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Activating Phosphorylation of the *Saccharomyces cerevisiae* Cyclin-dependent Kinase, Cdc28p, Precedes Cyclin Binding

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Eukaryotic cell cycle progression is controlled by a family of protein kinases known as cyclin-dependent kinases (Cdks). Two steps are essential for Cdk activation: binding of a cyclin and phosphorylation on a conserved threonine residue by the Cdk-activating kinase (CAK). We have studied the interplay between these regulatory mechanisms during the activation of the major *Saccharomyces cerevisiae* Cdk, Cdc28p. We found that the majority of Cdc28p was phosphorylated on its activating threonine (Thr-169) throughout the cell cycle. The extent of Thr-169 phosphorylation was similar for monomeric Cdc28p and Cdc28p bound to cyclin. By varying the order of the addition of cyclin and Cak1p, we determined that Cdc28p was activated most efficiently when it was phosphorylated before cyclin binding. Furthermore, we found that a $Cdc28p^{T169A}$ mutant, which cannot be phosphorylated, bound cyclin less well than wild-type Cdc28p in vivo. These results suggest that unphosphorylated Cdc28p may be unable to bind tightly to cyclin. We propose that Cdc28p is normally phosphorylated by Cak1p before it binds cyclin. This activation pathway contrasts with that in higher eukaryotes, in which cyclin binding appears to precede activating phosphorylation.

INTRODUCTION

Cdc28p, the Cdk that controls all cell cycle transitions in *Saccharomyces cerevisiae*, is regulated by a number of mechanisms, including cyclin binding, binding by inhibitors, and inhibitory and stimulatory phosphorylations (reviewed by Mendenhall and Hodge, 1998). Activation of Cdc28p requires binding of a cyclin subunit and activating phosphorylation on Thr-169 by the Cdk-activating kinase Cak1p. At least nine different cyclins interact with Cdc28p: Cln1p–Cln3p, which are required for the G1/S transition (START); Clb5p and Clb6p, which act during S phase; and Clb1p–Clb4p, which regulate events in G2 and mitosis (reviewed by Nasmyth, 1993). Cyclin expression and stability are tightly regulated so that cyclin levels oscillate during the cell cycle (reviewed by Nasmyth, 1993). Cyclin synthesis and degradation depend, in many cases, on the activity of other Cdc28p/cyclin complexes, ensuring that the appropriate cyclins accumulate sequentially as the cell cycle progresses (Amon *et al.*, 1993, 1994; Stuart and Wittenberg, 1995; Blondel and Mann, 1996; Lanker *et al.*, 1996; Amon, 1997; Schneider *et al.*, 1998; Koepp *et al.*, 1999).

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Cak1p, the protein kinase that phosphorylates Cdc28p on Thr-169, is a distant member of the Cdk family (Espinoza *et al.*, 1996; Kaldis *et al.*, 1996b; Thuret *et al.*, 1996). Unlike most kinases in this family, Cak1p is active as a monomer and does not appear to be regulated during the vegetative cell cycle (reviewed by Kaldis, 1999). Cak1p protein levels and kinase activity are constant throughout the cell cycle (Espinoza *et al.*, 1996; Sutton and Freiman, 1997), Cak1p is not posttranslationally modified, and its localization does not change in a cell cycle–dependent manner (Kaldis *et al.*, 1998a). However, activating phosphorylation of Cdc28p might still be regulated by controlling the access of Cdc28p to Cak1p or by the opposing action of a Thr-169 phosphatase.

Although the regulation of Cdc28p by cyclins and Cak1p has been studied intensively, it is not clear how these two regulatory mechanisms are coordinated. Cdc28p could be phosphorylated by Cak1p and then bound by cyclin, or, alternatively, cyclin binding could occur before activating phosphorylation. Additionally, it is possible that the activation pathway is different for different cyclins. The existing data do not unequivocally support either pathway. Several studies suggest that monomeric Cdc28p is phosphorylated. Experiments in which Cdc28p is labeled in vivo with radioactive phosphate indicate that the overall phosphorylation level of Cdc28p is similar in cycling cells, which contain both

Table 1. Plasmids used in this study

Plasmid	Relevant characteristics	Source
CWB174	*CEN TRP1 CDC28(HA)*	C. Wittenberg
CWB186	*CEN TRP1 CDC28(HA)^{T169A}*	C. Wittenberg
PKB289	*CEN TRP1 cak1-22*	A. Sutton
PKB340	*2μ leu2d GAL-CDC28(his$_6$)*	S. Reed
PKB341	*CEN URA3 CDC28*	A. Sutton
CDC28-169-43244	*CEN TRP1 CDC28(HA)^{43244}*	Cross and Levine (1998)
pAG5	*CEN LEU2 GAL1-CLN2(MYC$_9$)*	F. Cross
pWS945	*CEN URA3 GAL10-CLB2(HA)*	Seufert *et al.* (1995)
pKR153	*CEN URA3 GAL10-CLB2(MYC$_9$)*	This work

monomeric Cdc28p and Cdc28p/cyclin complexes, and in cells arrested in G1 by the mating pheromone α-factor, which contain only monomeric Cdc28p (Hadwiger and Reed, 1988; Wittenberg and Reed, 1988). Furthermore, monomeric Cdks are much better substrates for Cak1p in vitro than Cdk/cyclin complexes (Kaldis *et al.*, 1998b). Although these studies show that Cdc28p can be phosphorylated as a monomer and that cells contain a pool of phosphorylated monomeric Cdc28p, they do not rule out the possibility that there may also be a pool of unphosphorylated Cdc28p that is preferentially targeted by cyclins. There is some evidence that G1 cyclins may selectively bind to unphosphorylated Cdc28p. A mutant form of Cdc28p in which Thr-169 is replaced by glutamate (Cdc28p^{T169E}) to mimic constitutive phosphorylation binds to Clb2p but is unable to bind to Cln2p (Lim *et al.*, 1996). The interpretation of this result is not straightforward, however, because mutation of additional sites in Cdc28p^{T169E} allowed the protein to bind to both G1 and mitotic cyclins and to be active in the absence of Cak1p phosphorylation (Cross and Levine, 1998). This mutant supported cell growth, demonstrating that reversible phosphorylation of Thr-169 does not seem to be an absolute requirement for cell cycle progression.

Because many of these studies provided indirect evidence or used Cdks from organisms other than budding yeast, we decided to reexamine the Cdc28p activation pathway. In this paper, we report the results of two approaches. We developed an antibody specific for the Thr-169–phosphorylated form of Cdc28p and determined the level of Thr-169 phosphorylation during the cell cycle, and we used an in vitro system to study whether Cdc28p activation depends on the order of cyclin binding and Thr-169 phosphorylation.

MATERIALS AND METHODS

Yeast Strains and Media

Plasmids and genotypes of strains are listed in Tables 1 and 2. To isolate PKB340, DNA was prepared from a 5-ml culture of strain PKY171 by vortexing with glass beads in phenol:chloroform: isoamyl alcohol (25:24:1, vol/vol) and introduced into *Escherichia coli* by transformation (Ausubel *et al.*, 1995). Strain SY89 was transformed with PKB289, grown on 5-FOA to select against the 2μm *URA3 CAK1* plasmid, and then transformed with PKB340 to create YKR101. The Clb2p-MYC$_9$ expression plasmid was created by removing a *Not*I fragment encoding an HA$_3$ tag from plasmid pWS945 and replacing it with a *Not*I fragment encoding a MYC$_9$ tag obtained from plasmid pAG5.

Yeast media (YPD, complete minimal (CM), 5-FOA) were prepared as described (Ausubel *et al.*, 1995). For cell cycle arrests, YKR106 was grown to an OD$_{600}$ of 0.4–0.8 in CM/glucose, collected

Table 2. Strains used in this study

Strain	Relevant genotype	Source
W303-1A[a]	*MATa ade2-1 his3-11, 15 leu2-3,112 can1-100 ura3-1 trp1-1 ssd1-d*	Rothstein (1991)
1834-2A	*cak1::LEU2 cdc28::HIS3* [CDC28-169-43244]	Cross and Levine (1998)
1834-1B	*cdc28::HIS3* [CDC28-169-43244]	Cross and Levine (1998)
SY89	*cak1::HIS3 [2μm URA3 CAK1]*	Kaldis *et al.* (1996b)
PKY150	*cdc28::HIS3* [CWB174]	This work
PKY171	*pep4* [PKB340]	S. Reed
YKR101	*cak1::HIS3* [PKB289, PKB340]	This work
YKR102	[CWB174]	This work
YKR103	[CWB186]	This work
YKR104	1834-2A [PKB341]	This work
YKR105	1834-1B [PKB341]	This work
YKR106	PKY150 *bar1*Δ	This work
YKR107	[CWB174, pAG5]	This work
YKR108	[CWB174, pKR153]	This work
YKR109	[CWB186, pKR153]	This work

[a] All other strains except for PKY171 are derived from W303-1A.

by vacuum filtration with the use of a 0.45-μm cellulose acetate filter system (Corning, Corning, NY), resuspended at an OD_{600} of 0.2–0.3 in YPD containing 50 ng/ml α-factor (Sigma, St. Louis, MO), 50 μg/ml benomyl (DuPont, Wilmington, DE), or 400 mM hydroxyurea (Sigma), and incubated for 3–4 h at 30°C.

