Differential Regulation of Retinoblastoma Protein Function by Specific Cdk Phosphorylation Sites*

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The retinoblastoma tumor suppressor protein, RB, contains at least three distinct protein binding domains. The A/B pocket binds proteins with the LXCXE motif, the C pocket binds the nuclear c-Abl tyrosine kinase, and the large A/B pocket binds the transcription factor E2F. Dissociation of RB from its targets is observed as RB becomes phosphorylated during G1/S progression. There are 16 Cdk consensus phosphorylation sites in RB. It was previously unknown whether the many phosphorylation sites had redundant or distinct functions in the regulation of RB. Using RB mutant proteins lacking specific phosphorylation sites, we show that each of the binding domains is inhibited by different sites. Thr-821/826 phosphorylation is required to inhibit the binding to LXCXE containing proteins. Mutation of these two sites does not interfere with the hyperphosphorylation of RB. However, this phosphorylated mutant retains the ability to bind T-Ag, E7, and Elf-1, all of which contain the LXCXE motif. In contrast, Ser-807/811 phosphorylation is required to disrupt c-Abl binding. Mutation of Ser-807/811 and Thr-821/826 does not abolish the regulation of E2F binding. Taken together, these results show that the protein binding domains of RB are each regulated by distinct Cdk phosphorylation sites.

The retinoblastoma susceptibility gene, RB-1, was cloned on the basis of its biallelic inactivation in human retinoblastomas (1, 2). Rb-1 was subsequently observed to be mutated in a variety of tumor types, indicating that it may play a general role in the inhibition of the transformed phenotype (3, 4). Reintroduction of Rb-1 into some Rb—/— cells can lead to decreased tumorigenicity in nude mice or inhibition of growth in culture (5). Furthermore, ectopic expression of Rb-1 or microinjection of RB protein can block cell cycle progression at G1/S (6–8).

RB forms complexes with many proteins, and this protein binding activity is required for growth suppression. RB binds to its target proteins by several different mechanisms (1). Viral oncoproteins such as the SV40 large T-antigen (T-Ag), and several cellular proteins, e.g. D-type cyclins and Elf-1, contain the LXCXE motif that is important for binding to the A/B pocket of RB (9–11). The E2F transcription factors do not contain the LXCXE motif and their binding requires the A/B pocket and C-terminal amino acids, and this E2F binding site is called the "large A/B pocket" (7, 12–14). The C-terminal region of RB also contains an A/B pocket-independent binding domain, the C pocket, which binds to the nuclear c-Abl tyrosine kinase (15). The large A/B pocket and the C pocket of RB do not overlap, because RB can simultaneously bind to E2F and c-Abl in vitro and in vivo (16). In addition, complexes containing T-Ag/RB/c-Abl and cyclin D2/RB/c-Abl have been detected (15, 16). The protein assembly function of RB is known to be required for growth suppression, since overexpression of the individual domains can inactivate RB biological function (16).

The protein binding function of RB is regulated by phosphorylation (1). RB phosphorylation is observed as cells progress from G1 to S phase of the cell cycle, and this is correlated with the disruption of RB-assembled protein complexes. RB contains 16 Ser/Thr-Pro motifs which are potential Cdk phosphorylation sites. At least seven of these sites (Ser-249, -807, -811, and Thr-252, -373, -821, and -826) have been shown to be phosphorylated in vivo (17–19). A number of other sites are also phosphorylated in vivo, but the exact identity of these sites is unknown (17–19). In mitotic cells, cyclin B is the principle RB kinase (19). In interphase cells, RB is phosphorylated by other cyclin-cdk complexes, including cyclin D1/cdk4 and cyclin D2/cyclin A (2). Several Cdk consensus phosphorylation sites have been mutated in human and murine RB (9, 20–22). Elimination of eight Cdk consensus phosphorylation sites in murine RB abolishes phosphorylation in vivo, and correlates with a more effective suppression of E2F or Elf-1 activity in transfected cells (11, 22). The previous analysis of phosphorylation site mutants of RB did not address the question of whether the multiple Cdk phosphorylation sites of RB had redundant regulatory function.

