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Role of pRb-Related Proteins in Simian Virus 40 Large-T-Antigen-Mediated Transformation

JUAN ZALVIDE AND JAMES A. DECAPRIO*

Dana-Farber Cancer Institute and Harvard Medical School, Boston, Massachusetts 02115

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Simian virus 40 large T-antigen (TAg) transformation is thought to be mediated, at least in part, by binding to and modulating the function of certain cellular proteins, including the retinoblastoma tumor suppressor gene product, pRb. TAg can disrupt the inhibitory complexes formed by pRb with the oncogenic transcription factor E2F, and this mechanism has been suggested to be important for TAg-mediated transformation. Residues 102 to 114 of TAg (including the LXCXE motif) are required for binding to pRb. Mutations within this LXCXE motif abolish the ability of TAg to bind to pRb as well as to transform certain cell types. TAg can also bind to at least two other cellular proteins, p107 and p130, that are related to pRb by sequence homology and share the ability to bind E2F. However, whether p107 and p130 are also targets in TAg-mediated transformation is less clear. To assess the relative contribution of the inactivation of pRb, p107, and p130 to transformation by TAg, fibroblasts were prepared from embryos derived from matings of mice heterozygous for an *Rb* knockout allele. The ability of TAg to transform fibroblasts homozygous for either wild-type or knockout *Rb* alleles was evaluated. It is demonstrated that the integrity of the LXCXE motif provides a growth advantage in *Rb*+/+ and *Rb*−/− cells. Furthermore, wild-type TAg, but not the LXCXE mutants, could bind to p107 and p130 and disrupt p107-E2F and p130-E2F binding complexes. These results suggest that p107 and p130 participate in TAg-mediated transformation and that they may behave as tumor suppressors.

The transforming activity of simian virus 40 (SV40) large T antigen (TAg) has been linked to its ability to bind to and modulate the function of certain cellular proteins. Two such proteins are the products of the tumor suppressor genes *Rb-1* and the p53 gene. Mutations within TAg that disrupt its ability to bind to either pRb or p53 typically render it incapable of transforming cells (5, 10, 31, 46, 54). These cellular proteins are also involved in nonviral tumorigenesis. For example, loss of either pRb or p53 or both is observed in a high percentage of spontaneous human tumors (45, 58, 59). Both pRb and p53 can induce a growth arrest when introduced into transformed cells that lack *Rb* or the p53 gene, respectively (2, 24). Given the role of pRb and p53 in tumor suppression, other cellular targets of TAg may also play an important role in growth control.

Amino acid residues 102 to 114 of TAg are required for its binding to pRb, and point mutations or in-frame deletions within this domain disrupt TAg's ability to bind to pRb and to transform cells (5, 10, 54). This region of TAg includes the residues LXCXE, conserved in the transforming proteins of two other DNA tumor viruses, adenovirus E1a and human papillomavirus E7 (16, 32, 42). Integrity of the LXCXE motif is required for the full oncogenic potential of these viral proteins. Mutations that affect residues within this motif do not affect TAg's ability to immortalize primary mouse embryo fibroblasts (MEFs). However, by several criteria the resulting cell lines are not transformed. They show contact inhibition and are unable to grow in low serum concentrations or in an anchorage-independent manner (5).

The LXCXE domain also participates in binding to the two pRb-related proteins p107 and p130 (13, 36, 41). The cDNAs for p107 and p130 were cloned by virtue of their ability to bind to E1a (15, 36), and the predicted amino acid sequence demonstrates a high degree of homology with pRb. p107 and p130 may also behave as growth suppressors since they can, like pRb, induce a growth arrest in certain cell types (8, 63). pRb, p107, and p130 have been shown to bind to members of the E2F family of transcription factors and repress their transcriptional activity (22, 30, 49, 57, 63). The promoters of certain genes, including the dihydrofolate reductase gene (23), B-*Myb* (33), c-*myc* (23), and the E2F-1 gene itself (29, 44), contain specific E2F binding sites that have been shown to participate in the increased transcriptional activity observed during the transition from quiescence to proliferation. Therefore, binding of pRb, and possibly p107 and p130, to E2F may repress transcription of the genes during quiescence and growth arrest. It has been proposed that transformation by viral proteins is mediated in part by disruption of pRb binding to E2F, leading to deregulation of E2F activity and aberrant expression of genes that participate in proliferation. Notably, various E2F species have been shown to behave as transforming proteins. For example, it has been demonstrated that overexpression of E2F-1, -2, or -3 can transform rat embryo fibroblasts (28, 51) as well as mouse NIH 3T3 cells (61).

Since the LXCXE motif of TAg participates in binding to pRb, p107, and presumably p130, it has been difficult to assess the relative contributions of each of the pRb-related proteins to TAg-mediated transformation. The availability of mice with a targeted mutation in one allele of the *Rb-1* gene (7, 27, 34) makes it possible to generate cultures of MEFs (*Rb*−/− MEFs) which differ from normal cells only in the inactivation of the *Rb* gene. Therefore, the susceptibilities of *Rb*+/+ and *Rb*−/− MEFs to TAg-mediated transformation can be directly compared. Using this system, we can address the relative contribution of the inactivation of pRb and pRb-related proteins to the transforming activities of TAg. For example, if *Rb*−/− MEFs can be transformed by LXCXE mutants of TAg, it would suggest that the inactivation of pRb, and not that of p107 or p130, is necessary for transformation in this system. In

* Corresponding author. Mailing address: Dana-Farber Cancer Institute, 44 Binney St., Boston, MA 02115. Phone: (617) 632-3825. Fax: (617) 632-4381.

contrast, if *Rb*−/− fibroblasts cannot be transformed by the LXCXE mutants, then binding of the pRb-related proteins by TAg may be necessary for transformation. This result would suggest that p107 or p130 is a relevant target of TAg-mediated transformation.

MATERIALS AND METHODS

Cells. Rb^{x3t} heterozygous mice were generously provided by Tyler Jacks (Massachusetts Institute of Technology). The Rb^{x3t} allele has stop codons in all three reading frames of exon 3 that completely inactivate pRb function (27). Rb^{x3t} heterozygous mice were mated, and embryos were collected at 13.5 days postcoitum. MEFs were prepared by the cold trypsin method (17). After the head of the embryo was dissected and processed for genotyping (see below), the embryos were individually minced, transferred to a tube with cold phosphate-buffered saline (PBS), washed three times with PBS, and incubated with 0.25% trypsin–EDTA (Cellgro) for 6 h at 4°C. After the incubation, the supernatant was removed and the embryo pieces were incubated for 30 min at 37°C, resuspended in complete medium (Dulbecco modified Eagle's medium [Cellgro] with 10% fetal clone serum I [Hyclone], 100 U of penicillin per ml, 100 μg of streptomycin per ml, 2 mM glutamine, and 10 μg of gentamicin per ml), and plated on a 100-mm-diameter culture dish. These were considered passage 0 cells.

Genotyping. DNA was extracted from the heads of the embryos (1), and PCR was performed as previously described (27). The Rx3 oligonucleotide was modified to 5′-CTGGAAGGATATATTCAAAAG-3′ to improve the efficiency of amplification.

Establishment of MEFs. The spontaneous immortalization of primary MEFs was accomplished according to the 3T3 protocol (55). Passage 0 cells of each genotype (3×10^5) were seeded in a 60-mm-diameter plate. After the medium was changed the next day, cells were trypsinized 3 days after seeding and counted. Cells (3×10^5) were replated on another 60-mm-diameter plate. Cell growth was calculated by dividing the number of cells recovered after each passage by the number plated. This number was multiplied by the total obtained during the previous passage to yield the cumulative cell growth of the cells up to that passage.

Plasmids and transfections. Wild-type (WT) SV40 TAg cDNA or the cDNA constructs of the LXCXE mutants K1 (E107K), PVU-1 (*dl*-107-112Y), and C105G (3, 31, 40) were cloned into the *Bam*HI site of pSG5 (Stratagene). The plasmids pRc/CMV-wild type E7 and pRc/CMV-dlDLYC E7 express WT and mutant hemagglutinin-tagged E7 proteins.

