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The Retinoblastoma Protein Is Required for Ras-Induced Oncogenic Transformation†

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Most human cancers involve either mutational activation of the Ras oncogenic pathway and/or inactivation of the retinoblastoma tumor suppressor (RB) pathway. Paradoxically, tumors that harbor Ras mutations almost invariably retain expression of a wild-type pRB protein. We explain this phenomenon by demonstrating that Ras-induced oncogenic transformation surprisingly depends on functional pRB protein. Cells lacking pRB are less susceptible to the oncogenic actions of H-Ras^{V12} than wild-type cells and activated Ras has an inhibitory effect on the proliferation of pRB-deficient human tumor cells. In addition, depletion of pRB from Ras-transformed murine cells or human tumor cells that harbor Ras pathway mutations inhibits their proliferation and anchorage-independent growth. In sharp contrast to pRB$^{-/-}$ 3T3 cells, fibroblasts deficient in other pRB family members (p107 and p130) are more susceptible to Ras-mediated transformation than wild-type 3T3 cells. Moreover, loss of pRB in tumor cells harboring a Ras mutation results in increased expression of p107, and overexpression of p107 but not pRB strongly inhibits proliferation of these tumor cells. Together, these findings suggest that pRB and p107 have distinct roles in Ras-mediated transformation and suggest a novel tumor-suppressive role for p107 in the context of activated Ras.

Human tumors arise from an accumulation of genetic changes that result in a disruption of the normal control of several cellular processes, including proliferation, apoptosis, and differentiation. These genetic changes largely include loss-of-function mutations in tumor suppressor genes and gain-of-function mutations in proto-oncogenes. The retinoblastoma (*Rb*) gene was the first tumor suppressor gene to be cloned (22, 43) and loss of the *Rb* gene, originally described as the rate-limiting step in the development of retinoblastomas, has since been found in a variety of other tumor types (28, 34, 40, 74).

The retinoblastoma protein (pRB) has been implicated in the regulation of a multitude of cellular processes, such as cell cycle progression and cell death, as well as differentiation (44, 68, 73). The most clearly established role for pRB may be as a regulator of proliferation (10, 16, 20, 31, 36, 52, 56, 71). pRB is thought to control progression through the G_1 phase of the cell cycle by regulating the E2F transcription factor family and the recruitment of chromatin-remodeling complexes to promoter regions (4, 6, 26, 27, 65). The ability of pRB to repress E2F-mediated transcription is affected by phosphorylation of pRB by cyclin-dependent kinases, linking pRB to the intricate network that regulates the cell cycle (63). Significantly, alterations in several proteins (loss of the p16 cyclin-dependent kinase inhibitor and amplification of cyclin D or mutation of cdk4) that have been implicated in the regulation of pRB function are also commonly observed in a broad spectrum of tumor types, suggesting that deregulation of the normal pathway in which pRB functions is a common and important event in the development of many tumors (63, 64, 70).

The pRB protein is a member of a family of closely related mammalian proteins that also includes p107 and p130. Numerous studies of the pRB family of proteins have led to a rather extensive understanding of the biochemical properties of these proteins, and disruption of these genes in mice has helped to elucidate the role of pRB, p107, and p130 in embryonic development as well as in tumorigenesis. Intercrossing of pRB-, p107-, and p130-deficient mouse strains and the generation of chimeric animals that eliminate more than one protein has revealed significant functional overlap within this gene family in development and during tumor formation (7, 11, 13, 42, 58, 60, 69, 73). The phenotypes seen in these mice could potentially reflect cell cycle changes mediated by deregulation of E2F target genes but might also be the result of changes in other transcriptional programs involved in additional cellular processes, such as differentiation or apoptosis.

In order to identify potential functional distinctions among the closely related pRB family proteins, we have studied the biological properties of cells derived from mice harboring targeted disruptions of the RB family genes. Our initial studies of 3T3 fibroblasts derived from mouse embryos deficient in various combinations of pRB, p107, and p130 revealed overlapping roles for these proteins in cell cycle control but antagonistic functions for pRB and p107 in differentiation (9, 10). Given the observed deregulation of the cell cycle and the reduced differentiation potential of pRB-deficient 3T3 cells, we sought to investigate the effect of activating mutations in oncogenes on the tumorigenic potential of these cells. Since the E1A protein, and other tumor virus gene products (simian virus 40 T antigen and the papillomavirus E7 protein) that bind tightly to the members of the pRB family (pRB, p107, and

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p130) (8, 30) have been demonstrated in numerous settings to cooperate with activated Ras in assays of oncogenic transformation (25, 59), we focused our studies on the Ras oncoprotein.

Activating mutations of the *Ras* family of proto-oncogenes have been implicated in approximately 30% of human cancers. The encoded Ras proteins (K-Ras, H-Ras, and N-Ras) function as molecular switches, transducing signals in response to a variety of extracellular stimuli. Like pRB, Ras proteins appear to influence various cellular processes such as growth, proliferation, senescence, and differentiation in various contexts (3, 19, 46, 47, 53, 62). There are several identified direct effector targets of Ras that appear to be important for its oncogenic function, including the Raf family of protein kinases, phosphatidylinositol 3-kinase, and the Ral-guanine nucleotide dissociation stimulator proteins (19). Although many tumors have recently been shown to harbor mutations in B-Raf (15, 17), the specific contribution of each of the Ras targets to various aspects of the transformed phenotype remains somewhat unclear.

Even though many mouse studies have suggested cooperation between pRB pathway alterations and Ras activation, the effect of pRB loss on the ability of Ras to transform murine cells has never been directly investigated. We examined the ability of oncogenic Ras to transform mouse fibroblasts specifically lacking various pRB family members. Surprisingly, murine cells lacking pRB were found to be less susceptible to transformation by activated Ras than wild-type cells. In addition, expression of H-Ras^{V12} in human tumor cells carrying a deletion in the *Rb* locus results in decreased proliferation of these cells. Moreover, we find that pRB expression is required to maintain the proliferation of H-Ras^{V12}-transformed murine cells as well as human tumor cell lines harboring activating Ras mutations. We also provide evidence suggesting that pRB-dependent down-modulation of the related p107 protein is important for Ras-mediated oncogenic transformation.

MATERIALS AND METHODS

Cell culture, transfection, and proliferation assays. The generation and maintenance of wild-type, p107$^{-/-}$ p130$^{-/-}$, and pRB$^{-/-}$ 3T3 mouse fibroblasts has been previously described (10). Importantly, all these 3T3 cell lines harbor p53 mutations. Mouse embryo fibroblasts (MEFs) generated form day 13.5 wild-type and pRB-deficient embryos were a kind gift from Lili Yamasaki and E. Harrington (29) and were transfected at passage 2. Other cell lines were obtained from the American Type Culture Collection and maintained according to their recommendations. Lipid-based transfections of mouse embryo fibroblasts, 3T3 mouse fibroblast cells, HCT116, HT29, U2OS, 5637, and Saos-2 cells were performed with Lipofectamine Plus reagents according to the manufacturer's recommendations (Invitrogen).

Stable cell lines were generated by transfecting cells with recombinant plasmids (pBabe, pBabe-ras^{v12}, pBabe-pRB, pBabe-p107, MSCV, MSCV-p16sh, and MSCV-RB-sh) followed by selection in puromycin-containing medium (2 μg/ml; Sigma). Cells were assayed either as pools or as individual clones as specified in the figure legends. Cellular proliferation was assayed by seeding cells in medium containing 10% fetal bovine serum (FBS), and then counting cells 5 days postplating. Medium was replaced on day 3. Vector- or H-Ras^{V12}-transfected wild-type, p107$^{-/-}$/p130$^{-/-}$, and pRB$^{-/-}$ cells were seeded at 10^5 cells per 6-cm dish. In experiments where PD98050 was used, 10 μM of the pharmacological inhibitor was added every day with an equal volume of dimethyl sulfoxide added to the "untreated" control. Giemsa staining was performed according to the manufacturer's recommendations (Gibco) and quantification of stained plates were performed using a Odyssey infrared scanner (Li-cor) or by counting colonies (as specified in the figure legends).

Focus formation and soft agar colony formation. For focus-forming assays in MEFs, passage 2 to 3 cells were transfected with either vector, pBabe-HRas^{V12} or pBabe-HRas^{V12} and pCMV-E1A. Following 4 days of selection in puromycin-containing medium (2 μg/ml; Sigma) an equal number of cells (8×10^5/10-cm dish) were plated, medium was replaced every 3 days, and colonies were stained with Giemsa and counted 17 days later. For focus-forming assays in NIH 3T3 fibroblasts, cells were seeded at 3×10^5 cells per 3-cm plate in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS and transfected with 100 ng of the indicated plasmids. Medium was replaced every 2 to 3 days, and the cells were left for 14 days after reaching confluence. Macroscopically visible foci were then counted and photographed.

