

Cellular Response to Oncogenic Ras Involves Induction of the Cdk4 and Cdk6 Inhibitor p15^{INK4b}

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Received 26 July 1999/Returned for modification 13 September 1999/Accepted 27 January 2000

The cell cycle inhibitor p15^{INK4b} is frequently inactivated by homozygous deletion together with p16^{INK4a} and p19^{ARF} in some types of tumors. Although the tumor suppressor capability of p15^{INK4b} is still questioned, it has been found to be specifically inactivated by hypermethylation in hematopoietic malignancies in the absence of p16^{INK4a} alterations. Here we show that, in vitro, p15^{INK4b} is a strong inhibitor of cellular transformation by Ras. Surprisingly, p15^{INK4b} is induced in cultured cells by oncogenic Ras to an extent similar to that of p16^{INK4a}, and their expression is associated with premature G₁ arrest and senescence. Ras-dependent induction of these two *INK4* genes is mediated mainly by the Raf-Mek-Erk pathway. Studies with activated and dominant negative forms of Ras effectors indicate that the Raf-Mek-Erk pathway is essential for induction of both the p15^{INK4b} and p16^{INK4a} promoters, although other Ras effector pathways can collaborate, giving rise to a stronger response. Our results indicate that p15^{INK4b}, by itself, is able to stop cell transformation by Ras and other oncogenes such as Rgr (a new oncogene member of the Ral-GDS family, whose action is mediated through Ras). In fact, embryonic fibroblasts isolated from p15^{INK4b} knockout mice are susceptible to transformation by the Ras or Rgr oncogene whereas wild-type embryonic fibroblasts are not. Similarly, p15^{INK4b}-deficient mouse embryo fibroblasts are more sensitive than wild-type cells to transformation by a combination of the Rgr and E1A oncogenes. The cell cycle inhibitor p15^{INK4b} is therefore involved, at least in some cell types, in the tumor suppressor activity triggered after inappropriate oncogenic Ras activation in the cell.

The transforming activity of oncogenes has been extensively studied in the last 2 decades. Early on, transformation of primary cells was observed to require two cooperating oncogenes to convert normal cells to a tumorigenic state (27, 59). The resistance of primary cells to transformation by single oncogenes can now be explained as the effect of the induction of tumor suppressor genes by inappropriate oncogenic signals (reviewed in reference 73). Thus, prolonged oncogenic Ras activity produces in primary cells an increase in the levels of p16^{INK4a}, p21^{Cip1}, and p53, which, in turn, is dependent on the transcriptional induction of its positive regulator p19^{ARF} (32, 48, 64). Induction of the expression of these genes by Ras is accompanied by growth arrest in the G₁ phase of the cell cycle and a phenotype indistinguishable from premature senescence (33, 64). Both the p16^{INK4a} and p19^{ARF} proteins are expressed from a complex gene structure, the *INK4a* locus (for reviews, see references 13 and 67). Each of the two proteins uses a different exon 1, and both use the same exon 2, but each protein is translated in a different reading frame (53). Although their amino acid sequences are completely different, both proteins are cell cycle inhibitors. p16^{INK4a} is a potent inhibitor of cyclin-dependent kinases 4 and 6 (Cdk4/6) (61), whereas p19^{ARF} stabilizes the p53 tumor suppressor gene (for reviews, see references 7, 66, and 67).

In both humans and mice, the *INK4a* locus is located close to a second gene of the *INK4* family, p15^{INK4b}, which also func-

tions as a Cdk4/6 inhibitor and is strongly induced by transforming growth factor β (TGF- β) (14, 22, 54). Both loci, *INK4a* and *INK4b*, are frequently deleted in a variety of tumors and cell lines (22, 58). In addition, these proteins can also be inactivated by point mutations or methylation (reviewed in references 50 and 58). The expression of proteins p16^{INK4a}, p15^{INK4b}, and p19^{ARF} can be decreased by hypermethylation of the CpG island upstream of corresponding exon 1 in both humans (17, 41, 56) and rodents (36, 69). No clear tumor suppressor role has been assigned to the other two members of the *INK4* family, p18^{INK4c} and p19^{INK4d}.

Whereas the evidence for a tumor suppressor role of p16^{INK4a} is abundant, the role of p15^{INK4b} in tumor suppression is more controversial. In most tumors, homozygous deletions affect both the *INK4a* and *INK4b* loci or the *INK4a* locus alone. In only a few cases have specific deletions of p15^{INK4b} sequences been reported, i.e., leukemias and lymphomas, which are among the tumors with higher involvement of p15^{INK4b} deletions (58). Point mutations, which are relatively frequent in *INK4a*, only rarely occur in p15^{INK4b} (36, 50). In contrast, inactivation of p15^{INK4b} by hypermethylation seems to be selectively frequent in leukemias and lymphomas and does occur independently of p16^{INK4a} status (4, 17, 18, 36, 38), suggesting a tissue-specific tumor suppressor role for p15^{INK4b} in hematopoietic malignancies. In concordance with these data, Lois et al. (34) demonstrated an inverse relationship between p15^{INK4b} expression and proliferation of lymphocytes after mitogenic stimuli, suggesting a specific role for this gene in maintaining cell quiescence in lymphocytes.

Early studies on Ras mitogenic potential demonstrated that Ras induces and is required for DNA synthesis in serum-stimulated cells (44). Only recently have the pathways linking Ras activity with cell cycle control begun to be dissected. Ras acts on the cell cycle machinery by inactivating Cdk inhibitors

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such as p27^{Kip1} and inducing cyclins, giving rise to an increase in Cdk4/6 and Cdk2 kinase activities (for reviews, see references 11 and 35). Thus, Ras activity is linked directly to the G₁/S transition of the cell cycle and, in fact, G₁ is the only phase in which inhibition of Ras affects cell cycle progression. Ras is required for activation of both Cdk2 and Cdk4/6 complexes until 2 h before the G₁/S transition, a time corresponding to the so-called restriction point. Once cells have entered S phase, Ras becomes dispensable until the next cell cycle (19, 44). Although Ras signals through a growing number of different effector pathways, effects on both cyclin D induction and p27^{Kip1} degradation seem to be dependent on the Raf1-Erk pathway. The specific activation of the Erk pathway, however, is not sufficient to trigger p27^{Kip1} degradation, and it seems to be involved in a RhoA-associated pathway that could require a phosphatidylinositol 3'-kinase (PI3K)-dependent but protein kinase B-independent pathway (for a review, see reference 35).

Whereas different experiments have clearly shown that p16^{INK4a} is able to suppress cellular transformation by Ras and can contribute to cellular senescence (2, 20, 47, 62), the ability of p15^{INK4b} to inhibit cellular transformation has not been studied. In this article, we show that the cell cycle inhibitor p15^{INK4b} is able to produce cell cycle arrest and stop cellular transformation by Ras. Interestingly, this Cdk4/6 inhibitor is strongly induced in cultured cells by oncogenic Ras and, thus, can cooperate in causing the premature cellular senescence resulting from oncogenic signals. Using luciferase constructs carrying the p15^{INK4b} and p16^{INK4a} promoters, we show that the Raf-Mek-Erk pathway is the main effector pathway in the induction of the *INK4* promoters although other Ras effectors cooperate in the transcriptional induction of both p15^{INK4b} and p16^{INK4a} in NIH 3T3 cells. Finally, using mouse embryonic fibroblasts (MEFs) from p15^{INK4b} knockout mice, we show that the lack of p15^{INK4b} protein is sufficient to render these fibroblasts susceptible to transformation by the Ras or Rgr oncogene.

MATERIALS AND METHODS

Plasmids and DNA manipulation. Mouse genomic *N-ras* containing a codon 61 point mutation (pMZNT-17) or the wild-type sequence (pMZNN-1) was subcloned into the Zeocin-resistant vector pcDNA3.1/Zeo(+) (Invitrogen). Mouse p15^{INK4b} cDNA was isolated by reverse transcription-PCR and subcloned into the pCR3.1 (Invitrogen) or pMAMneo (Clontech) vector, giving rise to plasmids pHM414 (cytomegalovirus [CMV] promoter orientation), pHM411 (opposite orientation), and pMAMneo-p15. The same fragment was subcloned into the pBabe-puro vector for retroviral transduction of primary cells. A 6-kb genomic fragment containing the p15^{INK4b} and 5' upstream sequences was amplified by long-template PCR (Expand System; Boehringer Mannheim) using primers Mp15-P-1F (5'-GGC CAA AAC AGG ATC CCT TGG GAT GTG TTA-3') and Mp15-3'-1R (5'-TAA CCA TGG AGA TCT CTC CAG GCT CCA-3') as described previously (37). This fragment includes about 700 bp upstream of the p15^{INK4b} coding region and was subcloned into the bacterial plasmid pCR2.1 (Invitrogen) to generate pMM134. A different 8-kb genomic fragment containing the mouse p15^{INK4b} gene was obtained from pmp15 (36) and subcloned into pCR3.1 in the orientation opposite to that of the CMV promoter (pCRpmp15). The integrity and orientation of the inserts were confirmed by sequencing with an automatic 373 DNA Sequencer (Applied Biosystems). p16^{INK4a}, p27^{Kip1} and retinoblastoma expression plasmids were a gift from M. Pagano. Plasmids expressing wild-type, activated, or dominant negative forms of Raf (25), Mek1 (8), Erk1 and Erk2 (74), RalA (71, 76), p53 (70), or the PI3K p110 and p85 subunits (15, 55) were described previously. Ras effector domain mutants containing an activating G12V mutation (57) were used to specifically study the activity of the Raf, RalGDS, and PI3K effector pathways. The p16-luc5 plasmid contained a 1.1-kb promoter fragment of p16^{INK4a} upstream of the luciferase reporter gene (29). The plasmids pGal4-ElkC and p5xGal-luc were for assaying transactivation of Elk-1 (74). RhoA, Rac1, Cdc42, JNK1, PKC ζ , and MEK1 expression plasmids were graciously provided by P. Crespo. The mouse RalGDS gene (1) was subcloned into the pCR3.1 (Invitrogen) vector for expression in mammalian cells (pCR-RalGDS).

