

pRB binds to and modulates the transrepressing activity of the E1A-regulated transcription factor p120^{E4F}

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The retinoblastoma protein pRB is involved in the transcriptional control of genes essential for cell cycle progression and differentiation. pRB interacts with different transcription factors and thereby modulates their activity by sequestration, corepression, or activation. We report that pRB, but not p107 and p130, binds to and facilitates repression by p120^{E4F}, a ubiquitously expressed GLI-Kruppel-related protein identified as a cellular target of E1A. The interaction involves two distinct regions of p120^{E4F} and the C-terminal part of pRB. *In vivo* pRB–p120^{E4F} complexes can only be detected in growth-arrested cells, and accordingly contain the hypophosphorylated form of pRB. Repression of an E4F-responsive promoter is strongly increased by combined expression of p120^{E4F} and pRB, which correlates with pRB-dependent enhancement of p120^{E4F} binding activity. Elevated levels of p120^{E4F} have been shown to block growth of mouse fibroblasts in G₁. We find this requires pRB, because *RB*^{-/-} fibroblasts are significantly less sensitive to excess p120^{E4F}.

The members of the retinoblastoma protein family, pRB, p107, and p130 are key signal transducers connecting the cell cycle machinery to the transcriptional control of sets of genes whose products regulate cell growth and differentiation processes. Their cellular function depends on their ability to bind to and modulate the activity of different transcription factors (1–3). Most studies on the pRB family members have focused on their interaction with the E2F/DP family of transcription factors. During G₀ and G₁ phases of the mammalian cell cycle, the hypophosphorylated form of pRB interacts with E2F/DP bound to DNA, acting either as a sequestering factor by blocking the transactivating domain of E2Fs or as a corepressor by recruiting histone deacetylases (1–3). Before the G₁/S transition, cyclin-associated kinases further phosphorylate pRB, causing its dissociation from E2Fs and thus allowing transcription of E2F target genes. Beside E2Fs, there is also evidence that pRB binds to and promotes the transcriptional activity of many other factors (1–3), including MyoD (4), CCAAT/enhancer binding proteins (C/EBPs, NF-IL6) (5, 6), activator protein-1 (AP-1) factors (7–9), or HBP1 (10). pRB was proposed to enhance their DNA binding, acting as an activator required for setting up the correct assembly, conformation, or concentration of these factors (5, 6, 8, 9).

We now report physical and functional interactions between pRB and the E1A-regulated transcription factor p120^{E4F}. Adenovirus E1A proteins prepare the host cell for viral replication, stimulating cell cycling and viral transcription through the targeting of several critical cellular transcription factors, including E2F complexes and E4F (11, 12). Activation of E4F is essential for the transcription of the adenovirus E4 gene, a product of which (ORF 6/7) associates with E2Fs to drive efficiently the adenovirus E2 promoter (13, 14). Human E4F (13–15) and its mouse homologue, phiAP3 (16), are low abundance and ubiquitously expressed GLI-Kruppel-related zinc

finger phosphoproteins synthesized as a predominant 120-kDa species, p120^{E4F} (15–19). In E1A-infected cells, a proteolytic product of 50 kDa, p50^{E4F}, that retained the N-terminal region of p120^{E4F}, was also detected (17). Although both human p50^{E4F} and p120^{E4F} recognize the same DNA motifs (RTGACGTC/AAY) *in vitro*, they may differentially regulate gene expression in cells. Thus, while p50^{E4F} was shown to transactivate a reporter gene driven by the adenoviral E4 promoter, p120^{E4F} repressed the same reporter (17). In addition, phiAP3, the mouse homologue of p120^{E4F}, was initially identified as a cellular factor binding to and negatively regulating the promoter of the *E1A* oncogene (16, 20). Cellular genes regulated by p120^{E4F} and/or by p50^{E4F} are currently unknown, but it is likely that they are involved in pathways controlling cell cycle progression and apoptosis because it was reported that overexpression of p120^{E4F} in mouse fibroblasts induced cell cycle arrest in late G₁ (21) whereas that of p50^{E4F} in E1A-transformed cells accelerated apoptosis (22).

Here we report that cellular pRB–p120^{E4F} complexes are formed between the hypophosphorylated form of pRB and p120^{E4F} in growth-arrested but not in actively growing cells. pRB enhances both p120^{E4F} DNA binding activity and p120^{E4F}-mediated promoter repression. By using pRB-deficient cells we also present evidence that the antiproliferative effect of p120^{E4F} is, at least in part, dependent on the presence of a functional *RB* gene. Based on these findings we propose a novel role for pRB as a negative regulator of cell proliferation through its potentiating effect on p120^{E4F}.

