



Site-specific and temporally-regulated retinoblastoma protein dephosphorylation by protein phosphatase type 1

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pRb is dephosphorylated at mitotic exit by the type 1 serine/threonine protein phosphatases (PP1). Here we demonstrate for the first time that mitotic pRb dephosphorylation is a sequential, temporally-regulated event. We also provide evidence that the three mammalian isoforms of PP1, α , γ -1, and δ , differ in their respective preferences for site-specific pRb dephosphorylation and that the mitotic and G₁ PP1-isoform counterparts exhibit differential activities towards mitotic pRb. Finally, the physiological relevance of the striking contrast between the patterns of Thr821 and Thr826 dephosphorylation, sites known to be important for disrupting binding of LXCXE-containing proteins to pRb, is addressed. *Oncogene* (2001) 20, 3776–3785.

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Introduction

The identification of the role that the product of the retinoblastoma susceptibility gene, pRb, plays in cell cycle control has been crucial to understanding the cellular response to both growth-promoting and growth-inhibitory signals. One current model states that the restriction point, a time during which a cell commits to the division pathway (Pardee, 1974), is controlled by cell cycle-dependent pRb phosphorylation (reviewed in Bartek *et al.*, 1996; Herwig and Strauss, 1997). pRb phosphorylation peaks at S phase and diminishes at exit from mitosis (Buchkovich *et al.*, 1989; Chen *et al.*, 1989; DeCaprio *et al.*, 1989; Mihara *et al.*, 1989; Ludlow *et al.*, 1990). pRb is thus a substrate for both kinases and serine/threonine protein phosphatases.

As pRb contains 16 different consensus cdk sites (Kitagawa *et al.*, 1996), phosphorylation of pRb can be a complex matter. Phosphorylation of pRb is thought to be carried out by the sequential activation of Cyclin

D/cdk4 in mid-G₁ which precedes Cyclin E/cdk2 at the G₁/S boundary, and Cyclin A/cdk2 in S phase (Lundberg and Weinberg, 1998). Two-dimensional phosphopeptide mapping has shown that different cdks differentially target phosphorylation sites on pRb (Zarkowska and Mittnacht, 1997), and that phosphorylation at different sites interferes with distinct protein-binding functions of pRb (Knudsen and Wang, 1996; Zarkowska and Mittnacht, 1997).

The enzyme that dephosphorylates pRb at mitotic exit is a holoenzyme complex of serine/threonine protein phosphatase type 1 (PP1) (Alberts *et al.*, 1993; Ludlow *et al.*, 1993; Yan and Mumby, 1999) plus interacting subunits that are thought to regulate its activity (Nelson and Ludlow, 1997; Nelson *et al.*, 1997). PP1 itself is subject to regulation by phosphorylation which appears to temporally control its activity towards pRb, preventing its premature activation so that coordinated cell cycle progression occurs (Dohadwala *et al.*, 1994; Kwon *et al.*, 1997; Liu *et al.*, 1999; Puntoni and Villa-Moruzzi, 1997). We have previously shown that of the three widespread mammalian isoforms of PP1, the delta isoform has the greatest pRb-directed phosphatase activity (Nelson *et al.*, 1997).

In continuing our study into the regulation of pRb dephosphorylation, and in light of accumulating evidence for both specificity of cdks for subsets of pRb phosphorylation sites and sequential pRb phosphorylation, we sought to determine whether the activation of pRb following mitotic exit would also proceed sequentially. The purpose here is to help clarify the functional significance of individual phosphorylation sites. Towards this goal, we made use of a panel of phosphorylation site-specific antibodies which allowed for tracking levels of phosphorylation at specific ser/thr sites on pRb as cells were released from mitotic block. Use of phosphorylation site-specific antibodies to aid in creating protein functionality profiles is becoming increasingly popular, as evidenced by their use in studying p53 (Nakaya *et al.*, 2000). Our results support the notion that pRb is indeed dephosphorylated sequentially, in a temporal manner, and that Cyclin D/cdk4 sites are those initially dephosphorylated. Our results also indicate that isoforms of PP1, much like the different cdks, have preferences for targeting a subset of pRb phosphorylation sites, and that mitotic- and G₁ phase PP1 isoforms exhibit

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different activities towards each site on pRb. We also address the possible physiological significance of the striking contrast between the pattern of dephosphorylation of Thr821 and Thr826, sites known to be important for disrupting binding of LXCXE-containing proteins to pRb (Knudsen and Wang, 1996; Zarkowska and Mittnacht, 1997).

Results

pRb is sequentially dephosphorylated at mitotic exit

To determine whether pRb dephosphorylation at mitotic exit proceeded in a sequential, temporal manner, whole-cell lysates were prepared from CV1-P monkey kidney cells following a time course of release from a G₂/M block. As shown in Table 1, at least 95% of the starting cells were at the G₂/M phase following mitotic shake-off. Flow cytometric analysis at the indicated time points of release revealed an increasing population of G₁ cells after 50 min, indicative of mitotic completion. Western blot analyses using pRb phosphorylation site-specific antibodies (Tamrakar *et al.*, 1999) revealed that sequential, temporal dephosphorylation of individual cdk sites on pRb occurs during mitotic progression. Figure 1A illustrates this point. As shown, Western blot analyses from the same aliquot of whole-cell lysate reveals a decrease in phospho-Ser807 abundance after 30 min of mitotic release, coincident with an increase in abundance of the faster migrating, hypophosphorylated form of pRb. In contrast, abundance of phospho-Thr356 decreases after 60 min of mitotic release, and that of phospho-Ser608 persists until much later during the time course; 4 h, when a greater population of the cells are in G₁.

These data also reveal that the sites previously mapped as substrates for Cyclin D/cdk4 and those mapped to Cyclin E/cdk2 (Zarkowska and Mittnacht, 1997) appear to have different patterns for dephosphorylation at mitotic exit. As shown in Figure 1B, phosphorylation at the Cyclin D/cdk4 sites proceeds readily in one of two patterns. Ser249, Thr252, Thr356,

Ser788 and Ser807 are dephosphorylated at a rapid rate, completed by 50–60 min post mitotic block, when the first significant portion of cells has entered G₁. Dephosphorylation of Ser780 and Ser608, although proceeding at a slower rate, shows the same general pattern, but disappear much later; at 6 h following release from mitotic arrest (not represented on Figure 1B). The Cyclin E/cdk2 sites, Thr373 (which may also be targeted by Cyclin D/cdk4) and Ser795 show a markedly different course of progression (Figure 1C). Initial, rapid dephosphorylation by 40 min is followed by a rapid recovery of phosphorylated band intensity, eventually leveling off as the majority of the cells enter G₁. These data are in agreement with others who have shown that Ser795 is among the first sites phosphorylated in G₁ (Boylan *et al.*, 1999).

