Inhibitory Phosphorylation of PP1α Catalytic Subunit during the G₁/S Transition*

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We have shown earlier that, in cells expressing the retinoblastoma protein (pRB), a protein phosphatase (PP) α mutant (T320A) resistant to inhibitory phosphorylation by cyclin-dependent kinases (Cdks) causes G₁ arrest. In this study, we examined the cell cycle-dependent phosphorylation of PP1α in vivo using three different antibodies. PP1α was phosphorylated at Thr-320 during M-phase and again in late G₁ through early S-phase. Inhibition of Cdk2 led to a small increase in PP1 activity and also prevented PP1α phosphorylation. In vitro, PP1α was a substrate for Cdk2 but not Cdk4. In pRB-deficient cells, phosphorylation of PP1α occurred in M-phase but not at G₁/S. G₁/S phosphorylation was at least partially restored after reintroduction of pRB into these cells. Consistent with this result, PP1α phosphorylated at Thr-320 co-precipitated with pRB during G₁/S but was found in extracts immunodepleted of pRB in M-phase. In conjunction with earlier studies, these results indicate that PP1α may control pRB function throughout the cell cycle. In addition, our new results suggest that different subpopulations of PP1α regulate the G₁/S and G₂/M transitions and that PP1α complexed to pRB requires inhibitory phosphorylation by G₁-specific Cdks in order to prevent untimely reactivation of pRB and permit transition from G₁ to S-phase and/or complete S-phase.

In mammals, three genes encode four isozymes of serine/threonine-specific protein phosphatase 1 designated PP1α, PP1y1, PP1y2, and PP1β. Except for PP1y2, which is found only in testes, these isoforms are expressed in all tissues and cellular compartments and are playing important roles in many different aspects of cellular activities. Although the free catalytic subunit of PP1α can dephosphorylate multiple proteins in vitro, it is thought that due to its complex regulation PP1α is nonetheless capable of executing specific reactions upon receiving appropriate signals. This regulation typically involves interaction with inhibitory proteins or so-called targeting subunits that direct PP1 toward distinct cellular locales or even substrates (reviewed in Refs. 1 and 2). Both yeast two-hybrid screens (3, 4) and affinity chromatography on microcristin-Sepharose (5, 6) have recently led to the discovery of a number of proteins that appear to specifically bind PP1. Thus, PP1α is more adequately portrayed as an enzyme system rather than a single enzyme.

PP1 activity is crucial for cell cycle regulation (reviewed in Refs. 7 and 8). Genetic studies have shown that PP1α is required for progression through or exit from mitosis in Aspergillus nidulans (9) and fission yeast (10, 11), as well as in Drosophila (12). This was confirmed biochemically for mammalian cells in that microinjection of antibodies to PP1α causes mitotic arrest (13). However, essential mitotic substrates for PP1α have not unambiguously been identified. PP1α undergoes inhibitory phosphorylation by cyclin-dependent kinases (Cdks), both in vitro (14, 15) and in vivo during M-phase in Schizosaccharomyces pombe (15) and human cells (16, 17). Studies in S. pombe suggest that the phosphorylation of PP1α may be necessary to permit entry into M-phase (15). We have mapped the site of phosphorylation to Thr-320 in PP1α (14). PP1α activity also oscillates during the mammalian cell cycle. Cytoplasmic activity is maximal in quiescent cells and reduced up to 4-fold in the remaining phases of the cell cycle, whereas nuclear or chromatin-associated PP1α shows two similarly sized peaks of activity during G₂/G₁ and mitosis (14).

Another important role for PP1α in the cell cycle of mammalian cells may be the dephosphorylation of the retinoblastoma protein, pRB. Many pathways that regulate G₁ progression and the transition to S-phase converge on pRB (reviewed in Refs. 18–20). Ultimately, to permit passage through G₁/S, pRB has to be inactivated by Cdk phosphorylation in late G₁ (21). A variety of approaches has shown that PP1α interacts with and dephosphorylates pRB (3, 22–24). These findings raised the question whether PP1α controls the G₁/S transition and whether this putative function is linked to phosphorylation of PP1α. Following introduction of recombinant PP1α into synchronized cells by electroporation, we found that, unlike wild-type PP1α, a constitutively active mutant of PP1α that is resistant to Cdk phosphorylation in Thr-320 prevents cells from entering S-phase, provided they express functional pRB (25). This finding suggested that phosphorylation of PP1α somewhere in G₁ might be required to allow phosphorylation of pRB and initiation of S-phase. However, direct evidence for such a reaction was still missing.

