A potent transrepression domain in the retinoblastoma protein induces a cell cycle arrest when bound to E2F sites

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ABSTRACT An intact T/E1A-binding domain (the pocket) is necessary, but not sufficient, for the retinoblastoma protein (RB) to bind to DNA–protein complexes containing E2F and for RB to induce a G1/S block. Indirect evidence suggests that the binding of RB to E2F may, in addition to inhibiting E2F transactivation function, generate a complex capable of functioning as a transrepressor. Here we show that a chimera in which the E2F1 transactivation domain was replaced with the RB pocket could, in a DNA-binding and pocket-dependent manner, mimic the ability of RB to repress transcription and induce a cell cycle arrest. In contrast, a transdominant negative E2F1 mutant that is capable of blocking E2F-dependent transactivation did not. Fusion of the RB pocket to a heterologous DNA-binding domain unrelated to E2F likewise generated a transrepressor protein when scored against a suitable reporter. These results suggest that growth suppression by RB is due, at least in part, to transcription mediated by the pocket domain bound to certain promoters via E2F.

The smallest fragment of the retinoblastoma protein (RB) capable of inducing a G1/S block [i.e., the T/E1A-binding domain (or "pocket") and much of the adjacent C terminus] is also the smallest fragment of RB that can bind stably to an E2F–DNA complex. Further, all stable tumor-derived RB mutants fail to bind to E2F (1). E2F binding sites have been identified in a number of genes implicated in cell cycle control and/or differentiation (1). Each of the cloned E2F family members contains within its transactivation domain a colinear sequence that facilitates binding to RB or to the related proteins p107 and p130 (1). RB can inhibit E2F-dependent transactivation in cultured cells (1). Conversely, overproduction of E2F1 can induce quiescent cells to enter S phase and can overcome an RB-induced G1/S block (2–4). Similarly, viral oncoproteins, such as T and E1A, that disrupt RB–E2F complexes can induce entry into S phase (1). Thus, “inactivation” of E2F by RB might provide a mechanistic explanation for RB-mediated growth suppression.

While the above data suggest that regulation of E2F is necessary for RB to induce a G1/S block, they do not establish that such regulation is sufficient for this effect. Indeed, RB can bind, at least in vitro, to a number of cellular proteins known, or suspected, to play roles in cell growth control (2). Furthermore, data suggest that RB–E2F complexes, rather than representing “inactive” E2F, actively repress transcription in a DNA-binding-dependent manner. In particular, mutation of the E2F sites within certain cell cycle-regulated promoters leads to a loss of transcriptional repression in G1 (5–8). Conversely, the introduction of E2F sites within certain artificial promoters leads to RB-dependent transcriptional repression (9). A stable E2F1 mutant (aa 1–368) that can bind to DNA but can no longer transactivate can block the ability of RB to repress an E2F-responsive promoter (4). We therefore asked whether RB bound to an E2F-responsive promoter could repress the activities of adjacent transcriptional activators and whether the repression of E2F-responsive genes was sufficient to induce a G1/S block.

MATERIALS AND METHODS

Cell Culture, Transfection, and Metabolic Labeling. CV-1P monkey kidney cells and U-2 OS human osteosarcoma cells (RB+/–) were grown in Dulbecco’s modified Eagle’s medium (DMEM) with 10% FetalClone II (HyClone). Saos-2 (RB–/–) cells were grown in DMEM with 10% fetal bovine serum. Transfections using the 2× Bes-buffered saline (2×BBS)/calcium phosphate method and metabolic labeling with [35S]methionine were as described (10, 11).