Cells were transfected by the calcium phosphate precipitation method (19). After 16 h of exposure to the precipitate, cells were washed two times, refed with complete medium, allowed to grow for another 24 h, and then split into selection medium. Where indicated, individual colonies were isolated with cloning cylinders (Bellco).

Protein analysis and immunoprecipitations. Cells were washed two times with PBS and lysed in EBC buffer (50 mM Tris-HCl [pH 8.0], 120 mM NaCl, 0.5% NonidetP-40, 10 μg of aprotinin per ml, 10 μg of leupeptin per ml, 0.1 mM phenylmethylsulfonyl fluoride, 4 mM NaF, 0.1 mM $NaVO_4$) for 15 min on ice. Extracts were then cleared by centrifugation at 10,000 × *g* for 5 min and the protein concentration was quantitated by the Bradford assay (Bio-Rad). Equal protein quantities were incubated with the relevant antibodies for at least 1 h at 4°C. Immune complexes were collected with protein A-Sepharose beads, washed four times with NET-N (20 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 0.5% NonidetP-40), resuspended in sample buffer (50 mM Tris-HCl [pH 8.0], 1% sodium dodecyl sulfate [SDS], 10% glycerol, 0.01% bromophenol blue, 100 mM dithiothreitol), and separated in SDS-polyacrylamide gels. Proteins were transferred onto Polyscreen polyvinylidene difluoride membranes (NEN) in transfer buffer (25 mM Tris-HCl, 192 mM glycine, 20% [vol/vol] methanol, and 0.01% SDS) (56). Membranes were blocked for at least 30 min in Tris-buffered saline (10 mM Tris-HCl [pH 8.0], 150 mM NaCl) with 5% powdered nonfat milk (Carnation), after which the appropriate antibody was added and incubated for 4 to 14 h. Detection of the immune complexes was performed with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin (Boehringer Mannheim) and a colorimetric assay with Nitro Blue Tetrazolium–5-bromo-4-chloro-3-inolylphosphate toluidinium (BCIP) (Bio-Rad).

Growth in suspension. Dishes (35-mm diameter) were coated with 2 ml of Dulbecco modified Eagle's medium containing 10% fetal bovine serum (Hyclone) and 0.6% agarose (Gibco BRL). Cells were plated on top of this layer at a density of 5×10^4 cells per plate in Dulbecco modified Eagle's medium containing 10% fetal bovine serum and 0.3% agarose. Cells were fed every 4 to 5 days with the 0.3% agarose-containing medium. Colony formation was evaluated and photographed 3 weeks after the initial plating.

Gel retardation assays. Whole-cell extracts were prepared by a method previously described (43, 48, 50) from either subconfluent or confluent cells. Gel retardation assays were performed as described elsewhere (50). The oligonucleotide used as a labeled DNA probe includes the E2F binding site of the human c-Myc P2 promoter (9). The dihydrofolate reductase mutant oligonucleotide (DHFR MUT) has also been previously described (50). For competition experiments 100-fold excesses of unlabeled oligonucleotides were added prior to the addition of labeled probe. As described in the figure legends, different amounts of crude extracts of baculovirus-produced WT or K1 TAg were added to the binding reaction. For antibody perturbation experiments, 2 μl (200 ng) of rabbit polyclonal antibody C-20 (Santa Cruz) or normal rabbit polyclonal serum or 2 μl of tissue culture supernatant of the monoclonal antibody SD15 (12) or M73 (20) was added. The reaction products were separated in a 4% polyacrylamide gel run in 0.25× TBE (0.25 × TBE is 22.5 mM Tris-borate plus 0.5 mM EDTA) at 4°C. The gel was dried, and autoradiography with intensifying screens was performed.

RESULTS

Establishment of *Rb*−/− MEFs. MEFs were prepared from 13.5-day-postcoitus embryos derived from a mating between Rb^{x3t} heterozygote knockout mice (27). Cell extracts were prepared from third-passage MEFs of all three *Rb* genotypes (*Rb*+/+, *Rb*+/−, and *Rb*−/−), and expression of pRb was determined. Aliquots of lysates were immunoprecipitated with a cocktail of monoclonal anti-pRb antibodies, including XZ55, XZ77, XZ104, and G3-245 (10, 11, 26). Immune complexes were separated in an SDS–6% polyacrylamide gel, transferred to a polyvinylidene difluoride membrane, and incubated with the G3-245 anti-pRb antibody. As shown in Fig. 1B (lanes 1 and 2), *Rb*+/+ and *Rb*+/− MEFs expressed comparable levels of pRb, with similar phosphorylation patterns (4, 11). Conversely, *Rb*−/− cells did not express any detectable amounts of pRb (Fig. 1B, lane 3).

Primary MEFs of the *Rb*+/+ and *Rb*−/− genotype were subjected to a 3T3 protocol (55). By the fourth passage (Fig. 1A, 12 days), the growth rates of the cells of both genotypes appeared to decrease. This period of slowed cell growth is consistent with cells undergoing crisis (55). Similarly, when the 3T3 protocol was repeated in a separate experiment, MEFs from both genotypes again appeared to undergo crisis after the fifth passage (data not shown).

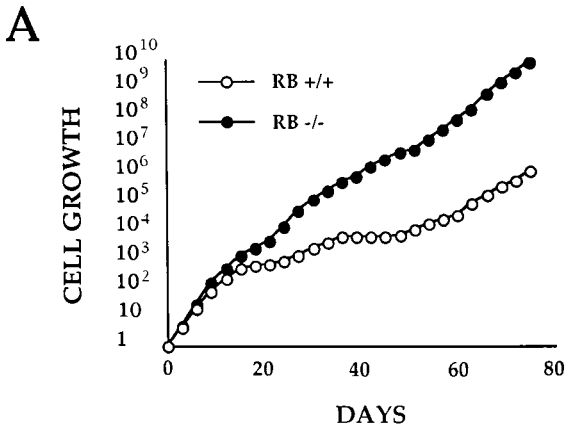
To assess pRb expression after establishment, extracts were prepared from 3T3 cells at the 37th passage. As shown in Fig. 1B, the *Rb*+/+ 3T3 cells expressed levels of pRb similar to those expressed by the primary *Rb*+/+ MEFs, and with similar phosphorylation patterns, suggesting that pRb was not grossly mutated during the immortalization process (Fig. 1B, compare lanes 1 and 4) (14, 25).

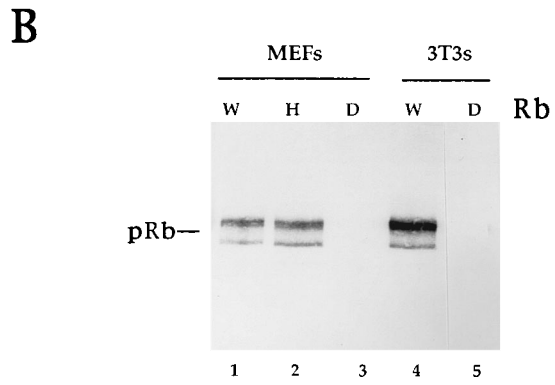
To test for the ability of established cells to undergo contact inhibition, 3T3 cells of either *Rb*+/+ or *Rb*−/− origin at passage 35 were seeded at a density of 5×10^5 cells per 100-mm-diameter plate. Cells from replica plates were trypsinized and counted at the indicated days thereafter (Fig. 1C). The growth rate of *Rb*+/+ and *Rb*−/− 3T3 cells appeared to decrease as cells reached confluence. When the DNA content was measured by flow cytometry analysis on days 2 and 9 of the experiment (Fig. 1D), cells appeared to accumulate in the G_0/G_1 phase of the cell cycle (Fig. 1D, compare days 2 and 9).

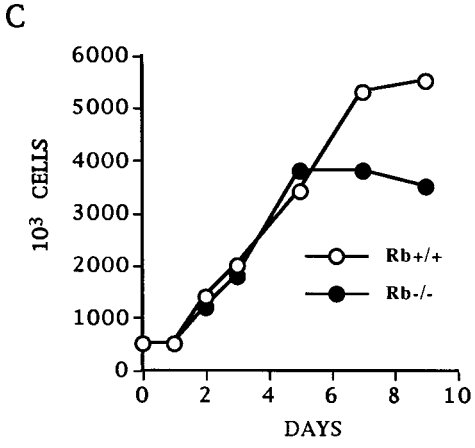
Rb+/+ and *Rb*−/− 3T3 cells were also seeded in medium containing 0.3% soft agarose to assay for their ability to grow in suspension. No colonies appeared from cells of either genotype during the course of the experiment (data not shown).

