The Carboxy-Terminal Region of the Retinoblastoma Protein Binds Non-Competitively
to Protein Phosphatase Type 1α and Inhibits Catalytic Activity

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¹Abbreviations used: pRB, retinoblastoma protein; PP1, protein phosphatase type 1,
alpha-isotype of protein phosphatase type 1, PP1α; protein phosphatase type 1 catalytic
subunit, PP1c; GST, glutathione-S-transferase.

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Abstract

pRB, a negative-growth regulatory protein, is a demonstrated substrate for type 1 serine/threonine protein phosphatases (PP1). In a recent report from this laboratory, we demonstrated that select forms of phosphorylated, as well as hypophosphorylated, pRB can be found complexed with the alpha-isotype of PP1 (PP1α). This complex can also be observed when PP1 is rendered catalytically dead by toxin-inhibition. These data suggested to us that pRB may bind to PP1 at one or more sites other than the catalytically active one on the enzyme, and that such binding may play a role other than bringing the substrate into contact with the enzyme to facilitate catalysis. To address this possibility, we utilized a series of pRB deletion mutants and coprecipitation studies to map the pRB domain involved in binding to PP1. Together with competition assays using in vivo expression of SV40 T-antigen, we show here that the carboxy-terminal region of pRB is both necessary and sufficient for physical interaction with PP1. Subsequent biochemical analyses demonstrated inhibition of PP1 catalytic activity towards the standard substrate phosphorylase a when this enzyme is bound to pRB containing this region. KM and Vmax calculations revealed that pRB binds to PP1 in a non-competitive manner. These data support the notion that pRB, in addition to being a substrate for PP1, also functions as a PP1 inhibitor. The significance of this finding with respect to the functional importance of this interaction is discussed.
Introduction

The nuclear phosphoprotein product of the retinoblastoma susceptibility gene, pRB\(^1\), has occupied a central position for investigations surrounding the mechanism of cell cycle progression. Numerous studies have shown that the growth suppressive property of this protein is dependent upon its cell cycle stage-dependent phosphorylation state, which affects the ability of pRB to complex with other cellular and viral proteins (1-8, reviewed in 9). Indeed, hypophosphorylated pRB found in early- to mid-G1 is capable of sequestering, and thereby functionally inactivating, transcription factors necessary for cell growth. Sequential serine/threonine phosphorylation of pRB by cyclin-dependent kinases has been shown to release these factors, after which cell proliferation commences. Beginning at anaphase, pRB becomes progressively dephosphorylated (10,11), and is returned to its growth suppressive, hypophosphorylated state by the next G1-phase. Our laboratory (11) as well as others (12) have reported that this mitotic-phase hypophosphorylation of pRB results, at least in part, by the activity of one or more members of the type 1 serine/threonine protein phosphatases (PP1). The role of PP1 in cell cycle regulation has been revealed by its requirement for successful exit from mitosis (13-17).

Considered together, pRB is a cell cycle regulatory protein whose function is modulated by cell cycle dependent serine/threonine phosphorylation. pRB enters mitosis hyperphosphorylated, yet hypophosphorylated pRB is maintained through mitosis and the subsequent G1-phase. The activity of PP1, a serine/threonine protein phosphatase, appears to be involved in M-phase progression, a time during which PP1 and pRB can be found complexed together. With such an apparent change during mitosis in the pRB phosphorylation state, together with the critical timing of PP1 activity for M-phase
progression, defining the biochemical and structural relationship between these two cellular proteins affords a unique opportunity for understanding the role of PP1 and pRB in cell cycle regulation.

Towards this goal, we have recently demonstrated that select forms of phosphorylated, as well as hypophosphorylated, pRB can be found complexed with the alpha-isotype of PP1 (PP1α) (18). This complex can also be observed when PP1 catalytic ability is toxin-inhibited. These data suggested to us that pRB may bind to PP1 at one or more sites other than the catalytically active one on the enzyme, and that such binding may play a role other than bringing the substrate into contact with the enzyme to facilitate catalysis. To address this possibility, we have undertaken a series of experiments in which we mapped the region of pRB involved in binding to PP1, tested the catalytic activity of PP1 when complexed with pRB, and analyzed the kinetics of the reaction. As presented in this report, these data support the notion that pRB, in addition to being a substrate for PP1, binds non-competitively to this enzyme. In so doing, the catalytic activity of PP1 is inhibited.