Plasmids. The plasmids encoding the chimeras of E2F1-(1–368)/RB-(379–792) and E2F1-(1–368)/Kox1, and the various derivatives thereof, were created by two-step sequential PCRs (12). The sense primers for the junctions of the E2F1/RB and E2F1/Kox1 chimeras were 5'-GGCGAGGCTGCGGCCATCCATGAAACACTATCCAAACA-3' and 5'-GGCGAGGCTGCGGCCATCCATGAAACACTATCCAAACA-3', respectively. pcDNA-HA-E2F1-(1–368) and pcDNA-HA-E2F1-(1–368;122), encoding hemagglutinin (HA) fusions, have been described (4). The E7 cDNAs from pGEX2T-E7 and pGEX2T-E7ΔCR2 were subcloned into pRC/CMV to create pRcCMV-HA-E7 and pRcCMV-HAE7ΔCR2. E7ΔCR2 lacks 4 aa (DLYC) beginning at residue 21. The E2F sites in the E2F1 promoter were changed to two tandem TETO sites by site-directed mutagenesis with the oligonucleotide 5'-AGTCCG GGCACCATTTCTCTATCAGTAAGGATCTCTATCAGTAAGGAGGCGCAGGCC-3' to create pGL2ANATETo. The Min 1/Bgl II-digested PCR amplification product of the TETr cDNA in pUHD15-1 (13) with primers 5'-GCCCCCAATTTGAATTTGGCTCGAGGATCTCTATCAGTAAGGAGGAGGCC-3' and 5'-CCCGTGAAGTCTGACTGTGTTGCT CTGAGGACCC-3' was ligated into EcoR1/BamH1-digested pSG5 (Stratagene) to create pSG5-TETr. The TETr–RB plasmids were made by standard techniques. Details are available upon request.

Antibodies. Anti-HA antibody 12CA5 was from Boehringer Mannheim. Anti-CD19 antibody B4 was provided by John Gribben (Dana–Farber Cancer Institute). N9 is a polyclonal anti-RB antisemur (14).

Fluorescence-Activated Cell Sorting (FACS). The cell cycle distribution of CD19+ cells was determined by propidium iodide staining followed by FACS (4). Cells were analyzed 48 hr (U-2OS) or 72 hr (Saos-2) after transfection.

Other Methods. β-Galactosidase and luciferase were assayed as described (4). In each transfection, 2 µg of a cytomegalovirus promoter–β-galactosidase expression plasmid was included. Luciferase values were normalized for β-galactosidase activity and expressed relative to the activity observed for the indicated reporters in the presence of backbone.
expression plasmid only. Immunoprecipitation, deoxocholate release, and gel shift experiments were performed as described (11). Immunoprecipitation was carried out with 1.5 μl of N9 anti-RB antiserum. E2F DNA binding activity was detected by gel shift assay using 2 ng of a 32P-labeled probe [E2F1 promoter E2F site B (5)].

RESULTS

We constructed full-length expression plasmids encoding chimeric proteins containing E2F1-(1-368) fused to various fragments of RB (Fig. 1B). In this manner we hoped to target fragments of RB to E2F DNA-binding sites (Fig. 1A) so that we could ask whether RB contained a domain capable of actively repressing transcription from known E2F-responsive promoters. We assumed that such a domain (if one existed) was located between RB residues 379 and 928, since this region is sufficient for repression of E2F activity in cultured cells and for the suppression of cell growth (16, 17). Each of the RB fragments tested here as E2F/RB chimeras, however, is unable to bind stably to an E2F–DNA complex or to arrest cell growth (16, 18). In particular, RB-(379–792) is defective for these functions (16). In parallel, we made plasmids encoding E2F1-(1–368) fused to the Kox1 transcriptional repression domain (15) (Fig. 1). Following transient transfection, each of these expression plasmids gave rise to a stable protein of the anticipated size in Western blot analyses, although the E2F1/Kox1 chimeras were reproducibly produced at higher levels than the E2F1/RB chimeras (Fig. 5D and data not shown).