Yeast Extracts

Arrested cells or exponential phase cells (OD_{600} = 0.3–0.8) were collected by centrifugation for 5 min at 2000 × *g* in a Sorvall (Newtown, CT) HB-6 or GS-3 rotor. Cells (0.2–0.3 g wet weight) were resuspended in 1 ml of buffer A (20 mM Tris-HCl, pH 7.9, 100 mM NaCl, 10 mM $MgCl_2$, 40 mM EDTA, 5% glycerol, 1 mM DTT, 1 mM PMSF, 1× protease inhibitors [10 μg/ml each leupeptin, chymostatin, and pepstatin {Chemicon, Temecula, CA}], 1 mM NaF, 0.1 mM Na_3VO_4). Glass beads (0.8 g of 0.5-mm beads; Biospec Products, Bartlesville, OK) were added, and cells were disrupted by bead beating (Klekamp and Weil, 1982) for 5 × 1 min at the highest setting in a Mini-Beadbeater-8 (Biospec Products) with 2 min of cooling in an ice water–NaCl bath (−5°C) after each round of bead beating. The crude extracts were centrifuged for 10 min at 15,000 × *g* in a microfuge at 4°C, and the supernatant was clarified for 15 min at 70,000 rpm in a TLA100.2 or TLA100.3 rotor in a Beckman (Fullerton, CA) Optima ultracentrifuge at 4°C. Extracts were frozen in liquid nitrogen and stored at −80°C. Protein concentrations, as determined by Bradford assay (Bio-Rad, Richmond, CA), ranged from 3.0 to 8.5 mg/ml.

Gel Filtration

Extracts were fractionated by gel filtration with the use of a Superdex 200 HR 10/30 column (Pharmacia, Piscataway, NJ) at a flow rate of 0.5 ml/min on a fast-protein liquid chromatography system as described (Kaldis *et al.*, 1996b, 1998a). Extract protein (4 mg) was passed through a 0.2-μm filter, loaded on the column, and run in buffer B (25 mM HEPES, pH 7.4, 100 mM NaCl, 10 mM EDTA, 1× protease inhibitors). One-milliliter fractions were collected.

Antibodies, Immunoblotting, and Immunoprecipitations

To raise the anti-phospho-Thr-169 (α-P-Thr-169) antibody, a 12-amino acid synthetic peptide derived from Cdc28p with a phosphothreonine at position 169 [CPLRAY(PO_4-T)HEIVT] was coupled to Keyhole Limpet Hemocyanine (Pierce, Rockford, IL) and used to immunize rabbits, as described previously (Schneider *et al.*, 1983; Kaldis *et al.*, 1996a). The serum was affinity purified against the phosphopeptide coupled to Sulfo-Link Coupling Gel (Pierce). The final concentration of purified immunoglobulin G (IgG) was 0.77 mg/ml. Immunoblots for Thr-169–phosphorylated Cdc28p were made with the use of α-P-Thr-169 (1.5 μg/ml), mouse anti-rabbit IgG (0.5 μg/ml; Pierce), and HRP-conjugated goat anti-mouse IgG (0.4 μg/ml; Pierce). For detection of total Cdc28p, affinity-purified α-PSTAIR antibody (0.5 μg/ml; Solomon *et al.*, 1990) and HRP-conjugated goat anti-rabbit IgG (0.4 μg/ml; Pierce) were used. For detection of influenza hemagglutinin (HA)-tagged proteins, 12CA5 antibody (0.5–1 μg/ml; Wilson *et al.*, 1984) and HRP-conjugated goat anti-mouse IgG (0.16–0.4 μg/ml) or affinity-purified rabbit α-HA antibody (0.1 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA) and HRP-conjugated goat anti-rabbit IgG (0.4 μg/ml) were used. To detect MYC-tagged proteins, protein A–purified 9E10 (5 μg/ml; a kind gift of J. Sedivy, Brown University, Providence, RI) and HRP-conjugated goat anti-mouse IgG (0.16 μg/ml) were used. All antibodies were diluted in buffer C (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween, 5% nonfat milk). Immunoblots were developed with the use of chemiluminescence reagents (SuperSignal ECL, Pierce). Blots were stripped by incubating for 30 min at 50°C in buffer D (62.5 mM Tris-HCl, pH 6.7, 100 mM 2-mercaptoethanol, 2% SDS) followed by two 10-min washes in buffer C without milk.

To immunoprecipitate Cdc28p-HA from gel filtration fractions, 1 ml of protein A–agarose beads (Life Technologies, Grand Island, NY) was preincubated with 100 μg of 12CA5 ascites fluid in buffer E (10 mM Na_2HPO_4, pH 7.4, 2 mM KH_2PO_4, 137 mM NaCl, 3 mM KCl, 0.5% NP-40, 1× protease inhibitors) for 1–2 h at 4°C. The beads were washed four times with buffer E, and 70 μl was incubated with 400 μl of gel filtration fraction (diluted to 800 μl with buffer E containing 40 mM EDTA) for 2 h at 4°C. Beads were washed four times with buffer E containing 40 mM EDTA and four times with buffer E without EDTA or NP-40.

To immunoprecipitate Clb2p-MYC_9, strains YKR108 and YKR109 were grown to an OD_{600} of 0.2–0.4 in CM/galactose. YKR107 was grown in CM/glucose as a "no-MYC" control. Extracts were prepared as described above except that buffer A contained only 1 mM EDTA and no DTT. Protein A–agarose beads (500 μl) were preincubated with 500 μg of protein A–purified 9E10 in buffer E for 2 h at 4°C. The beads were washed four times with buffer E, and 70 μl was incubated with 1 ml of extract (diluted to 1 mg/ml with buffer E) for 2 h at 4°C. The beads were then washed four times with 1 ml of buffer E and four times with 1 ml of buffer E without NP-40. Protein was eluted from the beads by incubation in 2× SDS-PAGE sample buffer lacking DTT at 65°C for 10 min and analyzed with SDS-PAGE.

Preparation of Recombinant Proteins

MBP-Clb2p (the construct was a kind gift of M. Olson and R. Deshaies, California Institute of Technology, Pasadena, CA) and MBP-Cln2p (the construct was a kind gift of E. Egan, Yale University, New Haven, CT) were expressed in *E. coli* and purified with the use of amylose resin according to the manufacturer's instructions (New England Biolabs, Beverly, MA). Approximately 0.5 mg of MBP-Clb2p was obtained from 1 l of culture, and ~0.5 mg of MBP-Cln2p was obtained from 500 ml of culture.

Cdc28p-HA was expressed in Hi5 insect cells. After 2 d of infection with a Cdc28p-HA recombinant baculovirus (a kind gift of J.W. Harper, Baylor College of Medicine, Houston, TX), cells were collected from nine 75-mm^2 culture flasks, harvested by centrifugation at 1000 × *g* for 4 min, resuspended in 4 ml of buffer F (10 mM HEPES, pH 7.4, 10 mM NaCl, 5 mM EDTA, 1× protease inhibitors, 0.1% Tween), and incubated on ice for 15 min. Buffer G (10 mM HEPES, pH 7.4, 900 mM NaCl, 5 mM EDTA; total, 450 μl) was added, and the lysate was centrifuged for 20 min at 10,000 × *g* at 4°C. The supernatant was diluted 1:2 with buffer H (10 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM EDTA, 1× protease inhibitors, 0.5% NP-40). For immunoprecipitation of Cdc28p-HA, 1 ml of protein A–agarose was incubated for 6 h with 200 μg of 12CA5 antibody in 10 ml of buffer H at 4°C. The beads were washed with buffer H, added to the diluted extract, and incubated for 3 h at 4°C. The beads were then washed four times with buffer H and four times with buffer H with no NP-40, divided into aliquots, frozen in liquid nitrogen, and stored at −80°C. The yield was ~1 ng of Cdc28p-HA per microliter of beads.

