

Interaction Between the Retinoblastoma Protein and Protein Phosphatase 1 During the Cell Cycle

Norbert Berndt and John W. Ludlow

Summary

The functions of the retinoblastoma protein (pRb) are in part regulated by reversible and cell cycle-dependent phosphorylation. While the regulation of pRb by cyclin-dependent kinases (Cdks) has been studied extensively, the role(s) of protein phosphatase 1 (PP1) in controlling pRb are only partially understood. In this chapter, we will describe experimental approaches to investigate the interactions between pRb and PP1. Methods will be presented to study the cell cycle-dependent dephosphorylation of pRb by various PP1 isozymes, the specificity of PP1 isozymes for distinct pRb phosphorylation sites, the dephosphorylation of pRb associated with apoptosis, and the cell cycle- and pRb-dependent phosphorylation of PP1.

Key Words: Retinoblastoma protein (pRb); protein phosphatase 1 (PP1); cyclin-dependent kinase (Cdk); tumor suppressor; protein dephosphorylation; protein complex; cell cycle; checkpoint control; restriction point; G1/S transition; mitosis; cancer; phosphorylation site-specific antibodies.

1. Introduction

The retinoblastoma protein (pRb) is the prototype of mammalian tumor suppressors and regulates the entry into and passage through the S phase of the cell cycle. pRb plus its upstream and downstream partners have recently been referred to as pRb pathway. Its established components are pRb itself; the cyclin-dependent kinases (Cdks), which phosphorylate and inactivate this protein as cells prepare for the G1/S transition; the regulators of these kinases, such as cyclins D, E, and A, as well as Cdk inhibitors; and finally, the downstream effectors of pRb, the transcription factors of the E2F family (for recent reviews, *see refs. 1–7*). This pathway is now being recognized as very important insofar as in the vast majority of human cancers, at least one member of the pRb pathway is malfunctioning (*8,9*). Since pRb is now being considered an integral part of the checkpoint controlling the G1/S transition, the original

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definition of a checkpoint bears repeating: The dependence of an event B upon the completion of a prior event A is due to a checkpoint, if a loss-of-function mutation exists that relieves that dependence (*10,11*).

Although the significance of each of the aforementioned proteins is not in dispute, we would like to suggest granting membership in this pathway to another protein—PP1, which has recently been shown to control the phosphorylation state of pRb. Support for this idea comes from several observations: PP1 dephosphorylates pRb at the end of mitosis (*12*) and apparently keeps pRb in a dephosphorylated state throughout G1 (*13*). Furthermore, PP1 α was found to interact with pRb in a two-hybrid screen and to co-immunoprecipitate with pRb (*14*). Consistent with these observations, PP1 α (*15*) as well as the other two PP1 isozymes γ and δ (Rui-Hong Wang and Norbert Berndt, unpublished) are found in a complex with pRb throughout interphase (*15*). Meanwhile, all three mammalian isoforms of PP1 dephosphorylate pRb at the end of mitosis; however, they prefer distinct subsets of phosphorylation sites (*16*). While these data strongly suggest that PP1 is an important regulator of pRb function, it is also evident that pRb can function as a PP1 inhibitor (*17*). In addition to the emerging role(s) of PP1 in the cell cycle, apoptotic stimuli induce PP1 α , which then appears to dephosphorylate pRb, presumably in order to recruit it to caspase-mediated destruction (*18*). We hope that this short introduction has convinced the reader that the interaction between pRb and PP1 is important for the regulation of both the cell cycle and apoptosis.

In this chapter, we will describe strategies and methods to analyze interactions between pRb and PP1. We will focus our attention on (a) the cell cycle-dependent dephosphorylation of pRb by PP1, (b) the apoptosis-associated dephosphorylation of pRb by PP1, and (c) the phosphorylation of PP1 by Cdk. Obviously, we have examined the PP1-pRb interactions only in a few cell lines, but we would like to point out that these techniques may be employed in or modified to accommodate a wide variety of experimental settings. There is increasing evidence that both regulation and functions of pRb as well as PP1 is context specific (for review, *see refs. 6,19–22*). Thus, it is likely that both the nature and extent of the PP1-pRb interactions vary with the cell type (i.e., the cell-specific proteome), the developmental status or previous history of the cell, or environmental signals. The techniques rely in large part on the use of phosphorylation site-specific antibodies.