For hybridizations, digested DNA or RNA was separated on agarose gels and transferred to nitrocellulose membranes (Schleicher & Schuell). DNA probes were labeled with [α -³²P]dCTP (3,000 Ci/mmol; Dupont-NEN, Boston, Mass.)

using a random primed labeling kit (Boehringer Mannheim, Indianapolis, Ind.) in accordance with the manufacturer's protocol. Hybridizations were visualized and quantified by use of a PhosphorImager (Molecular Dynamics) or by exposure of X-ray films (Kodak), digital scanning, and analysis with the NIH Image software.

Cell culture, transfection, and retroviral infection assays. All cultures were maintained in Dulbecco's modified Eagle medium (DMEM; Gibco) supplemented with 10% calf serum and 1% penicillin G-streptomycin sulfate (Gibco). Stable transfection of mammalian cells was performed using the calcium phosphate technique, and G418 (Gibco) at 400 μ g/ml or Zeocin (Invitrogen) at 500 μ g/ml was used for selection of clones. Expression of recombinant proteins in pooled transfected cells or in individual clones was analyzed by Western blotting. Inducible expression of p15^{INK4b} in NIH 3T3 clones containing pMAMneo-p15 was achieved after addition of 1 μ M dexamethasone dissolved in ethanol. Uninduced cells were treated with an equivalent amount of ethanol. For the cell cycle assay, cells were cotransfected with 1 μ g of pMZNT-17 (Ras) or pNM11 (a pMEXneo-derivative plasmid expressing the rabbit Rgr oncogene [9]) and 3 μ g of other expression plasmids or empty vectors. After transfection, cells were split 1:5 and maintained in DMEM with 5% calf serum for 2 weeks. Foci were scored after staining with cresyl violet.

For soft-agar assays, cells were resuspended in 0.33% agar in DMEM supplemented with 10% calf serum at a density of 5,000/65-mm-diameter plate and seeded onto solidified 0.5% agar-containing culture medium. Cultures were fed weekly, and photomicrographs of colonies were taken 2 weeks postplating.

MEFs were isolated from C57BL/6J \times DBA2 mice using E14.5 embryos. Embryonic tissues were disaggregated with trypsin, and primary fibroblasts were cultured at intermediate densities in DMEM with 10% calf serum. p15^{INK4b}-deficient MEFs and those of wild-type littermates were kindly provided by E. Latres and M. Barbacid (unpublished data). For the transformation assays, 10⁶ cells were plated and transfected with 10 μ g of a Ras (pMZNT-17), Rgr (pNM11), or E1A (a gift of M. Serrano) expression plasmid. When needed, transfection mixtures were supplemented with up to 20 μ g of mouse genomic DNA as carrier DNA. After transfection, MEFs were maintained in DMEM plus 10% fetal bovine serum and visible foci (>2 mm in diameter) were scored after 2 weeks of culture and cresyl violet staining. Infection of three-pass MEFs with a pZIPneoSV(X)-derivative retrovirus carrying the oncogenic *N-ras* cDNA (pZip4) was performed essentially as previously described (39) using clone Ψ 2A2. Mouse p15^{INK4b} and *N-ras* N61 cDNAs were cloned in the pBabe-puro retroviral vector. One day before the infection, MEFs were plated at 8 \times 10⁵/10-cm dish. Infections were performed by replacing the medium with the virus-containing supernatant which had previously been filtered (0.45- μ m-pore-size filter; Millipore) and supplemented with Polybrene (Sigma) at 4 μ g/ml. Infected cell populations were selected for 4 days using puromycin at 2 μ g/ml. At 1 and 3 days after selection, the cells were incubated for 5 h with 10 μ M bromodeoxyuridine (BrdU) (Boehringer Mannheim) and BrdU incorporation was analyzed by flow cytometry (see below).

The activity of senescence-associated β -galactosidase (SA- β Gal) was detected by following the original protocol described by Dimri et al. (10). About 100,000 MEFs were infected with the retrovirus carrying p15^{INK4b}, oncogenic Ras, or the empty vector, and SA- β Gal activity was assayed 7 days postinfection.

Protein expression. Western blotting was used to assess the level of p15^{INK4b} in cultured cells. Samples were homogenized with a Tissumizer in ice-cold, freshly prepared lysis buffer (1% NP-40, 20 mM HEPES [pH 7.5], 5 mM MgCl₂, aprotinin at 10 μ g/ml, leupeptin at 2 μ g/ml, pepstatin A at 1 μ g/ml, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol) and spun at 100,000 \times g for 45 min at 4°C. The amount of protein in the supernatants was quantified by the Bradford method using bovine serum albumin as the standard. Aliquots containing 50 μ g of protein per sample were subjected to sodium dodecyl sulfate-10 to 18% polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes, which were blocked with 5% nonfat dried milk in TBST buffer (0.1% Tween 20, 132 mM NaCl, 20 mM Tris [pH 7.5]) for 12 h. The polyclonal antibody that recognizes the murine p15^{INK4b} protein was kindly provided by E. Latres and M. Barbacid. A commercial antibody was used to detect the Erk proteins (C-16; Santa Cruz Biotechnologies) as a control for protein loading. Peroxidase signal was detected by the enhanced-chemiluminescence method (Amersham). Protein band intensity after different exposure times was quantified using a Umax PowerLook scanner and the NIH Image software.

Flow cytometry. The cell cycle status of mammalian cells was analyzed by propidium iodide staining of DNA. For double staining of DNA content and BrdU, cells were pulsed with 10 μ M BrdU, washed, and fixed in 70% ethanol. After a 30-min treatment with 2 N HCl and pepsin at 0.2 mg/ml, the pH was stabilized with 0.1 M sodium tetraborate (pH 8.5) and the cells were preincubated in 0.5% Tween 20-2% normal mouse serum in phosphate-buffered saline. Incorporated BrdU was detected with a fluorescein isothiocyanate-conjugated anti-BrdU antibody (Boehringer Mannheim) and resuspended in a solution containing propidium iodide at 50 μ g/ml and DNase-free RNase at 0.5 mg/ml in phosphate-buffered saline. Fluorescence was analyzed with a FACScan cytometer, and data were interpreted using the CellQuest and ModFitLT applications (Becton Dickinson).

Luciferase assays. The pLUC+ luciferase reporter plasmid used in this work and its derivatives were described previously (3). Mouse p15^{INK4b} genomic sequences upstream of the coding region were obtained from plasmid pmp15 (36).

For deletion mapping of the murine p15^{INK4b} promoter, different fragments carrying p15^{INK4b} upstream sequences were amplified by PCR and subcloned into pLUC+ digested with *Xho*I and *Hind*III. The following oligonucleotides that incorporate a *Hind*III (forward primers) or *Xho*I (reverse primers) recognition site were used for priming: 1F (5'-AGG GGA AGC TTT TAA AGA CAG GCC-3'), 2F (5'-TGC GCA AGC TTC TAA GAT CTT CCG AC-3'), 3F (5'-AAC AAG CTT GGG GGA GGG GTT AG-3'), 6F (5'-GCT AAG CTT CTG CGG GCT CCC C-3'), 1R (5'-CAG AAC TCG AGG TTT CCT AGT CTG GAA C-3'), and 2R (5'-CCC CTC TCG AGA CCC AGT AGC TTC GG-3'). DNA fragments were digested with *Hind*III and *Xho*I and subcloned into the pLUC+ vector to generate p15-1F1R-luc, p15-2F1R-luc, and so on. The identities and orientations of the new constructs were confirmed by DNA sequencing. About 500 ng of the pLUC+ constructs, 100 ng of β -galactosidase plasmid pCH110 (Pharmacia) or pQP-CH110 (a pCH110 derivative in which the CMV promoter has been replaced with the Q fragment of the mouse *N-ras* promoter [21]), and 3 μ g of the expression plasmids described above were used to cotransfect different cell lines. When several expression plasmids were used in one transfection, the total amount of the mixture was 3 μ g. Transient transfections were performed by the calcium phosphate method (NIH 3T3 and HaCaT cells and MEFs) or Lipofectin (A431 cells). Cells were plated onto six-well plates at a density of 100,000 per well, grown for 24 h, and transfected. Forty hours after transfection, cells were collected and lysed using the buffer provided in the Luciferase Assay System (Promega). In some cases, TGF- β 1 (Boehringer Mannheim) was added to transfected HaCaT cells to a concentration of 100 pM and luciferase activity was measured 20 h later, following the timing described by Li et al. (28). Luciferase was assayed in accordance with the manufacturer's recommendations (Luciferase Assay System; Promega Corp.). The same protein extracts were used then to measure the chemiluminescence produced by β -galactosidase using the Galacto-Light Plus system (Tropix). All of the luciferase experiments were performed at least in triplicate.