The above findings prompted us to re-investigate cell cycle-dependent phosphorylation of PP1α with emphasis on the G₁-phase. In this study, using novel antibodies that are capable of

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1 The abbreviations used are: PP1, protein phosphatase 1; pRB, retinoblastoma protein; Cdk, cyclin-dependent kinase; Ab, antibody; PAGE, polyacrylamide gel electrophoresis.
reacting with Cdk-phosphorylated PP1α, we demonstrate that PP1α is indeed phosphorylated in vivo before and after the G1/S transition. As we have seen with PP1α-mediated G1 arrest (25), G1/S phosphorylation of PP1α was not detectable in the absence of pRB. Consistent with this finding, most if not all of the G1/S phosphorylation in pRB-expressing cells affected PP1α that was physically associated with pRB. The implications of these findings will be discussed.

EXPERIMENTAL PROCEDURES

Materials—Olomoucine was from LC Laboratories, Woburn, MA, and histone H1 was from Sigma. Saos2 cells reconstituted with wild-type pRB were generated and provided by Yuen-Kai Fung (Childrens Hospital Los Angeles) (26). Sources of other cells and all other chemicals were given previously (25). The recombinant catalytic subunit of PP1α was expressed in Escherichia coli and purified as described previously (27). Recombinant baculoviruses encoding human Cdk5s and cyclins were kindly provided by David O. Morgan, University of California San Francisco (Cdk2 and cyclin A), and Charles J. Sherr, St. Jude Childrens Research Hospital, Memphis, TN (Cdk4, cyclin D1, D2, and E). Phosphorylase b and phosphorylase kinase were kindly provided by Balwant S. Khatra, California State University Long Beach, CA. Three different antipeptide antibodies specifically recognizing the C terminus of PP1α were used in this study. The first of these (Ab1), against residues 316–330 (28), only immunoprecipitates the dephosphorylated form of PP1α and weakly recognizes Thr-320-phosphorylated PP1α after denaturation in SDS-PAGE sample buffer (24). The second antibody (Ab2) was against residues 294–309 (29) and reacts with PP1α whether phosphorylated or not (17). Antibodies specific for PP1α phosphorylated at Thr-320 (Ab3) were obtained from Angus C. Nairn (Rockefeller University, New York). Antibodies to pRB (sc-102) and non-neutralizing antibodies to Cdk2 (sc-163G) were purchased from Santa Cruz Biotechnology, Santa Cruz, CA.

In Vitro Phosphorylation of PP1α—To phosphorylate PP1α, we used cytosolic lysates prepared from synchronized MG63 cells or Cdk-cyclin complexes that were expressed and reconstituted from recombinant baculoviruses (see above) in S9 insect cells as described (30). Serial dilutions of insect cell lysates were then used to determine their ability to label pRB with 32P. Aliquots corresponding to equal pRB phosphorylation were then used in the subsequent PP1α phosphorylation reactions. These were carried out under conditions described earlier (14) in the presence of [γ-32P]ATP (specific radioactivity ~2,000 cpm/pmole). Immunoprecipitation from Cells or in Vitro Phosphorylation Reactions—Cells were washed in phosphate-buffered saline and lysed with RIPA buffer (25). Each sample was adjusted to 1 mg/ml total protein by adding bovine serum albumin. Cell lysates were pre-cleared by a 30-min incubation on ice with 50 μl of protein A-Sepharose suspension (equal volumes of beads and buffer) which was removed by centrifugation. The supernatants were then mixed with 50 μl of protein A-Sepharose suspension and the appropriate antibody. The amounts of antibody used were as follows: 8 μg of Ab2 or 4 μg of Ab3 (for PP1α), 5 μg of sc-163G (for Cdk2), and 10 μg of sc-102 (for immunoprecipitation or immunodepletion of pRB). The mixtures were rotated at 4 °C for 2 h and processed further as described for pRB (25). To determine the activity status of cyclin E- and cyclin A-dependent Cdk2, immunoprecipitated Cdk2 was further incubated with histone H1 and 0.1 mM [γ-32P]ATP (specific radioactivity ~10,000 cpm/pmole) as described previously (31). 32P-Labeled histone, PP1α, pRB immune complexes, or pRB-immunodepleted supernatants were separated on 12% SDS-polyacrylamide gels.