2. Materials

1. Phosphate-buffered saline (PBS) (available in tablet form from Sigma-Aldrich). Dissolve one tablet in 200 mL of water to yield 137 mM NaCl, 2.7 mM KCl in 10 mM Na₂HPO₄/KH₂PO₄ phosphate buffer, pH 7.4. Store at room temperature.

2. Cytosine arabinoside (araC) (Sigma-Aldrich). Prepare a 100 mM stock solution in PBS. Store in small aliquots at -20°C .
3. Aphidicolin (Sigma-Aldrich). Prepare a stock solution of 5 mg/mL in DMSO and store at -20°C .
4. Mimosine (Sigma-Aldrich). Prepare a 10 mM stock solution in phosphate-buffered saline and store at -20°C .
5. Nocodazole (Sigma-Aldrich). Prepare a stock solution of 5 mg/mL in DMSO and store at -20°C .
6. Okadaic acid (Calbiochem). Prepare a stock solution of 5 μM in DMSO and store at -20°C .
7. EBC buffer: 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% NP-40. Store at 4°C .
8. Kinase reaction buffer: 20 mM Tris-HCl (pH 7.5), 20 mM MgCl_2 , 10 mM β -mercaptoethanol, 150 mM NaCl, 0.5% NP-40, supplemented with freshly added protease and phosphatase inhibitors: 2 $\mu\text{g/mL}$ aprotinin, 1 mM benzamidine, 0.5 $\mu\text{g/mL}$ leupeptin, 1 $\mu\text{g/mL}$ pepstatin A, 0.1 mM PMSF, 25 mM NaF, 1 mM β -glycerophosphate, 1 mM Na_3VO_4 (see **Note 1**). Store at 4°C .
9. NET-N buffer: 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, 4% bovine serum albumin (BSA). Store at 4°C .
10. Phosphatase reaction buffer: 20 mM imidazole (pH 7.0), 150 mM NaCl, supplemented with 1 $\mu\text{g/mL}$ aprotinin, 1 mM PMSF, and 1 mM MnCl_2 (see **Note 1**).
11. RIPA buffer: 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% (v/v) NP-40, 0.1% (w/v) SDS, supplemented with freshly added protease, protein kinase, and phosphatase inhibitors: 2 $\mu\text{g/mL}$ aprotinin, 1 mM benzamidine, 0.5 $\mu\text{g/mL}$ leupeptin, 1 $\mu\text{g/mL}$ pepstatin A, 0.1 mM PMSF, 0.5 mM EDTA, 100 mM NaF, 1 mM Na_3VO_4 , and 5 μM okadaic acid (see **Note 1**).
12. 2X SDS sample buffer: 125 mM Tris-HCl (pH 6.8), 4% (w/v) SDS, 20% (v/v) glycerol, 0.05% (w/v) bromophenol blue, 10% (v/v) 2-mercaptoethanol. Store at room temperature.
13. SDS-PAGE molecular weight standards: BioRad. The high and low molecular weight standards contain proteins with $M_r = 200, 116, 97, 66, 45, 36, 31, 21.5$, and 14.4 kDa, respectively.
14. Antibodies to PP1 α : Ab1, raised against residues 316–330 and thus recognizing the dephosphorylated form of PP1 α (**15,23**); Ab2, raised against residues 294–309 and thus recognizing PP1 α regardless of its phosphorylation state (provided by Emma Villa-Moruzzi, University of Pisa, Italy) (**24**); Ab3, raised against a peptide containing residues 316–323 and a chemically phosphorylated Thr320 (provided by Angus C. Nairn, Rockefeller University, New York, NY) (**25**).
Isoform-specific antibodies to PP1 α , PP1 γ 1, and PP1 δ are also available from Santa Cruz Biotechnology.
15. Antibodies to pRb: sc-102, specific for the centrally located A/B pocket region, and sc-50, specific for a peptide derived from the C terminus (both available from Santa Cruz Biotechnology). Phosphorylation site-specific antibodies for

Table 1
Phosphorylation Sites in pRb. Site Specificity
of Different Cdks and Effects of Phosphorylation

