Dephosphorylation of Human Cyclin-dependent Kinases by Protein Phosphatase Type 2Cα and β2 Isoforms*

Aiyang Cheng, Philipp Kaldis‡, and Mark J. Solomon§

From the Department of Molecular Biophysics and Biochemistry, Yale University School of Medicine, New Haven, Connecticut 06520-8114

We previously reported that the activating phosphorylation on cyclin-dependent kinases in yeast (Cdc28p) and in humans (Cdk2) is removed by type 2C protein phosphatases. In this study, we characterize this PP2C-like activity in HeLa cell extract and determine that it is due to PP2Cβ, a novel PP2Cβ isozyme, and to PP2Cα. PP2Cα and PP2Cβ co-purified with Mg2+-dependent Cdk2/Cdk6 phosphatase activity in DEAE-Sepharose, Superdex-200, and Mono Q chromatographies. Moreover, purified recombinant PP2Cα and PP2Cβ proteins efficiently dephosphorylated monomeric Cdk2/Cdk6 in vitro. The dephosphorylation of Cdk2 and Cdk6 by PP2C isoforms was inhibited by the binding of cyclins. We found that the PP2C-like activity in HeLa cell extract, partially purified HeLa PP2Cα and PP2Cβ isoforms, and the recombinant PP2Cs exhibited a comparable substrate preference for a phosphothreonine containing substrate, consistent with the conservation of threonine residues at the site of activating phosphorylation in CDKs.

Eukaryotic cell cycle progression is driven by the ordered activation and inactivation of cyclin-dependent protein kinases (CDKs).1 To precisely control the cell cycle engine, extracellular and intracellular signals control CDK activities through a variety of mechanisms, including association with regulatory proteins (cyclins, inhibitors, and assembly factors), subcellular localization, transcriptional regulation, selective proteolysis, and reversible protein phosphorylation (1–7). In the budding yeast Saccharomyces cerevisiae, Cdc28p is the main CDK involved in regulating the cell division cycle. On the other hand, Cdc2 (Cdk1), Cdk2, Cdk4, and Cdk6 control cell cycle progression in higher eukaryotes. Full activation of CDKs, which is necessary for normal cell cycle progression, requires binding of a cyclin, removal of inhibitory phosphorylations, and the presence of an activating phosphorylation. The cyclins are transcribed, synthesized, and degraded periodically during the cell cycle (1, 4, 8, 9). The inhibitory phosphorylations are carried out by the Wee1-like protein kinases, and removed by members of the Cdc25 family of dual specificity protein phosphatases (for reviews, see Refs. 6 and 7). Activating phosphorylation occurs within the “T-loop” (7, 10) on a conserved threonine residue corresponding to Thr161 in human Cdc2 and Thr160 in human Cdk2. Mutation of the equivalent site to alanine in Cdc2 from a variety of species abolishes kinase activity and biological function (11–16). This activating phosphorylation is carried out by Cdk-activating kinases (CAKs). Higher eukaryotic CAK, primarily localized to the nucleus, is composed of p40M015/ Cdk7, cyclin H, and an assembly factor, MAT1. These proteins also function as components of basal transcription factor III (17–21). In contrast to CAK in higher eukaryotes, CAK from budding yeast (Cak1p or Civ1p) is only distantly related to p40M015 (22–24), functions as a monomer, and is predominantly cytoplasmic (25).

Despite the large body of knowledge on CAK, there is relatively little information about the protein phosphatases that reverse the activating phosphorylation of the CDKs. A dual-specificity human protein phosphatase termed KAP (also Cdi1 and Cip2), which was identified by its interaction with Cdc2, Cdk2, and Cdk3 (26–28), was shown to dephosphorylate Thr160 on Cdk2 (29). However, there is no obvious KAP homologue in budding yeast. We found recently that two budding yeast type 2C protein phosphatases (PP2Cs), Ptc2p and Ptc3p, are the predominant and physiological enzymes that dephosphorylate Thr160 on the Cdc28p cyclin-dependent kinase (30). We also observed that PP2C-like activities were responsible for >99% of the phosphatase activity in HeLa cell extracts acting on Thr160 of Cdk2, indicating that the ability of type 2C protein phosphatases to remove the activating phosphorylation of CDKs is evolutionarily conserved (30).