Since RB can bind its target proteins through at least three different mechanisms, we hypothesized that each protein binding function might be regulated by distinct phosphorylation sites (1). This hypothesis would suggest that the multiple phosphorylation sites can establish a collection of functional states of RB, depending on the specific sites phosphorylated. To address this hypothesis, we developed in vitro binding assays to examine the requirement of specific phosphorylation sites on RB protein binding activity. We show here that different phosphorylation sites are required to inhibit the binding to T-Ag, c-Abl, and E2F. Furthermore, specific sites are also required for the efficient phosphorylation of RB in vivo and the reversal of RB-mediated growth suppression by cyclin A.

MATERIALS AND METHODS

Cell Culture—C33-A, HeLa, SAOS-2, and COS cells were obtained from the American Type Culture Collection. C33-A and SAOS-2 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% and 15% fetal calf serum, respectively, at 37°C. COS and HeLa cells were cultured in Dulbecco's modified Eagle's medium supple-
mented with 10% calf serum at 37°C. SF9 cells were cultured at 25°C in Grace's medium containing 10% heat inactivated fetal calf serum, yeastolate, lactalbumin hydrolysate, and gentamicin.

Plasmids—The RB phosphorylation site mutants PSM.2S and PSM.2T were constructed by oligonucleotide-directed mutagenesis using the Mutagen in vitro mutagenesis kit (Bio-Rad). PSM.2S was constructed by replacing restriction fragment 135–187 containing the Thr-821 and -826 consensus sites. For PSM.2T, Thr-821 and Thr-826 were mutated in the Thr-821/826 double mutant (PSM.2T, Fig. 1). The resulting site equals the 135–187 fragment containing the Ser-807 and -811 consensus sites. Both Ser-807 and -811 were mutated in the Ser-807/811 double mutant (PSM.2T, Fig. 1).

PSM.4 was constructed as described previously (8). PSM.2S, PSM.2T, and PSM.4 expression plasmids, containing the full-length RB cDNA were constructed by cloning into the unique BamHI site of pCMV-Neo (23). The CMV-CycA (8), pBABE-Puro (24), RSV-T-Ag (25), and GST-E2F-1 (13) plasmids have been previously described. Recombinant SV40 large T-ag baculovirus was described in Melendy and Stillman (26).

Binding Assays—GST fusion proteins were expressed in bacteria and purified on glutathione-agarose as described previously (15). The binding of soluble GST-AE to T-Ag was carried out as described previously (15). For the binding of soluble GST-AE to T-Ag, 1.0 μg of T-Ag was immobilized by immunoprecipitation with pAb 114. Approximately 200 ng of soluble GST-AE protein, obtained by elution with glutathione in 0.5 ml of NNT-N, was added to immobilized T-Ag and incubated for 35–45 min at 4°C, then washed four times with NNT-N. Bound protein was solubilized by boiling in SDS buffer and resolved by 7.5% SDS-PAGE. The GST-RB proteins were then detected by autoradiography and immunoblotting. For the binding of soluble T-Ag to GST-AE, approximately 30 ng of each of the GST-AE fusions (WT, PSM.2S, PSM.2T, and PSM.4) were immobilized on glutathione-agarose. SF9 cells infected with recombinant T-ag baculovirus were lysed in NNT-N supplemented with phosphatase and protease inhibitors, and clarified by centrifugation. Approximately 50 ng of T-Ag in 0.5 ml of NNT-N were added to the immobilized GST-AE and incubated at 4°C for 45 min. The GST-AE beads were then washed five times with NNT-N. The bound protein was solubilized by boiling in SDS buffer and resolved by 8% SDS-PAGE, and T-Ag was visualized by immunoblot. The E2F supershift assay was carried out using 30 ng of soluble of GST-AE and E2F purified from HeLa cells as described previously (16).

