Dephosphorylation of cyclin-dependent kinases by type 2C protein phosphatases

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Activating phosphorylation of cyclin-dependent protein kinases (CDKs) is necessary for their kinase activity and cell cycle progression. This phosphorylation is carried out by the Cdk-activating kinase (CAK); in contrast, little is known about the corresponding protein phosphatase. We show that type 2C protein phosphatases (PP2Cs) are responsible for this dephosphorylation of Cdc28p, the major budding yeast CDK. Two yeast PP2Cs, Ptc2p and Ptc3p, display Cdc28p phosphatase activity in vitro and in vivo, and account for ~90% of Cdc28p phosphatase activity in yeast extracts. Overexpression of *PTC2* or *PTC3* results in synthetic lethality in strains temperature-sensitive for yeast CAK1, and disruptions of *PTC2* and *PTC3* suppress the growth defect of a *cak1* mutant. Furthermore, PP2C-like enzymes are the predominant phosphatases toward human Cdk2 in HeLa cell extracts, indicating that the substrate specificity of PP2Cs toward CDKs is evolutionarily conserved.

[**Key Words:** Cyclin-dependent kinase; protein phosphatase type 2C; activating phosphorylation; Cdk-activating kinase (CAK)]

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Eukaryotic cell cycle progression is controlled by the sequential activation and inactivation of cyclin-dependent protein kinases (CDKs). To coordinate the cell cycle machinery, extracellular and intracellular signals regulate CDK activities through a variety of mechanisms, including association with regulatory subunits (cyclins, inhibitors, and assembly factors), subcellular localization, transcriptional regulation, selective proteolysis, and reversible protein phosphorylation (Pines 1995; Sherr and Roberts 1995, 1999; King et al. 1996; Morgan 1996, 1997; Solomon and Kaldis 1998). In the budding yeast *Saccharomyces cerevisiae*, Cdc28p is the major CDK involved in regulating the cell division cycle.

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 (Evans et al. 1983; Sherr 1994; Pines 1995; King et al. 1996). The inhibitory phosphorylations are carried out by the Wee1 and Myt1 protein kinases, and removed by the Cdc25 phosphatase family (for reviews, see Morgan 1997; Solomon and Kaldis 1998). Activating phosphorylation occurs within the so-called T-loop (Johnson et al. 1996; Solomon and Kaldis 1998) on a conserved threonine residue corresponding to Thr-169 in Cdc28p and Thr-160 in Cdk2. Mutation of the equivalent site to alanine in Cdc2 from a variety of species abolishes kinase activity and biological function (Booher

and Beach 1986; Gould et al. 1991; Lee et al. 1991; Krek and Nigg 1992; Solomon et al. 1992; Cismowski et al. 1995). Structural and biochemical studies have shown that the activating phosphorylation alters the CDK-substrate interface (Morgan 1996; Russo et al. 1996) and stabilizes the interaction between the cyclin and the CDK (Ducommun et al. 1991; Desai et al. 1992).

This activating phosphorylation is catalyzed by the Cdk-activating kinase (CAK). Higher eukaryotic CAK, whose subunits also function as components of basal transcription factor IIIH (TFIIH; Feaver et al. 1994; Roy et al. 1994; Serizawa et al. 1995; Shiekhata et al. 1995; Adamczewski et al. 1996), is composed of p40^{MO15}/Cdk7, cyclin H, and an assembly factor MAT1 (for review, see Solomon and Kaldis 1998; Kaldis 1999). In contrast to CAK in higher eukaryotes, the physiological CAK from budding yeast (Cak1p or Civ1p) is only distantly related to p40^{MO15} and functions as a monomer (Espinoza et al. 1996; Kaldis et al. 1996; Thuret et al. 1996).

Despite rapid progress in our understanding of CAK, Wee1, and Cdc25, much less is known about the protein phosphatases that reverse the activating phosphorylation. Studies in *Schizosaccharomyces pombe* and *Xenopus* egg extracts raised the possibility that the dephosphorylation of this residue may be required for exit from mitosis (Gould et al. 1991; Lorca et al. 1992), and implicated type 2A and type 1 protein phosphatases in the dephosphorylation of Cdc2 (Lee et al. 1991; Lorca et al. 1992). More recently, a dual specificity phosphatase KAP (also called Cdi1, Cip2) was identified by its interaction with Cdc2, Cdk2, and Cdk3 in a yeast two-hybrid system

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(Gyuris et al. 1993; Harper et al. 1993; Hannon et al. 1994). KAP dephosphorylated Thr-160 in human Cdk2 in vitro and preferred monomeric rather than cyclin-bound Cdk2 as a substrate (Poon and Hunter 1995), which is consistent with the observation that *Xenopus* Cdc2 is dephosphorylated only after cyclin degradation (Lorca et al. 1992). However, no obvious KAP homolog exists in the budding yeast genome.

To identify the Cdc28p phosphatase, we characterized the dephosphorylation of a Thr-169 phosphorylated form of Cdc28p in crude yeast lysates. Our biochemical studies showed that type 2C protein phosphatase (PP2C)-like activities are responsible for the dephosphorylation of Cdc28p in yeast extracts. Two of the five yeast PP2Cs, Ptc2p and Ptc3p, displayed Cdc28p phosphatase activity in vitro and in vivo, and were the predominant Cdc28p phosphatases in yeast extracts. Overexpression of *PTC2* or *PTC3* resulted in a synthetic lethal effect in a yeast strain containing a temperature-sensitive allele of *CAK1*, *cak1-22*, indicating that Ptc2p and Ptc3p oppose Cak1p in vivo. In contrast, disruption of *PTC2* and *PTC3* suppressed the growth defect of a *cak1-23* mutant at a semipermissive temperature. Like KAP, Ptc2p and Ptc3p preferred monomeric CDKs rather than cyclin-bound CDKs as substrates. Further studies revealed that type 2C protein phosphatases are also responsible for >99% of Cdk2 phosphatase activity in HeLa cell extracts, indicating that the ability of PP2Cs to reverse the activating phosphorylation of CDKs is evolutionarily conserved. The demonstration that PP2Cs are the main protein phosphatases acting to oppose CAK completes the identification of the basic kinases and phosphatases acting on the major phosphorylation sites of the CDKs controlling cell cycle progression.