Ser/Thr protein phosphatases (PPases) are classified into the PP1, PP2A, PP2B, and PP2C families based on their biochemical properties: PP1 and PP2A have no ion requirements and are sensitive to okadaic acid and microcystins; PP2B requires Ca2+ for full activity; and PP2C requires Mg2+ or Mn2+ (31). Molecular cloning revealed that PP1, PP2A, and PP2B families share homology to each other whereas the PP2C family is structurally distinct (32). In addition to their catalytic subunits, the PP1, PP2A, and PP2B holoenzymes also contain one or two regulatory subunits that appear to determine the substrate specificities of these phosphatases (31). Unlike PP1, PP2A, and PP2B, PP2C acts as a monomer. Thus, for PP2C, substrate specificity may result from the many PP2C family members, rather than from multiple regulatory subunits. In budding yeast, there are at least five PP2Cs (Ptc1p-Ptc5p) (30, 33). The mammalian PP2C family includes PP2Cα, PP2Cβ,
PP2Cγ (also called FIN13), PP2Cβ, Wip1, Ca2+-/calmodulin-dependent kinase II phosphatase, and NERP2-3C (43–44). In addition, human PP2Cα has two isoforms resulting from alternative splicing (44), and murine PP2Cβ has five isoforms (for comparison, see Ref. 45). In this study, we further characterized the PP2Cγ activities capable of dephosphorylating human Cdk2 and Cdk6 in a HeLa cell extract. Fractionation of proteins by DEAE-Sepharose, Superdex-200, and Mono Q chromatographies demonstrated that Cdk2 and Cdk6 phosphorylation activities co-purified with PP2Cα, a previously reported PP2C isoform, and PP2Cβ2, a novel human PP2Cβ isoform apparently resulting from alternative splicing. Recombinant PP2Cα and PP2Cβ2 effectively dephosphorylated monomeric- but not cyclin-bound Cdk2 and Cdk6 in vitro, confirming previous studies with yeast PP2Cα and human Cdk2 in a HeLa cell extract (30). Further enzymatic characterization showed that crude HeLa cell extract, partially purified PP2Cα and PP2Cβ2, and recombinant PP2Cα and PP2Cβ2 all exhibited a substrate preference for wild-type (Thr160) Cdk2 compared with a mutant Cdk2 protein containing an altered site of activating phosphorylation (Ser160) (40). These results support the conclusion that PP2Cα and PP2Cβ2 are the PP2C isoforms that dephosphorylate human CDKs in vivo.

MATERIALS AND METHODS

Reagents—Tissue culture medium, TRIZol, and SUPERSCRIPT II RNase H- reverse transcriptase were from Life Technologies (Grand Island, NY). DEAE-Sepharose Fast Flow, Superdex-200, and Mono Q HR5/5 were from Amersham Pharmacia Biotech. An antibody recognizing PP2Cα and PP2Cβ isoforms (catalog number 539548) was from Calbiochem (San Diego, CA). Sheep anti-PP2Cα antibodies (catalog number 06-523) were from Upstate Biotechnology (Lake Placid, NY). [γ-32P]ATP (3000 Ci/mmol) was from PerkinElmer Life Sciences. Horse-radish peroxidase-conjugated secondary antibodies and SuperSignal™ ECL reagents were from Pierce (Rockford, IL). Pho Turbo DNA polymerase and pBlueScript II KS(−) were from Stratagene (La Jolla, CA). All other chemicals were from Sigma unless indicated otherwise. 1× protease inhibitors contained 1 mM phenylmethylsulfonyl fluoride and 10 μg/ml each of leupeptin, chymostatin, and pepstatin.