We also observed a marked difference in the dephosphorylation rates and levels of two clustered phosphorylation sites. Thr821, a Cyclin E/cdk2 site, and Thr826, a Cyclin D/cdk4-specific site, are separated by 4 amino acids in the primary sequence of pRb, and both are part of the C-region of the large pocket domain of pRb. At release from mitotic block, Thr826 dephosphorylation is complete at 40 min, while Thr821 phosphorylation levels remain high, even as cells have entered G₁ after 60 min (Figure 1D).

Phosphorylation at Thr821 does not inhibit LXCXE protein binding

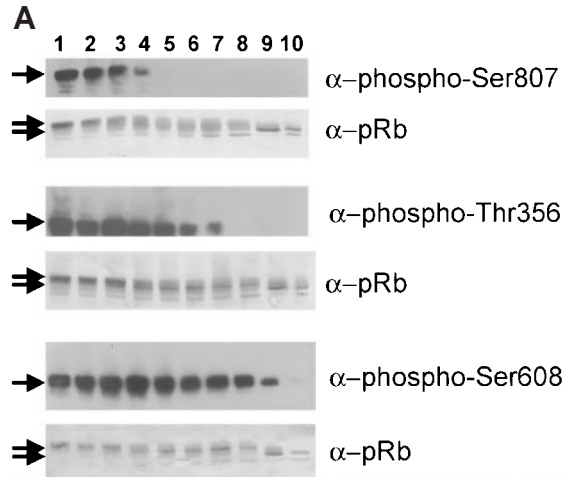
Since Thr821 and Thr826 phosphorylation has been shown in a number of different studies to be inhibitory to the interaction of GST-pRb with LXCXE-containing proteins such as SV40 T-antigen, Elf-1 and human papillomavirus E7 (Knudsen and Wang, 1996; Zarkowska and Mittnacht, 1997), we decided to address whether pRb phosphorylation at Thr821 would indeed inhibit LXCXE protein binding. Our approach was to perform coimmunoprecipitation studies using T-antigen expressing cells during a time course of release from a G₂/M block. This would allow us to obtain populations of cells with pRb differentially phosphorylated on Thr821 and Thr826. We reasoned that complex formation with T-antigen as a function of differential pRb phosphorylation would permit an assessment of the relative importance of these phosphorylated sites in T-antigen binding.

Towards this end, flow cytometric analysis of T-antigen expressing SV80 cells demonstrated that 50 min following the release from mitotic arrest, only 3% of the cells had reached the ensuing G₁ phase. However, 2 h after release from mitotic block, 51% of cells had entered G₁, much like CV1-P cells (Table 1). To compare the phosphorylation status of Thr821 and Thr826 between the SV80 and CV-1P cells, we chose to normalize to G₁ cell content. As such, SV80 cells released from mitotic block for 50 min or 2 h were subjected to separate immunoprecipitations with either antibody to pRb or T-antigen. When both Thr821 and Thr826 were phosphorylated (*t* = 50 min, 82% of cells in G₂/M), anti-T immunoprecipitates contained little, if

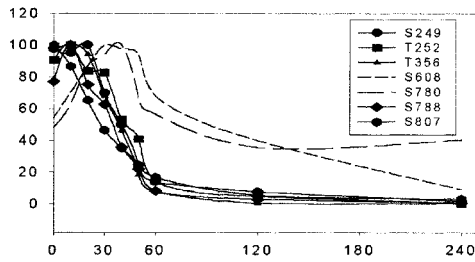
Table 1 Time course of CV1-P cells released from mitotic block

Time (min)	% G ₂ /M	% G ₁
0	95	0.5
10	96	0.3
20	93	0.7
30	93	0.7
40	91	1.0
50	55	35
60	63	29
120	39	51
240	43	43
360	31	56

CV1-P cells were nocodazole arrested in G₂/M, harvested, and a time course of release from mitosis carried out as described in Materials and methods. 0.5×10^6 cells were removed at the indicated times and cell cycle stage determined by flow cytometry and DNA histogram analysis. Results are representative of two different experiments

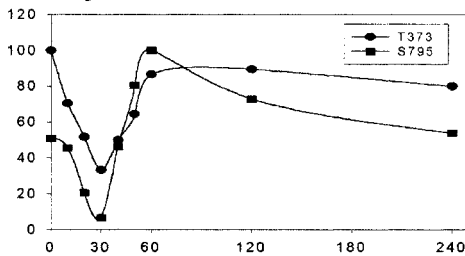


B. CyclinD/cdk4

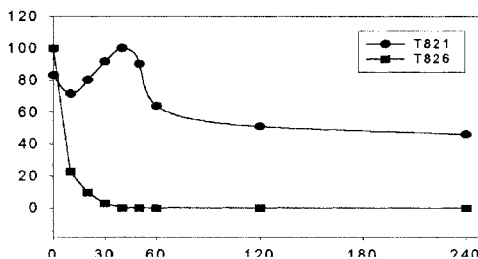


Relative phosphorylation levels

C. CyclinE/cdk2



D. Thr821 vs. Thr826



Time from G2/M arrest (min.)

Figure 1 Temporal regulation of pRb dephosphorylation at mitotic exit. (A) Protein extracts (100 μ g) from CV1-P cells following a time course of release from mitotic block were separated by SDS-PAGE, transferred to nitrocellulose, and incubated with phosphorylation site-specific antibodies to Ser807 (top panel), Thr356 (middle panel) or Ser608 (bottom panel) as indicated. Arrows indicate the positions of pRb phosphorylated at Ser807, Thr356 and Ser608, respectively. Blots were then re-probed with monoclonal antibodies to pRb (second panel from the top, fourth panel from the top, and bottom panel) to locate

any pRb (Figure 2, lane 3, anti-pRb blot). As expected, the reciprocal pRb immunoprecipitation contained a reduced amount of SV40 T-antigen (lane 5, anti-T-antigen blot). In contrast, 2 h post mitotic arrest, when 51% of cells have progressed into the ensuing G₁ phase, and Thr821 alone remains highly phosphorylated, abundant levels of pRb co-immunoprecipitate with T-antigen (Figure 2, lane 4, anti-pRb blot). The reciprocal immunoprecipitation also demonstrates T-antigen readily co-immunoprecipitating with pRb when Thr821 is phosphorylated (Figure 2, lane 6, anti-T-antigen blot). Taken together, it appears that little pRb-T-antigen complex exists in mitotic cells when Thr821 and Thr826 are both phosphorylated. However, when cells enter G₁ phase, Thr826 is dephosphorylated, while Thr821 is not. Nevertheless, pRb is capable of binding to this LXCXE-containing protein, suggesting that Thr821 phosphorylation does not interfere with LXCXE binding *in vivo*.