These results suggest that the loss of *Rb* in MEFs does not lead directly to immortalization or transformation. The period of senescence during the 3T3 passaging confirms that *Rb*−/− MEFs undergo crisis. Furthermore, the 3T3 cells established from *Rb*−/− MEFs exhibited contact inhibition with an arrest in the G_0/G_1 phase of the cell cycle and were unable to grow in soft agar. Therefore, events other than or in addition to the loss of *Rb* may be required for immortalization and transformation of MEFs.

WT and LXCXE mutants of TAg can immortalize *Rb*+/+, *Rb*+/−, and *Rb*−/− cells. MEFs of the three different *Rb* genotypes were cotransfected with a plasmid encoding puro-







D

GENOTYPE	G1	S	G2/M
Rb +/+			
d.2	52	21	27
d.9	68	11	20
Rb -/-			
d.2	56	22	23
d.9	85	9	5

FIG. 1. Establishment of *Rb*+/+ and *Rb*−/− MEFs. (A) Cumulative growth of primary *Rb*+/+ and *Rb*−/− MEFs. Passage 0 MEFs were subjected to a 3T3 protocol. Cell number was determined after each passage. The cumulative growth of primary fibroblasts is plotted against the number of days of passage. (B) Expression of pRb in primary and established MEFs. Cell lysates were prepared from primary MEFs (lanes 1 to 3) and 3T3 cells from the 37th passage (lanes 4 and 5) of the indicated *Rb* genotypes: W, *Rb*+/+; H, Rb+/−; D, Rb−/−. Protein (3 mg) was immunoprecipitated with a cocktail of monoclonal anti-pRb antibodies. Immune complexes were separated in an SDS–6% polyacrylamide gel, and pRb was detected by Western blot with G3-245 antibody. (C) Growth of 3T3 cells in 10% serum. 3T3 cells (5×10^5) of each genotype were plated on 100-mm-diameter plates. Replica plates were trypsinized, and the total number of cells was determined at the indicated days. (D) Cell cycle distribution of 3T3 cells at subconfluency and confluency. The DNA contents of *Rb*+/+ and *Rb*−/− 3T3 cells were determined by flow cytometry analysis at days 2 and 9 of the experiment shown in panel C.

TABLE 1. Immortalization of MEFs transfected with SV40 large TAg[a]

DNA	Cell genotype	No. of colonies established/no. picked[b] in:	
		Expt 1	Expt 2
Vector	*Rb*+/+	0/0	0/0
	Rb+/−	0/0	ND
	Rb−/−	0/0	0/0
WT TAg	*Rb*+/+	8/8	6/6
	Rb+/−	8/8	ND
	Rb−/−	8/8	6/6
K1 TAg	*Rb*+/+	5/8	5/6
	Rb+/−	6/8	ND
	Rb−/−	7/8	6/6
PVU-1 TAg	*Rb*+/+	6/8	ND
	Rb+/−	6/8	ND
	Rb−/−	8/8	ND
C105G TAg	*Rb*+/+	ND	5/5
	Rb−/−	ND	3/5

[a] Passage 3 MEFs were transfected with pEpuro together with SV40 large TAg or backbone vector alone. Resistant colonies were individually picked, and those that grew to confluency in a 100-mm-diameter plate were considered established.
[b] ND, not determined.

mycin resistance (pEpuro) and a second plasmid directing the expression of the cDNA for WT TAg, an LXCXE mutant (E107K [K1], *dl*107-112Y [PVU-1], or C105G), or empty vector (pSG5). Transfected cultures were selected for 2 to 3 weeks in media containing puromycin, and individual colonies were picked. Those clones that grew to confluence in a 100-mm-diameter plate were considered established (5, 53). No colonies appeared in cells of any genotype that were transfected with pEpuro and vector alone. However, cells transfected with plasmids encoding either WT or LXCXE mutant TAg produced colonies that established with high efficiency (Table 1). Cells transfected with WT TAg consistently generated 5- to 10-fold more colonies than those transfected with any of the LXCXE mutants. These results are consistent with previous work reporting that LXCXE mutants can establish primary MEFs less efficiently than WT TAg (5, 53, 62). In addition, cells transfected with WT TAg reached confluence in a 100-mm-diameter plate in slightly less time than their LXCXE mutant transfected counterparts (26 to 33 days from transfection to confluence in a 100-mm-diameter plate for WT TAg versus 28 to 36 days for LXCXE mutants in experiment 1; 36 to 43 days for WT TAg versus 43 to 47 days for LXCXE mutants in experiment 2). WT TAg-expressing clones of MEFs of all three *Rb* genotypes grew faster than their LXCXE counterparts.

All established clones were assessed for TAg expression. All 36 clones of WT TAg, 20 clones of K1 TAg, 14 clones of PVU-1 TAg, and 8 clones of C105G TAg that were isolated expressed detectable amounts of TAg when tested by Western blot (immunoblot) with a monoclonal anti-TAg antibody (data not shown). Levels of TAg were comparable between WT TAg and LXCXE mutants but varied slightly among the individual cell lines. Clones that expressed the highest levels of WT or

LXCXE mutant TAg were selected for further analysis. Immunofluorescence staining showed that both WT TAg and LXCXE mutants of TAg were detectable in every cell of a given clone and were localized in the nucleus (data not shown). Selected clones were metabolically labeled with [^{35}S]methionine, and immunoprecipitation for TAg was performed. Autoradiography showed that both WT TAg and the mutants K1 and PVU-1 were expressed at similar levels and could also coprecipitate p53 (data not shown) (5, 10).

LXCXE mutants of TAg cannot transform *Rb*−/− cells. To test the contribution of *Rb* inactivation to TAg-mediated transformation, the cell densities attained in medium containing 10% serum of *Rb*+/+ or *Rb*−/− cultures, expressing WT or LXCXE mutant TAg, were compared. WT TAg-expressing *Rb*+/+ MEFs have been reported to grow to a higher cell density in complete medium than comparable cells expressing LXCXE TAg mutants (5, 54). Two colonies of each genotype, stably expressing either WT TAg or the LXCXE mutant K1 or PVU-1, were plated at a low cell density in medium containing 10% serum. Replica plates of each clone were trypsinized in duplicate on day 1 after seeding and every 2 days thereafter, and their cell numbers were determined. Figure 2A shows that WT TAg-expressing cells grew to a higher cell density than those cells that expressed the LXCXE mutant K1 or PVU-1. When the cultures were subconfluent, i.e., up to day 3, there was not a clear difference in the growth rate between WT TAg and LXCXE mutant TAg cell lines, even though there was a certain degree of clonal variability in the rates of growth of the different cell lines. However, when the cultures reached confluence (i.e., by day 7), there was a clear growth advantage of the WT TAg-expressing cells. WT TAg-expressing cells grew to a much higher cell density than the LXCXE mutant TAg-expressing counterparts. Indeed, cells that expressed WT TAg did not appear to undergo contact inhibition during the course of this experiment. This growth advantage was evident in both the *Rb*+/+ and *Rb*−/− genetic backgrounds. It is noteworthy that the WT TAg lines in an *Rb*−/− genetic background could grow to a twofold-higher cell density 8 days after plating than *Rb*+/+ cells (about 6×10^6 versus 3×10^6 cells per plate). Conversely, both the *Rb*+/+ and *Rb*−/− K1- and PVU-1-expressing cells reached a plateau in cell number within 5 days after seeding. The cell density attained by the LXCXE mutants was twofold higher in the *Rb*−/− cells than in the Rb+/+ cells.