Materials and Methods

DNA Plasmids, cDNA Library. Details of all constructs are available on request. The serum-starved WI38 human fibroblast cDNA library in the multicopy prey plasmid pJG4–5 (23) was described previously (24, 25). Baits for the two-hybrid assays in pEG202 plasmid (23) are as follows: pEG202–RB (aa 2–928 of pRB), pEG202–RB661W [aa 2–894 of pRB pocket mutant RB661W (26)], and pEG202–p107 (aa 1–1068 of p107). Mammalian E4F expression constructs in pcDNA3 (Invitrogen) are as follows: pcDNA–p120^{E4F} (full-length E4F) pcDNA–p50^{E4F} (aa 1–357 of E4F), pcDNA3–E4F–Cter (aa 357–784) and pcDNA–E4F–Sma (aa 1–161). E4F glutathione S-transferase (GST) fusions in pGEX4T (Pharmacia): pGEX–p120^{E4F} (full-length E4F),

Abbreviations: C/EBP, CCAAT/enhancer binding proteins; AP-1, activator protein-1; GST, glutathione S-transferase; MEF, mouse embryo fibroblast; RT, room temperature; EMSA, electrophoretic mobility shift assays.

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pGEX-p50^{E4F} (aa 1–357), pGEX-E4F-Cter (aa 357–784). GST-RB constructs have been described previously (27). The reporter E4F-TK-LUC was constructed by cloning a single copy of the double-stranded oligonucleotide [(+) GATCCT-GACGTAACA/(-)GATCTGTTACGTCAG], corresponding to the E4F binding site of the adenoviral E4 promoter, upstream the HSV-TK (herpes simplex virus thymidine kinase) minimal promoter and the firefly luciferase gene in pGL2 (Promega).

Cell Culture, Protein Extracts, Transfections, and Luciferase Assays. U2OS and NIH 3T3 were grown in DMEM, 10% FCS (Biomed, Foster City, CA). Rb^{+/+}, Rb^{-/-}, and p107/p130^{-/-} 3T3 fibroblasts were established from mouse embryo fibroblasts (MEFs) by M. Classon (Massachusetts General Hospital, Boston) and maintained as described previously (28). Cells were growth arrested at confluence and by serum starvation for 48 h. Nuclear and whole cell extracts were prepared as previously described (28). All transfections were performed by using the Lipofectamine Plus reagent following the manufacturer's instructions (GIBCO/Life Technologies). For the E4F-RB-mediated repression assays, NIH 3T3 cells or C33A cells were cotransfected with 0.2 μ g of pCH110 (β -galactosidase expression vector) and 2 μ g of E4Fs-TK-LUC reporter vector alone or with various combinations of 100 ng of pcDNA-p120^{E4F} and pECE-RBp34 (29). β -Galactosidase and luciferase activity were measured 36 h later as previously described (28). Luciferase activity values were normalized to the β -galactosidase activity to account for variations in transfection efficiencies.

Two-Hybrid Screening. Two-hybrid screening in yeast was performed essentially as previously described (23, 24). The yeast strain is EGY048/lacZ (23) carrying two reporters whose expression is regulated by LexA-responsive promoters; i.e., a chromosomally integrated *LEU2* reporter gene (*LexA:LEU2*) and the 2 μ LacZ reporter plasmid pSH18-34 (23). pEG202-RB was used as a bait in EGY048/lacZ to screen the WI38 pJG4-5 prey library. A total of 2×10^6 yeast cotransformants were selected for galactose-induced reporter-dependent leucine prototrophy. Prey plasmids were rescued from positive clones and retransformed into EGY048/lacZ strains expressing pEG202-RB, pEG202-RB668W, pEG202-p107, pEG202-p130, or pEG202-bicoid. Cotransformants were re-selected for reporter-dependent leucine prototrophy and β -galactosidase production; positive clones were sequenced. β -Galactosidase activities of galactose-induced exponentially growing cultures were measured as previously described (23).

In Vitro and in Vivo Pull-Down Assays and Coimmunoprecipitation. *In vitro* translation reactions of E4F were performed by using [³⁵S]methionine (Amersham) in a TNT-coupled reticulocyte lysate (Promega) according to the manufacturer's instructions. The plasmids pcDNA-p120^{E4F}, pcDNA-p50^{E4F}, and pcDNA-E4F-Cter were used as templates to generate the fragment FL (aa 1–784), N (aa 1–357), and C (358–784), respectively. Template to generate fragment S of E4F (aa 358–486) was a PCR product amplified by using pcDNA-E4F-Cter as a matrix. Fragments F1 (aa 184–254), F2 (aa 428–521), and F3 (aa 515–584) of E4F were produced by using PCR templates containing the sequence of the T7 promoter, generated by using the following pairs of primers: F1, (+)TAATACGACTCACTATAGGGACATGAACAAGGATGGCCGC/(-)CTACTTTC-CACACTTGGAGCACTT; F2, (+)TAATACGACTCACTATAGGGACATGTCTAGCGTGCCAGG/(-)CTAGG-GACAAGGGTAGGGCCGCAC; F3, (+)TAATACGACTCACTATAGGGACATGGAGCGGCCCTACCCT/(-)CTAGGCGAAGCCACGGCCCACTT.