Thr821 is not targeted by PP1 isoforms *in vitro*

We were intrigued by the above observation that Thr821 does not exhibit dephosphorylation during our *in vivo* timecourse, and speculated that perhaps this phospho-residue is not a substrate for PP1. To

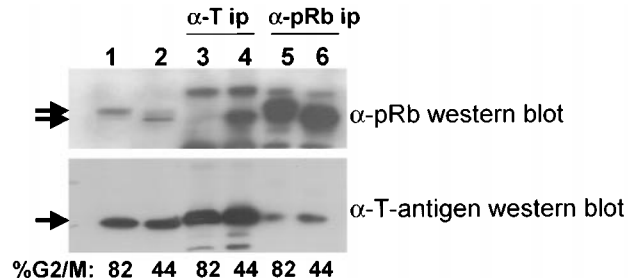


Figure 2 Thr821 phosphorylation does not hinder LXCXE binding. Western blots of anti-pRb (top panel) and SV40 T-antigen (lower panel) in co-immunoprecipitation experiments. SV80 cells were released from mitotic arrest for 50 min (lanes 1, 3, 5) or 2 h (lanes 2, 4, 6), and the resulting % of G₂/M cells was determined by flow cytometry and DNA histogram analysis. Lane 1, SV80 cell extracts after 50 min, and lane 2, after 2 h of release from G₂/M arrest. Positions of pRb isoforms and SV40 T-antigen are indicated by the arrows. Immunoprecipitations in lanes 3 and 4 were performed using antibody to T-antigen, which were then probed with anti-pRb (top panel). The reciprocal immunoprecipitation with anti-pRb and probing with anti-T-antigen (lower blot) is shown in lanes 5 and 6

both the hyperphosphorylated (top arrow) and hypophosphorylated (bottom arrow) forms. Extracts were prepared at 0 (lane 1), 10 (lane 2), 20 (lane 3), 30 (lane 4), 40 (lane 5), 50 (lane 6), 60 (lane 7), 120 (lane 8), 240 (lane 9), and 360 (lane 10) min of release. (B-D) Western blots of CV1-P cells released from mitotic block were probed with the indicated phosphorylation site-specific antibodies for Cyclin D/cdk4 sites (B), Cyclin E/cdk2 sites (C) and Thr826 (a Cyclin D/cdk4 site) and Thr821 (a Cyclin E/cdk2 site) (D). Band intensities were quantitated by densitometry and normalized for pRb content. Values were converted to % of peak levels and plotted

determine whether Thr821 on pRb can indeed serve as a substrate for PP1, the three widely distributed PP1 isoforms, α , γ -1, and δ , were immunoprecipitated from mitotic CV1-P cells for use in an *in vitro* dephosphorylation assay (Nelson *et al.*, 1997). Mitotic CV1-P extracts were incubated with mitotic PP1 isoform immune complexes for 60 min at 30°C, prior to preparation for gel electrophoresis. Western blots were probed with phospho-Thr821 or -Thr826 specific antibodies, or anti-pRb for total pRb content, then quantitated by densitometry. Densitometric analysis of these blots revealed that, unlike Thr826 (Figure 3A bars 4–6), Thr821 was not efficiently dephosphorylated by any mitotic PP1 isoform (Figure 3A bars 1–3), as band intensities did not diminish significantly compared to untreated controls. When PP1 isoforms from G₁-phase cells were tested for activity at these pRb sites, similar results were obtained (Figure 3B). These results are consistent with our findings that Thr821 phosphorylation levels are high following mitotic exit and during other phases of the cell cycle, and may suggest that failure of this site to be dephosphorylated by PP1 is at least partly responsible for this observation. To summarize these data, it appears that pRb phosphorylation at Thr821 does not disappear at mitotic exit (Figure 1D), is not targeted efficiently by

the various PP1 isoforms (Figure 3A,B), and does not interfere with the LXCXE protein binding function of pRb (Figure 2).

We did observe some differences in activities of the PP1 isoforms at these phosphorylation sites on pRb (Figure 3B compare lane 4 to lanes 5 and 6). This intrigued us in light of our earlier data showing that the mitotic δ isoform of PP1 was more efficient overall at dephosphorylating pRb *in vitro* than were the other isoforms (Nelson *et al.*, 1997). Also, it had recently been reported that different cdk's phosphorylated only subsets of sites on pRb (Connell-Crowley *et al.*, 1997; Knudsen and Wang, 1996; Zarkowska and Mittnacht, 1997). We speculated that perhaps the different PP1 isoforms could dephosphorylate subsets of the pRb phosphorylation sites.

The greater pRb-directed phosphatase activity of PP1 δ persists only until G₁ phase

To address whether the different PP1 isoforms demonstrated a preference for one or more subsets of pRb phosphorylation sites, we first measured overall pRb-directed phosphatase activity in extracts of cells released from mitotic exit. Consistent with our results shown in Table 1, release from mitotic block occurred by 60 min after replating nocodazole-treated cells in warm, serum-containing medium (Table 2). Figure 4A shows that 50 μ g of extracts prepared from these same cells released from mitotic arrest for 30 min (lane 1), 60 min (lane 2) or 2 h (lane 3) efficiently dephosphorylated radiolabeled pRb *in vitro*.

We had earlier shown that the δ isoform of mitotic PP1 dephosphorylated pRb *in vitro* more efficiently than did the other isoforms (Nelson *et al.*, 1997). In experiments presented here, cells were released into the ensuing G₁ phase in order to track the activation of pRb at mitotic exit. Therefore, to measure whether the δ isoform had greater pRb-directed phosphatase activity even upon release from mitotic arrest, PP1 was immunoprecipitated using antibodies previously shown to be isoform-specific (Tognarini and Villa-Moruzzi, 1998) from CV1-P cells released from mitotic block as represented in Table 2. Our results indicate that the δ isoform consistently dephosphorylated pRb more efficiently until the G₁ fraction of cells increased