It has been reported that cells expressing WT TAg have a growth advantage in low serum concentrations compared to cells expressing LXCXE mutants of TAg (5). To compare the growth of these TAg-expressing clones in medium containing low serum concentrations, we seeded cell lines under conditions identical to those for the previous experiment, but 1 day after seeding, the medium was changed to Dulbecco modified Eagle's medium with 1% serum. As shown in Fig. 2B, both *Rb*+/+ and *Rb*−/− WT TAg cells were capable of growing under these conditions, though at a rate lower than that in 10% serum (duplication time of 34 to 41 h in 1% serum versus 20 to 25 h in 10% serum). In contrast, cells expressing LXCXE mutants of TAg grew very slowly or not at all in medium containing 1% serum. All colonies except one had duplication times ranging from 51 to 98 h. One *Rb*−/− line, PVU-1 c1, grew almost as well as the WT TAg cells, with a doubling time of 37 h. This result may have reflected a clonal variation, since three other *Rb*−/− clones expressing PVU-1 TAg (PVU-1 c2, PVU-1 c4, and PVU-1 c8) grew with doubling times similar to that of the other LXCXE mutant-containing cells. Taken together, these two results indicate that cells expressing WT TAg had a growth advantage in complete medium as well as in low serum concentrations over those clones expressing any of the LXCXE mutants of TAg, independent of the presence of pRb.

Several of these clones were also tested for their ability to grow in an anchorage-independent manner. It has been reported that the LXCXE region of TAg is essential for the ability of this protein to promote growth of MEFs in suspension. To test whether the targeted inactivation of *Rb* could complement an LXCXE TAg mutant in this respect, 5×10^4 *Rb*+/+ or *Rb*−/− WT or mutant TAg-expressing cells were seeded in 0.3% agarose medium over a layer of 0.6% agarose. After 3 weeks, colonies of more than 16 cells were counted. As shown in Table 2, cells expressing WT TAg formed colonies in suspension with high efficiency. There were many more than 500 colonies per plate for each WT TAg-expressing cell line in soft agarose. We wanted to know with what efficiency these cells could form colonies under these circumstances. When we compared the number of colonies with the number of single cells in several microscopic fields of each plate of cells expressing WT TAg, we found that depending of the specific clone tested, between 2 and 15% of the cells seeded could grow in soft agar. WT TAg cells that formed colonies of fewer than 16 cells during the course of the experiment were not scored as positives in this assay. There were not clear differences in size, number, or morphology of the colonies formed between WT TAg-expressing cells of *Rb*+/+ and *Rb*−/− origin. In contrast, cells expressing the LXCXE mutants of TAg K1, PVU-1, or C105G, in either an *Rb*+/+ or *Rb*−/− background, failed to form colonies or did so very inefficiently (Table 2). The few colonies formed by these TAg mutants were smaller than those formed by the WT TAg-expressing cells and were typically smaller than 32 cells.

The previous data suggested that the transformation of cells expressing WT TAg was due to some function of the LXCXE motif independent of binding to pRb, since nearly identical results were obtained in the *Rb*+/+ and *Rb*−/− cells. If this is the case, then a heterologous protein carrying a wild-type LXCXE should be able to elicit the transformed phenotype when expressed in *trans* in either *Rb*+/+ or *Rb*−/− cells that express an LXCXE mutant of TAg. To demonstrate this, a plasmid encoding a hemagglutinin-epitope-tagged human papillomavirus type 16 E7 protein or the E7 LXCXE deletion mutant *dl*DLYC (47) was cotransfected with a hygromycin resistance marker into two K1-expressing cell lines, *Rb*+/+ K1 c1 and *Rb*−/− K1 c1. The hygromycin-resistant colonies were pooled, and E7 expression was evaluated by immunoprecipitation followed by Western blot analysis with 12CA5 anti-hemagglutinin antibody (60). Both WT and mutant E7 were expressed in the resistant pools (data not shown).

E7-expressing pools were seeded on soft agarose to score for their ability to grow in an anchorage-independent manner. Cells that had been transfected with wild-type E7 could form colonies in soft agarose (Fig. 3a and b). The colonies formed by these cells expressing WT E7 were indistinguishable in size and appearance from those formed by cells that expressed WT TAg (Table 2 and data not shown). However, cells that had been transfected with the LXCXE mutant of E7 were not able to form colonies (Fig. 3c and d). This effect of WT E7 was independent of the *Rb* genotype of the cell line transfected, showing that the LXCXE motif of a DNA tumor virus protein was required for anchorage-independent growth, even in an *Rb*−/− genetic background.

WT TAg but not an LXCXE mutant can bind pRb family proteins. The requirement for an LXCXE sequence in TAg-mediated transformation in both *Rb*+/+ and *Rb*−/− cells suggests that the binding of this motif to proteins other than pRb

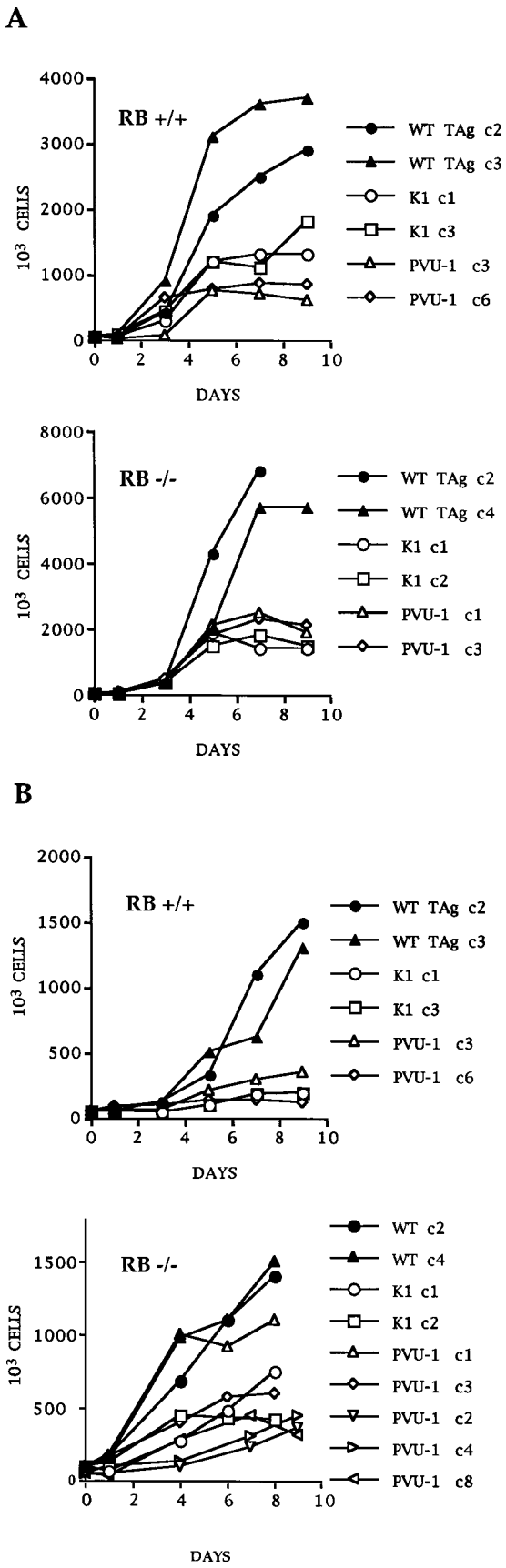


FIG. 2. Growth of *Rb*+/+ and *Rb*−/− cells expressing WT and mutant TAg in high and low serum concentrations. (A) Growth in a high serum concentration. Cells of each colony (5×10^4) were plated in 60-mm-diameter plates in the presence of 10% serum. Cells from two replica plates were trypsinized, and cells in each plate were counted in duplicate at the indicated days. The cDNA for TAg in each clone is identified at the right. Colonies (c) were derived from *Rb*+/+ or *Rb*−/− MEFs. (B) Growth in a low serum concentration. Cells of each colony (5×10^4) were plated in 60-mm-diameter plates in the presence of 10% serum. One day later the medium was washed two times and changed to 1% serum. Cells of replica plates were trypsinized and counted in duplicate at the indicated days. The colonies used were the same as in panel A, with the addition of *Rb*−/− PVU-1 c2, PVU-1 c4, and PVU-1 c8.

is important for transformation. The pRb-related proteins, p107 and p130, are obvious targets for TAg.