RESULTS

Cdk5 Phosphorylate PP1α Twice during the Cell Cycle—In order to test whether the observed cell cycle-dependent activity changes in PP1 (14) are due to regulated expression, we sub-jected cytoplasmic and nuclear lysates from synchronized MG63 cells to Western blotting with two antibodies specific for PP1α. This isoform is of special interest as it physically associates with pRB in a yeast two-hybrid screen and in co-immunoprecipitation experiments from G0 to S-phase (3). By using Ab2 for detection, the amounts of PP1α protein were apparently constant during all phases of the cell cycle (Fig. 1); this antibody recognizes residues 294–309 of PP1α (29) and therefore reacts with both unphosphorylated and phosphorylated forms of PP1α (17). This result indicated that PP1 activity during the cell cycle is more likely to be modified by i) interaction with regulatory subunits and/or ii) phosphorylation. The latter possibility appeared particularly intriguing as we have demonstrated that Cdk5s inhibit PP1α through phosphorylation at Thr-320 in vitro (14). To investigate the in vitro phosphorylation of PP1α was initially not possible as the PP1α-specific antibody we have generated (28) is unable to immunoprecipitate Cdk5-phosphorylated PP1α (24). This behavior of Ab1 is most likely due to the fact that it was raised against a peptide derived from a C-terminal sequence harboring Thr-320 (25); upon phosphorylation, the C terminus folds back to mask the catalytic center (32, 33). However, two recently described antibodies to PP1α put us in a position to address the question whether phosphorylation of PP1α occurs in vivo in a cell cycle-dependent manner. One of these antibodies (Ab2) was described above, and the second one (Ab3) was raised against a peptide comprising residues 316–323 that were phosphorylated inThr-320 (16). Both of these antibodies could immunoprecipitate PP1α that was phosphorylated by Cdk5s in vitro (data not shown). Initially, we performed two experiments to examine the cell cycle-dependent phosphorylation of PP1α. First, recombinant PP1α was mixed with cellular extracts derived from MG63 cells synchronized in different phases of the cell cycle and then immunoprecipitated with Ab2. As shown in Fig. 2, only mitotic extracts were able to phosphorylate PP1α. Next, to examine the in vivo phosphorylation of PP1α, MG63 cells were synchronized in different stages of the cell cycle and labeled with 32P, followed by immunoprecipitation with Ab3. Thr-320 was modified during two periods as follows: the first phosphorylation lasted from late G1 through early S-phase, and the second throughout M-phase (Fig. 3). Flow cytometry of identically treated sister cultures revealed that the cells were synchronized in the phases indicated (data not shown). Densi-tometric scanning of the individual bands showed that the level of phosphorylation during mitosis was approximately 2-fold higher than at G1/S. Similar results were obtained with Ab2 suggesting that Thr-320 is the only site that undergoes cell cycle-dependent phosphorylation. Based on these results and the previous characterization of Ab2 (16), in all subsequent experiments involving Ab2, 32P labeling was omitted. By using known amounts of phosphorylated and non-phosphorylated PP1α for comparison, we estimated that in late G1-early S-
Cytosolic lysates were prepared from synchronized MG63 cells in the presence of protease and phosphatase inhibitors (25), and 120 μg of cytosolic protein was mixed with approximately 6 μg of recombinant PP1α and 0.5 mM [γ-32P]ATP (2,000 cpm/pmol) and incubated for 45 min at 30 °C. PP1α was then immunoprecipitated with Ab2, separated by 12% SDS-PAGE, and visualized by overnight exposure to Kodak X-Omat AR film. The position of PP1α is given by the arrow. S, S-phase; M, M-phase.