For *in vitro* pull-down assays, the indicated GST-RB fusion proteins were purified from *Escherichia coli* (XL1Blue) by using

glutathione-Sepharose beads (Pharmacia) as described previously (30). GST-RB bound beads were incubated for 1 h at room temperature (RT) with the indicated ³⁵S-labeled *in vitro*-translated E4F constructs in pull-down buffer [50 mM Hepes (pH 7.8), 150 mM NaCl, 0.1% Nonidet P-40, 1 mM DTT, and 1 mM PMSF]. Proteins retained on the GST-RB beads were washed five times with 1 ml of pull-down buffer, solubilized in SDS-loading buffer, and analyzed on 8% acrylamide SDS/PAGE gels. For the *in vivo* pull-down assay, 2 μ g of GST-p120^{E4F} coupled to glutathione-Sepharose beads were incubated with 100 μ g of nuclear extracts prepared from NIH 3T3 cells (28) in pull-down buffer for 2 h at RT. After three washes, bound proteins were separated on 8% acrylamide SDS/PAGE gels, blotted, and probed with either anti-pRB, G3-245 (PharMingen), anti-p107, C18 (Santa Cruz Biotechnology), or anti-p130 (Transduction Laboratories, Lexington, KY) Abs.

For coimmunoprecipitation of cellular pRB by E4F, 200 μ g U2OS whole cell extracts were incubated with protein A beads coupled to either anti-E4F antibodies or preimmune serum in IP buffer [150 mM NaCl, 0.5% Nonidet P-40, and 50 mM Tris (pH 8)] for 1 h at RT. Bound proteins were washed three times in 1 ml IP buffer and separated on 8% acrylamide SDS/PAGE gels, blotted, and finally probed with anti-pRB (G3-245, Pharmingen). For coimmunoprecipitation of cellular E4F by pRB, 500 μ g U2OS nuclear cell extracts were incubated with agarose beads (AminoLink Coupling gel, Pierce) covalently coupled to either anti-pRB mAbs (21C9) or control mAbs [control 1: anti-HA tag, 12CA5; control 2: anti E2F5, 274 (46)] in IP buffer [150 mM NaCl, 0.5% Nonidet P-40, and 50 mM Tris (pH 8)] overnight at 8°C. Bound proteins were washed three times in 1 ml IP buffer and separated on 8% acrylamide SDS/PAGE gels, blotted, and finally probed with anti-E4F (88.2).

Electrophoretic Mobility Shift Assays (EMSA). Suboptimal amounts of purified GST-p120^{E4F} fusions (30) were incubated either alone or in combination with increasing amounts of recombinant human pRB purified from baculovirus-infected SF9 cells (31) for 15 min at RT in a total volume of 20 μ l binding buffer [10 mM Tris-HCl (pH 7.9), 40 mM KCl, 10% glycerol, 0.05% Nonidet P-40 and 1 mM DTT, and 1 μ g poly(dI-dC)] in presence of 1 ng of a T4-PNK end-labeled double-stranded oligonucleotide probe bearing the E4F binding site of the *E4* promoter [(+)GATCCCGGATGTGGCAAAGTGC]. DNA-protein complexes were separated by electrophoresis on a 4% polyacrylamide gel in 0.25 \times TBE buffer at RT and 10 V/cm.

Immunofluorescence. Cells were grown on cover-slips; 24 h after transfection with the indicated plasmids they were incubated for 12 h with BrdUrd. After fixation and permeabilization with methanol, cells were treated with 1.5 M HCl for 10 min at RT and incubated with a purified anti-E4F rabbit polyclonal Ab (E4F 88.2) together with an anti-BrdUrd mouse mAb (Dako). Preparations were then incubated with a combination of Texas Red-conjugated anti-rabbit IgG and FITC-conjugated anti-mouse IgG. E4F 88.2 Ab was raised against the E4F peptide EEDEDDVHRCGRCA (aa 50–64) and was affinity purified on an agarose-peptide column before use.

Results

Identification of p120^{E4F} as a pRB Interacting Protein. Our initial purpose was to use the yeast two-hybrid system to identify pRB interactors expressed in quiescent human fibroblasts. A LexA-pRB construct was used as a bait to screen a cDNA library of preys generated from confluent and serum-starved WI38 human fibroblasts (23–25). Two classes of clones were selected. One encoded E2Fs (E2F1 and E2F4). The second encoded the E1A-regulated transcription factor p120^{E4F}, which was represented by three independent positive clones containing full-

Table 1. Differential interactions of p120^{E4F}, E2F1, and E2F4 with pocket protein family members in a two-hybrid assay

Preys	Baits				
	pRB	RB61W	p107	p130	Bicoid
p120 ^{E4F}	+++	+++	-	+	-
E2F-1	++	-	+	+	-
E2F-4	++	-	++	++	-

A yeast strain containing a LacZ reporter plasmid driven by a lexA responsive promoter was cotransfected with indicated combinations of baits and preys (23–25). LexA-fusion baits: human pRB, the pRB mutant RB61W that fails to bind E1A and E2Fs (38), the pRB-related proteins p107 and p130, and bicoid as a control (23). B42-fusion preys: human p120^{E4F}, E2F1, and E2F4. β -Galactosidase activities that reflect the strength of the bait/prey interaction were measured on cultures of three isolates of each strain exponentially growing in 2% galactose. β -Galactosidase activities measured in Miller units were ordered as: +++, Miller units >1000; ++, >500; +, >50; and -, <50. Comparable levels of expression of the various baits were observed by using Abs directed against lexA (data not shown).

length p120^{E4F} cDNAs. We then compared the binding of E2Fs and p120^{E4F} to various pocket proteins in a two hybrid interaction assay by using a LacZ reporter strain (Table 1). E2F1 and E2F4 interacted, although with different intensities, with all three pocket proteins but not with a pocket mutant of pRB. By contrast to E2Fs, p120^{E4F} bound strongly to both wild-type and mutant pRB, but interacted very weakly with p130 and not at all with p107. Thus, at least in this yeast assay, p120^{E4F} displays a pocket protein binding specificity distinct from that of E2Fs.

