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Phosphorylation and inactivation of protein phosphatase 1 by cyclin-dependent kinases

(cell cycle/protein phosphorylation/*in vitro* expression/site-directed mutagenesis)

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ABSTRACT **Protein phosphatase 1 and protein phosphatase 2A contain potential phosphorylation sites for cyclin-dependent kinases. In the present study we found that rabbit skeletal muscle protein phosphatase 1, as well as recombinant protein phosphatase 1α and protein phosphatase 1γ1, but not protein phosphatase 2A, was phosphorylated and inhibited by cdc2/cyclin A and cdc2/cyclin B. Phosphopeptide mapping and phospho amino acid analysis suggested that the phosphorylation site was located at a C-terminal threonine. Neither cdc2/cyclin A nor cdc2/cyclin B phosphorylated an active form of protein phosphatase 1α in which Thr-320 had been mutated to alanine, indicating that the phosphorylation occurred at this threonine residue. Furthermore, protein phosphatase 1, but not protein phosphatase 2A, activity was found to change during the cell cycle of human MG-63 osteosarcoma cells. The observed oscillations in protein phosphatase 1 activity during the cell cycle may be due, at least in part, to phosphorylation of protein phosphatase 1 by cyclin-dependent kinases. Together, the results suggest a mechanism for direct regulation of protein phosphatase 1 activity.**

A family of serine/threonine-specific protein kinases termed cyclin-dependent protein kinases (cdks) plays a key role in driving the cell cycle (1–4). Distinct cyclins perform different tasks in specific phases of the cell cycle by binding to and activating different cdk catalytic subunits. Thus, cyclin B promotes the G_2/M transition and cyclin A is required for the onset or maintenance of S phase, while three G_1 cyclins (C, D, and E) may regulate the cell's commitment to S phase (5). Clearly, the phosphorylation of key proteins is required for cell cycle progression to occur. Since the level of protein phosphorylation depends on the ratio of protein kinase and protein phosphatase (PP) activities, the identification and regulation of PPs opposing the actions of cdks are of considerable interest.

Based on enzymatic and physicochemical properties, the major mammalian serine/threonine-specific PPs are designated PP1, PP2A, PP2B, and PP2C (6–8). A number of studies have indicated a potential role for PP1 and PP2A in the regulation of cell growth. Analyses of fungal mutants revealed that PP1 and PP2A are essential for mitosis (9–12), and the observation that okadaic acid is a powerful tumor promoter (13) and specific inhibitor of PP1 and PP2A (14) has led to suggestions that these two enzymes function as tumor suppressors in mammalian cells (15, 16).

All of the mammalian PP1 isoforms identified so far (17–20) bear a Thr-Pro-Pro-Arg sequence motif near the C terminus, corresponding to the preferred sequence for cdk phosphorylation, (Ser/Thr)-Pro-Xaa-(Lys/Arg) (21). A potential role for this site is suggested by the fact that it is also present in PP1 isoforms identified in *Aspergillus* (9), yeast (10), *Drosophila* (22), and plants (23), even though the C terminus is the least conserved region of the molecule (7). Here, we report that PP1α and PP1γ1 can be phosphorylated and inactivated *in vitro* by cdks. *In vitro* expression and site-directed mutagenesis of PP1α confirmed the cdk phosphorylation to occur exclusively at Thr-320. Finally, we demonstrate cell cycle-dependent changes in PP1 activity, raising the possibility that this may be attributed in part to direct phosphorylation of PP1 by cdks.

MATERIALS AND METHODS

Chemicals. Phenylmethylsulfonyl fluoride was from Pierce. [γ-^{32}P]ATP was obtained from ICN. Chromatography media and the *Escherichia coli* expression vector pDR540 were purchased from Pharmacia LKB. Okadaic acid was obtained from LC Services (Woburn, MA). *N*-chlorosuccinimide (NCS) and all other chemicals were from Sigma unless otherwise stated.

Proteins. The catalytic subunits of PP1 and PP2A were purified from rabbit skeletal muscle to near homogeneity essentially as described by Cohen *et al.* (24), with the following modifications: The initial ion-exchange chromatography was performed on DEAE-Sepharose fast flow, and the final gel filtration step was carried out on Sephacryl S-100 HR. cdc2/cyclin A was purified as described (25, 26).

Phosphorylase *b*, phosphorylase kinase, and inhibitor 2 were kindly provided by Balwant S. Khatra (California State University, Long Beach). cdc2/cyclin B was obtained from Steven L. Pelech (University of British Columbia, Vancouver).

Expression and Site-Directed Mutagenesis of PP1α. The *Nar* I–*Hin*dIII fragment of rabbit PP1α cDNA (27) was subcloned into the *Bam*HI site of pDR540 and transformed into *E. coli* DH5α. Cells were induced by the addition of isopropyl β-D-thiogalactopyranoside, and expressed PP1α was purified by chromatography on heparin-Sepharose and Sephacryl S-100 HR.