Site	Cdk/cyclin (<i>Reference</i>)	Effect of phosphorylation (<i>Reference</i>)	Antibody available
S005	4/D1-2/E-2/A (32)		
S249	4/D1 (32)		yes
T252	4/D1 (32)		
T356	4/D1 (32)	Decreases G1 arrest (33,34)	yes
T373	4/D1-2/E (32)		yes
S608	4/D1-2/A (32)		
S612	2/E-2/A (32)		yes
S780	4/D1 (35)	Disrupts binding to E2F (35)	yes
S788	4/D1 (32,36)		
S795	4/D1 (32,36); 2/E-2A (32)	Abolishes growth suppression (36)	yes
S807	4/D1 (32)	Decreases G1 arrest (33,34) Disrupts binding to Abl (37)	yes
S811	4/D1 (32)	Decreases G1 arrest (33,34) Disrupts binding to Abl (37)	yes
T821	2/E (32); 2/A (37)	Decreases G1 arrest (33,34) Disrupts binding to LXCXE motifs (32)	yes
T826	4/D1 (32,37)	Disrupts binding to LXCXE motifs (32)	yes

pRb were originally made in the laboratory of Sibylle Mittnacht (16) but are now available for various sites and from different companies: pS249, pT356, pS612, pS780, pS807, pS811, pT821, pT826 (BioSource); pS249, pT356, pT373, pS780, pS795 (Santa Cruz Biotechnology). This panel of antibodies covers 10 of the 14 commonly phosphorylated sites in pRb (*see also Table 1*).

- Proteins: The following colleagues kindly made available to us recombinant baculovirus preparations encoding human pRb (Barbara Driscoll, Children's Hospital Los Angeles); Cdk2, cyclin A, and cyclin E (David O. Morgan, University of California, San Francisco, CA); Cdk4 and cyclin D1 (Charles J. Sherr,

St. Jude's Children's Research Hospital, Memphis, TN). Expression vectors encoding PP1 α were generated in NB's laboratory; cDNAs encoding mammalian PP1 γ 1 and PP1 δ were kindly provided by Ernie Lee, Medical College of New York, Valhalla, NY.

3. Methods

3.1. Cell Synchronization

The protocols described here for cell synchronization are based on inducing specific cell cycle blocks and are guaranteed to work with the cell lines described here: MG63 human osteosarcoma cells and CV1-P monkey kidney cells. Although the principles of these techniques have proven effective in countless other cell types, the concentrations of the drugs and/or the length of exposure to them may have to be varied.

The success of any cell cycle experiment involving such synchronization procedures obviously depends on the degree of synchrony of a cell population. We recommend that with these or any other cell lines the cell cycle status of synchronized cells should always be verified by flow cytometric analysis of the DNA (also referred to as fluorescent-activated cell sorting, or FACS). This is typically being done by setting up sister cultures that are exposed to the same concentrations of the synchronizing agent as the experimental cultures. Detailed descriptions of methods related to cell synchronization and cell cycle analysis can be found in references (26) and (27).

3.1.1. CV1-P Cells

1. Synchronize cells in G0/G1 by incubation in methionine-free Dulbecco's modified Eagle's medium (DMEM) containing 2% fetal bovine serum (FBS) for 72 h.
2. To obtain cells enriched in S phase, incubate G0/G1 cells for 16 h in DMEM containing 0.5% hydroxyurea and 10% newborn calf serum (NCS). This arrests the cells at the G1/S boundary. Then release the cells for 3 h into S phase by using medium plus NCS but without the hydroxyurea.
3. To obtain cells in M phase, incubate cells arrested at G1/S for 18 h with DMEM plus 10% NCS and 0.4 μ g/mL nocodazole. Collect mitotic cells by a "shake-off."
4. Replate the cells in different dishes, incubate further for several hours, and collect cells at different time points.

3.1.2. MG63 Cells

1. Plate 1×10^6 cells in 75-cm² flasks (or approx 13,000 cells/cm²) in RPMI-160 medium containing 0.5% FBS, and incubate for 48 h at 37°C. These cells should be arrested in G0/G1 (*see Note 2*).
2. To stimulate re-entry into the cell cycle, add fresh medium containing 10% FBS to the cells. These cells may be harvested for up to 16 h to obtain cells in various stages of the G1 phase.