For soft agar proliferation assays, vector or H-Ras^{V12} transfected p107$^{-/-}$/p130$^{-/-}$, and pRB$^{-/-}$ cells were plated in agarose-containing medium (DMEM with 10% FBS and 0.45% SeaKem LE agarose) (BioWhittaker Molecular Applications), and a few drops of fresh medium (DMEM with 20% FBS) were added every 2 days for 14 days before cells were photographed. For soft agar assays in Saos-2 (pRB-deficient osteosarcoma) cells, cells were transfected with vector control, H-Ras^{V12}, or H-Ras^{V12C40} in combination with a plasmid that provides puromycin resistance, followed by puromycin selection in the presence of PD98059 (to retrieve a sufficient number of Ras-expressing cells). Notably, PD98059 does not inhibit the proliferation of pRB-deficient cells (see Fig. S2A in the supplemental material) (12).

Following 3 to 4 days of puromycin selection, protein expression was analyzed (see below for details), and an equal number of cells were seeded in soft agar (as above) in the presence or absence of PD98059 (10 μM). A few drops of fresh medium (DMEM with 20% FBS) with or without PD98059 were added every 2 to 3 days for 20 days, after which time the number of macroscopically visible colonies was counted. For transformation assays in the HT29 colorectal cell line, colonies from cells transfected with 1 μg MSCV, MSCV-p16-sh, or MSCV-pRB-sh (53) followed by puromycin selection. Clones with reduced pRB expression as well as controls were plated at a density of 5×10^4 cells/3-cm dish in soft agar (as above); 20 days postseeding colonies were photographed and the size of 50 colonies of each cell type was determined and averaged as presented in Fig. 6D.

Cell cycle analysis. Analysis of wild-type, p107$^{-/-}$/p130$^{-/-}$ and pRB$^{-/-}$ 3T3 mouse fibroblasts transfected with vector, H-Ras^{V12}, and H-Ras^{V12} plus E1A by fluorescence-activated cell sorting (FACS) was performed according to the manufacturer's protocol (Becton Dickinson). Cells were seeded in 10-cm dishes (DMEM with 1% FBS) for 2 days, at which time they were incubated with cell labeling reagent (bromodeoxyuridine; Amersham Pharmacia) at 37°C for 30 min.

FACS analysis of Saos-2 cells transfected with the vector control or H-Ras^{V12} was done as follows. Following 3 to 4 days of selection in the presence of PD98059 (to retrieve a sufficient number of Ras-expressing cells) an equal number of cells were seeded in 10-cm dishes (DMEM plus 10% FBS) for 2 days, at which time they were incubated with cell labeling reagent (bromodeoxyuridine, Amersham Pharmacia) at 37°C for 30 min. Before analysis cells were fixed and stained according to the manufacturer's recommendations (Becton Dickinson). In brief, all cells were washed with phosphate-buffered saline and trypsinized before being fixed in 80% ethanol. DNA was denatured for 30 min with 2 M HCl–0.5% Triton X-100 and neutralized with 0.1 M $NaB_4O_7 \cdot 10H_2O$ (pH 8.5), before incubation with antibromodeoxyuridine antibody (1:500, Becton-Dickinson) and a fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody (1:50, Vector Laboratories). Cells were stained with 5 μg/ml propidium iodide (Sigma) and treated with RNase A (Sigma) prior to two-dimensional FACS analysis using Cellquest software (Becton Dickinson). The number of gated cells incorporating bromodeoxyuridine at the time of analysis is presented as a percentage of cells in S phase and the number of cells in the sub-G_1 population is presented as the percentage of apoptotic cells (see Fig. 2D and 3C).

RNA interference studies. RNA inference (RNAi) was performed with custom-synthesized double-stranded RNA oligonucleotides (Xeragon/QIAGEN) introduced into cells with the Transmessenger reagent (QIAGEN) according to the manufacturer's protocol. In short, six-well dishes were seeded the day before transfection in DMEM containing 10% FBS. On the day of transfection, 2 μg total of a mixture of two target sequences of short interfering RNA (siRNA) were incubated with 4 μl of enhancer reagent in a total volume of 100 μl for 5 min followed by the addition of 8 μl of Transmessenger reagent and a 10-min incubation period before addition to the cells. After 3 h, the cells were washed and replenished with fresh medium.

The siRNA-treated cells were harvested 48 h after transfection for analysis. Target sequences are as follows: *Rb*-human 1, 5′-AAG TTT CAT CTG TGG ATG GAG-3′); *Rb*-human 2, 5′-AAT GGT TCA CCT CGA ACA CCC-3′); *Rb*-mouse 1, 5′-AAC CCA GCA GTG CGT TAT CTA-3′); *Rb*-mouse 2, 5′-AAC

TCT GGC TTC TTC AAA GCC-3′); and control nonsilencing, 5′-AAT TCT CCG AAC GTG TCA CGT-3′). For the analysis of HT29 cells stably expressing pRB-sh, the cells were transfected with 1 μg MSCV, MSCV-p16-sh, or MSCV-pRB-sh (53) selected with puromycin, and colonies were picked and analyzed for pRB, p107, and p130 expression as well as proliferation (Fig. 6 and 7B).

Protein expression analysis. To analyze expression levels of endogenous and exogenous recombinant proteins, cells were trypsinized, counted, and lysed in 2 g Laemmli's solution (5.5×10^5 cells/100 μl). Whole-cell lysates were homogenized by sonication and separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) in 8% (pRB, p107, p130, and p44/42) or 10 to 12% (Ras) polyacrylamide and transferred to a nitrocellulose membrane (Millipore Immobilon-P). Membranes were probed with antibodies directed against c-H-Ras (dilution 1:1,000; F235-1.7.1; Oncogene), pRB (1:1,000; G3-245; Pharmingen), p107 (SD9; 1:200; Santa Cruz), p130 (RB-2; Transduction Laboratories; 1:2,000), p44/42 mitogen-activated protein kinase (MAPK) (1:1,000; polyclonal; Cell Signaling Technologies), phosphorylation-specific pRB (Ser 780, Ser 795, and Ser 807/811, at 1:1,000; Cell Signaling Technologies), cyclin D1 antibody (1:2,000; DCS6; Cell Signaling Technologies), and E1A antibody (1:10; M73; Harlow). Secondary incubations were performed with horseradish peroxidase-conjugated anti-mouse (1:2,000; Amersham Pharmacia), anti-goat (1:2,000), or anti-rabbit (1:2,000; Amersham Pharmacia) antibodies, and visualization was achieved using an ECL kit.

For immunoprecipitations of p107 protein, cells were lysed in radioimmunoprecipitation assay (RIPA) buffer. Following centrifugation the supernatants were incubated with 2 μg p107 antibody (SD9; Santa Cruz) overnight at 4°C; protein G-Sepharose (Pharmacia) was added for 1 h and the precipitates were subsequently washed four times in RIPA buffer before the protein G pellets were boiled in sample buffer. Samples were separated on 8% polyacrylamide gels and Western blots were performed as described above.

Chromatin immunoprecipitation. Chromatin immunoprecipitations were performed using modified methods published previously (18, 48, 57). Briefly HCT116 or HT29 cells were grown to confluence and treated with formaldehyde (1% final) for 10 min. The cross-linking was stopped using 0.125 M glycine, the cells were trypsinized and washed twice with phosphate-buffered saline plus 10% FBS, and the pellets were subsequently freeze-thawed. Following lysis and sonication to generate DNA fragments of proper length, the equivalent of 0.8×10^7 cells were used for each immunoprecipitation reaction using antibodies against Rb (sc-50 from Santa Cruz and G3-245 from Becton Dickinson) and an E1A (Ab-1; Oncogene) used as a control.

Coprecipitated DNA as well as input DNA were analyzed in multiplex PCRs using 0.8 μCi [^{33}P]dCTP, with primers that amplify the p107 promoter (−170 to +96) and sequences within exon 22 of the p107 gene as a control. The presence of the p107 promoter sequence in the immunoprecipitation reaction is assayed as an enrichment relative to the exon 22 sequences, as well as enrichment relative to the nonspecific antibody (anti-E1A) immunoprecipitation. PCR products were resolved on 6% polyacrylamide gels, dried, and exposed to film. Each chromatin immunoprecipitation experiment was performed at least three times and the presence of pRB at the p107 promoter was confirmed using two different antibodies for immunoprecipitation.

Transcriptional assays. Reporter constructs carrying the *p107* gene promoter (10) driving the luciferase gene were stably integrated into HCT116 and HT29 cells using a cotransfected puromycin resistance marker (pBabe) for selection. The cells were selected in DMEM with 10% FBS containing 2 μg/ml puromycin, pooled, and used for transcriptional analysis. *Rb*-siRNA and PD98059 treatments were performed as described above on confluent cell populations to avoid effects that stem from proliferative changes. Luciferase assays (Promega) were performed according to the manufacturer on an equal number of cells and expressed as relative activity compared to untreated cells. Results were obtained using a luminometer from EG&G Berthold (Wallac, Gaithersburg, MD).