RESULTS

Construction and Characterization of RB Phosphorylation Site Mutants—We focused our analysis on four Cdk consensus phosphorylation sites in RB, Ser-807, Ser-811, Thr-821, and Thr-826. These four sites were chosen because they are phosphorylated to a higher level than other sites in HeLa and Molt4 cells, suggesting that they are phosphorylation sites of physiological relevance (19). These four sites can also be phosphorylated in vitro by cdk2/cyclin B or cdk2/cyclin A (19). We constructed double mutants of Ser-807/811 or Thr-821/826, because we were intrigued by the close proximity of the two Ser and the two Thr consensus sites. Both Ser-807 and -811 were mutated in Phosphorylation Site Mutant 2S (PSM.2S, Fig. 1). In PSM.2T, Thr-821 and -826 were mutated (Fig. 1). The PSM.4 combines the mutations in PSM.2S and PSM.2T. The WT and PSM proteins were expressed in bacteria as GST fusions in two forms: the GST-SE, which only contained the C pocket; or the GST-AE, which contained the A/B and C pockets of RB (Fig. 1). They were also expressed in mammalian cells as

Chromatography—Active Cdk/cyclin kinases used to phosphorylate RB were obtained from two sources. Cdk2/cyclin A was purified from recombinant baculovirus-infected SF9 cells (J. W. Harper). Alternatively, active cdk2 was immunopurified from nocodazole-arrested mitotic HeLa cells as described before (19). Kinase reactions with either active cdk2 (immune complexes from 5 to 10 × 10^5 mitotic cells) or purified Cdk2/cyclin A (final concentration 1–5 μM) were carried out in 50 μl of kinase buffer using 150 μM ATP, 80–200 μCi of [γ-32P]ATP, and 10–30 ng of RB as substrate. Reactions were allowed to proceed for 35–45 min at 4°C. The resulting 32P-labeled proteins were resolved by SDS-PAGE and detected by autoradiography, followed by immunoblotting. The autoradiograms (32P) and immunoblots (protein) were quantitated with an LKB densitometer, and the 32P/protein ratio was normalized to WT, which was arbitrarily set to 100.
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Summary of RB constructs. RB phosphorylation site mutants (PSM) were created by oligonucleotide-directed mutagenesis, as described under "Materials and Methods." RB contains 16 S/T-P sites (Thr-5, Ser-230, Ser-249, Thr-252, Thr-356, Thr-373, Ser-567, Ser-608, Ser-612, Ser-780, Ser-788, Ser-795, Ser-807, Ser-811, Thr-821, Thr-826) which are denoted by arrows (the filled arrows depict serine while the open arrows threonine sites). PSM.25 is a double substitution of Ser-807 and -811 with Ala and Leu, respectively. PSM.2T is a double mutant of Thr-821 and -826 substituted to Ala. PSM.4 is the combination of PSM.2S and PSM.2T. C706F is an A/B pocket mutant of RB. The AE proteins used for in vivo binding experiments were expressed in bacteria as glutathione S-transferase (GST) fusion proteins. The GST-AE fusion contains RB amino acids 384–928. WT AE contains 10 S/T-P sites and functional A/B and C pockets. The GST-SE contains RB amino acids 835–928, has no C-pocket activity and no S/T-P sites.

Stoichiometric Phosphorylation of RB in Vitro—The in vitro phosphorylation of WT RB with Cdk/cyclin could inhibit its protein binding activity as shown below. However, the mere incorporation of phosphate into RB did not necessarily cause an inhibition of binding, because some of the 32P-labeled WT RB was found to bind to its target proteins (data not shown). This result suggested that some of the phosphorylation sites might be irrelevant for the inhibition of protein binding. Therefore, we developed conditions to achieve the quantitative phosphorylation of RB in vitro at all Cdk consensus sites, using mitotic cdc2 kinase or purified Cdk2/cyclin. Quantitative phosphorylation was indicated by the complete conversion of RB to a single band with slower electrophoretic mobility on SDS-PAGE (Fig. 2). Stoichiometry of phosphorylation was measured for WT and PSM RB, and was found to be in agreement with the number of Cdk consensus phosphorylation sites present in each protein (Table IA). GST is not phosphorylated by Cdk2/cyclin (18). Along with the lower stoichiometry, the phosphorylated PSM proteins also exhibited a reduced shift in their electrophoretic mobility. The phosphorylated WT RB migrated to a higher position than its corresponding phosphorylation site mutants (Fig. 2, compare lanes 2–5 with lanes 6–8). Thus, the stoichiometry of phosphorylation appears to dictate the degree of mobility shift.