In Vivo Detection of p120^{E4F}-pRB Complexes. We next tested whether recombinant p120^{E4F} was able to bind cellular pocket proteins *in vitro*. Pull-down assays were performed with GST-p120^{E4F} or an unrelated protein, GST-ELK, and nuclear extracts prepared from confluent 3T3 fibroblasts established from RB^{+/+} and RB^{-/-} MEF (a gift from M. Classon; refs. 28 and 32). Bound proteins were then examined for the presence of pRB by immunoblotting. pRB was clearly detected on GST-p120^{E4F} beads and not on control beads (Fig. 1A). Furthermore, the pRB band was not observed using nuclear extracts from RB-deficient cells (Fig. 1A). Interestingly, we failed to detect GST-p120^{E4F}-pRB complexes in nuclear extracts prepared from exponentially growing NIH 3T3 fibroblasts (Fig. 1B) or from S phase-synchronized cells (data not shown), suggesting that the p120^{E4F}-pRB interaction might be cell-cycle dependent. Consistent with this, p120^{E4F}-bound pRB appeared to migrate as the hypophosphorylated pRB (Fig. 1B) which is the predominant and active form of the protein in growth-arrested cells (1–3).

Interestingly and in agreement with the result of our two-hybrid experiment (Table 1), the other members of the pRB family, p107 and p130, were not found to associate with the GST-p120^{E4F} beads (Fig. 1B). Thus, both sets of results suggest that pRB is the unique member of the pocket-protein family to associate with E4F in rodent fibroblasts.

We next explored whether crude nuclear extracts contained an endogenous p120^{E4F}-pRB complex. To perform this analysis, we developed and affinity purified an antibody specific for human p120^{E4F} (Fig. 1C). Coimmunoprecipitation experiments were not performed with mouse fibroblasts because endogenous p120^{E4F} was hardly detectable in these cells (data not shown). We used whole cell or nuclear extracts prepared from confluent human U2OS cells where endogenous p120^{E4F} and pRB were both easily visualized (Fig. 1C). On immunoprecipitation with the anti-p120^{E4F} Ab (88.2), pRB was clearly and specifically detected by immunoblotting (Fig. 1D). The other way around, p120^{E4F} was detected by immunoblotting among proteins bound to agarose beads covalently coupled to anti-pRB Abs (21C9)