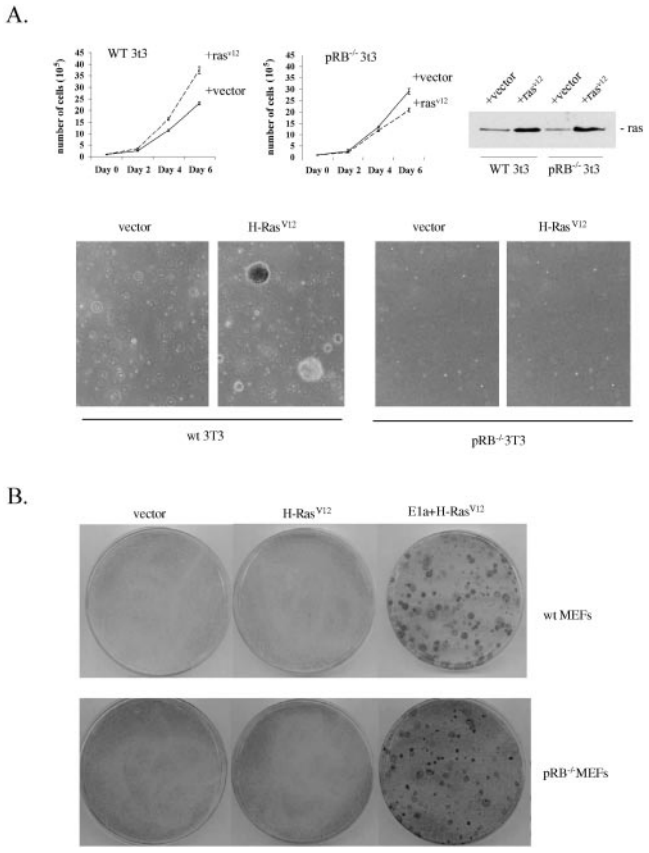

FIG. 1. pRB-deficient cells are not transformed by H-Ras^{V12}. (A) Pools of puromycin-selected wild-type 3T3 (WT) and pRB$^{-/-}$ 3T3 cells transfected with vector alone or with H-Ras^{V12} were assayed for proliferation. The proliferation rate of vector-transfected cells or H-Ras^{V12}-transfected cells was analyzed over a period of 6 days by counting cells every 2 days following plating. The results reflect the average of two independent experiments performed in triplicate. The right panel shows a Western blot verifying the expression of H-Ras^{V12} in wild-type and pRB$^{-/-}$ 3T3 cells as indicated. The bottom panels show the proliferation in soft agar of wild-type and pRB$^{-/-}$ 3T3 cells transfected with vector and H-Ras^{V12}. A representative example of soft agar colonies photographed 2 weeks postplating is shown. (B) pRB-deficient MEFs are not transformed by H-Ras^{V12} but requires a cooperating event. Early-passage wild-type and pRB$^{-/-}$ MEFs were transfected with vector, H-Ras^{V12} or H-Ras^{V12} plus adenovirus E1A as indicated in the figure. Infected cells were selected in puromycin containing medium and 4 days postselection an equal number of cells were plated on 10-cm dishes, medium was changed every 3 days, and the dishes were stained with Giemsa after 17 days. Representative images of two independent experiments in duplicate are shown.

RESULTS

Distinct requirements for pRB family members in the context of Ras-induced oncogenic transformation. To explore the relationship between the pRB family of proteins and Ras-mediated transformation, we compared the H-Ras^{V12} sensitivity of mouse 3T3 fibroblasts lacking various family members. Following transfection with an activated form of Ras (H-Ras^{V12}), we made the surprising observation that the proliferation of cells lacking pRB is reduced relative to that of wild-type cells transfected with H-Ras^{V12} (Fig. 1A). In contrast to wild-type 3T3 cells, pRB-deficient cells transfected with H-Ras^{V12} also failed to form colonies in soft agar (Fig. 1A, bottom panels) suggesting that that the pRB family member targeted by E1A and other viral oncoproteins may not be pRB.

As shown previously, pRB-deficient mouse embryo fibroblasts do not form foci in response to expression of activated Ras (54, 60) (Fig. 1B; see Fig. S1A in the supplemental material). Cotransfection of E1A and H-Ras^{V12} into wild-type and pRB$^{-/-}$ MEFs resulted in an equal number of foci in cells of both genotypes (Fig. 1B; see Fig. S1A in the supplemental

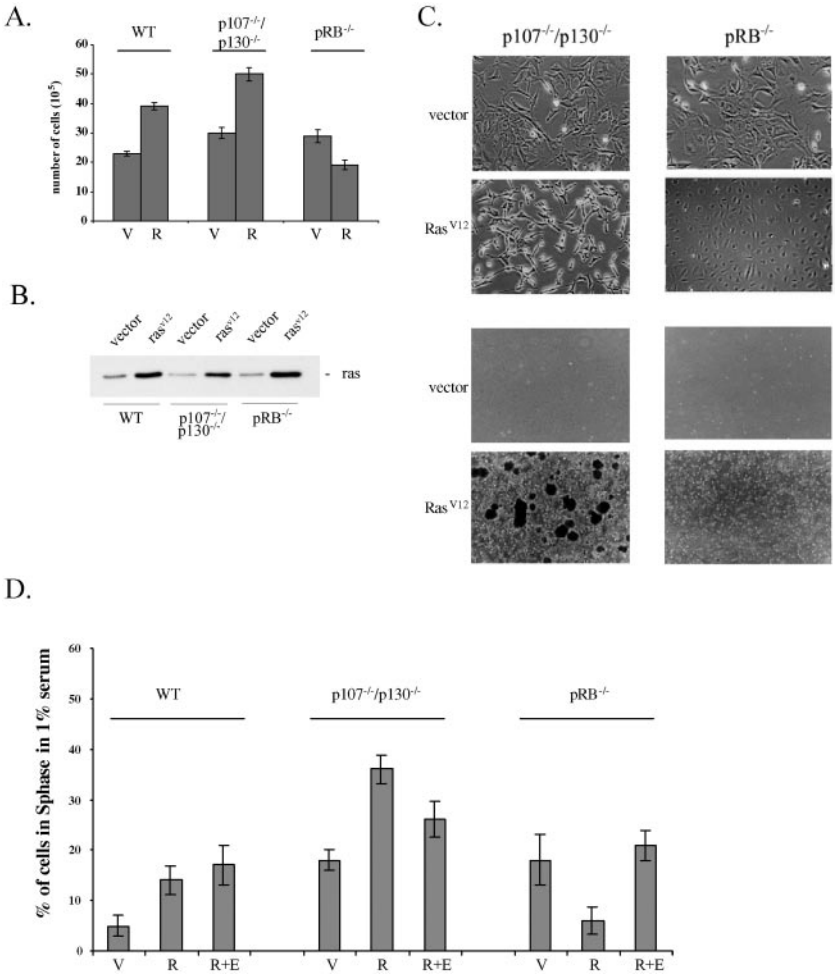

FIG. 2. Cells deficient in the pRB-related proteins p107 and p130 are more susceptible to H-Ras^{V12} transformation than wild-type and pRB-deficient cells. (A) Puromycin-selected pools of wild-type, p107$^{-/-}$/p130$^{-/-}$ or pRB$^{-/-}$ cells transfected with a vector control (V) or a H-Ras^{v12}-expressing plasmid (R) were assayed for proliferation 5 days after plating. The results are based on two independent experiments performed in triplicate. (B) Western blot analysis indicating the expression of Ras in wild-type, p107$^{-/-}$/p130$^{-/-}$ and pRB$^{-/-}$ cells transfected with vector or an H-Ras^{V12}-expressing plasmid. (C) Representative microscopy images of the morphology of p107$^{-/-}$/p130$^{-/-}$ and pRB$^{-/-}$ cells transfected with a vector control or a H-Ras^{V12}-expressing vector as indicated in the figure. The bottom panels show that p107$^{-/-}$/p130$^{-/-}$, but not pRB$^{-/-}$ cells transfected with H-Ras^{V12} proliferate in soft agar. A representative example of cells transfected with a vector control or an H-Ras^{V12}-expressing plasmid, plated in soft agar and photographed 2 weeks postplating, is shown. (D) The ability of p107$^{-/-}$/p130$^{-/-}$ and pRB$^{-/-}$ cells transfected with either vector (V), H-Ras^{V12} (R), or H-Ras^{V12} plus E1A (R+E)-expressing plasmids to proliferate after 2 days in low serum (1% FBS), as analyzed by FACS. Cells were labeled with bromodeoxyuridine for 30 min and the percentage of cells that incorporated bromodeoxyuridine is illustrated as percentage of S phase, and the data presented are based on three independent experiments.

material). Taken together, these experiments suggest that loss of pRB-expression does not result in increased proliferation in the context of a constitutively active Ras allele.

In contrast to the decrease in proliferation seen in response to the expression of activated Ras in pRB$^{-/-}$ 3T3 cells, the proliferation of 3T3 cells deficient in both p107 and p130 is potently stimulated by H-Ras^{V12} transfection (Fig. 2A). In fact, these cells proliferate somewhat faster than wild-type cells transfected with H-Ras^{V12}. When plated in medium with a low serum concentration, p107/p130-deficient cells transfected with H-Ras^{V12} have a high S-phase content compared to vector-transfected cells, whereas pRB$^{-/-}$ cells transfected with H-Ras^{V12} have a lower S-phase content than vector-transfected cells (Fig. 2D).