Results

A type 2C protein phosphatase dephosphorylates Thr-169 of Cdc28p in yeast extracts

To identify the Cdc28p phosphatase in budding yeast, we developed a conventional assay for Cdc28p phosphatase activity. Hexahistidine-tagged Cdc28p (Cdc28p-his₆) was overexpressed and purified from budding yeast and labeled with [γ -³²P]ATP using recombinant GST-Cak1p. The subsequent dephosphorylation of Cdc28p was assessed by autoradiography after SDS-PAGE (Fig. 1A, top). We analyzed the biochemical properties of the Cdc28p phosphatase in a yeast lysate using inhibitors of various classes of phosphatases. In budding yeast, ~31 phosphatases belong to the PPP, PPM, and dual specificity/tyrosine phosphatase families (Stark 1996). The PPP family includes PP1/PP2A/PP2B, whereas the PPM family includes PP2C (Cohen 1994). Different phosphatase families can be distinguished by their unique biochemical properties (Cohen 1989; Walton and Dixon 1993): PP1 and PP2A have no ion requirements and are sensitive to okadaic acid and microcystins; PP2B requires Ca²⁺ for full activity; PP2C requires Mg²⁺ or Mn²⁺; dual specificity/tyrosine phosphatases have no ion requirements but

Figure 1. PP2C-like activity dephosphorylates Thr-169 of Cdc28p in yeast extract. (A) Biochemical characterization of Cdc28p Thr-169 phosphatase activity. Fifty nanograms of ³²P-labeled Cdc28p-his₆ was incubated with 25 μ g of yeast extract with or without 5 mM Mg²⁺ or Ca²⁺ at room temperature for 15 min. The following phosphatase inhibitors were added to yeast extract 10 min before assay, as indicated: 5 μ M microcystin-LR (MC); 6 mM EDTA; 20 mM NaF; 1 mM Na₂WO₄; 1 mM Na₃VO₄; 10 mM sodium tartrate; 5 mM tetramisole (TMS). Cdc28p-his₆ was separated by 10% SDS-PAGE, transferred to a PVDF membrane, and analyzed by autoradiography (AR), and immunoblotting (IB) of the same filter with anti-PSTAIR antibodies. The lower band in the bottom panel is endogenous Cdc28p from the yeast extract. (B) MBP-Clb2p inhibits the dephosphorylation of Cdc28p-his₆ by yeast extract. Fifty nanograms of Cdc28p-his₆ was preincubated with MBP-Clb2p (lanes 3,4) or buffer alone (lanes 1,2) at room temperature for 30 min prior incubation with 25 μ g of yeast extract at room temperature for 15 min. Note that the presence of yeast extract alters the migration of Cdc28p-his₆ during electrophoresis.

are sensitive to inhibitors such as orthovanadate and tungstate.

As shown in Figure 1A (top), no dephosphorylation of Cdc28p occurred in the presence of buffer containing EDTA and EGTA (lane 2), which suggests that PP1/PP2A family and dual-specificity phosphatases play little role in dephosphorylating Thr-169 in the Cdc28p-his₆ substrate. However, the addition of Mg²⁺ potentially stimulated the dephosphorylation of Cdc28p (Fig. 1A, lane 3), suggesting that a PP2C-like activity was responsible for the dephosphorylation of Thr-169. Immunoblotting with an antibody to Cdc28p demonstrated that there was no proteolysis of the substrate during the assay (Fig. 1A, lower). Addition of Ca²⁺ failed to stimulate phosphatase activity, indicating that PP2B cannot dephosphory-

late Cdc28p-his₆ (Fig. 1A, lane 4). To further rule out the possibility that this Mg²⁺-activated phosphatase activity might belong to another phosphatase family, we tested the effects of adding various phosphatase inhibitors to a yeast extract in the presence of Mg²⁺ (Fig. 1A, lanes 4–11). Microcystin-LR can inhibit PP1 and PP2A completely in cell extracts at 5 μ M (Honkanen et al. 1990). Orthovanadate and tungstate are inhibitors of dual specificity/protein tyrosine phosphatases, including KAP (Walton and Dixon 1993; Hannon et al. 1994). Neither microcystin-LR, orthovanadate, nor tungstate had any effect on the Thr-169 phosphatase activity (Fig. 1A, lanes 5,8,9). The Cdc28p phosphatase activity was also insensitive to both tartrate and tetramisole (Fig. 1A, lanes 10,11), which inhibit certain broad specificity acid phosphatases and alkaline phosphatases, respectively (Abul-Fadl and King 1949; Van Belle 1972). Moreover, the dephosphorylation of Cdc28p was blocked completely by the Mg²⁺ chelator EDTA and by NaF (Fig. 1A, lanes 6,7), which is a nonselective inhibitor of serine/threonine protein phosphatases. These inhibitor sensitivities are fully compatible with a PP2C-like activity but inconsistent with PP1, PP2A, PP2B, or dual specificity/protein tyrosine phosphatase activities (for review, see Cohen 1989). Similar results were obtained using a ³²P-labeled human Cdk2 substrate in the yeast extract (data not shown).

We then tested whether dephosphorylation of Thr-169 was blocked in the presence of a cyclin. A previous study showed that KAP could only dephosphorylate Cdk2 in the absence of cyclin (Poon and Hunter 1995). We pre-incubated Cdc28p-his₆ with MBP-Clb2p (a yeast mitotic cyclin) or with buffer alone, before the dephosphorylation reaction. The presence of Clb2p decreased substantially the dephosphorylation of Cdc28p by the PP2C-like activity (Fig. 1B, top, cf. lane 2 with lane 4). Similar results were obtained using a ³²P-labeled human Cdk2 substrate and GST-cyclin A (data not shown).

Cloning and expression of yeast PP2Cs

There are six PP2C-like genes in *S. cerevisiae*, *PTC1*, *PTC2*, *PTC3*, *YBR125c*, *YCR079w*, and *YOR090c* (Fig. 2A) (Stark 1996). Ptc1p, Ptc2p, and Ptc3p have been previously identified or characterized as PP2C-like enzymes (Maeda et al. 1993, 1994). Ptc1p, which is slightly larger than the catalytic core of a PP2C, encodes one of the shortest PP2Cs. Ptc2p and Ptc3p share 62% identity and 77% similarity, and are more closely related than any other pair of yeast PP2Cs. Ptc2p shows 31% identity and 52% similarity to human PP2C α . Ptc3p shows 34% identity and 52% similarity to human PP2C α . The *YBR125c* gene product has 28% identity and 43% similarity to human PP2C α . Unlike the other yeast PP2Cs, *YOR090c* encodes a polypeptide with both amino-terminal and carboxy-terminal extensions surrounding the catalytic core. *YOR090c* protein shows 23% identity and 40% similarity to human PP2C α . Because the recombinant proteins encoded by *YBR125c* and *YOR090c* showed Mg²⁺/Mn²⁺-dependent phosphatase activities

Figure 2. Expression of budding yeast PP2Cs. (A) Comparison of the primary structures of PP2C-like enzymes in budding yeast. The catalytic domains of PP2Cs are depicted in gray, and the amino- and carboxy-terminal extensions are shown as solid lines. (B) Preparations of his₆-Ptc1p, GST-Ptc2p, GST-Ptc3p, his₆-Ptc4p, and GST-Ptc5p (10 μ g each) were subjected to 10% SDS-PAGE. Casein phosphatase activity was assayed in the presence of 20 mM Mg²⁺ or 20 mM Mn²⁺. One unit of phosphatase activity is that amount of enzyme that catalyzes the release of 1.0 nmole Pi/min in the standard assay.