To prepare unphosphorylated Cdc28p-his_6, 1 l of YKR101 was grown to an OD_{600} of 0.5 in CM/raffinose-LEU at 23°C. Thirty percent galactose was added to a final concentration of 2%, and the culture was shifted to 37°C for 8 h to induce expression of Cdc28p-his_6 and to inactivate Cak1p. Cells were harvested, and Cdc28p-his_6 was purified with the use of Talon metal affinity resin (Clontech, Palo Alto, CA) according to the manufacturer's instructions. Fractions containing the protein were further purified on a gel filtration column. Fractions 16 and 17 from the gel filtration column containing the monomeric Cdc28p-his_6 were pooled and concentrated with the use of Centricon-10 cells (Amicon, Beverly, MA). Approximately 5 μg of Cdc28p-his_6 was obtained. GST-Ptc2p and GST-Ptc3p were purified as described and were a kind gift of A. Cheng (Yale University, New Haven, CT) (Cheng *et al.*, 1999)

S. cerevisiae Cks1p (a kind gift of E. Egan) was expressed in *E. coli* from plasmid pRK171 (Tang and Reed, 1993). One liter of cells was

grown to an OD_{600} of 0.54, and protein expression was induced with 0.4 mM isopropylthio-β-galactoside for 2.5 h at 37°C. Cells were harvested, washed, and resuspended in 10 ml of buffer I (10 mM Na_2HPO_4, pH 7.4, 2 mM KH_2PO_4, 137 mM NaCl, 3 mM KCl, 10 mM DTT, 1× protease inhibitors). The suspension was sonicated twice for 30 s with the use of a microtip at its maximum setting. The crude lysate was boiled for 5 min and then clarified by ultracentrifugation for 30 min at 40,000 rpm in a Beckman 60 Ti rotor. Ammonium sulfate was added to 28% saturation. The extract was rotated for 30 min at 4°C and then centrifuged for 10 min at 15,000 × *g* in a Sorvall SA-600 rotor. The pellet was resuspended in 10 ml of buffer J (50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 100 mM NaCl) and dialyzed against 500 ml of the same buffer overnight at 4°C in Spectrapor dialysis tubing with a molecular weight cutoff of 3500. The dialyzed protein was centrifuged for 10 min at 15,000 × *g* in a Sorvall SA-600 rotor to remove insoluble material and was then divided into aliquots, frozen in liquid nitrogen, and stored at −80°C. The yield was ~20 mg of Cks1p.

GST-Cdc28p was expressed in *E. coli* and purified as described (Kaldis *et al.*, 1996b). GST-Cak1p was expressed in insect cells.

Phosphorylation and Phosphatase Treatment of Cdc28p-his$_6$

To demonstrate the specificity of the α-P-Thr-169 antibody (see Figure 1), Cdc28p-his_6 was either phosphorylated in vitro with GST-Cak1p or treated with phosphatase to reverse any phosphorylation that occurred during expression. For the phosphorylation reaction, 1 μg of Cdc28p-his_6 was incubated in a 40-μl reaction with 2.4 μg of GST-Cak1p and 500 μM ATP in buffer K (50 mM Tris-HCl, pH 8.0, 15 mM $MgCl_2$, 1 mM DTT, 1× protease inhibitors, 1 mg/ml ovalbumin). To dephosphorylate Cdc28p-his_6, 1 μg was incubated in a 40-μl reaction with 20 mM $MgCl_2$, 5 μg of GST-Ptc2p, and 5 μg of GST-Ptc3p in buffer L (20 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 0.1 mM EGTA, 20 mM $MgCl_2$, 1 mM DTT, 1× protease inhibitors, 1 mg/ml ovalbumin, 0.1% Tween). Both reactions proceeded for 2.5 h at room temperature and were stopped with 40 μl of 5× SDS-PAGE buffer.

To measure Cdc28p-his_6 phosphorylation in the presence of cyclin (see Figure 6B), 50 ng of Cdc28p-his_6 in 5 μl of buffer K was incubated with 5 μl of buffer M (10 mM sodium phosphate, pH 7.2, 500 mM NaCl, 1 mM EGTA, 1 mM DTT, 1× protease inhibitors, 10 mM maltose) containing various amounts of MBP-Clb2p (molar ratio of MBP-Clb2p to Cdc28p-his_6 ranged from 0 to 43) for 30 min at room temperature. Ten microliters of a mixture containing 7.5 ng of GST-Cak1p, 10 mM $MgCl_2$, 500 μM ATP, and 5 μCi of [γ-^{32}P]ATP in buffer K was added, and incubation was continued for 30 min at room temperature. Reactions were stopped by the addition of 10 μl of SDS-PAGE sample buffer and analyzed by SDS-PAGE and phosphorimaging.

To phosphorylate Cdc28p-HA produced in insect cells (see Figure 4A), 50 μl of Cdc28p-HA beads were incubated in a 30-μl reaction with 1.8 μg of GST-Cak1p and 500 μM ATP in buffer K for 2.5 h at room temperature.

Quantitation of Cdc28p-his$_6$ Phosphorylation

Three micrograms of Cdc28p-his_6 was phosphorylated in a 40-μl reaction with 2.4 μg of GST-Cak1p, 500 μM ATP, and 20 μCi of [γ-^{32}P]ATP in buffer K for 2.5 h at room temperature. The reaction was stopped by the addition of 20 μl of 5× SDS-PAGE buffer. Thirty microliters of the reaction mixture was run on SDS-PAGE alongside BSA standards of known concentrations. The gel was stained with Coomassie blue and dried, and the Cdc28p-his_6 band was phosphorimaged (Molecular Imager GS-250, Bio-Rad). The amount of phosphorylated Cdc28p-his_6 was calculated from the phosphorimager exposure, and the total Cdc28p-his_6 present was estimated by comparing the Coomassie blue–stained band with the BSA standards. The level of phosphorylation was ~90%. The remaining Cdc28p-his_6 from this reaction was run alongside dilutions of fraction 16 of the α-factor–arrested extract and blotted with α-P-Thr-169 and α-PSTAIR to determine the level of Cdc28p-HA phosphorylation in the yeast extract (see Figure 2B).

H1 Kinase Assays

To assay the histone H1 kinase activity of gel filtration fractions, immunoprecipitated Cdc28p-HA bound to protein A–agarose beads was divided into four 10-μl aliquots (aliquots 1–4). Aliquots 1 and 2 were incubated in 10 μl of "no-CAK" mix (1× ATP regenerating system [50 μg/ml creatine kinase, 35 mM phosphocreatine, 1 mM ATP, 2 mM HEPES, pH 7.2, 1 mM MgOAc], 500 μM ATP in buffer K), and aliquots 3 and 4 were incubated in 10 μl of "plus-CAK" mix (1× ATP regenerating system, 500 μM ATP, 50 ng of GST-Cak1p in buffer K) for 30 min at room temperature. Five microliters of buffer K (aliquots 1 and 3) or 5 μl of "Clb" mix (1 μg of MBP-Clb2p in buffer K) (aliquots 2 and 4) was added, and incubation was continued for another 30 min. Nine microliters of H1 mix (1 mM ATP, 10 mM $MgCl_2$, 2.25 μCi of [γ-^{32}P]ATP, 267 μg/ml histone H1 [stored in 0.2 M NaCl, 0.1 mM EDTA, 20 mM Tris-HCl, pH 7.4, 1 mM DTT, 1× protease inhibitors]) was added, and reactions were incubated for 30 min and stopped by the addition of 15 μl of 5× SDS-PAGE buffer.

In Vitro Phosphorylation and Activation of Cdc28p

For in vitro activation of baculovirus Cdc28p-HA (see Figure 4B), 5 μl of Cdc28p-HA beads was incubated in 10 μl of buffer K containing 500 μM ATP, 5 mM $MgCl_2$, with or without 100 nM Cks1p, with or without 9 ng of GST-Cak1p, and with 7.5 ng of MBP-Clb2p, 1.5 μg of MBP-Cln2p, or buffer for 30 min at room temperature. Beads were washed five times with 200 μl of buffer K and resuspended in 10 μl of buffer K. Six microliters of H1 mix (see above) was added, beads were incubated for 30 min at room temperature, and reactions were stopped by the addition of 10 μl of 5× SDS-PAGE sample buffer.

For order-of-addition experiments (see Figure 5), 10 μl of baculovirus Cdc28p-HA beads was incubated with 20 μl of buffer K containing 100 nM Cks1p, 500 μM ATP, 5 mM $MgCl_2$, with or without 12 ng of GST-Cak1p, and with 40 ng of MBP-Clb2p, 3 μg of MBP-Cln2p, or buffer M for 30 min at room temperature (first incubation). Beads were washed and incubated in the same way as for the first incubation (second incubation). Beads were washed again and assayed for H1 kinase activity.

To assay Cdc28p-HA phosphorylation (see Figure 6A), 10 μl of Cdc28p-HA beads was incubated in 20 μl of buffer K containing 100 nM Cks1p, 500 μM ATP, 5 mM $MgCl_2$, and either 40 ng of MBP-Clb2p, 3 μg of MBP-Cln2p, or buffer M for 30 min at room temperature. Five microliters of beads was incubated in 10 μl of buffer K containing 500 μM ATP, 10 μCi of [γ-^{32}P]ATP, 100 nM Cks1p, 5 mM $MgCl_2$, with or without 12 ng of GST-Cak1p for 30 min. Beads were pelleted, the mix was removed, and the beads were resuspended in 25 μl of 2.5× SDS-PAGE sample buffer.