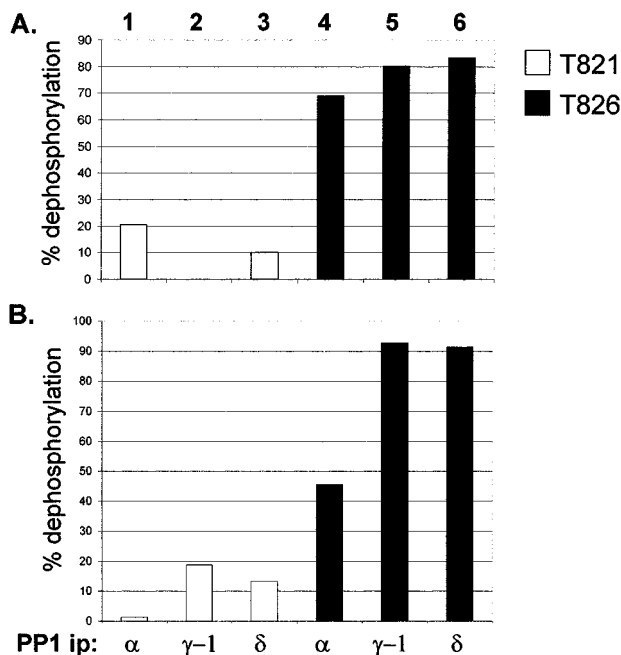


Figure 3 Thr821 is not dephosphorylated by PP1 isoforms *in vitro*. Mitotic cell extracts were mixed with immunoprecipitated PP1 isoforms from mitotic (A) or G₁ (B) cells followed by resolution by gel electrophoresis. Western blots were first probed with either phospho-Thr821 (lanes 1–3, white bars), or phospho-Thr826 (lanes 4–6, black bars) and then reprobed with anti-pRb. Band intensities were quantitated by densitometry and normalized for total pRb content. Per cent dephosphorylation was calculated by subtracting band intensities from values of untreated controls, dividing by the untreated control band intensity and then multiplying by 100. Results are representative of two different experiments

Table 2 Cell cycle profiles of CV1-P cells released from mitotic block for pRb phosphatase assays

Time (min)	% G ₂ /M	% G ₁
0	98.5	0.1
30	98.5	0.1
60	49	43
120	16	66

A timecourse of CV1-P cell release CV1-P from G₂/M was as described in Materials and methods. 0.5 × 10⁶ cells were removed at the indicated times following release and cell cycle stage determined by flow cytometry and DNA histogram analysis. Extracts from cells at the indicated stages of the cycle were used in all subsequent pRb-directed phosphatase assays

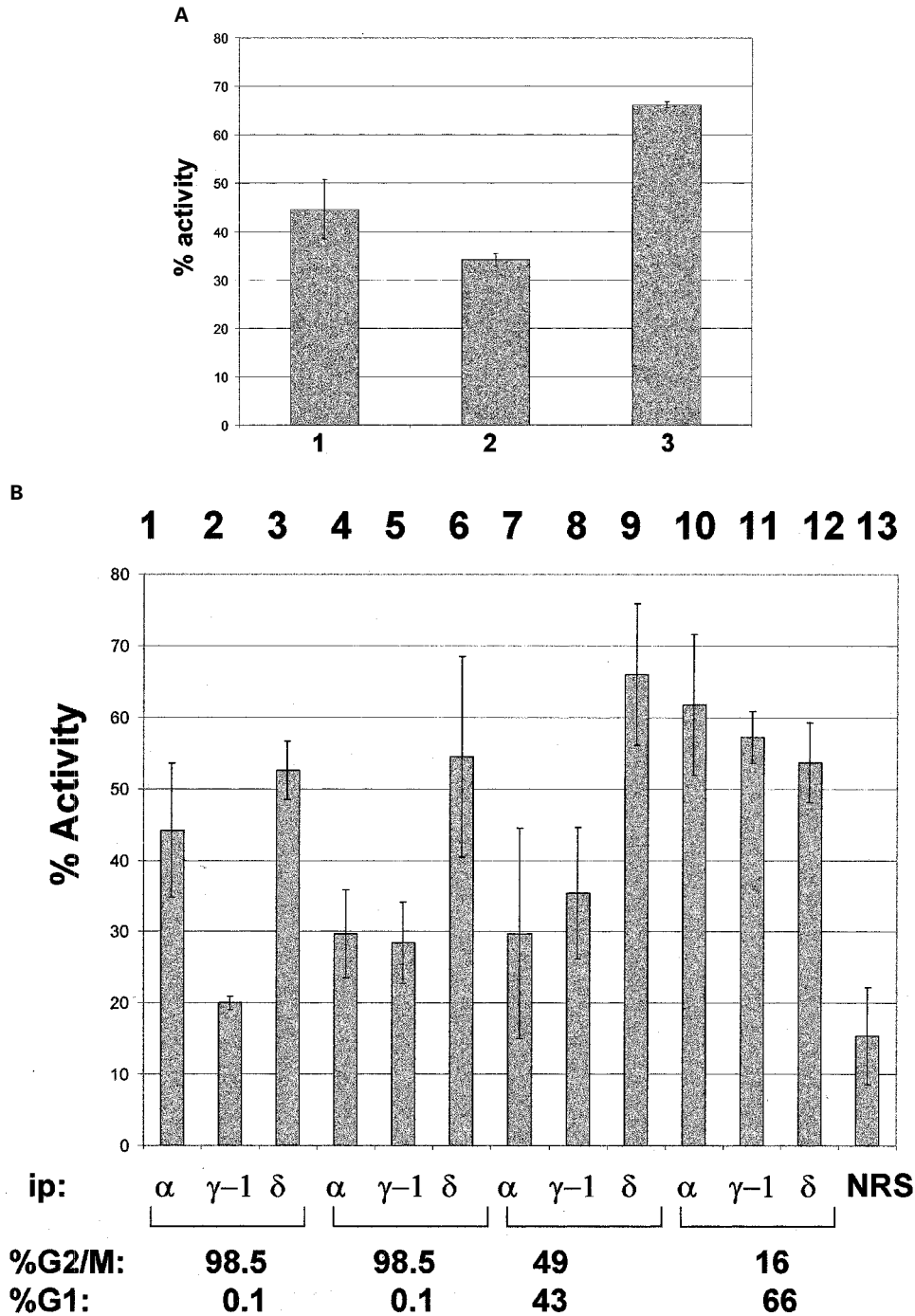


Figure 4 pRb dephosphorylation activity and isoform-specific pRb dephosphorylation in cells released from mitotic arrest. (A) Extracts (50 μ g) of cells released from mitotic arrest for 30 (lane 1), 60 (lane 2), and 120 (lane 3) min were mixed with 32 P-labeled immunocomplexed pRb substrate as described in Materials and methods. Proteins were resolved on 6% SDS polyacrylamide gels, dried, and radiolabeled bands were quantitated by phosphorimager analysis. (B) PP1 isoforms were immunoprecipitated from CV1-P cells released from mitotic arrest as per Table 2. Immune complexes were mixed with radiolabeled pRb and phosphatase activity measurements made as described in Materials and methods. Normal rabbit serum (NRS) immunoprecipitation served as a negative control. Per cent activity was calculated by subtracting the experimental 32 P-labeled pRb band intensity from untreated control intensity, then dividing by the untreated control intensity and multiplying by 100. Results are the average of three trials. Error bars represent the range of values for the three trials

