Identification of a growth suppression domain within the retinoblastoma gene product

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To date, all naturally occurring retinoblastoma susceptibility gene (RB) mutations known to be compatible with stable protein expression map to the T/E1A and cellular protein-binding region (the "pocket" domain). This domain extends from residue 379 to 792. When full-length RB and certain truncated forms were synthesized in human RB $-/-$ cells, we found that the minimal region necessary for overt growth suppression extended from residue 379 to 928. A functional pocket domain and sequences extending from the carboxy-terminal boundary of the pocket to the carboxyl terminus of the protein were both necessary for growth suppression. Both sets of sequences were also required for E2F binding; hence, the two functions may be linked.

[*Key Words*: RB; T/E1A-binding domain; growth suppression; E2F; phosphorylation]

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Genetic analyses have shown that inactivation of a cellular gene, RB-1, is regularly linked to human retinal tumorigenesis (Knudson 1971; Benedict et al. 1983; Cavenee et al. 1983; Godbout et al. 1983; Sparkes et al. 1983; Friend et al. 1986; Fung et al. 1987; Lee et al. 1987a). RB-1 has therefore been termed a tumor suppressor gene. In keeping with this designation, reintroduction of the wild-type retinoblastoma susceptibility gene (RB) allele into tumor cells lacking RB function typically leads to growth suppression, for example, as manifested by failure to replicate on a plastic surface (Huang et al. 1988; Bookstein et al. 1990; Takahashi et al. 1991; J. DeCaprio and S. Friend, unpubl.).

The RB gene product undergoes cell cycle-dependent phosphorylation and dephosphorylation (Buchkovich et al. 1989; Chen et al. 1989; DeCaprio et al. 1989; Mihara et al. 1989; Xu et al. 1989; Ludlow et al. 1990). These findings, and the preferential binding of the SV40-transforming protein, T, to unphosphorylated RB (Ludlow et al. 1989, 1990), suggest that unphosphorylated RB may have certain growth-regulating functions, which phosphorylated RB lacks, and that certain aspects of RB growth suppression function may be regulated by phosphorylation.

The minimal T/E1A-binding region of RB has been mapped to a segment spanning residues 379–792 (Hu et al. 1990; Huang et al. 1990; Kaelin et al. 1990). This segment of the protein sequence is sufficient for binding to certain cellular proteins in vitro (Kaelin et al. 1991). The suspicion that RB binding to cellular proteins constitutes at least part of the mechanism by which RB op-

erates in promoting growth control is underscored by the fact that naturally occurring, loss-of-function RB mutations, which do not destabilize the protein, regularly map to the T/E1A-binding region (the RB "pocket") and eliminate its known in vitro protein-binding function (Horowitz et al. 1989, 1990; Bookstein et al. 1990; Kaye et al. 1990; Shew et al. 1990a,b; Huang et al. 1991; Kaelin et al. 1991; Scheffner et al. 1991). Recent evidence suggests that the transcription factor E2F may form a complex with RB and that the pocket may play a major role in this process (Bagchi et al. 1991; Bandara and La Thangue 1991; Chellappan et al. 1991; Chittenden et al. 1991). This suggests that RB may function, at least in part, by regulating gene expression.

All of this notwithstanding, the suspicion that RB operates as a growth regulator by interacting with other proteins is based on circumstantial evidence obtained largely by in vitro experimental analyses or characterization of tumor-derived RB alleles. To provide a direct test of this hypothesis, we have performed experiments aimed at determining whether RB-mediated growth suppression is dependent on the function of its pocket, a known protein-binding domain.

Results

Expression of RB polypeptides in vivo

The RB segment extending from residue 379 to the natural carboxyl terminus (928) is active in binding T and a series of defined cellular proteins, as shown by in vitro assays (Hu et al. 1990; Huang et al. 1990, 1991; Kaelin et

Figure 1. In vivo synthesis of truncated RB polypeptides as determined by Western blot analysis. (A) Analysis of cell lysates prepared from U2OS cells transfected with pCMV-RB(379–928), pCMV-RB(379–928, 706 C → F), pCMV-RB, and pCMV-Neo-Bam (lanes 1–4, respectively), or SAOS2 cells transfected with the same set of plasmids (lanes 5–8, respectively) (7.5% SDS-polyacrylamide gel). (B) Analysis of cell lysates prepared from U2OS cells transfected with pCMV-RB(379–792), pCMV-RB(379–792, 706 C → F), and pCMV-RB(379–928) (lanes 1,2,5, respectively), or SAOS2 cells transfected with pCMV-RB(379–792, 706 C → F) and pCMV-RB(379–792) (lanes 3,4, respectively) (10% SDS-polyacrylamide gel). Filters were probed with an anti-RB antibody (XZ91), and antibody localization was determined colorimetrically.