To determine the effect of washing on cyclin binding (see Figure 6C), 10 μl of Cdc28p-HA beads was incubated in 20 μl of buffer K containing 100 nM Cks1p, 500 μM ATP, 5 mM $MgCl_2$, and either 40 ng MBP-Clb2p, 3 μg of MBP-Cln2p, or buffer M for 30 min at room temperature. Half of the reactions were washed and resuspended in 14 μl of buffer K containing 100 nM Cks1p, 500 μM ATP, 5 mM $MgCl_2$, and 6 μl of buffer M (final volume was 20 μl). Five microliters of beads from each reaction was then incubated in 10 μl of buffer K containing 500 μM ATP, 100 nM Cks1p, 5 mM $MgCl_2$, with or without 12 ng of GST-Cak1p. Beads were washed and H1 kinase assays were performed as described above.

For all of the baculovirus Cdc28p-HA activation experiments, GST-Cak1p and Cks1p were diluted in buffer K, and buffer K was used in place of these proteins in reactions in which they were omitted. MBP-Clb2p and MBP-Cln2p were diluted in buffer M, and buffer M was used in place of cyclin when it was omitted.

RESULTS

Detection of the Thr-169–phosphorylated Form of Cdc28p

To examine the phosphorylation of the activating threonine of Cdc28p (Thr-169) during the cell cycle, we raised a peptide antibody (α-P-Thr-169) against the Thr-169–phosphorylated form of Cdc28p. The α-P-Thr-169 antibody could detect as little as 3.7 ng of Cdc28p-his$_6$ phosphorylated in vitro by GST-Cak1p while not reacting with 300 ng of unphosphorylated Cdc28p-his$_6$ (Figure 1A, top). The membrane was reprobed with an α-PSTAIR antibody, which reacts with all forms of Cdc28p, to demonstrate that equivalent amounts of phosphorylated and unphosphorylated protein were loaded on the gel (Figure 1A, bottom). As a further negative control for the antibody, we used bacterially produced GST-Cdc28p. Up to 2 μg of this unphosphorylated GST-Cdc28p was still undetectable by the α-P-Thr-169 antibody (Figure 1A), indicating that the antibody is highly specific for the Thr-169–phosphorylated form of Cdc28p. The α-P-Thr-169 antibody also recognized recombinant human Cdk2 phosphorylated on its activating threonine but not phosphorylated human Cdk6 (P. Kaldis and M.J. Solomon, unpublished observations).

To further test the antibody, we used yeast strains that expressed both endogenous Cdc28p and either Cdc28p-HA or Cdc28p^{T169A}-HA, in which the activating threonine had been mutated to an alanine. The untagged and HA-tagged forms were distinguishable on SDS-PAGE due to their different sizes. The α-PSTAIR antibody (Figure 1B, top) recognized the endogenous and HA-tagged forms of Cdc28p in both strains, whereas the α-HA antibody (Figure 1B, middle) recognized only the more slowly migrating HA-tagged forms. The α-P-Thr-169 antibody (Figure 1B, bottom) recognized endogenous Cdc28p in both strains and the wild-type HA-tagged Cdc28p but did not react with Cdc28p^{T169A}-HA. These results demonstrate that the α-P-Thr-169 antibody can specifically recognize phosphorylated Cdc28p in yeast extracts.

We also tested the antibody on an extract that contained otherwise wild-type unphosphorylated Cdc28p. We used a strain that was deleted for *CAK1* (Figure 1C, Δ*cak1*) so that the endogenous Cdc28p was entirely unphosphorylated on Thr-169. Normally, deletion of *CAK1* is lethal; however, this strain was kept alive by a mutant form of Cdc28p with a threonine-to-glutamate mutation at position 169 (T169E) and a number of other point mutations that allow it to function in the absence of activating phosphorylation (Cdc28p^{43244}-HA; Cross and Levine, 1998). Because of the HA tag on Cdc28p^{43244}-HA, it could be distinguished from the untagged wild-type Cdc28p, which was also present in the cell. As a control, we used a strain that was identical except that it expressed Cak1p (Figure 1C, *CAK1*). Total extracts were loaded onto a gel filtration column, and fractions 16 and 17, which contained the majority of the Cdc28p, were immunoblotted with the α-P-Thr-169 and α-PSTAIR antibodies. The α-PSTAIR antibody (Figure 1C, top) recognized both forms of Cdc28p in both strains. The T169E mutant, despite functioning like a constitutively phosphorylated form of Cdc28p, was not recognized by the α-P-Thr-169 antibody (Figure 1C, bottom). Most importantly, the α-P-Thr-169 antibody recognized wild-type Cdc28p in the *CAK1* strain in which Thr-169 was presumably phosphorylated but did not recognize the unphosphorylated wild-type Cdc28p in the Δ*cak1* strain. Therefore, the α-P-Thr-169 antibody reacts specifically with Thr-169–phosphorylated Cdc28p in yeast extracts.

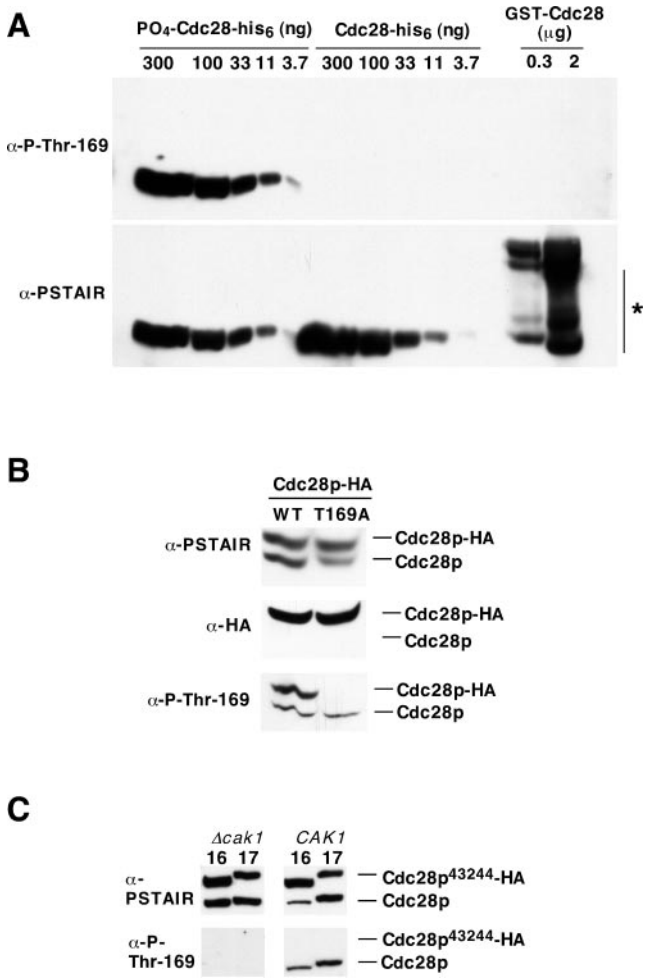

Figure 1. The α-P-Thr-169 antibody is specific for the Thr-169–phosphorylated form of Cdc28p. (A) Cdc28p-his$_6$ purified from YKR101 was phosphorylated in vitro by GST-Cak1p (PO$_4$-Cdc28p-his$_6$) or treated with GST-Ptc2p and GST-Ptc3p (Cdc28p-his$_6$). The indicated amounts of PO$_4$-Cdc28p-his$_6$, Cdc28p-his$_6$, and unphosphorylated GST-Cdc28p purified from *E. coli* were immunoblotted with the α-P-Thr-169 antibody. The membrane was then stripped and reprobed with an α-PSTAIR antibody to detect total Cdc28p. The asterisk (*) denotes degradation products of GST-Cdc28p. (B) Extracts (20 μg of protein) from strains YKR102 (Cdc28p-HA; WT) and YKR103 (Cdc28p^{T169A}-HA; T169A) were immunoblotted with the α-PSTAIR, α-HA, and α-P-Thr-169 antibodies. Note that both strains contain endogenous Cdc28p as well as the indicated form of HA-tagged Cdc28p. (C) Extracts from strains YKR104 (Δ*cak1*) and YKR105 (*CAK1*) were fractionated on a Superdex 200 column. Fractions 16 and 17, which contained the monomeric Cdc28p, were immunoblotted with the α-PSTAIR and α-P-Thr-169 antibodies. Cdc28p^{43244}-HA is a mutant form of Cdc28p that can function in the absence of activating phosphorylation (Cross and Levine, 1998).