To test whether TAg could bind to members of the pRb family in these cells, lysates were prepared from *Rb*+/+ or *Rb*−/− MEFs expressing either WT or K1 TAg and immunoprecipitations were performed with either anti-TAg or anti-pRb antibodies. The immunoprecipitates were separated in SDS-polyacrylamide gels and probed in Western blot analysis with the specific anti-pRb antibody RB-PMG3-245 (10) (Fig. 4, lanes 1 to 8). *Rb*+/+ but not *Rb*−/− cells expressing either WT or K1 TAg appeared to contain both phosphorylated and underphosphorylated pRb (Fig. 5, compare lanes 5 and 6 with lanes 7 and 8). Furthermore, as previously demonstrated (10, 38, 39), WT TAg but not K1 was capable of coprecipitating the underphosphorylated form of pRb. pRb has been difficult to detect in mouse 3T3 cell lines because of low levels of expression. For example, it was necessary to use up to eight times as

TABLE 2. Colony formation of TAg-expressing cells in soft agarose[a]

Genotype	TAg expressed	Cell line	No. of colonies
Rb+/+	WT	c1	>500
		c2	>500
		c3	>500
		c9	>500
	K1	c1	0
		c2	0
		c3	2
		c5	4
	PVU-1	c3	20
		c6	0
	C105G	c2	0
		c4	0
		c5	15
Rb−/−	WT	c1	>500
		c2	>500
		c3	>500
		c4	>500
		c12	>500
	K1	c1	0
		c2	3
		c3	6
		c6	4
		c7	0
	PVU-1	c1	10
		c3	18
		c4	0
		c5	3
		c8	2
	C105G	c1	6
		c3	7

[a] Cells (5×10^4) were seeded in 35-mm-diameter plates in medium containing 0.3% soft agarose. The number of colonies of more than 16 cells per plate was counted after 3 weeks.

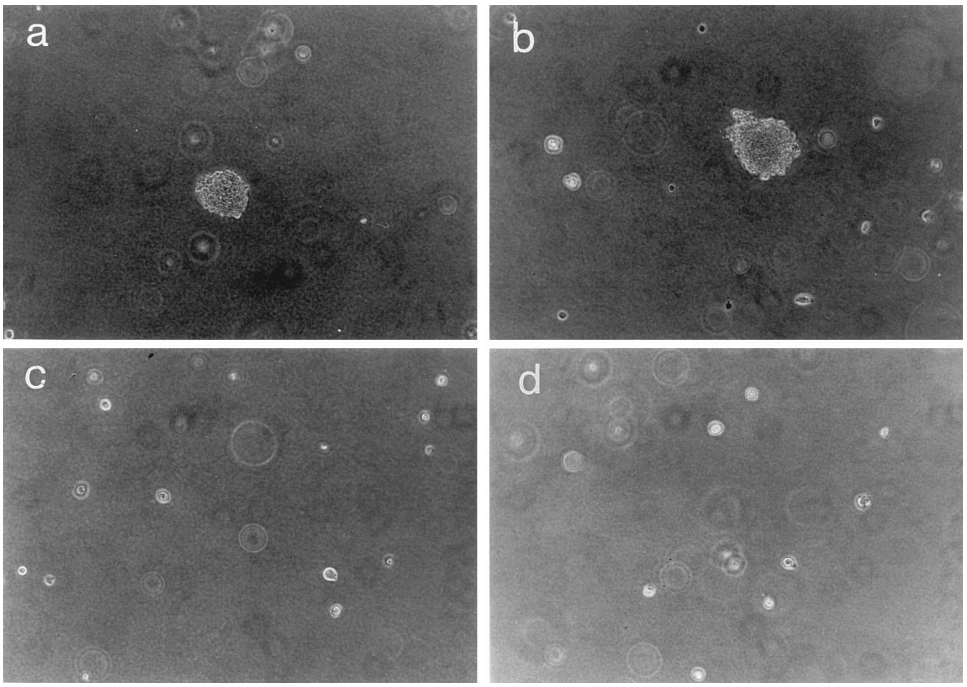


FIG. 3. Growth of E7-expressing cells in semisolid medium. Cells (5×10^4) were seeded in 1.5 ml of soft-agarose medium (final concentration, 0.3%) in 35-mm-diameter culture dishes. Cultures were fed with fresh soft agarose every 4 to 5 days. Three weeks after seeding, cells were photographed at a magnification of ×100. (a) *Rb*+/+ K1c1 cells expressing WT E7; (b) *Rb*−/− K1c1 cells expressing WT E7; (c) *Rb*+/+ K1c1 cells expressing *dl*DLYC E7; (d) *Rb*−/− K1c1 cells expressing *dl*DLYC E7.

much extract as that from NIH 3T3 cells or from comparable human *Rb*+/+ cell lines necessary to detect total pRb or TAg-associated pRb in these experiments.

TAg immunoprecipitates were also probed in Western blot analysis with the anti-p130 monoclonal antibody Z83. The Z83 antibody has been shown to cross-react with p107 in Western blots of extracts prepared from human (36) as well as mouse (52) cell lines. When the Western blots of the immunoprecipitations for WT TAg from *Rb*+/+ or *Rb*−/− cells were probed with Z83, both p107 and p130 were detected (lanes 9 and 11).

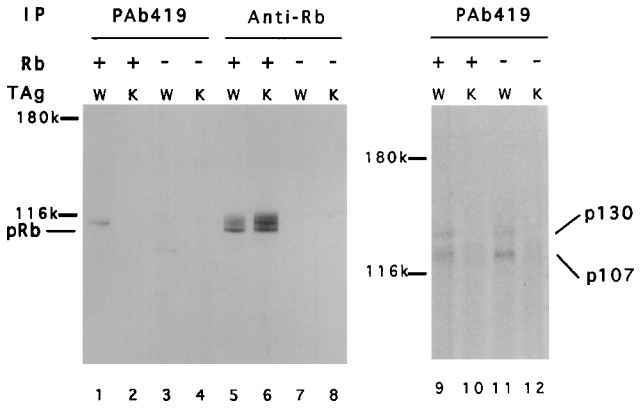


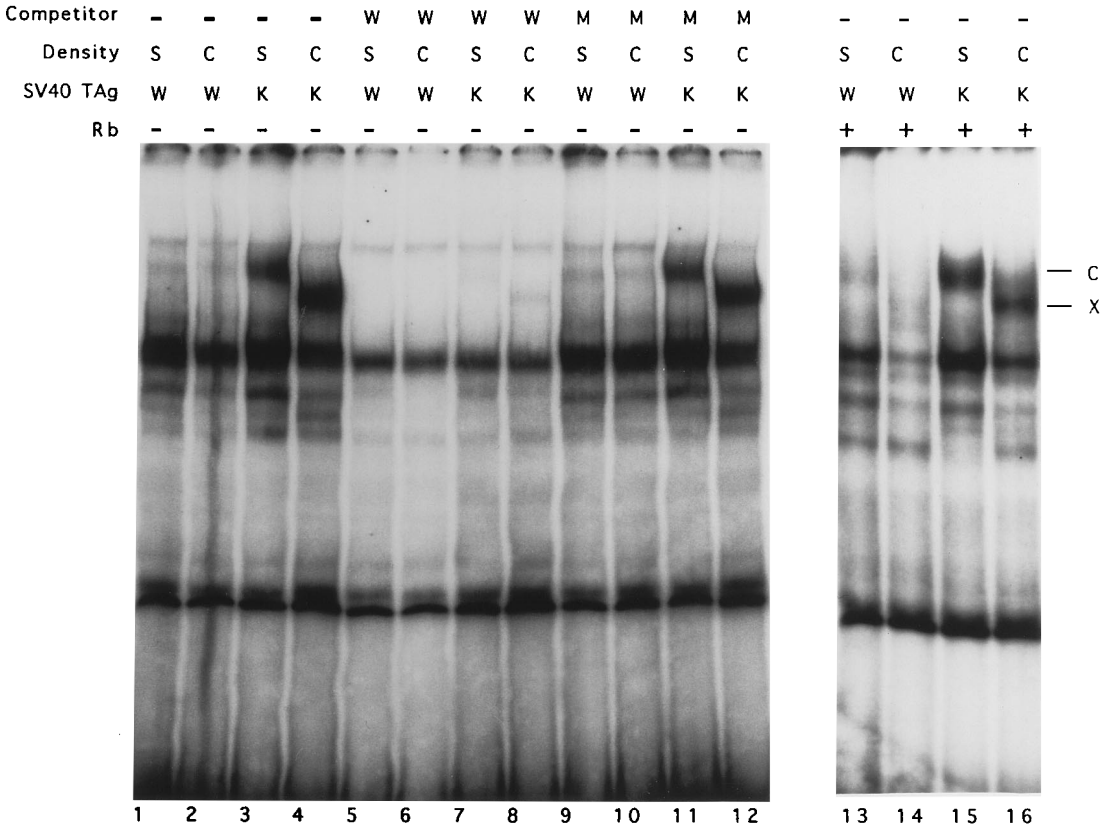
FIG. 4. Genetics of TAg binding to pRb family proteins. Extracts from *Rb*+/+ (lanes +) or *Rb*−/− (lanes −) MEFs, expressing either WT (lanes W) or K1 (lanes K) TAg, were prepared. Then 8 mg (lanes 1 to 4) or 500 μg (lanes 9 to 12) of protein was precipitated with the anti-TAg antibody pAb419 or 2 mg was precipitated with a cocktail of anti-pRb antibodies (lanes 5 to 8). Immune complexes were separated in an SDS–6% polyacrylamide gel. pRb was detected by Western blot with G3-245 antibody (lanes 1 to 8). p107 and p130 were detected with the Z83 antibody (lanes 9 to 12).