to 66% after 2 h (Figure 4B). At this point, the activity of all three isoforms was approximately equal (Figure 4B, bars 10–12). Enzymatic activity of the PP1

isoforms in this fraction of cells were all high, particularly for the α and $\gamma-1$ isoforms (compare bars 1 and 2, 4 and 5, 7 and 8, with bars 10 and 11),

consistent with the overall greater pRb-directed activity in extracts of these cells (Figure 4A, bar 3). We therefore conclude that PP1 δ has greater pRb-directed enzymatic activity throughout mitosis (Figure 4B, bars 3, 6, and 9), but this preferential activity does not persist in G₁.

Mitotic and G₁ PP1 isoforms exhibit different preferences for subsets of pRb phosphorylation sites

Since different cdks have specificity at different subsets of phosphorylation sites on pRb (Connell-Crowley *et al.*, 1997; Knudsen and Wang, 1996; Zarkowska and Mittnacht, 1997), we questioned whether the same would be true of the PP1 isoforms which dephosphorylate pRb. To address this, dephosphorylation assays were carried out using immunocomplexed PP1 isoforms from mitotic cells mixed with extracts from mitotic CV1-P cells. Proteins were resolved by gel electrophoresis and transferred to nitrocellulose for Western blotting with phosphorylation site-specific antibodies. Band intensities were quantitated by densitometry, normalized for total pRb content, and converted to per cent dephosphorylation compared to untreated controls. Figure 5A shows how mitotic PP1 isoforms differ in their preferences for one phosphorylation site on pRb. Ser608 is dephosphorylated to an equivalent degree by all mitotic PP1 isoforms compared to an untreated control (Figure 5A, top blot, compare lanes 2–4 with lane 1). In contrast, Ser249 appears to be dephosphorylated preferentially by the γ -1 isoform of mitotic PP1 (Figure 5A, third blot from the top, compare lane 3 with lanes 2 and 4), despite equivalent levels of total pRb (Figure 5A, α -pRb blots).

To determine per cent dephosphorylation, band intensities were quantitated by densitometry, normalized for total pRb content, and subtracted from those of untreated controls. Figure 5B shows that mitotic PP1 isoforms differ in their preferences at a subset of sites on pRb. While some phosphorylation sites, such as Ser608 and Ser807 are dephosphorylated to a relatively equivalent degree by all mitotic PP1 isoforms, this is not the case at a number of other phosphorylation sites on pRb. Most notably, Ser249 is targeted for dephosphorylation most efficiently by the γ -1 isoform of mitotic PP1 (as in Figure 5A) while Ser788 is dephosphorylated least efficiently by the α isoform. We had previously reported on the differential activity of the mitotic PP1 isoforms toward pRB *in vitro* (Nelson *et al.*, 1997). In this earlier study, we were assaying for the overall PP1 activity towards pRb phosphorylated *en toto*; we were not able at that time to assay for PP1 activity towards individual phosphorylated residues on pRb. In this present report, we now show that individual PP1 isoforms do indeed differ in their preferences for specific phosphorylated residues on pRb.

When mitotic cell extracts were treated with immune complexes of PP1 isoforms from G₁-phase cells, another observation was made. Some sites, such as Ser807 in Figure 5C were dephosphorylated completely

by all PP1 isoforms, both mitotic (lanes 2–4) and G₁-derived (lanes 5–7). Other sites, such as Thr356, are dephosphorylated to different degrees by each PP1 preparation. Comparing lanes 5–7 with lanes 2–4 in Figure 5C, it appears that Thr356 is dephosphorylated with greater efficiency by the G₁-phase enzyme preparations.

Quantitative analysis of these phosphatase reactions revealed that G₁ phase PP1 isoforms also display differences in dephosphorylation efficiencies at a number of sites on pRb, again most notably at Ser249 and Ser788 (Figure 5D). In addition, a comparison of isoform-specific dephosphorylation in Figure 5B,D reveals a difference in the observed patterns. If mitotic and G₁ phase PP1 holoenzymes are identical, the graphs in Figure 5B,D should be very similar. As can be seen, this is not the case. While Ser249 is dephosphorylated most efficiently by mitotic PP1 γ -1, the reverse is true of the G₁ phase γ -1 isoform. Differences in mitotic and G₁ phase PP1 isoform activity are also observed at Ser788 where PP1 α derived from G₁ but not mitotic cells displays greater phosphatase activity at Ser788 than the other isoforms. Consistent with our results in Figure 4A, where cell extracts containing a greater G₁ population dephosphorylated pRb more efficiently, we infer that mitotic and G₁-phase PP1 isoforms are differentially regulated in their activities towards pRb, which contributes to the apparent temporal and sequential dephosphorylation of pRb.

Discussion

In this study we have undertaken the most comprehensive analysis of the dephosphorylation pattern of pRb at mitotic exit that we are aware of, by utilizing a panel of pRb phosphorylation site-specific antibodies. Use of phosphorylation site-specific antibodies to aid in creating protein functionality profiles is becoming increasingly popular, as evidenced by their use in studying p53 (Nakaya *et al.*, 2000). To summarize our data, we demonstrate, for the first time, that the dephosphorylation of pRb occurs in a sequential, temporally-regulated manner. Dephosphorylation at the sites on pRb studied does not occur at the same rate, nor to the same extent, up to 4 h following the release from G₂/M arrest, when the majority of cells have entered G₁. What is the biological significance of this finding? It has been known that phosphorylation at different sites affects different protein binding functions of pRb (Kitagawa *et al.*, 1996; Knudsen and Wang, 1996; Zarkowska and Mittnacht, 1997), as well as its nuclear sublocation (Mittnacht *et al.*, 1994) and intermolecular interactions (Harbour *et al.*, 1999) thought to be important for its cell cycle regulatory effects (Chew *et al.*, 1998; Connell-Crowley *et al.*, 1997). Regulated dephosphorylation of pRb supports the idea that the protein-binding functions of pRb are reactivated at different times during the return to G₁. Our data supports the idea that the Cyclin D/cdk4 sites