Our previous studies of pRB family-deficient 3T3 cells had shown that the cell cycle properties of wild-type 3T3 cells are different from those of cells deficient in pRB, p107, and/or p130 (10) as well as S-phase content in low serum (shown in Fig. 2D), complicating comparisons between them. Therefore, we chose the p107$^{-/-}$/p130$^{-/-}$ and the pRB$^{-/-}$ 3T3 cells for further analysis, since their cell cycle properties as well as morphological features are virtually indistinguishable (10) (Fig. 2C, upper panels). Whereas, neither the p107/p130-deficient nor the pRB-deficient 3T3 cells that we generated form colonies in soft agar (Fig. 2C, bottom panels), H-Ras^{V12}-transfected p107$^{-/-}$/p130$^{-/-}$ cells, but not H-Ras^{V12}-transfected pRB$^{-/-}$ cells, readily form colonies in soft agar (Fig. 2C).

Taken together, these findings suggest that loss of pRB does not increase the transforming ability of activated Ras in immortalized 3T3 cells. In addition, our findings suggest that the

pRB family member target for E1A, in the context of Ras transformation, may be p107 and/or p130 rather than pRB, since the proliferation of H-Ras^{V12}-transformed p107$^{-/-}$/p130$^{-/-}$ cells is inhibited by E1A, whereas the proliferation of H-Ras^{V12}-transfected pRB-deficient 3T3 cells is stimulated by E1A expression (Fig. 2D).

In summary, these experiments reveal a functional distinction between the closely related pRB family proteins in the presence of activated Ras in immortalized mouse fibroblasts, whereas in other contexts, these proteins seem to exhibit substantial functional redundancy (14, 60).

Oncogenic Ras inhibits proliferation of human tumor cells that lack pRB. To clarify the observed antagonistic relationship between activated Ras and loss of pRB in oncogenic transformation, we took advantage of human tumor cells deficient in pRB. To determine whether activated Ras negatively affects the proliferation of human tumor cells that lack pRB, as seen in pRB-deficient 3T3 cells (Fig. 1 and 2), we transfected activated H-Ras^{V12} into *Rb*-deficient human osteosarcoma cells (Saos-2). These experiments show that H-Ras^{V12} substantially inhibits the proliferation of these cells (Fig. 3A and B). The same decrease in proliferation is seen in 5637 cells (a pRB-deficient bladder tumor cell line) (Fig. 3A, bottom panels). The decreased number of Saos-2 cells seen in response to activated H-Ras is due to a decrease in the S-phase population as well as an increase in apoptosis (Fig. 3C).

To further analyze the effect of activated Ras on the tumorigenic potential of the osteosarcoma cells, we plated vector- and H-Ras^{V12}-transfected Saos-2 cells in soft agar. These experiments show that H-Ras^{V12} also inhibits the ability of Saos-2 cells to form colonies in semisolid medium (Fig. 3D). The inhibition of anchorage-independent growth by H-Ras^{V12} in Saos-2 cells is partially reversed by the pharmacological Mek inhibitor PD98059 (an inhibitor of the Ras-MAPK pathway). Similarly, an H-Ras^{V12} mutant (H-Ras^{V12} C40) that fails to activate the MAPK cascade exhibits a reduced ability to suppress the colony-forming efficiency of Saos-2 cells, indicating that the inhibitory effects of oncogenic Ras in cells lacking pRB require MAPK activation. Notably, PD98059 does not inhibit the proliferation of pRB-deficient cells (see Fig. S2A in the supplemental material) (12).

To verify that the observed effect of activated Ras is dependent on the absence of pRB we investigated whether reexpression of pRB in these cells would reverse the inhibitory effect that activated Ras has on their proliferation. Even though it is well established that pRB overexpression inhibits the proliferation of Saos-2 cells (56), low levels of pRB (not growth inhibitory) partially rescue the growth-inhibitory effect that is seen when activated H-Ras^{V12} is expressed in Saos-2 cells (Fig. 3E). Taken together, these results suggest that in the absence of pRB, mutationally activated Ras has an unexpected inhibitory effect on the proliferative capacity of human tumor cells.

pRB expression is required to maintain proliferation in H-Ras^{V12}-transformed mouse fibroblasts. Tumors carrying Ras pathway mutations almost uniformly maintain an intact *Rb* gene but carry alterations in other pRB pathway components. The lack of *Rb* mutations in these tumors has been previously explained by the observation that activation of Ras results in increased levels of cyclin D (21), which, according to current dogma would phosphorylate and inactivate pRB. However, it

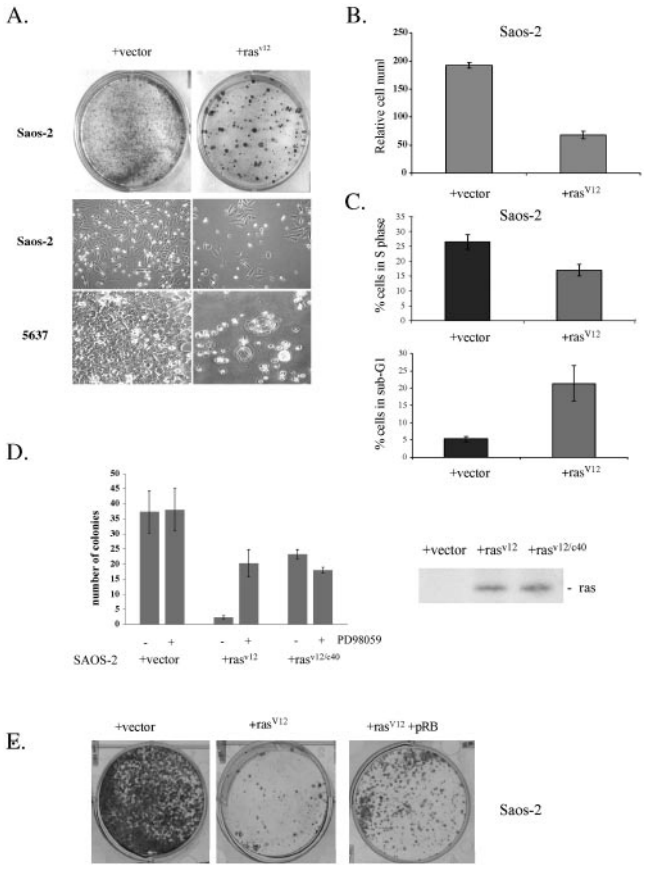

FIG. 3. Constitutively active Ras inhibits the proliferation of *Rb*-deficient human tumor cells (A) Giemsa-stained plates of Saos-2 (Rb-deficient osteosarcoma, top two panels) cells transfected with vector- or H-Ras^{V12}-expressing plasmid followed by selection in puromycin. The middle panels are representative microscope images of pools of Saos-2 cells transfected with a vector control, and H-Ras^{V12}- or pRB-expressing vectors. The transfected cells were plated at the same density and photographed 3 days after plating. The two bottom panels are representative images of 5637 (*Rb*-deficient bladder tumor) cells transfected with a vector control or a H-Ras^{V12}-expressing plasmid after selection for 2 weeks. (B) The relative number of Saos-2 cells following transfection with a vector and an H-Ras^{v12}-expressing plasmid as indicated in the figure. Giemsa-stained plates were quantified using an Odyssey image reader (Li-Cor) and the numbers presented are based on three independent experiments in duplicate. (C) Saos-2 cells transfected with a vector control or H-Ras^{V12}-expressing plasmid were selected for 5 days in puromycin (2 μg/ml) in the presence of PD98059 (10 uM), to retrieve a sufficient number of H-Ras^{V12}-expressing cells. Cells were labeled with bromodeoxyuridine for 30 min 2 days after plating at 3×10^5 cells in a 3-cm dish. The percentage of cells that incorporated bromodeoxyuridine is illustrated % of cells in S phase and the percentage of cells that have less than a 2N DNA (cells undergoing apoptosis) content is illustrated as % cells in sub-G_1. The results presented are based on two experiments in duplicates. (D) Saos-2 cells transfected with a vector control, and H-Ras^{v12}- or H-Ras^{V12}C40-expressing plasmids were selected for 3 to 4 days in puromycin in the presence of PD98059 (to obtain a sufficient number of cells transfected with activated Ras), and equal numbers of cells were plated in soft agar for 18 to 20 days after which colonies were counted. The results presented in the figure are derived from two independent experiments in which five separate fields were counted under the microscope. The panel to the right of the graph shows a representative Western analysis verifying Ras expression in transfected Saos-2 cells. (E) Giemsa-stained plates of Saos-2 cells transfected with vector (100 ng), H-Ras^{V12} (100 ng), or H-Ras^{V12} (100 ng) plus pRB (10 ng)-expressing plasmids followed by selection in puromycin.

