MOLECULAR AND CELLULAR BIOLOGY, Oct. 1997, p. 5771–5783 Vol. 17, No. 10
0270-7306/97/$04.00+0


# Dual Mechanisms for the Inhibition of E2F Binding to RB by Cyclin-Dependent Kinase-Mediated RB Phosphorylation

ERIK S. KNUDSEN AND JEAN Y. J. WANG*

*Department of Biology and Center for Molecular Genetics, University of California at San Diego, La Jolla, California 92093-0322*

Received 10 April 1997/Returned for modification 15 May 1997/Accepted 14 July 1997

**The growth suppression function of RB is dependent on its protein binding activity. RB contains at least three distinct protein binding functions: (i) the A/B pocket, which binds proteins with the LXCXE motif; (ii) the C pocket, which binds the c-Abl tyrosine kinase; and (iii) the large A/B pocket, which binds the E2F family of transcription factors. Phosphorylation of RB, which is catalyzed by cyclin-dependent protein kinases, inhibits all three protein binding activities. We have previously shown that LXCXE binding is inactivated by the phosphorylation of two threonines (Thr821 and Thr826), while the C pocket is inhibited by the phosphorylation of two serines (Ser807 and Ser811). In this report, we show that the E2F binding activity of RB is inhibited by two sets of phosphorylation sites acting through distinct mechanisms. Phosphorylation at several of the seven C-terminal sites can inhibit E2F binding. Additionally, phosphorylation of two serine sites in the insert domain can inhibit E2F binding, but this inhibition requires the presence of the RB N-terminal region. RB mutant proteins lacking all seven C-terminal sites and two insert domain serines can block Rat-1 cells in $G_1$. These RB mutants can bind LXCXE proteins, c-Abl, and E2F even after they become phosphorylated at the remaining nonmutated sites. Thus, multiple phosphorylation sites regulate the protein binding activities of RB through different mechanisms, and a constitutive growth suppressor can be generated through the combined mutation of the relevant phosphorylation sites in RB.**

The retinoblastoma susceptibility gene product, RB, is a suppressor of $G_1$/S progression. Loss of RB compromises the $G_1$/S regulation, which is characterized by a shorter $G_1$ phase and reduced responses to antimitogenic factors (17, 18). Ectopic expression of RB can arrest some, but not all, tumor cell lines in $G_1$ (10, 39, 44, 47). This growth-inhibitory activity of RB depends on its ability to bind cellular proteins and to assemble protein complexes (47, 48, 51). Biochemical analysis has revealed the presence of at least three distinct protein binding functions in RB.

The first protein binding function identified in RB was the A/B pocket, which is comprised of two noncontiguous sequences of amino acids called the A domain (amino acids 379 to 572) and B domain (amino acids 646 to 772) (21, 23, 26). The spacer sequence (amino acids 573 to 645), which is called the insert domain, is required for the proper folding of the A/B pocket but can be exchanged for random amino acids without inactivating A/B pocket activity (21, 23). The A/B pocket binds the viral oncoproteins of DNA tumor viruses (e.g., simian virus 40 [SV40] large T antigen or adenovirus E1A), which each contain a conserved LXCXE motif (21, 23, 26). Subsequent analysis revealed that the A/B pocket of RB also interacts with cellular proteins which contain the LXCXE motif (47). It has been hypothesized that viral oncoproteins disrupt RB protein binding activity in a competitive manner and in this way lead to the inactivation of RB, thus promoting cellular transformation. A majority of RB mutant proteins found in human tumors are defective for LXCXE binding function, suggesting that the A/B pocket activity is required for RB to suppress cell growth (15, 39, 40, 41).

A second protein binding function, the C pocket, was identified as a binding site for the c-Abl tyrosine kinase (50). The C pocket resides within the C-terminal amino acids 772 to 870 (50, 52a). The proto-oncoprotein Mdm-2 also interacts with the C-terminal region of RB (53), although it is unclear whether the binding sites for Mdm-2 and c-Abl are the same. Overexpression of either Mdm-2 or c-Abl protein has been shown to overcome RB-mediated growth arrest (52, 53), suggesting that the C pocket function also contributes to growth inhibition.

The third RB protein binding function is the large A/B pocket (LP). This function requires the A, B, and C regions (amino acids 379 to 870) (19, 39). The LP is the binding site for the E2F family of transcription factors (19, 39). The E2F, LXCXE, and C pocket binding activities of RB are functionally distinct. This is supported by the finding that the LXCXE and E2F binding sites of RB can be co-occupied (8, 22, 24). Likewise, RB can form ternary complexes containing both E2F and c-Abl (51).

E2F is a family of DNA-binding transcription factors which play an important role in the regulation of $G_1$/S progression (43). E2F binds DNA as a heterodimer composed of E2F and DP subunits. To date, five E2F (E2F-1 to E2F-5) and two DP (DP-1 and DP-2) genes have been cloned (43). Consensus E2F DNA-binding sites (TTTCGCGC) are found in the promoters of growth-promoting genes, such as c-Myc, B-Myb, cyclin E, and cyclin A (7, 43). Furthermore, E2F sites are also present in the promoters of genes involved in DNA replication, such as dihydrofolate reductase, thymidine kinase, and DNA polymerase α (7, 43). The finding of E2F binding sites in the promoters of such genes suggests that the E2F transcription factors play important roles in the regulation of entry into S phase.

Accumulated evidence indicates that one of the functions of E2F is to assemble RB-dependent repressor complexes which inhibit the expression of genes containing E2F binding sites in their promoters (43, 46). Supporting this is the finding that mutation of E2F DNA-binding sites in the promoters of cell cycle-regulated genes, such as B-Myb and cyclin A, does not

* Corresponding author. Phone: (619) 534-6253. Fax: (619) 534-2821. E-mail: jywang@ucsd.edu.

prevent the expression of these genes but allows for expression in quiescent and early $G_1$ cells when transcription is normally repressed (30, 43, 56). In addition, it has recently been found that the E2F DNA-binding site of the B-Myb promoter is occupied in $G_1$ but not S phase, showing that E2F binding is correlated with repression (57).

Repression of E2F-regulated transcription is dependent on the direct binding of RB and related proteins, p107 and p130 (1, 9, 14, 49). RB functions as a transcriptional repressor when brought to promoters via E2F (1, 41, 49). Derepression of these promoters can be achieved by viral oncoproteins E1A and large T antigen, which disrupt the RB-E2F association (4, 43). Derepression may also be achieved through the overproduction of E2F, presumably by titrating RB from the relevant promoters and thus leading to inappropriate entry into S phase (25). During normal $G_1$/S progression, derepression of E2F-regulated promoters is achieved by the phosphorylation of RB, which inhibits its binding to E2F.

RB is phosphorylated in a cell cycle-dependent manner by cyclin-dependent protein kinases (cdk's) (2, 31, 32, 34). In quiescent and early $G_1$ cells, RB exists in a predominantly unphosphorylated state. As cells progress toward S phase, RB becomes phosphorylated. Initial phosphorylation of RB is most likely catalyzed by cdk4-cyclin D or cdk6-cyclin D complexes; thereafter, RB phosphorylation is maintained by other cdk-cyclins (47, 48). During mitosis, RB is rapidly dephosphorylated by an anaphase-specific phosphatase (34). Sixteen potential sites for cdk-mediated phosphorylation (Ser/Thr-Pro motifs) exist in RB, and 11 of these sites have been shown to be phosphorylated in vivo (6, 12, 27, 31, 33, 54). Different cdk-cyclin complexes preferentially phosphorylate RB at distinct sites in vitro, suggesting that different cdk-cyclin complexes may exert distinct effects on RB function in vivo (6, 27, 54).

Phosphorylation of RB inactivates its growth suppression activity. In general, agents which block RB phosphorylation arrest cell cycle progression in $G_1$, while agents which stimulate RB phosphorylation promote cell cycle progression (47). Evidence that phosphorylation of RB inactivates its growth-inhibitory activity came from a study with the RB-deficient osteosarcoma cell line SAOS-2 (20). In SAOS-2 cells, ectopically expressed RB is not phosphorylated, and it arrests cells in $G_1$. However, when RB is coexpressed with cyclin E or cyclin A, it fails to arrest SAOS-2 cells, and this is correlated with its becoming phosphorylated (20). Another line of evidence supporting the idea that phosphorylation of RB is required for $G_1$/S progression comes from studies of $p16^{INK4}$, an inhibitor of cdk4-cdk6 (29, 35, 36). This protein inhibits the phosphorylation of RB and arrests only RB-positive cells in $G_1$. The biological significance of RB phosphorylation and its impact on the ordered progression through $G_1$/S are exemplified by the frequent mutations that affect this process in human tumors. For example, amplification of the cyclin D1 gene is observed in breast cancer and esophageal cancer cells, in which it is believed to promote RB phosphorylation (42). Likewise, $p16^{INK4}$, which acts to prevent RB phosphorylation, is lost in a large fraction of human tumors, again leading to the deregulated phosphorylation of RB (42).

We have previously shown that distinct phosphorylation sites are responsible for disrupting the LXCXE and c-Abl binding activities of RB (28). Mutation of the Thr821 and Thr826 phosphorylation sites abolishes the inhibition of LXCXE binding by phosphorylation, whereas mutation of Ser807 and Ser811 disrupts regulation of the C pocket activity (28). In this report, we identified the cdk phosphorylation sites in RB which are required to inhibit E2F binding. Our results show that phosphorylation at several of the seven cdk sites encoded in RB exon 23 can inhibit E2F binding. In addition, phosphorylation of two serine sites in the insert domain can inhibit E2F binding. These results show that a majority of the cdk phosphorylation sites in RB are involved in the regulation of RB-E2F interaction.

## MATERIALS AND METHODS

**Cell culture.** The C33-A cervical carcinoma cell line (from the American Type Culture Collection), the immortalized rat fibroblastic cell line Rat-1 (from D. Green, La Jolla Institute of Allergy and Immunology), and the adenovirus-transformed 293 cell derivatives BOSC293 and BING293 (from D. Baltimore, Massachusetts Institute of Technology) were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum. Human foreskin fibroblasts (HFF), passages 11 to 25 (from D. Spector, University of California, San Diego), were grown in Earle's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum. Amphitropic recombinant retroviruses were obtained from transiently transfected BING293 cells as previously described (37, 52). Viral infections were carried out as previously described (52). Rat-1 cells were selected in 2-μg/ml puromycin (Sigma), and HFF were selected with 1.0-μg/ml puromycin. For growth curves, cell counting was performed with hemocytometers, and all plates were counted in triplicate. Metabolic labeling was carried out with [$^{32}$P]phosphoric acid at 1.0 mCi/ml in phosphate-free medium with 10% dialyzed calf bovine serum for 3 h. Treatment with nocadazole was carried out with 0.1-μg/ml medium for 8 h.

**Plasmids.** The human RB phosphorylation site mutants were constructed by either oligonucleotide-directed mutagenesis or subcloning by standard procedures. The PSM.4 construct has been previously described (28). For the construction of PSM.3, the following two mutagenic oligonucleotides were utilized: CATTCCTCGAGACCCTTACAAGTTTCCTAGTGCACCCTTACGGATTC CTGG and GTAAGGGTCTCGAGGAATGTGAGGTATTGGTGCCAAGG TAGGGGG. PSM.3 was spliced with PSM.4 to generate PSM.7. PSM.6 was generated by subcloning. The Ser788Asp mutation introduced a unique *Xho*I site in RB. PSM.7 and the single-site mutant PSM.1 (Ser788Asp) were cut with *Xho*I, and the N-terminal sequences were exchanged, thereby reintroducing Ser780 and generating PSM.6.

The murine pΔ34-RB and WT(mu)-RB, which are tagged with a hemagglutinin (HA) epitope, were generous gifts of P. Hamel, University of Toronto. The mutations generated in pΔ34-RB have been previously described (13). The human-mouse chimera PSM.11 was constructed by swapping the PSM.7 C terminus with that of the pΔ34-RB construct described by Hamel et al., by subcloning from the conserved *Ssp*I site in RB. PSM.4NI was constructed by swapping the wild-type (WT) human RB (WT-RB) C terminus with that of pΔ34-RB at the *Ssp*I site. The PSM.9N mutant was made by swapping the N terminus of pΔ34-RB with PSM.7 at the unique *Bsa*BI site within RB. The PSM.9I construct was made by digesting PSM.11 with *Bsa*BI and replacing the N terminus with human WT-RB sequence. The mutants generated are shown schematically in Fig. 1. The phosphorylation sites mutated are conserved between murine RB and human RB with the numbering as follows (human-murine) Thr252-Thr246, Thr356-Thr350, Ser608-Ser601, Ser612-Ser605, Ser780-Ser773, Ser788-Ser781, Ser795-Ser788, Ser807-Ser800, Ser811-Ser804, Thr821-Thr814, and Thr826-Thr819).