RESULTS

p15^{INK4b} inhibits cellular transformation by Ras. NIH 3T3 fibroblasts, similar to many other immortal cell lines, lack the *INK4a* and *INK4b* loci due to homozygous deletion of their chromosomal region (31, 54). In order to analyze the effect of the reintroduction of p15^{INK4b}, NIH 3T3 cells were stably transfected with pMAMneo-p15, where p15^{INK4b} expression is controlled by the dexamethasone-responsive mouse mammary tumor virus promoter. Addition of 1 μ M dexamethasone produces enforced expression of p15^{INK4b} compared to cells grown in the presence of the solvent (ethanol). The effect of p15^{INK4b} induction was investigated by growth curve analysis of transfected clones and double staining for BrdU incorporation and DNA content. NIH 3T3 cells expressing p15^{INK4b} grew more slowly than noninduced cells or nontransfected NIH 3T3 cells (Fig. 1).

The growth-inhibitory effect of p15^{INK4b} expression was maintained even in Ras-transformed cells. NIH 3T3 cells were transfected with pMZNT-17, containing the activated *N-ras* gene in a Zeocin-resistant vector. Transformed clones that expressed the Ras oncogene were selected with Zeocin. Clones expressing the *N-ras* oncogene and showing transformed morphology were chosen and transfected with pMAMneo-p15. Zeocin- and G418-resistant pooled cells were treated with dexamethasone or left untreated. Induction of p15^{INK4b} by dexamethasone was able to provoke cell cycle arrest in the presence of activated Ras, as in untransformed NIH 3T3 cells. Ras-transformed NIH 3T3 cells grew more slowly, and the percentage of BrdU-incorporating cells decreased from 25 to 16% when p15^{INK4b} was induced. However, the morphology of Ras-transformed NIH 3T3 cells remained unchanged in the presence of p15^{INK4b}. These cells maintained the typical morphology of Ras-transformed cells, being small and highly refractile and presenting long, thin cellular prolongations (Fig. 1).

In the focus formation assay, p15^{INK4b} was also able to inhibit the formation of foci by Ras to an extent similar to that of the p16^{INK4a} or p27^{Kip1} protein (Fig. 2A and B). Only about 30% of the foci produced by oncogenic Ras were scored when Ras was cotransfected with pHM414, a G418-resistance plas-

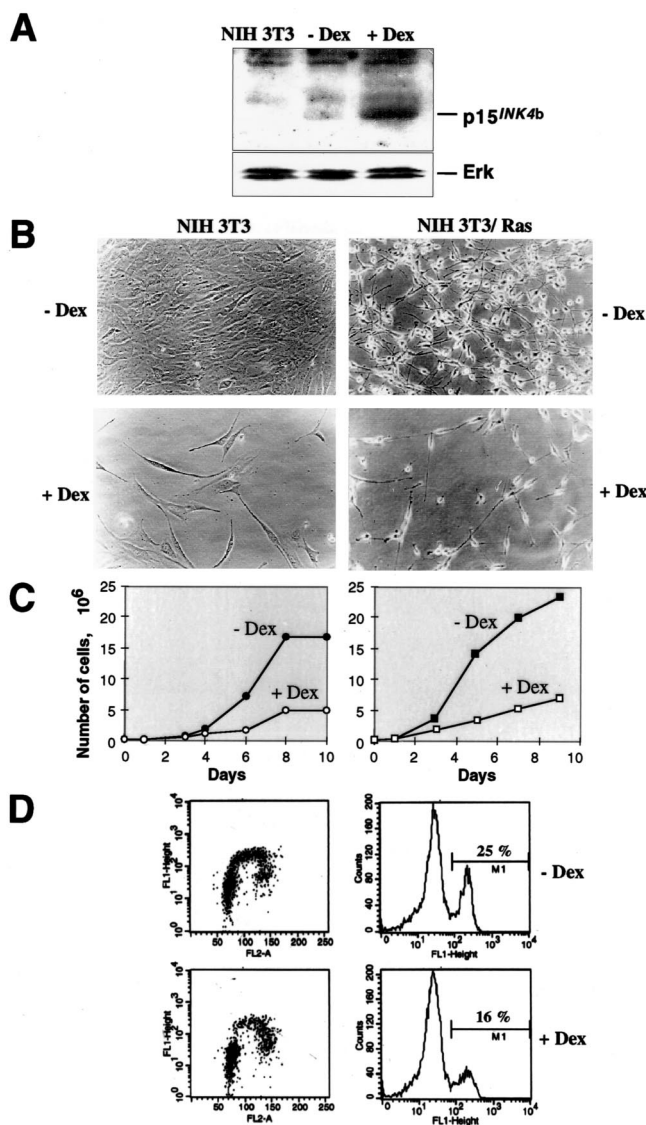


FIG. 1. Cell cycle-inhibitory activity of p15^{INK4b} in Ras-transformed or untransformed NIH 3T3 cells. (A) Western blot of NIH 3T3 cell lysates. Control NIH 3T3 cells have no p15^{INK4b} protein, whereas fibroblasts containing pMAMneo-p15 express the protein at low levels without dexamethasone treatment (- Dex). Twenty hours after the addition of dexamethasone (+ Dex), the levels of p15^{INK4b} are strongly increased. Erk proteins were detected as loading controls. (B) Photomicrographs of untransformed or Ras-transformed NIH 3T3/pMAMneo-p15 fibroblasts. About 100,000 cells were seeded and grown in DMEM-10% calf serum without or with dexamethasone. Pictures were taken after 7 days of culture. (C) Growth curve of the same cultures. (D) Double staining for BrdU incorporation and DNA content using a flow cytometer. Ras-transformed NIH 3T3 cells containing plasmid pMAMneo-p15 were grown in the absence or presence of dexamethasone for 24 h, pulsed with BrdU for 2 h, and fixed in 70% ethanol. Incorporation of BrdU was measured in a FACScan cytometer (FL1-Height) and plotted against propidium iodide staining (FL2-A) (left panels). BrdU-positive cells (M1 marker in the right panels) decreased from 25 to 16% after p15^{INK4b} induction.

mid in which p15^{INK4b} is driven by the CMV promoter. The reduction in the number of foci was similar to that found when Ras was cotransfected with the p16^{INK4a} or p27^{Kip1} expression plasmid but not when p15^{INK4b} was placed in the orientation opposite to that of the CMV promoter (pHM411). Individual clones resistant to Zeocin and G418 were selected, and Ras and p15^{INK4b} expression was determined by Western blotting.

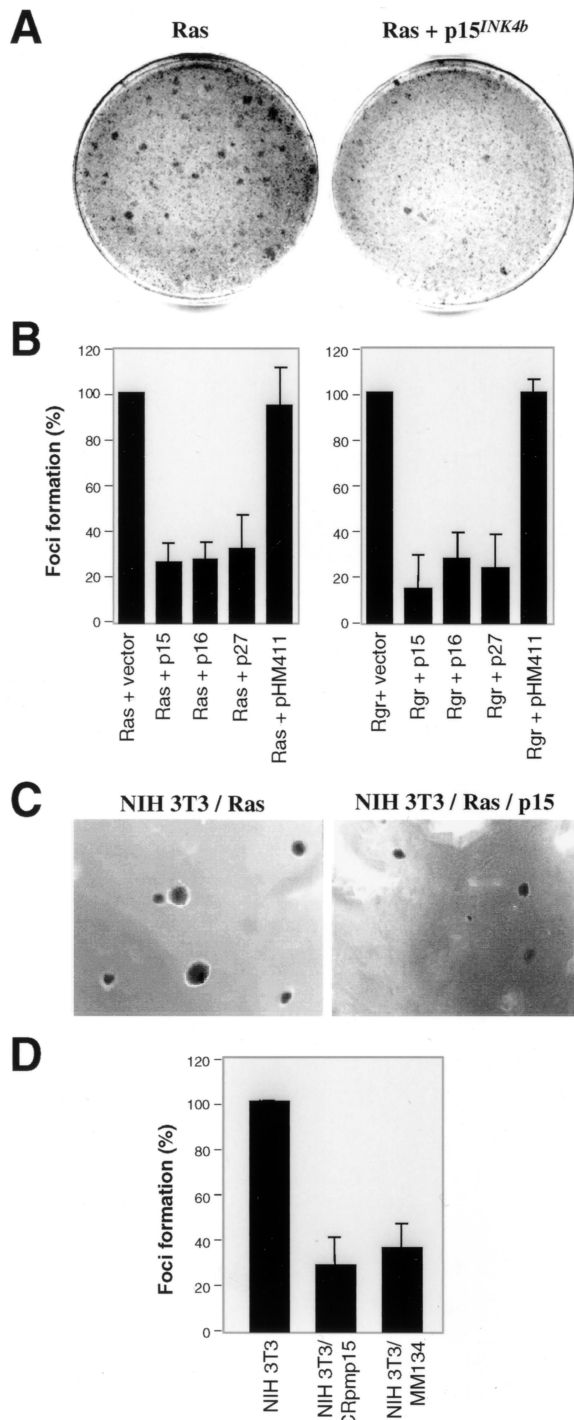


FIG. 2. Effects of several cell cycle inhibitors on NIH 3T3 cellular transformation by Ras. (A) Photograph of foci obtained after cotransfection of NIH 3T3 cells with pMZNT-17 (expressing oncogenic *N-ras* N61) and the empty vector pCR3.1 (left) or pMZNT-17 and pHM414 (expressing p15^{INK4b}) (right). (B) Suppression activity of p15^{INK4b} and other cell cycle inhibitors (p16^{INK4a} and p27^{Kip1}) on focus formation by Ras (left panel) or Rgr (right panel) in NIH 3T3 cells. Cells were cotransfected with pMZNT-17 (Ras) or pNM11 (Rgr) and the expression plasmid for p15^{INK4b}, p16^{INK4a}, or p27^{Kip1}. The empty vector or the vector containing p15^{INK4b} in the opposite orientation (pHM411) was used as a control. Results are represented as percentages of the number of foci obtained with Ras or Rgr and the empty vector (taken as 100%). The mean and standard error were calculated from three independent experiments. (C) Effect of p15^{INK4b} expression on anchorage-independent growth of Ras-transformed NIH 3T3 cells. Transformed cells expressing or not expressing p15^{INK4b} were seeded in agar-containing medium and grown for 2 weeks. The presence of this cell cycle