(see below) we named these genes *PTC4* and *PTC5*, respectively. Although a sixth open reading frame, *YCR079w*, encodes a protein with only one mismatch to the Prosite motif for a PP2C (Stark 1996), a GST fusion protein failed to show any casein phosphatase or Cdc28p phosphatase activity in the presence of Mg²⁺ or Mn²⁺, and disruption of *YCR079w* had no effect on the Cdc28p phosphatase activity in a yeast extract (data not shown). Further work will be required to establish whether *YCR079w* actually encodes a PP2C.

Because PP2C enzymes function as monomers (for review, see Cohen 1989), we expected that recombinant PP2C proteins could help us identify which PP2C dephosphorylates Cdc28p. We cloned and expressed *PTC1* through *PTC5* as recombinant proteins in *Escherichia coli*. Ptc2p, Ptc3p, and Ptc5p were expressed and purified as GST fusion proteins. Although Ptc1p and Ptc4p were initially expressed as GST fusion proteins, we found that amino-terminal hexahistidine-tagged forms of Ptc1p and Ptc4p had higher casein phosphatase activities. Purified Ptc1p through Ptc5p are shown in Figure 2B. Like other type-2C phosphatases, these recombinant enzymes dem-

onstrated Mg^{2+} - or Mn^{2+} -dependent casein phosphatase activities, with specific activities ranging from 0.3 to 2.3 U/mg protein (Fig. 2C). (One unit of phosphatase activity is the amount of enzyme that catalyzes the release of 1.0 nmoles of P_i /min in the standard assay.) GST-Ptc2p, GST-Ptc3p, and GST-Ptc5p were more active with Mg^{2+} than with Mn^{2+} , whereas his₆-Ptc1p and his₆-Ptc4p had the opposite ion preference. GST-Ptc2^{D234A} and GST-Ptc3^{D234A}, which contain mutations predicted to abolish metal binding, showed no detectable casein phosphatase activity (data not shown).

Dephosphorylation of Cdc28p Thr-169 by recombinant Ptc2p and Ptc3p in vitro

We then examined whether these catalytically active PP2C enzymes could dephosphorylate Cdc28p on Thr-

169. Preliminary studies with Ptc2p and Ptc3p showed that both Mg^{2+} and Mn^{2+} supported the dephosphorylation of Cdc28p, and that the choice of ion had little effect on the ratio of Cdc28p phosphatase activity to casein phosphatase activity (data not shown). Therefore, the five recombinant phosphatases were analyzed for Cdc28p phosphatase activity in the presence of whichever ion gave the higher casein phosphatase activity. Recombinant GST-Ptc2p and GST-Ptc3p showed the greatest Cdc28p phosphatase activity (Fig. 3A, top, and 3B), and were able to dephosphorylate efficiently substrates in 15 min. Titration experiments showed that Ptc1p had <10% the specific Cdc28p phosphatase activity of Ptc2p or Ptc3p (Fig. 3B). Ptc4p had very weak Cdc28p phosphatase activity, as did Ptc5p, although it showed the greatest activity toward ³²P-labeled casein of the recombinant enzymes. For comparison, GST-KAP

Figure 3. Recombinant Ptc2p and Ptc3p dephosphorylate Cdc28p-his₆ in vitro. (A) Cdc28p Thr-169 phosphatase activity of recombinant PP2C enzymes. his₆-Ptc1p, GST-Ptc2p, GST-Ptc3p, his₆-Ptc4p, and GST-Ptc5p (1 μg each) were assayed for Cdc28p phosphatase activity at room temperature for 15 min. ³²P-labeled Cdc28p-his₆ 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 reaction extends until ~30% of the substrate has been dephosphorylated. (C,D) Cyclins inhibit the dephosphorylation of CDKs by recombinant GST-Ptc2p, GST-Ptc3p, and GST-KAP. Fifty nanograms of ³²P-labeled Cdc28p-his₆ (C) or ³²P-labeled Cdk2 (D) was preincubated with 300 ng of MBP-Clb2p (C), GST-cyclin A (D), or buffer alone at room temperature for 30 min. The samples were then incubated with buffer or 1 μg of GST-Ptc2p, GST-Ptc3p, or GST-KAP at room temperature for 15 min. Cdc28p-his₆ and Cdk2 were separated by 10% SDS-PAGE, transferred to a PVDF membrane, and analyzed by autoradiography (AR) and immunoblotting (IB) with anti-PSTAIR antibodies.

sayed for Cdc28p-his₆ phosphatase activity in the presence of 20 mM Mg^{2+} (lanes 2,6,8,12,14) or 20 mM Mn^{2+} (lanes 4,10). The labeled substrate was detected as described in Fig. 1. (B) Comparison of the relative Cdc28p-his₆ phosphatase activities of recombinant PP2Cs. Increasing amounts (0.1, 0.2, 0.5, 1, 2, 5 μg) of his₆-Ptc1p (●), GST-Ptc2p (□), GST-Ptc3p (○), his₆-Ptc4p (▲), GST-Ptc5p (■), and GST-KAP (△) were assayed for Cdc28p phosphatase activity at room temperature for 15 min. ³²P-labeled Cdc28p-his₆ 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 reaction extends until ~30% of the substrate has been dephosphorylated. (C,D) Cyclins inhibit the dephosphorylation of CDKs by recombinant GST-Ptc2p, GST-Ptc3p, and GST-KAP. Fifty nanograms of ³²P-labeled Cdc28p-his₆ (C) or ³²P-labeled Cdk2 (D) was preincubated with 300 ng of MBP-Clb2p (C), GST-cyclin A (D), or buffer alone at room temperature for 30 min. The samples were then incubated with buffer or 1 μg of GST-Ptc2p, GST-Ptc3p, or GST-KAP at room temperature for 15 min. Cdc28p-his₆ and Cdk2 were separated by 10% SDS-PAGE, transferred to a PVDF membrane, and analyzed by autoradiography (AR) and immunoblotting (IB) with anti-PSTAIR antibodies.

showed weaker phosphatase activity against Cdc28p than Ptc2p or Ptc3p (Fig. 3A,B). The relative Cdc28p phosphatase activity of KAP was similar to that of Ptc1p (Fig. 3B). Neither Mg^{2+} nor Mn^{2+} stimulated the dephosphorylation of Cdc28p or Cdk2 by GST-KAP (data not shown), confirming that its phosphatase activity is Mg^{2+}/Mn^{2+} independent.