The murine WT(mu)-RB and pΔ34-RB were subcloned into the pBABE-puro vector for the production of recombinant retrovirus. The full-length WT, phosphorylation site-mutated (PSM), and pΔ34-RB constructs were cloned into the unique *Bam*HI site of the pCMV-Neo plasmid for expression in cells. The LP proteins were made by subcloning into LP-CMV-Neo, which was obtained from W. Kaelin (39). The glutathione *S*-transferase (GST)-LP mutants were also constructed by subcloning into the WT-GST-LP plasmid, which was previously described (28).

The CMV-CD20 and CMV-E2F-1 expression plasmids were generous gifts of E. Harlow (Massachusetts General Hospital). The cyclin D1 expression plasmid was from C. Sherr (St. Jude's Hospital), the cyclin E expression plasmid was from J. Roberts (Fred Hutchinson Cancer Research Center), and the cyclin A expression plasmid was from S. Reed (Scripps Research Institute). The Gal4-E2F-1 (E2F-1 amino acids 368 to 437) and LP-CMV plasmids were from W. Kaelin (Dana-Farber Cancer Institute). WT(mu)-RB and pΔ34-RB were obtained from P. Hamel (University of Toronto). The E2F-4 and Mdm-2 expression plasmids were gifts of D. Livingston (Dana-Farber Cancer Institute).

**Transfections.** Transfections of all cells were carried out by *N*,*N*-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES)-calcium phosphate precipitation as previously described (28). For the expression of RB proteins in BOSC293 cells, 16 μg of LP or 4 μg of full-length expression plasmid was transfected. BOSC293 cells were utilized for metabolic labeling studies, since these cells are transfected efficiently, allowing for cleaner results. For the expression of RB proteins in C33-A cells, 6 μg of RB was cotransfected with 3.3 μg each of cyclin E, cyclin A, and cyclin D1 expression plasmids. The cyclins were cotransfected to promote RB phosphorylation, which is relatively inefficient in C33-A cells. For coimmunoprecipitation of RB with E2F-1, 3 μg of RB and 3 μg of E2F-1 expression plasmid were cotransfected with the cyclin plasmids. For chloram-

phenicol acetyltransferase (CAT) assays, C33-A cells were transfected with 0.2 μg of cytomegalovirus (CMV) β-galactosidase (β-Gal), 0.2 μg of Gal4-E2F-1, 0.2 μg of 5×Gal4-CAT, and 2.0 μg of RB and either 15.0 μg of vector or 5.0 μg each of cyclin E, cyclin A, and cyclin D1. CAT assays were carried out by standard procedures, as previously described (50). For the cell cycle assays in Rat-1 cells, cells were transfected with 12.5 μg of vector, RB, or p16 expression plasmid and 2.5 μg of CD20 expression plasmid. For the rescue experiments, 2.5 μg of CD20, 5.0 μg of PSM-RB, and 10 μg of the effector expression plasmid were transfected. Each of the effector expression plasmids used (T-Ag, E2F-1, E2F-4, c-Myc, Mdm-2, and kinase-defective c-Abl) produce functional proteins.

**Flow cytometry.** Transfected Rat-1 cells were harvested by trypsinization, washed once with phosphate-buffered saline, and stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD20 antibody (PharMingen) for 20 min on ice (55). The cells were then washed twice with phosphate-buffered saline, fixed with ethanol, and stained with propidium iodide, as previously described (52). Analysis of the processed cells was carried out with a FACScan (Becton Dickinson) equipped with CellFit software. Transfected cells were detected based on their FITC labeling, and DNA content was determined by propidium iodide staining (55). The net change in percent $G_1$ was obtained by subtracting the percent $G_1$ content of $CD20^+$ cells from the percent $G_1$ of the $CD20^+$ vector-transfected cells.

**Immunoprecipitation, binding assays, and antibodies.** In vitro binding assays were carried out with GST-LP proteins purified from bacteria on glutathione agarose as previously described (28). Stoichiometric phosphorylation of the GST-LP proteins was carried out using active cdc2/cyclinB isolated by immunoprecipitation as previously described (28). A 50-ng amount of GST-LP protein either mock phosphorylated or phosphorylated in 50 μl of kinase buffer was diluted with 250 μl of NET-N (100 mM NaCl, 1 mM EDTA, 20 mM Tris [pH 8.0], 0.1% Nonidet P-40) supplemented with protease (1,10-phenanthroline at 10 μg/ml, aprotinin at 10 μg/ml, leupeptin at 10 μg/ml, and 1 mM phenylmethylsulfonyl fluoride) and phosphatase inhibitors (10 mM sodium fluoride, 10 mM sodium pyrophosphate), and the dilution was then applied to 100 ng of immobilized E2F-1. E2F-1 was produced in bacteria as a GST fusion protein which was immobilized by immunoprecipitation with anti-E2F-1 monoclonal antibody (PharMingen) and protein G-Sepharose (Pharmacia). Binding was carried out for 1 h at 4°C with rotation. Complexes were washed four times with NET-N, boiled in sodium dodecyl sulfate (SDS)-loading buffer, resolved by SDS-7.5% polyacrylamide gel electrophoresis (PAGE), and transferred to Immobilon P (Millipore). GST-LP protein was then detected by autoradiography or immunoblotting. The input loaded was known to be 15% of the GST-LP protein, which was applied to immobilized E2F-1. Percent binding was determined by comparing the fraction of GST-LP protein bound with the input into the binding reaction. Quantitation of phosphorylated GST-LP was carried out with a PhosphorImager (Molecular Dynamics), while quantitation of the mock-phosphorylated GST-LP protein was carried out from the immunoblot with a personal densitometer (Molecular Dynamics).

The binding of RB produced in cells to GST–E2F-1 was carried out as previously described (28). For coimmunoprecipitation, transfected C33-A cells were lysed in NET-N and clarified by centrifugation for 15 min at 4°C. Soluble protein was then subjected to immunoprecipitation with either anti-RB or anti-E2F-1 antibodies, which were recovered on protein A or G Sepharose (Pharmacia). Immune complexes were washed four times with NET-N, denatured by boiling in SDS sample buffer, resolved by SDS-PAGE, and transferred to Immobilon P (Millipore). RB proteins were then detected by immunoblotting.

Immunoprecipitation and immunoblotting of RB proteins were carried out by standard methods and as previously described (28). The relative labelings of in vivo-phosphorylated proteins were determined by calculating the $^{32}P$-to-protein ratio. $^{32}P$ incorporation was determined with a PhosphorImager (Molecular Dynamics), and the protein was quantitated from the immunoblot with a personal densitometer (Molecular Dynamics). The $^{32}P$-to-protein ratio of WT-LP or WT RB was set to 100. The antibodies for E2F-1 used were either monoclonal anti-E2F-1 (PharMingen) (for the in vitro binding reactions) or polyclonal anti-E2F-1 (a gift of Amy Yee ([Tufts University] used in the coimmunoprecipitation reactions). The anti-RB antibody used for immunoblotting was the 851 (50) polyclonal antibody. For immunoprecipitation, the XZ91 (PharMingen) or 851 (50) antibody was utilized. The anti-HA antibody used for immunoprecipitation was obtained from BABCO Scientific. Immunoprecipitation of monoclonal antibodies was carried out with protein G-Sepharose (Pharmacia), while for polyclonal antibodies protein A-Sepharose (Pharmacia) was used.

## RESULTS

**PSM RB constructs.** The RB protein contains 16 Ser-Pro or Thr-Pro motifs that are potential sites of phosphorylation by cdk's. Six of the cdk sites are in the N-terminal region, one is in the A domain, two are in the insert domain, and seven are clustered in exon 23-encoded sequences in the C-terminal region (Fig. 1). All 10 cdk sites in the A, B, and C regions of RB (LP) can be stoichiometrically phosphorylated by cdk's in vitro (28). We have previously demonstrated that binding of RB to the LXCXE motif is regulated by two threonine sites, Thr821 and Thr826, and that binding of RB to the c-Abl tyrosine kinase is regulated by two serine sites, Ser807 and Ser811 (28). In the present study, we investigated how phosphorylation regulates the binding of E2F to RB. The mutants that we used to elucidate the regulation of RB-E2F binding are summarized in Fig. 1. Each PSM construct is named by the number of mutated sites. For example, PSM.3 contains 3 mutated serine sites, Ser780, Ser788, and Ser795, and retains the other 13 Ser-Pro and Thr-Pro motifs. PSM.4 contains 4 mutated sites at the extreme C terminus of RB, Ser807, Ser811, Thr821, and Thr826, and retains the other 12 Ser-Pro and Thr-Pro motifs. PSM.7 is a combination of PSM.3 and PSM.4, lacking all seven of the C-terminal exon 23-encoded cdk phosphorylation sites. The pΔ34 mutant was constructed by Hamel et al. in murine RB (13); it lacks eight cdk phosphorylation sites, which are the equivalent of human phosphorylation sites Ser246, Thr356, Ser608, Ser612, Ser788, Ser795, Ser807, and Ser811 (Fig. 1). The pΔ34 backbone was used to generate a number of murine-human chimeric PSM constructs. PSM.11 contains the C-terminal region of human PSM.7 fused in frame to the N-terminal and A/B pocket regions of pΔ34, resulting in the elimination of 11 phosphorylation sites (Fig. 1). PSM.4NI contains the C-terminal region of human WT RB fused to the N-terminal and A/B pocket regions of pΔ34, resulting in the loss of four phosphorylation sites. PSM.9N has the N-terminal region and A domain of pΔ34 fused to the insert B and C regions of PSM.7, resulting in the loss of two N-terminal sites, Thr252 and Thr356, and the seven exon 23-encoded sites. PSM.9I has the insert and B domain of pΔ34 in the human PSM.7 backbone, thereby lacking Ser608 and Ser612 plus the C-terminal phosphorylation sites. The murine RB is 91% identical to human RB, and pΔ34 has been previously shown to suppress the growth of human cell lines (3, 53). All of the PSM proteins, including the human-murine hybrids, bind to E2F-1 in the unphosphorylated form and all are capable of suppressing the growth of human osteosarcoma SAOS-2 cells. Although most of the phosphorylation sites are mutated to alanine, Ser788 is substituted with aspartic acid and Ser811 is substituted with leucine. The substitution of the negatively charged aspartic acid residue does not disrupt RB protein binding or biological activity.

**RB-E2F interaction can be disrupted by phosphorylation at multiple C-terminal sites.** We have previously shown that PSM.4-RB binds to E2F and that this interaction can be disrupted by phosphorylation (28). Thus, we reasoned that the cdk sites involved in the inhibition of E2F binding must still be present in PSM.4-RB. Therefore, we began mapping the phosphorylation sites that regulate E2F binding by mutating the other three exon 23-encoded phosphorylation sites (Ser780, -788, and -795) either individually or in combinations (Fig. 1). These PSM-RB proteins were first tested for E2F binding by an in vitro assay (28). In this assay, the RB mutants were expressed in *Escherichia coli* as GST fusion proteins containing the large A/B pocket fragment, from RB amino acids 379 to 928 (Fig. 1 [LP]). Each GST fusion was purified and then stoichiometrically phosphorylated as previously described (28). A cdc2-cyclin B complex was used as the kinase (32). As a control, GST fusion proteins were incubated with cdc2-cyclin B in the absence of ATP (Fig. 2). The mock-phosphorylated and phosphorylated GST fusion proteins were then incubated with E2F-1 that was immobilized by immunoprecipitation with anti-E2F-1 antibodies. The amount of RB protein bound to E2F-1 was then quantitated (see Materials and Methods).

With the WT-GST-LP fragment, 5.5% of the input RB was bound to E2F-1 after mock phosphorylation (Fig. 2A, lane 2).