al. 1990, 1991; Chittenden et al. 1991; Ewen et al. 1992). The minimal pocket domain, RB(379–792), is an internal segment of this RB fragment and is sufficient for T and cellular protein–RB complex formation in vitro (Kaelin et al. 1990, 1991). In preparation for the first set of experiments, RB(379–928) and a mutant derivative RB(379–928, 706 C → F), the minimal pocket domain (379–792) and its corresponding mutant derivative RB(379–792, 706 C → F), and full-length RB, were subcloned into a eukaryotic expression vector, pCMV-Neo-Bam (Baker et al. 1990). After transfection into two human osteosarcoma cell lines, U2OS (RB +/+) and SAOS2 (RB –/–) (Lee et al. 1987b; Shew et al. 1990b), each plasmid gave rise to the synthesis of the expected RB product (Fig. 1A,B), as measured in a transient expression assay. In U2OS cells, RB and Rb(379–928) appeared as a collection of differentially migrating SDS-gel bands (Fig. 1A, lanes 3 and 1, respectively). In contrast, only one major band of each species, that is, the fastest migrating one, was detected in SAOS2 cells (Fig. 1A, lanes 7 and 5, respectively). Unlike RB(379–928) and full-length RB, the products of both the wild-type and mutant minimal pocket domain-encoding plasmids [RB(379–792) and RB(379–792, 706 C → F), respectively] migrated largely as single bands after extraction from both cell lines (Fig. 1B, lanes 1–4; see Fig. 3B, lane 2, below).

Phosphorylation of RB derivatives expressed in RB +/+ and RB –/– cell lines

To probe the nature of the differentially migrating RB species, immunoprecipitation and Western blot analyses were performed after parallel [32 P]orthophosphate (Fig. 2A,B) and [35 S]methionine (Fig. 2C) labeling of tran-

Figure 2. 32 P-labeling and phosphatase assay. U2OS cells were metabolically labeled with either [32 P]orthophosphate (A,B) or [35 S]methionine (C). After immunoprecipitation with an anti-RB antibody (XZ91), protein A–Sepharose-bound proteins were resolved in SDS-polyacrylamide gels, transferred to PVDF membranes, and detected either by immunoblotting with XZ91 (A) or by autoradiography after blotting (B,C). (A) (Lane 1) U2OS cells transfected with pCMV-Neo-Bam alone; (lane 2) pCMV-RB(379–792); (lane 3) pCMV-RB(379–928, 706 C → F); (lane 4) pCMV-RB(379–928); and (lane 5) pCMV-RB. (B) Autoradiograph of the blot described in A. The film was exposed for 20 min without an intensifying screen. (C) Phosphatase assay. Aliquots of a cell lysate prepared from U2OS cells transfected with pCMV-RB(379–928) were immunoprecipitated with XZ91. Alkaline phosphatase treatment was performed in the presence or absence of phosphatase inhibitors (lanes 1,2, respectively). (Lane 3) A control in which no enzyme was added. The heavy, ~48- to 52-kD triplet noted in A is immunoglobulin heavy chain.

siently transfected U2OS cells. The results indicate that the slower, and not the fastest, migrating RB(379–928) and full-length RB species were apparently phosphorylated (cf. Fig. 2A and B). In contrast, as expected from an earlier study with full-length RB (Kaye et al. 1990), there was no apparent phosphorylation of RB(379–928, 706 C → F), which migrated as a single band in these gels (Fig. 1A, lane 2; Fig. 2A, lane 3, and data not shown). Moreover, there was also no apparent phosphorylation of the RB(379–792) species in U2OS cells, as shown in Figure 2, A and B (lane 2 in each case).

The ^{32}P -labeling experiment did not exclude the possibility that additional forms of post-translational modification contributed to the appearance of the slower migrating RB species. Therefore, the truncated RB species extending from residue 379 to 928 was subjected to exogenous phosphatase treatment in an effort to determine whether phosphate removal would eliminate any of the slower migrating bands observed in Figures 1 and 2. As shown in Figure 2C, such treatment specifically eliminated the slower migrating species. As demonstrated previously (Ludlow et al. 1989), phosphorylation is the basis for heterogenous migration of the full-length protein. The data shown in Figure 2C indicate that the same is true for the truncated RB product. It should also be noted that all newly synthesized RB species detected in SAOS2 cells appeared to be unphosphorylated, because they migrated as single bands (Fig. 1A, lanes 5–7), which comigrated with the apparently unphosphorylated RB species observed in U2OS cells. They also failed to label with ^{32}P , and their migration was not affected by phosphatase treatment (data not shown). Similar results were noted in two other RB $^{-/-}$ cell lines—the human osteosarcoma cell line, OHS50-T (Diller et al. 1990), and the human breast carcinoma cell line, MDA-MB-468

(Lee et al. 1988; T'Ang et al. 1988; Horowitz et al. 1990, and data not shown). We conclude from these findings that none of these three RB $^{-/-}$ cell lines efficiently phosphorylated the newly synthesized RB derivatives described here.