There are differences in the phosphorylation of PP1α at the Thr-320; MG63 cells were synchronized at various stages of the cell cycle corresponding to G0, G1, G1/S boundary, and G2/M as described (14, 25) and released from drug-induced cell cycle arrest for up to 7.5 h (the last 2.5 h in the presence of [32P]ATP and lysed with RIPA buffer. PP1α phosphorylated at Thr-320 was immunoprecipitated with Ab3, separated by 12% SDS-PAGE, and visualized by autoradiography. The amount of cellular protein used for immunoprecipitation and loaded per lane was equal within each group, 0.6 mg for G0, late G1, and G1/S and 0.3 mg for G2/M. The position of PP1α is given by the arrow.

Thus, it was important to examine whether the inhibitory phosphorylation of PP1α was equally dependent on pRB. We therefore compared the cell cycle-dependent phosphorylation pattern of Thr-320 in MG63 cells with that in Saos2 cells as well as Saos2 cells that had been stably transfected with wild-type pRB. This experiment was conducted in a manner analogous to that described in Fig. 3. With regard to MG63 cells, this experiment confirmed that Thr-320 was increasingly phosphorylated during the G1/S transition and M-phase when compared with cells in G0/G1. However, in cells lacking pRB, only mitotic phosphorylation could be detected, whereas stable re-
introduction of pRB into Saos2 cells restored phosphorylation of PP1α in S-phase (Fig. 6A). Considering that both cyclin E (36) and cyclin A (37) in concert with Cdk2 are required for the G1/S transition and that Cdk2 is a prime candidate for phosphorylating PP1α (compare Figs. 4 and 5), we determined the histone H1 kinase activity of Cdk2 was determined as described (31). Shown here are the histone H1 bands stained with Coomassie Blue R (left-hand panels) or visualized by autoradiography (right-hand panels).

To see whether phosphorylation was affecting or, perhaps, limited to PP1α that was associated with pRB, we immunoprecipitated PP1α from cells in late G1- through M-phase, and we examined the distribution of PP1α and PP1α phosphorylated at Thr-320 (compare Figs. 4 and 5). We determined the presence of functional pRB-primarily occurs at G1/S, when it is in a complex with pRB, and at the beginning of M-phase, when it is not in a complex with pRB.

**DISCUSSION**

**G1/S Phosphorylation**—In this paper, we demonstrate that PP1α catalytic subunit is phosphorylated at Thr-320, a Cdk consensus site, as cells approach and traverse S-phase. We had proposed such a mechanism following our previous study showing that a phosphorylation-resistant, constitutively active PP1α causes G1 arrest (25). This PP1α-mediated G1 arrest is dependent on the presence of functional pRB (25). Two lines of evidence suggested that phosphorylation of Thr-320 during the G1/S transition depends on functional pRB as well. First, phosphorylation of this site could not be detected in pRB-deficient cells but was at least partially restored in the same cell line that was stably transfected with pRB. Second, G1/S phosphorylation of Thr-320 was apparent in PP1α that was in a complex with pRB but was virtually absent in cell extracts immunodepleted of pRB (see Figs. 6 and 7). The co-immunoprecipitation/immunodepletion experiments depicted in Fig. 7 may also explain the initially puzzling failure of G1/S cell extracts to phosphorylate the free catalytic subunit of PP1α (see Fig. 2); Cdk S phase, as they take much longer to traverse S-phase than MG63 cells (25). B, the histone H1 kinase activity of Cdk2 was determined as described (31). Shown here are the histone H1 bands stained with Coomassie Blue R (left-hand panels) or visualized by autoradiography (right-hand panels).