Fractionation of HeLa Cell Extracts—HeLa S3 cells were lysed with a Dounce homogenizer in hypotonic buffer (10 mM Hepes, pH 7.9 (at 4 °C), 1.5 mM MgCl2, 10 mM KCl, 1 mM dithiothreitol, 1× protease inhibitors) and centrifuged at 100,000 × g for 1 h at a 60-Ti rotor (46). The supernatant was frozen in liquid nitrogen and stored at −80 °C until use. Clarified extract (25 ml, protein concentration 6.3 mg/ml) was applied to a DEAE-Sepharose Fast Flow column (1.5 × 10 cm) pre-equilibrated with buffer A (20 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% β-mercaptoethanol, 1× protease inhibitors) at a flow rate of 0.5 ml/min. The column was washed with buffer A until the absorbance of the elute at 280 nm was <0.05. Bound proteins were eluted with a 20-ml linear gradient from 0 to 1.0 M NaCl at a flow rate of 1 ml/min. One-ml fractions were collected, and Cdk2/Cdk6 phosphorylation activities in the fractions were assayed (see below). The active fractions were pooled, concentrated using a Vivaspin concentrator (10,000 MWCO; Vivascience LTD, Binbrook Hill, United Kingdom), and loaded onto a Superdex-200 column pre-equilibrated with buffer B (20 mM triethanolamine-HCl, pH 7.0 (at 25 °C), 5% glycerol, 0.01% Chaps, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% β-mercaptoethanol, 1× protease inhibitors) at a flow rate of 0.5 ml/min. One-ml fractions were collected, and Cdk2/Cdk6 phosphorylation activities in the fractions were assayed (see below). The active fractions (~45–kDa) were pooled, loaded onto a Mono Q HR5/5 column pre-equilibrated with buffer B, and developed with a linear salt gradient from 0 to 700 mM NaCl in buffer B at a flow rate of 0.5 ml/min as described (47). 0.5-ml fractions were collected, assayed for activity, and immunoblotted (see below).

Partial Purification of Human PP2Cα and PP2Cβ2 Isoforms—To identify novel PP2Cβ isoforms, a BLASTN search of a human EST data base was performed using the last 420 nucleotides in the 3′-coding region of human PP2Cβ as the query. In addition to PP2Cβ2, a second group of EST clones was found that matched the first ~120 nucleotides of the query, indicating that they encode a novel PP2Cβ isoform, which was named PP2Cβ2. Total RNA was isolated from HeLa cells with TRIzol reagent according to the manufacturer’s protocol. The full-length coding regions of human PP2Cα and PP2Cβ2 isoforms were amplified from total RNA by RT-PCR with the following primers (start and stop codons are underlined): PP2Cα: 5′-CCCAATATGGGAGACATTTTACAACAG-3′ and 5′-CCCAAGGTTTTACCAATACGGTC-3′; PP2Cβ2: 5′-GCCCAAGGTTTTACCAATACGGTC-3′ and 5′-CGGGCTGTCGAGCCTCCATTGCTTCTGAC-3′. PCR fragments were inserted into pBlueScript II KS(−) and sequenced. For PP2Cβ2, a second COOH-terminal site was used to eliminate an internal NolI site before the stop codon without changing the amino acid residue. Recombinant Protein Expression and Purification—To express non-tagged proteins, the full-length coding regions of PP2Cα and PP2Cβ2 were inserted into the NdeII/HindIII sites of pET21a and into the NolI/Xhol sites of pET21d, respectively. For expression of an N-terminal histidine-tagged fusion protein, PP2Cβ2 (without its stop codon) was amplified by PCR and inserted into the BamHI-HindIII sites of pET28a, which resulted in an NH2-terminal histidine-tagged fusion protein. PP2Cβ2 was expressed in LBL21(DE3) cells containing pET19b-PP2Cγ (provided by M. V. Murray and A. R. Kainer) and purified as described (39). The GST-Wip1 plasmid was provided by E. Appella and GST-Wip1 was expressed in E. coli and purified as described (41). GST-cyclin A733–432 (30), GST-Cak1p (23), and GST-Cdk6 (63) have been described previously. Purified GST-KSHV-cyclin is a gift from L. Tong and N. Pavletich.

Preparation of 32P-Labeled CDKs and Protein Phosphatase Assays—Cdk2, GST-Cdk2, GST-Cdk6, and GST-Cdk2β2 isoforms were phosphorylated by GST-Cak1p in the presence of [γ-32P]ATP as described (30). 1× TBS (40) and GST-Cdk6β2-160 were labeled reproducible and nearly saturated levels. The CDK phosphorylation activity and casein phosphorylation activity were determined as described (30, 47). The protein phosphatase activities of HeLa cell lysate and recombinant PP2Cα were assayed in the presence of 5 and 20 mM MgCl2, respectively. Briefly, 5 μl of each fraction was incubated with 50 ng of 32P-labeled CDKs in a 20-μl reaction for 15 min at room temperature. Reactions were terminated by addition of 10 μl of 3× sample buffer separated by 10% SDS-PAGE, and analyzed by autoradiography and PhosphorImager (Molecular Imager GS-250; Bio-Rad).