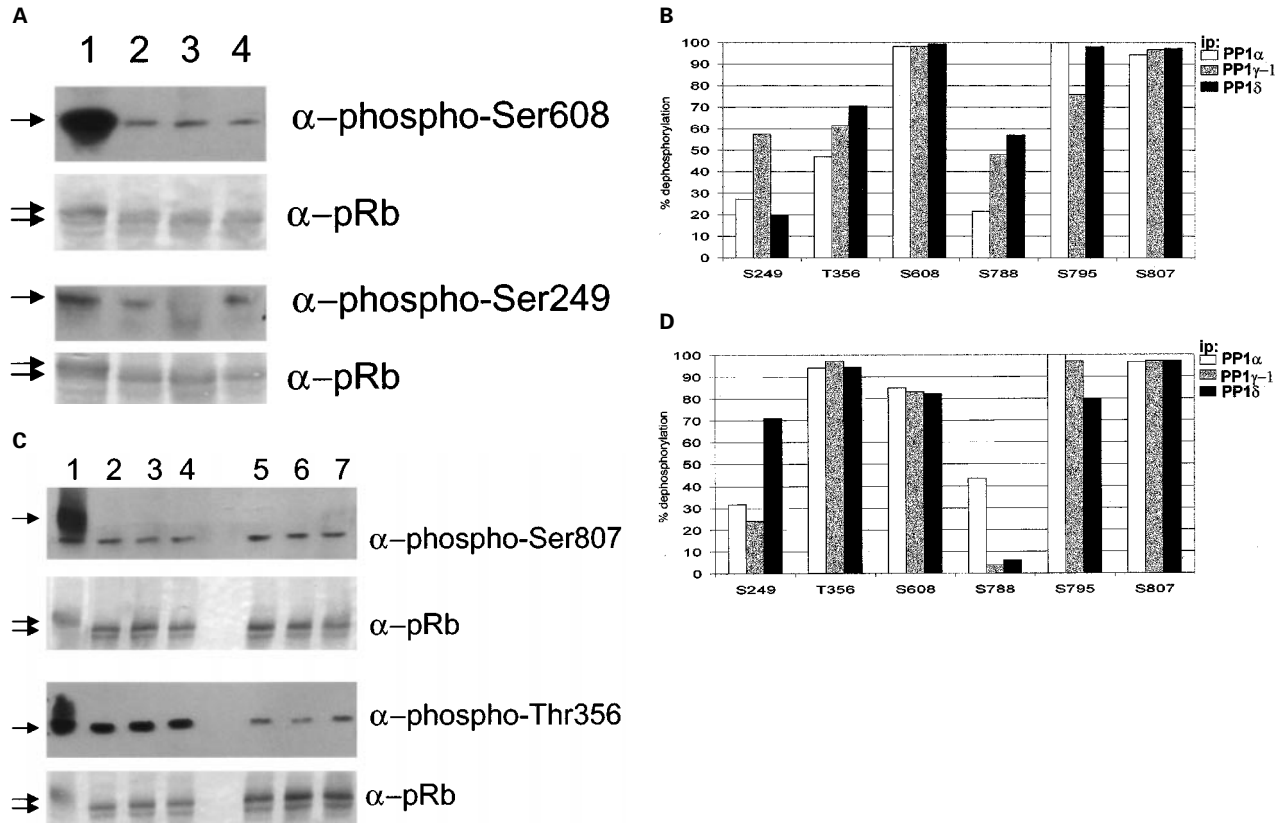


Figure 5 Specificity of mitotic and G₁-phase PP1 isoforms for phosphorylation sites on pRb. Results shown are representative of two different experiments. (A) Mitotic PP1 isoforms α (lane 2), $\gamma-1$ (lane 3) and δ (lane 4) were immunoprecipitated from mitotic cells and mixed with mitotic cell extracts (lane 1 is an untreated control). Two hundred μ g of total protein were separated by gel electrophoresis and transferred to nitrocellulose. Western blots were probed with phosphorylation site-specific antibodies as indicated, and re-probed with anti-pRb antibodies. Positions of the hyper- and hypo-phosphorylated bands of pRb are as indicated by arrows. (B) Band intensities from Western blots probed with phosphorylation site-specific antibodies as indicated were quantitated by densitometry and normalized for total pRb content. Percent dephosphorylation by mitotic PP1 isoforms was calculated by subtracting band intensity of each assay from band intensity of untreated control, then dividing by untreated control intensity and multiplying by 100. (C) Comparison between mitotic and G₁-phase PP1 isoforms is shown in this panel. PP1 isoforms α (lanes 2 and 5), $\gamma-1$ (lanes 3 and 6) and δ (lanes 4 and 7) were immunoprecipitated from either mitotic (lanes 2–4) or G₁-phase (lanes 5–7) cells mixed with mitotic cell extracts. Lane 1 is an untreated control. (D) Band intensities from Western blots probed with phosphorylation site-specific antibodies as indicated were quantitated by densitometry and normalized for total pRb content. Percent dephosphorylation by G₁-phase PP1 isoforms was calculated by subtracting band intensity of each assay from band intensity of untreated control, then dividing by untreated control intensity and multiplying by 100.

are the first to undergo dephosphorylation upon release from mitotic block. Since these are the sites first targeted for phosphorylation upon mitogenic stimulation, the functions that these sites mediate must be crucial to pRb's overall growth inhibitory program. The Cyclin E/cdk2 sites, on the other hand, may maintain high levels of phosphorylation, at least until early G₁, as this will not hinder maintenance in G₁ until further stimulation. As shown here for these cell lines, this is the case for Thr373, Ser795 and Thr821.

One prominent example of the way in which dephosphorylation at different sites may activate different protein-binding functions of pRb comes from an examination of Thr821 and Thr826 dephosphorylation patterns. While Thr826, a Cyclin D/cdk4 site, is readily and rapidly dephosphorylated following passage through mitosis, Thr821 is not, nor is it targeted by PP1 isoforms in our *in vitro* experiments. In fact,

Thr821 phosphorylation appears at all phases of the cell cycle. Since these sites had earlier been implicated in LXCXE protein binding (Knudsen and Wang, 1996; Zarkowska and Mittnacht, 1997), we could not readily explain how Thr821 could remain phosphorylated following entry into G₁. One possibility was that phosphorylation at Thr821 did not impair pRb function. We sought to test this with respect to the LXCXE-protein binding function of pRb. We were able to co-immunoprecipitate pRb with SV40 T-antigen from SV80 cells following G₂/M arrest when Thr821 alone remains highly phosphorylated. The pRb/T-antigen complex was not evident by Western blot when both Thr826 and Thr821 are phosphorylated. However, when Thr826 phosphorylation abates, complex formation can be demonstrated. Therefore, Thr821 dephosphorylation does not appear to be required to reactivate pRb's ability to complex with

an LXCXE-containing protein. In contrast to these results (Zarkowska and Mittnacht, 1997), others have observed that phosphorylation of either Thr821 with Cyclin A/cdk2 or Thr826 with Cyclin D/cdk4 inhibited SV40 T-antigen binding to GST-pRb. These experiments, however, were performed using GST pull-down assays *in vitro*, which may not mirror the results we obtained tracking the reactivation of pRb at mitotic exit *in vivo*.