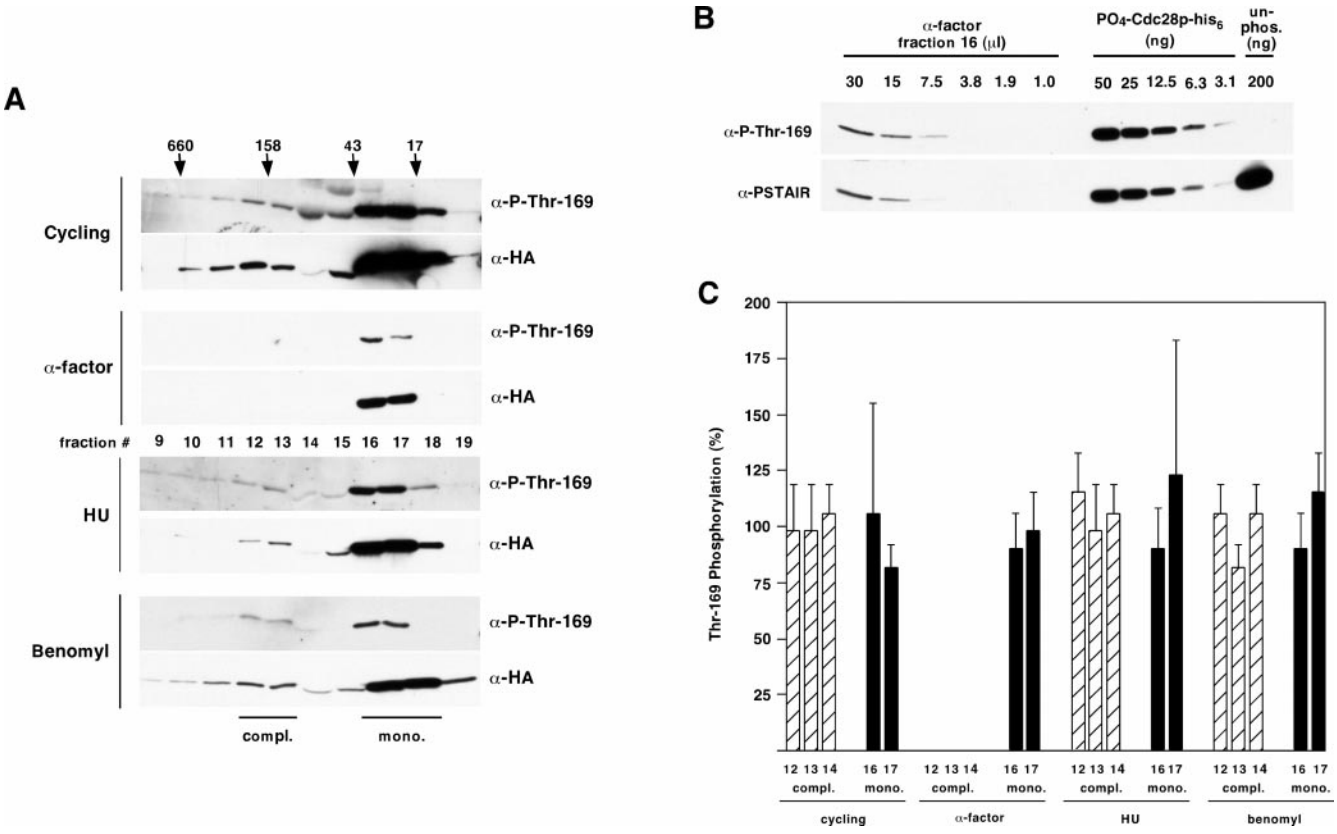

Figure 2. The majority of Cdc28p-HA is phosphorylated throughout the cell cycle. (A) Extracts were prepared from asynchronous YKR106 cells (cycling) and YKR106 cells arrested with α-factor, HU, or benomyl and gel filtered on a Superdex 200 column. Fractions 9–19 were immunoblotted with the α-P-Thr-169 and α-HA antibodies. The peaks of Cdc28p-HA complexes (compl.) and monomer (mono.) are indicated. Gel filtration standards included bovine thyroglobulin (660 kDa), bovine gamma globulin (158 kDa), chicken ovalbumin (43 kDa), and horse myoglobin (17 kDa). (B) To determine the absolute level of phosphorylation of Cdc28p-HA in fraction 16 of the α-factor–arrested extract, dilutions of this fraction were run alongside the indicated amounts of phosphorylated Cdc28p-his$_6$ (PO_4-Cdc28p-his$_6$) and immunoblotted with the α-P-Thr-169 antibody. The membrane was stripped and reprobed with the α-PSTAIR antibody. Unphosphorylated Cdc28p-his$_6$ (200 ng; unphos.) was included to demonstrate the specificity of the α-P-Thr-169 antibody. The PO_4-Cdc28p-his$_6$ was 90% phosphorylated (see MATERIALS AND METHODS). (C) The relative levels of Cdc28p-HA phosphorylation in the cycling and α-factor–, HU-, and benomyl-arrested extracts were determined by running serial dilutions of fractions containing Cdc28p-HA complexes (12, 13, and 14 compl.) and monomer (16 and 17 mono.) alongside each other, immunoblotting with the α-P-Thr-169 antibody, stripping, and reprobing with the α-PSTAIR antibody to detect total Cdc28p-HA. Relative phosphorylation was converted to an absolute percentage of phosphorylation by comparison with fraction 16 of the α-factor–arrested extract. Results are averages of two to five repeats for each fraction. Error bars represent SDs.

Cdc28p Phosphorylation during the Cell Cycle

We next determined whether the level of Thr-169 phosphorylation varied during the cell cycle and whether the phosphorylation of monomeric Cdc28p differed from that of active Cdc28p complexes. Using a strain that expressed Cdc28p-HA as its only form of Cdc28p, we prepared extracts from cycling cells and from cells arrested in G1 by the mating pheromone α-factor, in S phase by the DNA replication inhibitor hydroxyurea (HU), and in mitosis by the spindle-depolymerizing agent benomyl (see MATERIALS AND METHODS). We added 40 mM EDTA to the lysis buffer to inhibit phosphorylation of Cdc28p by Cak1p and its dephosphorylation by the Cdc28p phosphatases Ptc2p and Ptc3p (Cheng *et al.*, 1999) during extract preparation. To separate monomeric Cdc28p from its cyclin-bound form, we fractionated the extracts on a Superdex 200 column. Fractions 9–19 were immunoblotted with the α-P-Thr-169 antibody to determine the level of Thr-169–phosphorylated Cdc28p-HA and with the α-HA antibody to determine the total amount of Cdc28p-HA present (Figure 2A). Cycling, HU-arrested, and benomyl-arrested extracts had similar distributions of Cdc28p-HA. The majority of Cdc28p-HA was monomeric and eluted in fractions 16 and 17. There was also a second peak of Cdc28p complexes in fractions 12–14. These results agree with a previous gel filtration study of the distribution of Cdc28p (Wittenberg and Reed, 1988). In the α-factor–arrested extract, all of the Cdc28p-HA was monomeric, consistent with the fact that G1-arrested cells contain very little cyclin (Amon *et al.*, 1994). In all four extracts, the α-P-Thr-169 and the α-HA immunoblots looked qualitatively similar. This finding suggested that the proportion of Cdc28p-HA phosphorylated on Thr-169 was similar

throughout the cell cycle and did not change when Cdc28p-HA was incorporated into complexes.

To quantitate the level of Cdc28p-HA phosphorylation in the gel filtration fractions, we first compared the level of Cdc28p-HA phosphorylation in fraction 16 of the α-factor–arrested extract with that of recombinant Cdc28p-his$_6$ that had been 90% phosphorylated by GST-Cak1p in vitro (see MATERIALS AND METHODS; Figure 2B). We immunoblotted serial dilutions of fraction 16 and Cdc28p-his$_6$ with α-P-Thr-169 and α-PSTAIR to determine the levels of phosphorylated and total Cdc28p, respectively. For both fraction 16 and Cdc28p-his$_6$, the ratio of Thr-169–phosphorylated Cdc28p (α-P-Thr-169 signal) to total Cdc28p (α-PSTAIR signal) was the same, indicating that the Cdc28p-HA in fraction 16 was also ~90% phosphorylated. Next, we immunoblotted fractions 12–14 (complexes peak) and fractions 16 and 17 (monomeric peak) from the four extracts with the α-P-Thr-169 and α-PSTAIR antibodies and estimated the level of Cdc28p-HA phosphorylation relative to that of fraction 16 of the α-factor–arrested extract. Using the fact that the Cdc28p-HA in fraction 16 was 90% phosphorylated, we then computed the absolute level of Cdc28p-HA phosphorylation in each fraction (Figure 2C). Cdc28p-HA was nearly 100% phosphorylated at all times (mean, 100%; SD, 12%). We found no significant variations between monomeric Cdc28p-HA (mean, 99%; SD, 14%) and Cdc28p-HA in complexes (mean, 102%; SD, 9%) or among any of the cell cycle phases that we examined. Our results indicate that both monomeric Cdc28p-HA and Cdc28p-HA in complexes are essentially completely phosphorylated throughout the cell cycle.