Some of these clones presented a typical Ras-transformed morphology even in the presence of the p15^{INK4b} protein. However, clones expressing both oncogenic Ras and p15^{INK4b} grew more slowly than the Ras-positive, p15^{INK4b}-deficient clones (data not shown). In addition, the presence of p15^{INK4b} decreased the number and size of colonies formed in soft agar by Ras-transformed NIH 3T3 cells (Fig. 2C).

p15^{INK4b} and the other Cdk inhibitors (p16^{INK4a} and p27^{Kip1}) are also able to inhibit transformation by Rgr (Fig. 2B), an oncogene belonging to the Ral family of guanine nucleotide exchange factors (9). This oncogene has recently been shown to activate the Erk kinases and to induce Fos expression and cyclin D1 transcription by using pathways similar to those used by Ras (M. I. Hernández and A. Pellicer, unpublished data).

Oncogenic Ras induces p15^{INK4b} expression and growth arrest. Since oncogenic Ras produces transformation in NIH 3T3 cells but G₁ arrest in primary fibroblasts, we analyzed the effect of oncogenic Ras on NIH 3T3 cells containing a p15^{INK4b} genomic fragment. NIH 3T3 cells were transfected with pCRpmp15 or pMM134, containing an 8- or 6-kb genomic p15^{INK4b} fragment, respectively, and selected with G418. Both NIH 3T3/CRpmp15 and NIH 3T3/MM134 cells are highly resistant to focus formation when transfected with the oncogenic Ras plasmid pMZNT-17, compared to parental NIH 3T3 cells in the focus formation assay (Fig. 2D). The expression of p15^{INK4b} in NIH 3T3 cells decreased the tumorigenic potential of Ras in these cells similarly to what happens in primary cultures. Expression of the oncogenic *N-ras* N61 mutant in MEFs produced the same morphological changes and cell cycle arrest found previously by Serrano et al. (64) using *H-ras* V12. Indeed, similar results were observed when MEFs were infected with a p15^{INK4b}-expressing retrovirus (Fig. 3A). p15^{INK4b}-transduced cells became flat and enlarged, stained positive for SA-βGal, and grew more slowly than cells infected with the Ras retrovirus or an empty vector. BrdU incorporation and cell cycle analysis by flow cytometry revealed that this phenomenon was produced through arrest in the G₁ phase of the cell cycle and a decrease in the number of cells that progressed to S phase (Fig. 3B). Thus, similar to that of Ras, retroviral expression of p15^{INK4b} in primary fibroblasts produces a senescence morphology accompanied by an arrest in cell proliferation. These results indicate that Ras-dependent induction of p15^{INK4b} is physiologically relevant, since only overexpression of p15^{INK4b} is able to produce G₁ arrest and a senescence phenotype in primary cells similar to that described for oncogenic Ras.

We next measured the expression of the p15^{INK4b} and Ras proteins in transfected NIH 3T3 cells or infected primary cultures. Surprisingly, when analyzing NIH 3T3/MM134 cells, carrying the genomic p15^{INK4b} region, we observed strong induction of p15^{INK4b} expression in Ras-transfected cells (Fig. 4A). A similar induction was not found when a p15^{INK4b} cDNA driven by the CMV promoter (pHM414) was used instead of the genomic fragment, suggesting the presence of Ras-responsive sequences in the p15^{INK4b} genomic region.

inhibitor was accompanied by a decrease in the number and size of colonies in two separate experiments. A sample of the colonies obtained in cells expressing Ras or Ras plus p15^{INK4b} is shown. (D) Focus formation by oncogenic Ras in NIH 3T3 cells containing a p15^{INK4b} genomic fragment. The same number of NIH 3T3, NIH 3T3/CRpmp15, or NIH 3T3/MM134 cells was transfected with pMZNT-17, and the number of foci was scored after 15 days. Results are presented as percentages of the number of foci obtained with Ras in NIH 3T3 cells (taken as 100%). The mean and standard error were calculated from three independent experiments.

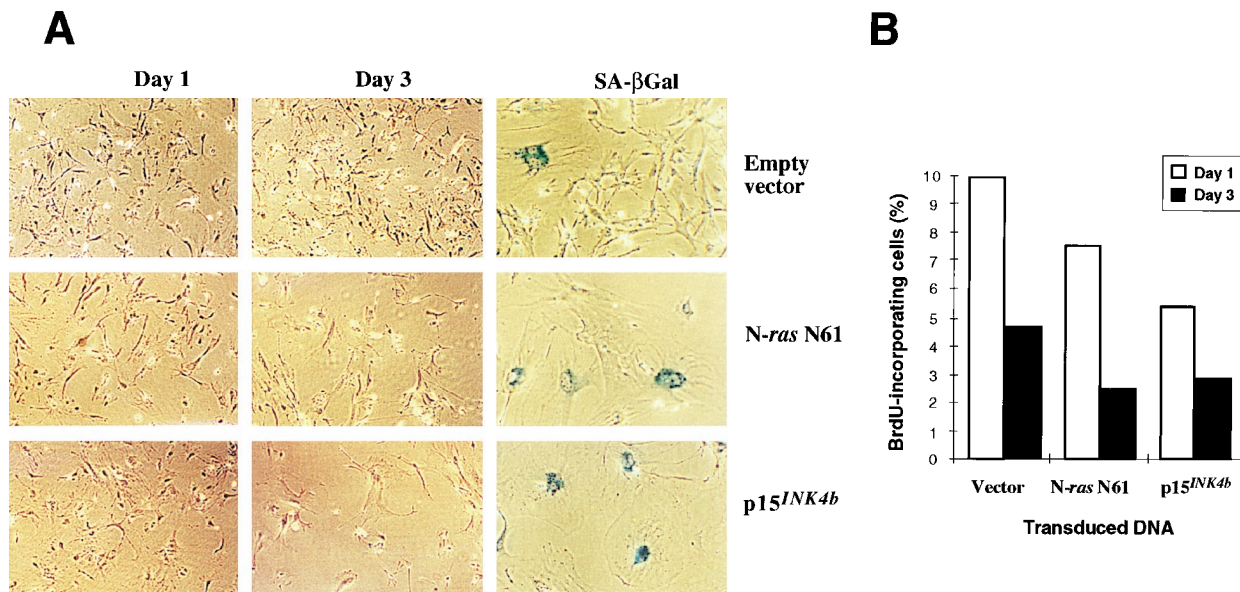


FIG. 3. Retrovirus-mediated ectopic expression of N-ras N61 and p15^{INK4b} in mouse primary fibroblasts. Early-passage MEFs were transduced with a retrovirus expressing p15^{INK4b} or N-ras N61 (as a positive control for premature cell senescence) or with an empty pBabe-puro vector. Infected cells were selected with puromycin for 4 days. (A) Cell morphology at days 1 and 3 after selection with puromycin and staining for SA-βGal. SA-βGal panels were photographed at an original magnification of ×1,000 to show in detail the morphology of the β-Gal-positive cells. All of the other panels were photographed at an original magnification of ×400. (B) Levels of BrdU incorporation by these MEFs on days 1 and 3. The decrease in BrdU-incorporating cells is correlated with G₁ arrest, as shown previously by others (72). These results are representative of three separate experiments.

We then analyzed the induction of p15^{INK4b} in early-passage MEFs, where both p15^{INK4b} and p16^{INK4a} are expressed. MEFs were infected with pZip4, a retroviral vector containing the N-ras oncogene, and after 48 h, cell lysates were analyzed for protein expression. As shown in Fig. 4B, p15^{INK4b} was overexpressed after infection of MEFs with the Ras-expressing retrovirus. This overexpression is similar to that obtained when p15^{INK4b} was retrovirally transduced into the same cells.