Immunoblotting—Samples were resolved by SDS-PAGE (10% total acrylamide) and transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore) with a semi-dry blotting apparatus (Trans-Blot SD, Bio-Rad). After blocking at room temperature for 2 h in Blotto (5% nonfat dry milk), the membranes were incubated with anti-PP2C polyclonal antibody (1 μg/ml in Blotto) overnight at 4 °C followed by horseradish peroxidase-conjugated goat anti-rabbit secondary antibody or rabbit anti-sheep secondary antibody (Pierce; 1:2000 dilution in Blotto) at room temperature for 2 h. Antibodies were detected with SuperSignal ECL reagents (Pierce).

RESULTS

Partial Purification of CDK Phosphatases from HeLa Cell Extract—We previously found that PP2Cα are responsible for the vast majority (99%) of phosphatase activity toward Thr160 of Cdk2 in a HeLa cell extract (30). The activity required Mg2+– and was insensitive to okadaic acid or sodium vanadate. To identify the responsible phosphatase(s), we fractionated a HeLa cell lysate on DEAE-Sepharose Fast Flow, Superdex-200, and Mono-Q columns and detected residual activities in the fractions by their abilities to dephosphorylate Cdk2β2 phosphorylated on Thr160 (Fig. 1) and Cdk6 phosphorylated on Thr177 (data not shown). The GST-Cdk2 and Cdk6 phosphorylation activities were present in the same fractions and showed only a single sharp peak in the first two columns. Mono-Q chromatography, however, showed a broader distribution of Cdk2/Cdk6 phosphorylation activity (Fig. 1D), suggesting the presence of more than one CDK phosphatase.

PP2Cα and PP2Cβ isoforms are the mammalian PP2Cs most
closely related to Ptc2p and Ptc3p, the budding yeast phosphatases responsible for dephosphorylating Thr\(^{160}\) of Cdc28p. We speculated that the CDK phosphatases in HeLa cells might be PP2Ca and/or PP2Cb isoforms. This suggestion was supported by the observation that overexpression of human PP2Ca in yeast led to synthetic lethality in cac1-22\(^{\alpha}\) cells at a semi-permissive temperature.\(^2\) We determined whether the active Mono Q fractions contained PP2Ca and/or PP2Cb proteins by immunoblotting. 0.5-ml fractions were collected to provide higher resolution than in Fig. 1D. Affinity purified antibodies that specifically recognize both PP2Ca and β detected two proteins in a HeLa cell lysate (Fig. 2B). Their sizes of about 45 and 42 kDa were similar to the calculated molecular mass of PP2Ca (~42 kDa). Immunoblotting of Mono-Q fractions showed that the 45- and 42-kDa PP2Ca/β co-purified with the Cdk2/Cdk6 phosphatase activities (Fig. 2, A and B). The total amounts of these isoforms correlated well with Cdk2/Cdk6 phosphatase activity, suggesting that they were likely candidates for the CDK phosphatases in HeLa cells. The elution profiles of the 45- and 42-kDa PP2Ca/β on Mono Q chromatography were similar to those of the previously described rabbit PP2C1 and PP2C2 isoenzymes, respectively (48). In addition, the 45- and 42-kDa PP2Ca/β isoforms were partially separable by Mono-Q chromatography (Fig. 2B): fractions 30 and 31 contained only the 45-kDa PP2Ca/β and fractions 34 and 35 contained almost exclusively the 42-kDa PP2Ca/β. Since the Cdk2 phosphatase activity in fraction 34 was similar to that in fraction 30 (Fig. 2A), it appears that both the 45- and 42-kDa PP2Ca/β proteins are capable of dephosphorylating Cdk2 and Cdk6.