**Materials and Methods**

**Cell culture, transfection, and extract preparation**

CV1-P cells, a monkey kidney epithelial line, were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) (GIBCO) supplemented with 10% newborn calf serum (GIBCO). The human cervical carcinoma cell line C33A, the SV40-transformed human cell line SV80, and the human retinoblastoma cell line Weri-1, were all maintained in DMEM supplemented with 10% fetal calf serum. All cultures were maintained at 37°C in a humidified, 5% CO₂-containing atmosphere.
Exogenous expression of pRB, SV40 wild-type T-antigen, and SV40 mutant T-antigen, was accomplished by transfecting cells using the Lipofectin reagent (Gibco BRL) mixed with the corresponding full-length cDNA (2) cloned into the pSG5 mammalian cell expression vector (Stratagene). Wild-type T-antigen has an intact LXCXE motif necessary for efficient binding to the pocket region of pRB (2, 19), while the mutant T-antigen used here, which does not bind to pRB, has an E to K change in this motif.

All cells were lysed for 15 min. at 4°C in EBC buffer (50mM Tris-HCl, pH 8.0, 120mM NaCl, 0.5% Nonidet P-40) containing 10µg/mL of the protease inhibitors aprotinin, leupeptin, and phenylmethylsulfonylfluoride (PMSF). The lysates were cleared by centrifugation at 14,000 x g for 10 minutes.

**Bacterially-expressed fusion proteins**

GST-PP1α, and various deletion mutants of GST-pRB (big pocket, GST-pRB^{BP}, aa. 379-928; small pocket, GST-pRB^{SP}, aa. 379-792; N-terminus, GST-pRB^{NT}, aa. 1-380; C-terminus, GST-pRB^{CT}, aa. 793-928) (20) were produced in *E.coli* DH5α as described previously (18, 20, 21). Full-length GST-pRB (aa. 1-928) was produced in Bl21pLys bacteria as previously described (18). Cell lysates were centrifuged at 10,000 xg for 30 minutes, and the clarified supernate mixed with glutathione-Sepharose beads and rocked overnight at 4°C. The beads were then washed three times with wash buffer (150mM NaCl, 25mM Tris-HCl pH 7.5, 1mM DTT) supplemented with the protease inhibitors leupeptin (10µg/ml), aprotinin (1% v/v final concentration), and PMSF (1mM final concentration) before use. The protein bound to the beads was then used for
coprecipitation experiments. For activity assays and immunoprecipitation, the fusion proteins were eluted with 25mM glutathione solution in 20mM Tris-HCL pH 8.0, 100mM NaCl, 1mM EDTA, pH8.0, and 0.5% Nonidet P-40 (NET-N) supplemented with these same protease inhibitors. Both bound and eluted proteins were quantitated by comparing their band intensities with those of known amounts of bovine serum albumin on silver stained polyacrylamide gels.

His$_6$PP1$\alpha$ was constructed by subcloning the $Bam$HI fragment encoding cDNA of PP1$\alpha$ into the His$_6$ expression vector, pQE, (Qiagen) so that the His$_6$ tag is on the N-terminal end of the protein. The resulting plasmid was used to transform E.coli DH5$\alpha$. Affinity purification of His$_6$PP1$\alpha$ was carried out according to the manufacturer, using Ni-NTA agarose beads.

Coimmunoprecipitation assays

Into 500$\mu$l of boiled Weri-1 cell lysate (approximately 1mg), 37nM of full length GST-pRB and various concentrations of GST-PP1$\alpha$ (1$\mu$M to 5$\mu$M) were added. Following a 15 min. incubation at $4^0$C, 2$\mu$g of monoclonal antibody against pRB (RB-PMG3-245; Pharmingen) was added into each reaction for immunoprecipitation, as we have previously described (22). Weri-1 cells express PP1$\alpha$, but do not express pRB. Thus, competition between endogenous pRB and GST-pRB for binding to GST-PP1$\alpha$ was avoided. By boiling the lysate, competition between endogenous PP1 and GST-PP1$\alpha$ was reduced, while still allowing this lysate to serve as a mammalian cell protein-containing diluent for the immunoprecipitation.
Binding of GST-pRB and His6PP1α

Approximately 5μg of GST-pRB bound to glutathione beads was incubated with 1μM or 0.1μM of purified His6PP1α. Control reactions included 1μM of His6PP1α mixed with 5μg of GST-alone bound to glutathione-Sepharose beads, and 50μl of glutathione-Sepharose beads alone. The final volume of each sample was brought to 500μl with NET-N. Binding was facilitated by rocking at 4°C for 2 hours. The beads were then washed with NET-N to remove any unbound proteins before use.