Because the binding of MBP-Clb2p to Cdc28p blocked the dephosphorylation of Cdc28p by yeast extract (see Fig. 1B), we tested whether cyclin binding could also inhibit the dephosphorylation of CDKs by purified Ptc2p and Ptc3p. Preincubation of Cdc28p-his₆ with MBP-Clb2p or of Cdk2 with GST-cyclin A blocked the dephosphorylation of Cdc28p and Cdk2 by Ptc2p and Ptc3p (Fig. 3C,D, top). Consistent with a previous report (Poon and Hunter 1995), the dephosphorylations of Cdc28p and Cdk2 by KAP were also blocked by cyclin binding (Fig. 3C,D). In contrast, MBP-Clb2p had no effect on the casein phosphatase activity of GST-Ptc2p (data not shown).

Ptc2p and Ptc3p are the major Cdc28p phosphatases in yeast extracts

To determine the contributions of Ptc2p and Ptc3p to Cdc28p phosphatase activity, we analyzed Cdc28p phosphatase activity in extracts from yeast cells disrupted for the corresponding phosphatases genes. Disruption of *PTC2* and *PTC3*, either singly or together, had no obvious phenotypic effects, and the growth rate of the double deletion strain was almost identical to that of a wild-type strain during exponential growth at 30°C. Extracts from wild-type, $\Delta ptc2$, $\Delta ptc3$, and $\Delta ptc2 \Delta ptc3$ strains were prepared and analyzed for Cdc28p phosphatase activity (Fig. 4A, lanes 2–5). Compared to the wild-type strain (Fig. 4A, lane 2), Cdc28p phosphatase activity in the $\Delta ptc2$ strain and the $\Delta ptc3$ strain decreased 77% and 78%, respectively (Fig. 4B). Furthermore, 90% of Cdc28p phosphatase activity was eliminated in extracts from the double deletion cells (Fig. 4B). These results indicated that Ptc2p and Ptc3p are responsible for the bulk of the Cdc28p phosphatase activity in yeast extracts.

Overexpression of *PTC2* or *PTC3* from a galactose-inducible promoter on a high copy plasmid increased Cdc28p phosphatase activity about seven to eightfold compared to a control strain (Fig. 4A, cf. lanes 7 and 8 with lane 2; Fig. 4B). The overexpression of predicted catalytically inactive forms of *PTC2*^{D234A} or *PTC3*^{D234A} (Das et al. 1996) did not increase Cdc28p phosphatase activity (Fig. 4A, lanes 9,10). Interestingly, overexpression of wild-type Ptc2p and Ptc3p increased the cell doubling time by ~200%, whereas overexpression of the mutant forms of Ptc2p or Ptc3p had no effects (data not shown).

Genetic interaction between CAK1 and PTC2/PTC3

We tested whether any of the five *PTC* genes could oppose Cak1p activity in vivo. Given the fact that the phos-

Figure 4. Ptc2p and Ptc3p are the predominant Cdc28p Thr-169 phosphatases. Comparison of Cdc28p phosphatase activities in yeast extracts after the alteration of *PTC2* and *PTC3* expression. Yeast extracts were prepared from exponential phase wild-type, $\Delta ptc2$, $\Delta ptc3$, and $\Delta ptc2 \Delta ptc3$ cells grown in YPD. (A) Cdc28p phosphatase activities were analyzed in the presence of 5 mM Mg^{2+} : (lane 1) buffer alone; (lane 2) wild-type; (lane 3) $\Delta ptc2$; (lane 4) $\Delta ptc3$; (lane 5) $\Delta ptc2 \Delta ptc3$; (lane 6) $\Delta ptc2 \Delta ptc3$ YEplac112GAL; (lane 7), $\Delta ptc2 \Delta ptc3$ YEplac112GAL-*PTC2*; (lane 8) $\Delta ptc2 \Delta ptc3$ YEplac112GAL-*PTC3*; (lane 9) $\Delta ptc2 \Delta ptc3$ YEplac112GAL-*PTC2*^{D234A}; (lane 10) $\Delta ptc2 \Delta ptc3$ YEplac112GAL-*PTC3*^{D234A}. (B) Quantitation of the experiment shown in A by PhosphorImager analysis. Each point represents the mean from three independent experiments. For the overexpression of Ptc2p and Ptc3p, $\Delta ptc2 \Delta ptc3$ strains containing *PTC2*, *PTC3*, *PTC2*^{D234A}, or *PTC3*^{D234A} in YEplac112GAL were grown in raffinose-containing CM lacking tryptophan, and shifted to galactose-containing medium for 8 hr. Fifty nanograms of ³²P-labeled Cdc28p-his₆ was incubated with 25 μ g of yeast extract in the presence of 5 mM Mg^{2+} at room temperature for 15 min. The relative CDK phosphatase activities in $\Delta ptc2 \Delta ptc3$ YEplac112GAL-*PTC2*^{D234A} and $\Delta ptc2 \Delta ptc3$ YEplac112GAL-*PTC3*^{D234A} extracts were determined by a time-course experiment. ³²P-labeled Cdc28p-his₆ was separated by 10% SDS-PAGE and analyzed by autoradiography and PhosphorImager analysis.

phorylation of Thr-169 in Cdc28p by Cak1p is necessary for normal cell cycle progression (Cismowski et al. 1995; Espinoza et al. 1996; Kaldis et al. 1996; Thuret et al. 1996), we asked whether increasing PP2C activity had any effect on the viability of temperature-sensitive *cak1*-

22 cells. The *PTC* genes were expressed in the *cak1-22* strain (SY143) or an isogenic *CAK1* strain (SY162) from a galactose-inducible promoter on a high-copy plasmid. The strains were grown at 35.5°C, a semipermissive temperature for growth of a *cak1-22* strain. Overexpression of *PTC2* or *PTC3* in *cak1-22* cells resulted in cell inviability at 35.5°C, whereas the temperature-sensitive *cak1-22* cells with an empty plasmid continued to grow at this temperature (Fig. 5). In contrast, overexpression of *PTC2* or *PTC3* had only modest effects on the growth of isogenic *CAK1* cells. Because the sole essential function of Cak1p is to phosphorylate Cdc28p (Cross and Levine 1998), the synthetic lethality between *cak1-22* and overexpression of *PTC2* or *PTC3* is presumably due to inactivation of Cdc28p. In contrast, overexpression of *PTC1*, *PTC4*, or *PTC5* had no detrimental effect on the growth of *cak1-22* cells at any temperature (data not shown), in agreement with our observation that Ptc1p, Ptc4p, and Ptc5p were weak Cdc28p phosphatases in vitro.