**Identification of PP2Ca and PP2Cβ Isoforms in HeLa Cell Lysate**—We identified the 45- and 42-kDa PP2Ca isoforms using a combination of immunoblotting and molecular cloning. An antibody specific for PP2Ca isoforms recognized the 45-kDa PP2C but not the 42-kDa PP2C (Fig. 2C), indicating that the 45-kDa protein is a PP2Ca isoform and that the 42-kDa protein is a PP2Cβ isoform. Moreover, both the 45-kDa PP2Ca isoform and recombinant PP2Ca exhibited the same mobility in SDS-PAGE (Fig. 2D, lanes 1 and 2), indicating that the 45-kDa PP2C was likely the previously reported human PP2Ca.

In contrast, the 42-kDa PP2Cβ isoform was much smaller than the previously reported human PP2Cβ, which has 479 amino acid residues and an apparent molecular mass of ~55 kDa in SDS-PAGE (49). Instead, the size of the 42-kDa PP2Cβ isoform was similar to those of reported PP2Cβs from rabbit, mouse, and rat (34–36). We speculated that the 42-kDa PP2Cβ might be an unreported human ortholog of PP2Cβs in these other mammals. By searching an EST database, we identified and cloned a novel human PP2Cβ isoform ("PP2Cβ2") from HeLa cells by RT-PCR. The encoded protein is predicted to be 387 amino acids, compared with 479 amino acids for human PP2Cβ and 390 amino acids for mouse and rat PP2Cβ. Since the first 1134 nucleotides (378 amino acids) in the human PP2Cβ and PP2Cβ2 coding regions were identical, PP2Cβ and PP2Cβ2 appear to arise via alternative splicing. Human PP2Cβ2 showed ~95% identity to mouse and rat PP2Cβ (Fig. 3). Recombinant human PP2Cβ2 exhibited the same mobility as the 42-kDa PP2C on SDS-PAGE (Fig. 2D, lanes 3 and 4), suggesting that the 42-kDa HeLa PP2C is PP2Cβ2. Interestingly, the mobility of PP2Ca was less than that of PP2Cβ2 on

---

\(^2\) A. Cheng and M. J. Solomon, unpublished data.

---

**Fig. 1.** Partial purification of Cdk2/Cdk6 phosphatase activity from HeLa S3 cell extracts. A, flow chart of the partial purification of CDK phosphatase activities by chromatography on DEAE-Sepharose, Superdex-200, and Mono Q. B–D, chromatography on DEAE-Sepharose Fast Flow, Superdex-200, and Mono Q. The peak of activity on DEAE-Sepharose Fast Flow was at 0.37 M NaCl, the peak of activity on Superdex-200 was at ~45 kDa, and the peak of activity of activities on Mono Q was at 0.38 M NaCl.
Dephosphorylation of Human CDKs by PP2Ca and PP2Cβ2

Dephosphorylation of CDKs by Recombinant PP2Ca and PP2Cβ—We next determined whether recombinant human PP2Cs could dephosphorylate human Cdk2 and Cdk6 in vitro. PP2Ca and PP2Cβ2 were expressed in E. coli and purified as NH2-terminal and COOH-terminal hexahistidine-PP2C. The results were obtained with PP2Cβ2 (GenBank™ accession number AF294792), murine PP2Cβ (GenBank™ accession number P36993), and rat PP2Cβ (GenBank™ accession number P35813), human PP2Cβ (GenBank™ accession number A35012), mouse PP2Cβ (GenBank™ accession number P36993), and PP2Cβ2 (GenBank™ accession number P35815) were aligned by the blast method using the LASERGENE program.

SDS-PAGE even though PP2Ca (382 aa) is slightly smaller than PP2Cβ2 (387 amino acids).