Ras-dependent induction of p15^{INK4b} is mediated by sequences located in the promoter region. The effect of Ras activation on the p15^{INK4b} promoter was analyzed next using luciferase reporter plasmids. Since the sequences needed for the Ras-dependent induction of p15^{INK4b} were present in pMM134, we subcloned the 720-bp DNA fragment upstream of the p15^{INK4b} coding region (fragment 1F2R) present in pMM134 into the pLUC+ vector. Plasmid p15-1F2R-luc was then cotransfected into NIH 3T3 cells with an expression plasmid for the N-ras proto-oncogene (pMZNN-1) or N-ras oncogene N61 (pMZNT-17), and luciferase activity was measured after 40 h. Whereas the Ras proto-oncogene had a negligible effect on the p15^{INK4b} promoter in the assay, luciferase activity was increased 12-fold in cotransfections with the Ras oncogene (Fig. 5). This effect was independent of the Ras isoform used, and similar results were obtained with oncogenic N-RasN61, H-RasV12, and K-RasV12 constructs (data not shown). Similar induction was also found when the Rgr oncogene was used instead of Ras. However, an expression plasmid carrying the retinoblastoma (pCMV-Rb) or p53 (p11-4) gene had no effect on the p15^{INK4b} promoter under these conditions. To analyze the effect of Ras on different cell lines, luciferase constructs were cotransfected with the Ras expression plasmids in the A431 (human squamous carcinoma cells) and HaCaT (human normal keratinocytes) cell lines and in primary MEFs (Fig. 5). In all cases, the p15^{INK4b} promoter was induced by oncogenic Ras and this induction was similar to that obtained with p16-

luc5, a reporter plasmid carrying the p16^{INK4a} promoter (see below).

In order to identify the sequence elements involved in the response to Ras, progressive deletions of the murine p15^{INK4b} promoter were amplified by PCR and subcloned into the

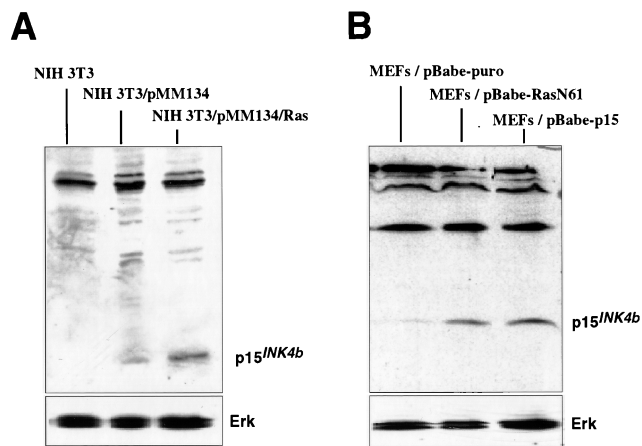


FIG. 4. Oncogenic Ras induces the expression of p15^{INK4b} in MEFs. (A) NIH 3T3 cells were cotransfected with pMM134, carrying p15^{INK4b} genomic DNA with 5'-upstream sequences, and pMZNT-17, containing oncogenic N-ras. Forty-eight hours after the transfection, cell lysates were prepared and p15^{INK4b} expression was analyzed by Western blotting. The levels of p15^{INK4b} protein were greatly increased in cells expressing activated Ras (NIH 3T3/pMM134/Ras cells) compared to those in fibroblasts transfected with pMM134 and an empty pcDNA3.1 vector (NIH 3T3/pMM134 cells). (B) Expression of p15^{INK4b} in MEFs infected with an oncogenic Ras retrovirus (pBabe-RasN61), a p15^{INK4b} retrovirus (pBabe-p15), or the empty vector (pBabe-puro). Ras activity induced endogenous expression of p15^{INK4b} at levels similar to those obtained with the p15^{INK4b} retrovirus. As in Fig. 1, detection of the Erk proteins was used as a loading control.

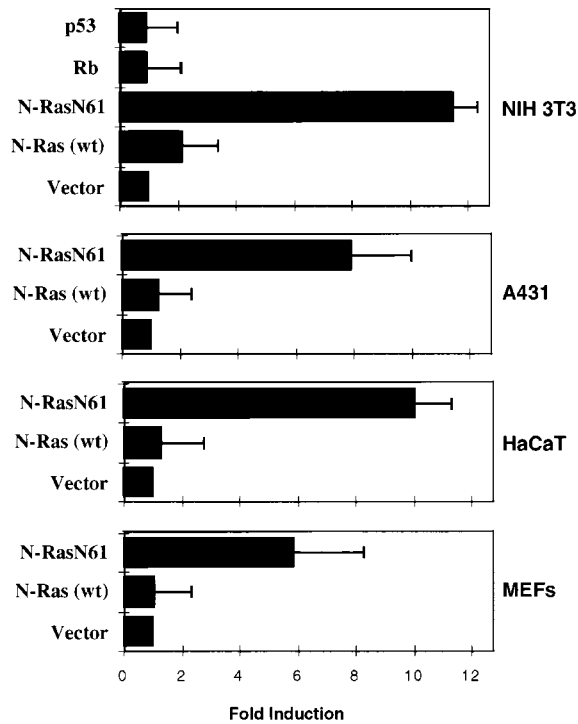


FIG. 5. Induction of the murine $p15^{INK4b}$ promoter by oncogenic Ras in cultured cells. Plasmid p15-1F2R-luc, containing 720 bp of $p15^{INK4b}$ 5' sequences driving a luciferase reporter gene, was cotransfected with different expression plasmids in NIH 3T3, A431, or HaCaT cells or in primary MEFs. Luciferase activities from three separate experiments with each cell line were measured. The promoter activity of p15-1F2R-luc cotransfected with an empty vector was arbitrarily chosen as 1. Cotransfection with an *N-ras* proto-oncogene, p53, or retinoblastoma expression plasmid had little or no effect on promoter induction. However, there was a 12-fold increase in luciferase activity when an oncogenic *N-ras* plasmid (pMZNT-17) was used in NIH 3T3 cells and similar results were obtained with the other cells. wt, wild type.

pLUC+ reporter plasmid. These constructs were cotransfected with pMZNT-17 or the empty vector (pcDNA3.1), and luciferase activity was assayed (Fig. 6). Induction by Ras was obtained with all constructions carrying at least 120 bp proximal to exon 1. However, no response was found with p15-1F1R-luc, carrying a distal fragment, or p15-6F2R-luc, containing only 55 bp upstream of exon 1. Thus, some Ras response elements must be located at positions -122 to -65 . Computer searches revealed the presence of three Sp1 consensus sites between these positions (Fig. 6A), in an organization similar to that of the human sequences (28). The Sp1 and Sp3 sites present in the human gene seem to be responsible for its induction by TGF- β (28). To determine whether these homologous sequences are involved in the induction by TGF- β in the murine promoter, the deletion constructs described above were transfected into HaCaT keratinocytes. Twenty hours after transfection, cells were treated with 100 pM TGF- β 1 or left untreated and luciferase activity was assayed 20 h later. Only construct p6F2R-luc had lost inducibility by TGF- β , showing that the same region responsible for Ras induction seems to be involved, at least partially, in the TGF- β response. These results agree with those of Li et al. (28) showing that the promoter response to TGF- β resides in Sp1 sites proximal (positions -79 to -55) to the $p15^{INK4b}$ gene. However, when we used a 720-bp fragment upstream of the mouse $p15^{INK4b}$ coding sequence (similar to the human fragment used as described in reference 28), only a threefold induction of $p15^{INK4b}$ could

be observed after TGF- β treatment. Since a 30-fold induction of $p15^{INK4b}$ was observed in keratinocytes after TGF- β treatment (14), other elements needed for a complete TGF- β response could be located outside this promoter region (our results and A. Iavarone and J. Massagué, personal communication).

The Raf-Mek-Erk effector pathway is essential for Ras-dependent *INK4* induction. Cellular effects of Ras activation are diverse and are mediated by several different effector pathways (5, 24, 35). In order to investigate which of the signaling cascades downstream of Ras are involved in the induction of the $p15^{INK4b}$ and $p16^{INK4a}$ cell cycle inhibitors, several expression plasmids containing activated versions of signaling proteins were cotransfected with the $p15$ -1F2R-luc ($p15^{INK4b}$ promoter) and $p16$ -luc5 ($p16^{INK4a}$ promoter) plasmids. Both oncogenic Ras and Rgr induce the *INK4* promoters to similar extents (about 12-fold). Activated versions of Raf1 (RafBxB)