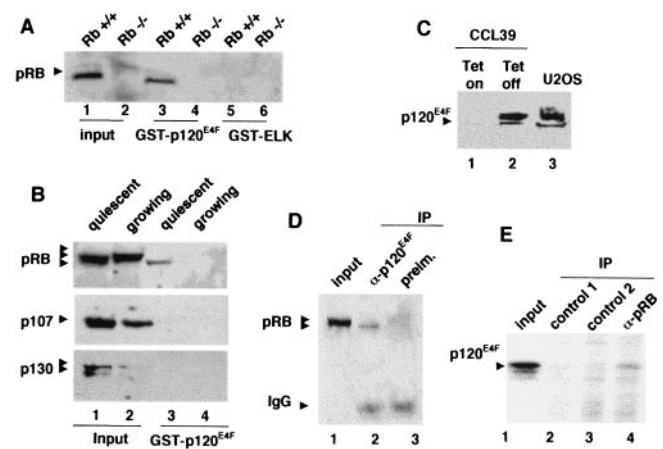


Fig. 1. p120^{E4F} associates with pRB. (A) Recombinant p120^{E4F} associates with cellular pRB. GST pull-down assays were performed with nuclear extracts prepared from confluent cultures of either RB^{+/+} (lanes 1, 3, and 5) or RB^{-/-} NIH 3T3 mouse fibroblasts (lanes 2, 4, and 6). Extracts were incubated with either Sepharose-bound GST-p120^{E4F} (lanes 3 and 4) or GST-ELK (negative control) (lanes 5 and 6). Bound proteins were released and probed for the presence of pRB by immunoblotting. One-third of the input extract was loaded in lane 1 and 2. (B) Recombinant p120^{E4F} associates with the hypophosphorylated form of pRB present in arrested cells and does not associate at all with cellular p107 and p130. GST pull-down assays were performed as above by using nuclear extracts from either quiescent (lanes 1 and 3) or exponentially growing (lanes 2 and 4) NIH 3T3 fibroblasts. Bound proteins (lanes 3 and 4) were probed for the presence of pRB (Top), p107 (Middle), or p130 (Bottom) by immunoblotting. One-third of the input extract was loaded in lane 1 and 2. (C) p120^{E4F} is easily detectable in U2OS cells. Nuclear extract from U2OS cells is probed by immunoblotting by using an affinity-purified rabbit polyclonal antibody (E4F-88) (lane 3). Specificity of this Ab is tested on nuclear extracts prepared from a Chinese hamster cell line (CCL39-tet/E4F) expressing a tetracycline inducible p120^{E4F} gene (lanes: 1, + tetracycline; 2, - tetracycline). (D and E) Coimmunoprecipitation of cellular p120^{E4F} and pRB proteins from U2OS cells extracts. (D) p120^{E4F} is immunoprecipitated from confluent U2OS nuclear extracts by using the E4F 88.2 Ab, and precipitate is probed for the presence of pRB by immunoblotting (lane 2). The same experiment is performed with the corresponding preimmune serum (lane 3). One-tenth of the input extract was loaded in lane 1. (E) pRB is immunoprecipitated from confluent U2OS nuclear extracts by using the anti-pRB mAb coupled to agarose beads and precipitate is probed for the presence of p120^{E4F} by immunoblotting (lane 4). The same experiment is performed with control beads coupled to unrelated mAbs (lanes 2 and 3). One-thirtieth of the input extract was loaded in lane 1.

(Fig. 1C). Similar coimmunoprecipitation were also observed by using nuclear extracts from MCF7 cells blocked in G₁ by leucine and serum depletion (data not shown). Together, these results strongly suggest that p120^{E4F}-pRB complexes do exist in living cells.

Mapping the Binding Domains of pRB and p120^{E4F}. Several distinct protein-binding domains have been identified in pRB: The “large pocket,” composed of subdomains A and B, that binds E2Fs and proteins containing an LXCXE-motif, and the C-terminal domain that binds the nuclear c-Abl tyrosine kinase, UBF, and Jun proteins (Fig. 2B) (1–3). Pull-down assays were performed by using *in vitro*-translated radiolabeled p120^{E4F} and GST fusion proteins encoding various domains of pRB (Fig. 2A). No binding was detected with pocket domain A alone, whereas GST-RB proteins containing either the three domains, A, B, and C, or only domains B and C, exhibited strong binding to p120^{E4F}.

A weak binding was also observed with domain B alone. Nevertheless, p120^{E4F} was still able to bind to pRB_{C706F} which bears a naturally occurring mutation in the pocket B of pRB that disrupts the binding to the transcription factor E2F (33). This

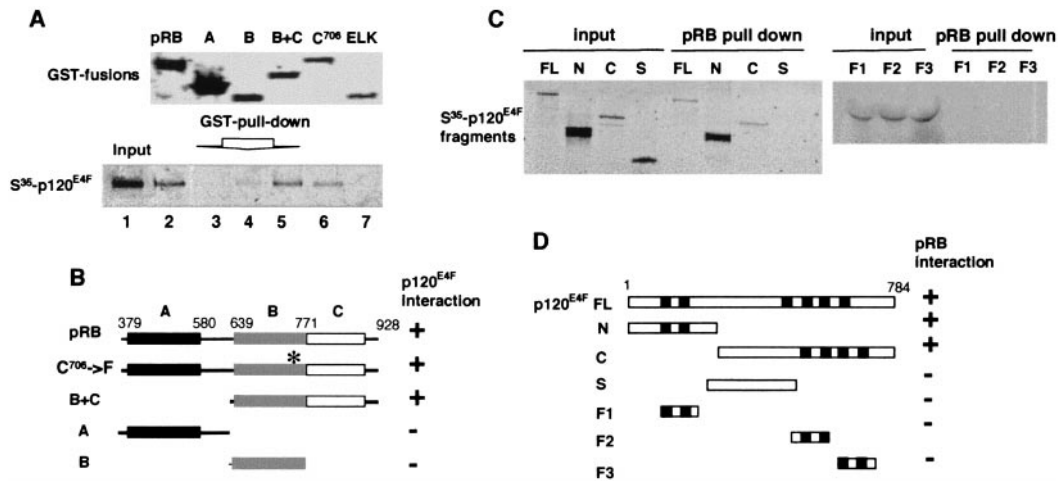


Fig. 2. Mapping of the binding domains of pRB and p120^{E4F}. (A) GST pull-down assays using the indicated GST-pRB constructs (Upper) and ³⁵S-labeled *in vitro* translated p120^{E4F} (full-length) (lanes 2–6, Lower). Input of radiolabeled p120^{E4F} is loaded lane 1 (Lower). As a control, GST pull-down assay is done with GST-ELK protein (lane 7). (B) Schematic representation of the various pRB protein constructs and summary of their capacity to bind p120^{E4F}. Pocket domains A (aa 379–580), B (aa 639–771), C (aa 771–928) are indicated. The asterisk indicates the C→F mutation of pRB^{C706-F}. (C) GST pull-down assays using the indicated ³⁵S-labeled *in vitro*-translated p120^{E4F} fragments and the GST-pRB construct containing domains A, B, and C of pRB. (D) Schematic representation of the various p120^{E4F} fragments used in the assay and summary of their capacity to bind pRB. The zinc finger domains are shown as black boxes. FL, full length (aa 1–784); N/p50^{E4F}, aa 1–357; C, aa 358–784; S, aa 358–486; F1, aa 184–254; F2, aa 428–521; F3, aa 515–584.