In Vitro Binding Assays—The quantitatively phosphorylated WT and PSM RB were used in two in vitro assays to examine the regulation of protein binding activity. In the first assay (Fig. 3), GST-RB proteins were labeled with 32P. Each labeled sample was combined with a known amount of its unphosphorylated counterpart to give a constant 32P/protein ratio, and then applied to immobilized c-Abl or T-Ag. The 32P/protein ratio of the input and the bound fraction was compared for each sample. If phosphorylation inhibited binding, the 32P/protein ratio of the bound fraction would be lower than that of the input. However, if phosphorylation did not affect binding, the 32P/protein ratio of the bound fraction would be equal to that of the input. In the second assay (Fig. 4), GST-RB proteins were quantitatively phosphorylated. The RG proteins were then used either in an immobilized form to bind soluble c-Abl or T-Ag (Fig. 4, B and C), or in a soluble form to interact with E2F in gel shift assays (Fig. 4D).

Table I

| GST-AE | GST-SE |
|--------|--------|
| Consensus Cdk sites | Stoichiometry, phosphate/molecule | Consensus Cdk sites | Stoichiometry, phosphate/molecule |
| WT | 10 | 10.5 ± 0.7 | 7 | 7.3 |
| PSM.2S | 8 | 7.5 ± 0.7 | 5 | 4.9 |
| PSM.2T | 8 | 7.9 ± 0.8 | 5 | ND |
| PSM.4 | 6 | 5.7 ± 0.7 | 3 | 2.7 |

**Table I Continued**

| GST-AE | GST-SE |
|--------|--------|
| Consensus Cdk sites | Relative phosphorylation |
| WT-FL | 16 | 100 |
| PSM.2S-FL | 14 | 39 ± 2.5 |
| PSM.2T-FL | 14 | 82 ± 1.5 |
| PSM.4-FL | 12 | 41 ± 1.5 |

*a* The Cdk consensus phosphorylation site is defined as any S/T-P motif, within the given RB sequence.

*b* Each given GST fusion was phosphorylated under conditions of quantitative conversion to a single hyperphosphorylated band (Fig. 2A). The stoichiometry for each hyperphosphorylated species was determined as described under "Materials and Methods." The stoichiometry for GST-AE proteins was determined in two independent experiments. ND, not determined.

*c* The given RB proteins were coexpressed with cyclin A in C33-A cells and labeled with phosphate. The 32P/protein ratio for each protein was determined as described above, and normalized to the WT protein which was set to be 100. The relative phosphorylation of the proteins was determined in two independent experiments.
were analyzed, PSM.2S, PSM.4, lane 4 with protein A-Sepharose. The input and the bound fraction of WT (binding to SV40 large T-antigen immobilized by immunoprecipitation cdc2 kinase. Each phosphorylated protein was combined with a 25-fold excess of its unphosphorylated counterpart, and this was utilized as the Input. Each sample was assayed for binding to GST-A1 (the ATP-binding lobe of c-Abl fused with GST (15)). The input (upper panels) represent 10% of the sample applied to the binding reaction. Protein fractions were resolved by 15% SDS-PAGE and transferred to Immobilon-P. Phosphorylated SE fragments were visualized by autoradiography (32P, left panels), and the unphosphorylated SE or ME were revealed by anti-RB immunoblot (αRB, right panels). B, phosphorylation inhibits binding to T-Ag. Soluble GST-AE proteins were quantitatively phosphorylated with mitotic cdc2 kinase. Each phosphorylated protein was combined with a 25-fold excess of its unphosphorylated counterpart, and this was assayed for binding to SV40 large T-antigen immobilized by immunoprecipitation with protein A-Sepharose. The input and the bound fraction of WT (lane 1), PSM.2S (lane 2), PSM.4 (lane 3), and C706F (lane 4) were analyzed by autoradiography (32P, left panels) to detect phosphorylated RB and anti-RB immunoblot (αRB, right panels) to detect the excess unphosphorylated GST-AE. 15% of the input and all of the bound fraction was loaded. Samples were resolved by 7.5% SDS-PAGE.