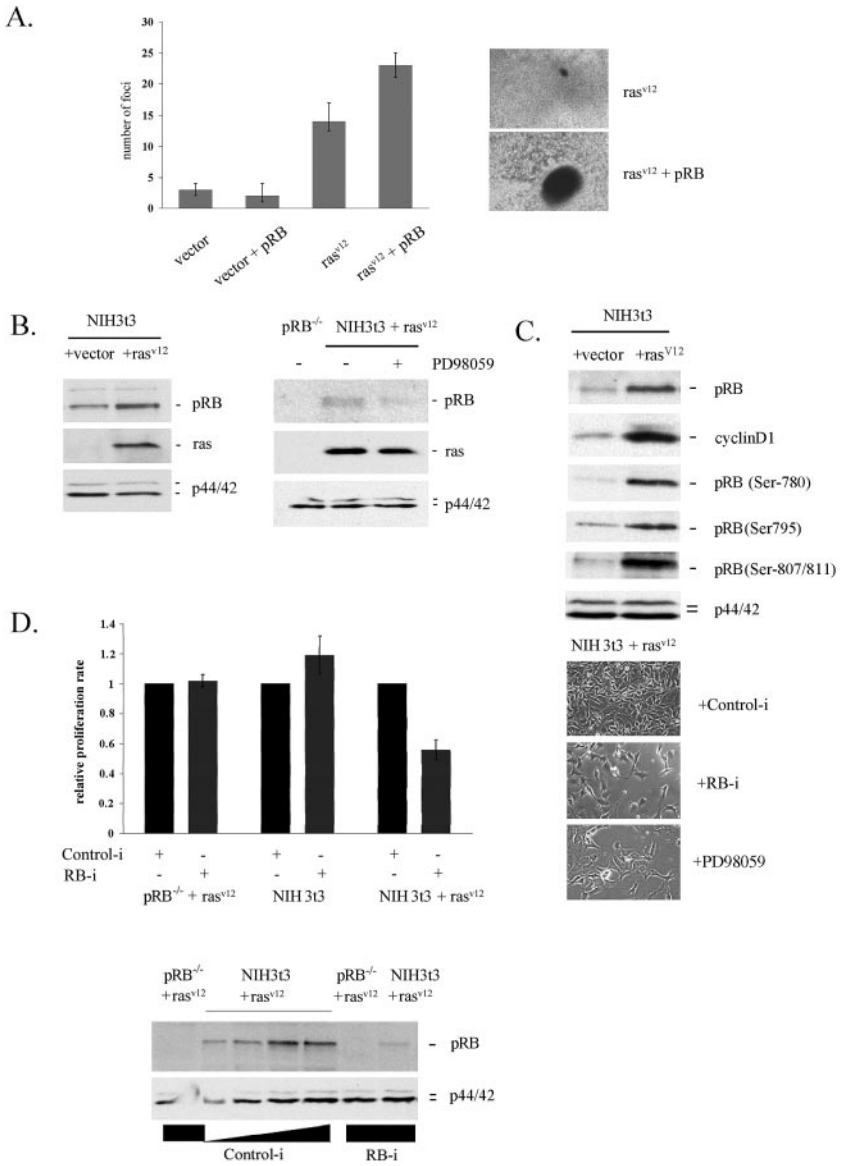

FIG. 4. Maintenance of H-Ras^{V12}-induced proliferation in 3T3 fibroblasts is dependent on pRB expression. (A) NIH 3T3 cells were transfected with a vector control (vector), a pRB-expressing plasmid (pRB), an H-Ras^{V12}-expressing vector (ras^{V12}), or the combination of H-Ras^{V12} and pRB (Ras^{V12} plus pRB), and colonies were counted 2 to 3 weeks postconfluence. The bar graphs are based on the results of two independent experiments. The microscopy images on the right illustrate the increased size of foci seen following transfection with a combination H-Ras^{V12} and pRB, as indicated. (B) Western blot analysis of pRB protein levels in pools of NIH 3T3 cells transfected with vector or H-Ras^{V12} as indicated in the left panel of the figure. The right panel shows the level of pRB protein in confluent H-Ras^{V12}-transfected NIH 3T3 cells that have been treated with the dimethyl sulfoxide control or PD89059 for 15 h. Confluent cells were used to minimize cell cycle effects. (C) Western blot analysis of levels of total pRB protein, cyclin D, as well as the levels of pRB protein phosphorylated on various sites using phospho-specific antibodies as indicated in the figure, in pools of NIH 3T3 cells transfected with vector or H-Ras^{V12}. (D) The proliferation of NIH 3T3, NIH 3T3 plus H-Ras^{V12}, and pRB$^{-/-}$ plus H-Ras^{V12} cells transfected with *Rb* siRNA oligonucleotides (RB-i) and control siRNA oligonucleotides (Control-i) 2 days posttransfection. Illustrated in the figure is the relative proliferation of cells transfected with *Rb*-siRNA oligonucleotides compared to cells transfected with control RNAi oligonucleotides. The results are based on three independent experiments. The microscopy images show representative examples of the reduced proliferation seen in H-Ras^{V12}-transformed NIH 3T3 cells treated with *Rb*-siRNA oligonucleotides or PD98059 2 days posttransfection or addition of drug. The bottom panel illustrates the decrease in pRB levels following transfection of pRB siRNA oligonucleotides (RB-i) or control RNAi (Control-i), as indicated. The extract from cells transfected with the control siRNA oligonucleotide was titrated to better illustrate the loss of pRB in the *Rb*-siRNA-treated samples, using total p44/42 as a loading control.

has also been reported previously that some cancers that exhibit activation of the Ras pathway, such as human colon tumors, frequently express very high levels of pRB protein (2, 24, 75), raising the possibility that increased pRB expression is important for the transformed phenotype of these cells.

Consistent with such a scenario, we find that when NIH 3T3 cells are transfected with a vector that expresses pRB together with H-Ras^{V12}, an increase in the number of transformed foci compared to cells transfected with H-Ras^{V12} alone is observed (Fig. 4A). Moreover, the average size of foci transfected with

both H-Ras^{V12} and pRB, is substantially greater than that of foci seen with H-Ras^{V12} alone (Fig. 4A, right panels). We determined that cells from each of six independent H-Ras^{V12}/Rb clones (foci) express variable levels of Ras protein; however, there is no correlation between Ras levels and focus size (data not shown), indicating that this effect is not simply a consequence of pRB-induced increased Ras expression.

We also observed that all H-Ras^{V12}-transformed foci express elevated levels of pRB protein compared to vector-transfected NIH 3T3 cells (Fig. 4B). Treatment of H-Ras^{V12}-transformed NIH 3T3cells (Fig. 4B, right panel) with the Mek inhibitor PD98059 decreases pRB protein levels, indicating that the increase in pRB expression is at least partially due to increased Ras-MAPK signaling. Increased pRB levels have also been reported in K-Ras-transformed rat enterocytes (1). Moreover, a microarray gene expression screen identified *Rb* as a transcriptional target of activated Ras (78). These results are consistent with a role for Ras in regulating pRB levels as an essential component of Ras transforming activity.

Since it is well established that cyclin D1 levels are increased by activated Ras (21, 23) (Fig. 4C), and this can lead to pRB "inactivation" by Cdk-mediated phosphorylation, it is possible that pRB protein in H-Ras^{V12}-transformed cells is inactive. Using phospho-RB-specific antibodies, we found no significant increase in the proportion of phosphorylated pRB relative to the increased total levels of pRB in the Ras-transformed cells (Fig. 4C), suggesting that the pool of active pRB is not significantly affected by the activation of Ras.

To directly determine whether the pRB protein is important to maintain Ras-mediated proliferation we utilized siRNA technology to specifically reduce pRB expression in H-Ras^{V12}-transformed NIH 3T3 cells (Fig. 4D; see Fig. S1C in the supplemental material). While transfection of nontransformed NIH 3T3 cells with siRNA corresponding to the *Rb* gene mildly stimulates cell proliferation, transfection of H-Ras^{V12}-transformed 3T3 cells with *Rb*-siRNA causes a substantial reduction in their proliferative rate. Importantly, *Rb*-siRNA has no effect on pRB-deficient 3T3 cells, excluding potential nonspecific effects resulting from treatment with *Rb*-siRNA. These results indicate that pRB expression is required to maintain the proliferation of H-Ras^{V12}-transformed NIH 3T3 cells. Taken together with the data showing that activation of Ras in pRB-deficient 3T3 cells results in reduced proliferation (Fig. 1 and 2), these findings further support the seemingly antagonistic relationship between Ras activation and pRB-loss.

pRB expression is required to maintain the transformed phenotype in human tumor cells harboring Ras pathway mutations. In light of these observations, we next tested the hypothesis that the proliferation of naturally arising human tumors that harbor Ras mutations depends on the maintenance of pRB expression. For this analysis, we used human colon cancer cell lines in which the Ras pathway is mutationally activated. Ras and Raf mutations are seen with high frequency in colorectal cancers, and such tumors often exhibit a relatively high level of pRB protein (2, 24, 32, 35, 75).