3. To obtain a cell population arrested in late G1, incubate serum-deprived cells obtained in **step 1** for 24 h in the presence of fresh medium containing 10% FBS and 600 μM mimosine (*see Note 2*).
4. To obtain a cell population arrested at G1/S, incubate serum-deprived cells obtained in **step 1** for 24 h in the presence of fresh medium containing 10% FBS and 2.5 $\mu\text{g/mL}$ aphidicolin. For S-phase-enriched cells, wash aphidicolin-treated cells repeatedly with PBS to remove the drug and finally incubate for another 4 h in the presence of 10% FBS (*see Note 2*).
5. To obtain mitotic cells, incubate serum-deprived cells obtained in **step 1** for 30 h in the presence of fresh medium containing 10% FBS and 50 ng/mL nocodazole.

3.2. Cell Cycle-Dependent Dephosphorylation of pRb by PP1

The observation that the phosphorylation status of pRb is directly or indirectly controlled by PP1 was first reported in 1993 by several groups using different approaches (*12,14,28*). While these studies suggested that pRb undergoes dephosphorylation during mitosis, experiments with phosphatase inhibitors also indicated that PP1 activity might be required during G1 to maintain pRb in the dephosphorylated form (*13*). Apart from the dephosphorylation of pRb itself, there is also the interesting issue of complex formation between pRb and PP1. Studies in a number of cell types suggest that various forms of PP1 bind to pRb in a cell cycle-dependent manner (*14,15,17,29–31*), and that this binding involves the participation of a third protein (*29*). The question whether different PP1 isoforms dephosphorylate specific sites in pRb is important, because the phosphorylation of pRb at different sites has distinct functional consequences (examples are given in **Table 1**). Until recently, investigating the phosphorylation or dephosphorylation of pRb had to rely on techniques that require metabolic labeling of cells with rather large amounts of radioactivity. With the availability of phospho-specific antibodies to a large subset of pRb phosphorylation sites, this appears no longer necessary.

3.2.1. Dephosphorylation of pRb During Exit From Mitosis

1. Prepare mitotic CV1-P cells as described in **Subheading 3.1.1.**
2. Release the cells from the nocodazole-induced mitotic block and collect them for up to 6 h.
3. Separate equal amounts of protein (100 μg) per time point via SDS-PAGE and transfer them to Immobilon-P membranes. Probe the membranes with the appropriate antibodies (*see Note 3* for our Western blotting protocol).
4. Incubate the membranes with either sc-102 (to quantitate the overall amount of pRb) or the various phospho-specific antibodies (use 1 μg of antibody per 100 μg of cellular protein) (*see Note 4*).

3.2.2. Specificity of PP1 Isoforms for Different pRb Phosphorylation Sites In Vitro

1. Express mammalian recombinant PP1 in *E.coli* and purify as described (38).
2. Use recombinant baculoviruses encoding pRb, Cdk2/cyclin A, Cdk2/cyclin E, or Cdk4/cyclin D1 to infect 10^7 Sf9 cells (18). A detailed protocol for generating pRb and Cdks from baculovirus infected insect cells can be found in references (39) and (40), respectively.
3. Lyse cells by incubating them for 10 min at 4°C in kinase reaction buffer and immunoprecipitate pRb from pRb-overexpressing cells as described in **Subheading 3.3.1.**. Retain a small portion of nonphosphorylated pRb as a control.
4. Mix the lysates from Cdk/cyclin-overexpressing cells with the immunoprecipitated pRb, add 1 mM ATP, and incubate for up to 45 min at 30°C with occasional mixing (see **Note 5**).
5. After the phosphorylation reaction, it is crucial to wash the protein G beads exhaustively (at least six times) with ice-cold EBC buffer to remove the protein kinases and ATP. Retain one-fifth of the sample to examine the efficiency of pRb phosphorylation.
6. Divide the remainder of sample into four equally sized aliquots. Incubate equal amounts of the sample with either approx 20 U of PP1 α , PP1 γ , or PP1 δ individually, or approx 20 U of a mixture or all three isoforms at 30°C for 20 min.
7. Wash the dephosphorylated pRb again at least six times with ice-cold EBC buffer, and finally resuspend the sample in SDS sample buffer, separate by SDS-PAGE, and perform western blotting with the appropriate phospho-pRb antibodies (see **Notes 3** and **6**).