**Fig. 3.** In vitro phosphorylation inhibits the protein binding function of RB. A, phosphorylation inhibits binding to c-Abl. RB-SE or ME fragments (lanes 1–4) obtained by thrombin cleavage were quantitatively phosphorylated with mitotic cdk2 kinase. Each phosphorylated protein was combined with a 10-fold excess of its unphosphorylated counterpart, and this was utilized as the Input. Each sample was assayed for binding to GST-A1 (the ATP-binding lobe of c-Abl fused with GST (15)). The Input (upper panels) represent 10% of the sample applied to the binding reaction. Protein fractions were resolved by 15% SDS-PAGE and transferred to Immobilon-P. Phosphorylated SE fragments were visualized by autoradiography (32P, left panels), and the unphosphorylated SE or ME were revealed by anti-RB immunoblot (αRB, right panels). B, phosphorylation inhibits binding to T-Ag. Soluble GST-AE proteins were quantitatively phosphorylated with mitotic cdc2 kinase. Each phosphorylated protein was combined with a 25-fold excess of its unphosphorylated counterpart, and this was assayed for binding to SV40 large T-antigen immobilized by immunoprecipitation with protein A-Sepharose. The input and the bound fraction of WT (lane 1), PSM.2S (lane 2), PSM.4 (lane 3), and C706F (lane 4) were analyzed by autoradiography (32P, left panels) to detect phosphorylated RB and anti-RB immunoblot (αRB, right panels) to detect the excess unphosphorylated GST-AE. 15% of the input and all of the bound fraction was loaded. Samples were resolved by 7.5% SDS-PAGE.

**Ser-807/811 and Thr-821/826 Are Not Required to Inhibit E2F Binding—To assay the E2F binding activity, the unphosphorylated or phosphorylated GST-AE fragments were added to purified E2F and an E2F-oligonucleotide (Fig. 4D). In the unphosphorylated form, the WT and the PSM fragments tested all bound to E2F, as indicated by the “supershift” of the E2F-DNA complex (lanes 1–4). All four proteins failed to complex with E2F-DNA when phosphorylated (lanes 5–8). As a negative control, the GST-SE fragment of RB did not supershift the E2F-DNA complex (lane 10). These results showed that Cdk sites within the AE fragment of RB were sufficient to inhibit E2F binding. However, phosphorylation of Ser-807/811 and Thr-821/826 are not required to inhibit E2F binding.**