All RB species containing an intact pocket and synthesized in vivo bind to SV40 T

To determine whether these various species, synthesized in vivo, could bind specifically to T, cell lysates were prepared from transiently transfected, [^{35}S]methionine-labeled SAOS2 cells (Fig. 3A). Identical amounts of each lysate were mixed with aliquots of a lysate of MEF Ψ T cells (Ewen et al. 1989; Chen and Paucha 1990), a murine cell line that overproduces intact SV40 T. Immunoprecipitation with PAb419 (Harlow et al. 1981), an anti-T monoclonal antibody, showed that full-length RB, RB(379–928), and RB(379–792) all coprecipitated with T (Fig. 3A, lanes 3, 5, and 1, respectively). In another experiment, none of the products of transfection noted above were found to coprecipitate when incubated with lysates of the mouse cell lines A31 and MEF Ψ K1 (data not shown). The former cell line produces no T, and the latter produces a mutant T species (K1T) that can neither transform nor bind to RB (Kalderson and Smith 1984; DeCaprio et al. 1988; Chen and Paucha 1990). Finally, where tested, the 706 C → F derivatives of these truncated RB species also failed to bind to T (data not shown), in keeping with past results (Kaye et al. 1990; Kaelin et al. 1991). Therefore, binding of T to RB depended on the integrity of the RB pocket. Similar results were obtained using the cell lines U2OS and OHS50-T (data not shown). Further immunoprecipitation and Western blot analysis using U2OS cells, in which overt RB phosphorylation

Figure 3. T binding. (A) [^{35}S]Methionine-labeled lysates prepared from various SAOS2 transfectants were incubated in the presence (lanes 1,3,5,7) or absence (lanes 2,4,6,8) of aliquots of unlabeled T-containing cell lysate and immunoprecipitated with a monoclonal antibody directed against T (PAb419). (Lanes 1,2) pCMV–RB(379–792); (lanes 3,4) pCMV–RB; (lanes 5,6) pCMV–RB(379–928); (lanes 7,8) pCMV–Neo–Bam. (B) T binds only unphosphorylated RB(379–928). [^{35}S]Methionine-labeled lysates, prepared from U2OS cells transfected with pCMV–RB(379–792) (lanes 1,2) and pCMV–RB(379–928) (lanes 3,4) were immunoprecipitated with PAb419 in the presence of T, as described above (lanes 1,3), or immunoprecipitated with XZ91 (lanes 2,4). Bound proteins were resolved in 7.5% polyacrylamide gels and detected by autoradiography.

could be readily detected, indicated that T bound only the unphosphorylated species of RB(379–928) (Fig. 3B, cf. lanes 3 and 4). The preference of T for unphosphorylated RB has been described previously for full-length RB (Ludlow et al. 1989, 1990). Taken together, these results indicate that after de novo synthesis, the specifically truncated RB species possessing an intact pocket can serve as specific targets for T binding. Furthermore, T can discriminate between the phosphorylated and unphosphorylated forms of either RB or RB(379–928), implying that the latter retains those sequences needed to render RB immune to T binding after phosphorylation.

Nuclear localization of RB and its truncated derivatives

Full-length RB is a nuclear protein whose growth suppressor functions may require interactions with nuclear elements such as certain transcription factors and nuclear tethering proteins not yet identified (Mittnacht and Weinberg 1991; Templeton et al. 1991). To determine the intracellular location of the truncated RB polypeptides after in vivo expression, immunofluorescence was performed with an anti-RB monoclonal antibody (XZ91 or XZ56). All of the wild-type and mutant RB derivatives described above were found to be primarily nuclear in location (Fig. 4).