We have previously reported that the holoenzyme composition of mitotic PP1 isoforms differ from one another and that this may alter their activity towards a single substrate, pRb. In that study (Nelson *et al.*, 1997), the δ isoform of mitotic PP1 dephosphorylated pRb more efficiently *in vitro* than did PP1 α or γ -1. PP1 δ alone was unique in binding a 111-kD interacting subunit which may be the regulatory subunit called PNUTS (Allen *et al.*, 1998). Our current data suggests that this preferential activity may extend into the ensuing G₁ phase, at which point, the relative catalytic efficiencies of each of these isoforms are equalized. When examining relative activities of each of the isoforms on particular phosphorylation sites on pRb, we observed that each enzyme complex has particular specificities at a number of sites. Thus, much like the cdk2s that phosphorylate pRb in G₁- through S-phase, the enzyme that dephosphorylates pRb at mitotic exit has certain specificities for a subset of sites on pRb. Furthermore, for a given PP1 isoform, preferential dephosphorylation at one site will not necessarily be the same for the mitotic- and G₁-phase form of the enzyme. Since PP1 exists in multimeric complexes with interacting subunits that are not alike for each isoform, there are at least two possible explanations. Either the holoenzyme composition of PP1 plus interacting subunits is different for the G₁ and mitotic forms of these phosphatases, or that these noncatalytic subunits are themselves regulated, modulating their activity towards pRb. Some of the PP1-interacting subunits identified to date have been shown to be regulated by phosphorylation (Umstott-Hall *et al.*, 1999; Wera and Hemmings, 1995), and this may be the case here as well. Preliminary investigation into the subunit compositions of the G₁ form of PP1 seems to suggest that the overall molecular weight of the holoenzyme, 166 kD, is identical to that of its mitotic counterpart (N Krucher and JW Ludlow, unpublished observations). The 111 kD subunit, however, did not appear to be part of the holoenzyme complex. This subunit may be supplanted by the presence of pRb binding to PP1, which is known to occur upon G₁ entry (Durfee *et al.*, 1993; Nelson and Ludlow, 1997), to ensure that premature pRb phosphorylation and cell cycle progression does not occur. Further investigations to determine PP1-interacting subunits in G₁ phase and their regulation of enzymatic activity are necessary to distinguish between the two possibilities.

In closing, we would like to suggest that the differential preferences that each mitotic PP1 isoform exhibits towards subsets of phosphorylation sites on pRb may well play a role in reactivation of the

suppressive function of pRb. Understanding this process will lead us towards a more complete knowledge of the steps that go awry during oncogenic transformation.

Materials and methods

Cell culture and synchronization

CV1-P cells were synchronized in G₁-phase by incubation in methionine-free Dulbecco's modified Eagle's medium (DMEM) containing 2% dialyzed calf serum as previously described (Nelson and Ludlow, 1997). All incubations were carried out at 37°C in a humidified, 5% CO₂-containing atmosphere. A time course of release from G₁ was accomplished by replating at a density of 1.5×10^6 cells per 100 mm culture dish in 10% newborn bovine serum-containing DMEM (Gibco) and incubating for 3, 6, 9, or 12 h. For G₁-phase CV1-P lysates used in the phosphatase targeting experiments, cells were arrested by contact inhibition when reaching 100% confluence in 100 mm plates. Flow cytometric analysis of contact-inhibited cells confirmed the arrest in G₁.

CV1-P cells were synchronized in G₂/M using a nocodazole block (Nelson and Ludlow, 1997; Nelson *et al.*, 1997). Cells from the mitotic shake-off were elutriated and mitotic cells were pooled and counted. Mitotic cells were replated at a density of 1.0×10^6 cells per 10 ml of warm DMEM containing 10% newborn bovine serum. 1.0×10^6 cells were removed every 10 min until 60 min and then every 2 h until 6 h. After each interval, cells were prevented from further cell cycle progression by cooling on ice. 0.5×10^6 cells were removed for flow cytometry, while the remaining cells were lysed for protein analysis.

SV80 human cells, stably transformed with SV40 large T-antigen (Todaro *et al.*, 1966), were synchronized and elutriated as described above, and the G₂/M cells pooled. Mitotic cells were released from G₂/M arrest by replating at a density of 1.0×10^6 cells per 10 ml of warm 10% fetal bovine serum-containing DMEM and incubated for 50 min or 2 h. 0.5×10^6 cells were removed for flow cytometry, while the remaining cells were lysed for protein analysis.

pRb phospho-specific antibody production

Antibody to specific phosphorylated residues of pRb were raised against 11-mer peptides coupled to tuberculin purified protein derivative (PPD). Using the amino acid sequence of pRb as a guide, peptides were synthesized such that the specific phosphorylated residue was located approximately in the middle of the sequence. These coupled peptides were injected into BCG treated rabbits as described by Lussow *et al.* (1991). The resulting serum was tested against the peptide used for immunization in an ELISA assay to ensure specificity, as well as recombinant GST-pRB protein phosphorylated in a site-specific manner by cyclin-dependent kinases (Zarkowska and Mittnacht, 1997). These antibodies have been successfully used by us in defining differentially phosphorylated forms of pRb complexing with type 1 protein phosphatases (Tamrakar *et al.*, 1999).