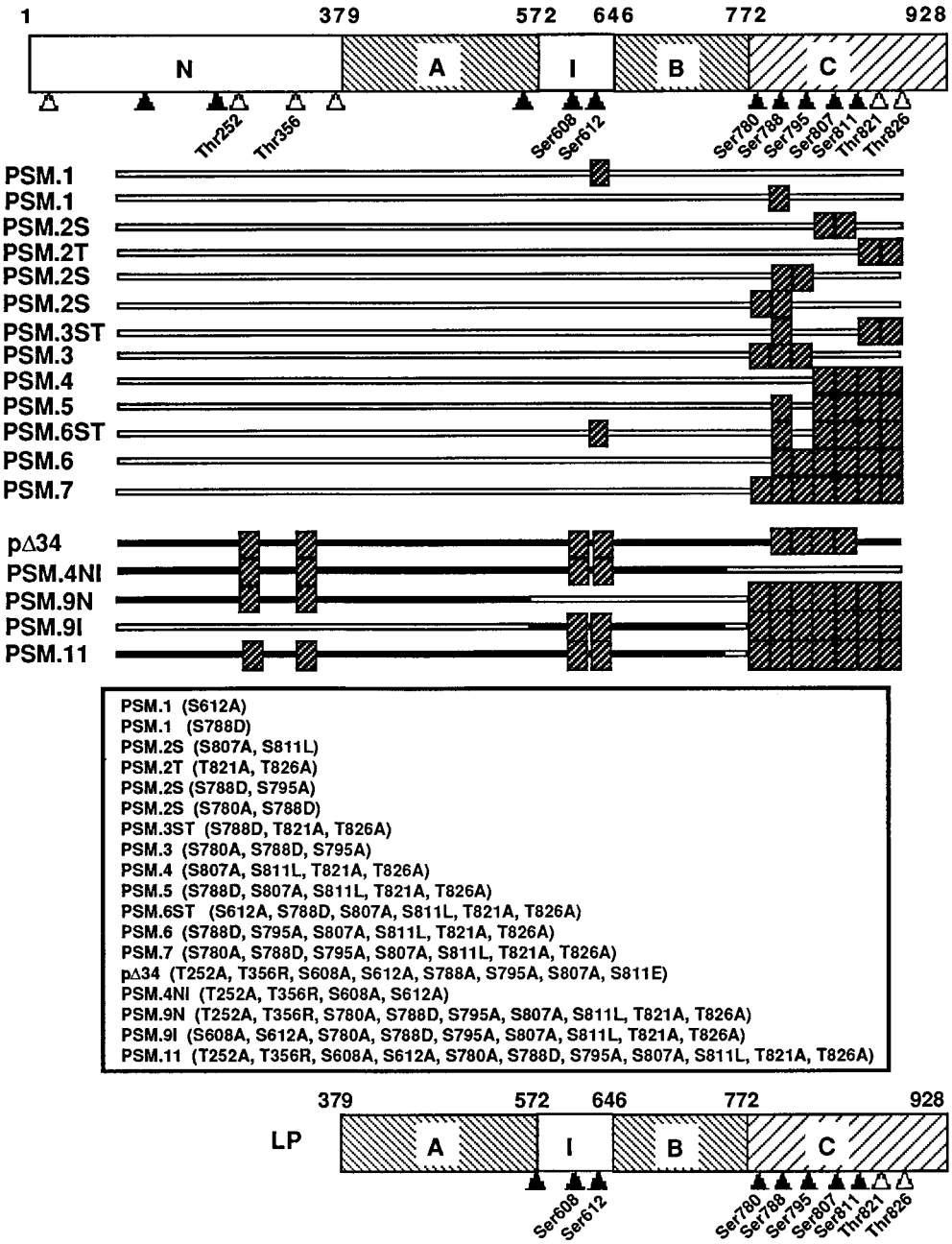


FIG. 1. Summary of RB constructs. PSM-RB proteins were generated as described in Materials and Methods. Full-length human RB contains 16 Ser/Thr-Pro motifs (Thr5, Ser230, Ser249, Thr252, Thr356, Thr373, Ser567, Ser608, Ser612, Ser780, Ser788, Ser807, Ser811, Thr821, and Thr826), which are denoted by arrowheads (the filled arrows represent serine, and the open arrows represent threonine). The phosphorylation site mutants described in this paper are shown schematically and are listed inside the box. Some of the mutants were hybrids of human and murine RB. Open bars, human RB sequences; shaded bars, murine RB sequences. PSM.3, PSM.4, PSM.6, and PSM.7 were made in human RB. The pΔ34 was constructed in murine RB (13). The PSM.9N, PSM.9I, PSM.4NI, and PSM.11 mutants are chimeras, as shown schematically. The RB mutant proteins were expressed either as full-length proteins (amino acids 1 to 928) or as the LP fragment (amino acids 379 to 928). The LP fragment contains only 10 of the 16 Ser/Thr-Pro phosphorylation sites. All phosphorylation sites targeted for mutagenesis are conserved between human RB and murine RB, with the numbering as follows, respectively: Thr252 = Thr246, Thr356 = Thr350, Ser608 = Ser601, Ser612 = Ser605, Ser780 = Ser773, Ser788 = Ser781, Ser795 = Ser788, Ser807 = Ser800, Ser811 = Ser804, Thr821 = Thr814, and Thr826 = Thr819.

When WT-GST-LP was stoichiometrically phosphorylated prior to binding, only 0.3% of the input RB was bound to E2F-1 (Fig. 2B, lane 2). Thus, phosphorylation inhibits RB-E2F interaction in vitro. As shown previously, PSM.4-GST-LP did not bind to E2F-1 after phosphorylation (Fig. 2, lane 6). The combination of three mutations in PSM.3 also did not abolish regulation (Fig. 2, lane 4). However, when PSM.3 and PSM.4 were combined, as in PSM.7-GST-LP, regulation of E2F binding was lost. PSM.7-GST-LP was phosphorylated at the remaining three sites in vitro. However, it bound to E2F-1 equally well in the mock-phosphorylated and stoichiometrically phosphorylated state (Fig. 2, lane 6). This result shows that phosphorylation at the three remaining sites in PSM.7-GST-LP (i.e., Ser567, Ser608, and Ser612) must not be able to

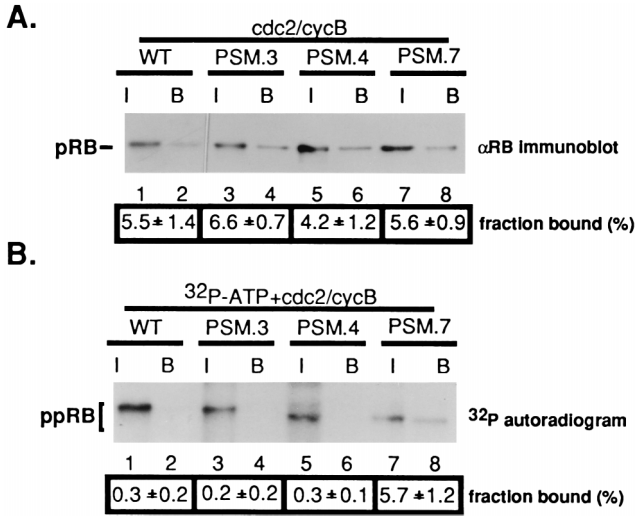


FIG. 2. In vitro-phosphorylated PSM.7-LP binds to E2F-1. (A) PSM proteins bind to E2F-1 in vitro. Purified GST-LP proteins, WT (lanes 1 and 2), PSM.3 (lanes 3 and 4), PSM.4 (lanes 5 and 6), or PSM.7 (lanes 7 and 8), were mock phosphorylated by incubation with active cdc2-cyclinB in the absence of ATP. Resulting unphosphorylated GST-LP proteins were applied to E2F-1 immobilized on beads by immunoprecipitation. The beads were washed, and either 15% of the input (I [odd lanes]) or 100% of bound protein (B [even lanes]) was resolved by SDS-7.5% PAGE. The GST-LP protein (pRB) was detected by immunoblotting, with the 851 polyclonal anti-RB antibody. The fraction of input protein bound to E2F-1 as described in Materials and Methods. The values shown are from three independent experiments. (B) Phosphorylated PSM.7-LP binds to E2F-1. Purified GST-LP proteins, WT (lanes 1 and 2), PSM.3 (lanes 3 and 4), PSM.4 (lanes 5 and 6), or PSM.7 (lanes 7 and 8), were phosphorylated by incubation with active cdc2-cyclinB in the presence of 150 μM ATP and 80 μCi of [γ-$^{32}$P]ATP. The resulting phosphorylated proteins were applied to E2F-1 immobilized by immunoprecipitation; 15% of the input (I [odd lanes]) or 100% of bound protein (B [even lanes]) was resolved by SDS-7.5% PAGE. Phosphorylated GST-LP protein (ppRB) was detected by autoradiography. The fraction of phosphorylated GST-LP protein bound to E2F-1 was determined. The values shown are from three independent experiments.

disrupt the binding of the LP fragment to E2F-1. As summarized in Table 1, a total of 14 PSM-GST-LP proteins were tested in this assay, and of these, only PSM.7-LP bound E2F when phosphorylated. Taken together, these results suggest that phosphorylation at several of the seven exon 23-encoded phosphorylation sites can inhibit E2F binding.

To confirm the binding results obtained with the in vitro-phosphorylated RB, we expressed the LP fragments in cells. We first measured the relative stoichiometry of in vivo phosphorylation by labeling transfected cells with $^{32}P_i$. The LP proteins were isolated by immunoprecipitation, and the $^{32}$P-to-protein ratio for each protein was determined (Fig. 3A). The WT-LP protein migrated as several electrophoretically distinct bands. The most slowly migrating band contained the majority of the radioactive phosphate (Fig. 3A, lane 2). The PSM.4-LP protein migrated as a doublet, and the majority of the label was present in the upper band (Fig. 3A, lane 3). The relative labeling efficiency of PSM.4-LP was 40% of that of WT LP. The electrophoretic mobility of PSM.7-LP resembled that of PSM.4-LP (compare lanes 3 and 4), but the incorporation of phosphate was reduced to 23% of WT LP (Fig. 3A, lane 4). It is important to note that both PSM.4-LP and PSM.7-LP were still phosphorylated in vivo, albeit to a lower level, consistent with the loss of phosphorylation sites.

These in vivo-phosphorylated RB-LP proteins were assayed for binding to E2F-1 by passing lysates from transfected cells through GST–E2F-1 immobilized on glutathione agarose. The bound fraction was resolved by SDS-PAGE, and RB was detected by immunoblotting. This binding reaction is specific, as previously characterized RB mutants, e.g., R661W and C706F, fail to bind GST–E2F-1 in this assay (52a; data not shown). In keeping with previously published results, of the multiple bands in the input pool (Fig. 4A, lane 1), only the lower band of WT-LP was retained by GST–E2F-1 (Fig. 4A, lane 2). The phosphorylated upper band of PSM.4-LP also failed to bind GST-E2F-1 (Fig. 4A, compare lanes 3 and 4). PSM.3-LP behaved in the same manner as PSM.4-LP, with only the lower band retained by GST–E2F-1 (not shown). In contrast, the phosphorylated upper band of PSM.7-LP bound to GST–E2F-1 (Fig. 4A, compare lanes 5 and 6). These results are consistent with those obtained with in vitro-phosphorylated RB (Fig. 2) and further indicate that phosphorylation at several of the seven C-terminal phosphorylation sites may disrupt the binding of E2F-1.

To test if phosphorylation at a single exon 23-encoded site can inhibit E2F binding, we reverted amino acid 780 back to serine to generate PSM.6-LP (Fig. 1). The binding of in vivo-phosphorylated PSM.6-LP to GST–E2F-1 was then compared to that of PSM.7-LP. While both the phosphorylated and unphosphorylated forms of PSM.7-LP bound to GST–E2F-1 with equal affinities (Fig. 4B, compare lanes 1 and 2), the phosphorylated form of PSM.6-LP was not retained by the immobilized GST–E2F-1 (Fig. 4B, compare lanes 3 and 4). Thus, the in vivo phosphorylation of Ser780 is able to inhibit binding of RB-LP to E2F-1. Because PSM.3-LP, PSM.4-LP, and PSM.6-LP are each sensitive to inhibition by phosphorylation, Ser780 and at least one other cdk site among the four mutated in PSM.4-LP must be able to inhibit E2F-1 binding when phosphorylated.