A truncated RB polypeptide containing residues 379–928 can suppress cell growth

To determine whether one or more of the various wild-type and mutant RB alleles described above could suppress the growth of a series of RB $+/+$ and RB $-/-$ cell lines, we used an assay described earlier by Baker et al. (1990) to measure growth suppression by p53 alleles. Here, the question was whether various RB cDNAs, when transfected in the presence of a neomycin resistance gene, would suppress the ability of recipient cells to form macroscopic, G418-resistant colonies. In the first group of growth-suppression assays, we transfected four plasmids: pCMV(Neo–Bam), the vector alone; pCMV–RB; pCMV–RB(379–928); and pCMV–RB(379–928, 706 C $\rightarrow$ F) into SAOS2 and U2OS cells. In the RB-containing plasmids, the cDNA-coding unit was linked to a cytomegalovirus (CMV) promoter. As shown in Figure 5A, dramatic suppression of G418^r colony formation was observed in SAOS2 cells transfected by plasmids encoding either full-length RB or RB(379–928). No significant inhibition was noted with the 706 C $\rightarrow$ F derivative of the latter. Furthermore, none of the relatively few colonies observed among the wild-type RB(379–928) transfectants gave rise to stable cell lines, whereas the colonies obtained with the point mutant derivative did so readily (data not shown). Finally, cells transfected with pCMV–RB or pCMV–RB(379–928), but not pCMV–RB(379–928, 706 C $\rightarrow$ F), repeatedly exhibited an increase in size, a phenotype suggestive of possible cell cycle arrest (Fig. 6, and data not shown). Pulse-chase experiments indicated that there was no significant dif-

ference in the half-lives of the wild-type and the 706 C $\rightarrow$ F mutant derivative of RB(379–928) studied here (data not shown). Therefore, stability differences do not likely explain the observed differences in biological activity. To eliminate the possibility that these gross differences in growth inhibition were the result of more wild-type than mutant RB(379–928) being present in these cells after transfection, the experiment was repeated under conditions where similar amounts of the two proteins were present in transfected SAOS2 cells, as detected by Western blotting. Again, the wild-type RB(379–928)-encoding plasmid suppressed colony formation, and the mutant RB(379–928, 706 C $\rightarrow$ F) did not (data not shown).

The growth suppression assay was repeated using a plasmid containing a full-length RB cDNA encoding an RB protein with a pocket bearing a deletion that results from the loss of exon 22 (Horowitz et al. 1990; Kaelin et al. 1991). In keeping with the work of others (Horowitz et al. 1990; Templeton et al. 1991), this plasmid directed the synthesis of an apparently unphosphorylated nuclear protein as determined by Western blotting, orthophosphate labeling, and immunofluorescence (Fig. 4H, and data not shown). No growth inhibition of SAOS2 was observed (data not shown), despite levels of exogenous RB expression equaling that seen with the RB(379–928)-encoding plasmid (data not shown). Because previous results indicated that mutations in the pocket region inactivate its protein-binding function (Kaye et al. 1990; Chittenden et al. 1991; Kaelin et al. 1991), these data imply that this particular function of the pocket is linked to its growth-inhibiting action.

Analogous results were obtained with full-length RB, RB(379–928), and its 706 C $\rightarrow$ F derivative in OHS50-T cells (RB $-/-$) (data not shown). Unlike SAOS2 cells, in which an RB protein truncated at the carboxyl terminus can be detected (Shew et al. 1990b), this latter cell line produces no detectable RB protein (data not shown, and S. Friend, pers. comm.). Thus, the growth suppression observed in SAOS2 cells is not likely the result of complementation between the newly introduced, truncated RB species and the endogenous mutant RB protein.

Similar transfections were performed in U2OS cells (an RB $+/+$ osteogenic sarcoma line) with pCMV(Neo–Bam), pCMV–RB, pCMV–RB(379–928), and pCMV–RB(379–928, 706 C $\rightarrow$ F). In contrast to the results described above, none of the four plasmids transfected under conditions identical to those used in the SAOS2 experiments suppressed colony formation (data not shown).

The minimal RB T/E1A-binding domain is not sufficient to suppress cell growth

We then asked whether the pocket alone (RB residues 379–792) could suppress the growth of an RB $-/-$ cell line. To this end, plasmids encoding wild-type and the 706 C $\rightarrow$ F mutant derivative of the minimal pocket domain (379–792) were transfected into SAOS2 cells along with a plasmid encoding the full-length RB protein. As

Figure 4. Immunofluorescence micrographs of transfected SAOS2 cells doubly stained with DAPI for DNA (A,C,E,G,I,K) and with an antibody to RB (B,D,F,H,J,L). (A,B) SAOS2 cells transfected with pCMV-RB(379–928); (C,D) pCMV-RB(379–928,706 C → F); (E,F) pCMV-RB(379–792); (G,H) pCMV-RB(d122); (I,J) pCMV-RB(773–928); (K,L) pCMV-RB(379–817).

shown in Figure 5B, the minimal pocket was inactive in this assay, unlike its wild-type, full-length RB counterpart. The same was true in OSH50-T cells (data not

shown). This difference cannot be ascribed to failure to produce enough nuclear RB(379–792), because, as shown earlier (Fig. 1B), the relevant plasmids led to the synthe-