**FIG. 6. Phosphorylation of PP1α at Thr-320 in pRB+ and pRB− cells.** MG63 cells (pRB+), Saos2 cells (pRB−), or Saos2 cells reconstituted with pRB (26) were synchronized as in Fig. 2. The cells were then lysed with RIPA buffer (25) and subjected to different analyses. A, the phosphorylation state at Thr-320 was analyzed by Western blotting with Ab3. Each lane contained 105 cells. Note that a longer observation period was chosen for Saos2 cells released from G1/S arrest, as they take much longer to traverse S-phase than MG63 cells (25). B, the histone H1 kinase activity of Cdk2 was determined as described (31). Shown here are the histone H1 bands stained with Coomassie Blue R (left-hand panels) or visualized by autoradiography (right-hand panels).

**FIG. 7. Thr-320 phosphorylation of PP1α in association with pRB.** Synchronized MG63 cells were lysed in RIPA buffer, and pRB was immunoprecipitated (IP) from 2 × 10⁵ cells with 10 μl of sc-102 as described (25). To remove pRB, lysates corresponding to 4 × 10⁵ cells were mixed with the same amount of sc-102. The resulting immunoprecipitates (left-hand panels) or immunodepleted extracts (right-hand panels) were then separated by 12% SDS-PAGE, transferred to Immobilon® membranes, and probed with sc-102, Ab1, or Ab3. The membranes from immunoprecipitations were exposed to film for 30 s and those from immunodepletions for 2 min. **WB,** Western blot.

As expected, extracts depleted of pRB revealed similar amounts of PP1α from late G1- through M-phase; however, PP1α phosphorylated at Thr-320 could only be detected in M-phase. This phosphorylation (estimated to affect approximately 25–30% of PP1α, see above) may explain the small but discernible decrease in signal obtained for PP1α in M-phase (lane 5, center right panel). As the amount of cellular protein used for the immunodepletion experiments (50 μg or approximately 10⁵ cells) was identical to that used in the straight Western blot experiments shown in Fig. 6A, these data suggest that most if not all of PP1α that was associated with pRB from late G1-
growth-suppressing function of pRB. The persistent PP1α-pRB association might have a dual purpose. (i) Before PP1 kinases inactivate PP1α, it may be crucial for maintaining cells in G1-phase. (ii) After PP1 kinases have inactivated PP1α, it may enable cells that are now committed to initiate or complete S-phase to re-activate PP1α which in turn could re-activate pRB. This would allow cells to quickly respond to signals (such as DNA damage) that require a temporary cell cycle arrest.

Evidence from other laboratories has suggested that pRB has anti-apoptotic function (38), and indeed, we have observed earlier that, in pRB-positive cells, PP1αThr320A induces G1 arrest and then cell death (25) by apoptosis, whereas in pRB-negative cells, both wild-type PP1α and PP1αThr320A can trigger cell death without prior G1 arrest (25). Thus, as has been proposed by others (39), it may be the phosphorylated form of pRB rather than pRB per se that protects cells from apoptosis. The lack of Thr-320 phosphorylation at G1/S in the absence of pRB reported here suggests that this phosphorylation event is primarily related to the cell cycle function of PP1α. Our study does not preclude the possibility that PP1α also controls the phosphorylation state of pRB indirectly, e.g. by acting on the Cdk inhibitors p21Cip1 or p27Kip1. Both of these proteins have recently been shown to be subject to phosphorylation, which possibly triggers their proteolytic degradation (40–42). De-phosphorylation of these two proteins would be predicted to have a stabilizing effect, thus perpetuating Cdk inhibition and preventing pRB phosphorylation.

M-phase Phosphorylation—Cell cycle-dependent phosphorylation of PP1α has been demonstrated before in S. pombe, where it occurs only at the onset of mitosis (15). Subsequently, mitotic phosphorylation of human PP1α has also been established (16, 17), although the reasons for this are less clear. It may be required to support the phosphorylation of multiple proteins that usually accompanies mitosis (15, 16). In the present paper, we confirmed that Thr-320 is phosphorylated during mitosis, regardless of whether pRB is present. Phosphorylation occurred in pRB-positive and pRB-negative cells, and in pRB-free cell lysates (see Figs. 6 and 7). This indicates that the phosphorylation of PP1α permitting S-phase or M-phase entry is likely to involve two different subpopulations of the enzyme. As PP1α starts to dephosphorylate pRB in mid-to-late mitosis (22), at a time when PP1α is phosphorylated as well (see Fig. 3), even in cells lacking pRB (see Fig. 6), it is likely that the pRB-directed activity of PP1α and the phosphorylated PP1α represent distinct subpopulations of the enzyme or, alternatively, that mitotic dephosphorylation of pRB is catalyzed by another PP1 isoform. The latter explanation would be favored by the results of our co-immunoprecipitation and immunodepletion experiments (compare Fig. 7). At the onset of M-phase, neither PP1α nor phosphorylated PP1α could be detected in a complex with pRB.