K1 TAg failed to coprecipitate either p107 or p130 (lanes 10 and 12), despite expression of p107 and p130 (data not shown). WT TAg also appeared to coprecipitate the underphosphorylated forms of p107 and p130 (52). Therefore, it appears that an intact LXCXE motif is necessary for the ability of TAg to coprecipitate pRb, p107, and p130.

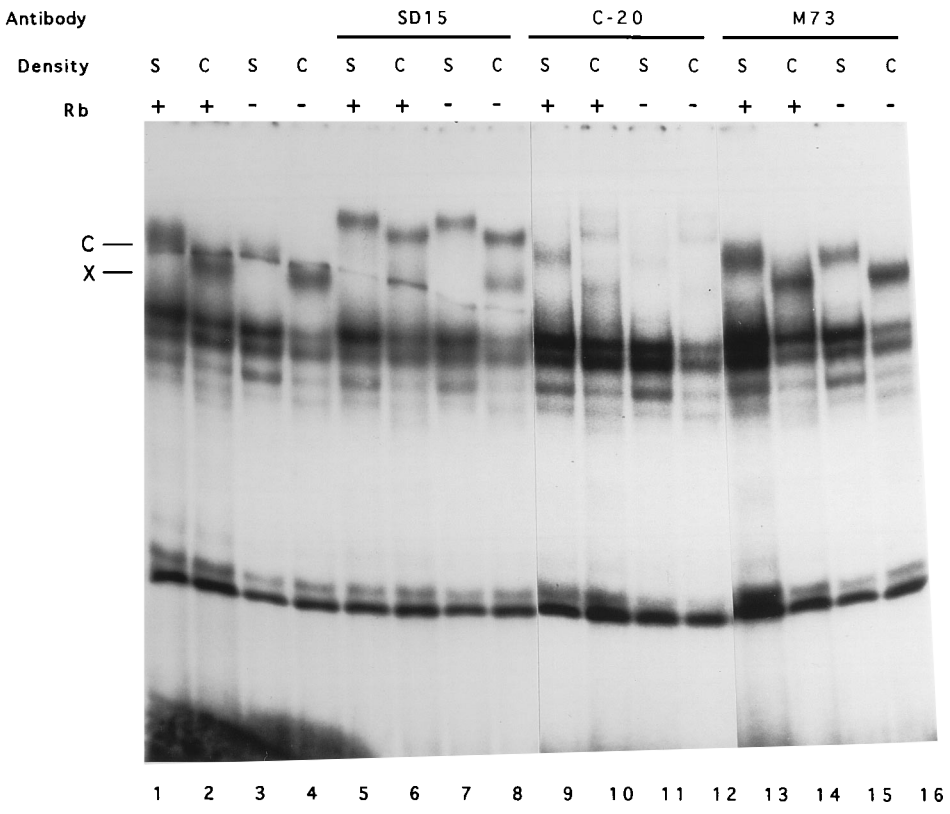
WT TAg can disrupt p107-E2F and p130-E2F DNA binding complexes. pRb can bind to members of the E2F transcription factor family and repress their transcriptional activity. Given that the E2F family can transactivate several genes important for cell proliferation, it has been suggested that E2F is an important target for pRb-mediated growth suppression (22, 30, 35). TAg can disrupt pRb-E2F complexes and therefore inactivate pRb's repression of E2F activity. It has also been shown that p130 and p107 can bind to and repress members of the E2F transcription factor family (6, 9, 50, 57). Furthermore, deletion analysis has suggested that p107's ability to suppress growth when overexpressed cosegregates with its binding to E2F (63). p107 and p130 may also regulate cell growth at least in part through repression of E2F activity. If this is the case, disruption of p107-E2F and p130-E2F complexes may also be a component of TAg-mediated transformation. Given this, we wanted to know whether TAg could affect the E2F–pRb-related protein complexes in these cells under various growth conditions.

Whole-cell extracts were prepared from proliferating or confluent *Rb*+/+ and *Rb*−/− cells expressing either WT or K1 TAg (43, 48, 50) and tested for the presence of E2F DNA binding complexes. Extracts were prepared from proliferating cells when subconfluent, at a density comparable to that of day 4 in the experiment shown in Fig. 2A. Extracts were also prepared from the same clone of cells when confluent at a density comparable to that of day 7 in Fig. 2A. Under these conditions, K1-expressing cells were clearly contact inhibited

A


Competitor - - - - W W W W M M M M - - - -
Density S C S C S C S C S C S C S C S C
SV40 TAg W W K K W W K K W W K K W W K K
Rb - - - - - - - - - - - - + + + +
C
X
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

B


Antibody SD15 C-20 M73
Density S C S C S C S C S C S C S C S C
Rb + + - - + + - - + + - - + + - -
C
X
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