In the first set of experiments we transfected asynchronous CV1-P (RB+/+) cells with a reporter plasmid in which the E2F1 promoter was cloned upstream of a luciferase cDNA (5). The E2F1 promoter contains four E2F sites that act as transcriptional repressor elements in some cancer cells (5, 6). The E2F1 promoter–luciferase reporter was profoundly repressed by either the E2F1-(1–368)/RB-(379–792) or the E2F1-(1–368)/Kox1 expression plasmid (Fig. 2B), whereas DNA-binding-defective point mutant derivatives thereof had no apparent effect. As anticipated, a subtle mutation in the Kox1 domain known to interfere with its ability to repress transcription (15) inactivated the E2F1-(1–368)/Kox1 chimeras in these assays. Likewise, the introduction of a naturally occurring RB mutation [deletion of exon 22 (Δ22; ref. 16)] gave rise to an E2F1/RB chimera that was inactive in these assays although it retained, as predicted, the ability to bind to E2F

![Fig. 1.](image)

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![Fig. 2.](image)

DNA-binding sites (data not shown). Complementation experiments performed to date suggest that the E2F and RB functions being assayed here cannot be provided in trans (data not shown). In particular, we have not observed transcriptional repression following cotransfection of the plasmids encoding E2F1-(1–368)/RB-(379–792) and E2F1-(1–368)/RB-(379–928;Δ21). Note that an intact E2F1-(1–368) moiety was necessary but not sufficient for transcriptional repression in these assays. Thus, the effects observed appear to reflect more than competition between the chimeras and endogenous E2F for binding to the reporters. Indeed, elimination of the E2F sites in the E2F1 promoter led to an ~5-fold increase, rather than a decrease, in basal activity, in keeping with earlier studies (5, 6) (Fig. 2A). E2F1-(1–368)/RB-(379–792), but not E2F1-(1–368)/RB-(379–792), also repressed transcription when the DNA polymerase α (19), B-myb (7), and DHFR (20) promoters were similarly assayed (Fig. 2C). E2F1-(1–368)/RB-(379–792) appeared to be as potent, and in some cases more potent, than E2F1-(1–368)/Kox1 when the two were scored at various concentrations for their ability to repress transcription from a panel of E2F-responsive promoters (Fig. 2D and data not shown).

pRB-(379–792) (the pRB pocket) contains the minimal fragment of pRB protein required for binding to viral oncoproteins such as T and E1A (1). That the E2F1-(1–368)/RB-(379–928) chimera with a pocket mutation [deletion of exon 22 (Δ22)] failed to repress the E2F1 promoter suggested that repression might be linked to the integrity of this region. We therefore tested three additional, naturally occurring RB
pocket mutations [61W, 706F, and deletion of exon 21(A21)] (18, 21) (Fig. 3A). All of the pocket mutations, whether in the context of RB-(379–792) or RB-(379–928), led to a marked loss of transcriptional repression function relative to E2F1-(1–368)/RB-(379–792). Thus, the integrity of the pocket appeared to be essential for transcriptional repression by RB.

To test this further, we asked whether viral oncoproteins that bind to the pocket could block the ability of E2F1-(1–368)/RB-(379–792) to repress the E2F1 promoter. Coproduction of wild-type E7 or T blocked the ability of E2F1-(1–368)/RB-(379–792) to repress transcription, whereas RB-binding-defective E7 and T mutants had no effect (Fig. 3B and data not shown). E7 had no discernible effect on the ability of E2F1-(1–368)/Kox1 to repress transcription (Fig. 3B). Thus, pocket-binding viral oncoproteins, as well as naturally occurring pocket mutations, led to a decrease in RB-mediated transcriptional repression.