Site-directed mutagenesis was initiated by annealing a mismatched oligonucleotide, 5′-GTTGCGGGGTGGGGC-GATGGGTCGGCCTCC-3′, to the coding strand of the PP1α cDNA. The underlined codon (where C was the only mismatch) replaced the original Thr-320 by alanine. The reaction was completed by using the "Transformer" site-directed mutagenesis kit (Clontech) according to the manufacturer's instructions. Mutant clones were selected by discriminatory

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Abbreviations: cdk, cyclin-dependent protein kinase; NCS, *N*-chlorosuccinimide; PP, protein phosphatase.

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restriction mapping, since the desired mutation eliminated an *Hph* I site present in the parent plasmid, and then confirmed by DNA sequencing.

PP Assays. The substrate [^{32}P]phosphorylase *a* was prepared by incubating phosphorylase *b* and phosphorylase kinase in a molar ratio of 200:1 in the presence of 0.2 mM [γ-^{32}P]ATP (28). PP activity was determined with 10.2 μM [^{32}P]phosphorylase *a* (1 mg/ml) in a total volume of 30 μl (24). To distinguish between PP1 and PP2A activities, the assays were carried out in the absence and presence of 2 nM okadaic acid (29), which selectively inhibits PP2A. One unit of activity is defined as the amount that catalyzes the release of 1 nmol of P_i from phosphorylase *a* per minute at 30°C.

Phosphorylation Assays, Phosphopeptide Mapping, and Phospho Amino Acid Analysis. The purified catalytic subunit of PP1 or a synthetic C-terminal peptide of PP1α (Gly-Arg-Pro-Ile-Thr-Pro-Pro-Arg-Asn-Ser-Ala-Lys-Ala-Lys-Lys) was incubated with purified cdk for up to 60 min in 50 mM Tris·HCl, pH 7.5/1 mM dithiothreitol/0.1 mM EDTA/100 mM NaCl/10 mM $MgCl_2$/0.1 mM [γ-^{32}P]ATP (specific radioactivity $\geq$ 1000 cpm/pmol) and then analyzed as follows. When intact PP1 or PP2A was used as substrate, the samples were resolved by SDS/12% PAGE (30) and analyzed by autoradiography. When the peptide was used as substrate, the samples were spotted onto phosphocellulose paper, and their ^{32}P content was determined (25). For phosphopeptide mapping, cdk-phosphorylated PP1 was resolved by SDS/PAGE and digested with NCS within the excised gel slice (31). The resulting peptides were separated by SDS/15% PAGE, the gel was stained with silver and dried, and autoradiography was performed. Phospho amino acid analysis was carried out as described (32).

Cell Synchronization. Human MG-63 osteosarcoma cells (8×10^5) were plated in 80-cm^2 flasks, cultured, and synchronized essentially as described (33). Asynchronous cells were cultured for 48 hr in the presence of reduced serum (0.5% fetal bovine serum) in order to obtain G_0/G_1 samples. These cells were then grown in the presence of aphidicolin (2.5 μg/ml) for 24 hr to arrest them at the G_1/S boundary. Cells in S phase were obtained 4 hr after the release of the aphidicolin block by washing. Nocodazole (50 ng/ml) was used for 30 hr to synchronize cells at G_2/M. The cellular synchrony was monitored and characterized by fluorescence-activated cell sorter (FACS) analysis (34). At the appropriate times, cells were collected by scraping and washed in 5 ml of Hanks' balanced salt solution. The cells were centrifuged for 10 min at 700 $\times$ *g*, suspended in 1 ml of ice-cold 50 mM Tris·HCl, pH 7.5/0.1 mM dithiothreitol/0.1 mM EDTA/0.1 mM EGTA/0.1 mM phenylmethylsulfonyl fluoride/1 mM benzamidine/150 mM NaCl with leupeptin at 5 μg/ml, and homogenized in a Dounce tissue grinder. The nuclear fraction was collected by centrifugation for 15 min at 1500 $\times$ *g* and 4°C. The resulting supernatant was taken as the cytosolic fraction. The nuclear fraction was resuspended in 0.3 ml of the above buffer and mixed with 33 μl of 4 M NaCl, vortexed, and briefly sonicated. Protein concentrations were determined according to Bradford (35).

RESULTS

Phosphorylation and Inactivation of PP1 Catalytic Subunit *in Vitro*. The presence of a consensus phosphorylation site for cdk in PP1 prompted us to investigate whether PP activity might be controlled by phosphorylation. Although PP2A does not contain a preferred cdk site as defined by Moreno and Nurse (21), it has three (Ser/Thr)-Pro motifs that could potentially be phosphorylated by proline-directed kinases (2). However, only PP1 was phosphorylated following the incubation of rabbit skeletal muscle PP1 or PP2A with cdc2/cyclin A. The phosphorylation of PP1 proceeded in a time-dependent manner and was accompanied by an inhibition of PP1 activity (Fig. 1). In parallel experiments, PP1 was phosphorylated to 0.5 mol of phosphate per mol of protein within 30 min (Fig. 1*A*), and its activity decreased to 50% of the initial value (Fig. 1*B*). The inactivation of PP1 by cdc2/cyclin A required the presence of both ATP and the kinase (Fig. 1*B*).