If Ptc2p and Ptc3p are physiological Cdc28p phosphatases,

we expected that deletion of *PTC2* and *PTC3* might lessen the growth defect of a *cak1* mutant. We compared the growth of *cak1-23* cells (FHE56; Espinoza et al. 1998) with *cak1-23 Δptc2 Δptc3* cells (ACY171) at different temperatures. Although both strains grew well at 23°C, disruption of *PTC2* and *PTC3* suppressed the growth defect of *cak1-23* at a semipermissive temperature (32°C) (Fig. 5B). Disruption of *PTC2* and *PTC3* had no effect on the growth of wild-type *CAK1* cells at any temperature (data not shown). Therefore, Ptc2p and Ptc3p physiologically oppose the biological functions of Cak1p in budding yeast.

Ptc2p and Ptc3p dephosphorylate Thr-169 in Cdc28p in vivo

We then investigated whether overexpression of Ptc2p and Ptc3p affected the phosphorylation of Cdc28p in vivo. *PTC2* and *PTC3* were expressed from a galactose-inducible promoter on a high-copy plasmid. To monitor Cdc28p activity and phosphorylation, a low-copy plasmid containing a *CDC28-HA* gene under its own promoter was also introduced into the cells. After growth of these strains in galactose-containing medium for 16 hr, 35% of the cells with the *PTC2* plasmid and 37% of the cells with the *PTC3* plasmid were elongated, had elongated buds, or both (for examples, see Fig. 6A), similar to the phenotype of *cak1-22* cells grown at a semipermissive temperature (30°C, data not shown). Extracts were prepared from cells grown in either glucose-containing medium or in galactose-containing medium. Cdc28p-HA was immunoprecipitated from cell lysates with the 12CA5 antibody to the HA tag and analyzed for histone H1 kinase activity (Fig. 6B). The histone H1 kinase activity was barely affected by overexpression of Ptc2p or Ptc3p, consistent with our earlier observation that the presence of cyclins protects Thr-169 from dephosphorylation.

The extent of Thr-169 phosphorylation of cyclin-free Cdc28p could be assayed indirectly after the addition of cyclins to immunoprecipitated Cdc28p-HA and determination of the resulting histone H1 kinase activity. A significant fraction of monomeric Cdc28p is phosphorylated on Thr-169 (Espinoza et al. 1998; K. Ross, P. Kaldis, and M.J. Solomon, unpubl. data). Therefore, the increase in H1 kinase activity after the addition of cyclin should reflect the amount of phosphorylated monomeric Cdc28p-HA. In control cells bearing an empty plasmid, the addition of MBP-Clb2p to Cdc28p increased the histone H1 kinase activity ~130% (Fig. 6B). Similarly, when the expression of Ptc2p and Ptc3p was repressed in glucose-containing medium, preincubation with MBP-Clb2p increased the histone H1 kinase activity by ~100%. In contrast, overexpression of Ptc2p and Ptc3p blocked the increase in histone H1 kinase activity by exogenous MBP-Clb2p (Fig. 6B), indicating that most of the previously phosphorylated monomeric Cdc28p became unphosphorylated.

Next, we directly assessed the phosphorylation of Cdc28p on Thr-169 using a phospho-specific antibody

Figure 5. Genetic interactions between *CAK1* and *PTC2/PTC3*. (A) Synthetic lethality between *cak1-22* and overexpression of *PTC2* and *PTC3*. Growth characteristics of isogenic *CAK1* and *cak1-22* strains transformed with the following plasmids: YEplac195GAL, YEplac195GAL-*PTC2*, and YEplac195GAL-*PTC3*. Cells were grown at 35.5°C on glucose-containing or galactose-containing plates for 3 days. (B) Suppression of the growth defect of *cak1-23* cells by deletion of *PTC2* and *PTC3*. Growth characteristics of FHE56 (*cak1-23*) and ACY171 (*cak1-23 Δptc2 Δptc3*) at a permissive temperature (23°C) and a semipermissive temperature (32°C). Cells were grown on YPD plates at 23°C for 2 days or at 32°C for 3 days.

Figure 6. Dephosphorylation of Cdc28p Thr-169 by Ptc2p and Ptc3p in vivo. The *CAK1* strain SY162 (Kaldis et al. 1996) was transformed with a high-copy plasmid (YEplac112GAL) containing no insert, *PTC2*, or *PTC3*. To monitor Cdc28p activity and the phosphorylation of Cdc28p in vivo, a low-copy plasmid containing *CDC28-HA* with its own promoter was also present. Cells were grown in raffinose-containing CM lacking uracil and tryptophan to early exponential phase, then shifted to either glucose-containing or galactose-containing medium for 16 hr. (A) The morphology of typical cells is shown using Nomarski optics. The overexpression of Ptc2p and Ptc3p induced elongated buds. (B) Cdc28p-HA was immunoprecipitated from cell extracts using the 12CA5 antibody and histone H1 kinase activities were analyzed directly (open bars) or after addition of MBP-Clb2p (solid bars). (C) Cdc28p-HA was immunoprecipitated from cell lysates using the 12CA5 antibody. Thr-169 phosphorylated Cdc28p and total Cdc28p-HA were detected by immunoblotting with antibodies specific for the Thr-169 phosphorylated form of Cdc28p (top) or with anti-PSTAIR antibodies specific for Cdc28p (bottom), respectively. Note that strain SY162 expresses *CAK1* from a plasmid, resulting in about one-third as much CAK activity as when *CAK1* is expressed from its normal chromosomal location (Kaldis et al. 1996). The *TRP1/cen-CDC28-HA* plasmid was obtained from Curt Wittenberg.

raised against a Thr-169 phosphorylated peptide, PLRAY(T^{PO4})HEIVT. The affinity-purified antibody specifically recognized phosphorylated Cdc28p rather than the unphosphorylated form as demonstrated by ELISA and immunoblotting (P. Kaldis, K. Ross, and M.J. Solomon, unpubl. data). Cdc28p-HA was immunoprecipitated from yeast extracts and the extent of Thr-169 phosphorylation was analyzed by immunoblotting analysis. Overexpression of Ptc2p and Ptc3p significantly reduced the phosphorylation of Thr-169 (Fig. 6C, top) but had no effect on the amount of Cdc28p (Fig. 6C, bottom) or Cak1p (data not shown) present. Thus, Ptc2p and Ptc3p can dephosphorylate Thr-169 in Cdc28p in vivo.