finding suggested that intact pocket B was not required for interaction with p120^{E4F}. No binding was observed with GST-ELK (Fig. 2A) or GST alone (data not shown), demonstrating the specificity of these p120^{E4F}-pRB interactions. Together, these results suggest that the p120^{E4F}-pRB interaction is mainly pocket A/B-independent and requires the pocket C-terminal region (Fig. 2B).

p120^{E4F} is a GLI-Kruppel-related transcription factor containing six C2H2 zinc finger motifs (Fig. 2D) which exists as a predominant 120-kDa protein p120^{E4F}, and a less abundant, proteolytically derived 50-kDa N-terminal fragment (p50^{E4F}) (15, 16). *In vitro*-translated radiolabeled p50^{E4F} (N terminus) and p120^{E4F} exhibited strong GST-pRB binding (Fig. 2C). Interestingly, the C-terminal domain of p120^{E4F}, which is not present in p50^{E4F}, also interacted with pRB. In contrast, no binding was detected with the various zinc finger motifs and with the central domains of p120^{E4F} (Fig. 2C and D). Thus p120^{E4F} can interact with pRB through at least two domains located in the C- and N-terminal parts of the protein, respectively.

pRB Up-Regulates the Transcriptional Activity of p120^{E4F}. Next we assessed what were the functional consequences of the p120^{E4F}-pRB interaction on the binding and transrepression capabilities of p120^{E4F}. Transient transfection assays, performed in NIH 3T3 cells, show that increasing amounts of p120^{E4F} progressively decreased the activity of an E4F-responsive element-driven luciferase reporter construct, E4-TK-Luc (Fig. 3A). We then transfected a phosphorylation-defective constitutively active pRB (29) together with the maximum amount of p120^{E4F} plasmid that was unable to inhibit the reporter activity (Fig. 3B, striped bar). This restored a strong reporter repression (Fig. 3B, dotted bar). This potentiating effect was specific for the presence of p120^{E4F} because pRB alone had no effect on reporter activity (Fig. 3B, open bar). This effect was also specific for the E4F DNA-binding site because the same p120^{E4F}-pRB combination did not affect a control reporter construct (Fig. 3B). To test whether this might be caused by an indirect effect of pRB on the cell cycle distribution of the transfected cells, we also performed similar reporter assays were in cervical carcinoma cell line C33A,

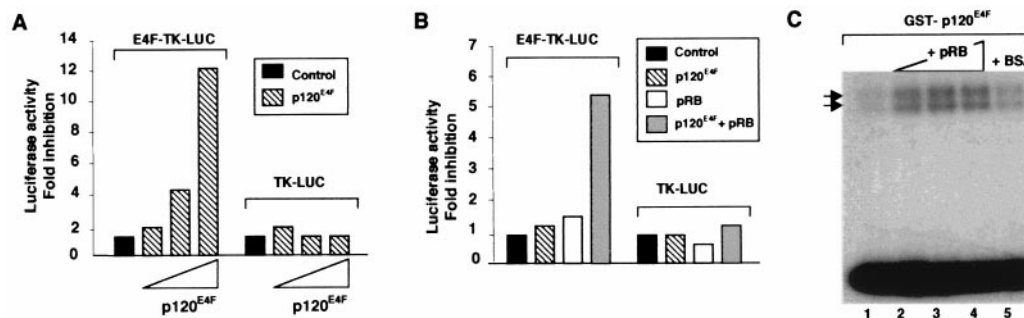


Fig. 3. pRB up-regulates the DNA binding and transrepression capabilities of p120^{E4F}. (A) p120^{E4F} is a site-dependent transcriptional repressor. NIH 3T3 cells are transiently cotransfected with an E4F-responsive reporter gene E4F-TK-Luc and increasing amounts of the pcDNA-p120^{E4F} plasmid. TK-Luc (without E4F site) was used as a control. Luciferase activity is shown as fold inhibition of the activity obtained with E4F-TK-Luc or TK-Luc constructs transfected alone. (B) pRB up-regulates the transrepression capabilities of p120^{E4F}. The maximum amount of p120^{E4F} plasmid that was unable to inhibit the reporter activity as determined in A is cotransfected with either E4F-TK-Luc or TK-Luc and with (dotted bars) or without (striped bars) a phosphorylation-defective constitutively active pRB (p34RB). As a control, E4F-TK-Luc and TK-Luc reporters were also transfected alone (filled bars) or with p34RB (open bars). (C) pRB up-regulates the DNA binding activity of p120^{E4F} *in vitro*. EMSAs are performed with a probe containing an E4F DNA binding site. Purified GST-p120^{E4F} is added alone (lane 1) or together with increasing amounts of baculovirus-expressed purified pRB protein (lanes 2–4) or BSA (lane 5).