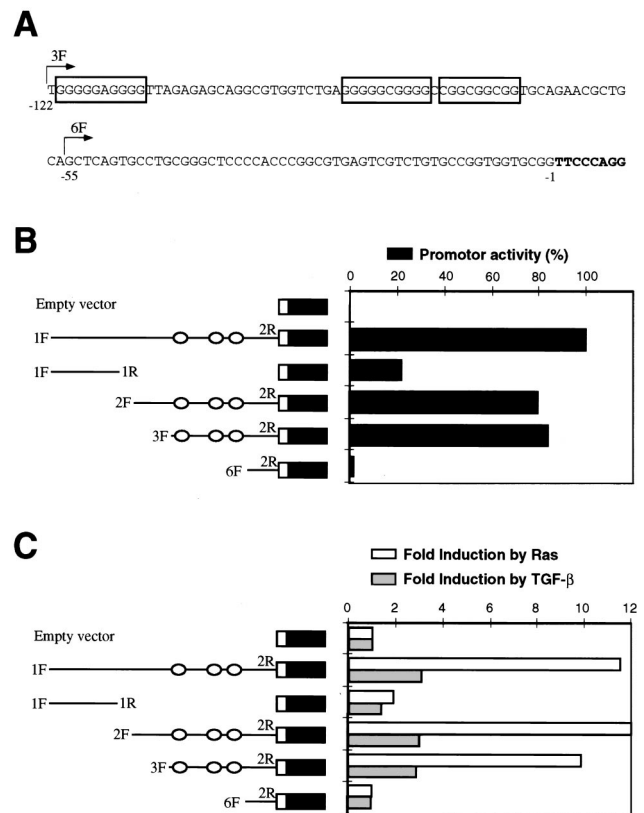


FIG. 6. Deletion analysis of the mouse $p15^{INK4b}$ promoter. (A) Proximal sequence upstream $p15^{INK4b}$ exon 1. Nucleotide positions are numbered backward from the first base of the cDNA sequence (nucleotides in boldface). Different fragments upstream of the $p15^{INK4b}$ gene were amplified by PCR and subcloned into the luciferase reporter vector. The 5'-end position of some primers used for amplification (3F and 6F) is shown by arrows. Primer 2R (not shown) is a reverse oligonucleotide inside the cDNA sequence. Boxes indicate putative recognition sites for the Sp1 family of transcription factors. (B) Scheme of the deletion fragments and promoter activity (black bars). Circles indicate the positions of putative Sp1-binding sites. The data shown are as percentages of the activity of p15-1F2R-luc (100%) carrying a 720-bp insert. (C) Induction of promoter activity by Ras and TGF- β . The constructs were cotransfected with an activated Ras plasmid (pMZNT-17) or the vector pcDNA3.1. Fold induction by oncogenic Ras (white bars) was compared to the luciferase activity of the same fragment cotransfected with the empty vector. Fold induction by TGF- β (gray bars) corresponded to the increase in luciferase activity after TGF- β treatment of HaCaT cells transfected with the constructs. All of the data shown here were normalized to β -galactosidase activity as described in Materials and Methods and are representative of three separate experiments.

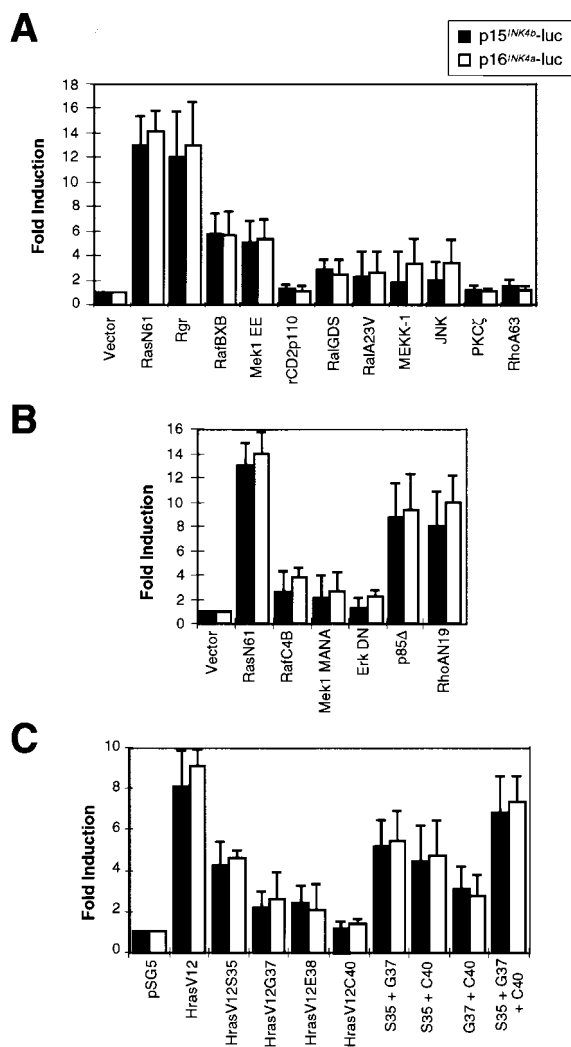


FIG. 7. Analysis of the Ras effector pathways involved in p15^{INK4b} and p16^{INK4a} promoter induction. The p15^{INK4b} (p15-1F2R-luc; black columns) and p16^{INK4a} (p16-luc5, white columns) reporter plasmids were cotransfected into NIH 3T3 cells with empty plasmids (Vector) or with expression plasmids carrying wild-type, activated, or dominant negative forms of molecules involved in the pathways downstream of Ras. Luciferase activity was then compared to the effect of oncogenic Ras (NrasN61 in pMZNT-17 or HrasV12 in pSG5), and the data are shown as fold induction with respect to the effect of the empty vector. Averages and standard errors from at least three different experiments are shown. Luciferase activity was normalized to β -galactosidase activity as a control for transfection efficiency. (A) Effect of overexpression of wild-type or activated proteins in p15^{INK4b} and p16^{INK4a} promoters. Whereas the Ras or Rgr oncogene produced a 12-fold increase in luciferase activity, only partial induction was found with single molecules downstream of Ras. (B) NIH 3T3 cells were cotransfected with the p15^{INK4b} and p16^{INK4a} reporter plasmids, oncogenic Ras (pMZNT-17), and the dominant negative forms of Raf1, Mek1, Erk1, PI3K (p85 Δ), and RhoA. All of the Raf1-Mek1-Erk inhibitory forms suppressed Ras-dependent induction, whereas the other proteins had little or no effect. (C) p15^{INK4b} and p16^{INK4a} promoter induction by effector domain mutant forms of Ras. Only partial induction was achieved with HrasV12S35 (Raf1-interacting mutant), and a very modest effect was obtained with the other forms. However, the three different forms cooperated to produce a stronger response.

and Mek1 (Mek1 EE) showed partial induction of p15^{INK4b} and p16^{INK4a} compared with Ras (Fig. 7A). Other activated proteins, such as RalA (RalA23V), RhoA (RhoA63), and the catalytic subunit of PI3K (rCD2p110), or overexpression of the wild-type RalGDS, JNK, or PKC ζ protein had a reduced or no effect on the p15^{INK4b} or p16^{INK4a} promoter. To ensure the

functional integrity of the Raf1 and Mek1 constructs, these proteins were used in Elk-1 transactivation assays using luciferase reporters (74). Although the three proteins strongly induced Elk-1 transactivation (data not shown), only a fivefold increase on p15^{INK4b} or p16^{INK4a} promoter activity was observed (Fig. 7A).

Using a similar experimental approach, dominant negative variants of several proteins involved in the signaling cascades downstream of Ras were cotransfected with the luciferase reporter plasmids and the oncogenic Ras construct (pMZNT-17). Only the dominant negative forms of Raf1 (RafC4B), Mek1 (Mek1 MANA), and Erks (KR-Erk1 and K52R-Erk2) were able to suppress the luciferase activity induced by Ras (Fig. 7B). No significant effect was observed with a RalA (RalAN28), Rac1 (Rac1N17), Cdc42 (Cdc42N17), or PKC ζ (PKC ζ W281) inhibitory mutant protein (data not shown), and only a slight decrease in luciferase activity was detected when the PI3K (p85 Δ) and RhoA (RhoAN19) dominant negative proteins were used. We also took advantage of several effector domain mutant forms of Ras which are impaired in specific downstream pathways. Thus, S35 Ras mutant proteins signal through the Raf1 pathway but not through the RalGDS or PI3K effector. G37 and E38 mutant proteins are specific for the RalGDS pathway, and C40 interacts with PI3K but not with the other two effectors (57, 75). We cotransfected activated forms (V12) of these mutant proteins with a p15-1F2R-luc or p16-luc5 reporter using the HrasV12 mutant protein (with full effector response) or the empty vector as a control. As shown in Fig. 7C, HrasV12 produced 8- to 10-fold induction of the promoters whereas Raf1-interacting mutant HrasV12S35 mutant showed partial (3-fold) induction. A slight increase in luciferase activity was found with HrasV12G37 and HrasV12E38 (less than twofold induction in the RalGDS-interacting forms). No significant increase in luciferase activity was found with PI3K-interacting Ras mutant HrasV12C40. Interestingly, higher induction of the INK4 promoters was observed when combinations of these mutants were used, especially in the case of Raf1- and RalGDS-interacting proteins or when a mixture of the three Ras mutant proteins was used, indicating that, at least in NIH 3T3 cells, several Ras effector pathways can cooperate in p15^{INK4b} and p16^{INK4a} induction.

p15^{INK4b}-deficient MEFs are susceptible to transformation by a single oncogene. Since the in vitro results described above suggested a role for p15^{INK4b} in suppressing the oncogenic activity of Ras in cultured fibroblasts, we decided to analyze the effect of Ras activation in MEFs obtained from p15^{INK4b} knockout mice. These MEFs and those from wild-type littermates were transfected at early passages with plasmids expressing oncogenic Ras, Rgr, or the E1A protein. After transfection, cells were cultured for 2 weeks and visible foci were scored. Whereas no foci were observed in wild-type MEFs transfected with either the Ras or the Rgr oncogene, a few foci were scored in p15^{INK4b}-deficient MEFs transfected with either of these proteins (Fig. 8). The efficiency of transformation of p15^{INK4b}-deficient MEFs (averages of five foci per assay with Ras and seven foci per assay with Rgr) was, however, lower than that of INK4 Δ 2,3 knockout MEFs lacking both the p16^{INK4a} and p19^{ARF} proteins (an average of 36 foci per assay; data not shown). These results are similar to those obtained by Latres and Barbacid (unpublished) showing that p15^{INK4b} knockout MEFs are more susceptible to transformation by Ras or a combination of the Ras and Myc oncogenes. The numbers of foci obtained with Ras and Rgr were very similar, supporting an oncogenic mechanism common to both proteins (Hernández and Pellicer, unpublished). When Rgr was cotransfected with the E1A oncoprotein, transformed foci were observed in