In Vivo Phosphorylation of PSM RB—With the in vitro quantitatively phosphorylated RB, we showed that different Cdk sites were required to regulate binding to c-Abl, T-Ag, and E2F. Because not all of the Cdk sites were quantitatively phosphorylated in vivo, we wished to determine the protein binding activity of in vivo phosphorylated WT and PSM RB. The WT and PSM proteins were transiently expressed in the human cervical carcinoma cell line, C33-A, which contains a truncated RB that is unstable. Phosphorylation of the exogenously expressed RB was enhanced by the cotransfection with cyclin A. The relative extent of phosphorylation was determined by the appearance of slower migrating bands on anti-RB immunoblots and by 32P labeling (Fig. 5). The WT and PSM 2T were phosphorylated in vivo, as indicated by the slower migrating bands which were 32P-labeled (Fig. 5, lanes 1 and 3, upper and lower). The PSM 2S and PSM 4 proteins were labeled with 32P in vivo (Fig. 5, lanes 2 and 4, upper panel), but did not generate the characteristic mobility shift (Fig. 5, lanes 2 and 4, lower panel). The relative level of in vivo phosphorylation was determined for each protein (Table I). PSM 2T was phosphorylated to 80–90% that of WT, consistent with the mutation of two out
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FIG. 4. Differential protein binding activities of in vitro phosphorylated RB. A, GST-AE fragments used in binding assays. GST-AE proteins WT (lanes 1 and 5), PSM.2S (lanes 2 and 6), PSM.2T (lanes 3 and 7), and PSM.4 (lanes 4 and 8), were immobilized on GSH-agarose and incubated with Cdk2/cyclin A in the absence (lanes 1–4) or presence (lanes 5–8) of 150 μM ATP. A portion of each sample was resolved by 8% SDS-PAGE, transferred to Immobilon-P, then immunoblotted with anti-RB antibody. B, phosphorylation of Ser-807/811 and Thr-821/826 is dispensible for inhibiting c-Abl binding. Equal amounts of GST-AE proteins, phosphorylated (lanes 3 and 5, 7) or unphosphorylated (lanes 2 and 4, 8), were incubated with lysate of Sf9 cells containing wild-type c-Abl, labeled with [35S]methionine. The c-Abl bound to immobilized GST-AE, WT (lanes 1 and 5), PSM.2S (lanes 2 and 6), PSM.2T (lanes 3 and 7), or PSM.4 (lanes 4 and 8), was recovered and resolved by 6.5% SDS-PAGE. The amount of c-Abl bound was determined by autoradiography. C, phosphorylation of Thr-821/826 is only required for the inhibition of T-Ag binding. Since T-Ag interacts with RB through an LXCXE motif, we tested whether phosphorylation of Thr-821/826 was required to regulate the binding of other LXCXE containing proteins, e.g. E7 and Elf-1. As with T-Ag, the phosphorylated WT RB did not bind to E7 or Elf-1 (Fig. 6, A and C, lanes 1 and 2), while the phosphorylated upper bands of PSM.2T did bind to these two proteins (Fig. 6, B and D, lanes 2). Thus, phosphorylation cannot inhibit the LXCXE binding function of RB when Thr-821/826 are mutated. To determine whether the GST pull-down assays reflected the regulation of T-Ag binding inside the cell, we performed communoprecipitation. In C33-A cells cotransfected with T-Ag and RB, only the underphosphorylated form of WT was coprecipitated with T-Ag (Fig. 7A, lane 1). In contrast, the hyper-

FIG. 5. Phosphorylation of PSM RB in vivo. C33-A cells were cotransfected with plasmids expressing cyclin A and the full-length (FL) RB proteins: WT-FL (lane 1), PSM.2S-FL (lane 2), PSM.2T-FL (lane 3), and PSM.4-FL (lane 4). The transfected cells were metabolically labeled with [35S]phosphoric acid, lysed, and RB was immunoprecipitated with anti-RB antibodies. Immunocomplexes were recovered, resolved by 7.2% (lanes 1–4) SDS-PAGE, and transferred to Immobilon-P. RB was detected by autoradiography ([35S], upper panel), followed by immunoblotting with anti-RB (αRB, lower panel) antibodies.