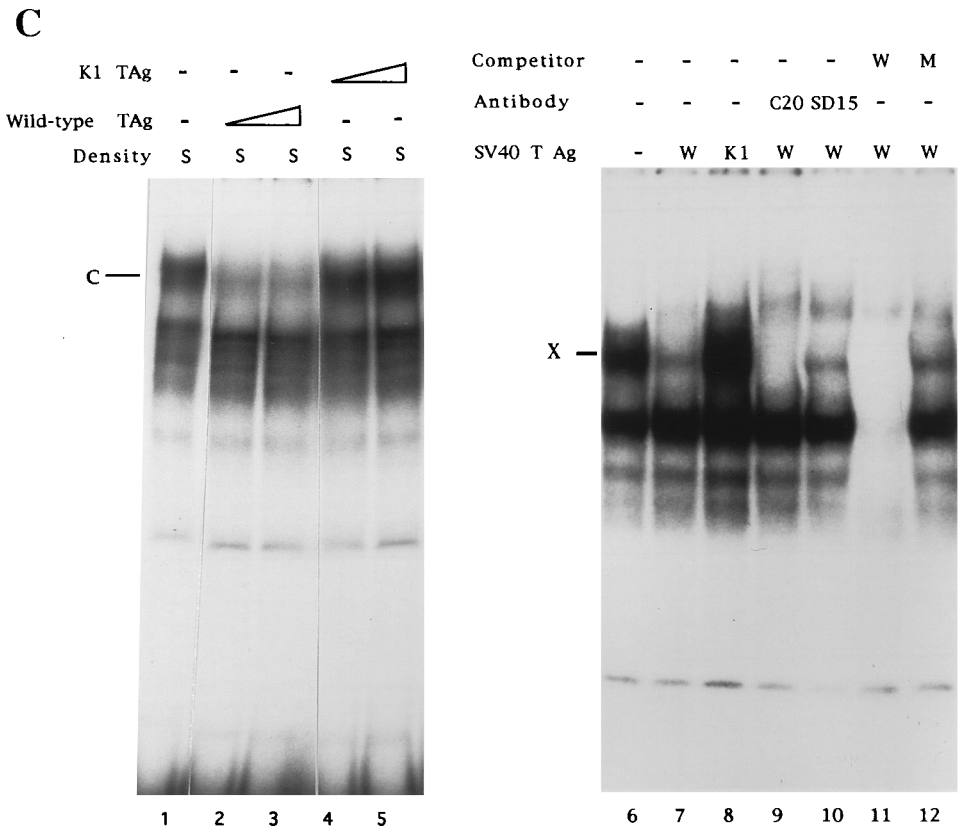


FIG. 5. Effect of WT and K1 TAg on E2F DNA binding complexes. (A) E2F complexes in WT and K1 TAg-expressing cells. Gel retardation assays were performed with extracts from cells expressing WT (TAg lanes W) or K1 (TAg lanes K) TAg derived from MEFs of the *Rb*+/+ (lanes 13 to 16) or *Rb*−/− (lanes 1 to 12) genotype. Extracts were prepared from subconfluent (lanes S) or confluent (lanes C) cultures. The probe used included the E2F binding site of the c-*myc* P2 promoter. WT (competitor lanes W) or mutant (competitor lanes M) competitor was added in the indicated lanes. The positions of complexes C and X are marked. (B) p107-E2F and p130-E2F DNA binding complexes in K1 TAg-expressing cells. Gel retardation experiments were performed with extracts from cells expressing K1 TAg derived from *Rb*+/+ (lanes +) or *Rb*−/− (lanes −) MEFs. Extracts were prepared from subconfluent (lanes S) or confluent (lanes C) cultures. Anti-p107 (SD15), anti-p130 (C-20), and anti-E1A (M73) antibodies were added to the binding reactions as indicated. The positions of complexes C and X are marked. (C) Effect of WT and K1 TAg on E2F DNA binding complexes in vitro. Extracts prepared from subconfluent cells expressing K1 TAg (lanes 1 to 5) were mixed with either WT (1 μl [lane 2] or 2 μl [lane 3]) or K1 (1 μl [lane 4] or 2 μl [lane 5]) baculovirus-expressed TAg. Extracts from confluent cells (lanes 6 to 12) were mixed with baculovirus-produced WT TAg (TAg lanes W) or K1 TAg (lane 8). The anti-p130 C-20 antibody (lane 9), anti-p107 SD15 antibody (lane 10), and WT (lane 11) or mutant (lane 12) competitor were added as indicated. Gel retardation assays were performed as in panel A. The positions of complexes C and X are marked.

while WT TAg-expressing cells were still capable of increasing in number. As shown in Fig. 5A, extracts prepared from cells that expressed K1 TAg contained slowly migrating complexes that were greatly reduced in the WT TAg-expressing cells (Fig. 5A, compare lanes 1, 2, 13, and 14 with lanes 3, 4, 15, and 16). Subconfluent, proliferating cultures of K1-expressing cells contained an E2F complex noted as C (Fig. 5A, lanes 3 and 15). Extracts prepared from K1 cells that had been confluent for 2 days contained a complex noted as X (lanes 4 and 16). In contrast, extracts prepared from cells expressing WT TAg had greatly decreased amounts of both C and X complexes (lanes 1, 2, 13, and 14). The specificity of these complexes for the E2F sites is shown by competition with an unlabeled DNA probe (lanes 5 to 8). The C and X complexes were not eliminated by a dihydrofolate reductase probe with a mutant E2F binding site (lanes 8 to 12). In addition, several faster-migrating specific E2F DNA binding complexes were also detected, but there were no apparent differences between WT TAg- and K1-expressing lines in this regard. We were not able to detect specific differences in the DNA binding complexes between the *Rb*+/+ and *Rb*−/− cells (compare lanes 1 through 4 and 13 through 16).

The presence of the X and C E2F-containing complexes has been previously reported (6, 50). For example, it has been demonstrated that MEFs, as well as BALB/c 3T3 A31 cells, contain p107-E2F and p130-E2F DNA binding complexes (9, 50). To determine if the C and X complexes of these cells contained p107 and p130, specific antibody supershift analysis was performed. As shown in Fig. 5B, the anti-p107 monoclonal antibody SD15 could supershift both the C and the X complexes from the K1-expressing *Rb*+/+ and *Rb*−/− cells (lanes 5 to 8). This antibody was able to supershift all of the C complex but only about half of the X complex (compare lanes 5 and 7 with lanes 6 and 8). The control monoclonal antibody, M73, had no effect on either C or X (lanes 13 to 16). Conversely, the polyclonal anti-p130 antibody (C-20) appeared to be more effective at supershifting the X complex than the C complex (lanes 9 to 12). In addition to supershifting, this antibody preparation also had the effect of reducing the relative amounts of specific DNA binding complexes. A rabbit polyclonal antibody to mouse immunoglobulin G failed to have any effect on the C or X complexes (data not shown). It is notable that whereas SD15 was specific for p107 when tested by immunoprecipitation or Western blot of whole-cell lysates, the anti-p130 antibody could immunoprecipitate both p130 and p107 (52). Since SD15 completely supershifts the C complex

(Fig. 5B, lanes 5 and 7), it is likely that C contains exclusively p107 and very little, if any, p130. Conversely, since the X complex can be supershifted partially by SD15 and very efficiently by C20, it probably contains both p107 and p130. Therefore, in subconfluent, proliferating cultures, the predominant pRb-related protein that binds to E2F is p107 as the C complex, whereas in confluent, growth-arrested cultures, p107 and p130 both can contribute to the formation of X.

While extracts prepared from cell lines that expressed WT TAg had greatly reduced p107-E2F and p130-E2F DNA binding complexes (Fig. 5A, lanes 1, 2, 13, and 14), small amounts of C and X could still be observed. Indeed, C was observed in the *Rb*−/− cell extracts prepared from subconfluent and confluent cultures (Fig. 5A, lanes 1 and 2), whereas X was observed only in the confluent *Rb*−/− fibroblasts (lane 14). The lack of X in the confluent *Rb*−/− WT TAg extracts may reflect the ability of these cells to continue to proliferate at cell densities even higher than their *Rb*+/+ WT TAg-expressing counterparts.

To determine whether WT TAg could disrupt the C and X complexes in vitro, crude lysates of baculovirus-produced WT or K1 TAg were added to whole-cell extracts prepared from *Rb*+/+ K1 TAg-expressing cells before the addition of labeled probe. As shown in Fig. 5C, WT TAg, but not the K1 mutant, could disrupt both the C and the X E2F-containing complexes (compare lanes 2 and 3 with lanes 4 and 5 and lane 7 with lane 8). A small fraction of the X and C complexes appeared resistant to disruption by TAg in vitro. The specificity of the remaining fraction is demonstrated by its disappearance with wild-type but not mutant E2F site-containing competing DNA (lanes 11 and 12) and by supershift with the C-20 antibody (lane 9). The continued presence of C or X was not due to limiting amounts of TAg, since the degree of disruption did not increase with increasing amounts of the protein (Fig. 5C, lanes 2 and 3, and data not shown). Therefore, WT TAg, but not an LXCXE mutant, could disrupt most of the p107-E2F and p130-E2F complexes both in vivo and in vitro.

No specific pRb-containing E2F DNA binding complex could be detected in these gel retardation experiments. Indeed, the E2F binding patterns of *Rb*+/+ and *Rb*−/− cells appeared similar if not identical (Fig. 5A [compare lanes 3 and 4 with lanes 15 and 16] and B [compare lanes 1 and 2 with lanes 3 and 4). Several monoclonal anti-pRb antibodies, including XZ55, XZ104, and RB-PMG3-245, were tested for their ability to supershift any of the specific E2F complexes. No specific interaction was observed when they were added to extracts prepared from either *Rb*+/+ or *Rb*−/− cells. The apparent lack of pRb-E2F complexes was also observed when extracts from primary MEFs were tested in gel retardation assays with anti-pRb monoclonal antibodies (data not shown).