Gel electrophoresis and western blotting

Electrophoresis was performed in 10% SDS-polyacrylamide gels (23) using 100μg of total cell protein for each sample lane (24). After electrophoresis, the proteins were transferred to nitrocellulose paper in buffer containing 25mM Tris-HCl, 192mM glycine, 20% v/v methanol, and 0.01% SDS (pH 8.5) (25). Residual protein binding sites on the nitrocellulose were blocked by incubation for 30 minutes in TBST (25mM Tris-HCl, pH 8.0, 150mM NaCl, 0.5% Tween-20) containing 4% non-fat dry milk. Next, the nitrocellulose was incubated in TBST containing 2% non-fat dry milk containing either monoclonal antibody to pRB (RB-PMG3-245), or polyclonal rabbit antibody to a PP1α-specific peptide (26). Following three washes of 10 minutes each with TBST, the nitrocellulose was probed with horse-radish peroxidase-conjugated anti-IgG (Promega, Madison, WI) and developed using chemiluminescence detection (Pierce, Rockford, IL) according to manufacturer’s instructions.

GST-pRB binding assays
100nM of each GST-pRB fusion protein (full length GST-pRB, GST-pRB<sup>BP</sup>, GST-pRB<sup>SP</sup>, GST-pRB<sup>NT</sup>, GST-pRB<sup>CT</sup>) bound to glutathione Sepharose beads was mixed with 1mg of Weri-1 lysate, which was used as a source of endogenous PP1. Total volume of each reaction was brought to 500 µl. After rocking at 4°C for 2 hr, the beads were washed with NET-N and boiled in SDS-sample buffer (23) before loading onto a SDS-polyacrylamide gel to separate the bound proteins. Following transfer to nitrocellulose, the imprinted membrane was probed with polyclonal rabbit anti-PP1α (26).

**GST-PP1α coprecipitation experiments**

From each of the pRB or T-antigen transfected or control cultures of CV1P, C33A, or SV80, cells were harvested by scraping into 200 µl of EBC buffer supplemented with protease inhibitors. Cell lysis was facilitated by rocking at 4°C for 15 minutes. The supernatant from each transfection was mixed with 5µg of GST-PP1α bound to glutathione-Sepharose beads. After rocking at 4°C for 2 hours, the beads were washed with protease inhibitor -supplemented EBC buffer. The washed beads were boiled in SDS-sample buffer and the bound proteins separated using a 6% SDS-polyacrylamide gel. The protein was then transferred to nitrocellulose and probed with antibody to pRB and T-antigen (PAB 419) (2) for detection by chemiluminiscence or alkaline phosphatase.

**Phosphorylase a phosphatase assays.**

These assays were performed as previously described (27). Briefly,
phosphorylase b (Sigma) was first *in vitro* labeled using $\gamma^{32}$P-ATP (NEN) to obtain phosphorylase a with specific radioactivity of $1 \times 10^6$ cpm/nmol of protein. Each phosphatase reaction utilized 30$\mu$g (315 pmol) of this substrate, with a constant amount of enzyme and varying amounts of the indicated fusion-proteins. The final volume of each reaction was 30$\mu$l. Following incubation at 30$^0$C for 10 min, the reactions were stopped by 10% trichloroacetic acid (TCA) precipitation. After removing the precipitated protein by centrifugation, the phosphatase activity was determined by the amount of soluble $^{32}$P released. These experiments were also performed using recombinant PP1 catalytic subunit alone (PP1c) by cleaving the GST portion using thrombin. While we observed comparable activities for PP1c and GST-PP1, any remaining thrombin cleaved GST-pRB. Thus, PP1c was not used in order to avoid this possible protease action.