Dephosphorylation of Thr-160 in Cdk2 by a PP2C-like phosphatase activity in a HeLa cell extract

Because the basic cell cycle machinery is highly conserved among species, we characterized the phosphatase activities in a HeLa cell extract that could dephosphorylate Thr-160 of Cdk2 (Fig. 7). Cdk2 phosphatase activity was Mg²⁺ dependent and insensitive to inhibitors of PP1/PP2A/PP2B and of dual specificity/tyrosine phosphatases (Fig. 7A, top), indicating that the major Thr-160 phosphatase activity is a type 2C protein phosphatase. We quantitated the relative levels of Mg²⁺-dependent and Mg²⁺-independent Thr-160 phosphatase activity by performing a time-course assay using HeLa cell extracts (Fig. 7B,C). Note that much less HeLa cell extract was needed to show detectable phosphatase activity in the presence of Mg²⁺ than in its absence. On the basis of PhosphorImager analysis of the linear phases of these time courses (when <30% of the substrate had been dephosphorylated) we estimate that the PP2C-like (Mg²⁺-dependent) activity is ~120-fold greater than the Mg²⁺-independent Cdk2 phosphatase activity. Thus, as in yeast, PP2Cs appear to be the major phosphatases that dephosphorylate the activating phosphorylation site in human Cdk2.

Discussion

To identify the Cdc28p Thr-169 phosphatases, we performed a biochemical characterization of phosphatase activity in yeast extracts using ³²P-labeled Cdc28p as a substrate. PP2C-like enzymes, which require Mg²⁺ or Mn²⁺ for activity, were found to be responsible for the dephosphorylation of Cdc28p in yeast extracts. Two of the budding yeast PP2Cs, Ptc2p and Ptc3p, specifically dephosphorylated Thr-169 in vitro, and this dephosphorylation was inhibited by cyclin binding. Ptc2p and Ptc3p together accounted for ~90% of the Cdc28p phosphatase activity in yeast extracts. These phosphatases dephosphorylated Cdc28p in vivo, and their overexpression was toxic to a strain carrying a *cak1-22* mutation. Moreover, deletion of *PTC2* and *PTC3* rescued the growth defect of a *cak1-23* mutant at a semipermissive temperature. Thus, Ptc2p and Ptc3p act physiologically in opposition to Cak1p by dephosphorylating Thr-169 of

Figure 7. Thr-160 of Cdk2 is dephosphorylated by a PP2C-like activity in a HeLa cell extract. (A) Biochemical characterization of Cdk2 Thr-160 phosphatase activity in a HeLa cell extract. Fifty nanograms of ³²P-labeled Cdk2 was incubated with 2.5 μg of HeLa cell extract with or without 5 mM Mg²⁺ and Ca²⁺ at room temperature for 15 min. The following phosphatase inhibitors were added 10 min before assay, as indicated: 5 μM microcystin-LR (MC); 6 mM EDTA; 20 mM NaF; 1 mM Na₂WO₄; 1 mM Na₃VO₄; 10 mM sodium tartrate; 5 mM tetramisole (TMS). ³²P-labeled Cdk2 was detected as described in Fig. 1 for Cdc28p. (B) Comparison of Mg²⁺-dependent and Mg²⁺-independent phosphatase activity against Cdk2 in HeLa cell extract. Fifty nanograms of Cdk2 was incubated with 2.5 μg of cell extract with 5 mM Mg²⁺ or with 40 μg of cell extract with 1 mM EDTA. Reactions were stopped at the indicated times and analyzed by SDS-PAGE and autoradiography. (C) Quantification of B by Phosphor-Imager analysis. Note that the dephosphorylation reaction is within the linear range when <30% substrate has been used.

Cdc28p. Finally, PP2C-like enzymes were also found to be the predominant human phosphatases acting on Thr-160 of Cdk2 in a HeLa cell extract, indicating that the substrate specificity of PP2Cs against CDKs is evolutionarily conserved. Indeed, both Cak1p-labeled Cdk2 and Cdk6 are excellent substrates for recombinant human PP2Cα and PP2Cβ isoforms (A. Cheng and M.J. Solomon, in prep.). This work completes the identification of the basic enzymes controlling the major phosphorylations of CDKs: Wee1-like protein kinases and Cdc25 pro-

tein phosphatases add and remove phosphates at inhibitory phosphorylation sites, and CAK and PP2Cs do the same for the activating phosphorylation site.

PP2C-like enzymes have been implicated in negatively regulating stress-responsive protein kinase cascades in eukaryotic cells. In both budding yeast and fission yeast, genetic studies show that PP2C-like enzymes oppose the activation of the MAP kinase pathway that is activated in response to osmotic and heat shocks (Maeda et al. 1994; Shiozaki et al. 1994; Shiozaki and Russell 1995). In plants, a Ca²⁺-regulated PP2C is required for signal transduction by the plant hormone abscisic acid, leading to stomatal closure, seed dormancy, and growth inhibition (Leung et al. 1994; Meyer et al. 1994). The ability of PP2C enzymes to inhibit the stress-activated signal transduction cascade is evolutionarily conserved as human PP2Cα can reverse the activation of the p38 and JNK MAPKs induced by stresses and cytokines (Takekawa et al. 1998).

Human KAP has also been shown to dephosphorylate Thr-160 of Cdk2 (Poon and Hunter 1995). KAP is a dual specificity phosphatase that can interact with Cdc2, Cdk2, and Cdk3 (Gyuris et al. 1993; Harper et al. 1993; Hannon et al. 1994). KAP dephosphorylates human Cdk2 on Thr-160 and was found to be responsible for ~45% of such phosphatase activity in a HeLa cell extract (Poon and Hunter 1995). In contrast, our studies show that PP2C-like enzymes account for >99% of the phosphatase activity against human Cdk2 in a HeLa cell extract. This discrepancy is probably due to whether Mg²⁺-dependent or Mg²⁺-independent phosphatase activities were assayed: Mg²⁺ was included in the phosphatase reactions in this study, whereas the previous study used a Mg²⁺-free buffer (Poon and Hunter 1995) in which the total Thr-160 phosphatase activity was much lower due to the absence of PP2C activities. Although both phosphatases specifically reversed the activating phosphorylation in CDKs, KAP and PP2C might be regulated differently. Because KAP is expressed at the G₁/S transition, it is possible that KAP temporally or spatially dephosphorylates CDKs. On the other hand, the expression of PP2Cs is constitutive (Takekawa et al. 1998), indicating that PP2C-like enzymes are likely to dephosphorylate CDKs throughout the cell cycle.