Similar to findings in H-Ras^{V12}-transformed 3T3 cells, the pRB levels in these cells seem to be induced by activation of Ras-MAPK signaling since treatment of colorectal tumor cell lines (Fig. 5A, left panel) with the Mek inhibitor PD98059 decreases pRB protein levels. Using phospho-RB-specific antibodies, we found no significant increase in the proportion of phosphorylated pRB relative to increased total levels pRB in the colorectal tumor cells (Fig. 5A, right panel), indicating that the pool of active pRB, similar to the findings in H-Ras^{V12}-transformed NIH 3T3cells (Fig. 4C), is not significantly affected by activation of the Ras pathway.

Using siRNA directed against human *Rb*, we determined that reduction of pRB expression in colorectal tumor cells leads to a substantial inhibition of their proliferation (Fig. 5B and C). These findings reveal a requirement for continuous pRB expression in Ras-induced proliferation, similar to the results seen in H-Ras^{V12}-transformed NIH-3T3 cells (Fig. 4D). As a control for nonspecific effects of the *Rb*-RNAi oligonucleotides, we determined that transfection of *Rb*-siRNA has no effect on the proliferation of the pRB-deficient, Saos-2 osteosarcoma cell line (Fig. 5B). Notably, antisense RB has previously been shown to result in a reduced number of cells in S phase as well as the induction of apoptosis in HCT116 colorectal tumor cells (75). Although we did not detect any significant induction of apoptosis in our siRNA experiments in HCT116 cells and Ras-transformed 3T3 cells, we did see apoptosis in Saos-2 cells expressing constitutively active H-Ras^{V12} (Fig. 3C), indicating that in addition to a decrease in S phase, there may be induction of apoptosis in response to activated Ras in the absence of pRB.

In addition to the inhibitory effect on proliferation seen in colorectal tumor cells following pRB depletion (Fig. 5B), we also determined that transfection with *Rb*-siRNA significantly inhibits the proliferative rate of each of several other human tumor cell lines known to harbor Ras mutations, including pancreatic cancer, breast cancer, and small cell lung carcinoma cell lines harboring Ras pathway mutations, indicating that pRB is required for Ras-induced tumorigenesis in multiple tissue types (see Fig. S2B in the supplemental material). Notably, similar to the high levels of pRB seen in colorectal tumors, high levels of pRB expression have been reported in pancreatic carcinoma compared to normal pancreatic tissue (55).

To determine whether loss of pRB expression in colorectal tumor cells affects their tumorigenic potential, we investigated the ability of these cells to form colonies in soft agar. For these experiments we stably transfected these cells with retroviral vectors expressing short hairpin interfering RNAs targeting the *Rb* gene (pRB-sh) (53). In both HCT116 and HT29 colon carcinoma cell lines, transfection with the pRB-sh plasmid results in a reduced number of colonies relative to the number seen in cells transfected with a vector control or a short hairpin plasmid that targets p16 (p16-sh) (53) (data not shown).

From the colonies that did grow we were unable to establish HCT116 lines that had a substantial reduction in pRB protein levels. However, we generated three separate HT29 lines that exhibit a reduction in pRB protein levels (Fig. 6A). HT29 cells that express the pRB-sh exhibit a decrease in their proliferative rate compared to cells transfected with the vector control or a vector expressing p16-sh (Fig. 6B and C). The decrease in cell number is similar to that seen in HT29 cells transiently transfected with RNAi oligonucleotides against pRB (Fig. 5B). These cell lines were then plated in soft agar to determine whether HT29 cells with reduced pRB levels also have a di-

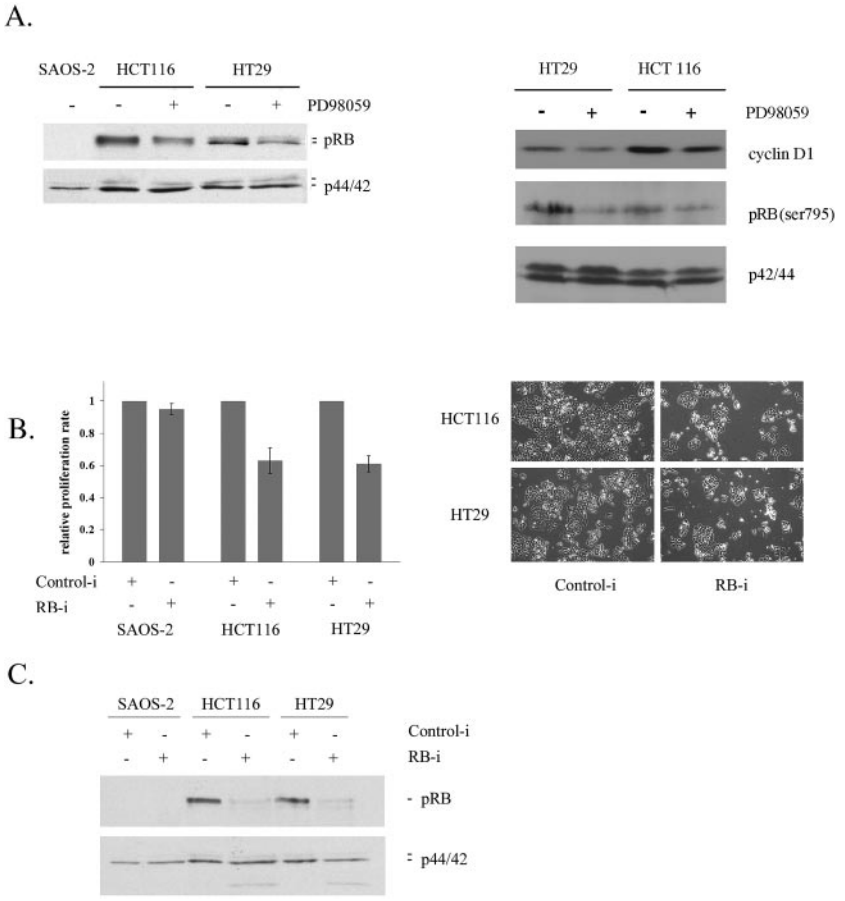

FIG. 5. pRB is required for the proliferation of human tumor cells harboring Ras mutations. (A) pRB protein levels in colorectal tumor lines are decreased by inhibition of MAPK signaling. The right panel is a Western blot of pRB protein levels in confluent HCT116 and HT29 colorectal tumor lines after treatment with dimethyl sulfoxide control or PD98059 for 15 h, as indicated. An equivalent number of cells were used for Western blot analysis, using the total amount of p44/42 as an internal control. The right panel show an analysis of cyclin D and phosphorylated pRB as indicated in the figure. Confluent cell populations were used for these experiments to minimize cell cycle effects. (B) Saos-2 cells (human osteosarcoma deficient in pRB, used as a control for nonspecific siRNA effects), HCT116 and HT29 (human colorectal tumor lines with normal pRB and activating K-Ras and Raf mutations, respectively) cells were transfected with control siRNA oligonucleotides or *Rb*-siRNA oligonucleotides. Cells were counted two days posttransfection and the bar graphs illustrate the relative rate of proliferation of each cell line following *Rb*-siRNA transfection (RB-i) compared to that of cells transfected with control siRNA (Control-i). The results are based on three independent experiments, performed in duplicate. To the right of the graphs are representative microscopy images of HCT116 and HT29 cells transfected with control or *Rb*-siRNA oligonucleotides 2 days posttransfection, as indicated. (C) Western blot analysis illustrating the decrease in pRB protein seen after transfection with *Rb*-siRNAi oligonucleotides compared to controls, using total p44/42 as a loading control.

minished ability to proliferate in soft agar. As seen in Fig. 6D (average size of soft agar colonies) and 6E (representative images), HT 29 clones expressing the pRB-sh form substantially smaller colonies than HT29 cells transfected with the vector control or the p16-sh plasmid, suggesting that cells with an activated Ras pathway are less tumorigenic when pRB levels are reduced. Taken together with our observations that activation of Ras results in reduced proliferation in soft agar of pRB-deficient osteosarcoma cells (Fig. 3D), these results indicate that pRB is required for the transforming effects of activated Ras in human tumor cells.

Increased levels of pRB in colorectal tumor cells repress p107 expression. Surprisingly, our studies suggest that Ras-mediated transformation is increased in the presence, and not the absence, of the pRB tumor suppressor protein. As mentioned above, colorectal tumors express relatively high levels of pRB protein and this increase in pRB expression is at least partially due to increased Ras-MAPK signaling (Fig. 5A, left panel). The most clearly established cellular function of the pRB protein is that of transcriptional regulation via its interaction with the E2F-family proteins (for review see reference 65). One of the identified downstream transcriptional targets of pRB is its close relative, p107, whose expression is repressed by pRB (36, 51, 67).

Interestingly, our experiments in 3T3 cells deficient in various family members had suggested that loss of p107 and p130 results in increased sensitivity to H-Ras^{V12}-mediated transformation (Fig. 2). We also found that H-Ras^{V12}-transfomed p107/p130-deficient cells were relatively insensitive to pRB loss (data not shown), suggesting that an important function of pRB in Ras-mediated transformation could be its suppressive effect on p107 transcription.