Dephosphorylation of CDKs by Recombinant PP2Ca and PP2Cβ—We next determined whether recombinant human PP2Cs could dephosphorylate human Cdk2 and Cdk6 in vitro. PP2Ca and PP2Cβ2 were expressed in E. coli and purified as NH2-terminal and COOH-terminal hexahistidine-tagged (His6) proteins, respectively. His6-PP2Ca was expressed and purified from BL21(DE3) cells bearing pET19b-PP2Ca as described (39). Since the COOH-terminal portions of PP2Cs may regulate substrate specificity (45), we also prepared His6-tagged COOH-terminal deletions of these proteins, PP2CaΔC (PP2Ca1–308) and PP2Cβ2ΔC (PP2Cβ21–312), which only contained the minimal regions necessary for activity (45, 50). The casein phosphatase activities of recombinant PP2Ca, PP2Cβ2, PP2Cα2, and PP2Cβ2ΔC were comparable to each other and ranged from 91 to 109 milliunits/mg in the presence of 20 mM MgCl2 as described previously (30, 47). Means and standard deviations from three experiments are shown. B, dephosphorylation of 32P-Cdk2 by PP2Cs. Increasing amounts (1, 2, 5, 10, 20, 50, and 100 ng) of PP2Ca (○), PP2Cβ2 (●), PP2Cγ (▲), PP2Cα2 (▲), and PP2Cβ2ΔC (▲) were assayed for Cdk2 phosphatase activity at room temperature for 15 min. 32P-Cdk2 was separated by 10% SDS-PAGE and analyzed by PhosphorImager analysis. Each point represents the mean from three experiments. Note that the linear range for the dephosphorylation reactions extends until ~30% of the substrate has been dephosphorylated. C, dephosphorylation of 32P-GST-Cdk6 by PP2Cs. Increasing amounts (1, 2, 5, 10, 20, 50, and 100 ng) of PP2Ca (○), PP2Cβ2 (●), PP2Cα2 (▲), and PP2Cγ (▲) were assayed for Cdk6 phosphatase activity as described in B. Each point represents the mean from three experiments.

2C protein phosphatase, was incapable of dephosphorylating Cdk2 (data not shown).

Substrate Preference of PP2Ca and PP2Cβ2—PP2Ca has been observed to show a 20-fold preference for a phosphothreonine peptide substrate compared with an equivalent phosphoserine substrate in vitro (51), leading to the suggestion that PP2C substrates are generally phosphorylated on threonine residues (32). We, therefore, compared the abilities of PP2Cs to dephosphorylate wild-type Cdk2 and mutant Cdk2307Ser-160 and mutant Cdk2307Thr-160. We previously showed that a HeLa cell extract dephosphorylated Cdk2Thr-160 about 4 times as fast as Cdk2Ser-160 (52). Fig. 5A confirms this preference. Fig. 5B shows that the partially purified HeLa PP2Cs (Mono-Q fractions 31 and 34) exhibited the same qualitative preference for Cdk2Thr-160. To quantitate this effect, we used recombinant PP2Ca and PP2Cβ2 and performed assays within the linear range (when less than 30% of the substrate was dephosphorylated). This analysis showed that PP2Ca and PP2Cβ2 have 3- and 2.7-fold preferences for Cdk2 Thr-160 over Cdk2 Ser-160 (Fig. 5C), confirming the qualitative observations with the native enzymes (Fig. 5B).

Binding of Cyclin Prevents the Dephosphorylation of CDKs by PP2Cs—Since the binding of cyclins to CDKs blocked the dephosphorylation of yeast Cdc28p and human Cdk2 by Ptc2p, Ptc3p, and KAP (29–30), we tested whether the binding of cyclin could also inhibit the dephosphorylation of Cdk2 and Cdk6 by human PP2C isoforms. Preincubation of Cdk2 with excess GST-cyclin A blocked the dephosphorylation of Cdk2 by...
using Cdk2Thr-160 and Cdk2Ser-160 as substrates. 50 ng of 32P-Cdk2Thr-160 A, kinetics of dephosphorylation by Cdk2 Thr-160 and Cdk2 Ser-160 by PP2C

Clearly, the dephosphorylation of GST-Cdk6 by PP2C a forms, PP2C

KSHV-cyclin (Fig. 6 in vitro on the casein phosphatase activities of PP2C a PP2C

PP2C a

KSHV-cyclin ("V-cyc") (B) at room temperature for 30 min. The samples were then incubated with buffer or 100 ng of PP2Cs at room temperature for 15 min. Cdk2 (A) and Cdk6 (B) were separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and analyzed by autoradiography (AR) and immunoblotting (IB) with antibodies (A). C, cyclins have no effect on the casein phosphatase activity of PP2Cs. 50 ng of PP2Ca was preincubated with 250 ng of cyclin A or KSHV-cyclin ("V-cyc") at room temperature for 30 min, followed by determination of casein phosphatase activities.