As a second assay for the level of Thr-169 phosphorylation, we immunoprecipitated the Cdc28p-HA from the gel filtration fractions and determined its histone H1 kinase activity in the presence and absence of added cyclin and Cak1p. If there were a significant amount of unphosphorylated Cdc28p-HA in the immunoprecipitates, incubation with Cak1p and cyclin should result in a higher level of histone H1 kinase activity than incubation with cyclin alone, whereas if the Cdc28p-HA were already highly phosphorylated, Cak1p should have little additional effect. The results support the conclusion that the majority of Cdc28p-HA was phosphorylated (Figure 3). In the absence of cyclin (squares), there was a peak of H1 kinase activity in fractions 13 and 14 of the cycling, HU-arrested, and benomyl-arrested extracts, indicating that these fractions contained active Cdc28p-HA/cyclin complexes. Addition of an excess of MBP-Clb2p generated a peak of H1 kinase activity in the monomeric fractions (fractions 16 and 17) in all four extracts (circles). However, the addition of GST-Cak1p (closed symbols) had no significant effect on the H1 kinase activity of any of the fractions in the presence or absence of cyclin (compare open and closed symbols). We conclude that Cdc28p-HA was highly phosphorylated on Thr-169 under all conditions that we tested.

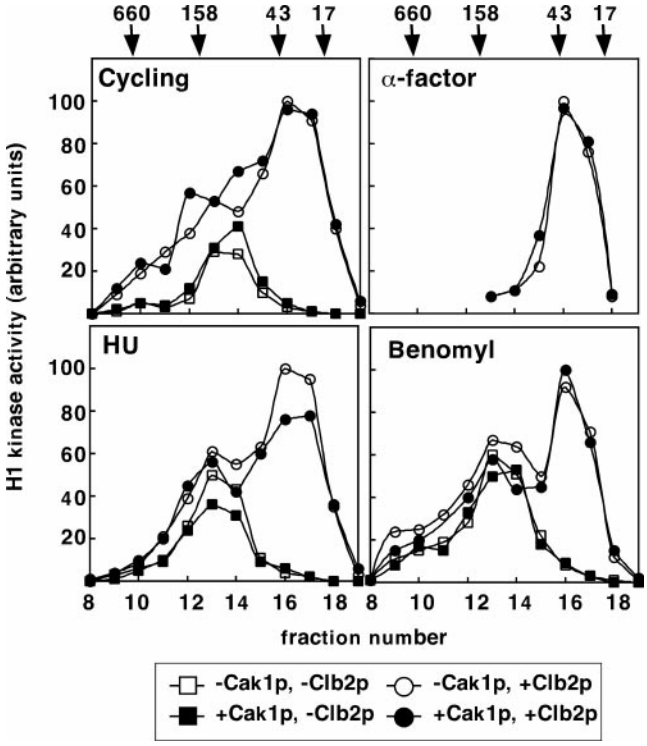

Figure 3. Treatment with GST-Cak1p does not increase the H1 kinase activity of Cdc28p-HA from yeast extract. Cdc28p-HA was immunoprecipitated from fractions 13–18 of the α-factor–arrested extract and fractions 8–19 of the cycling, HU-arrested, and benomyl-arrested extracts (Figure 2A). Cdc28p-HA–containing beads were divided into four aliquots, incubated with buffer (□), GST-Cak1p only (■), MBP-Clb2p only (○), or GST-Cak1p and MBP-Clb2p (●), and assayed for histone H1 kinase activity. Activity was quantitated with the use of a phosphorimager and plotted with the highest level of activity for each extract normalized to 100%. The activity of Cdc28p-HA from the α-factor–arrested extract was undetectable in the absence of cyclin. Gel filtration standards in kDa are as in Figure 2A.

Activation of Recombinant Cdc28p-HA In Vitro

To determine whether the minimal events required for Cdc28p activation—cyclin binding and Thr-169 phosphorylation—have to occur in a particular order, we developed an in vitro system for activation of Cdc28p. This system used only recombinant proteins and allowed us to incubate Cdc28p individually with each of the components required for its activation. We expressed Cdc28p-HA in insect cells and bound it via α-HA antibodies to protein A–agarose beads. Because the Cdc28p-HA was immobilized on beads, we were able to perform sequential incubations with cyclin and Cak1p with intervening washes.

We first tested the insect cell–produced Cdc28p-HA to ensure that it was not significantly phosphorylated on Thr-169 by endogenous insect cell kinases and that it could be phosphorylated in vitro by recombinant GST-Cak1p (Figure 4A). We incubated Cdc28p-HA beads with either GST-Cak1p (Figure 4A, PO_4-Cdc28p-HA) or buffer (Figure 4A, Cdc28p-HA) and immunoblotted with α-P-Thr-169 antibodies to determine the extent of Thr-169 phosphorylation (top) and with α-HA antibodies to measure the total amount of Cdc28p-HA present (bottom). The α-P-Thr-169 antibody was unable to detect 20 μl of untreated Cdc28p-HA beads, demonstrating that Cdc28p-HA is not highly phosphorylated in insect cells. After incubation with GST-Cak1p, as little as 3 μl of Cdc28p-HA beads reacted with the α-P-Thr-

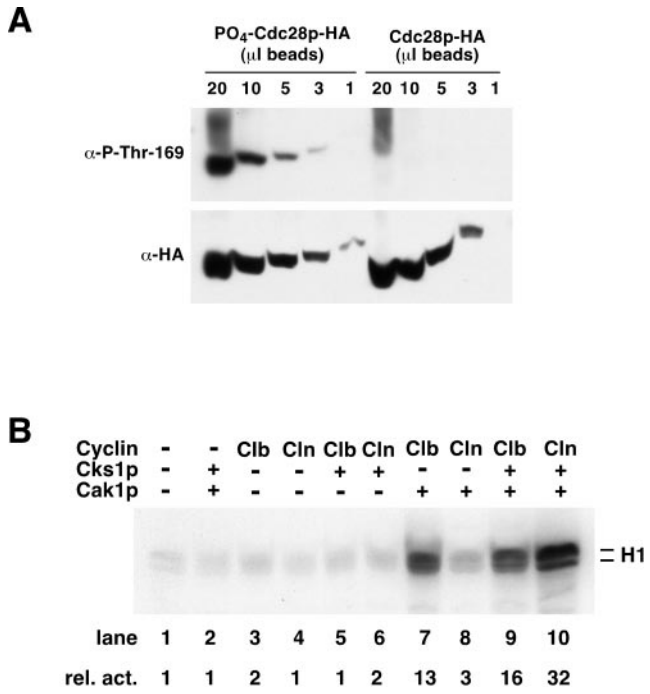

Figure 4. In vitro activation of Cdc28p-HA. (A) Beads containing Cdc28p-HA immunoprecipitated from insect cells were phosphorylated in vitro with GST-Cak1p (PO_4-Cdc28p-HA) or left untreated (Cdc28p-HA). The indicated amounts of beads were immunoblotted with the α-P-Thr-169 (top) and α-HA (bottom) antibodies. The smear in the 20-μl lane of the α-P-Thr-169 blot is due to IgG present in the immunoprecipitates. (B) Cdc28p-HA beads were incubated with MBP-Clb2p (Clb), MBP-Cln2p (Cln), GST-Cak1p, and Cks1p in the indicated combinations. Beads were washed five times with 200 μl of buffer K and then assayed for histone H1 kinase activity. Activity was quantitated with the use of a phosphorimager, and the relative activity (rel. act.) is listed beneath each lane, with the lowest activity normalized to 1.

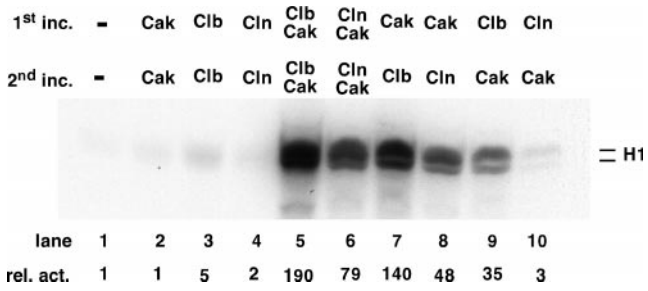

Figure 5. Dependence of Cdc28p activity on the order of addition of cyclin and Cak1p. Beads containing Cdc28p-HA immunoprecipitated from insect cells were incubated with MBP-Clb2p (Clb), MBP-Cln2p (Cln), and GST-Cak1p (Cak) as indicated for 30 min (first incubation [1st inc.]). Beads were washed five times with 200 μl of buffer K and then incubated for 30 min with MBP-Clb2p (Clb), MBP-Cln2p (Cln), and GST-Cak1p (Cak) as indicated (second incubation [2nd inc.]). Beads were again washed five times with 200 μl of buffer K and finally assayed for histone H1 kinase activity. All samples contained 100 nM Cks1p and 500 μM ATP during the first and second incubations. Activity was quantitated with the use of a phosphorimager, and the relative activity (rel. act.) is listed beneath each lane, with the lowest activity normalized to 1.