Purified, recombinant mammalian PP1 isoforms expressed in the baculovirus system (unpublished work) were also used to investigate the phosphorylation of individual isoforms (Fig. 2). These data show (*i*) that PP1 also served as a substrate for cdc2/cyclin B, and (*ii*) that both PP1α and PP1γ1 were phosphorylated to similar levels. When the incubation was carried out in the presence of 20 μM okadaic acid, thereby completely inhibiting PP1 activity, the phosphorylation of PP1 was stimulated to a greater extent, suggesting that the catalytic subunit of PP1 is capable of autodephosphorylation (Fig. 2, lane 2 vs. lane 3).

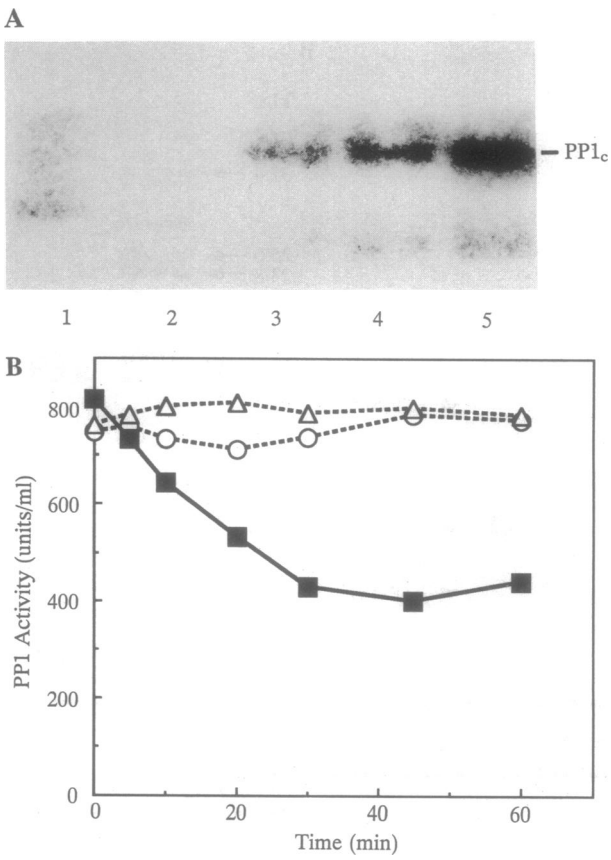

FIG. 1. Phosphorylation and inactivation of PP1 by cdc2 kinase. The purified catalytic subunit of rabbit skeletal muscle PP1 ($PP1_c$, 780 ng, corresponding to $\approx$20 pmol) was incubated for various times at 30°C with $\approx$5 milliunits of purified cdc2/cyclin A in a total volume of 50 μl. (*A*) Time course of phosphorylation. The reaction was stopped by the addition of 10 μl of 1% bovine serum albumin and 40 μl of 25% trichloroacetic acid. Precipitated and washed proteins were analyzed by SDS/PAGE and autoradiography. Lane 1, kinase only; lane 2, PP1 only; lanes 3–5, kinase plus PP1 (10, 20, and 30 min of incubation, respectively). The phosphorylation of PP1 at 30 min represented a stoichiometry of 0.5 mol/mol. (*B*) Effect of phosphorylation on the activity of PP1. Incubation conditions were identical to those described in *A*, except that radiolabeled ATP was omitted. At the indicated times, aliquots of the reaction mixture were diluted 1:600 with ice-cold 50 mM Tris·HCl, pH 7.0/1 mM dithiothreitol/0.1% bovine serum albumin. Phosphatase activity was determined immediately. Control incubations lacking either cdc2/cyclin A or ATP were carried out simultaneously. Data represent the mean values of three independent experiments. ■, PP1 plus kinase plus ATP; △, PP1 plus kinase; ○, PP1 plus ATP.

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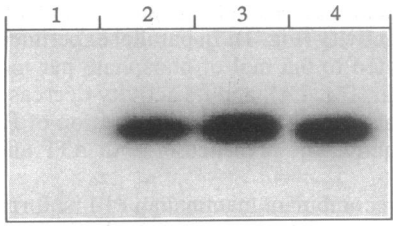

FIG. 2. Phosphorylation of recombinant PP1 isoforms by cdc2/cyclin B. PP1 isoforms were phosphorylated with cdc2/cyclin B and [γ-^{32}P]ATP and analyzed by SDS/PAGE and autoradiography. Lane 1, kinase only; lane 2, kinase plus PP1α; lane 3, kinase plus PP1α plus 20 μM okadaic acid; lane 4, kinase plus PP1γ1. The amount of phosphate incorporated into PP1α was increased $\approx$1.5-fold in the presence of okadaic acid (compare lanes 2 and 3).