DISCUSSION

The transforming proteins of DNA tumor viruses have long been recognized as systems for the study of tumorigenesis (37). This effect of TAg correlates with its ability to bind to and presumably inactivate the products of at least two tumor suppressors, pRb and p53. TAg-dependent immortalization of MEFs has been very closely linked to its ability to bind to p53. The ability to transform cells is linked to TAg's ability to bind pRb and p53. The LXCXE domain of TAg is required for binding to pRb and for transformation and can participate in binding to two pRb-related proteins, p107 and p130. We have attempted to determine whether the LXCXE domain of TAg was required for transformation of cells in which *Rb* had been inactivated by gene targeting but were not otherwise transformed.

Our data suggest that the targeted inactivation of *Rb* does not directly lead to the immortalization or transformation of MEFs. Indeed, early-passage *Rb*−/− MEFs were not spontaneously immortalized when evaluated by several criteria. *Rb*−/− primary cells appeared to undergo a period of crisis when subjected to a 3T3 protocol. Furthermore, neither *Rb*+/+ nor *Rb*−/− MEFs appeared to generate spontaneously immortalized cells when transfected with a selectable marker only (Table 1). In addition, when clones were established from primary cells by transfection of TAg, the LXCXE domain was not required for immortalization of primary MEFs. These results are consistent with reports that the LXCXE motif of TAg is not required for immortalization of either primary MEFs or rat embryo fibroblasts (5, 54, 62).

The *Rb*+/+ and *Rb*−/− MEFs, as well as the established 3T3 cells, did not appear to be transformed by several criteria. Both the *Rb*+/+ and *Rb*−/− 3T3 cells appeared to undergo contact inhibition and accumulate in G_0/G_1 while growing in complete medium. As shown in Fig. 1C and D, 3T3 cells reached a saturation cell density with an enrichment for cells in the G_0/G_1 phase of the cell cycle. These cells were also incapable of anchorage-independent growth when plated in soft agarose. Together, these observations suggest that the targeted inactivation of *Rb* does not lead directly to immortalization or transformation of MEFs.

Given that the *Rb*−/− MEFs and the 3T3 cells were not transformed, we asked whether the LXCXE domain of TAg was required for transformation in these cells. As previously reported by others, we confirmed that the LXCXE motif is essential for TAg-mediated transformation in *Rb*+/+ MEFs as assessed by several assays. Surprisingly, however, the LXCXE motif of TAg was also required for transformation of *Rb*−/− MEFs. These observations suggest that this motif of TAg targets at least one cellular protein in addition to pRb to elicit transformation.

Transformation was assayed by several criteria. *Rb*+/+ and *Rb*−/− cells expressing WT TAg, but not those expressing the LXCXE mutants, were able to form colonies in soft agarose. In an attempt to demonstrate that this effect was specific for the LXCXE region, K1-expressing *Rb*+/+ and *Rb*−/− cell lines were transfected with a second viral protein, human papillomavirus type 16 E7, that contains an intact LXCXE motif. The WT E7, but not a mutant deleted in the LXCXE motif, could transform these cells when assayed by soft-agarose growth.

The growth rates in 10 and 1% serum also demonstrated an advantage for cells expressing an intact LXCXE domain in both *Rb*+/+ and *Rb*−/− cells. In 10% serum, the WT TAg clones were able to increase their cell number upon reaching confluence and resist contact inhibition. In 1% serum conditions, the WT TAg clones were able to grow significantly faster than the LXCXE derivatives. The ability of WT TAg-expressing clones to grow in conditions under which the LXCXE mutant TAg-expressing clones or the untransformed primary and 3T3 cells become growth arrested is clear evidence of the role of the LXCXE motif in transformation of both *Rb*+/+ and *Rb*−/− cells. We do not think that the deficiencies in transformation of the LXCXE mutants are due to long-range effects of the mutations that affect transforming activities of TAg outside the LXCXE motif. Three different mutants displayed the same phenotype. All three proteins were stable, were expressed in the cells at similar levels, and could bind efficiently to at least one other cellular protein, p53. Furthermore, a completely different DNA tumor virus protein, human papillomavirus type 16 E7, could also rescue the phenotype of

the LXCXE mutants only when its own LXCXE motif was intact.

The ability of WT TAg to promote growth in conditions under which untransformed cells cannot grow also correlates with its effect on E2F DNA binding complexes. Specifically, WT TAg, but not the K1 mutant, could disrupt p107- and p130-containing complexes both in vivo and in vitro. The X complex was detected in extracts prepared from growth-arrested K1-expressing cells cultured under contact inhibition conditions. The reduced presence of X in WT TAg-expressing cells correlates with the ability of these cells to continue growing under similar conditions. Given that X has been suggested to repress E2F activity and might contribute to the growth arrest state, WT TAg might prevent this growth arrest by specifically disrupting the X complex.

Curiously, in the gel shift experiments reported here, a small fraction of the C and X complexes was not disrupted by WT TAg. Although WT TAg could disrupt most of the complexes in vivo and in vitro (Fig. 5A and C), a small fraction appeared to be resistant to TAg disruption. Whether this small fraction is relevant is not clear. However, both p107 and p130 are phosphoproteins, and the specific phosphorylation states of p107 and p130 may affect E2F and TAg binding. Experiments to define the role of phosphorylation of p107 and p130 are under way.

An unexpected result in our experiments was the low levels of pRb present in both primary and immortal *Rb*+/+ MEFs. We do not think that this was due to a lower immunoreactivity of mouse compared to human pRb to the antibodies used in these experiments, because pRb was readily detected in other cell types derived from mice, including NIH 3T3 and NS1 cells (52). This low level of expression of WT pRb is consistent with our inability to detect pRb-E2F DNA binding complexes in *Rb*+/+ MEFs and cells expressing LXCXE mutants of TAg. Given this low level of pRb, it is not surprising that a considerable amount of extract was required to coimmunoprecipitate a detectable amount of pRb with TAg in our cells.

Deregulated E2F activity can lead to transformation. Overexpression of E2F-1, E2F-2, and E2F-3 can transform rat embryo fibroblasts and mouse NIH 3T3 cells. Interestingly, E2F-4, an E2F species that binds preferentially to p130 and p107 rather than to pRb is not oncogenic in BALB/c 3T3 cells. However, E2F-4 mutated in the region required for binding to p107 and p130 can transform these cells, which suggests that p107 and/or p130 may act normally to repress the E2F oncogenic capabilities (18). The mechanism of E2F-mediated transformation is not known, but E2F can transactivate genes that are important for cell cycle progression, and this may lead to transformation.

Even if TAg is affecting the E2F binding activities of p107 and p130, it is difficult to be certain whether this effect contributes directly to the transformation in *Rb*−/− MEFs. The LXCXE motif of TAg may bind to other proteins in addition to pRb, p107, and p130. For example, other pRb-related proteins that bind to E1a have been described previously (21). Even though the C and X E2F-containing complexes could be supershifted completely by antibodies that recognized p107 and p130, it is possible that other pRb-related proteins participate in E2F DNA-binding complexes. Alternatively, the LXCXE motif might also bind other proteins unrelated to pRb, and these may also be the targets relevant for transformation.

It is not clear if p107 and p130 are true tumor suppressors. Despite an extensive search of human tumor cell lines and tissue samples, there have been no reports of a spontaneously occurring, inactivating mutation of p107 and there has been only one report of a mutation in the p130 protein (8). Nevertheless, p107 and p130 overexpression have been reported to induce growth arrest in certain cell types (8, 63). These results suggest that these proteins have an antiproliferative action in these cells. This antiproliferative potential in these experiments could be a pharmacological rather than physiological effect, and p107 and p130 might be mimicking pRb in this setting. In this report, we demonstrate that p107 and p130 can bind to WT TAg, that p107-E2F and p130-E2F DNA binding complexes can be disrupted by TAg, and that both activities are dependent on an intact LXCXE domain. These results suggest that pRb is not the only target of the LXCXE domain in TAg-mediated transformation.

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