**Kinetic analysis**

Unlabeled phosphorylase a was prepared by using the same method as described above, except $\gamma^{32}$P ATP was omitted from the reaction. Phosphorylase a dephosphorylation by 50ng of GST-PP1$\alpha$ was carried out with 1$\mu$M of radiolabeled phosphorylase a in the presence of increasing concentration, ranging from 0.5 to 30$\mu$M, of the competing unlabeled phosphorylase a. The assays were also carried out in the presence of different concentrations of GST-RB$^{CT}$. Rate of the reaction was determined for each concentration of phosphorylase a and was plotted as a function of substrate concentration [phos a]. The data from each set was fitted into the curves using Sigma Plot version 4.0. for $K_m$ and $V_{max}$ determinations.
Results

Coimmunoprecipitation of GST-PP1α with full-length GST-pRB

We have previously demonstrated that GST-PP1α can bind to both hypophosphorylated and select forms of hyperphosphorylated pRB endogenously expressed by mammalian cells (18). Building upon our earlier successes in achieving complex formation between recombinant and native forms of both proteins (18), we next addressed whether recombinant PP1 and recombinant pRB can specifically associate. Once achieved, further characterizing this interaction by mapping the pRB region involved in this association, as well as analyzing the effect of pRB binding on PP1 catalytic activity, can be more effectively pursued. Towards this goal, GST-PP1α and full-length GST-pRB were first tested for their ability to form a complex at typical intracellular concentrations (6, 28-32). Immunoprecipitation of full-length GST-pRB at a constant concentration of 37nM was carried out in the presence of varying amounts of GST-PP1α, ranging from 1 to 5 μM. As shown in Figure 1A, lanes 4-6, immunoprecipitation of full-length GST-pRB with antibody to pRB coimmunoprecipitates GST-PP1α, as evidenced by western blotting. This binding was also observed when GST-PP1α concentrations were 10 and 100 nM (data not shown).

We can also demonstrate the association of these two recombinant proteins using an alternately- tagged form of PP1. As shown in Figure 1B, His6PP1α is also able to bind to full-length GST-pRB bound to glutathione-Sepharose beads (lanes 5 and 6). In contrast, His6PP1 does not bind GST-alone or glutathione-Sepharose beads-alone (lanes 3 and 4, respectively). Taken together, these data support the conclusion that complex formation between recombinant pRB and PP1α is dependent upon the pRB and PP1α portions of these fusion proteins, and that such binding can...
occur when these proteins are present at physiologically-relevant levels. We chose to continue these analyses using the GST-fusion proteins, since the catalytic activity of His6PP1 was consistently found to be less than GST-PP1α (data not shown).

**Carboxy-terminal domain of pRB is sufficient for binding to PP1.**

To determine the region on pRB required for complex formation with PP1, we used a coprecipitation approach employing deletion mutants of GST-pRB and cell lysate containing endogenous PP1α. This general approach has worked well for us in the past, when we used GST-PP1α and cell lysates containing endogenous pRB to detect select forms of phosphorylated pRB binding to PP1 (18). Figure 2A illustrates the GST-pRB fusion protein constructs used in this study as PP1 affinity reagents.

Glutathione-Sepharose beads with full-length GST-pRB (aa 1-928), amino terminus (GST-pRB NT, aa 1-380), big-pocket (GST-pRB BP, aa 379-928), small-pocket (GST-pRB SP, aa 379-793), and carboxy-terminus (GST-pRB CT, aa 793-928) were each incubated with Weri-1 cell lysate, which does not contain endogenous pRB but does contain endogenous PP1α. The bound protein was separated by SDS-PAGE and then transferred to nitrocellulose. When probed with antibody to PP1α, the recombinant fusion proteins harboring the carboxy-terminus of pRB (i.e. full length GST-pRB, GST-pRB BP, and GST-pRB CT) were able to form a complex with PP1α (Fig 2B lanes 2, 3, and 5). In contrast, recombinant proteins lacking this region (GST-pRB SP and GST-pRB NT) were unable to bind PP1α. We conclude from these data that the carboxy-terminal region of pRB, aa 793-928, is both necessary and sufficient for
complex formation with PP1.