Figure 5. Growth assay of transfected SAOS2 cells. (A,B) Cells were transfected with 30 μ g of the indicated plasmids and grown in the presence of G418 for ~2 weeks. G418^r colonies were then stained with crystal violet.

sis of comparable amounts of RB(379–792) and RB(379–928) and both of the proteins were similarly concentrated in the nucleus as determined by immunofluorescence (Fig. 4F and B, respectively). To exclude the possibility that the addition of a few residues carboxy-terminal to the RB pocket would enable the T/E1A-bind-

ing domain to suppress cell growth in this assay or that the carboxyl terminus alone is sufficient to suppress cell growth, we repeated the above experiments with pCMV plasmids encoding RB residues 379–816 and 773–928. Both plasmids directed the synthesis of apparently unphosphorylated nuclear proteins, as determined by West-

Figure 6. Phase-contrast micrographs (340 $\times$) 12 days after G418 selection showing morphologies of SAOS2 cells transfected with the indicated plasmids.

ern blot and immunofluorescence analysis, and were expressed at levels equal to or exceeding those observed with RB(379–928) (Fig. 4L and J, respectively, and data not shown). In the case of RB(379–816), the absence of phosphorylation was confirmed in orthophosphate-labeling experiments (data not shown). As was demonstrated with RB(379–792), no evidence of growth suppression in SAOS2 cells was observed for either RB(379–816) or RB(773–928) (data not shown). Cotransfection of plasmids encoding RB(379–928, 706 C → F) and RB(379–792), at varying molar ratios, likewise failed to lead to growth suppression in this assay (data not shown). This suggests that the pocket and the carboxyl terminus may not operate in *trans*.

In vitro binding of truncated RB proteins to E2F

The failure of RB(379–792) and RB(379–816) to suppress colony formation in the transfection assays described above suggests that carboxy-terminal residues outside of the minimal pocket region (i.e., residues 816–928) may contribute an important component of RB growth suppression function. As RB interacts with a transcription factor, E2F, thought to play a role in regulating cell growth, we asked whether the ability of the RB derivatives, described above, to suppress cell growth might correlate with their ability to bind to E2F *in vitro*. Gel-retardation assays were performed using nuclear extracts prepared from an RB $-/-$ cell line (SAOS2) and a 32 P-labeled oligonucleotide probe corresponding to a known E2F-binding site within the dihydrofolate reductase (DHFR) promoter (Hiebert et al. 1991). Approximately equal amounts of bacterially produced glutathione S-transferase (GST)–RB fusion proteins, containing RB residues 379–792; 379–816; 379–928; 379–928, 706 C → F; RB(dl22) (deletion of exon 22); or 773–928, were added to the reaction mixtures, and protein–DNA complexes were then electrophoresed in a nondenaturing polyacrylamide gel (Fig. 7). The complex indicated by the arrow in Figure 7 was shown previously to be “free” E2F (Bagchi et al. 1990; Shirodkar et al. 1992). Addition of GST–RB(379–928) resulted in a quantitative supershift of the free E2F species to yield a new complex of slower mobility (Fig. 7), indicating that the RB(379–928) segment can bind E2F efficiently in this assay. In contrast, other truncated or mutated GST–RB fusion proteins either failed to alter the mobility of E2F or did so less efficiently, suggesting that these RB species do not form stable complexes with E2F. It should also be noted that a longer exposure of the experiment in Figure 7 revealed a faint supershifted E2F band with GST–RB(379–792). Additional *in vitro*-binding assays, in which GST–RB(379–792) and GST–RB(379–928) were assayed for E2F-binding activity, demonstrated levels of E2F binding to the former that were significant but ~10-fold lower than that observed for the latter (data not shown).

The results of these binding assays (summarized in Fig. 8) suggest that part or all of the 792–928 segment of RB is required for efficient *in vitro* association with the transcription factor E2F. However, the carboxy-terminal

Figure 7. Binding of RB fusion proteins to E2F, as detected by gel retardation analysis. DNA-binding reactions contained nuclear extract prepared from SAOS2 cells and a 32 P-labeled oligonucleotide probe encoding the E2F DNA-binding site within the DHFR promoter. Specificity of the complexes was demonstrated by the addition of a 100-fold molar excess of unlabeled competitor oligonucleotide DNA representing either the wild-type (WT) or mutant (MUT) DHFR E2F-binding site. (Lane M) A control binding reaction with a previously characterized nuclear extract prepared from U937 (human) cells, which serves as a mobility marker for E2F complexes. The arrow indicates the position of a band corresponding to free E2F; the asterisk (*) denotes a nonspecific band. Purified, bacterially expressed GST or the indicated GST–RB fusion protein species was added to the DNA-binding reactions. Protein–DNA complexes were resolved by electrophoresis on 4% polyacrylamide gels. (Lane CTL) A control binding reaction containing GST–RB(379–928) and the probe without any SAOS2 nuclear extract.