Localization of the cdk Phosphorylation Site. To localize the phosphorylation site, PP1 was phosphorylated by cdk, and phospho amino acid analysis and peptide mapping were performed (Fig. 3). When phosphorylated PP1 was subjected to phospho amino acid analysis, only phosphothreonine was detected (Fig. 3*A*). Cleavage of phosphorylated PP1 with NCS generated four fragments of 25.0, 23.8, 13.7, and 12.5 kDa, as detected by silver staining (Fig. 3*B*, lane 1). The bulk of radioactivity was associated with the smaller peptide (Fig. 3*B*, lane 2). The peptide pattern obtained with NCS treatment is consistent with preferential cleavage at Trp-206 and Trp-216 rather than at Trp-149 (Fig. 3 *B* and *C*). Of the four threonine residues that occur within the 12.5-kDa C-terminal peptide, only Thr-320, in the sequence Thr-Pro-Pro-Arg, corresponds to the cdk consensus phosphorylation site, (Ser/Thr)-Pro-Xaa-(Lys/Arg) (21). These data strongly suggested Thr-320 as the site that was phosphorylated by the cdks *in vitro*. This conclusion was supported by two additional findings. First, cdc2/cyclin A was found to utilize a PP1 C-terminal peptide as a substrate. With 25, 50, and 100 μM peptide the phosphate incorporation was 6.3, 11.4, and 19.8 pmol/30 min, rates that were well within the range reported earlier with a number of other synthetic peptide substrates (25). In addition, the presence of the peptide lowered substantially the amount of phosphate incorporated into PP1 (data not shown).

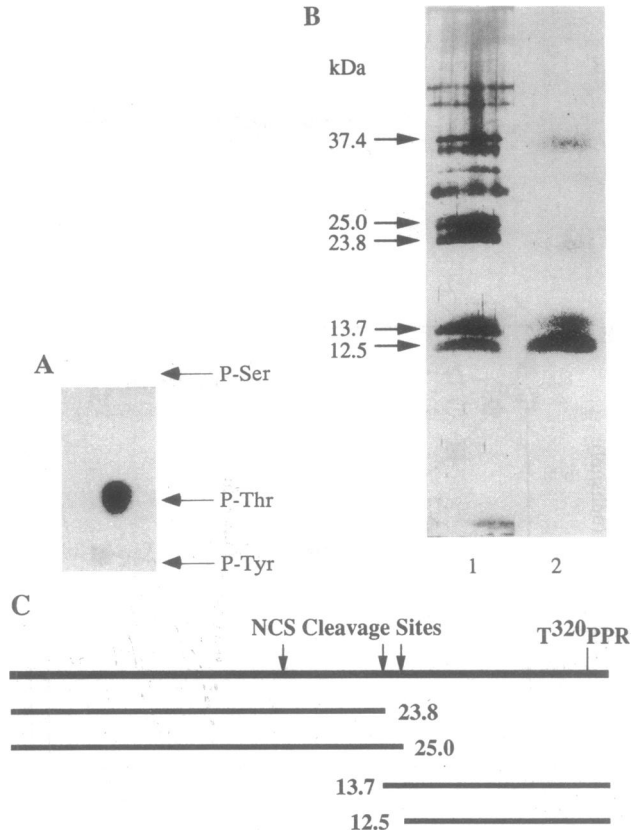

FIG. 3. Localization of the cdk phosphorylation site. (*A*) Phospho amino acid analysis of PP1. *In vitro* phosphorylated PP1 was subjected to phospho amino acid analysis as described (32). Positions of the standard phospho amino acids were established by ninhydrin staining and are indicated with arrows. (*B*) Phosphopeptide mapping of PP1 after phosphorylation by cdc2/cyclin A *in vitro*. Purified PP1 was phosphorylated, separated by SDS/PAGE, excised from the gel, and digested with NCS (31). The resulting peptides were resolved in an SDS/15% polyacrylamide gel and detected by silver staining, and the gel was dried and exposed to film for 24 hr. Lane 1, silver-stained peptides derived from PP1 (gel); lane 2, phosphopeptides derived from PP1 (autoradiograph). (*C*) PP1 peptides obtained by NCS cleavage. The top bar represents the full-length PP1 protein. The tryptophan residues potentially cleaved by NCS (Trp-149, Trp-206, and Trp-216) are indicated by the vertical arrows. The consensus sequence Thr-Pro-Pro-Arg (T^{320}PPR) for phosphorylation by cdks, located near the C terminus of PP1, is also indicated. A schematic representation of the peptides recovered after NCS cleavage is presented. The numbers correspond to the calculated polypeptide molecular mass in kilodaltons.