3.2.3. Specificity of PP1 Isoforms for Different pRb Phosphorylation Sites In Vivo

1. Synchronize CV1-P cells as described in **Subheading 3.1.1.**
2. Lyse 5×10^5 cells by sonication in 1 mL EBC buffer supplemented with 1 μ g/mL aprotinin and 1 μ g/mL PMSF (see **Note 1**).
3. Preclear the lysates by adding 75 μ L protein A Sepharose diluted 1:1 in NET-N.
4. Immunoprecipitate the PP1 isoforms from cells corresponding to 100 μ g of protein.
5. Wash the immunoprecipitates three times with 1 mL NET-N and twice with phosphatase reaction buffer.
6. To assess the overall pRb-directed phosphatase activity of distinct PP1 isoforms, mix immunoprecipitated PP1 with 32 P-labeled immunocomplexed pRb (this can be prepared as described in **Subheading 3.3.1.**) and incubate at 30°C for 30 min.
7. Stop the reaction by adding 2X SDS sample buffer, boil for 10 min, spin for 1 min at full speed, separate labeled proteins by SDS-PAGE, and visualize by autoradiography (see **Note 7** for a recommendation regarding quantitation of band intensities).

8. To assess the activity of individual PP1 isozymes against specific pRb phosphorylation sites, mix immunoprecipitated PP1 isoforms with lysates obtained from mitotic cells, carry out the phosphatase reaction as described previously, and analyze the reaction products by western blotting with antibodies to the pRb phosphorylation sites in question. It is worth mentioning that in our study, PP1 isoforms isolated from G1 or M phases markedly differed in their ability to dephosphorylate certain sites (pS807 and pT356) in pRb (**16**).

3.3. Apoptosis-Associated Dephosphorylation of pRb by PP1

Several lines of evidence suggest that pRb plays a negative role in apoptosis (for reviews, *see refs. 5,41*). Consistent with this function, pRb is being destroyed by a caspase in response to apoptotic stimuli (**42–44**): the major cleavage reaction liberates a 5 kDa fragment from the C-terminus of pRb. More recent results suggest that for pRb to be proteolyzed, it has to be present in dephosphorylated form (**18**). The reader will recall that traditionally, countless laboratories assessed the overall phosphorylation state of pRb by simple Western blot, assuming that phosphorylated pRb produces a band corresponding to 110 kDa, whereas dephosphorylated pRb produces a band of 105 kDa. Therefore, the larger of the two pRb fragments resulting from caspase-mediated cleavage can very easily be confused with the dephosphorylated version of pRb. To distinguish between proteolysis and dephosphorylation, a combination of two antibodies may be used. One of these (sc-50) reacts with the C-terminus of pRb and will thus reveal the amount of intact pRb, regardless of the phosphorylation state.

3.3.1. Analysis of pRb Following Induction of Apoptosis in Synchronized HL-60 Cells

1. To obtain HL-60 cells synchronized at the G1/S boundary, incubate the cells for 16 h in RPMI-1640 medium supplemented with 10% FBS and antibiotics (100 U/mL of penicillin G and 100 µg/mL of streptomycin) and 5 µg/mL aphidicolin.
2. Wash the cells repeatedly to remove aphidicolin. To induce the apoptotic response, add araC to a final concentration of 10 µM. Incubate the cells for 2 h.
3. Preincubate 1×10^6 cells with 2 mL of phosphate-free medium for 30 min, then replace with 2 mL of fresh phosphate-free medium containing 200 µCi ^{32}P -inorganic phosphate and incubate for 2 h in the presence or absence of araC (*see Note 8*).
4. Remove the drug and the remaining radioactive label by washing the cells at least four times in complete medium. Then incubate the cells further in complete medium. Collect the cells at different time points for up to 6 h, and wash at least four times with PBS (*see Note 9* for a comment on the time course of pRb dephosphorylation). Set aside a small aliquot for protein determination. Cells can be stored frozen at -20°C at this stage.
5. Using the small aliquot from **step 4**, lyse the washed cells in EBC buffer and determine the protein concentration. Then lyse the frozen cells as before, and if

necessary, increase the protein concentration to approx 1 mg/mL by adding BSA (see **Note 10**).