To investigate this possibility, we first analyzed the levels of p107 in colorectal tumor cell lines (HCT116 and HT29) com-

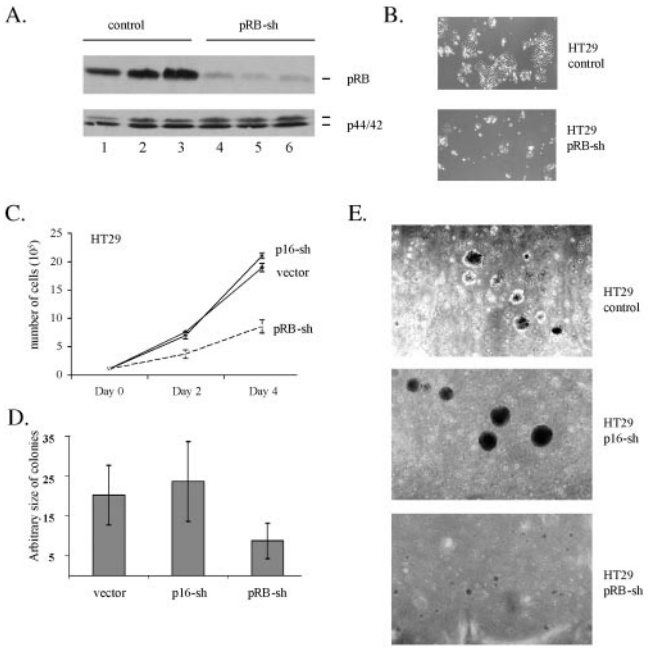

FIG. 6. pRB depletion inhibits soft agar proliferation of human tumor cells with an activated Ras pathway. (A) Western blot illustrating the decrease in pRB protein levels seen in clones of HT29 cells expressing and pRB-sh (lanes 4 to 6) compared to a MSCV vector (lane 1) or a p16-sh (lanes 2 and 3), using total p44/42 as a loading control. (B) These panels show representative microscopy images of HT29 cells stably expressing a control vector or pRB-sh vector 4 days after plating at 4×10^5 cells/10-cm dish. (C) Clones of HT29 cells transfected with MSCV (vector), p16-sh, or pRB-sh were assayed for proliferation. The empty retroviral vector and a p16-sh expressing vector were used as controls in these experiments since HT29 cells exhibit undetectable levels of expression of p16 due to methylation of its promoter (76). The number of cells in each subline was analyzed at day 2 and day 4 following plating as indicated in the figure. The results reflect the average of two independent experiments performed in duplicate. (D) Analysis of the colony diameter of HT29 foci in cells containing a control vector (one clone), a p16-sh-expressing vector (two clones), or a pRB-sh-expressing vector (two clones). Equal numbers of cells were plated in soft agar for 18 to 20 days after which colonies were photographed. The results presented in the figure are derived from two independent experiments in which the diameter of 50 separate colonies was measured and expressed as an arbitrary size (vector, 20.2 ± -0.5; p16-sh, 23.7 ± -10; pRB-sh, 8.75 ± -0.5). (E) Representative images of HT29 colonies in soft agarose from cells containing a control plasmid, a p16-sh-expressing plasmid, or a pRB-sh-expressing plasmid.

pared to tumor cell lines that are deficient in pRB (Saos-2 and 5637). These experiments show that p107 levels are much lower in the colorectal tumor lines that have high levels of pRB expression compared to pRB-deficient tumor lines (Fig. 7A). In addition, we determined that loss of pRB expression in human colorectal tumor cells results in an increase in the expression of p107. All of the HT 29 clones that express the pRB-sh plasmid exhibit an increase in p107 protein levels but no change in their p130 levels (Fig. 7B).

We also find that expression from a p107 promoter reporter is relatively low in the colorectal tumor lines compared to tumor lines that lack pRB expression, suggesting that the high level of pRB in these tumor cells results in reduced transcription from the p107 promoter. To test this hypothesis further, we used chromatin immunoprecipitation methodology as well as assays in which the effects of reduced pRB levels on p107 transcription could be measured. Using chromatin immunoprecipitations we show that the pRB protein is bound to the p107 promoter in colorectal tumor cells (Fig. 7C). In addition, the expression from a p107 promoter reporter integrated within the genome of human colon cancer cells is significantly increased following transfection with *Rb*-siRNA or if Ras signaling is blocked using PD98059 (Fig. 7D), circumstances under which pRB expression is reduced (Fig. 5A and C). Taken together, these findings suggest that the high levels of pRB protein seen in these tumor cells repress p107 transcription.

Considering the reported proliferation-inhibitory activity of p107 (8, 77), we tested the possibility that increased p107 levels in colorectal tumor cells that harbor Ras pathway mutations would inhibit their proliferation. Indeed, a very potent inhibitory effect of p107 is observed in transfected HCT116 and HT29 cells (Fig. 7E and data not shown), leading to a virtually complete cessation of proliferation. In contrast, transfection of the same tumor cells with pRB leads to a slight increase in proliferation (Fig. 7E and data not shown), suggesting not only that pRB is required for Ras-induced oncogenic transformation, but that levels of pRB protein may limit the proliferative rate of Ras-transformed human tumor cells, as we had observed in the focus formation assays in NIH 3T3 cells (Fig. 4A). Taken together, these observations suggest that the ability of pRB to repress levels of p107 mediates its role in oncogenic transformation by Ras. Moreover, they suggest that the level of p107 protein may play a role in human tumorigenesis.

DISCUSSION

The products of the Ras proto-oncogenes as well as the retinoblastoma tumor suppressor gene have been implicated in the regulation of a variety of cellular processes. Even though both Ras and pRB have each been placed in somewhat linear pathways, it is becoming increasingly clear that the signals that govern cellular processes such as entry and exit from the cell cycle, differentiation, and apoptosis are under the control of complex regulatory networks rather than simple linear pathways. In addition, these pathways are likely to be wired differently in different tissue contexts, and in various tumor types.

Although many human tumors exhibit deregulation of pRB pathway components, in general, a relatively small subset of tumor types exhibit pRB loss. Several previous studies have led to the suggestion that activated Ras cooperates with a disruption of the RB pathway in tumorigenesis. However, in those cases, RB pathway disruption does not involve the direct loss of the pRB protein, but instead involves either loss of the Cdk inhibitor p16, amplification of cyclin D1, or mutation of cdk4 (33, 37, 39, 61, 72, 79). Although it is commonly accepted that this is a linear pathway, it is not entirely clear that cyclin D amplification or p16 loss is equivalent to pRB loss in oncogenesis. In fact, our findings suggest that the expression of the pRB tumor suppressor protein is important for the proliferation of human tumor cells that harbor an activated Ras pathway and is not equivalent to loss of p16 or activation of cyclin D in this setting.

It has been previously suggested that *Rb*-deficient tumors have increased levels of active, GTP-bound Ras (41), which