Simultaneous binding of PP1 and SV40 T-antigen to pRB

It has been speculated that one or both LXSXE motifs found in PP1 are involved in binding to pRB (33). Precedent for this speculation comes from previous work in which LXSXE (34) or a similar LXCXE (3,7,28,35) motif in viral oncoproteins were found to be critical for binding to pRB. Since an in vitro peptide competition assay yielded inconclusive results, we decided to use an in vivo competition approach to address this issue. We reasoned that if PP1 binds to pRB through this LXSXE-motif, then overexpression of SV40 T-antigen, which contains an LXCXE-motif which is similar to LXSXE-motif with respect to pRB binding (34), should compete with PP1 binding to pRB. Alternatively, if PP1 binding to pRB does not involve this motif, then PP1 and SV40 T-antigen should both be found complexing with pRB. To test this, C33A cells, which express low levels of non-functional pRB of abnormal size, were transfected with wild-type or mutant (E to K change at amino acid position 107) SV40 T-antigen-expressing plasmids in the presence or absence of a wild-type pRB-harboring plasmid.

Successful expression of each protein was demonstrated by western blotting (Figure 3 top and middle panels). Lysates from these transfected cells were then incubated with GST-PP1α bound to glutathione-Sepharose beads. As expected, exogenous wild-type pRB does bind to GST-PP1α (lane 2). As shown, both wild-type and mutant T-antigen do not associate with GST-PP1α (bottom panel, lanes 3 and 4). In contrast, when pRB was cotransfected with wild-type T-antigen, this viral oncoprotein can be found in a complex containing GST-PP1α (lane 5). Mutant T-antigen, which is incapable of binding to pRB due to a mutation in the LXCXE region, was not found in
complex with GST-PP1α even when the cultures were co-transfected with pRB (lane 6). These data suggest that T-antigen binding to GST-PP1α is indirect, requiring another PP1 interacting protein, in this case pRB, to serve as a bridge. Identical results were observed when these transfection experiments were performed with another cell line, CV-1P, which expresses endogenous wild-type pRB (lanes 7-9); wild type T-antigen was found among the proteins bound to GST-PP1α (lane 8) while mutant T-antigen was not (lane 9). These data argue against the notion that PP1 binds to pRB through its LXSXE-motif. In addition, since T-antigen uses this motif to interact with the A-B region of pRB (36-38), these data are in agreement with, and further support, our conclusion above that it is the carboxy-terminus of pRB which binds to PP1.

Carboxy-terminal domain binding of GST-pRB to GST-PP1α inhibits catalytic activity

We (21,39) and others (33) have previously reported on the binding of hypophosphorylated pRB to PP1. Although we have recently isolated a subpopulation of phosphorylated pRB bound to PP1 (18), we found it curious that a non-substrate form of pRB binds so efficiently to PP1. It has long been known that several PP1-associated proteins function to regulate its catalytic activity towards specific substrates (reviewed in 30, 40, 41). We therefore decided to address the question of whether binding of unphosphorylated pRB to PP1 affects the catalytic activity of PP1. This notion of pRB regulating enzymatic activity is not without precedent. A report by Siegert and Robbins (42) has shown direct interaction of pRB with the TATA-binding protein associated factor TAFII250, results in inhibition of the ability to phosphorylate itself and other targets.
The effect of pRB binding on the activity of PP1 was examined using standard phosphorylase a phosphatase activity assays. Increasing amount of the aforementioned GST-pRB proteins were allowed to bind to GST-PP1α before the addition of radiolabeled phosphorylase a. As summarized in Figure 4A, all GST-pRB fusion proteins containing the carboxy-terminal amino acids 793-928, which are capable of binding to PP1 (see Figure 2 above), reduced the amount of phosphorylase a dephosphorylation by GST-PP1α compared to that of GST-PP1α alone. This decrease in phosphorylase a-directed activity of PP1 can be observed using physiologically-relevant concentrations of pRB (6, 28). When the construct bearing only these carboxy-terminal amino acids was tested for its ability to inhibit GST-PP1α activity, it was also found to decrease the phosphorylase a-directed activity of GST-PP1α in a physiologically-relevant, and concentration dependent, manner (Figure 4B). These data suggest that unphosphorylated pRB association with PP1 can modulate the activity of this enzyme.