6. To preclear the lysate, add 1 μ g of mouse IgG plus a 1:1 mixture of 40 μ L of protein G-sepharose:EBC buffer in 1 mL of EBC buffer to 1 mg of protein.
7. Centrifuge the suspensions and mix the supernatants with 2 μ g of the pRb antibody sc-102; rotate overnight at 4°C. Add 20 μ L of protein G-Sepharose beads and incubate for another 4 h. Centrifuge the beads and wash at least six times with EBC buffer at 4°C.
9. Separate the immunocomplexed pRb via SDS-PAGE.
10. Carry out immunoblotting with the two pRb antibodies sc-102 and sc-50 (see **Note 3**). sc-102 reacts with all of the pRb present. sc-50 reacts only with intact pRb. A shift to a smaller apparent molecular mass as revealed by sc-102 thus indicates dephosphorylation, as long as sc-50 produces pRb bands of the same intensity.

3.4. Cell Cycle-Dependent Phosphorylation of PP1 by Cdk

Shortly after mammalian PP1 α was found to be inhibited in vitro by Cdk-mediated phosphorylation at a unique residue, Thr320 (45), it was shown that the *S. pombe* homolog of PP1, dis2, is also phosphorylated at the corresponding Thr in vivo at the onset of mitosis (46). Using an antibody specific for the phosphorylated form of PP1 α , Kwon et al. demonstrated a few years later that mammalian PP1 α undergoes mitotic phosphorylation at Thr320 (25). Our finding that a phosphorylation-resistant, constitutively active PP1 α mutant causes pRb-dependent cell cycle arrest in G1 (47) suggested that PP1 α is also phosphorylated in late G1. And indeed, Thr320 in pRb-associated PP1 α was shown to be phosphorylated before the G1/S transition (15). The techniques used in this study form the basis for the methods described herein.

3.4.1. Immunoprecipitation of PP1 From Radioactively Labeled Cells

1. Plate and synchronize MG63 cells in 75 cm² flasks as described in **Subheading 3.1.2.**
2. Once the desired point in the cell cycle has been reached, preincubate the cells with 2 mL of phosphate-free medium containing 5% FBS for 30 min, then replace with fresh phosphate-free medium containing 1.0–1.5 mCi of ³²P_i per 1 \times 10⁶ cells and incubate for 2.5 h (see **Note 8**).
3. To harvest the cells, remove the radioactive medium and discard it into appropriately shielded radioactive waste containers. Rinse the monolayer of cells three times with serum-free medium and discard as above. Lyse the cells in the flask with 1.2 mL ice-cold RIPA buffer. This is sufficient to just cover the cells evenly (if, for any reason, the size of the flask or well has to be changed, you should aim for at least 15 μ L per cm²). Leave on ice for 10 min, and then tilt the flask so that the liquid can be collected from one corner of the flask. Transfer lysates to Eppendorf tubes. If convenient, lysates can be stored at –20°C until further use.

4. Determine the protein concentration and adjust each sample to about 1 mg/mL of protein by adding BSA (*see Note 10*). Pre-clear the lysates by incubating them for 30 min on ice with 50 μ L of a 1:1 suspension of protein A–Sepharose:RIPA buffer. Then remove the Sepharose A beads by a short centrifugation at 1500g for 2 min at 4°C and carefully transfer the supernatants to fresh tubes on ice. Discard the pellet into a radioactive waste container.
5. For immunoprecipitation, add a fresh 50 μ L of Sepharose A suspension and the PP1 α antibody Ab2 (8 μ g) or the phospho-PP1 α antibody Ab3 (4 μ g). Rotate the samples end-over-end for 2 h at 4°C and collect the beads by centrifugation for 15 sec at 4°C at top speed (14,000g). Aspirate the supernatants with a 25-gage needle and discard into radioactive waste. Add fresh RIPA buffer, mix gently, and centrifuge. Repeat three times.
6. Finally, mix the beads containing radioactively labeled PP1 with 50 μ L of 2X concentrated SDS sample buffer and boil for 5 min. Samples are now ready to be examined by SDS-PAGE.
7. With radioactively labeled samples, expose the dried gel to x-ray film and visualize phosphorylated PP1 by autoradiography. With nonlabeled samples, perform a Western blot with Ab3 (*see Notes 3 and 11*).