RB segment, alone, is not sufficient for the interaction with E2F because GST–RB(773–928) failed to bind E2F in this assay (Fig. 7). In addition, consistent with previous findings (Bandara and La Thangue 1991; Chittenden et al. 1991; Hiebert et al. 1992), the integrity of the RB pocket is also required for efficient E2F binding, because GST–RB proteins harboring mutations within the pocket domain, RB(379–928, 706 C → F) and RB(dl22), were defective for binding E2F (Figs. 7 and 8). Therefore, the ability to bind E2F efficiently *in vitro* correlates, at least in the cases examined here, with the ability to suppress cell growth *in vivo* (see Fig. 8).

Discussion

These findings indicate that more than the minimal

Figure 8. Summary of growth suppression, phosphorylation, T binding, and E2F binding data.

pocket domain is needed for RB-mediated growth suppression in cells that lack RB function. The additional sequences lie at the carboxyl terminus of the protein and are not essential for the binding of T, E1A, and most of the cellular proteins that have been identified as pocket-binding elements to date (Hu et al. 1990; Huang et al. 1990; Kaelin et al. 1991). However, we must add that the *in vitro* assays performed thus far (Kaelin et al. 1991) may have failed to detect physiologically important differences in the ability of one or more cellular proteins to bind to the minimal pocket domain and to this domain linked to a normal carboxy-terminal segment. In this regard, the gel-shift assays described above suggest that the transcription factor E2F binds less efficiently to RB(379–792) than to RB(379–928). Similar results have been obtained by others (Hiebert et al. 1992). E2F is strongly suspected of regulating the G_1 exit process (Mudryj et al. 1990; Shirodkar et al. 1992), and indirect evidence suggests that RB may modulate its function (Hiebert et al. 1992). Thus, given the correlation between E2F binding and growth suppression, one might hypothesize that the ability of the RB polypeptides studied here to suppress cell growth is the result of their ability to bind to and modulate E2F *in vivo*. It is also possible that the carboxyl terminus provides a function essential to RB growth suppression that is unrelated to E2F binding and separate from that of the pocket.

The data also indicate that sequences outside of the minimal pocket domain are essential for overt RB phos-

phorylation. This observation is consistent with earlier work by Hamel et al. (1990). Yet, it is obvious that lack of phosphorylation is not the sole basis for the absence of minimal pocket growth suppression, for the un(der)phosphorylated RB species is the predominant one observed in the RB $-/-$ cells transfected with (and growth arrested by) either full-length RB or RB(379–928). On the other hand, it is currently not possible to rule out the hypothesis that cell cycle-dependent phosphorylation contributes to an orderly expression of RB functions over time. In this regard, it is noteworthy that to date, all spontaneously occurring, loss-of-function RB mutants, which encode stable proteins, are hypophosphorylated *in vivo* (Horowitz et al. 1989, 1990; Bookstein et al. 1990; Kaye et al. 1990; Shew et al. 1990a,b; Scheffner et al. 1991). Given the results of the growth suppression assay, perhaps the simplest explanation for the apparent lack of phosphorylation of either RB(379–928) or full-length RB in the RB $-/-$ cells is that the recipient cells are growth arrested in G_1 , that is, that time in the cell cycle when RB normally exists in an un- or underphosphorylated form (Buchkovich et al. 1989; Chen et al. 1989; DeCaprio et al. 1989; Mihara et al. 1989; Xu et al. 1989). In support of this hypothesis, Goodrich et al. (1991) have recently demonstrated that microinjection of a recombinant RB protein spanning residues 394–928 is sufficient to induce G_1 growth arrest in RB $-/-$ cells. Among several alternative explanations would be an inherent inability of the RB $-/-$ cells chosen for this study to

phosphorylate an exogenous RB substrate. Experiments are under way to address these possibilities.

As has been observed previously upon reintroduction of a wild-type p53 allele into p53 +/+ cells (Baker et al. 1990; Johnson et al. 1991) and after reintroducing full-length RB into RB +/+ cells (Huang et al. 1988), we failed to detect any growth perturbation when the various wild-type RB-encoding plasmids were introduced into U2OS (RB +/+) cells. Although the molecular explanation for this phenomenon has not been elucidated, it remains a formal possibility that the transformed cells studied here have sustained one or more mutations that render them immune to the growth suppression functions of RB.