**GST-RBCT inhibits PP1 in a non-competitive manner**

Since pRB in its hyperphosphorylated form is a substrate for PP1, and while only unphosphorylated, non-substrate forms of pRB were used in the assays above, perhaps unphosphorylated GST-pRBCT is binding to the active site of GST-PP1α. If so, then the observed inhibition may not be due to an intrinsic inhibitory property of GST-pRBCT, but may be due to competition between unphosphorylated GST-pRBCT and phosphorylase a. In order to determine if pRB was acting as an inhibitor without affecting the ability of PP1 to bind phosphorylase a, the mechanism of PP1 inhibition upon pRB binding was examined. GST-pRBCT was chosen for these experiments since
this region appears to be necessary and sufficient for PP1 inhibition. Phosphorylase a assays were carried out in the presence of varying concentrations of GST-pRBCT. Increasing concentration of substrate was used to determine the rate of the reaction as a function of substrate concentration. A graph of this rate plotted against the corresponding substrate concentration revealed that the maximum velocity decreases as the concentration of inhibitor (GST-pRBCT) increases, while the $K_m$ appeared to remain unchanged. Such a plot would be expected for a protein binding non-competitively to the enzyme. Conversion of these data into a Lineweaver-Burk plot (Figure 5) further supports this suggestion that GST-pRBCT and phosphorylase a are not competing for the active site on PP1. Taken together, we conclude that unphosphorylated pRB association with PP1 inhibits the catalytic activity of this enzyme in a non-competitive manner.

**Discussion**

The motive behind this study comes from the observation that hypophosphorylated pRB, a presumably poor substrate for dephosphorylation by PP1, can be readily found in a complex with this enzyme. We decided to first investigate the functional significance of this interaction by utilizing a series of pRB deletion mutants and coprecipitation studies to map the pRB domain involved in PP1 binding. Previous work by others has shown that the A-B pocket of pRB, which is conserved among members of the pRB family of proteins, is required for binding with LXCXE / LXSXE motif bearing proteins such as SV40 T-Ag (36-38). The A-B pocket along with C-pocket in the carboxy-terminus is essential for complex formation between pRB and E2F, or cyclin D
The C-pocket has also been shown to bind the c-abl tyrosine kinase (47, 48). As we report here, the carboxy-terminal region of pRB, containing amino acids 793-928, is both necessary and sufficient for physical interaction with PP1. In support of these data, a similarly-sized region of pRB (aa 773-928) appears to be required for PP1 binding when assaying by a yeast two-hybrid system (33). Previous studies by others have suggested that a motif comprised of the amino acids R/K I/V X F are involved in the binding of PP1-associated proteins (49). pRB sequence analysis has revealed close matches to this motif; residues 46-49 (RLEF) and residues 874-877 (KLRF). In both cases, there is a conservative substitution in the second position of I/V to L. Interestingly, the fragment of pRB (residues 1-380) that contains the N-terminal occurrence of this motif does not complex with PP1. The significance of this observation must await further mutagenesis studies.

Competition assays using in vivo expression of SV40 T-antigen indirectly supports the aforementioned fusion-protein binding data, while providing further insight into the intrinsic properties of PP1 needed for binding to pRB. Both T-antigen and PP1 contain LXCXE / LXSXE motifs which are found in several, but not all, pRB binding proteins. Proteins that use this motif generally bind to the A-B pocket region of pRB, while our evidence indicates that PP1 binds to the carboxy-terminal region of pRB. The concurrent binding of PP1 and T-antigen to pRB supports the idea that these two proteins use different, non-overlapping regions on pRB to form a ternary complex. Although not directly addressed here, these data also support the notion that PP1 does not utilize this LXSXE motif in binding to pRB. Indeed, a recent report by Dick et al. (50) supports the notion that this motif, and the corresponding binding region on pRB, is more important for viral oncoprotein binding than for cellular protein binding. In contrast, the
aforementioned yeast two-hybrid analysis reported by others (33) includes mutagenesis data suggesting that the A-B region of pRB may also be involved in binding both PP1 and SV40 T-antigen. However, these data contain exceptions in which deletion mutagenesis of the B-region results in obliteration of T-antigen binding while PP1 binding is reduced, and deletion of the C-terminus obliterates PP1 binding while T-antigen binding is unaffected. Perhaps, reconciliation of these apparent discrepancies lies in the difference in the type and sensitivity of the experimental assays, and also their use of truncated (aa. 1-273), instead of full-length, T-antigen.