3.4.2. Co-Immunoprecipitation of PP1 and pRb From Nonlabeled Cells

1. Prepare cells as described in **Subheading 3.4.1.**, except that no metabolic labeling of cells is required.
2. For immunoprecipitation of pRb from 2×10^6 MG63 cells, use 10 μ g of sc-102; otherwise follow the protocol described in **Subheading 3.3.1.**
3. Separate the immunocomplexes via SDS-PAGE, and carry out Western blotting (*see Note 3*) with three antibodies—sc-102 for pRb, Ab1 for PP1 α , and Ab3 for phosphorylated PP1 α (*see Note 12*).

4. Concluding Remarks

Although a cell cycle function for pRb has been known since 1989, the study of this important protein is still attractive to many laboratories. One major reason for the continued interest in pRb is the intriguing idea to target pRb for novel cancer therapies, and another may be that the presumably many functions and interactions of pRb still pose severe puzzles. It is obvious that, therefore, pRb is being studied in a wide variety of model systems and techniques. In this chapter, we have described several approaches to study the interaction between PP1 and pRb, which has been neglected so far. Given that further conceptual and technical breakthroughs (such as the realization that pRb and PP1 are involved in apoptosis, and the availability of phosphospecific antibodies for the majority of sites phosphorylated in pRb) will occur, new and important questions will undoubtedly arise. Therefore, the methods described herein have to be considered as guidelines.

Table 2
Preparation of Concentrated Stock Solutions

Reagent	Concentration	Times final	Solvent	Store at
Aprotinin	2 mg/ μ L	1000	H ₂ O	−20°C
Benzamidine	1 M	1000	H ₂ O	RT
Leupeptin	50 μ g/mL	100	H ₂ O	−20°C
Pepstatin	1 mg/mL	1000	H ₂ O	−20°C
PMSF	100 mM	1000	DMSO	RT
EDTA	0.5 M	1000	H ₂ O	RT
NaF	1 M	10–40	H ₂ O	RT
Na ₃ VO ₄	100 mM	10	H ₂ O	RT

5. Notes

1. For all supplements to be added freshly it is most convenient to prepare concentrated stock solutions as follows, and according to **Table 2**:
2. Serum deprivation, mimosine, and aphidicolin typically arrest eukaryotic cells with the same DNA content: FACS analysis reveals that these cells contain a G1 amount of DNA—i.e., these cells have not yet replicated their DNA. FACS analysis of cells that have been released from these various blocks for 4–24 h reveals that these three conditions are not equivalent. Thus, cells released from serum deprivation need the most time to reach S phase, cells released from mimosine need a few hours to reach S phase, whereas aphidicolin-treated cells enter S phase almost immediately. In summary, serum-deprived cells are believed to be in G0/G1. Mimosine—chemical name: α -amino- β -(*N*-[3-hydroxy-4-pyridone])propionic acid—is a plant amino acid that arrests cells late in G1 (**48**), and its mechanism of action may involve induction of p27^{Kip1} (**49**). Aphidicolin arrests cells at the G1/S boundary by inhibiting DNA polymerase (**50**).
3. We have used the following protocol for Western blotting experiments involving pRb and PP1 in the past: After electrophoresis, transfer proteins to Immobilon-P membranes at a constant voltage of 30 V. Using the Novex Minicell, this should be accomplished in 1–2 h. Incubate the membrane overnight in 5% non-fat dry milk in TBS (25 mM Tris-HCl, pH 8.0, 137 mM NaCl, 2.7 mM KCl). Incubate the membrane for 2 h with the primary antibodies diluted in TBS containing 0.2% Tween-20 (TBS-T) and 2% BSA. If convenient, this step can be performed overnight as well. Wash the membrane with TBS-T three times for 15 min, and incubate for 1 h with the secondary antibody diluted with 2% BSA in TBS-T. Wash again three times for 15 min and visualize immunoreactive bands with the ECL reagents (Amersham). All incubations are at room temperature.
4. Make sure to harvest sufficient sample so that each pRb phosphorylation site can be examined individually in a separate western blotting experiment. It is not recommended to “strip” the membranes and reprobe with another pRb antibody.