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of 16 phosphorylation sites (Table IB). The relative phosphorylation for PSM.2S or PSM.4 was only about 40% that of WT (Table IB). The inefficient phosphorylation of the Ser-807/811 mutant RB was consistent with a previous report which showed that mutation of the murine equivalent of Ser-807/811 prevented the hyperphosphorylation of murine RB in vivo (21). Hyperphosphorylated PSM.2T binds T-Ag—Because WT and PSM.2T were both hyperphosphorylated in vivo (Fig. 6A, lanes 1 and 2), we compared their binding to c-Abl, E2F, and T-Ag (Fig. 6B–D). Since PSM.2S was not hyperphosphorylated in vivo, it was not used in the subsequent assays. The hyperphosphorylated WT and PSM.2T could not bind to c-Abl (Fig. 6B), nor did they bind to E2F-1 (Fig. 6C). In contrast, the hyperphosphorylated bands of PSM.2T could bind to T-Ag (Fig. 6D). These results were consistent with those obtained with in vitro phosphorylated RB, showing that phosphorylation at Thr-821/826 is only required for the inhibition of T-Ag binding. Since T-Ag interacts with RB through an LXCXE motif, we tested whether phosphorylation of Thr-821/826 was required to regulate the binding of other LXCXE containing proteins, e.g. E7 and Elf-1. As with T-Ag, the phosphorylated WT RB did not bind to E7 or Elf-1 (Fig. 6F, lanes 1 and 2), while the phosphorylated upper bands of PSM.2T did bind to these two proteins (Fig. 6, E and F, lane 2). Thus, phosphorylation cannot inhibit the LXCXE binding function of RB when Thr-821/826 are mutated.
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FIG. 6. Hyperphosphorylated PSM.2T binds to LXCCXE containing proteins. A, in vivo phosphorylated WT and PSM.2T RB. C33-A cells were cotransfected with cyclin A and WT-FL (lane 1) and PSM.2T-FL (lane 2). Lysates from the transfected cells were immunoprecipitated with anti-RB antibody, and resolved by 7.2% SDS-PAGE. RB proteins were then detected by anti-RB immunoblot. B, hyperphosphorylated PSM.2T does not bind c-Abl. Lysates from C33-A transfected with WT-FL (lane 1) and PSM.2T-FL (lane 2) were applied to immobilized GST-Abl (containing the SH2 and tyrosine kinase domains). Proteins bound to GST-Abl were recovered and resolved on 7.2% SDS-PAGE. The RB proteins were then visualized by anti-RB immunoblot. C, hyperphosphorylated PSM.2T RB does not bind E2F-1. The full-length RB proteins (WT-FL, lane 1, and PSM.2T-FL, lane 2) expressed in C33-A cells were used in binding reactions with GST-E2F-1, immobilized on GSH-agarose. Proteins bound to E2F-1 were recovered and resolved by 7.2% SDS-PAGE. RB was visualized by anti-RB immunoblot. D, in vivo hyperphosphorylated PSM.2T binds T-Ag. Both the WT and PSM.2T (lanes 1 and 2) proteins were expressed in C33-A cells, and used in binding reactions with T-Ag. The fraction bound to T-Ag was recovered and resolved by 7.2% SDS-PAGE. The RB proteins were then visualized by anti-RB immunoblot. E, in vivo hyperphosphorylated PSM.2T binds HPV-16-E7. The WT and PSM.2T (lanes 1 and 2) proteins coexpressed with cyclin A were used in binding reactions with GST-E7. The fraction bound to E7 was recovered and resolved by 7.2% SDS-PAGE. RB bands were revealed by immunoblotting with anti-RB antibodies. F, in vivo hyperphosphorylated PSM.2T binds Elf-1. The WT and PSM.2T (lanes 1 and 2) proteins produced in C33-A cells were assayed for binding to GST-Elf-1. RB proteins bound to Elf-1 were recovered and resolved by 7.2% SDS-PAGE. RB was detected by anti-RB immunoblot.

Phosphorylated PSM.2T bands were brought down by anti-T-Ag immunoprecipitation (Fig. 7A, lane 2). Similar results were also obtained when WT and PSM.2T RB proteins were expressed in COS cells and phosphorylated by the endogenous Cdk/cyclins (Fig. 7B, lanes 1 and 2). Thus, PSM.2T binds to T-Ag irrespective of its phosphorylation status.