PP2Ca, PP2Cβ2, PP2CaΔC, and PP2Cβ2ΔC (Fig. 6A). Similarly, the dephosphorylation of GST-Cdk6 by PP2Ca and PP2Cβ2 was blocked by the binding of a viral D-type cyclin, KSHV-cyclin (Fig. 6B). In contrast, these cyclins had no effects on the casein phosphatase activities of PP2Ca, PP2Cβ2, PP2Cγ, PP2CaΔC, and PP2Cβ2ΔC (Fig. 6C), indicating that cyclins did not inhibit PP2C activity directly.

DISCUSSION

We previously reported that two yeast PP2Cs, Ptc2p and Ptc3p, are the major physiological protein phosphatases for the Cdc28p cyclin-dependent kinase in budding yeast and that PP2C-like activity was also responsible for >90% of the Cdk2 phosphatase activity in a HeLa cell extract (30). We now provide evidence that the PP2C-like activities in HeLa cell extract are due to PP2Cβ2, a novel PP2Cβ isofrom, and to PP2Ca. PP2Ca/β-specific antibodies detected two proteins with apparent molecular masses of 45- and 42-kDa in HeLa cell lysate. Mg2+-dependent Cdk2 and Cdk6 phosphatase activity co-purified with 45- and 42-kDa PP2Ca/β isoforms during chromatography on DEAE-Sepharose, Superdex-200, and Mono-Q columns. The 45-kDa PP2C was also recognized by a PP2Ca-specific antibody and had the same electrophoretic mobility as recombinant PP2Ca. The 42-kDa PP2Ca/β did not react with the PP2Ca-specific antibody and exhibited the same electrophoretic mobility as a novel PP2Cβ isoform (PP2Cβ2). PP2Cβ2 possesses 387 amino acid residues and shows ~95% identity to PP2Cβ's from mouse, rat, and rabbit. We found that recombinant PP2Ca and PP2Cβ2 efficiently dephosphorylated monomeric Cdk2 and Cdk6 in vitro but that two other PP2C isoforms, PP2Cγ and Wip1, did not. Further biochemical analysis demonstrated that Cdk2Thr-160 was a relatively poor substrate compared with wild-type Cdk2Thr-160 for HeLa cell extract, the partially purified 45- and 42-kDa PP2Cs, and recombinant PP2Ca and PP2Cβ2. Similar to budding yeast Ptc2p and Ptc3p, PP2Ca and PP2Cβ2 could not dephosphorylate cyclin-bound CDKs. These results indicate that human PP2Ca and PP2Cβ2 represent the PP2C activity responsible for removing the activating phosphorylation from Cdk2 in HeLa cells. These studies also provide evidence that Cdk6, like Cdk2, is a substrate for PP2Cs.

Besides CDKs, a number of MAP kinases appear to be substrates for type 2C protein phosphatases. For instance, PP2Cs have been implicated in negatively regulating stress-responsive protein kinase cascades in eukaryotic cells. In both budding yeast and fission yeast, genetic studies have shown that PP2C-like enzymes oppose the activation of the MAP kinase pathway that is activated in response to osmotic and heat shocks (53–55). In human cells, PP2Ca can reverse the activation of the p38 and JNK MAPKs induced by stress and cytokines (44). Biochemically, a human PP2Cβ isoform dephosphorylated a similar threonine within the activation loop of the p38 MAPK (44). Recently, Ptc1 and Ptc3 in Schizosaccharomyces pombe were shown to dephosphorylate Thr172 of the p38 homolog (Sp1) in its activating loop (56). Given the similarity between the sites of activating phosphorylation in MAPKs and CDKs, we have proposed that PP2C-like enzymes could be general T-loop protein phosphatases (30).