5. The aim of this experiment is to generate pRb that has been phosphorylated by a distinct agent or a “cocktail” of Cdk/cyclin complexes. Therefore, the Cdk/cyclin complexes should be in excess of pRb to shorten the reaction time, while the overall amounts of protein should be adjusted such that there is sufficient sample to visualize by western blotting. Assuming that (1) 50 ng of pRb phosphorylated in a particular site is enough for this purpose (*see also Note 5*), (2) this experiment involves at least 10 phospho-specific antibodies, and (3) the phosphorylation reactions proceed to only 50% completion, one would have to use at least 1 μ g of pRb per PP1 isoform to be tested in this experiment.
6. Originally, the phosphorylation of pRb was performed in the presence of [γ - 32 P]ATP (**51**). In that case, in order to address the question of site specificity, 32 P-labeled pRb had to be digested by a protease (we used chymotrypsin successfully [Barbara Driscoll and Norbert Berndt, unpublished]), and the peptides were separated by two-dimensional phosphopeptide mapping. Nowadays however, while it may still be a good idea to include radioactively labeled ATP to assess the overall level of phosphorylation, the phosphorylation site-specific antibodies are more convenient to address the issue of site specificity.
7. If necessary, the band intensities of the Western blot can be analyzed with a scanning densitometer (Hoefer Scientific Instruments) and quantitated with GS370 v.3 software. This approach should yield an accurate assessment of the time course of pRb dephosphorylation for different sites.
8. Phosphate-free medium is easily available commercially—for example, from Irvine Scientific, Santa Ana, CA. To obtain phosphate-free FBS, dialyze the required amount against an excess of phosphate-free medium overnight. To minimize the pipetting steps and, therefore, possible contamination of the workspace with radioactivity, it is best to prepare metabolic labeling experiments as follows: Determine the number n of samples/wells/flasks, the volume V of the medium that is needed per sample, and the amount of radioactivity R needed per sample. Prepare $(n + 1) \times V = a$ mL of medium, add $(n + 1) \times R = b$ μ Ci, and mix well. Then add the predetermined volume of medium containing the radioactivity to each sample.

As already pointed out in **Subheading 3.1.**, in order to double-check the degree of synchrony of metabolically labeled cells, it is advisable to set up identically treated sister cultures (except that radioactive label is omitted) and examine them by FACS analysis.

9. This protocol describes the procedure for araC-induced apoptosis in HL-60 cells synchronized at the G1/S boundary. In this case, dephosphorylation of pRb was maximal 45 min after terminating the exposure to araC, whereas caspase-mediated cleavage of pRb was not detectable until 105 min after terminating the exposure to araC (**18**). Assuming that this protocol may be of interest for researchers studying other experimental models of apoptosis (cell types, cell cycle phase, apoptosis-inducing drugs, “death substrates” that are also substrates for PP1), these times may vary. Therefore, we recommend performing a time course of dephosphorylation of the protein in question.

10. In very dilute protein solutions, proteins often adsorb to tube walls, or aggregate, or become degraded. The addition of BSA is recommended to protect precious sample proteins from these unspecific processes. If the protein concentration is higher than 1 mg/mL, then this is not necessary; instead dilute all samples to 1 mg/mL with the appropriate buffer (e.g., the one the samples are stored in).
11. It would be of interest to also determine the phosphorylation of the other two PP1 isozymes, γ and δ . As phosphorylation site-specific antibodies for these are not yet available, an alternative approach would be to immunoprecipitate these PP1 isoforms, transfer them to membranes, and then probe them with phosphothreonine antibodies (PharMingen).
12. It is recommended to run this experiment in duplicate. After electrotransfer of the proteins, cut the membranes in two pieces along the BSA molecular weight standard (66 kDa), with pRb ($M_r = 110$ kDa) on the upper half and PP1 ($M_r = 37$ kDa) on the lower half. Done this way, you can avoid "stripping" membranes and reprobing them with a different primary antibody. Probe the upper halves with sc-102, and the lower halves with Ab1 and Ab3, respectively.

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