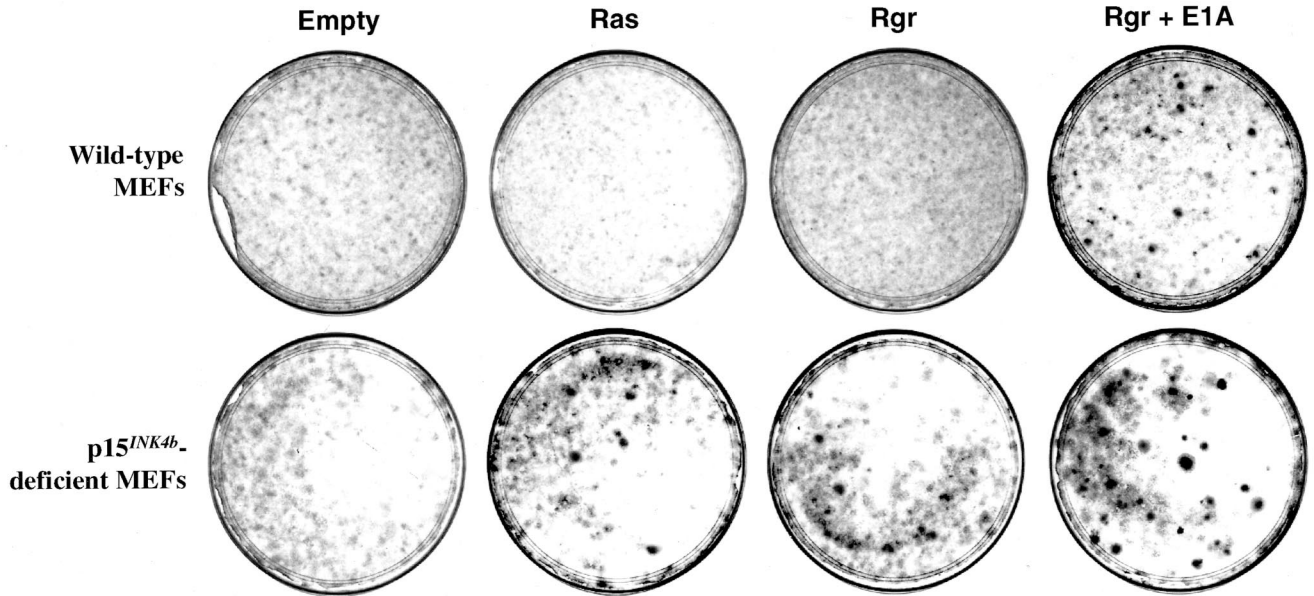


FIG. 8. Transformation assays with MEFs derived from $p15^{INK4b}$ knockout mice. $p15^{INK4b}$ -deficient MEFs or those from wild-type littermates were transfected with a plasmid expressing the Ras or Rgr oncogene or a combination of Rgr plus E1A. Whereas no foci were observed in wild-type MEFs after transfection with Ras or Rgr, foci developed in cells lacking $p15^{INK4b}$. Similarly, $p15^{INK4b}$ -deficient MEFs were more sensitive than wild-type cells to cotransfection with both the Rgr and E1A oncogenes.

wild-type MEFs (an average of 10 foci per assay). However, the lack of $p15^{INK4b}$ in the fibroblasts resulted in higher susceptibility to transformation, since the number of foci increased (about 27 per assay) and they were larger than those obtained with wild-type MEFs (Fig. 8).

DISCUSSION

Members of the two families of Cdk inhibitors, such as $p16^{INK4a}$ (62), $p21^{Cip1}$ (42), and $p57^{Kip2}$ (72), have been previously shown to suppress cell transformation by oncogenic Ras or other oncogenes. Our results obtained with NIH 3T3 cells show that $p15^{INK4b}$ is as potent an inhibitor of Ras transformation as $p16^{INK4a}$ or $p27^{Kip1}$. $p15^{INK4b}$ produced a similar G_1 arrest in Ras-transformed cells and decreased the tumorigenic potential of Ras or other oncogenes such as Rgr in the focus formation assay (Fig. 1 and 2). BrdU incorporation assays show that $p15^{INK4b}$ is able to suppress the entry into S phase of the cell cycle induced by oncogenic Ras, as previously reported for $p16^{INK4a}$ (62). In addition, cells expressing both Ras and $p15^{INK4b}$ formed fewer and smaller colonies in soft agar, indicating that ectopic expression of $p15^{INK4b}$ inhibits Ras-induced anchorage-independent growth of NIH 3T3 cells (Fig. 2). A similar effect has also been described for $p21^{Cip1}$ (42). Interestingly, the morphology of Ras-transformed NIH 3T3 cells did not change after the ectopic introduction of $p15^{INK4b}$. Cells expressing both Ras and $p15^{INK4b}$ maintained the characteristic phenotype of Ras-transformed cells (small and refractile) even when they entered $p15^{INK4b}$ -induced G_1 arrest (Fig. 1B). These results suggest that the effect of $p15^{INK4b}$ on cell transformation by Ras is exclusively suppression of mitogenic activity. A dissociation between Ras-dependent growth arrest and cell morphology has been recently described in the premature-senescence phenotype of primary fibroblasts. Whereas Ras-arrested cells became large and flat (64), cells arrested by an activated Mek protein were small and refractile (30). In this system, the Raf-Mek-Erk pathway provides the antiprolifera-

tive signals to arrest growth without modifying cell morphology. Thus, our data support a specific role for $p15^{INK4b}$ in the mitogenic arrest of Ras-transformed NIH 3T3 cells without affecting other Ras-dependent pathways involved in producing the transformed morphology.

$p15^{INK4b}$ induction by Ras. Transfection of oncogenic Ras into NIH 3T3 cells carrying a $p15^{INK4b}$ genomic fragment resulted in inhibition of focus formation compared to control NIH 3T3 cells (Fig. 2D). We realized that $p15^{INK4b}$ is overexpressed in Ras-transfected cells (Fig. 4), and since this effect is not observed with a $p15^{INK4b}$ cDNA, it is therefore induced by Ras through noncoding genomic sequences. As we have shown in this work, overexpression of $p15^{INK4b}$ is able to suppress Ras transformation and induction of $p15^{INK4b}$ is likely to antagonize the oncogenic effects of Ras.

Although $p15^{INK4b}$ is not expressed in mouse embryos, this gene is strongly induced in the first 4 days after embryos are disrupted and cultured as MEFs (47, 81). This behavior is similar to that described for $p16^{INK4a}$, although its expression is delayed compared to that of $p15^{INK4b}$. Two days after MEF plating, $p15^{INK4b}$ is highly expressed while the expression of $p16^{INK4a}$ is only barely detectable. After 4 days, $p16^{INK4a}$ continues accumulating as the MEFs divide in culture and approach crisis whereas $p15^{INK4b}$ expression only slightly increases (47, 81). We have shown here that the retroviral overexpression of $p15^{INK4b}$ is sufficient to cause G_1 arrest and senescence morphology (Fig. 3). In fact, it has been recently reported that $p15^{INK4b}$ can induce G_1 arrest and senescence even more effectively than $p16^{INK4a}$ or the Cip and Kip proteins (40). The Ras-dependent induction of $p15^{INK4b}$ described above is also similar to that described for $p16^{INK4a}$ in primary fibroblasts (64). Ras oncogenic activity in human and murine primary fibroblasts provokes the induction of $p16^{INK4a}$ and p53, which is associated with premature senescence of these cells. As shown in Fig. 4, $p15^{INK4b}$ is also induced in mouse primary fibroblasts after infection with an oncogenic Ras retrovirus.

This effect on cell cycle arrest and senescence could be even more dramatic in lymphoid tissues, where p15^{INK4b} has been shown to play an important role in lymphocyte activation (34) or tumorigenesis (4, 17, 36). T lymphocytes accumulate p15^{INK4b} protein during successive population doublings and display high levels of this molecule as they enter replicative senescence (12). Accumulation of INK4 proteins is accompanied by their increased binding to Cdk6 and decreased Cdk6 and Cdk2 activity. Thus, absence of the p15^{INK4b} protein could make it possible for tumoral lymphocytes to proliferate, avoiding replicative senescence.

Transfection experiments using pMM134 (p15^{INK4b} genomic DNA containing about 720 bp of the promoter sequence) and luciferase reporter assays show that Ras-dependent induction of p15^{INK4b} and p16^{INK4a} occurs through sequences located in the promoter region. In the case of p15^{INK4b}, induction by Ras is blocked when 60 bp located between positions -122 and -55 are deleted from the promoter. Similar sequences also seem to be necessary for response to TGF- β in the human p15^{INK4b} promoter (28). Sp1-binding sites are also important for the regulation of the p19^{ARF} promoter (56), and since p19^{ARF} is also induced by Ras activation (48), we are currently analyzing whether these sequences are also involved in Ras-dependent expression of the p19^{ARF} protein. Ras, therefore, could use the same sequence motifs that TGF- β uses for induction of p15^{INK4b}. In fact, a role for Ras in TGF- β transcriptional activation has been suggested (16, 45, 78). In epithelial cells, growth inhibition by TGF- β 1 and TGF- β 2 is associated with rapid activation of both Ras and Erk1 (45). Moreover, expression of the Ras dominant negative form (RasN17) partially abrogates Erk1 activation and inhibition of DNA synthesis by TGF- β (16) and Ras activation is also needed for TGF- β upregulation of p21^{Cip1} and p27^{Kip1} (78). Since p27^{Kip1} upregulation by TGF- β has been described as an indirect effect of p15^{INK4b} induction and displacement of p27^{Kip1} from cyclin D-Cdk4/6 complexes (49, 51, 60), Ras could be physiologically involved in p15^{INK4b} induction. If this is the case, the ability of Ras to induce the expression of some Cdk inhibitors could not only be a protective or stress response of the cell but have a function in normal physiological stages.