In summary, much of the RB-coding sequence (residues 1–379) is not necessary for either overt RB phosphorylation, the presence of differentially phosphorylated RB species, T and E2F binding, or the ability of RB to grossly suppress the growth of certain RB (–/–) cell lines. In contrast, the action of the RB pocket and, presumably, its ability to form complexes with one or more specific cellular proteins is necessary, in support of the prior speculation on this matter. However, it is not sufficient for RB-mediated growth suppression, as assayed here. A second function, provided by sequences carboxy-terminal to the pocket, is also required, although the nature of this function is presently unclear. In this regard, we have demonstrated that the carboxyl terminus of RB is required for the pocket to interact efficiently with E2F, at least as measured by gel-shift activity. Hence, given the correlations described in Figure 8, E2F binding might provide one potential explanation for the requirement of the carboxyl terminus in the biological assays described here.

Importantly, in comparing the sequences of the RB proteins of several species—human, mouse, and *Xenopus* (Bernards et al. 1989; S. Friend and O. Destree, pers. comm.), it is clear that there is greater homology among the pocket and carboxy-terminal segments of all of these species than among the amino-terminal portions, suggesting that whatever its mode of action, the carboxyl terminus contributes to a core RB function. Of immediate interest will be determination of whether the carboxyl terminus operates by contributing to the function of the pocket or by performing a wholly different function.

Materials and methods

Cells

U2OS, SAOS2, and MDA-MB-468 cells were obtained from the American Type Culture Collection. MEF Ψ T, MEF Ψ K1, and A31 cells were our own laboratory stock. OHS50-T cells were kindly provided by Steve Friend. All of the cells were grown in Dulbecco's modified Eagle medium (DMEM) (Sigma) containing 12% fetal calf serum in a 10% CO₂-containing atmosphere.

Construction of plasmids

The generation of cDNAs encoding RB residues 379–792, 379–817, and 379–928, as well as a 706 C $\rightarrow$ F mutant derivative of

the latter, has been described previously (Kaelin et al. 1990, 1991). An RB cDNA encoding 379–792 with the 706F mutation was also generated by polymerase chain reaction (PCR) by use of the primers and PCR conditions described previously (Kaelin et al. 1990). These cDNAs were subcloned into the unique *Bam*HI site of the pCMV–Neo–Bam vector (Baker et al. 1990). The construction of pGEX plasmids encoding pGT–RB(379–792), pGT–RB(379–928), and pGT–RB(379–928,706 C $\rightarrow$ F) has been described previously (Kaelin et al. 1991). A full-length RB cDNA, a mutant version in which exon 22 was deleted, as well as the 379–817 cDNA described above, was also subcloned into pCMV–Neo–Bam and a modified pGEX plasmid, pGEX-2TK (W. Kaelin and M. Blonar, unpubl.). The exon 22 mutant was generated by cutting the full-length RB cDNA at unique *Nco*I and *Bsm*I sites, and inserting the *Nco*I–*Bsm*I fragment from pGEX-2T–RB(379–928; dl22) (Kaelin et al. 1991). An RB cDNA encoding 773–928 was generated by PCR using the primers 5'-GCGCGGATCCTCATTCTCTTCCTTGTTGAGG-3' (DN74) and 5'-GCGCGGATCCGCCACCATGTCCAC-CAGGCCCCCTACCTTG-3' (Rb773S). The PCR conditions for generating this cDNA were as described (Kaelin et al. 1990), except that the annealing temperature was 55°C and the amplification proceeded for 35 cycles. This cDNA was subcloned into pCMV–Neo–Bam vector and sequenced by DyeDeoxy Terminator method (Pharmacia).

Transfection

Cells were transfected at ~70% confluence with 30 μ g of plasmid DNA by the calcium phosphate method, as described by DeCaprio et al. (1988). The medium was changed twice, 16 hr after transfection.

Western blot analysis

Thirty-six hours after transfection, cells were lysed in EBC buffer (50 mM Tris at pH 8.0, 120 mM NaCl, 0.5% NP-40), as described previously (DeCaprio et al. 1988), and protein concentrations were determined by the Bio-Rad protein assay. Approximately 60 μ g of total protein was loaded per lane, resolved in a SDS-polyacrylamide gel, and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore) in Tris/glycine/methanol buffer for 4 hr (DeCaprio et al. 1988). Membranes were incubated overnight with a monoclonal antibody directed against human RB (XZ91 or XZ56) [1 : 3 dilution of hybridoma supernatant in TBS (10 mM Tris at pH 8.0, 150 mM NaCl) containing 5% BSA]. The membranes were washed with TBS plus 0.5% BSA and probed with an alkaline phosphatase-conjugated goat anti-mouse antibody (Fisher), as described by the manufacturer.

³⁵S-labeling and immunoprecipitation

For ³⁵S-labeling, cells were starved in methionine-free DMEM for 0.5 hr and then fed with DMEM containing [³⁵S]methionine (1140 Ci/mmol, New England Nuclear) at a concentration of 200 μ Ci/ml for 3 hr.