The ability of E2F1-(1–368)/RB-(379–792) to repress transcription left open the possibility that E2F1-(1–368), in addition to providing DNA-binding activity, also contained residues essential for RB-dependent transcriptional repression. To address this, we replaced the four E2F sites in the E2F1 promoter with two tandem tetracycline repressor binding sites (TETo) (13) while maintaining the distance between the Sp1 sites and the transcription start site. Cotransfection of this TETo-containing promoter (pGL2ANATeTo) fused to luciferase with an expression plasmid encoding the DNA-binding region of the tetracycline repressor (TETr) fused to the E2F1 transactivation domain led to an increase in promoter activity (data not shown). Conversely, TETr fused to RB-(379–928), but not to the pocket mutant RB-(379–928;Δ21) or RB-(379–928;Δ22), led to marked repression (Fig. 4A). TETr/RB-(379–792), TETr/RB-(379–928;Δaa803–806), and TETr/RB-(379–928;Δaa785–806), all of which retain pocket function but lack residues critical for stable binding to E2F–DNA complexes (1), also repressed transcription. The ability of these chimeras to bind to E2F was tested directly by a sensitive gel shift assay (Fig. 4B). Cells were transfected with mammalian expression plasmids encoding the TETr/RB/chimeras described above, radiolabeled with [35S]methionine, lysed, and immunoprecipitated with a polyclonal anti-RB antiserum (N9). The recovery of each protein was confirmed by autoradiography (Fig. 4C) and Western blot analysis (data not shown). Associated proteins were released with deoxycholate and assayed for E2F activity in gel shift experiments. As expected, E2F activity com Immunoprecipitated with TETr/RB-(379–928) whereas no E2F activity was detected in association with TETr/RB-(379–792) (Fig. 4B). TETr binding to TETr/RB-(379–928;Δaa803–806) and TETr/RB-(379–928;Δaa785–806) was diminished relative to TETr/RB-(379–928) but was still detectable by this approach. Thus, E2F binding would not appear to underlie the ability of RB to repress transcription when fused to TETr.

These results suggest that RB-(379–792) contains a transcriptional repression domain which can function when fused to a heterologous DNA-binding domain unrelated to E2F. This property may be shared by other pocket proteins as well, as a TETr/p130 chimera containing the p130 region homologous to the RB pocket could likewise repress transcription (data not shown).

The reagents allowed us to ask whether repression of E2F-responsive promoters is sufficient to account for the ability of RB to induce a G1/S arrest. As expected, wild-type RB led to a significant increase in the percentage of cells in G1 when reintroduced into Saos-2 (RB–/–) cells (Fig. 5A). RB(A22) and RB-(379–792), in contrast, were enfeebled in this regard, in keeping with earlier studies (16). E2F1-(1–368)/RB-(379–792) did, however, induce a G1/S block comparable to wild-type RB, whereas the DNA-binding-defective [E2F1-(1–368;132)/RB-(379–792)] and pocket function-defective [E2F1-(1–368)/RB-(379–928;Δ22)] chimera did not. Thus, targeting RB-(379–792) to E2F DNA-binding sites appeared to
restore its ability to suppress cell growth. In contrast, E2F1-(1–368)/Kox1, a potent transcriptional repressor when tested in these cells, failed to induce a G1/S block.

U-2 OS (RB+/−) osteosarcoma cells do not produce detectable amounts of the cyclin-dependent kinase inhibitor p16/MTS1/INK4A (22) and become blocked at the G1/S boundary following its reintroduction (22, 23). The loss of p16 in these cells, and consequent deregulation of one or more cyclin-dependent RB kinases, may account for the earlier observation that these cells contain exclusively phosphorylated pRB and appear to be inured to the effects of wild-type RB (16). The E2F1-(1–368)/RB(379–792) chimera, however, lacks the RB phosphorylation sites required for inhibition of RB growth-suppression function and, perhaps as a result, induced a G1/S block in U-2 OS cells in a DNA-binding and RB-pocket-dependent manner (Fig. 5B). Overproduction of wild-type RB, as expected, did not induce a G1/S block (data not shown), whereas a nonphosphorylatable and, hence, constitutively active RB mutant (24) did (Fig. 5B). The E2F1/Kox1 chimera did not efficiently induce a G1/S block, in keeping with the results obtained in Saos-2 cells. Note that an intact E2F1-(1–368) moiety was not sufficient to induce a G1/S block in either cell type, even though this fragment of E2F1 can, as predicted, block E2F-dependent transactivation (see Fig. 5C).