Stoichiometry of Phosphorylation—Both G1/S and mitotic phosphorylation events affected only a minor portion of the total PP1α present. This is in agreement with our previous study showing that, following electrophoretic separation of recombinant PP1α into synchronized cells, the activity of wild-type PP1α decreases only slightly when compared with constitutively active PP1α (25). Furthermore, the degree of phosphorylation seen at G1/S, as well as the PP1 activity increase upon inhibition of Cdks acting in late G1 (see Fig. 4A), is smaller than the drop in PP1 activity we reported earlier (14). This could be explained by several possibilities as follows: (i) the activity measured earlier did not distinguish between PP1 isoforms, and (ii) PP1 is down-regulated not only by phosphorylation of the catalytic subunit but also by interaction with one or more inhibitory proteins. In particular, inhibitor-2 oscillates during the cell cycle (43) and translocates to the nucleus at the G2/S transition, thereby providing another means to inhibit nuclear PP1 (44). Our results are in agreement with the current model that recognizes PP1α as an enzyme that performs multiple tasks in cells (see the Introduction); this means that only a subpopulation of PP1α can perform functions related to the cell cycle (8). If this model is true, then small and even statistically insignificant changes of the overall activity can trigger significant effects on a particular process. Therefore, the low in vivo stoichiometry notwithstanding, phosphorylation of Thr-320 is expected to be physiologically relevant.

Do pRB Kinases Also Phosphorylate PP1α?—To inactivate pRB requires the activity of both cyclin D- and then cyclin E-Cdk complexes (45–47); furthermore, continued phosphorylation of pRB by Cdk2/cyclin A appears to be required during S-phase (48). Exposure of cells to olomoucine before and at the G2/S transition slightly increased PP1 activity and prevented Thr-320 phosphorylation (see Fig. 4), indicating that PP1α may be inhibited by Cdk2-mediated phosphorylation in vivo during the G2/S transition. Cdk2 is known to associate with cyclin E and cyclin A (18–21). Both Cdk2/cyclin E and Cdk2/cyclin A were able to phosphorylate PP1α in vitro (see Fig. 5), in addition to Cdk1/cyclin A (14). The only physiologically relevant substrate for cyclin D-dependent kinases that has been identified is pRB (49). Our observation that Cdk4/cyclin D apparently does not phosphorylate PP1α in vitro is consistent with this model. Cdk2/cyclin E is also required to fully inactivate pRB (47); however, this enzyme is required for the G2/S transition even in the absence of pRB (36) implying that Cdk2/cyclin E must be involved in the phosphorylation of other substrates. Although PP1α may be phosphorylated by Cdk2/cyclin E in vivo, it cannot be the only critical one, as the phosphorylation of PP1α was only detectable in pRB-expressing cells. Nonetheless, with the possible exception of Cdk4/cyclin D, the kinases phosphorylating pRB may be the same that phosphorylate PP1α.

In conclusion, this work demonstrates for the first time that PP1α is down-regulated by Cdk phosphorylation shortly before and during the G2/S transition. In conjunction with our previous study, these data suggest that a subpopulation of PP1α plays a pivotal role in inhibiting the G2/S transition via controlling the pRB activity status. As the pRB pathway is malfunctioning in virtually every human cancer studied (50, 51), further investigations will be important to characterize the interaction between the different PP1 isoforms, putative regulatory subunits of PP1, and pRB or upstream regulators of pRB.

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