Phosphorylation of Wild-Type and Mutant PP1α. Both wild-type PP1α and the PP1αT320A mutant were expressed in *E. coli* and yielded soluble, active proteins that, like rabbit skeletal muscle PP1, were sensitive to inhibition by okadaic acid and inhibitor 2 (data not shown). Wild-type PP1α, but not PP1αT320A, was phosphorylated by cdc2/cyclin A (Fig. 4). The lack of phosphorylation of PP1αT320A, coupled with the identification of a single major phosphopeptide by two-dimensional mapping (unpublished work) and the recovery of phosphothreonine by phospho amino acid analysis, identifies Thr-320 as the only site in PP1 phosphorylated by cdk.

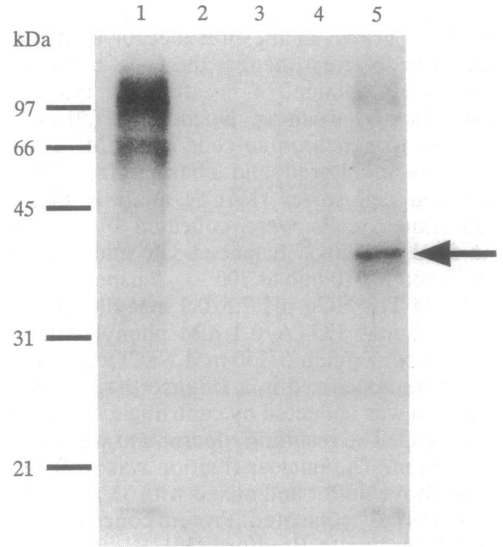

FIG. 4. Determination of the cdk phosphorylation site in PP1α. Approximately 1 μg of recombinant wild-type or mutant PP1α was incubated with cdc2/cyclin A for 30 min as described in *Materials and Methods*. Lane 1, kinase only; lane 2, PP1α only; lane 3, PP1αT320A only; lane 4, kinase plus PP1αT320A; lane 5, kinase plus PP1α. Since the kinase preparation used in this experiment was somewhat less pure than that employed in earlier experiments, it contained a number of other proteins that were phosphorylated by the kinase (lane 1). These were virtually completely dephosphorylated in the presence of the mutant PP1αT320A (lane 4), while in the presence of the same amount of wild-type PP1α a significant fraction of phosphoproteins persisted (lane 5). This observation is consistent with phosphorylation of PP1 inhibiting its activity. The arrow indicates the position of PP1.

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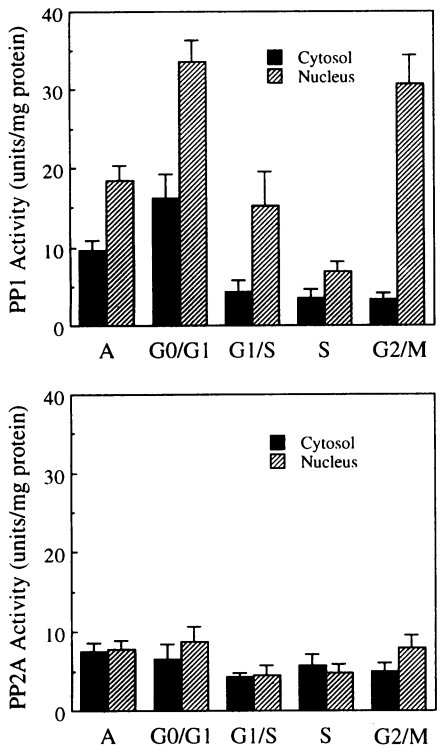

FIG. 5. PP1 and PP2A activities during the course of the cell cycle of MG-63 cells. Cytosolic (filled bars) and nuclear (hatched bars) extracts were prepared from synchronized MG-63 cells and subsequently assayed for PP1 (*Upper*) and PP2A (*Lower*). "A" represents asynchronous cells. The activity measured in "nuclear" extracts of M-phase cells represents PP associated with chromatin. The error bars represent the standard error of the mean ($6 \leq n \leq 8$).

One problem that we encountered was the varying degree of phosphate incorporation. With highly purified cdc2/cyclin A, the maximal stoichiometry was about 0.5 mol of phosphate per mol of PP1α, whereas less-pure cdc2/cyclin A preparations and also highly purified cdc2/cyclin B resulted in a stoichiometry of only about 0.1–0.2 (even in the presence of okadaic acid). This suggested that efficient phosphorylation of PP1 may be dependent not only on the purity of the kinase preparation but also on the type of cyclin involved.