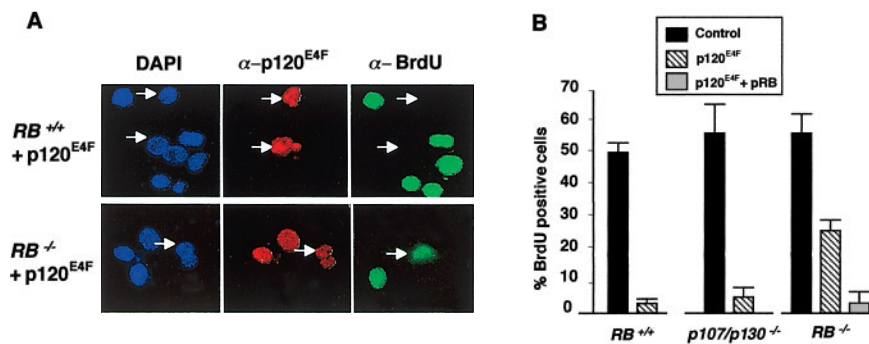


Fig. 4. p120^{E4F}-mediated growth arrest is pRB-dependent. (A) Rb^{+/+} (Upper) or Rb^{-/-} (Lower) mouse fibroblasts were transfected with the pcDNA-p120^{E4F} plasmid. Twenty-four hours after transfection with the indicated plasmids, cells were assessed for BrdUrd uptake in their DNA (14 h in BrdUrd) as a marker of cell proliferation. Cells were fixed and labeled with 4',6'-diamidino-2-phenylindole (DAPI) (blue). p120^{E4F}-expressing cells were identified by immunofluorescence by using anti-human E4F (E4F 88.2) (red) and monitored for BrdUrd incorporation by using an anti-BrdUrd-specific Ab (green). (B) Diagram showing the average of four independent experiments (200 cells counted per experiment) performed as reported in A. Average of similar experiments performed in p107/p130^{-/-} cells are also shown as indicated. As a control, Rb^{-/-} cells are also transfected with p34(RB).

which was previously shown to be resistant to pRB growth suppression (47). Results were identical to those obtained by using NIH 3T3 fibroblasts (data not shown).

One possibility was that pRB potentiated E4F-mediated repression through stimulation of DNA binding by p120^{E4F}. To test this, EMSAs were performed by using a probe containing the p120^{E4F} responsive element of the adenoviral E4 promoter and recombinant p120^{E4F} and pRB proteins expressed in bacteria and baculovirus-infected cells, respectively (Fig. 3C). We used suboptimal concentrations of p120^{E4F} (Fig. 3C, lane 1) with increasing amounts of pRB or of control proteins (BSA, GST-ELK) (Fig. 3C). Addition of pRB increased the binding of p120^{E4F} to the probe, unlike BSA (Fig. 3C) or GST-ELK (data not shown). Recombinant pRB alone did not bind to the E4-probe and binding of p120^{E4F} was competed by an excess of wild-type, but not of mutated, competitor (data not shown). However, several different pRB-specific antibodies did not supershift the p120^{E4F}-specific complexes. Similar effects of pRB have been described previously with c-Jun, C/EBPs and NF-IL6 transcription factors (5, 6, 8). Thus, pRB somehow strongly facilitates and/or stabilizes binding of p120^{E4F} to an E4F-site, as with the factors described above.

p120^{E4F}-Mediated Growth Arrest Is pRB-Dependent. Elevated levels of p120^{E4F} inhibit the growth of mouse fibroblasts by blocking the cells in the G₁ phase of their cell cycle (21). We explored whether the interaction with pRB plays a role in this p120^{E4F}-mediated G₁ arrest. To this end, we transfected a p120^{E4F} expression vector into mouse NIH 3T3 fibroblasts derived from Rb^{-/-} MEFs, from p107/p130^{-/-} MEFs, or from the corresponding normal cells (Rb^{+/+}), and measured their proliferation by BrdUrd incorporation (Fig. 4). As expected, Rb^{+/+} cells expressing p120^{E4F} failed to incorporate BrdUrd (Fig. 4A, Upper), quantitation of several experiments clearly showing that p120^{E4F} blocked almost all transfected cells (Fig. 4B). Similarly, p107/p130^{-/-} cells were also blocked efficiently by p120^{E4F} (Fig. 4B). In contrast, a significant proportion of p120^{E4F}-positive Rb^{-/-} cells were also positive for BrdUrd labeling (Fig. 4A, Lower), an effect reproducibly observed in four independent experiments (Fig. 4B). Notably, this p120^{E4F}-mediated arrest did not correspond to increased apoptosis, as shown by the lack of correlation between annexin-FITC staining and p120^{E4F} overexpression (unpublished data). These results show that p120^{E4F} overexpression can partially suppress cell growth in an Rb^{-/-} background but that this effect is strongly enhanced in the presence of functional pRB. This observation is consistent with

the results presented in Fig. 3 showing that pRB potentiates the p120^{E4F}-mediated transcriptional repression.