To further confirm the GST-binding results, the in vivo associations of the RB-LP proteins with E2F-1 were assayed by coimmunoprecipitation (Fig. 5A). C33-A cells were cotransfected with RB-LP and E2F-1 expression plasmids. Lysates prepared from these cells were subjected to immunoprecipitation with either anti-RB or anti-E2F-1 antibodies, and the precipitated RB-LP was then detected by immunoblotting. Coimmunoprecipitation of WT-LP with E2F-1 was observed, but the phosphorylated forms of WT-LP were excluded from the anti-E2F-1 immunoprecipitate (Fig. 5A, compare lanes 1 and 2). Similar exclusion of phosphorylated PSM.3-LP, PSM.4-LP, and PSM.6-LP was also observed (not shown). In contrast, both phosphorylated and unphosphorylated forms of PSM.7-LP were present in the E2F-1 immunoprecipitate (Fig. 5A, compare lanes 3 and 4). Thus, mutation of all seven cdk sites encoded in RB exon 23 can abolish the phosphorylation-mediated inhibition of RB-LP binding E2F-1.

RB binding leads to the inhibition of E2F transcription-activating activity, and this inhibition is alleviated by the cdk-cyclin-catalyzed phosphorylation of RB (1, 9). The binding results suggest that PSM.7-LP should inhibit E2F activity even after it is phosphorylated. To test this prediction, we cotransfected RB-LP with a Gal4–E2F-1 fusion protein and examined the transactivation of a 5×Gal4 promoter (9). WT-LP inhibited the activity of Gal4-E2F-1 by approximately fivefold (Fig. 5B). Coexpression of cyclins D1, E, and A reversed the WT-LP-induced inhibition (Fig. 5B). As predicted, PSM.7-LP also inhibited Gal4-E2F-1, but its effect was not reversed by the combined coexpression of these three cyclins (Fig. 5B). This result further demonstrates that the association of PSM.7-LP with E2F-1 is not disrupted by cdk-cyclin-catalyzed phosphorylation.

**E2F binding to RB can also be disrupted by insert domain cdk phosphorylation sites.** The LP fragment contains 10 of the 16 cdk phosphorylation sites in full-length RB (Fig. 1). To

TABLE 1. Summary of PSM.RB binding to E2F and suppression of Rat-1 cell proliferation

| RB construct[a] | No. of S/P or T/P[b] | Regulation of E2F binding[c] | Rat-1 growth suppression[d] |
|---|---|---|---|
| WT | 16 | + | − |
| WT-LP | 10 | + | − |
| PSM.1-612 | 15 | + | ND |
| PSM.1-LP | 9 | + | ND |
| PSM.1-788 | 15 | + | ND |
| PSM.1-LP | 9 | + | ND |
| PSM.2S-807/811 | 14 | + | − |
| PSM.2S-LP | 8 | + | − |
| PSM.2T-821/826 | 14 | + | − |
| PSM.2T-LP | 8 | + | − |
| PSM.2S-788/795 | 14 | ND | ND |
| PSM.2S-LP | 8 | + | ND |
| PSM.2S-780/788 | 14 | ND | ND |
| PSM.2S-LP | 8 | + | ND |
| PSM.3ST-788/821/826 | 13 | + | ND |
| PSM.3ST-LP | 7 | + | ND |
| PSM.3-780/788/795 | 13 | ND | ND |
| PSM.3-LP | 7 | + | − |
| PSM.4-807/811/821/826 | 12 | + | − |
| PSM.4-LP | 6 | + | − |
| PSM.5-788/807/811/821/826 | 11 | + | − |
| PSM.5-LP | 5 | + | ND |
| PSM.6ST-612/788/807/811/821/826 | 10 | + | − |
| PSM.6ST-LP | 4 | + | ND |
| PSM.7-780/788/795/807/811/821/826 | 9 | + | − |
| PSM.7-LP | 3 | − | + |
| PSM.6-788/795/807/811/821/826 | 10 | ND | ND |
| PSM.6-LP | 4 | + | − |
| pΔ34-252/356/608/612/788/795/807/811 | 8 | − | − |
| PSM.4NI-252/356/608/612 | 12 | + | − |
| PSM.9N-252/356/780/788/795/807/811/821/826 | 7 | + | − |
| PSM.9I-608/612/780/788/795/807/811/821/826 | 7 | − | + |
| PSM.11-252/356/608/612/780/788/795/807/811/821/826 | 5 | − | + |

[a] The RB constructs, either in the full-length or in the LP form, are diagrammed in Fig. 1.
[b] The total number of Ser-Pro (S/P) or Thr-Pro (T/P) motifs present in each construct.
[c] Phosphorylation-mediated regulation of E2F binding was determined in vitro and in vivo. Examples of these assays are shown in Fig. 2 to 6. +, phosphorylation regulates E2F binding (i.e., E2F binding is lost when RB protein is phosphorylated); −, phosphorylation does not regulate E2F binding (i.e., phosphorylated RB binds to E2F); ND, not determined.
[d] Suppression of Rat-1 cell proliferation was determined as described in the legend to Fig. 8. −, expression of the mutant RB does not affect Rat-1 cell cycle progression; +, expression of the mutant RB blocks Rat-1 cells in $G_1$; ND, not determined.

investigate the regulation of E2F binding to full-length RB, we expressed PSM-RB proteins in cells and assayed for binding to GST–E2F-1. As expected, the phosphorylated WT-RB failed to bind immobilized GST–E2F-1 (Fig. 6A, compare lanes 1 and 2). Phosphorylated PSM.4-RB also failed to bind GST–E2F-1 (Fig. 6A, compare lanes 3 and 4). To our surprise, the phosphorylated PSM.7-RB did not bind to E2F-1 (Fig. 6A, compare lanes 5 and 6). This is in sharp contrast to the PSM.7-LP protein, which bound to E2F-1 in its phosphorylated form (Fig. 4A, lanes 5 and 6). This result indicates that phosphorylation of sites other than the C-terminal seven sites can also disrupt the binding of full-length RB to E2F-1.

Mutations were then introduced into PSM.7-RB to identify the additional sites that can inhibit E2F-1 binding to the full-length RB. Initially, we suspected the additional sites to reside in the N-terminal region which was missing from the LP fragment (Fig. 1). We generated PSM.11-RB with the following four additional sites lost: two in the insert domain (Ser608 and Ser612) and two in the N-terminal region (Thr252 and Thr356) (Fig. 1). The PSM.11 protein was poorly phosphorylated in vivo, migrating as a single band, the $^{32}$P-to-protein ratio being less than 5% that of WT-RB (Fig. 3B, compare lanes 2 and 4). This single band bound to GST–E2F-1 (Fig. 6A, lanes 7 and 8). To further identify the relevant sites, we separated the four mutated sites to generate PSM.9N and PSM.9I (Fig. 1).

In PSM.9N, the two N-terminal phosphorylation sites (Thr252 and Thr356) and the seven exon 23-encoded phosphorylation sites were mutated (Fig. 1). This mutant was phosphorylated in vivo and migrated as a doublet (Fig. 6B, lane 1). Surprisingly, the phosphorylated upper band of PSM.9N-RB, like PSM.7-RB, did not bind GST–E2F-1 (Fig. 6B, compare lanes 1 and 2). Therefore, phosphorylation of the N-terminal Thr252 and Thr356 must not be responsible for the disruption of PSM.7-RB binding to E2F-1.

In PSM.9I, the two insert domain phosphorylation sites (Ser608 and Ser612) and the seven exon 23-encoded phosphorylation sites were mutated (Fig. 1). This protein was also phosphorylated in vivo and migrated as a doublet (Fig. 6B, lane 3). Interestingly, both bands of PSM.9I-RB bound to GST–E2F-1 with equal avidity (Fig. 6B, lanes 3 and 4). The phosphorylated forms of PSM.9I-RB also associated with E2F-1 in vivo, as determined by coimmunoprecipitation experiments (Fig. 6C, compare lanes 3 and 4). Thus, phosphorylation at Ser608 and/or Ser612 is required for the disruption of PSM.7-RB

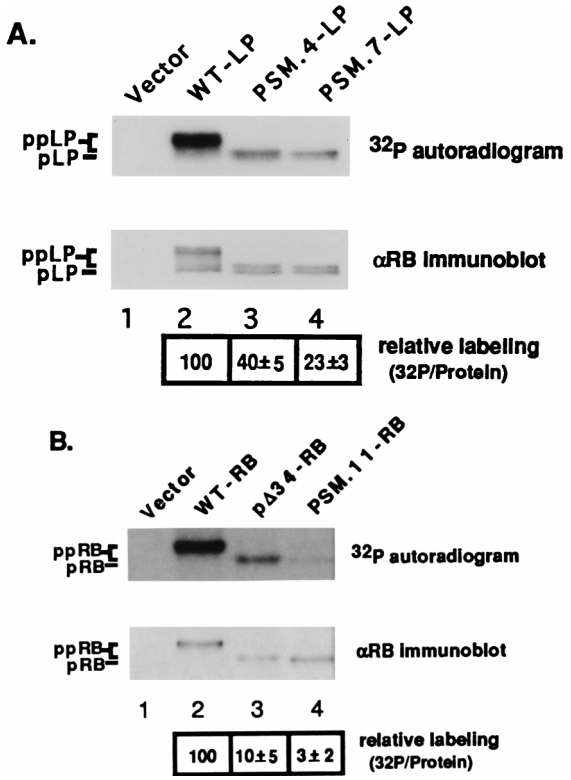


FIG. 3. In vivo phosphorylation of PSM-RB proteins. (A) Relative efficiency of PSM-LP phosphorylation in vivo. BOSC293 cells were transfected with either a vector control (lane 1) or plasmids expressing the following LP-RB proteins: WT-LP (lane 2), PSM.4-LP (lane 3), and PSM.7-LP (lane 4). The transfected cells were metabolically labeled with [$^{32}$P]phosphoric acid and lysed, and LP protein was immunoprecipitated with the anti-851 antibody. Immunocomplexes were recovered, resolved by SDS-7.5% PAGE, and transferred to Immobilon P. Phosphorylated LP was detected by autoradiography (upper panel), and total LP protein was detected by immunoblotting with anti-RB antibodies (lower panel). The ratio of $^{32}$P to protein for each LP protein was determined as described in Materials and Methods and normalized to WT-LP, which was arbitrarily set to 100. (B) Relative efficiency of PSM-RB phosphorylation in vivo. BOSC293 cells were transfected with either a vector control (lane 1) or a plasmid expressing the full-length WT(mu)-RB (lane 2), pΔ34-RB (lane 3), or PSM.11-RB (lane 4) proteins which were tagged with the HA epitope. The transfected cells were metabolically labeled with [$^{32}$P]phosphoric acid and lysed, and full-length proteins were immunoprecipitated with anti-HA antibody. Immunocomplexes were recovered, resolved by SDS-7.5% PAGE, and transferred to Immobilon P. Phosphorylated RB was detected by autoradiography (upper panel), and total RB protein was detected by immunoblotting with anti-RB antibodies (lower panel). The ratio of $^{32}$P to protein for each full-length protein was determined as described in Materials and Methods and normalized to WT-RB, which was arbitrarily set to 100. ppLP, hyperphosphorylated LP; ppRB, hyperphosphorylated full-length RB.

binding to E2F-1. Because phosphorylated PSM.9I-RB bound to E2F-1, phosphorylation of any of the six remaining sites (Thr5, Ser230, Ser249, Thr252, Thr356, and Ser567) must not be able to inhibit the E2F binding function of RB.