PP1 and PP2A Activities During the Cell Cycle. Since PP1 and PP2A have been implicated in cell cycle regulation and our observations suggest that PP1 may be directly inhibited by one or more members of the cdk family, we examined PP1 and PP2A activity in extracts from synchronized MG-63 cells. The nature of the enzyme assay employed was such that the absence or presence of 2 nM okadaic acid was used to determine both PP1 and PP2A activities (29). Neither aphidicolin nor nocodazole, the drugs used to obtain cells arrested at the G_1/S and G_2/M boundaries, had any direct effect on PP1 or PP2A activity *in vitro* (data not shown).

Cytosolic PP1 activity was maximal (16.1 ± 2.8 units/mg) during quiescence, dropped sharply to about one-fourth of that activity (4.3 ± 1.3 units/mg) at the G_1/S boundary, and then persisted at levels corresponding to about one-fifth of maximum (3.6 ± 1.1 and 3.3 ± 0.7 units/mg, respectively) for the remainder of the cell cycle (Fig. 5 *Upper*). A very similar pattern was seen with nuclear PP1 activity, except for mitosis. From high levels (33.6 ± 2.7 units/mg) in G_0/G_1, the nuclear PP1 activity decreased to 15.2 ± 4.3 units/mg at the G_1/S boundary, further decreasing (to 6.8 ± 1.3 units/mg) during S phase. During M phase, where nuclei *per se* no longer exist, a relatively high level of PP1 activity (30.7 ± 3.6 units/mg) was associated with pelleted chromatin (Fig. 5 *Upper*). In the case of PP2A, both the cytosolic and the nuclear or chromatin-associated activity levels remained fairly constant in all four phases examined (Fig. 5 *Lower*).

DISCUSSION

The results of the present study allow several conclusions. First, the catalytic subunit of PP1 can be phosphorylated on threonine *in vitro* by both cyclin A and cyclin B kinase complexes. Second, *in vitro* phosphorylation of PP1 leads to a decrease in phosphatase activity. Third, cdk phosphorylation of PP1α occurs exclusively at Thr-320. Fourth, cdks also phosphorylate PP1γ1, presumably at Thr-311, which corresponds to Thr-320 in PP1α. It is interesting that a similar consensus site for cdk phosphorylation is found in fungal (9, 10), *Drosophila* (22), and plant (23) PP1 isoforms. Although the specific functions of the various PP1 isoforms are unknown at present, PP1α has been implicated in the control of cell proliferation (19), mitosis (9–12, 36), and phosphorylation of the retinoblastoma protein (20). Furthermore, the pattern of PP1 activity changes that are observed during the cell cycle of somatic cells is consistent with the notion that phosphatases may function as cell cycle regulators. Indeed, our data are consistent with a number of findings by other workers: (*i*) serum withdrawal leads to an increase in the ribosomal protein S6 phosphatase activity of PP1 (37); (*ii*) the levels and activity of inhibitor 2 oscillate during the cell cycle, exhibiting one peak during S phase and another peak during M phase (38), possibly contributing to the cytosolic activity profile reported here; and (*iii*) PP1 translocates from the cytoplasm to the nucleus during G_2 (39), possibly explaining the high level of nuclear PP1 activity measured in G_2/M (Fig. 5 *Upper*). In contrast, PP1 has also been reported to show peaks of activity during interphase and M phase in *Xenopus* egg extracts (40). However, that finding may not be directly comparable with the data presented here, as in the early embryonic cell cycle of *Xenopus* there is virtually no G_1 phase and associated growth controls. In addition to the known mechanisms for regulating PP1 activity by translocation of the catalytic subunit or its interaction with inhibitory subunits, our data provide evidence for the direct inhibition of PP1 by cdk phosphorylation. It should be possible to address the question of the biological role of PP1 phosphorylation by cdks, by transfecting cDNA encoding the active, phosphorylation-resistant mutant PP1αT320A into mammalian cells.

In vitro phosphorylation of PP1 as well as PP2A has been previously observed, albeit on C-terminal tyrosine residues, by the oncogenic kinase pp60^{src} (41, 42). In both cases, a concomitant decrease in phosphatase activity was also reported. Those data and the data reported here support the notion of the existence of a regulatory domain within the C terminus of PP1. An important question is whether PP1 is phosphorylated *in vivo*, and if so, which isoforms are regulated by which cyclin/cdks. The observed differences in phosphorylation of PP1 by cdc2/cyclin A and cdc2/cyclin B may represent yet another example of substrate targeting that appears to reside in the cyclin rather than the cdk subunit (43). Possibly, PP1α is a better substrate for cyclin C-, D-, or E-dependent kinases than the enzymes used in the present study. Alternatively, since a number of cdc2-like kinases have been identified by recombinant DNA technology, it is possible that one of these may phosphorylate PP1 more efficiently, although there is virtually no information available on the substrate specificity and cyclin association of these proteins. A third possibility would be that tyrosine kinases and serine/threonine kinases controlled by cyclins [which also have been implicated in oncogenesis (44–47)] may act synergistically to inhibit PP1 activity.