Preparation of cell extracts and immunoprecipitation reactions were performed as described previously (DeCaprio et al. 1988).

³²P-Labeling and alkaline phosphatase analyses

Cells were metabolically labeled in 3 ml of serum-free, phosphate-free DMEM supplemented with 1 mCi of [³²P]orthophosphate (New England Nuclear) per milliliter of culture medium for 3 hr at 37°C. Aliquots of cell lysate were immunoprecipi-

tated with XZ91 (Hu et al. 1991) (hybridoma supernatant). Protein A-Sepharose bead-bound proteins were resolved in 7.5% SDS-polyacrylamide gels, transferred to PVDF membranes, and detected by immunoblotting with XZ91 followed by autoradiography.

For phosphatase treatment experiments, the protein A-Sepharose was resuspended in 50 μ l of 1 $\times$ alkaline phosphatase buffer (50 mM Tris at pH 8.5, 0.1 mM EDTA) to which was added 6 units of alkaline phosphatase (Boehringer Mannheim) and the following protease inhibitors: 2 μ g/ml of aprotinin, 2 μ g/ml of phenylmethylsulfonylfluoride, and 20 μ g/ml of leupeptin. After a 2-hr incubation at 37°C, an additional 6 units of enzyme were added to the reaction mix, and the reaction was allowed to proceed for an additional hour at 56°C. Control reaction mixtures contained either no enzyme or the phosphatase inhibitors sodium orthovanadate (10 mM) and sodium fluoride (2 mM).

Immunofluorescence

Cells were grown on glass coverslips in 60-mm dishes before transfection. After transfection, cells were washed with PBS (137 mM NaCl, 3 mM KCl, 5 mM Na₂HPO₄, and 2 mM KH₂PO₄ at pH 7.4), fixed with 0.5% paraformaldehyde solution for 15 min on ice, and permeabilized with methanol at 25°C. After three washes in PBS, cells were incubated with an appropriate dilution (1 : 1) of primary antibody (hybridoma supernatants: XZ91 for RB(379–792), RB(379–928), their 706 C $\rightarrow$ F mutant derivatives, and 379–816; XZ56 (Hu et al. 1991) for RB(773–928) and RB(dl22) in PBS containing 5% goat serum at 25°C for 1 hr. The second antibody (FITC-conjugated goat anti-mouse antibody (Boehringer Mannheim), 1 : 10 dilution in PBS) incubation was performed at 25°C for 45 min. Cells were then washed with PBS, stained for DNA with 0.5 μ g/ml of 4'-6-diamidino-2-phenylindole hydrochloride (DAPI), mounted, and examined by fluorescence microscopy.

Colony formation assay

Cells that had achieved ~70% confluence were transfected with either the pCMV-Neo-Bam parental plasmid or the various RB-encoding derivatives. The cell growth media were supplemented with G418 (300 μ g/ml) 48 hr later. Approximately 2 weeks later, when macroscopic colonies became detectable, the cells were washed with PBS, fixed in 10% HOAc/10% MeOH for 15 min, and stained with 0.4% crystal violet, 20% EtOH, for 15 min.

E2F mobility-shift assay

Nuclear extracts from SAOS2 cells were prepared by the procedure of Schreiber et al. (1989). E2F gel mobility-shift assays were performed essentially as described by Shirodkar et al. (1992). GST-RB fusion proteins were synthesized in bacteria (200-ml cultures) as described by Kaelin et al. (1991), purified by adsorption to 100 μ l of glutathione-Sepharose beads, and eluted by incubation (5 min at 4°C) in 100 μ l of elution buffer (100 mM Tris at pH 8.0, 120 mM NaCl, 20 mM reduced glutathione). Two microliters of each purified fusion protein, containing approximately equal amounts (~30 ng) as estimated by Coomassie blue-stained gels, was added to DNA-binding reactions just before the addition of the probe. The E2F probe was a synthetic oligonucleotide corresponding to the E2F-binding site defined within the DHFR promoter (Hiebert et al. 1991). It was ³²P-end-labeled by T4 polynucleotide kinase. The DNA sequences of the wild-type DHFR oligonucleotide and a corresponding oligonucleotide containing mutations within the E2F DNA-binding se-

quences are as follows (+ strand): DHFR WT, CTAGTGAATTTTCGCGCCAAACTTG; DHFR MUT, CTAGTGCAATTGCTCGACCAACTTG. Binding reactions were analyzed by electrophoresis in 4% polyacrylamide gels run in 0.25 $\times$ TBE (22 mM Tris, 22 mM boric acid, 0.5 mM EDTA) at 4°C.

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