Growth Suppression by PSM RB—Phosphorylation of RB has been correlated with the inactivation of its growth suppression function in SAOS-2 cells (8). Exogenous RB does not become phosphorylated in SAOS-2 cells and causes a cell cycle block at G1/S which generates growth-arrested flat cells. Cotransfection of cyclin A can drive RB phosphorylation in SAOS-2 cells and alleviate the cell cycle block (8). In keeping with previous reports, WT RB gave rise to numerous flat cells, and the number of flat cells was reduced 8-fold with the cotransfection of cyclin A (Table II). PSM.2T induced a similar number of flat cells and the number was reduced 6-fold by the cotransfection of cyclin A. With PSM.2S, however, cyclin A only caused a 1.6-fold reduction in the number of flat cells (Table II). To determine if PSM.2S could be inactivated by other means, we cotransfected it with T-Ag (Table II). The wild type RB and PSM.2S were both sensitive to T-Ag, which caused a 33-fold reduction in the number of flat cells. Cotransfection with cyclin A drove the phosphorylation of WT and PSM RB, and the relative level of phosphorylation was similar to that observed with C33-A cells (Fig. 5 and Table 1B). PSM.2S was poorly phosphorylated in SAOS-2 cells, and this might account for its resistance to the inactivation by cyclin A.

**DISCUSSION**

Under conditions where every Cdk consensus site is phosphorylated, we have shown that in vitro phosphorylation does inhibit the protein binding function of RB. Using mutants lacking specific Cdk phosphorylation sites, we also demonstrated that different Cdk sites are required for inhibiting distinct RB protein binding activities. Specifically, Ser-807/811 is required for the inhibition of c-Abl binding, while phosphorylation of Thr-821/826 is required for the inhibition of binding to T-Ag, Elf-1, and E7 (all of which contain the LXCCXE motif). However, none of these sites are required for the inhibition of E2F binding. We find that the mutation of Ser-807/811 prevents the efficient phosphorylation of RB in vivo, and cyclin A cannot overcome the growth suppressing activity of this mutant in SAOS-2 cells. In contrast, mutation of Thr-821/826 does not prevent RB hyperphosphorylation nor the inactivation of its growth suppressing activity by cyclin A.

Regulation of the A/B Pocket by Phosphorylation—Both T-Ag and E2F interact with RB through the A/B pocket. However, the LXCCXE motif that mediates the T-Ag binding is not found in E2F (12, 13). Furthermore, the CR1I fragment of E1A, which contains the LXCCXE motif, cannot disrupt the RB/E2F complex (28). Thus, the A/B pocket of RB may contain two binding sites, one for the LXCCXE motif, and another for E2F. This idea is consistent with our finding that the regulation of T-Ag and E2F binding to RB requires different Cdk phosphorylation sites. Phosphorylation of Thr-821/826 is required for the disruption of the T-Ag/RB complex. In addition to T-Ag, we have found that the inhibition of binding to Elf-1 and E7, both of which contain the LXCCXE motif, also required Thr-821/826. This finding strongly supports that the LXCCXE binding site is regulated by Thr-821/826 phosphorylation. We cannot determine if both threonine residues or only one of them is required for the regulation of LXCCXE binding. Single site mutants would have to be constructed to resolve this issue. Since mutation of Thr-821/826 does not interfere with the regulation of E2F bind-
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Effect of cyclin A on the growth suppressing activity of WT and PSM RB

| Plasmids       | Flat cells (>10^-5) | % Vector control |
|----------------|---------------------|-----------------|
|                | Exp. 1 | Exp. 2 | Exp. 3 | Exp. 4 |                   |
| WT-FL plus CMV-vector | 7.0    | 7.0    | 5.2    | 8.1    | 100               |
| CMV-CycA       | 0.8    | 1.1    | 0.6    | 0.6    | 12 ± 4            |
| RSV-T-Ag       | 0.2    |         |         |         | 3                 |
| PSM.2S-FL plus CMV-vector | 10.0   | 5.1    | 4.0    | 9.9    | 100               |
| CMV-CycA       | 7.0    | 2.7    | 3.6    | 3.7    | 63 ± 20           |
| RSV-T-Ag       | 0.2    |         |         |         | 3                 |
| PSM.2T-FL plus CMV-vector | 7.9    | 9.1    | 1.4    | 1.5    | 100               |
| CMV-CycA       |         |         | 1.4    | 1.5    | 17 ± 1            |

The number of puromycin resistant growth arrested flat cells arising from the transfection of 5 x 10^5 SAOS-2 cells.

The relative amount of flat cells was determined for each set of transfections, with RB + CMV vector set to 100%.

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