**DISCUSSION**

RB-(379–792) lacks the ability to bind stably to E2F–DNA complexes and cannot induce a G1/S block (16). We demonstrate here that restoring the ability of RB-(379–792) to bind to E2F-responsive promoters, as an E2F/RB chimera, likewise restores its ability to induce a G1/S block. Further, the ability of such E2F/RB chimeras to induce a G1/S block correlated with their ability to bind to DNA and to actively repress transcription. In particular, an E2F1 mutant that blocked E2F-dependent activation but could not actively repress transcription failed to induce a G1/S block. These results provide direct evidence that the induction of a G1/S block by RB is due, at least in part (see below), to active transcriptional repression by RB/ E2F complexes bound to E2F-responsive promoters. In contrast, neutralization or “sequestration” of E2F by RB would not appear to account for the ability of pRB to induce a G1/S block.

Others have shown that GAL4/RB chimeras can actively repress transcription from artificial reporters containing tandem GAL4 binding sites in a RB pocket-dependent manner (25–27). Our results extend these observations by showing that active transcriptional repression by RB-(379–792) can be measured in the context of naturally occurring E2F-responsive promoters. Further, using a sensitive gel shift assay, we provide data which strongly suggest that residual E2F binding activity does not underlie the ability of RB-(379–792) to actively repress transcription from E2F-responsive promoters. Thus, DNA binding (via E2F) and transcriptional repression by RB appear to be dissociable functions. Finally, and perhaps most important, our results link this activity to the ability of RB to arrest cell growth.

At a minimum, RB, as an RB–E2F complex, would appear to act as a transcriptional repressor in the course of regulating certain E2F-responsive promoters. In this regard, many E2F-responsive promoters share certain features, including Sp1 and/or ATF sites in proximity to their E2F site(s) (which, in turn, are frequently located near the transcription start site) and the lack of a recognizable TATA box (28). It is possible that this organization in some way facilitates the action of RB–E2F complexes upon adjacent transcription factors. RB is in vast excess of E2F in mammalian cells and can bind, at least in vitro, to other transcription factors. Thus, RB might also serve to repress certain non-E2F-dependent promoters, although this remains to be proven.

How does RB repress transcription? Our genetic analysis, coupled with the ability of viral oncoproteins to block the action of the E2F/RB chimera, suggests that this function is linked to the integrity of pRB residues 379–792 (the RB pocket). The pocket is necessary, but not sufficient for stable binding to E2F–DNA complexes and for growth suppression (1). Nonetheless, the pocket will, when produced in bacteria and present in very high concentration, bind to E2F in solution and in filter binding assays (11). Thus, it at first seemed possible that the TETr/- and E2F/RB-(379–792) chimeras bound to a cellular E2F, thus reconstituting a DNA-bound RB–E2F complex. If so, this would not have undermined the conclusions that (i) pRB–E2F complexes actively repress transcription in a DNA-binding-dependent manner and (ii) this activity, may account, at least in part, for the ability of RB to induce a G1/S block. To date, however, we have been unable to detect a stable interaction between E2F and RB-(379–792) when the latter is produced in mammalian cells. Furthermore, the ability of RB mutants to repress transcription in our assays did not parallel their ability to bind to E2F. Thus, as stated
tumors, either genetically or epigenetically, would be predicted to lead to both a loss of RB–E2F repressor complexes and to an increase in free, transcriptionally active E2F. Small molecules that inhibit the ability of free E2F to transactivate yet fail to lead to repression of E2F-responsive promoters may fail to inhibit cell growth. Furthermore, small molecules which directly or indirectly target the ability of E2F to bind to DNA may actually lead to a loss of cell cycle control in RB+/− cells by inhibiting the ability of RB-E2F complexes to bind to E2F-responsive promoters.

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