Discussion

pRB interacts with a variety of transcription factors and thereby regulates both cell growth and differentiation through its ability to coordinate multiple transcriptional events, acting as a competence factor to allow or impede various cellular transitions. Here we show that pRB also binds the E1A-regulated p120^{E4F} transcription factor both *in vitro* and *in vivo*. Recombinant p120^{E4F} and pRB proteins bind each other directly *in vitro*. In cells, the pRB-p120^{E4F} complex was detected only in growth-arrested cells where it contained the hypophosphorylated form of pRB. Thus, as previously observed for most pRB-binding proteins, p120^{E4F} interacts preferentially with the transcriptionally active form of pRB. An open question also remains about the role of p120^{E4F} phosphorylation in this interaction because it has been reported that serum stimulation or adenovirus infection of arrested cells affects p120^{E4F} phosphorylation (13, 15, 16, 18).

p120^{E4F} displays pRB binding properties distinct from that of E2Fs or of LXCXE-containing proteins. Indeed, we found that mutations or deletions within the pocket region of pRB that abrogate pRB binding to E2F did not affect interactions with p120^{E4F} *in vitro*. Moreover, we failed to detect any interaction between p120^{E4F} and the other member of the pRB protein family, p107 or p130. Consistent with both observations, we found that p120^{E4F}-pRB interaction involved the C-terminal part of pRB, downstream of the A/B pocket domains, which is not related in structure and sequence to that of p107 and p130. Beside p120^{E4F}, this C-terminal region of pRB was reported to bind other proteins important for cell proliferation, including the high mobility group (HMG) box-containing transcription factor UBF (37), the c-Abl tyrosine kinase (34-36) and the transcription factor c-Jun (8). Consistent with the key role played by these factors in growth control, it was also shown that the C-terminal part of pRB contributes to RB-mediated growth suppression (1, 3, 34-37).

There are increasing evidence that the biological activity of pRB not only depends on the inhibition of its targets but also on its ability to properly assemble specific protein complexes on DNA. As is the case for C/EBPs, NF-IL6, ATF2, myoD, and c-Jun (1, 3, 5-8), we found that pRB stimulates the binding of p120^{E4F} to its cognate DNA site. Surprisingly, it is worth to note here that five of the bZIP factors mentioned above are members of protein families, i.e., ATF/CREB, C/EBPs, and AP-1, that can also bind the core sequence of the E4F site with however, a lower affinity than p120^{E4F} (13-15, 19). Although the biological

significance of this observation remains to be explored, it is conceivable that pRB could control a specific class of regulatory elements through its association with multiple and nonrelated factors that recognize these elements. Nevertheless, we could not detect pRB in p120^{E4F}-DNA complexes measured by gel shift assays, as has also been observed with C/EBPs, NF-IL6, and c-Jun in presence of pRB (4–9). The biochemical basis of this facilitation process is not understood, although it has been proposed in the case of C/EBPs, NF-IL6, and c-Jun, that pRB might promote conformational changes, dimerization and increased concentration of dimerized factors, resulting in a more efficient binding of the low abundance active form of the transcription factor (5, 6, 8). A similar mechanism occurs in our system as we found that pRB associates with the N-terminal region of p120^{E4F}, which contains a domain required for its stable association to DNA (18).

The presence of pRB but not of the other pocket proteins strongly enhances the negative effect of p120^{E4F} on transcription and proliferation. Consistent with this, overexpression of p120^{E4F} blocks the cell cycle more efficiently in RB^{+/+} or p107/p130^{-/-} cells than in RB^{-/-} cells and, importantly, this block is not associated with increased apoptosis. Similarly, pRB interacts with and triggers activation of the negative regulators of transcription, BRCA1 (39), HBP1 (40), and p202 (41), whose overexpression in cells also induces cell cycle arrest in G₁. Thus, it is conceivable that one of the cellular functions of pRB could be to act as a competence factor for multiple growth suppressor, including p120^{E4F}. A question then arises about the physiological function of these factors. Interestingly, one possible shared

downstream effector for all these growth suppressors, and therefore for pRB, might be the cyclin-cdk inhibitor p21^(WAF1/CIP1/SDI1). Indeed, it has been reported that p120^{E4F}-, BRCA1-, HBP1-, and p202-arrested cells contain elevated levels of p21 protein (21, 42–44). The mechanism by which p120^{E4F} exerted a control on p21 remains unclear. It was first reported to result from a p53-independent posttranscriptional stabilization of the p21 protein (21). However, others have found recently that p120^{E4F}-induced arrest requires p53 (48). Anyhow, this is likely not the only mechanism for p120^{E4F} induced growth arrest because we have recently observed that p21^{-/-} and p53^{-/-} primary fibroblasts (45) are still blocked in G₁ on ectopic expression of p120^{E4F} (L.F. and C.S., unpublished observation). Further studies are currently underway in our laboratory aiming to identify *bona fide* target genes of p120^{E4F} involved in cell cycle regulation.

In summary, we provide evidence for a novel function of pRB that might promote cell-cycle arrest, namely potentiating the repressive effect of the ubiquitous cellular factor p120^{E4F}.

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