169 antibody. Thus, treatment with GST-Cak1p increased Thr-169 phosphorylation of Cdc28p-HA at least sevenfold and perhaps much more.

Cdc28p-HA beads had only background levels of histone H1 kinase activity in the absence of cyclin and GST-Cak1p (Figure 4B, lane 1) or when incubated with cyclin alone (lanes 3 and 4). Incubation of Cdc28p-HA beads with MBP-Clb2p and GST-Cak1p produced readily detectable levels of H1 kinase activity (lane 7). Incubation of Cdc28p-HA with MBP-Cln2p and GST-Cak1p failed to produce activity above background unless Cks1p was included (compare lanes 8 and 10). Cks1p is the homologue of *Schizosaccharomyces pombe* Suc1 (Hadwiger *et al.*, 1989). The function of these Cdk-binding proteins is not well understood, but Cks1p has been used previously to improve the activity of recombinant Cdc28p/Cln2p complexes (Feldman *et al.*, 1997; Skowyra *et al.*, 1997). Cks1p did not activate Cdc28p-HA in the absence of cyclin (lane 2) or GST-Cak1p (lanes 5 and 6) and did not significantly improve the activation of Cdc28p-HA by MBP-Clb2p and GST-Cak1p (compare lanes 7 and 9). Because of its strong effect on Cdc28p-HA/MBP-Cln2p activity, we included Cks1p in all of our subsequent experiments.

To determine whether activation of Cdc28p-HA depended on the order of addition of cyclin and Cak1p, we incubated Cdc28p-HA beads with cyclin, GST-Cak1p, or both (Figure 5, first incubation), washed the beads thoroughly with buffer, incubated the beads again with cyclin, GST-Cak1p, or both (Figure 5, second incubation), washed the beads a second time, and finally assayed for histone H1 kinase activity. ATP and Cks1p were included in all assays during both incubations. The highest levels of activity were generated when cyclin and GST-Cak1p were included in both incubations (lanes 5 and 6). H1 kinase activity was almost as high when Cdc28p-HA was incubated with GST-Cak1p in the first incubation and with cyclin in the second incubation (lanes 7 and 8). This result indicated that both MBP-Clb2p and MBP-Cln2p could bind to and activate Cdc28p-HA that had been phosphorylated previously on Thr-169. It is unlikely that a significant fraction of the activity resulted from phosphorylation of Cdc28p-HA/cyclin complexes during the second incubation by GST-Cak1p left over from the first incubation because the washing conditions were sufficient to remove 80–90% of the Cak1p activity (our unpublished observation). In contrast, activation of Cdc28p-HA was weak when cyclin was added in the first incubation and GST-Cak1p was added in the second incubation (lanes 9 and 10). The H1 kinase activity of Cdc28p-HA incubated first with MBP-Cln2p and then with GST-Cak1p was 95% lower than when MBP-Cln2p and GST-Cak1p were added together (compare lanes 6 and 10). Cdc28p-HA/MBP-Clb2p activity was reduced by 80% but was still significantly above background (compare lanes 5 and 9).

To explore this result further, we first considered the possibility that prebound cyclin might interfere with the phosphorylation of Cdc28p-HA by GST-Cak1p. Such an effect has been observed with the use of budding yeast Cak1p, human Cdk2, and human cyclin A; monomeric Cdk2 is an excellent substrate for Cak1p, but Cdk2/cyclin A complexes

are phosphorylated inefficiently (Kaldis *et al.*, 1998b). By analogy, preincubation of cyclin with Cdc28p-HA might lead to the formation of unphosphorylated Cdc28p-HA complexes that would resist phosphorylation by GST-Cak1p. To examine this possibility, we incubated Cdc28p-HA beads with or without cyclin and assayed the subsequent phosphorylation of Cdc28p-HA by GST-Cak1p in the presence of radiolabeled ATP (Figure 6A). Surprisingly, we found that our standard concentration of cyclin did not inhibit the phosphorylation of Cdc28p-HA by GST-Cak1p under these conditions (Figure 6A, compare lane 1 with lanes 2 and 3). Although this result could imply that GST-Cak1p phosphorylates Cdc28p-HA/cyclin complexes (unlike the situation with Cdk2 and cyclin A), it is also possible that cyclin binds so weakly to unphosphorylated Cdc28p-HA that little Cdc28p is bound to cyclin in the steady state. If the failure of cyclin to block Cdc28p phosphorylation by GST-Cak1p were due to its weak binding to Cdc28p, we should be able to drive complex formation and induce inhibition of the phosphorylation of Cdc28p at higher ratios of cyclin to Cdc28p. To test this idea, we incubated Cdc28p-his$_6$ with increasing amounts of MBP-Clb2p (from no cyclin to a 43-fold molar excess) and measured the phosphorylation of Cdc28p-his$_6$ by GST-Cak1p (Figure 6B). Cdc28p-his$_6$ phosphorylation was unaffected by as much as an 11-fold excess of Clb2p; however, in the presence of higher levels of MBP-Clb2p, Cdc28p-his$_6$ phosphorylation was inhibited. A 43-fold excess of Clb2p inhibited phosphorylation by 65%. In our standard assay (Figures 5 and 6A), we used equimolar amounts of Clb2p and Cdc28p, well below the threshold necessary to induce inhibition of Thr-169 phosphorylation. We concluded that, like Cdk2/cyclin A, Cdc28p/Clb2p was inefficiently phosphorylated by Cak1p, but cyclin had no effect on phosphorylation under our normal assay conditions because it was not present in high enough amounts to form stable complexes with unphosphorylated Cdc28p.

A second explanation for the poor activation of Cdc28p in the order-of-addition experiment (Figure 5) when cyclin was preincubated with Cdc28p is that cyclin dissociated from Cdc28p during washing. This explanation would be consistent with the apparently weak binding inferred from our previous experiment (Figure 6B). To determine whether washing was responsible for the low Cdc28p activity, we incubated Cdc28p-HA beads with cyclin and then with GST-Cak1p with or without an intervening wash to remove unbound cyclin (Figure 6C). When the beads were washed between the cyclin and GST-Cak1p incubations, Cdc28p-HA activation by both MBP-Clb2p and MBP-Cln2p was poorer than when the wash was omitted (Figure 6C, compare lanes 10–12 with lanes 7–9). Cdc28p-HA/MBP-Cln2p activity was much more sensitive to washing than was Cdc28p-HA/MBP-Clb2p activity. Thus, cyclin, particularly MBP-Cln2p, appears to bind weakly to unphosphorylated Cdc28p-HA. It should be emphasized that all samples in the H1 kinase assays shown in both Figures 5 and 6 were washed immediately before the H1 kinase assay step, demonstrating that complexes of cyclin with phosphorylated Cdc28p-HA were not easily disrupted by washing.