Cell lysis, gel electrophoresis and Western blotting

At the indicated times, 0.5×10^6 of the harvested cells were lysed by sonication in EBC (50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 0.5% NP-40) supplemented with $1 \mu\text{g/ml}$

aprotinin (Sigma) and 1 $\mu\text{g}/\text{ml}$ of phenylmethylsulfonylfluoride (PMSF) (Sigma). Protein assays were performed using the BioRad protein assay system according to the manufacturer's instructions and protein concentrations were determined by the software included in a Biorad Smartspec 3000 Spectrophotometer. One hundred μg of total protein per sample was separated by 6% SDS-PAGE and transferred to nitrocellulose in buffer containing 25 mM Tris-HCl, pH 8.5, 192 mM glycine, 20% v/v methanol and 0.01% SDS (Towbin *et al.*, 1979). The imprinted membrane was then blocked for 30 min with TBST (20 mM Tris-HCl, pH 8.0, 120 mM NaCl, 0.01% Tween-20) containing 4% nonfat dry milk. Detection of phosphorylated Ser249, Thr252, Thr356, Thr373, Ser608, Ser780, Ser788, Ser795, Ser807, Thr821 and Thr826 was performed by overnight incubation with phosphorylation site-specific antibodies, described above and in a previous report by us (Tamrakar *et al.*, 1999), diluted 1:2000 in TBST containing 2% nonfat dry milk. Total pRb was detected using monoclonal antibody RB-PMG3-245 (Pharmingen). Blots were developed using either horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibodies (Southern Biotechnology Associates) and enhanced chemiluminescent detection (Pierce) or alkaline phosphatase-conjugated anti-mouse secondary antibodies (Promega) and NBT-BCIP (Sigma). Detection of SV40 large T-antigen using monoclonal antibody pAB-419 (Harlow *et al.*, 1981) was performed as described previously (Ludlow *et al.*, 1990).

Cell counting, flow cytometry and DNA histogram analysis

For each cell synchronization condition, harvested cells were counted by Coulter Counter (Model ZM, Coulter Electronics, Inc., Miami, FL, USA). Each count and condition was performed in duplicate. 0.5×10^6 cells were removed and fixed in 70% ethanol and stored at 4°C until flow cytometric analysis. Fixed cells were then centrifuged, resuspended in 1 ml of phosphate buffered saline containing 1 mg/ml RNase A, and incubated at room temperature for 30 min. Propidium iodide was added to a final concentration of 10 $\mu\text{g}/\text{ml}$ and flow cytometry was performed on a Profile II flow cytometer (Coulter Electronics, Inc., Hialeah, FL, USA). DNA histogram analysis was performed using the cytologic DNA analysis program provided by Coulter.

Immunoprecipitation

For phosphatase-targeting experiments, PP1 isoforms were immunoprecipitated using isoform-specific antibodies (Tognarini and Villa-Moruzzi, 1998) from either mitotic- or G₁-phase CV1-P cells synchronized as described above, following lysis in EBC supplemented with 10 $\mu\text{g}/\text{ml}$ each of aprotinin and PMSF. Isoform-specific PP1-immune complexes demonstrated identical phosphorylase *a*-directed activity and PP1 catalytic subunit abundance as previously determined (Nelson *et al.*, 1997; Nelson and Ludlow 1997; Tognarini and Villa-Moruzzi, 1998; Tamrakar *et al.*, 1999; Tamrakar and Ludlow, 2000). Subunit composition of mitotic PP1 isoforms (Nelson *et al.*, 1997) has been demonstrated previously. Preliminary experiments indicated that equivalent amounts of each isoform could be immunoprecipitated from CV1-P cells with 4 μl of isoform specific antibody and that each immune complex had identical activity towards phosphorylase *a*, a standard substrate used for quantitating phosphatase activity.

Cell extracts were precleared by incubation with 75 μl of protein A Sepharose (Sigma) diluted 1:1 in NET-N (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40) containing 4% bovine serum albumin. Rabbit antibody to

PP1 α , γ -1 or δ (4 μl) prepared as described in Tognarini and Villa-Moruzzi (1998) was added to lysates together with 100 μl of a 1:1 mixture of protein A-Sepharose:NET-N and rocked overnight. Immunoprecipitates were washed three times with 1 ml NET-N and twice with 1 ml phosphatase buffer containing 20 mM imidazole, pH 7.0, and 150 mM NaCl, supplemented with 10 $\mu\text{l}/\text{ml}$ aprotinin, 1 mM PMSF and 1 mM MnCl₂. Immunocomplexed PP1 isoforms were detected by Western blot using anti-PP1 antibody FL-18 (Santa Cruz Biotechnology). Activity of PP1 immunoprecipitates was tested using glycogen phosphorylase *a* (Sigma) as a substrate, as described previously (Nelson and Ludlow, 1997).

For co-immunoprecipitation of pRb and SV40 T-antigen, monoclonal antibody RB-PMG3-245, rabbit anti-mouse IgG (Cappell) and protein A-Sepharose were used to precipitate pRb complexes. Monoclonal antibody pAb-419 (Harlow *et al.*, 1981) and protein A-Sepharose were used to precipitate SV40 T-antigen complexes. Immune complexes were washed three times with 1 ml NET-N prior to boiling in 100 μl of sample buffer and separation by 6% SDS-PAGE.

Measure of total and isoform-specific pRb-directed phosphatase activity

Mitotic CV1-P cells were prepared and released from nocodazole-induced arrest as described above for 30, 60 or 120 min. At the indicated times, total pRb-directed phosphatase activity in protein extracts of each fraction of cells was measured by mixing ³²P-labeled immunocomplexed pRb substrate with 50 μg of cell extract for 40 min at 30°C as described previously (Ludlow *et al.*, 1993; Nelson and Ludlow, 1997).

To measure isoform-specific pRb-directed phosphatase activity, PP1 isoforms were immunoprecipitated as described above and mixed with ³²P-labeled immunocomplexed pRb for 30 min at 30°C with occasional mixing. Reactions were terminated by the addition of 3XSDS-PAGE sample buffer and boiled for 10 min. Labeled proteins were separated by 6% SDS-PAGE, fixed, dried, visualized by autoradiography and quantitated by phosphorimager analysis on a Molecular Dynamics Storm 860 phosphorimager. Results are presented as per cent activity by quantitating decrease in pRb band intensity relative to controls.

Phosphatase targeting assays

Mitotic CV1-P cell lysates were mixed with mitotic- or G₁-phase PP1 isoform immunoprecipitates originating from matched amount of protein extract. Reactions were carried out at 30°C for 60 min with occasional mixing. Protein aliquots of each reaction (200 μg) were boiled in 3XSDS-PAGE sample buffer prior to separation on 6% SDS gels and analysed by Western blotting.

Densitometric analysis of Western blots and quantitation

Western blots were scanned on a Biorad GS-700 scanner and band intensities quantitated by Biorad Quantity One software. Band intensities were normalized by correcting for any differences in total pRb content. Results are presented as per cent dephosphorylation relative to controls. For sequential activation of pRb at mitotic exit, normalized band intensities at homologous time points from two experiments were averaged and moving averages plotted as a function of both time of release and mean G₂/M content. For PP1 targeting experiments, band intensities from a representative of two

experiments were normalized by correcting for differences in total pRb content, and converted to per cent of untreated control. Per cent dephosphorylation was calculated by subtracting this value from 100%.

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