The activities of PP2Cs toward a variety of substrates can be affected by whether the site to be dephosphorylated is a serine or a threonine and by regulatory factors that bind to the substrates. Biochemically, PP2Ca has been shown to dephosphorylate a phosphothreonine substrate 20-fold more efficiently than the corresponding phosphoserine substrate in vitro (51) and PP2C substrates have been proposed to be phosphorylated on threonine residues in vitro (32). Using human Cdk2, a likely physiological substrate for PP2Cs, we confirmed that PP2Ca
and PP2Cβ2 removed the phosphate from a phosphoserine substrate slower (~3-fold) than from the phosphothreonine substrate (Fig. 5). Indeed, many PP2C substrates, including all known CDKs undergoing activating phosphorylation, AMPK (57–59), moesin (62), and p38 MAPK (44), are phosphorylated on threonine residues. However, some PP2C substrates such as axin (60) and CFTR (61) may be phosphorylated on serines. Since exchanges of serines and threonines often have little effect on protein functions, the replacement of a threonine with a serine could be used to control the duration of phosphorylation. In addition, the binding of ligands or regulatory proteins to substrates also influences the rate of dephosphorylation by PP2Cs. For example, the dephosphorylation of AMPK is inhibited in the presence of 5'-AMP (59), and the dephosphorylation of CDKs is blocked by the binding of cyclins (Fig. 6, A–B, and Ref. 30). The binding of ligands and regulatory proteins could induce conformational changes, block dephosphorylation, and preserve the phosphorylated state of the substrate for a period of time. Dephosphorylation could occur rapidly following removal of the ligand or of the regulatory proteins, such as happens with CDKs after cyclin degradation.

Given that no regulatory subunits for PP2Cαβ have been found and that PP2Cαβ isoforms only differ significantly in their COOH-terminal segments, it is tempting to speculate that the diverse COOH-terminal regions are involved in regulating PP2C activity. Our studies showed that the COOH-terminal truncated forms of PP2Cα and PP2Cβ2 dephosphorylated Cdk2 and Cdk6 as well as the full-length enzymes (Fig. 4), indicating that the carboxyl regions may not directly regulate enzymatic activity or substrate specificity. Additional work will be required to determine whether these segments might facilitate interactions with substrates, localization within the cell, or other properties of these enzymes. In addition to the COOH-terminal regions, mammalian PP2Cs and -β isoforms possess potential N-myristoylation sites similar to those in budding yeast Ptc2p and Ptc3p. In the crystal structure of human PP2Cα, the NH2-terminal glycine residue is close to its catalytic center (32), therefore, it is possible that mammalian PP2Cs and PP2Cβ are regulated by N-myristoylation in vivo. Further experiments will be necessary to test this possibility.

Acknowledgments—We thank L. Tong and N. Pavletich for GST-KSHV cyclin, M. V. Murray and A. R. Krainer for PP2Cγ, E. Appella for GST-Wip1, and A. Natrillo for technical assistance. For helpful discussions and critical reading of the manuscript, we thank J. Burton, D. Ostapenko, K.E. Ross, and V. Tsakraklides.

REFERENCES

1. Pines, J. (1995) Biochem. J. 308, 697–711
2. Sherr, C. J., and Roberts, J. M. (1999) Genes Dev. 13, 1011–1014
3. Sherr, C. J., and Roberts, J. M. (1995) Science 267, 26705–26712
4. Morgan, D. O. (1996) Science 275, 1731–1735
5. Morgan, D. O. (1996) Science 275, 242–250
6. Kaldis, P., and Solomon, M. J. (2000) Eur. J. Biochem. 267, 4213–4221
7. Kaldis, P., and Solomon, M. J. (2000) Mol. Cell. Biol. 20, 791–803
8. Kipreos, E. T., Rosenthal, E. T., Youngblom, J., Distel, D., and Hunt, T. (1983) Cell 33, 389–396
9. Sherr, C. J. (1994) Cell 79, 551–555
10. Johnson, L. N., Noble, M. E. M., and Owen, D. J. (1996) Cell 85, 149–158
11. Boor, R., and Beach, D. (1986) Mol. Cell. Biol. 6, 3523–3530
12. Gould, K. L., Moreno, S., Owen, D. J., Sazer, S., and Nurse, P. (1991) EMBO J. 10, 3287–3300
13. Lee, T. H., Solomon, M. J., Mummy, M. C., and Kirschner, M. W. (1991) Cell 64, 415–423
14. Krek, W., and Nigg, E. A. (1992) The New Biologist 4, 323–329
15. Solomon, M. J., Lee, T., and Kirschner, M. W. (1992) Mol. Biol. Cell 3, 13–27
16. Ostapenko, K. E., Ross, A., and V. Tsakraklides. Ostapenko, K. E., Ross, A., and V. Tsakraklides.