CAK vs. PP2C

The balance between activating phosphorylation and dephosphorylation of CDKs appears to vary greatly between species. For example, studies from *S. pombe* and *Xenopus* show that the activating phosphorylation of Cdc2 is removed rapidly either during or at the end of mitosis (Gould et al. 1991; Lorca et al. 1992). In *S. cerevisiae*, however, Cdc28p remains largely phosphorylated at Thr-169 throughout the cell cycle (Morgan 1997; Espinoza et al. 1998; K. Ross, P. Kaldis, and M.J. Solomon, unpubl. data). This difference is likely due to the different substrate specificities of the respective CAKs and to the relative activities of CAK and PP2C in the different species: (1) Budding yeast Cak1p phosphorylates pref-

entially monomeric CDKs rather than cyclin-bound CDKs, whereas p40^{MO15}/cyclin H/MAT1 prefers cyclin-bound CDKs as substrates (Kaldis et al. 1998). However, PP2C dephosphorylates CDKs only upon cyclin degradation. (2) The CDK phosphatase activity in HeLa cell extracts is much higher than in budding yeast extracts (25 μ g yeast extract used in Fig. 1A vs. 2.5 μ g HeLa cell extract used in Fig. 7A). Therefore, Cak1p plays a dominant role in determining the phosphorylation of monomeric Cdc28p in budding yeast, whereas PP2Cs wins the battle in human cells.

PP2C substrate specificity: general T-loop phosphatases for CDKs and MAPKs?

Although genetic studies had provided some insights into the biological functions of PP2C, the identification of substrates of PP2C in vivo has been hindered by the absence of specific inhibitors. Nevertheless, PP2C exhibits a 20-fold preference for phosphothreonine-containing substrates compared with equivalent phosphoserine substrates in vitro (Donella Deana et al. 1990) and substrates of PP2C in vivo have been proposed to be phosphorylated on threonine residues (Das et al. 1996). Human PP2C α 2 was found to dephosphorylate a MAPK (p38) on a similar threonine residue in its activation loop (T-loop) (Takekawa et al. 1998). Recently, Ptc1 and Ptc3 in *S. pombe* were shown to dephosphorylate Thr-171 of the Spc1 protein kinase (a homolog of human p38) in its T-loop (Nguyen and Shiozaki 1999). Given that a number of PP2C substrates are CDKs and MAPKs, and that these kinases are typically regulated by activating phosphorylations on threonines within their T-loops, an interesting possibility is that many PP2C-like enzymes could be general T-loop phosphatases.

Materials and methods

Yeast strains and plasmids

The yeast strains used in this study are based on W303-1A: *MATa ade2-1 his3-11,15 leu2-3,112 can1-100 ura3-1 trp1-1 ssd1-d* (Rothstein 1991). The genotypes of the specific strains used in this study are as follows: ACY102, *ptc2::HIS3*; ACY103, *ptc3::HIS3*; ACY140, *ptc2::URA3 ptc3::HIS3*; ACY141, *ptc2::URA3 ptc3::HIS3 (PTC2 in YEplac112GAL)*; ACY142, *ptc2::URA3 ptc3::HIS3 (PTC2^{D234A} in YEplac112GAL)*; ACY143, *ptc2::URA3 ptc3::HIS3 (PTC3 in YEplac112GAL)*; ACY144, *ptc2::URA3 ptc3::HIS3 (PTC3^{D234A} in YEplac112GAL)*; ACY145, *ptc2::URA3 ptc3::HIS3 (YEplac112GAL)*; ACY150, *cak1::HIS3 (LEU/cen-cak1-22, YEplac195GAL)*; ACY152, *cak1::HIS3 (LEU/cen-cak1-22, PTC2 in YEplac195GAL)*; ACY153, *cak1::HIS3 (LEU/cen-cak1-22, PTC3 in YEplac195GAL)*; ACY160, *cak1::HIS3 (LEU/cen-CAK1, YEplac195GAL)*; ACY162, *cak1::HIS3 (LEU/cen-CAK1, PTC2 in YEplac195GAL)*; ACY163, *cak1::HIS3 (LEU/cen-CAK1, PTC3 in YEplac195GAL)*; ACY171, *cak1-23 ptc2::URA3 ptc3::HIS3*. FHE56, *cak1-23, SY143, cak1::HIS3 (LEU/cen-cak1-22)*, and SY162, *cak1::HIS3 (LEU/cen-CAK1)* were described previously (Kaldis et al. 1996; Espinoza et al. 1998).

YPD medium contained 1% yeast extract, 2% bacto-peptone,

and 2% glucose; YPGal contained 2% galactose instead of glucose; complete minimal (CM) medium included all essential amino acids, uracil, adenine, and 2% glucose or galactose (Ausubel et al. 1995).

The full-length coding regions of *PTC1*, *PTC2*, *PTC3*, *YBR125c*, *YCR079w*, and *YOR090c* were amplified from yeast genomic DNA by PCR, cloned, and sequenced. QuikChange mutagenesis (Stratagene) was used to create the Ptc2p^{D234A} and Ptc3p^{D234A} mutations. To overexpress PP2Cs in budding yeast, open reading frames were inserted into modified YEplac112 and YEplac195 vectors (YEplac112GAL and YEplac195GAL, respectively; Gietz and Sugino 1988; Kolman et al. 1992), in which protein expression is controlled by the galactose-inducible promoter. For disruption of *PTC1*, *PTC2*, *PTC3*, and *PTC4*, the coding regions of these genes from the ATG to 250 bp upstream of the stop codon were replaced with a *HIS3* fragment or a *URA3* fragment. For disruption of *PTC5*, a *SpeI*-*BglII* fragment of *PTC5* (from 140 to 470 bp of the coding region), was replaced with a 1.2-kbp fragment of *HIS3*. The *YCR079w* disruption plasmid was created by replacing a 1.0-kbp *NcoI*-*XcmI* fragment of *YCR079w* (from 200 to 1230 bp of the coding region) with an 0.9-kbp fragment of *TRP1*. Strains carrying null alleles of the PP2Cs were created by one-step gene disruption (Rothstein 1991) and confirmed by PCR.