While mutation of Ser608 and Ser612 can abolish the phosphorylation-mediated inhibition of E2F-1 binding to PSM.7-RB, mutations of these two sites alone does not abolish regulation. The PSM.4NI-RB protein lacks the two N-terminal and the two insert domain phosphorylation sites (Fig. 1), but the binding of PSM.4NI to E2F-1 is still inhibited by phosphorylation (Fig. 6B, compare lanes 5 and 6). Therefore, when the exon 23-encoded sites are intact, phosphorylation can inhibit E2F binding even when Ser608 and Ser612 are not phosphorylated. Taken together, these results indicate that phosphorylation at either the exon 23-encoded sites or at Ser608 and

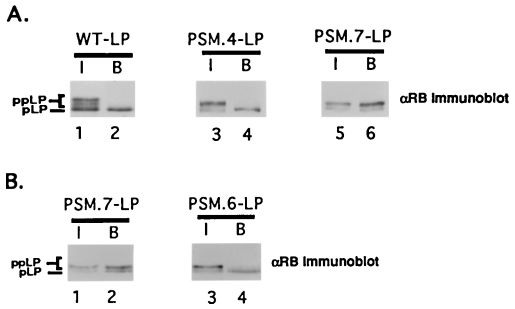


FIG. 4. In vivo-phosphorylated PSM.7-LP but not PSM.6-LP binds to E2F-1. (A) In vivo-phosphorylated PSM.7-LP binds to E2F-1. C33-A cells were cotransfected with cyclin D1, E, and A expression plasmids (Materials and Methods) and plasmids expressing either WT-LP (lanes 1 and 2), PSM.4-LP (lanes 3 and 4), or PSM.7-LP (lanes 5 and 6). Lysates prepared from the transfected cells were subjected to immunoprecipitation with anti-RB antibody (I) or applied to immobilized GST–E2F-1 (B). Proteins bound to E2F-1 were recovered and resolved by SDS-7.5% PAGE. LP proteins bound to E2F-1 were detected by anti-RB immunoblotting. (B) Phosphorylation at Ser780 is able to disrupt RB–E2F-1. C33-A cells were cotransfected with cyclin D1, E, and A expression plasmids and with either PSM.7-LP (lanes 1 and 2) or PSM.6-LP (lanes 3 and 4) expression plasmids. Lysates prepared from the transfected cells were either subjected to immunoprecipitation with anti-RB antibodies (lanes 1 and 3) or applied to immobilized GST–E2F-1 (lanes 2 and 4). Proteins recovered were resolved by SDS-7.5% PAGE, and LP protein was detected by immunoblotting with anti-RB antibodies.

Ser612 can inhibit E2F binding to the full-length RB. However, phosphorylation at Ser608 and Ser612 cannot inhibit the binding of E2F to the LP fragment (Fig. 2 and 4). The difference between the LP fragment and the full-length RB is the N-terminal region. Both the N-terminal region and the insert domain are not involved in the formation of the E2F binding site (47). However, the results shown here suggest that the

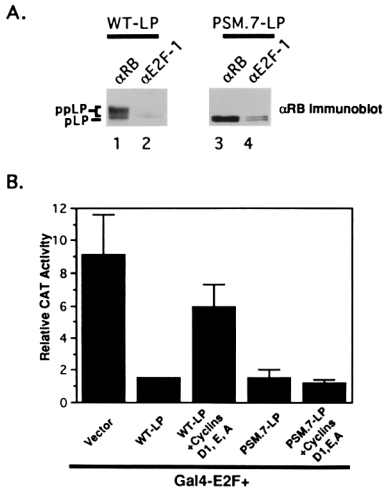


FIG. 5. Phosphorylated PSM.7-LP coprecipitates with E2F-1 and inhibits E2F-1 activity. (A) Phosphorylated PSM.7-LP coimmunoprecipitates with E2F-1. C33-A cells were cotransfected with cyclin D1, E, and A expression plasmids in conjunction with plasmids expressing E2F-1 and either WT-LP (lanes 1 and 2) or PSM.7-LP (lanes 3 and 4). Lysates from the transfected cells were prepared and either subjected to immunoprecipitation with anti-RB (lanes 1 and 3) or anti-E2F-1 (lanes 2 and 4) antibodies. The recovered proteins were resolved by SDS-7.5% PAGE, and LP proteins were visualized by immunoblotting with anti-RB antibodies. (B) Phosphorylated PSM.7-LP inhibits E2F-1 activity. C33-A cells were cotransfected with β-Gal and Gal4–E2F-1 expression plasmids, a Gal4-CAT reporter construct, and the indicated expression plasmids. Cells were harvested and assayed for CAT activities. Results show percentages of acetylation of [$^{14}$C]chloramphenicol normalized to β-Gal activity for transfection efficiency. The data shown are from two independent experiments.

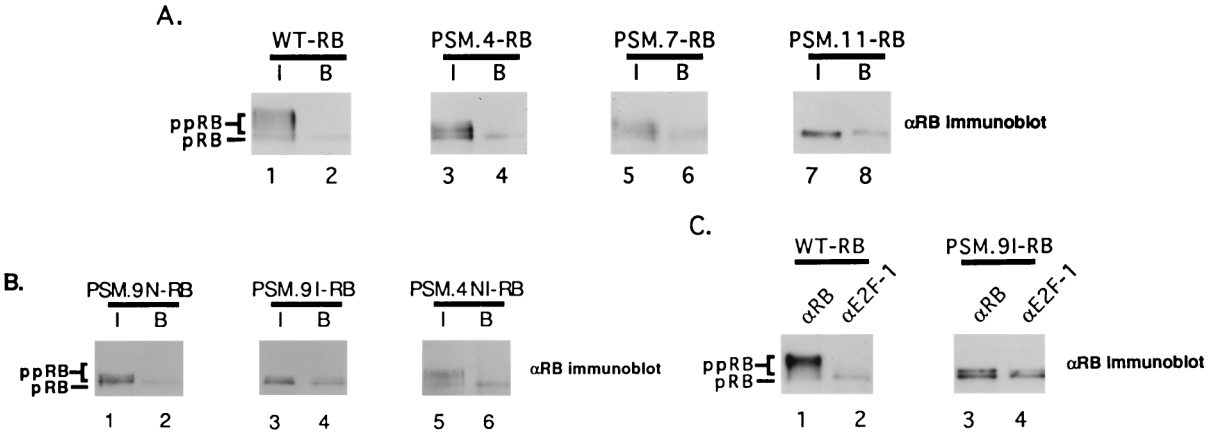


FIG. 6. Association of full-length RB and E2F-1 is disrupted by phosphorylation of Ser608 and/or Ser612. (A) Phosphorylated full-length PSM.7-RB does not bind E2F-1. C33-A cells were cotransfected with cyclin D1, E, and A expression plasmids and expression plasmids for the full-length RB proteins WT-RB (lanes 1 and 2), PSM.4-RB (lanes 3 and 4), PSM.7-RB (lanes 5 and 6), and PSM.11-RB. Lysates prepared from the transfected cells were either subjected to immunoprecipitation with anti-RB antibodies (lanes 1, 3, 5, and 7) or assayed for binding to immobilized GST–E2F-1 (lanes 2, 4, 6, and 8). Proteins recovered were resolved by SDS-6.5% PAGE, and LP protein was detected by immunoblotting with anti-RB antibodies. (B) Phosphorylated PSM.9I-RB binds E2F-1. C33-A cells were cotransfected with cyclin D1, E, and A expression plasmids and expression plasmids for the full-length RB protein PSM.9N-RB (lanes 1 and 2), PSM.9I-RB (lanes 3 and 4), or PSM.4NI (lanes 5 and 6). Lysates prepared from the transfected cells were either subjected to immunoprecipitation with anti-RB antibodies (lanes 1, 3, and 5) or assayed for binding to immobilized GST–E2F-1 (lanes 2, 4, and 6). Proteins recovered were resolved by SDS-6.5% PAGE, and LP protein was detected by immunoblotting with anti-RB antibodies. (C) Phosphorylated PSM.9I-RB coimmunoprecipitates with E2F-1. C33-A cells were cotransfected with cyclin D1, E, and A expression plasmids, E2F-1, and either WT-RB (lanes 1 and 2) or PSM.9I-RB (lanes 3 and 4) expression plasmids. Lysates from the transfected cells were prepared and subjected to immunoprecipitation with either anti-RB (lanes 1 and 3) or anti-E2F-1 (lanes 2 and 4) antibodies. The recovered proteins were resolved by SDS-6.5% PAGE, and RB proteins were visualized by immunoblotting with anti-RB antibodies.

phosphorylation sites in the insert domain can inhibit E2F binding when the N-terminal region is present. Because the regulatory role of Ser608 and Ser612 is observed only with full-length RB, while the regulatory role of exon 23 sites applies to both full-length and LP-RB, these two sets of phosphorylation sites are likely to regulate E2F binding through distinct mechanisms.

**Growth-suppressing activity of PSM-RB.** Release of E2F from RB is proposed to be an important step in the progression from $G_1$ into S phase (48). If this hypothesis is correct, an RB mutant that does not release E2F after phosphorylation should irreversibly block cells in $G_1$. Previously, Hamel et al. described an RB mutant, pΔ34-RB, which does not release E2F even after it is phosphorylated (1, 13, 14). pΔ34-RB has been shown to suppress cell proliferation (3, 53). However, in our hands, pΔ34-RB does not suppress the proliferation of Rat-1 cells or primary HFF (Fig. 7). Rat-1 cells were infected with recombinant retrovirus, producing either WT-RB or pΔ34-RB. During selection with puromycin, there was no apparent inhibition of colony formation, and equal numbers of drug-resistant clones were obtained with vector, WT-RB, or pΔ34-RB. The infected cells expressed the WT-RB and pΔ34-RB proteins at approximately the same level (Fig. 7A, lanes 2 and 3), and this expression level was stable for numerous passages over a period of several months. Cells expressing WT-RB or pΔ34-RB showed no growth disadvantage (Fig. 7B) or change in cell cycle distribution (Fig. 7C) compared with vector control-infected cells. To determine whether this result was specific for immortalized Rat-1 cells, we also expressed pΔ34-RB in HFF cells with amphitropic retroviruses. Drug-resistant HFF cells which stably expressed pΔ34-RB (Fig. 7D, lane 3) were again generated. The expression of pΔ34-RB had no discernible effect on cell growth (Fig. 7E) or cell cycle distribution (Fig. 7F) in these diploid human fibroblasts.

The above finding seemed contrary to the idea that the phosphorylation of RB is required for $G_1$/S progression (20, 29, 35, 36). We therefore assessed the in vivo phosphorylation level of pΔ34-RB and compared it to that of WT-RB and PSM.11-RB (Fig. 3B). We found that pΔ34-RB is phosphorylated in vivo (Fig. 3B, lane 3) to a lesser extent than WT-RB (lane 2) but more than PSM.11-RB (lane 4). Our results sug-

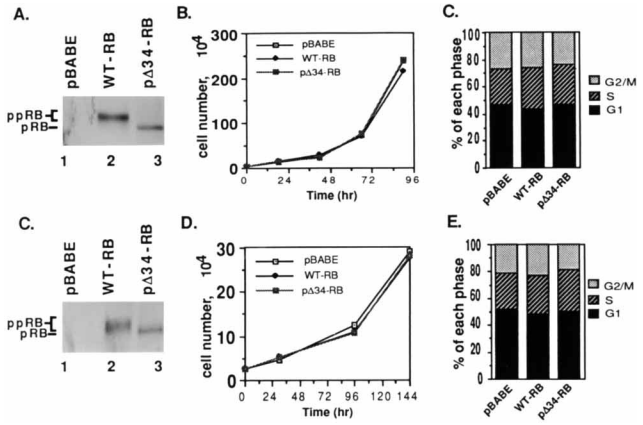


FIG. 7. pΔ34-RB does not arrest the growth of Rat-1 cells or HFF. Rat-1 cells (A to C) or primary HFF (D to F) were infected with amphitropic virus produced in BING293 cells (37). Selection of the infected cells was carried out with puromycin as described in Materials and Methods. (A and D) pΔ34-RB can be stably expressed. Lysates from the infected cells were prepared 2 weeks postinfection, and the ectopically expressed WT-RB (lane 2) or pΔ34-RB (lane 3) was immunoprecipitated with anti-HA antibody. Recovered protein was resolved by SDS-6.5% PAGE, and RB proteins were visualized by immunoblotting with anti-RB antibodies. (B and E) Expression of pΔ34-RB does not affect cell growth. Growth curves of vector (pBABE)-, WT-RB-, and pΔ34-RB-infected cells were determined by counting cells at the indicated times. Values are from three independent plates for each time point. (C and F) Expression of pΔ34-RB has no effect on cell cycle. Exponentially growing cells were harvested, fixed with ethanol, and stained with propidium iodide. The DNA content of the stained cells was determined with a FACScan cytometer with Cellfit software. The values shown are from two experiments.