The mitosis-specific form of cdk, cdc2/cyclin B, has been reported to activate PP1 *in vitro* by phosphorylating inhibitor

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2 (48). This observation appears to be in contrast with the work presented here and previous findings discussed above. However, these results are not contradictory, since PP1 appears to be required for the completion of mitosis in *Aspergillus* (9), yeast (10–12), and *Drosophila* (36). If this also holds true for mammalian cells, activation of the inhibitor 2-bound PP1 by cdk could possibly provide the PP1 activity required for exit from mitosis. The inactivation of PP1 observed here occurs at or near the G_1/S transition and, provided that phosphorylation of the catalytic subunit contributes to this phenomenon, most likely involves a kinase other than cdc2/cyclin B.

One protein whose function is known to be mediated by phosphorylation and believed to play a central role in maintaining cells in G_1 is the prototypical tumor suppressor, the retinoblastoma protein (RB). RB is largely dephosphorylated throughout G_1 and becomes phosphorylated near the G_1/S boundary. It is thought that transition to S phase requires inactivation of RB by hyperphosphorylation and that the dephospho form of RB actively blocks cell cycle progression (49, 50). RB is phosphorylated and inactivated by members of the cdk family *in vivo*, most likely by cyclin A- and/or cyclin E-dependent kinases (26, 51, 52). Furthermore, *in vitro* studies demonstrated that RB is dephosphorylated by an inhibitor 2-sensitive phosphatase present in mitotic extracts (53). Using the yeast two-hybrid system, Durfee *et al.* (20) cloned a gene encoding a phosphatase, possibly an alternatively spliced isoform of PP1, PP1α2, that can associate with RB. These findings, coupled with the characteristic changes in PP1 activity observed during the cell cycle, suggest a potential role for PP1 at or after the M/G_1 boundary, where RB dephosphorylation takes place.

In summary, our data provide biochemical evidence for a direct inhibition of PP1 activity by a cyclin-dependent protein kinase, a mechanism which bypasses the known regulatory subunits of PP1 and which may have important implications for the regulation of the mammalian cell cycle, as well as other cellular processes involving PP1.