What is the biochemical significance of pRB binding to PP1? Taking a cue from Siegert et al. (42), in which they reported inhibition of kinase activity when pRB is bound to the amino terminal kinase domain of TAFII250, we tested whether PP1 catalytic activity can be altered upon pRB binding. Enzymatic analyses of pRB/PP1α complexes reveals an inhibition of catalytic activity towards the standard substrate phosphorylase a. This inhibition appears to be dependent upon an intact carboxy-terminal region of pRB, the same region required for pRB binding to PP1. K_M and V_max calculations revealed that pRB binds to PP1 in a non-competitive manner. This argues against the suggestion that the observed inhibition of PP1 activity is due to pRB occupying the active site, thereby preventing dephosphorylation of other substrates. In light of these data, we would suggest that, in addition to being a substrate for PP1, pRB also functions as an inhibitor of PP1. As additional support for this suggestion, the possibility of pRB binding to PP1 and serving as a regulator was first discussed by Durfee et al. (33). This discussion followed their observation that hypophosphorylated pRB is readily detectable in a complex with PP1, while hyperphosphorylated pRB is not. Building upon these observations, we recently reported that the interaction between pRB and PP1 is not
dependent upon PP1 being catalytically active (18). In fact, inhibitory toxin-binding to the PP1 active site does not alter the ability of hypo- or hyper-phosphorylated pRB to bind this enzyme (18). The non-competitive inhibition data presented here validates this observation.

What could be the biological significance of a potential substrate also serving as a negative regulatory protein upon association with an enzyme? As shown previously (33, 21), interaction between PP1 and pRB occurs during early to mid G1. Perhaps accumulation of the hypophosphorylated pRB facilitates complex formation with PP1 during this period of the cell cycle, and signals a decrease in the need for PP1 activity directed towards pRB. Such an inhibitory effect on PP1 could be part of a negative feedback mechanism; pRB itself playing an important role in the regulation of pRB dephosphorylation. While speculative at this point, binding of pRB to PP1 may also affect catalytic activity towards other substrates involved in cell cycle regulation. Owing to the presence of multiple phosphorylated residues on pRB, it is also likely that several modes of PP1 regulation towards pRB take place. As reported by Liu et al. (51), inactivating phosphorylation of the PP1 catalytic subunit itself occurs in a cell cycle stage-dependent manner, which no doubt also affects the phosphorylation state of pRB. With this newly reported function of pRB, we now have another avenue of pursuit in defining the function of this protein in cell cycle regulation.

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Figure Legends

**Figure 1A.** Coimmunoprecipitation of GST-PP1α with full-length GST-pRB. A mixture of full-length GST-pRB (37nM) and GST-PP1α (5µM, 2µM, and 1µM) were immunoprecipitated with antibody to pRB antibody. After separation of the immunoprecipitated proteins by SDS-PAGE and transfer to nitrocellulose, the blot was probed with antibody to PP1α and pRB. Immunoprecipitations were from mixtures containing GST-pRB without the addition of GST-PP1α (lane 3), with the addition of 5µM, 2µM, and 1µM of GST-PP1α (lanes 4, 5, and 6, respectively), and a mixture containing GST-PP1α without the addition of GST-pRB (lane 7). Controls include 0.1µg of GST-pRB alone (lane 1) and 0.1µg of GST-PP1α alone (lane 2). Positions of GST-pRB, GST-PP1α, and IgG are indicated. **B.** His$_6$PP1α (1µM) was mixed with glutathione-Sepharose beads alone (lane 3), GST- alone bound to glutathione-Sepharose beads (lane 4), or GST-pRB bound to glutathione-Sepharose beads (lane 5). For lane 6, 0.1µM of His$_6$PP1α was mixed with GST-pRB bound to glutathione-Sepharose beads. After separation of the proteins by SDS-PAGE and transfer to nitrocellulose, the blot was probed with antibody to PP1α. Controls include 1µg of GST-pRB (lane 1) and 1µg of His$_6$PP1α (lane 2).