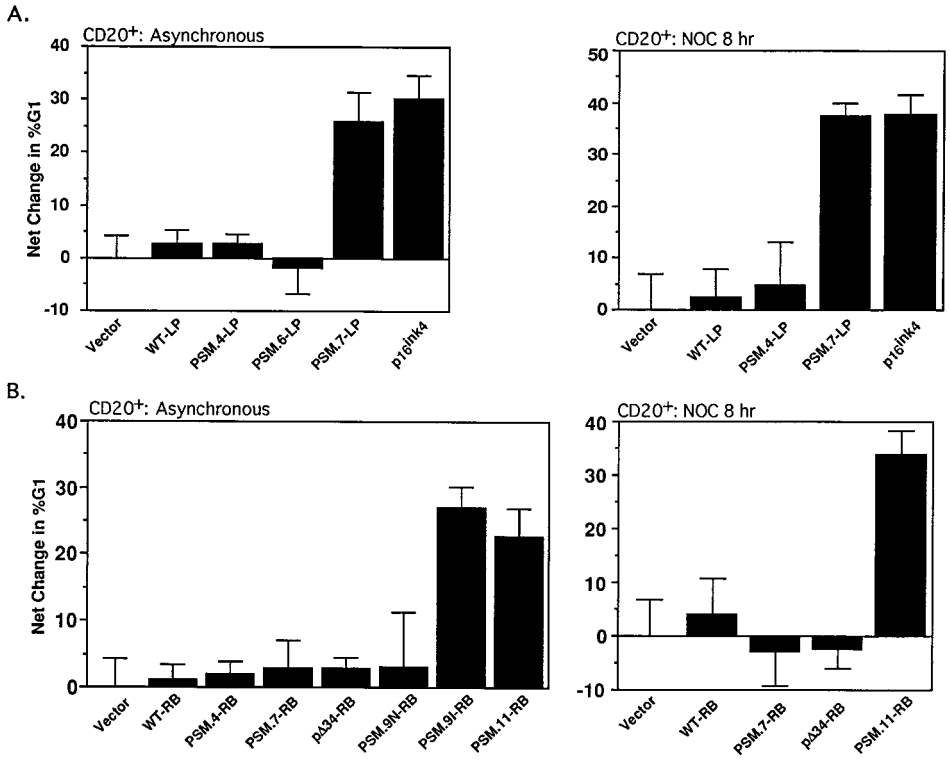


FIG. 8. Growth arrest of Rat-1 cells by PSM-RB proteins. Rat-1 cells were cotransfected with the indicated expression plasmids and a plasmid expressing the CD20 cell surface marker. Forty-eight hours posttransfection, the cells were either harvested without treatment (left panels) or were treated with NOC (0.1 μg/ml) for an additional 8 h (right panels). The cells were stained with FITC-labeled anti-CD20 antibodies, fixed in 80% ethanol, and then stained with propidium iodide. The DNA content of the CD20-positive cells was determined by analyzing the propidium iodide staining of FITC-positive cells with a FACScan cytometer with Cellfit software. The net change in percent $G_1$ was calculated by subtracting the percentage of CD20-positive $G_1$ cells in vector-transfected cells from that of PSM-RB-transfected culture. The values shown are from at least two independent experiments. (A) LP-RB-transfected cells; (B) full-length RB-transfected cells.

gest that the growth suppression function of Δ34-RB may be inactivated by this low level of phosphorylation.

In contrast to pΔ34-RB, several of the PSM mutants that we prepared could suppress Rat-1 cell proliferation. To demonstrate this, we utilized a transient assay in which WT, pΔ34, or PSM expression plasmids were each cotransfected with a plasmid that expresses the cell surface marker CD20. Transfected cells were identified by the expression of CD20, and the cell cycle distribution was determined by propidium iodide staining of DNA content (55). WT, pΔ34, and each of the PSM proteins induced a $G_1$ arrest in SAOS-2 cells, in which RB cannot become phosphorylated (not shown). Therefore, each of the PSM proteins has a growth suppression function in its unphosphorylated form.

In Rat-1 cells, which are capable of phosphorylating RB, neither WT-RB nor pΔ34-RB induced $G_1$ arrest (Fig. 8B), which was consistent with the results described above (Fig. 7). In vector-transfected control, approximately 45% of the $CD20^+$ cells were in $G_1$. A similar $G_1$ content was observed with $CD20^+$ cells cotransfected with WT-, PSM.4-, PSM.7-, pΔ34-, and PSM.9N-RB (Fig. 8B, left panel). However, the $G_1$ content increased to 70 to 75% with the expression of PSM.9I-RB and PSM.11-RB (Fig. 8B, left panel). To demonstrate that the observed $G_1$ increase was due to an arrest in $G_1$, transfected cells were treated with a microtubule poison, nocodazole (NOC), for 8 h (55). NOC-treated cells cannot complete mitosis and thus accumulate in culture with a 4 N DNA content. When cells are arrested in $G_1$, the DNA content will remain 2 N, whereas $G_1$-delayed cells will accumulate with 4 N DNA. With vector-transfected cells, the percentage of $G_1$ cells decreased to approximately 35%, with a concomitant increase in the population of cells in $G_2$ upon NOC treatment (not shown). A similar $G_1$ decrease was observed with WT-, PSM.7-, and pΔ34-RB transfected cells, while the $G_1$ content of PSM.11-RB transfected cells did not change upon NOC treatment (Fig. 8B, right panel). PSM.9I-RB was similarly found to induce $G_1$ arrest (not shown). These results show that PSM.9I-RB and PSM.11-RB, but not pΔ34-RB, can suppress Rat-1 cell proliferation.

The RB-LP constructs were also tested in Rat-1 cells. WT-, PSM.4- and PSM.6-LP did not cause a $G_1$ increase (Fig. 8A, left panel) or $G_1$ arrest (right panel). However, PSM.7-LP induced $G_1$ increase (Fig. 8A, left panel) and $G_1$ arrest (right panel) in Rat-1 cells. This is in contrast to the full-length PSM.7-RB, which does not function as a growth suppressor (Fig. 8B). The extent of $G_1$ increase induced by PSM.7-LP was similar to that induced by $p16^{INK4}$, which is known to block cells in $G_1$ by inhibiting the phosphorylation of RB (29, 35, 36).

**Rescue of PSM-mediated cell cycle arrest by E2F-1 and large T-antigen.** The growth suppression function of RB can be disrupted by viral oncoproteins such as the SV40 large T-antigen (46). Coexpression of T-antigen completely alleviated the $G_1$ arrest caused by either PSM.7-LP (Fig. 9A) or PSM.11-RB (Fig. 9B). In SAOS-2 cells, expression of several cellular proteins can also disrupt RB function; these include cyclins A and E (20), E2F-1 (40), c-Myc (11), kinase-defective c-Abl (52), and Mdm-2 (53). Therefore, we challenged the PSM-mediated arrest of Rat-1 cells with expression plasmids

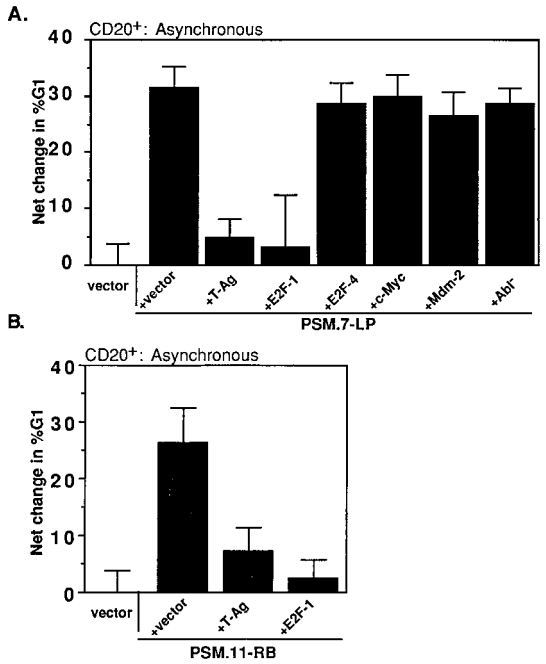


FIG. 9. Rescue of PSM-RB-induced $G_1$ arrest. Rat-1 cells were cotransfected with the indicated expression plasmids and a plasmid expressing the CD20 cell surface marker. Forty-eight hours posttransfection, the cells were harvested and stained with FITC-labeled anti-CD20 antibodies, fixed in 80% ethanol, and stained with propidium iodide. The DNA content of the CD20-positive cells was determined by analyzing the propidium iodide staining of FITC-positive cells with a FACScan flow cytometer with Cellfit software. $Abl^-$, kinase-defective c-Abl; c-Myc, E2F-1, and E2F-4 are the given transcription factors; Mdm-2 is the proto-oncoprotein; T-Ag, SV40 large T-antigen. The net change in percent $G_1$ was calculated as described in the legend to Fig. 8. Data shown are from at least two independent experiments. (A) LP-RB-transfected cells; (B) full-length RB-transfected cells.

which produce these proteins. When it was tested in Rat-1 cells, we found that cotransfection with E2F-1 expression plasmid rescued the $G_1$ arrest induced by either PSM.7-LP or PSM.11-RB (Fig. 9). The expression of E2F-4 did not reverse the cell cycle arrest. Expression of c-Myc, Mdm-2, or kinase-defective c-Abl also failed to rescue the PSM-induced $G_1$ block (Fig. 9). Cotransfection with cyclin D1, E, or A also did not efficiently reverse the block induced by PSM.11-RB or PSM.7-LP (not shown). Thus, only E2F-1 and T-antigen are able to abrogate the growth suppression function of PSM-RB in Rat-1 cells.

## DISCUSSION

**Regulation of RB-E2F interaction by phosphorylation. (i) Regulation of E2F binding to the LP fragment of RB.** The E2F binding site in RB is composed of amino acids in the A, B, and C regions. The RB-LP fragment, which binds E2F, is a functional growth suppressor (19, 20, 39). The binding of RB-LP to E2F is disrupted by phosphorylation at the seven phosphorylation sites clustered in the 55-amino-acid sequence of exon 23 (Fig. 1). The combined mutations of four of the seven sites in PSM.4-LP (lacking Ser807 and Ser811 and Thr821 and Thr826) or the reciprocal combined mutations of the other three sites in PSM.3 (lacking Ser780, 788, and 795) cannot abolish phosphorylation-mediated inhibition of E2F binding to LP (Table 1). Even with the mutant PSM.6-LP, which contains only one of the seven exon 23-encoded sites (Ser780), phosphorylation can still inhibit the binding of E2F. Consistent with this finding is the result reported by Kitagawa et al. (27). Using a monoclonal antibody against the phosphorylated Ser780 epitope, they showed that Ser780-phosphorylated RB does not bind to E2F (27). In the RB-LP context, elimination of all seven exon 23-encoded sites can abolish the regulation of E2F binding, as demonstrated by the binding of phosphorylated PSM.7-LP to E2F (Fig. 2, 4, and 5). Thus, phosphorylation at the three remaining sites, Ser567, Ser608, and Ser612, cannot disrupt RB-LP–E2F interaction when all seven exon 23-encoded sites are lost. However, at least two combinations of the insert domain and exon 23-encoded sites can disrupt E2F binding, as revealed by the two PSM.6-LP mutants. In PSM.6-LP, the combination of insert domain sites and Ser780 can disrupt E2F binding upon phosphorylation. In PSM.6ST-LP, the combination of one insert domain site (Ser608) and Ser780 and Ser795 can also disrupt E2F binding. Taken together, these results suggest that RB-LP–E2F interaction can be disrupted by a number of phosphorylation sites in the insert domain and exon 23-encoded region. These phosphorylation sites are likely to have redundant function in the regulation of RB-LP binding to E2F.