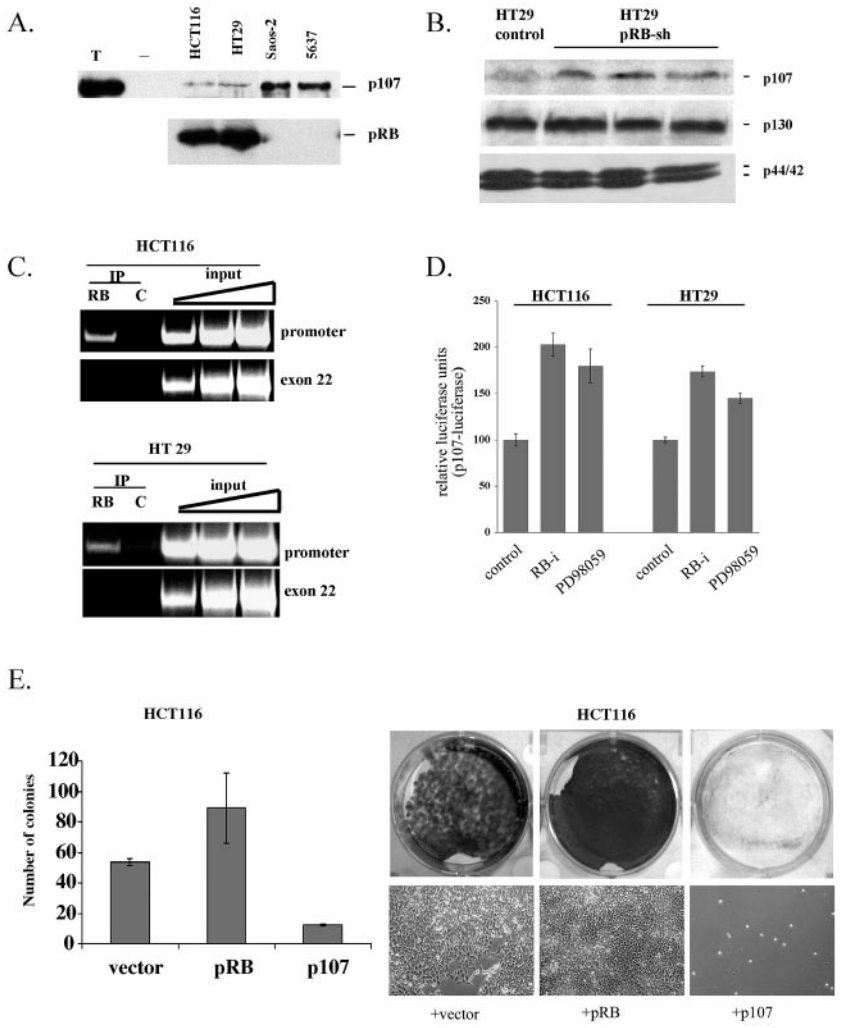

FIG. 7. Increased pRB levels in cell lines with an activated Ras pathway mediates its oncogenic actions via effects on p107. (A) Tumor cells deficient in pRB have higher p107 levels. The top panel illustrates the p107 protein levels in HCT 116 and HT29 as well as pRB-deficient Saos-2 and 5637 cells following immunoprecipitation and Western blot analysis using a p107-specific antibody (SD9). Cells transfected with a p107 expressing plasmid (T) was used as a control, and − represents a control immunoprecipitation without the addition of p107 antibody. The bottom panel shows the pRB protein levels in the same cells as measured by Western blot. An equivalent number of cells from each cell line were used for immunoprecipitations or Western blot analysis, using the total amount of p44/42 as an internal control (not shown). (B) Western blot analysis of p107 and p130 protein expression in HT29 cells transfected with a control vector (one subline) or three sublines of HT29 cells carrying a pRB-sh construct resulting in reduced pRB protein levels (Fig. 5A, lanes 1 and 4 to 6), using p44/42 as a loading control. (C) Chromatin immunoprecipitations using pRB or control antibodies (E1A) as indicated in the figure followed by amplification using primers in the promoter region as well as from exon 22 of the p107 gene as a negative control. Serial dilutions of input DNA (1/1,000, 1/500, and 1/300) were used to ensure the linearity of amplification. (D) Transcription from a genome-integrated p107-luciferase reporter in HCT116 and HT29 cells following control (c-i) or *Rb* (RB-i) siRNA transfection, or treatment with PD98059 for 15 h as indicated. The results are based on two independent experiments performed in duplicate using an equal number of cells. (E) The photographs on the left are representative images of Giemsa-stained plates of HCT116 cells transfected with 1 μg of vector or pRB- or p107-expressing plasmids followed by selection in puromycin. The three lower panels contain microscopic images of the same cells, as indicated. The bar graph on the right shows the average number of colonies in three separate experiments of HCT116 cells transfected with 1 μg of vector or p107- and pRB-expressing constructs (quantification was achieved using an Odyssey plate reader).

could bypass the requirement for Ras activation in these tumors. Our results (Fig. 1 to 3) suggest an additional explanation for the absence of Ras mutations in pRB-deficient tumors since activation of Ras has an inhibitory effect on the proliferation of pRB-deficient cells. The phenomenon that tumors carrying Ras mutations typically maintain an intact *Rb* gene and, in some cases, exhibit very high levels of pRB expression (2, 24, 75) has been explained by experiments in which it was shown that activation of Ras results in increased levels of cyclin D1 (21). The higher levels of cyclin D in these tumor cells would result in increased activity of cdk4 and cdk6, which according to current models inactivates pRB function. Thus, if constitutive activation of Ras results in inactivation of the pRB protein there would be no pressure to lose the *Rb* gene in tumors harboring an activated Ras pathway. However, applying the same logic, one can imagine that there should be no

pressure to lose p16 in tumors where cyclin D expression is up-regulated by activating mutations in the Ras pathway; nevertheless, p16 expression is frequently lost in tumors harboring Ras mutations.

Also, we and others find that p16 overexpression (50) (data not shown) is inhibitory when expressed in colorectal tumor cells, whereas pRB overexpression is not (Fig. 7E). In addition, depletion of pRB in tumors harboring Ras pathway mutations would be inconsequential, whereas our studies demonstrate that depletion of pRB surprisingly results in decreased proliferation in these tumor cells (Fig. 5 and 6). Thus, loss of p16 and loss of pRB clearly has distinct consequences for cells in the context of Ras transformation. It is also worth noting that our studies show that activated Ras, in addition to increasing the levels of cyclin D, stimulates the expression of pRB, indicating that the pool of active pRB is not significantly affected by Ras activation.

Taken together, our findings imply that although p16, cyclin D, cdk4, and pRB are largely regarded as components of the same linear pathway, loss of p16 expression or gain of cyclin D kinase activity is not equivalent to losing pRB in the context of activated Ras. Importantly, pRB is not the only cellular substrate of cyclin D-dependent kinases, and our data in murine cells suggests that the other members of the pRB family, p107 and/or p130, may be the relevant substrates in the presence of activating mutations in the Ras pathway. Notably, it has been shown that, in addition to pRB, there is a requirement for p107 and p130 in p16-induced arrest (5), suggesting that p107 and p130 are important downstream targets in p16-induced proliferative arrest.

Although it is generally believed that the cooperativity between activated Ras and E1A (and other viral oncoproteins) is associated with their ability to bind and neutralize the actions of pRB, our data also suggest that p107 and p130 might be important targets for these viral oncoproteins in this context. Significantly, a recent study has demonstrated that simian virus 40 infection of growth-arrested monkey kidney epithelial cells results in the specific disruption of p130-E2F and p107-E2F complexes, whereas pRB-E2F complexes are not affected (66). Previous experiments have also found evidence to suggest that the targeting of all three members of the pRB family of proteins is required for the cooperativity between Ras and simian virus 40 T antigen (49).

Overall, our results are consistent with a requirement for pRB for the full transforming activity of H-Ras^{V12} and suggest that the ability of E1A or p16 to target p107 and/or p130 is in fact its relevant oncogenic function in the context of its ability to cooperate with activated Ras. Taken together, these observations raise the possibility that in the context of an activated Ras pathway, loss of p16, similar to the expression of viral oncoproteins, is equivalent to inactivation of all three members of the pRB family of proteins (Fig. 8). It may also be interesting to speculate that the requirement for pRB in Ras-mediated transformation is tumor specific and does not occur in primary cells where Ras has been show to induce premature senescence (53, 62). The effects we see may require mutations in other pathways, such as the p53 pathway, which is mutated in most tumor cells and extensive cross talk has been seen between the p53 and pRB tumor suppressor pathways (for a review see reference 45). Finally, it is formally possible that there are rare

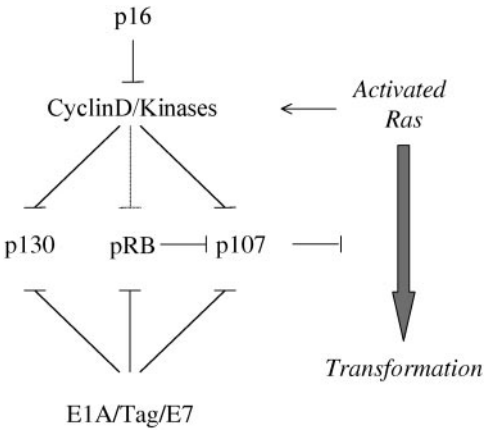

FIG. 8. Loss of pRB protein is distinct from the "pRB pathway" disruption in the context of activated Ras. Taken together, our results suggest that the ability of viral oncoproteins and cyclin D-dependent kinases to target p107 and/or p130 is important for their oncogenic functions in cooperation with activated Ras. In addition, the suppression of p107 expression by pRB and the strong inhibitory effect that p107 has on proliferation in this setting suggest a tumor-suppressive role for p107, rather than pRB, in Ras-mediated transformation.

tumors that harbor both Ras and *Rb* mutations, but which compensate for the *Rb* loss through disruption of p107 and/or p130 function or activation of oncogenes that will overcome the negative effect that losing pRB would have on these tumors.

Our collective observations also point to a clear functional distinction between the closely related pRB and p107 proteins in the presence of activated Ras, whereas in other contexts, these proteins seem to exhibit substantial functional redundancy (14, 60). The pRB-mediated repression of p107 expression seen in colorectal tumor cell lines provide a potential mechanistic explanation for the requirement for pRB in Ras-mediated oncogenic transformation. Furthermore, the strong inhibitory effect that p107, but not pRB, exerts on the proliferation of tumor cells harboring an activated Ras pathway (Fig. 6E) suggests that p107 provides a tumor-suppressive activity in this context.

In conclusion, the finding that loss of the *Rb* tumor suppressor leads to a proliferative disadvantage in tumor cells harboring activating mutations in the Ras pathway is a previously unrecognized and "counterintuitive" relationship between two of the most intensively investigated cancer genes and highlights the context-dependent nature of oncogene and tumor suppressor function. These findings are particularly surprising when considering that pRB is widely recognized as a proliferation inhibitor, and potentially explain why human tumors do not exhibit simultaneous loss of pRB and activating Ras mutations (35, 38, 39).

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ADDENDUM

Interestingly, it has recently been shown that loss of pRB in a model of skin carcinogenesis that is associated with H-Ras activation results in fewer and smaller tumors (58a).

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