**Figure 2.** Fusion-protein constructs of GST-pRB. **A.** Full-length wild-type GST-pRB (aa 1-928) shows the positions of the A-B pocket domain, along with C-pocket. Other GST-fusion protein constructs used for these studies include big pocket (GST-RBBP; aa 379-928), small pocket (GST-RBSP; aa 379-793), amino-terminus (GST-pRBNT;
aa 1-380), and carboxy-terminus (GST-RBCT; aa 792-928). B. 1mg of Weri-1 lysate was incubated with 100nM each of the GST-pRB constructs bound to glutathione-Sepharose beads. Following separation of the bound proteins by SDS-PAGE and transfer to nitrocellulose, the blot was probed with antibody to PP1α. The position of PP1α is indicated by the arrow. Lane 1 - Weri-1 lysate alone (100µg); Lanes 2 through 6 – PP1α bound to full-length GST-pRB, GST-pRBBP, GST-pRBSP, GST-pRBCT, GST-pRBNT, respectively. Lane 7 - a positive control of PP1α binding to glutathione-Sepharose containing GST-PNUTS, a demonstrated PP1α binding protein (52); Lane 8 – a negative control showing the absence of PP1α binding to GST-alone bound to glutathione-Sepharose beads.

**Figure 3.** Simultaneous binding of SV40 T-antigen and PP1α to pRB in vivo. **Top and middle panels.** Following transfection with the pSG5 mammalian expression vector containing wild-type pRB, SV40 T-antigen or mutant T-antigen, protein expression in 100µg of C33A and CV-1P cell lysate was detected by western blotting. As indicated above each lane, C33A cells, which do not express wild type, endogenous pRB or SV40 T-antigen, were transfected with vector-alone (lane 1), pRB (lane 2), wild-type T-antigen (lane 3), mutant T-antigen (lane 4), pRB and wild-type T-antigen (lane 5), pRB and mutant T-antigen (lane 6). CV1-P cells, which express endogenous, wild-type pRB but do not express SV40 T-antigen, were also transfected with vector-alone (lane 7), wild-type T-antigen (lane 8), or mutant T-antigen (lane 9). Lysate from untransfected SV80 cells, which express endogenous wild-type pRB and SV40 T-antigen, was used as a control (lane 10). The positions of pRB (top panel) and T-antigen (middle panel)
are indicated by the arrows. **Bottom Panel.** 1mg of whole cell lysate from each of these transfections were mixed with 5µg of GST-PP1α bound to glutathione-Sepharose beads, the proteins separated by SDS-PAGE, transferred to nitrocellulose, and the blot probed with antibody to pRB and SV40 T-antigen.

**Figure 4. Biochemical activity of PP1α when bound to pRB.** **A.** Phosphorylase a phosphatase assay reaction consists of 0.2µg of GST-PP1α and 30µg of 32P-labeled phosphorylase a, as described in Materials and Methods. As indicated, glutathione-Sepharose affinity-purified fusion proteins were added at concentrations of 40, 80 and 160nM. The activity indicated is relative to that of the control (GST-PP1α alone) which was designated as 100%. GST-alone, which does not bind to PP1α, served as a negative control. GST-PNUTS, which binds to PP1α and downregulates the catalytic activity of this enzyme, served as the positive control. The results shown are representative of three individual experiments. **B.** The effect of GST-pRBCT on phosphorylase a-directed PP1α activity was quantitated by increasing the concentration of GST-pRBCT in the reactions. The activity of GST-PP1α in the presence or absence of GST-pRBCT is reported as the percent of 32P released from the radiolabeled phosphorylase α. The reactions were designed to keep the percent of 32P released within the linear range of the reaction, typically around 30% (27). Results are presented as the mean percent values obtained in triplicate assays. Standard error of the mean bars are included.

**Figure 5.** Kinetics of Phosphorylase a dephosphorylation by GST-PP1α. GST-PP1α
(50ng) dephosphorylation of radiolabeled phosphorylase a (1µm) in the presence of increasing amounts of competing unlabeled phosphorylase a and different concentrations (100nM and 1µM) of GST-pRBCT. A double reciprocal plot of the velocity and substrate concentration is shown.
GST  pRB

GST-pRB
full length

GST-pRB

GST-pRB^P

GST-pRB^P

GST-pRB^N

GST-pRB^C

1  379  572  646  772  870  928

379

379

380

792  928
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