**(ii) Regulation of E2F binding to full-length RB.** We observed a major difference between the full-length RB versus the LP fragment with respect to the effect of insert domain phosphorylation sites (Ser608 and Ser612) on E2F binding. In the LP context, as discussed above, phosphorylation at the insert domain sites is not sufficient to inhibit E2F binding. At least one exon 23-encoded site needs to be present in order for phosphorylation to disrupt E2F binding to the RB-LP. However, in the full-length context, the insert domain sites can regulate E2F binding because mutation of all seven exon 23-encoded sites does not abolish regulation. The combined mutations of all seven exon 23-encoded sites and Ser608 and Ser612 are required to disrupt regulation of E2F binding by phosphorylation. With full-length RB, we also observed redundant roles for the multiple phosphorylation sites. This is demonstrated by the two reciprocal mutants PSM.7-RB and PSM.4NI-RB (Table 1). Both PSM.7-RB and PSM.4NI-RB are regulated for E2F binding, but the combined mutant PSM.11-RB is not regulated. The minimal combination is found with PSM.9I-RB, in which the seven exon 23-encoded sites and two insert domain sites (Ser608 and Ser612) are lost (Table 1; Fig. 1). Taken together, these results suggest that when either the exon 23-encoded sites or the insert domain sites are phosphorylated, they can disrupt the RB-E2F interaction. Others have shown that phosphorylated pΔ34-RB binds E2F (1, 13), and we could confirm this result with pΔ34-RB expressed in Rat-1 cells (data not shown). Eight phosphorylation sites are lost in pΔ34-RB (Fig. 1). Mutations that are common between pΔ34-RB and PSM.9I-RB are (i) the two insert domain sites (Ser608 and 612) and (ii) four of the seven exon 23-encoded sites (Ser788, 795, 807, and 811). Phosphorylation at these six serine sites is likely to inhibit E2F binding either alone or in combination. Three of the exon 23-encoded sites are intact in pΔ34-RB (the murine equivalent of human Ser780, Thr821, and Thr826). Because phosphorylated pΔ34-RB binds to E2F, phosphorylation of Ser780 and Thr821 and 826 in the context of pΔ34-RB must not be able to inhibit E2F binding. Alternatively, Ser780 and Thr821 and 826 may not be efficiently phosphorylated in pΔ34-RB. Nevertheless, studies of PSM mutants of RB have identified the insert domain and exon 23-encoded cdk sites as the regulators of RB-E2F binding.

**(iii) Dual mechanisms for the regulation of E2F binding to RB.** With full-length RB, our results suggest that E2F binding

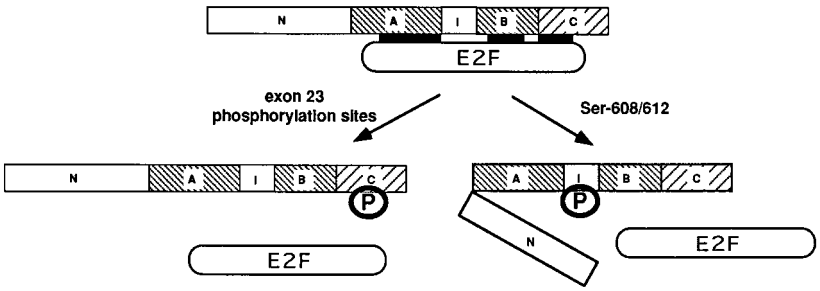


FIG. 10. Dual mechanisms for inhibition of E2F binding by RB phosphorylation. The E2F binding site in RB involves sequences in the A, B, and C regions. The phosphorylation of RB inhibits E2F binding (bottom). The present study has identified two distinct mechanisms for this inhibition. First, phosphorylation of RB at several exon 23-encoded cdk sites can disrupt binding to E2F. Second, phosphorylation of Ser608 and/or Ser612 in the insert domain also inhibits binding; however, this occurs with the full-length RB, but not with the LP fragment. Because this second mechanism is dependent on the N-terminal region, a conformational change involving the N-terminal region may underlie phospho-Ser608 and -Ser612-dependent inhibition of E2F binding to RB.

can be regulated by either the insert domain or the exon 23-encoded phosphorylation sites (Fig. 10). Because exon 23-encoded amino acids are required for the formation of the E2F binding site (19, 39, 51), it is conceivable that the E2F binding site can be directly affected by phosphorylation at the exon 23-encoded cdk sites. However, phosphorylation at these exon 23-encoded sites is not the only way to inhibit E2F binding. This is indicated by PSM.7-RB, which lacks all seven exon 23-encoded sites, but its binding to E2F is still inhibited by phosphorylation through the phosphorylation of the insert domain sites Ser608 and 612. The insert domain is not directly involved in E2F binding, since this domain can be replaced with random amino acids without affecting E2F binding (47). It is interesting to find that phosphorylation at the insert domain Ser608 and Ser612 can disrupt full-length RB binding to E2F even when all the exon 23-encoded sites are mutated (compare results obtained with PSM.7-RB and PSM.9I-RB). This inhibitory effect appears to be dependent on the N-terminal region of RB, because insert domain phosphorylation is not sufficient to disrupt the binding of RB-LP to E2F (PSM.7-LP [Table 1]). The N-terminal region of RB is also dispensable for E2F binding (19, 39). However, the N-terminal region and the insert domain may play modulatory roles in the conformation of the E2F binding site. This idea is supported by three lines of evidence. (i) While the complete deletion of the N region has no effect on E2F binding, small internal deletions within this region can inhibit RB-E2F interaction (38). (ii) The A and B domains of RB, separated by the insert, interact with each other to form the E2F binding site (5). (iii) The N and C regions of RB may interact with each other (16). We propose that phosphorylation at the insert domain sites may affect one or more of these intramolecular interactions to inhibit E2F binding (Fig. 10).

In summary, the binding of E2F to RB is regulated by a number of cdk sites in RB through two distinct mechanisms. This is in contrast to the regulation of RB binding to T-antigen and c-Abl tyrosine kinase. We have previously shown that Thr821 and Thr826 are required for the inhibition of T-antigen binding by phosphorylation. PSM.2T-RB, lacking these two sites, binds to T-antigen in its phosphorylated form (28). The regulation of c-Abl binding to RB requires Ser807 and Ser811, and PSM.2S-RB lacking these two sites binds to c-Abl in its phosphorylated form (28). The recent finding that different cdk-cyclin complexes preferentially phosphorylate specific phosphorylation sites within RB (6, 27, 54) raises the possibility that different protein binding activities of RB may be specifically targeted by a particular cdk-cyclin. Our finding that multiple sites regulate E2F binding suggests that the inactivation of E2F-binding activity can be achieved by a number of different cdk-cyclin complexes, whereas the inactivation of T-antigen or c-Abl binding may be cdk-cyclin specific. Taken together, the cdk sites in RB are not functionally equivalent, but a majority of them are involved in the regulation of E2F binding.

**PSM-RB proteins as constitutive growth suppressors.** The inactivation of RB by phosphorylation has been proposed to be a requirement for cell cycle progression through the $G_1$ restriction point (48). A strong prediction of this model is that phosphorylation-defective RB proteins should block cells in $G_1$. The pΔ34-RB protein has a stronger growth suppression function than wild-type RB (3, 53) but is not a constitutive gain-of-function mutant. We have shown that pΔ34-RB does not arrest the growth of Rat-1 cells or HFF (Fig. 7 and 8). In contrast, PSM.7-LP, PSM.11-RB, and PSM.9I-RB behave as constitutive suppressors of Rat-1 cell growth (Fig. 8). Because PSM.3-LP, PSM.4-LP, PSM.6-LP, PSM.7-RB, and PSM.9N-RB are regulated for E2F binding and do not block Rat-1 cells in $G_1$, E2F binding is clearly required for RB-induced $G_1$ arrest. However, because pΔ34-RB, which binds to E2F irrespective of phosphorylation (1, 13), cannot arrest Rat-1 cells in $G_1$, binding to E2F alone must not be sufficient for RB to induce a $G_1$ block. That E2F binding is necessary but not sufficient for RB to suppress growth is discussed in a recent review (46).

The pΔ34-RB protein contains intact Thr821 and Thr826 sites. Phosphorylation at these two Thr sites inhibits RB binding to proteins with the LXCXE motif (28). The failure of pΔ34-RB to arrest cells in $G_1$ could, therefore, be due to the release of critical LXCXE proteins upon phosphorylation at Thr821 and Thr826. PSM.9I-RB, which differs from pΔ34-RB in that it lacks Thr821 and Thr826, can arrest cells in $G_1$. This strongly suggests that phosphorylation of Thr821 or Thr826 can inactivate the growth suppression function of RB.

The $G_1$ arrest induced by PSM-RB in Rat-1 cells can be overcome by E2F-1 and SV40 large T-antigen, but not by E2F-4 (Fig. 9). This observation is similar to the previously reported disruption of WT-RB growth-inhibitory activity by E2F-1 and T-antigen, but not by E2F-4 in SAOS-2 cells (28, 40, 45). However, the effect of PSM-RB in Rat-1 cells cannot be rescued by ectopic expression of c-Myc, Mdm-2, or a kinase-defective c-Abl, while the growth suppression function of WT-RB in SAOS-2 cells is reversed by these proteins (11, 50, 53). The reasons for the discrepancy observed with these two cell systems are not clear at this time. The $G_1$ arrest induced by PSM-RB in Rat-1 cells is also not efficiently overcome by the ectopic expression of cyclin D1, cyclin E, or cyclin A, consistent with the fact that PSM-RB cannot be inactivated by phosphor-

ylation. In any event, we have succeeded in preparing phosphorylation site-defective RB proteins that function as constitutive inhibitors of $G_1$/S progression. These PSM-RB proteins are phosphorylated to a low level in vivo; however, their protein binding functions cannot be inactivated by phosphorylation at the remaining sites. Our results support the idea that inactivation of the protein binding functions of RB by phosphorylation is a necessary step for entry into S phase.

## ACKNOWLEDGMENTS

We thank K. E. Knudsen, W. Arap, L. L. Whitaker, H. Su, X. Tan, and R. Baskaran for reagents, helpful suggestions, and critical reading of the manuscript. We also thank the following for the provision of reagents: D. Baltimore (MIT), D. Green (La Jolla Institute of Allergy and Immunology), S. I. Reed (Scripps Research Institute), D. Spector (UCSD), W. G. Kaelin (Dana-Farber Cancer Institute), D. M. Livingston (Dana Farber Cancer Institute), E. Harlow (Massachusetts General Hospital), J. Roberts (Fred Hutchinson Cancer Research Center), P. Hamel (University of Toronto), D. Green (Burnham Institute), A. Yee (Tufts University), and C. Sherr (St. Jude's Hospital).

This work was supported by a grant to J.Y.J.W. from the National Institutes of Health (CA58320).