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1. Pines, J. & Hunter, T. (1990) *New Biol.* **2**, 389–401.
2. Hall, F. L. & Vulliet, R. P. (1991) *Curr. Opin. Cell Biol.* **3**, 176–184.
3. Norbury, C. & Nurse, P. (1992) *Annu. Rev. Biochem.* **61**, 441–470.
4. Wang, J. Y. J. (1992) *Biochim. Biophys. Acta* **1114**, 179–192.
5. Pines, J. (1993) *Trends Biochem. Sci.* **18**, 195–197.
6. Cohen, P. (1989) *Annu. Rev. Biochem.* **58**, 453–508.
7. Shenolikar, S. & Nairn, A. C. (1991) in *Advances in Second Messenger and Phosphoprotein Research*, eds. Greengard, P. & Robison, G. A. (Raven, New York), Vol. 23, pp. 1–123.
8. Bollen, M. & Stalmans, W. (1992) *Crit. Rev. Biochem. Mol. Biol.* **27**, 227–281.
9. Doonan, J. H. & Morris, N. R. (1989) *Cell* **57**, 987–996.
10. Ohkura, H., Kinoshita, N., Miyatani, S., Toda, T. & Yanagida, M. (1989) *Cell* **57**, 997–1007.
11. Booher, R. & Beach, D. (1989) *Cell* **57**, 1009–1016.
12. Kinoshita, N., Ohkura, H. & Yanagida, M. (1990) *Cell* **63**, 405–415.
13. Suganuma, M., Fujiki, H., Suguri, H., Yoshizawa, S., Hirota, M., Nakayasu, M., Ojika, M., Wakamatsu, K., Yamada, K. & Sugimura, T. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 1768–1771.
14. Bialojan, C. & Takai, A. (1988) *Biochem. J.* **256**, 283–290.
15. Cohen, P. & Cohen, P. T. W. (1989) *J. Biol. Chem.* **264**, 21435–21438.
16. Haystead, T. A. J., Sim, A. T. R., Carling, D. A., Honnor, R. C., Tsukitani, Y., Cohen, P. & Hardie, D. G. (1989) *Nature (London)* **337**, 78–81.
17. Berndt, N., Campbell, D. G., Caudwell, F. B., Cohen, P., da Cruz e Silva, E. F., da Cruz e Silva, O. B. & Cohen, P. T. W. (1987) *FEBS Lett.* **223**, 340–346.
18. Cohen, P. T. W. (1988) *FEBS Lett.* **232**, 17–23.
19. Sasaki, K., Shima, H., Kitagawa, Y., Sugimura, T. & Nagao, M. (1990) *Jpn. J. Cancer Res.* **81**, 1272–1280.
20. Durfee, T., Becherer, K., Chen, P.-L., Yeh, S.-H., Yang, Y., Kilburn, A. E., Lee, W.-H. & Elledge, S. J. (1993) *Genes Dev.* **7**, 555–569.
21. Moreno, S. & Nurse, P. (1990) *Cell* **61**, 549–551.
22. Dombradi, V., Axton, J. M., Brewis, N. D., da Cruz e Silva, E. F., Alphey, L. & Cohen, P. T. W. (1990) *Eur. J. Biochem.* **194**, 739–745.
23. Smith, R. D. & Walker, J. C. (1993) *Plant Mol. Biol.* **21**, 307–316.
24. Cohen, P., Alemany, S., Hemmings, B. A., Resink, T. J., Strålfors, P. & Tung, L. (1988) *Methods Enzymol.* **159**, 390–408.
25. Hall, F. L., Braun, R. K., Mihara, K., Fung, Y.-K. T., Berndt, N., Carbonaro-Hall, D. A. & Vulliet, P. R. (1991) *J. Biol. Chem.* **266**, 17430–17440.
26. Williams, R. T., Carbonaro-Hall, D. A. & Hall, F. L. (1992) *Oncogene* **7**, 423–432.
27. Berndt, N. & Cohen, P. T. W. (1990) *Eur. J. Biochem.* **190**, 291–297.
28. Shenolikar, S. & Ingebritsen, T. S. (1984) *Methods Enzymol.* **107**, 102–129.
29. Cohen, P., Klumpp, S. & Schelling, D. L. (1989) *FEBS Lett.* **250**, 596–600.
30. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
31. Lischwe, M. A. & Ochs, D. (1982) *Anal. Biochem.* **127**, 453–457.
32. Nairn, A. C. & Greengard, P. (1987) *J. Biol. Chem.* **262**, 7273–7281.
33. Carbonaro-Hall, D. A., Williams, R. T., Wu, L., Warburton, D., Zeichner-David, M., MacDougall, M., Tolo, V. & Hall, F. L. (1993) *Oncogene* **8**, 1649–1659.
34. Adams, R. L. P. (1990) *Cell Culture for Biochemists* (Elsevier, Amsterdam).
35. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254.
36. Axton, J. M., Dombradi, V., Cohen, P. T. W. & Glover, D. M. (1990) *Cell* **63**, 33–46.
37. Olivier, A. R. & Thomas, G. (1990) *J. Biol. Chem.* **265**, 22460–22466.
38. Brautigan, D. L., Sunwoo, J., Labbé, J.-C., Fernandez, A. & Lamb, N. J. C. (1990) *Nature (London)* **344**, 74–78.
39. Fernandez, A., Brautigan, D. L. & Lamb, N. J. C. (1992) *J. Cell Biol.* **116**, 1421–1430.
40. Walker, D. H., DePaoli-Roach, A. A. & Maller, J. L. (1992) *Mol. Biol. Cell* **3**, 687–698.
41. Johansen, J. W. & Ingebritsen, T. S. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 207–211.
42. Chen, J., Martin, B. L. & Brautigan, D. L. (1992) *Science* **257**, 1261–1264.
43. Peeper, D. S., Parker, L. L., Ewen, M. E., Toebes, M., Hall, F. L., Xu, M., Zanterna, A., Van der Eb, A. J. & Piwnica-Worms, H. (1993) *EMBO J.* **12**, 1947–1954.
44. Hunter, T. & Pines, J. (1991) *Cell* **66**, 1071–1074.
45. Hall, F. L., Williams, R. T., Wu, L., Wu, F., Carbonaro-Hall, D. A., Harper, J. W. & Warburton, D. (1993) *Oncogene* **8**, 1377–1384.
46. Jiang, W., Kahn, S. M., Zhou, P., Zhang, Y.-J., Cacace, A. M., Infante, A. S., Doi, S., Santella, R. M. & Weinstein, I. B. (1993) *Oncogene* **8**, 3447–3457.
47. Hinds, P. W., Dowdy, S. F., Eaton, E. N., Arnold, A. & Weinberg, R. A. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 709–713.
48. Villa-Moruzzi, E. (1992) *FEBS Lett.* **304**, 211–215.
49. Weinberg, R. A. (1991) *Science* **254**, 1138–1146.
50. Hamel, P. A., Gallie, B. L. & Phillips, R. A. (1992) *Trends Genet.* **8**, 180–185.
51. Lin, B. T.-Y., Gruenwald, S., Morla, A. O., Lee, W.-H. & Wang, J. Y. J. (1991) *EMBO J.* **10**, 857–864.
52. Hinds, P. W., Mittnacht, S., Dulic, V., Arnold, A., Reed, S. I. & Weinberg, R. A. (1992) *Cell* **70**, 993–1006.
53. Ludlow, J. W., Glendening, C. L., Livingston, D. M. & DeCaprio, J. A. (1993) *Mol. Cell. Biol.* **13**, 367–372.

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