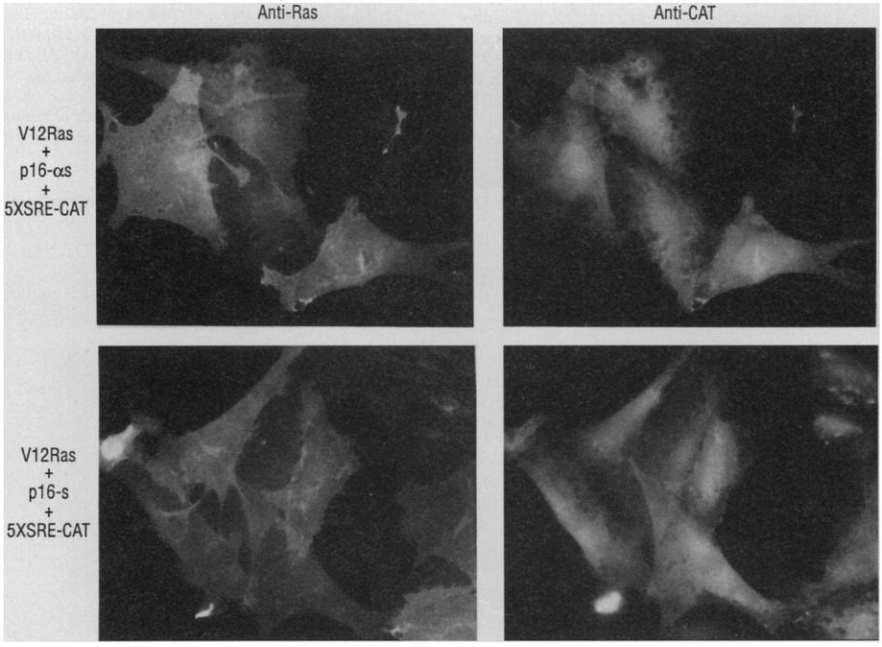

Fig. 3. Lack of effect of $p16^{INK4}$ on induction of 5XSRE-CAT by V12Ras. Double immunofluorescence staining of REF-52 cells with antibody to Ras (Anti-Ras, left panels) or antibody to CAT (Anti-CAT, right panels) 3 hours after injection of the indicated expression plasmids (*7*, *10*). (**Top panels**) V12Ras (5 ng/ml), 5XSRE-CAT (50 ng/ml), and $p16^{INK4}$-αs (40 ng/ml). (**Bottom panels**) V12Ras (5 ng/ml), 5XSRE-CAT (50 ng/ml), and $p16^{INK4}$-s (40 ng/ml).

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7. REF-52 cells were plated onto gridded glass cover slips and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal bovine serum (FBS, 10%). The cells were grown to confluency, then placed in starvation medium (DMEM with 0.5% FBS) for 24 hours before microinjection. A mixture containing the indicated plasmids in microinjection buffer [50 mM Hepes (pH 7.2), 100 mM KCl, 5 mM $NaHPO_4$] was microinjected into the nuclei of all the cells present in a chosen square of the gridded cover slip. The cover slips were then processed according to the corresponding immunological procedure. V12Ras was cloned into the expression vector pDCR under a cytomegalovirus (CMV) promoter; $p16^{INK4}$ was cloned into the pRc/CMV vector (Invitrogen) in either sense ($p16^{INK4}$-s) or antisense ($p16^{INK4}$-αs) orientation with respect to the CMV-derived promoter; and CDK4-K35M was cloned into the CMV expression vector pCEP4 (Invitrogen).
8. For monitoring DNA synthesis, BrdU was added to the cell culture medium at 10 μM immediately after microinjection (*7*). After 30 hours, cells were fixed and permeabilized in acid alcohol (ethanol:water:acetic acid, 90:5:5) for 1 hour at −20°C. The cover slips were incubated with mouse antibody to BrdU and then with horseradish peroxidase-conjugated goat antibody to mouse immunoglobulin G (IgG). Immunocytochemical color development was done according to the manufacturer's instructions (Cell Proliferation Kit, Amersham). At least 200 injected cells were scored in each assay for quantitation.
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10. At the indicated time after injection (*7*), cells were fixed in 3.7% formaldehyde in phosphate-buffered saline (PBS) for 1 hour, and then permeabilized with 0.1% Triton X-100 in PBS for 3 min at room temperature. For V12Ras and $p16^{INK4}$ detection, the cover slips were incubated with a mixture of rat antibody to Ras (Y13-259) [M. E. Furth, J. L. Davis, B. Fleurdelys, E. M. Scolnick, *J. Virol.* **43**, 294 (1982)] and rabbit antibody to $p16^{INK4}$ (*5*) in PBS containing bovine serum albumin (2 mg/ml) and then with a mixture of fluorescein-conjugated goat antibody to rat IgG (Cappel) and rhodamine-conjugated goat antibody to rabbit IgG (Cappel). For detection of V12Ras and CAT, the cover slips were incubated with Y13-259 and rabbit antibody to CAT (5′ → 3′ Co.) and then incubated with a mixture of secondary antibodies as indicated above. The expression of the endogenous rat $p16^{INK4}$ cannot be detected because the antibody to human $p16^{INK4}$ used in this study does not cross-react with the rat $p16^{INK4}$. The nuclear localization of $p16^{INK4}$ in the injected cells is consistent with that observed for the endogenous protein in human cell lines (*23*).
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17. After transfection of REF cells with plasmids encoding Myc and V12Ras in the absence or presence of a $p16^{INK4}$ expression plasmid (*16*), foci were isolated, and their ability to form subclonal cell lines was tested as described (*25*). When foci were obtained from a Myc-V12Ras transformation assay, 92% of the foci tested (11 out of 12) grew as independent subclones. In contrast, when foci were obtained from a Myc-V12Ras-$p16^{INK4}$ transformation assay, only 36% of the foci tested (4 out of 11) grew as independent subclones. Three of these subclones were analyzed by protein immunoblotting, and expression of $p16^{INK4}$ was observed in all of them. The genetic alterations that allowed these subclones to overcome the growth inhibition exerted by $p16^{INK4}$ are not known.
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