## REFERENCES

1. **Bremner, R., B. L. Cohen, M. Sopta, P. A. Hamel, C. J. Ingles, B. L. Gallie, and R. A. Phillips.** 1995. Direct transcriptional repression by pRB and its reversal by specific cyclins. Mol. Cell. Biol. **15:**3256–3265.
2. **Buchkovich, K., L. A. Duffy, and E. Harlow.** 1989. The retinoblastoma protein is phosphorylated during specific phases of the cell cycle. Cell **58:**1097–1105.
3. **Chang, M. W., E. Barr, J. Seltzer, Y. Q. Jiang, G. J. Nabel, E. G. Nabel, M. S. Parmacek, and J. M. Leiden.** 1995. Cytostatic gene therapy for vascular proliferative disorders with a constitutively active form of the retinoblastoma gene product. Science **267:**518–522.
4. **Chellappan, S., V. B. Kraus, B. Kroger, K. Munger, P. M. Howley, W. C. Phelps, and J. R. Nevins.** 1992. Adenovirus E1A, simian virus 40 tumor antigen, and human papillomavirus E7 protein share the capacity to disrupt the interaction between transcription factor E2F and the retinoblastoma gene product. Proc. Natl. Acad. Sci. USA **89:**4549–4553.
5. **Chow, K. N., P. Starostik, and D. C. Dean.** 1996. The Rb family contains a conserved cyclin-dependent-kinase-regulated transcriptional repressor motif. Mol. Cell. Biol. **16:**7173–7181.
6. **Connell-Crowley, L., J. W. Harper, and D. W. Goodrich.** 1997. Cyclin D1/Cdk4 regulates retinoblastoma protein-mediated cell cycle arrest by site-specific phosphorylation. Mol. Biol. Cell **8:**287–301.
7. **DeGregori, J., T. Kowalik, and J. R. Nevins.** 1995. Cellular targets for activation by the E2F1 transcription factor include DNA synthesis- and $G_1$/S-regulatory genes. Mol. Cell. Biol. **15:**4215–4224.
8. **Fattaey, A. R., E. Harlow, and K. Helin.** 1993. Independent regions of adenovirus E1A are required for binding to and dissociation of E2F-protein complexes. Mol. Cell. Biol. **13:**7267–7277.
9. **Flemington, E. K., S. H. Speck, and W. G. Kaelin, Jr.** 1993. E2F-1-mediated transactivation is inhibited by complex formation with the retinoblastoma susceptibility gene product. Proc. Natl. Acad. Sci. USA **90:**6914–6918.
10. **Goodrich, D. W., N. P. Wang, Y. W. Qian, E. Y. Lee, and W. H. Lee.** 1991. The retinoblastoma gene product regulates progression through the G1 phase of the cell cycle. Cell **67:**293–302.
11. **Goodrich, D. W., and W. H. Lee.** 1992. Abrogation by c-myc of G1 phase arrest induced by RB protein but not by p53. Nature **360:**177–179.
12. **Hamel, P. A., B. L. Cohen, L. M. Sorce, B. L. Gallie, and R. A. Phillips.** 1990. Hyperphosphorylation of the retinoblastoma gene product is determined by domains outside the simian virus 40 large-T-antigen-binding regions. Mol. Cell. Biol. **10:**6586–6595.
13. **Hamel, P. A., R. M. Gill, R. A. Phillips, and B. L. Gallie.** 1992. Regions controlling hyperphosphorylation and conformation of the retinoblastoma gene product are independent of domains required for transcriptional repression. Oncogene **7:**693–701.
14. **Hamel, P. A., R. M. Gill, R. A. Phillips, and B. L. Gallie.** 1992. Transcriptional repression of the E2-containing promoters EIIaE, c-*myc*, and *RB1* by the product of the *RB1* gene. Mol. Cell. Biol. **12:**3431–3438.
15. **Hamel, P. A., R. A. Phillips, M. Muncaster, and B. L. Gallie.** 1993. Speculations on the roles of RB1 in tissue-specific differentiation, tumor initiation, and tumor progression. FASEB J. **7:**846–854.
16. **Hensey, C. E., F. T. Hong, T. Durfee, Y. W. Qian, E. Y. Lee, and W. H. Lee.** 1996. Identification of discrete structural domains in the retinoblastoma protein. Amino-terminal domain is required for its oligomerization. J. Biol. Chem. **269:**1380–1387.
17. **Herrera, R. E., T. P. Mäkelä, and R. A. Weinberg.** 1996. TGF beta-induced growth inhibition in primary fibroblasts requires the retinoblastoma protein. Mol. Biol. Cell **7:**1335–1342.
18. **Herrera, R. E., V. P. Sah, B. O. Williams, T. P. Mäkelä, R. A. Weinberg, and T. Jacks.** 1996. Altered cell cycle kinetics, gene expression, and $G_1$ restriction point regulation in *Rb*-deficient fibroblasts. Mol. Cell. Biol. **16:**2402–2407.
19. **Hiebert, S. W.** 1993. Regions of the retinoblastoma gene product required for its interaction with the E2F transcription factor are necessary for E2 promoter repression and pRb-mediated growth suppression. Mol. Cell. Biol. **13:**3384–3391.
20. **Hinds, P. W., S. Mittnacht, V. Dulic, A. Arnold, S. I. Reed, and R. A. Weinberg.** 1992. Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. Cell **70:**993–1006.
21. **Hu, Q. J., N. Dyson, and E. Harlow.** 1990. The regions of the retinoblastoma protein needed for binding to adenovirus E1A or SV40 large T antigen are common sites for mutations. EMBO J. **9:**1147–1155.
22. **Huang, P. S., D. R. Patrick, G. Edwards, P. J. Goodhart, H. E. Huber, L. Miles, V. M. Garsky, A. Oliff, and D. C. Heimbrook.** 1993. Protein domains governing interactions between E2F, the retinoblastoma gene product, and human papillomavirus type 16 E7 protein. Mol. Cell. Biol. **13:**953–960.
23. **Huang, S., N. P. Wang, B. Y. Tseng, W. H. Lee, and E. H. Lee.** 1990. Two distinct and frequently mutated regions of retinoblastoma protein are required for binding to SV40 T antigen. EMBO J. **9:**1815–1822.
24. **Ikeda, M. A., and J. R. Nevins.** 1993. Identification of distinct roles for separate E1A domains in disruption of E2F complexes. Mol. Cell. Biol. **13:**7029–7035.
25. **Johnson, D. G., J. K. Schwarz, W. D. Cress, and J. R. Nevins.** 1993. Expression of transcription factor E2F1 induces quiescent cells to enter S-phase. Nature **365:**349–352.
26. **Kaelin, W. G., Jr., M. E. Ewen, and D. M. Livingston.** 1990. Definition of the minimal simian virus 40 large T antigen- and adenovirus E1A-binding domain in the retinoblastoma gene product. Mol. Cell. Biol. **10:**3761–3769.
27. **Kitagawa, M., H. Higashi, H. K. Jung, I. Suzuki-Takahashi, M. Ikeda, K. Tamai, J. Kato, K. Segawa, E. Yoshida, and S. Nishimura.** 1996. The consensus motif for phosphorylation by cyclin D1-Cdk4 is different from that for phosphorylation by cyclin A/E-Cdk2. EMBO J. **15:**7060–7069.
28. **Knudsen, E. S., and J. Y. Wang.** 1996. Differential regulation of retinoblastoma protein function by specific Cdk phosphorylation sites. J. Biol. Chem. **271:**8313–8320.
29. **Koh, J., G. H. Enders, B. D. Dynlacht, and E. Harlow.** 1995. Tumour-derived p16 alleles encoding proteins defective in cell-cycle inhibition. Nature **375:**506–510.
30. **Lam, E. W., and R. J. Watson.** 1993. An E2F-binding site mediates cell-cycle regulated repression of mouse B-myb transcription. EMBO J. **12:**2705–2713.
31. **Lees, J. A., K. J. Buchkovich, D. R. Marshak, C. W. Anderson, and E. Harlow.** 1991. The retinoblastoma protein is phosphorylated on multiple sites by human cdc2. EMBO J. **10:**4279–4290.
32. **Lin, B. T., S. Gruenwald, A. O. Morla, W. H. Lee, and J. Y. Wang.** 1991. Retinoblastoma cancer suppressor gene product is a substrate of the cell cycle regulator cdc2 kinase. EMBO J. **10:**857–864.
33. **Lin, B. T., and J. Y. Wang.** 1992. Cell cycle regulation of retinoblastoma protein phosphorylation. Ciba Found. Symp. **170:**227–241.
34. **Ludlow, J. W., C. L. Glendening, D. M. Livingston, and J. A. DeCarprio.** 1993. Specific enzymatic dephosphorylation of the retinoblastoma protein. Mol. Cell. Biol. **13:**367–372.
35. **Lukas, J., D. Parry, L. Aagaard, D. J. Mann, J. Bartkova, M. Strauss, G. Peters, and J. Bartek.** 1995. Retinoblastoma-protein-dependent cell-cycle inhibition by the tumour suppressor p16. Nature **375:**503–506.
36. **Medema, R. H., R. E. Herrera, F. Lam, and R. A. Weinberg.** 1995. Growth suppression by p16ink4 requires functional retinoblastoma protein. Proc. Natl. Acad. Sci. USA **92:**6289–6293.
37. **Pear, W. S., G. P. Nolan, M. L. Scott, and D. Baltimore.** 1993. Production of high-titer helper-free retroviruses by transient transfection. Proc. Natl. Acad. Sci. USA **90:**8392–8396.
38. **Qian, Y., C. Luckey, L. Horton, M. Esser, and D. J. Templeton.** 1992. Biological function of the retinoblastoma protein requires distinct domains for hyperphosphorylation and transcription factor binding. Mol. Cell. Biol. **12:**5363–5372.
39. **Qin, X. Q., T. Chittenden, D. M. Livingston, and W. G. Kaelin, Jr.** 1992. Identification of a growth suppression domain within the retinoblastoma gene product. Genes Dev. **6:**953–964.
40. **Qin, X. Q., D. M. Livingston, M. Ewen, W. R. Sellers, Z. Arany, and W. G. Kaelin, Jr.** 1995. The transcription factor E2F-1 is a downstream target of RB action. Mol. Cell. Biol. **15:**742–755.
41. **Sellers, W. R., J. W. Rodgers, and W. G. Kaelin, Jr.** 1995. A potent transrepression domain in the retinoblastoma protein induces a cell cycle arrest when bound to E2F sites. Proc. Natl. Acad. Sci. USA **92:**11544–11548.
42. **Sherr, C. J.** 1996. Cancer cell cycles. Science **274:**1672–1677.
43. **Slansky, J. E., and P. J. Farnham.** 1996. Introduction to the E2F family: protein structure and gene regulation. Curr. Top. Microbiol. Immunol. **208:**1–30.
44. **Templeton, D. J., S. H. Park, L. Lanier, and R. A. Weinberg.** 1991. Non-

functional mutants of the retinoblastoma protein are characterized by defects in phosphorylation, viral oncoprotein association, and nuclear tethering. Proc. Natl. Acad. Sci. USA **88:**3033–3037.

45. **Vairo, G., D. M. Livingston, and D. Ginsberg.** 1995. Functional interaction between E2F-4 and p130: evidence for distinct mechanisms underlying growth suppression by different retinoblastoma protein family members. Genes Dev. **9:**869–881.
46. **Wang, J. Y.** 1997. Retinoblastoma protein in growth suppression and death protection. Curr. Opin. Genet. Dev. **7:**39–45.
47. **Wang, J. Y., E. S. Knudsen, and P. J. Welch.** 1994. The retinoblastoma tumor suppressor protein. Adv. Cancer Res. **64:**25–85.
48. **Weinberg, R. A.** 1994. The retinoblastoma protein and cell cycle control. Cell **81:**323–330.
49. **Weintraub, S. J., C. A. Prater, and D. C. Dean.** 1992. Retinoblastoma protein switches the E2F site from positive to negative element. Nature **358:**259–261.
50. **Welch, P. J., and J. Y. Wang.** 1993. A C-terminal protein-binding domain in the retinoblastoma protein regulates nuclear c-Abl tyrosine kinase in the cell cycle. Cell **75:**779–790.
51. **Welch, P. J., and J. Y. Wang.** 1995. Disruption of retinoblastoma protein function by coexpression of its C pocket fragment. Genes Dev. **9:**31–46.
52. **Welch, P. J., and J. Y. Wang.** 1995. Abrogation of retinoblastoma protein function by c-Abl through tyrosine kinase-dependent and -independent mechanisms. Mol. Cell. Biol. **15:**5542–5551.

52a. **Whitaker, L. L., H. Su, and J. Y. J. Wang.** Unpublished data.

53. **Xiao, Z. X., J. Chen, A. J. Levine, N. Modjtahedi, J. Xing, W. R. Sellers, and D. M. Livingston.** 1995. Interaction between the retinoblastoma protein and the oncoprotein MDM2. Nature **375:**694–698.
54. **Zarkowska, T., and S. Mittnacht.** 1997. Differential phosphorylation of the retinoblastoma protein by G(1)/S cyclin-dependent kinases. J. Biol. Chem. **272:**12738–12746.
55. **Zhu, L., S. van den Heuvel, K. Helin, A. Fattaey, M. Ewen, D. Livingston, N. Dyson, and E. Harlow.** 1993. Inhibition of cell proliferation by p107, a relative of the retinoblastoma protein. Genes Dev. **7:**1111–1125.
56. **Zwicker, J., F. C. Lucibello, L. A. Wolfraim, C. Gross, M. Truss, K. Engeland, and R. Müller.** 1995. Cell cycle regulation of the cyclin A, cdc25C and cdc2 genes is based on a common mechanism of transcriptional repression. EMBO J. **14:**4514–4522.
57. **Zwicker, J., N. Liu, K. Engeland, F. C. Lucibello, and R. Müller.** 1996. Cell cycle regulation of E2F site occupation in vivo. Science **271:**1595–1597.