Yeast extracts

For small-scale yeast extracts, cells were grown in 100 ml of medium at the indicated temperatures and harvested in exponential phase ($A_{600} = 0.3$ – 0.6) by centrifugation for 5 min at 5000g. The cell pellet (0.3–0.4 grams) was washed once with sterile water and resuspended in 1 ml of lysis buffer A, which contained 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5% glycerol, 2 mM DTT, and 1 $\times$ protease inhibitors (1 mM PMSF and 10 μ g/ml each of leupeptin, chymostatin, and pepstatin). Cell extract was prepared by bead beating as described previously (Kaldis et al. 1996). Typical extract concentrations were 4–6 mg/ml. HeLa 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 DTT, 1 $\times$ protease inhibitors] and clarified by centrifugation at 100,000g for 1 hr as described (Ausubel et al. 1995).

Protein expression and purification

To construct his₆-tagged Ptc1p and Ptc4p, the coding regions of Ptc1p and Ptc4p were inserted into the *Bam*HI-*Hind*III sites in pET28a (Novagen). Two-litre cultures in LB/Kan were grown at 37°C until the OD₆₀₀ reached 0.4. IPTG was added to 0.4 mM, and growth was continued for 3 hr at 23°C. Cells were harvested and his₆-tagged proteins were purified with metal affinity resin following the manufacturer's instructions (TALON, Clontech). The yields of recombinant Ptc1p and Ptc4p were ~2 mg/liter. The full-length coding regions of *PTC2*, *PTC3*, and *PTC5* were inserted into the *Bam*HI-*Hind*III sites in the pGEX-KG expression vector (Guan and Dixon 1991). The GST-KAP expression vector was kindly provided by R.Y.C. Poon. GST-cyclin A contained human cyclin A from amino acids 173–432 inserted into the *NcoI*-*SacI* sites of the pGEX-KG vector (Guan and Dixon 1991). GST-Ptc2p, GST-Ptc3p, GST-Ptc5p, GST-KAP, and GST-cyclin A were expressed and purified as described for GST-cyclin B (Solomon et al. 1990). The yields of these GST fusion proteins were ~1 mg/liter.

A yeast *pep4* strain with Cdc28p-his₆ under a *GAL* promoter on a high copy plasmid was provided by S.I. Reed (Scripps Research Institute, La Jolla, CA). Cdc28p-his₆ was purified using a

metal affinity resin following the manufacturer's instructions (TALON, Clontech). Other recombinant proteins were expressed in insect cells (Cdk2 and GST-Cak1p) and *E. coli* (MBP-Clb2p), and purified as described previously (Kaldis et al. 1996, 1998).

Protein phosphatase assays

Partially dephosphorylated and hydrolyzed casein (Sigma, C-4765) was phosphorylated using the cAMP-dependent protein kinase (Sigma, P-2645) as described (McGowan and Cohen 1988). ^{32}P -Labeled casein was purified with three rounds of TCA precipitation (final concentration 20%) and an acetone wash, and resuspended into storage buffer [100 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 0.1 mM EGTA, 150 mM NaCl, 2 mM DTT]. Casein phosphatase activity was measured in the presence of 20 mM Mg^{2+} or Mn^{2+} using an organic extraction method (Cheng et al. 1998).

For the preparation of ^{32}P -labeled CDK substrates, 2 μg of human Cdk2 or Cdc28p-his₆ was incubated with 60 ng of purified GST-Cak1p in the presence of 60 μCi [γ - ^{32}P]ATP, 30 μM ATP, and 10 mM MgCl_2 in buffer A [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM DTT, 1 mg/ml ovalbumin, 0.1% Tween 20, 1 $\times$ protease inhibitors] in a total volume of 30 μl . The reactions were terminated after 2 hr at room temperature by the addition of 30 μl of buffer B (buffer A containing 11 mM EDTA). The ^{32}P -labeled substrates were passed through a spin column (Chroma Spin-10, Clontech) pre-equilibrated with buffer C [20 mM Tris-HCl (pH 7.4), 100 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.1% Tween-20]. Glycerol was added to a final concentration of 20%. The labeled CDKs were stored at -20°C .

To measure CDK phosphatase activity, 50 ng of ^{32}P -labeled CDK was incubated with 25 μg of yeast extract, 2.5 or 40 μg of HeLa cell extract, or 0.1–5 μg of recombinant PP2Cs in buffer A (final volume 20 μl). Divalent metal ions or inhibitors were included as described in the text and figure legends. The reaction was terminated after 15 min at room temperature by the addition of 10 μl of 3 $\times$ SDS-PAGE sample buffer. Samples were separated in 10% SDS-PAGE, transferred to a PVDF membrane using a semi-dry blotting apparatus (TransBlot-SD, Bio-Rad), and analyzed by PhosphorImager (Molecular Imager GS-250, Bio-Rad) and autoradiography.

To quantitate the relative CDK phosphatase activity in yeast and HeLa cell extracts, the dephosphorylation reactions were performed as described above except that the reactions were terminated at different times (1, 2, 5, 10, 15 min). Samples were separated in 10% SDS-PAGE, the gels were fixed and dried, and the phosphorylation of the CDK was quantitated using a GS-250 Molecular Imager (Bio-Rad).

Histone H1 kinase assay

Cell lysates were prepared by bead beating in buffer D, which contained 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 40 mM EDTA, 1 mM EGTA, 20 mM NaF, 1 mM Na_3VO_4 , 5% glycerol, 2 mM DTT, 0.1% Triton X-100, and 1 $\times$ protease inhibitors. Cdc28p-HA was immunoprecipitated from 1 mg of yeast extract using 10 μg of 12CA5 antibody (Wilson et al. 1984) and 20 μl of protein A-agarose beads. Seven microliters of Cdc28p-HA beads was assayed for histone H1 kinase activity as described previously (Kaldis et al. 1996). The gels were fixed and dried, and the phosphorylation of histone H1 was quantitated using a GS-250 Molecular Imager (Bio-Rad). To assay phosphorylated Cdc28p indirectly, 10 μl of Cdc28p-HA bound to protein A beads was preincubated with 5 μg of MBP-Clb2p at room tem-

perature for 30 min, then assayed for histone H1 kinase activity as above.

Immunoblotting analysis

Samples were separated on a 10% SDS-PAGE gel and transferred to a PVDF membrane with a semi-dry transfer apparatus (Bio-Rad). After blocking overnight in Blotto (1 $\times$ TBST containing 5% nonfat dry milk), the membranes were incubated with an affinity-purified anti-phospho-T169 polyclonal antibody (1.5 $\mu\text{g}/\text{ml}$ in Blotto) or an anti-PSTAIR antibody (1:1000 in blotto), followed by HRP-conjugated goat anti-rabbit secondary antibody (Pierce, 1:2000 dilution in blotto). Signals were detected with SuperSignal ECL reagents (Pierce).

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