The constant levels of p15^{INK4b} expression during the cell cycle and its induction by TGF- β have suggested a role for p15^{INK4b} as an important effector in TGF- β -induced growth arrest, rather than in regulation of the timing of events in the cell cycle itself (14, 68). Our results suggest that p15^{INK4b} could also provoke G₁ arrest in response to cellular signals mediated by not only TGF- β but also other stimuli depending on Ras signaling. When Ras is required to send a mitogenic stimulus, other signals must cooperate to suppress the induction of p15^{INK4b} and other inhibitors. For instance, in Ras-dependent induction of p21^{Cip1}, once Ras has been activated, Rho signaling is required for suppression of p21^{Cip1} induction (46). When signaling through Rho is inhibited, constitutively active Ras induces p21^{Cip1} and entry into the DNA synthesis phase of the cell cycle is blocked.

Ras effector pathways and cell cycle control. Since p15^{INK4b} is also induced by Ras in NIH 3T3 cells containing the genomic *INK4b* gene, the downstream effector pathways used by Ras seem to be intact in these cells. Among the Ras effectors, the Raf-Mek-Erk pathway has been shown to function not only in the stimulation of cellular proliferation but also in growth arrest. There is growing evidence that a sustained increase in Erk activity can lead to inhibition of Cdk activity and cell cycle arrest (30, 33, 52, 77). In some systems, these inhibitory effects have been attributed to induction of the Cdk inhibitor p21^{Cip1} or a decrease in cyclin A levels (32, 65). This induction has

been proposed to occur in a p53-dependent manner in primary rat Schwann cells (33) but independently of p53 in mouse primary fibroblasts (77). In other cases, the increase in p16^{INK4a} expression seems more relevant and activated Raf or Mek protein is sufficient to provoke its induction and premature senescence in primary fibroblasts (30, 79).

In our study, analysis of the pathways involved in the Ras-dependent transcriptional activation of the p15^{INK4b} and p16^{INK4a} promoters showed that in NIH 3T3 cells, different cooperating pathways can be required to emulate the effect of oncogenic Ras. Activated forms of the Raf1 and Mek (MEK EE) kinases only partially increased luciferase activity from the p15^{INK4b} and p16^{INK4a} promoter, whereas the effect was almost null in other pathways (Fig. 7). Interestingly, a recently described oncogene named Rgr (9) is able to induce the INK4 proteins to an extent similar to that of oncogenic Ras. Rgr is a strong oncogene belonging to the RaIGDS family of exchange factors. Rgr is able to activate RalA, similar to the other members of the family, but is also able to activate some Rho-mediated pathways and the mitogen-activated protein kinases, and this activation is at least partially dependent on Ras (Hernández and Pellicer, unpublished). All of these pathways are strongly related to transcriptional activation and could therefore account for the induction of the INK4 inhibitors.

The use of dominant negative proteins shows that the Raf-Mek-Erk pathway is essential for Ras-dependent activation of p15^{INK4b} and p16^{INK4a}. These results agree with the recent reports by Lin et al. (30) and Zhu et al. (79) showing that activation of the mitogen-activated protein kinase cascade is essential for induction of Cdk inhibitors and promotion of premature senescence. However, while they described comparable data on growth arrest and senescence after Ras, Raf1, or Mek activation, we repeatedly found only partial induction of the INK4 promoters when using activated Raf or Mek protein. These results can be explained by differences between the experimental approaches. While we analyzed the transcriptional activation of individual promoters, the work of Lin et al. (30) was based mainly on measurement of growth arrest and cellular senescence. Thus, small increases in the individual levels of p16^{INK4a}, p15^{INK4b}, and perhaps p21^{Cip1} induced by H-RasV12S35 could still cooperate and produce cell cycle arrest. On the other hand, strong mutant proteins used in their work, such as MekQ56P, could produce a stronger signal than the activated forms used in the present work. In fact, activated MekQ56P causes cell cycle arrest even more rapidly than oncogenic Ras (30) and differences in the strength of the signal have been previously shown to be essential in the stimulation of the Raf-Mek-Erk cascade outcome (26, 77). It has been suggested that the ultimate cellular response (cell cycle arrest or cellular proliferation) to activation of the Mek-Erk pathway can depend on the strength and duration of the Erk signal, which is the transient or cyclical activation responsible for the proliferative output, while sustained high levels of Erk activity could result in cell cycle arrest (52, 77). Thus, low levels of Raf activity lead to activation of cyclin D1-Cdk4 and cyclin E-Cdk2 complexes and cell cycle progression, whereas higher Raf activity elicited cell cycle arrest correlating with p21^{Cip1} induction and inhibition of cyclin-Cdk activity (77). Finally, the signaling pathways from Ras to p16^{INK4a} and p15^{INK4b} induction could be modified in NIH 3T3 cells, so that the Raf and Mek proteins might not be able to produce as high levels of INK4 induction as Ras does. We therefore do not exclude the possibility that different pathways could cooperate with the Raf-Mek-Erk cascade in NIH 3T3 and other cell types to produce growth arrest mediated by the Cdk inhibitors. In fact, in NIH 3T3 cells expressing human TrkA, PD98059 (a Mek inhibitor) blocked

the ability of nerve growth factor (NGF) to inhibit Cdk2 and Cdk4 activities but only partially prevented the NGF induction of p21^{Cip1}. Thus, NGF-dependent up-regulation of the p21^{Cip1} protein appears to be only partially mediated through the Mek-Erk pathway (52), suggesting that other pathways can also be involved.

Susceptibility of p15^{INK4b}-deficient MEFs to cell transformation by single oncogenes. The need for oncogene cooperation in the transformation of primary cells was reported almost 20 years ago (27, 59). In the last few years, it has been demonstrated both in vitro and in vivo that oncogenic activation and loss of inhibitory effects might cooperate in cell transformation. In fact, primary cells from *INK4a*Δ2,3-, p19^{ARF}-, p21^{Cip1}-, or p53-deficient mice can be transformed by Ras alone (23, 43, 63). On the other hand, Ras transgenics in an *INK4a*Δ2,3 background have a significantly higher tumor incidence (6). We show here that p15^{INK4b}-deficient MEFs are susceptible to transformation by the Ras or Rgr oncogene, although with a lower efficiency than *INK4a*Δ2,3 MEFs. These MEFs lack both the p16^{INK4a} and p19^{ARF} proteins, and therefore, the contribution of each protein to this phenotype has not been assessed yet. Moreover, MEFs lacking only p19^{ARF} are also susceptible to transformation by Ras (23) and the effect of p16^{INK4a} inactivation in primary MEFs remains to be determined. The absence of the other INK4 proteins (p18^{INK4c} and p19^{INK4d}) in the corresponding knockout MEFs does not result in increased sensitivity to transformation by Ras (82; Latres and Barbacid, unpublished). Our results and those obtained by Latres and Barbacid (unpublished) indicate that p15^{INK4b} has a suppressor effect on MEF transformation by either the Ras or the Rgr oncogene or by the combination of Ras and different oncogenes such as Myc or E1A.

The role of p15^{INK4b} as a tumor suppressor in lymphocytes has also been recently highlighted as a result of methylation studies with both human and mouse cells (4, 17, 18, 36, 38, 80). Thus, p15^{INK4b} is frequently (up to 88%) inactivated by methylation of the promoter region in both humans (4, 17, 18) and experimental systems (36). In agreement with these observations, p15^{INK4b} knockout mice frequently develop lymphoproliferative disorders (Latres and Barbacid, unpublished). The fact that oncogenic Ras can activate p16^{INK4a} and p15^{INK4b} therefore provides a new insight into their role as tumor suppressor genes and suggests that Ras oncogenic signals might cooperate with p15^{INK4b} inactivation in tumorigenesis in vivo. These INK4 proteins have similar activities in vitro and are likely to participate in the growth arrest response to oncogenic signals. Their different role in vivo could be dependent on their specific pattern of expression or in their ability to be induced by different stimuli. Among them, Ras seems to be a strong INK4 inducer and an important one due to its ability to participate in physiological and tumorigenic processes.

ACKNOWLEDGMENTS

We thank J. Altschmied, M. Barbacid, J. L. Bos, D. A. Brenner, P. Crespo, C. J. Der, L. A. Feig, M. Kasuga, D. Levy, J. Massagué, M. Pagano, U. R. Rapp, K. Reif, P. Rodríguez-Viciana, M. Serrano, R. A. Weinberg, and Y. Xiong for kindly providing some of the plasmids and reagents used in this work. We are specially indebted to E. Latres and M. Barbacid for providing us with the p15^{INK4b}-deficient MEFs.

M.M., M.I.H., and I.P.C. received fellowships from the Ministerio de Educación (Madrid, Spain). This work was supported by grants CA 36327 and CA 50434 from NIH to A.P.

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