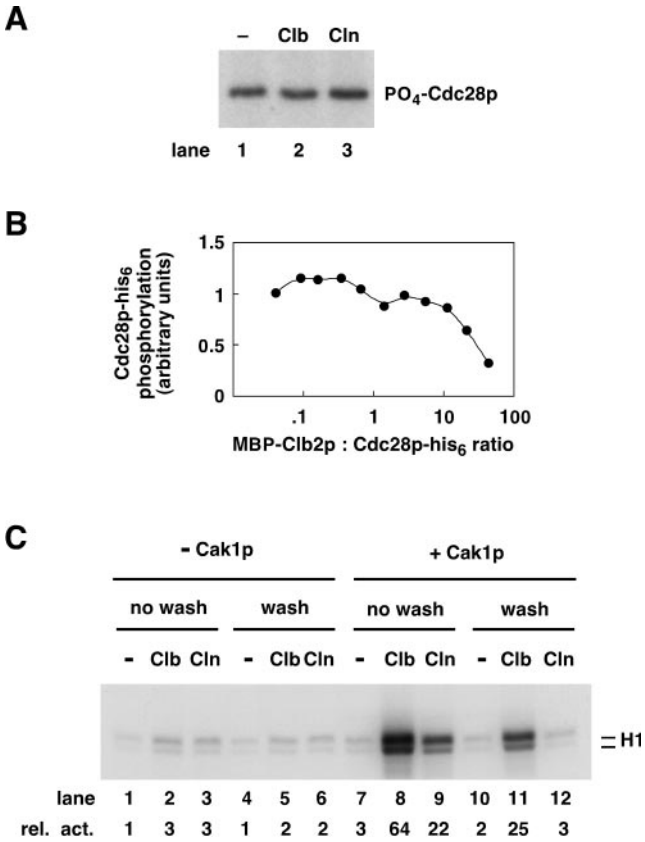

Figure 6. Cyclin binds weakly to unphosphorylated Cdc28p-HA in vitro. (A) Beads containing Cdc28p-HA immunoprecipitated from insect cells were preincubated with no cyclin (lane 1), MBP-Clb2p (lane 2), or MBP-Cln2p (lane 3) for 30 min. GST-Cak1p and [γ-^{32}P]ATP were added, and the beads were incubated for 30 min to assay phosphorylation of Cdc28p-HA. (B) Cdc28p-his$_6$ (50 ng) was preincubated with MBP-Clb2p in the indicated molar ratios for 30 min; 7.5 ng of GST-Cak1p and [γ-^{32}P]ATP were added, and incubation was continued for another 30 min. Phosphorylation of Cdc28p-his$_6$ was quantitated by SDS-PAGE and phosphorimaging. (C) Beads containing Cdc28p-HA immunoprecipitated from insect cells were incubated with no cyclin (−; lanes 1, 4, 7, and 10), MBP-Clb2p (Clb; lanes 2, 5, 8, and 11), or MBP-Cln2p (Cln; lanes 3, 6, 9, and 12) for 30 min. The beads were washed in assays 4–6 and 10–12 five times with 200 μl of buffer K and resuspended in buffer. Beads from all assays were then incubated with unlabeled ATP in the absence (−Cak1p; lanes 1–6) or presence (+Cak1p; lanes 7–12) of GST-Cak1p for 30 min, washed five times with 200 μl of buffer K, and assayed for histone H1 kinase activity. H1 kinase activity was quantitated with the use of a phosphorimager, and the relative activity (rel. act.) is listed beneath each lane, with the lowest activity normalized to 1. During the incubations with cyclin and Cak1p, all assays contained 100 nM Cks1p and 500 μM ATP.

Dependence of Cyclin Binding on Thr-169 Phosphorylation In Vivo

Our in vitro results suggested that cyclin might bind preferentially to phosphorylated Cdc28p. To test this prediction in vivo, we performed gel filtration on extracts of cells expressing endogenous Cdc28p together with Cdc28p-HA or Cdc28p^{T169A}-HA and immunoblotted with the α-PSTAIR

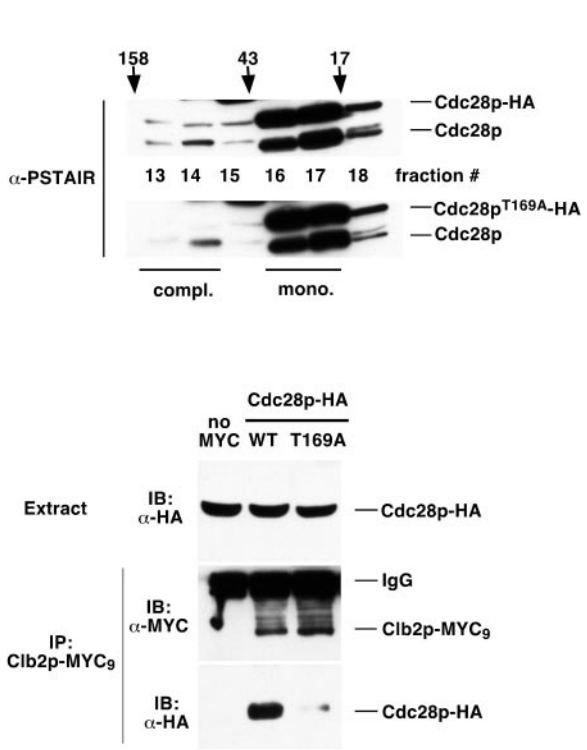

Figure 7. Cdc28p^{T169A}-HA is defective at binding cyclin in vivo. (A) Extracts were prepared from YKR102 (Cdc28p-HA; top) and YKR103 (Cdc28p^{T169A}-HA; bottom) and gel filtered on a Superdex 200 column, and fractions 13–18 were blotted with the α-PSTAIR antibody. The faint band just above the untagged Cdc28p appears to be a degradation product of the HA-tagged Cdc28p. Note the decreased amount of Cdc28p^{T169A} in fraction 14, indicating reduced cyclin binding. Gel filtration standards of 158, 43, and 17 kDa, and the peaks of Cdc28p-HA complexes (compl.) and monomer (mono.), are indicated. (B) Extracts were prepared from YKR108 [*CDC28(HA) GAL-CLB2(MYC$_9$)*; WT] and YKR109 [*CDC28(HA)^{T169A} GAL-CLB2(MYC$_9$)*; T169A] grown in galactose to induce expression of Clb2p-MYC$_9$ and from YKR107 grown in glucose to repress expression of the MYC-tagged protein (no MYC). Total extracts were immunoblotted (IB) with α-HA antibodies to detect Cdc28p-HA (top). Clb2p-MYC$_9$ was immunoprecipitated (IP) with α-MYC antibodies, and the pellets were immunoblotted with α-MYC to detect Clb2p-MYC$_9$ (middle) and with α-HA to detect coprecipitating Cdc28p-HA (bottom).

antibody to detect both the tagged and untagged forms of Cdc28p. If cyclin bound less well to unphosphorylated Cdc28p in vivo, we would expect to see that Cdc28p^{T169A}-HA would not form complexes as efficiently as wild-type Cdc28p-HA. In both strains, the untagged wild-type Cdc28p eluted in two peaks: a peak of monomer in fractions 16 and 17 and a peak of Cdc28p-containing complexes in fractions 13 and 14 (Figure 7A). Wild-type Cdc28p-HA had a similar distribution (Figure 7A). Although Cdc28p^{T169A}-HA was expressed at least as well as wild-type Cdc28p-HA (compare the amounts of HA-tagged protein in fractions 16 and 17 in the top and bottom panels), less of the T169A mutant formed complexes (compare fractions 13 and 14 in the top and bottom panels). This result suggests that Thr-169 phosphorylation enhances cyclin binding, although we cannot exclude a subtle effect of the T169A mutation on the folding of Cdc28p.

To examine the ability of Cdc28p^{T169A}-HA to interact with a particular cyclin in vivo, we performed a coprecipitation experiment (Figure 7B). We prepared extracts from strains expressing Clb2p tagged with the MYC epitope (Clb2p-MYC$_9$) along with wild-type Cdc28p-HA (WT) or Cdc28p^{T169A}-HA (T169A), immunoprecipitated the Clb2p-MYC$_9$ with antibodies against the MYC epitope tag, and compared the levels of Cdc28p-HA and Cdc28p^{T169A}-HA that coprecipitated with the cyclin by immunoblotting. We found that the WT and T169A forms of Cdc28p-HA were expressed at similar levels in the total extracts (Figure 7B, top) and that Clb2p-MYC$_9$ was immunoprecipitated from both strains with equal efficiency (7B, middle). However, although we found significant amounts of wild-type Cdc28p-HA associated with Clb2p-MYC$_9$, Cdc28p^{T169A}-HA coprecipitated poorly. There was no Clb2p-MYC$_9$ or Cdc28p-HA in the immunoprecipitate from a strain that did not express MYC-tagged Clb2p (no MYC). Thus, the T169A mutation seems to impair the ability of Cdc28p to associate with Clb2p in vivo. These results are consistent with our in vitro findings, and together, our data support a model in which Thr-169 phosphorylation of Cdc28p increases its affinity for cyclins.

DISCUSSION

We studied the regulation of Cdc28p by Cak1p and cyclins. In agreement with previous data, we found that most of the Cdc28p in the cell was monomeric and that the small percentage that was in complexes contributed all of the histone H1 kinase activity (Wittenberg and Reed, 1988). However, essentially all of the Cdc28p, including monomeric Cdc28p, was highly phosphorylated on Thr-169 throughout the mitotic cell cycle. Cells in stationary phase and cells undergoing synchronous meiosis, two conditions in which Cak1p levels change dramatically (Kaldis *et al.*, 1998a), also exhibited no change in the stoichiometry of Cdc28p phosphorylation (our unpublished observations). Our in vitro assays revealed that Cdc28p prephosphorylated on Thr-169 was efficiently activated by both Clb2p and Cln2p; in contrast, cyclin bound weakly to unphosphorylated Cdc28p. Furthermore, monomeric Cdc28p was efficiently phosphorylated by Cak1p, whereas Cdc28p/cyclin complexes, formed when cyclin was present in high enough amounts to drive complex formation, were not. Together, our data indicate that the activation pathway for the bulk of Cdc28p in the cell involves phosphorylation by Cak1p followed by binding to cyclin. It appears that Cak1p maintains a large pool of highly phosphorylated Cdc28p, which is available for immediate activation by cyclins.

Previous studies with the Cdc28p^{T169E} mutant had reached contradictory conclusions regarding the role of Thr-169 phosphorylation in cyclin binding (Lim *et al.*, 1996; Cross and Levine, 1998). Lim *et al.* (1996) found that Cdc28p^{T169E} bound poorly to Cln2p and proposed that G1 cyclins preferentially bound to unphosphorylated Cdc28p. On the other hand, Cross and Levine (1998) identified additional mutations in Cdc28p^{T169E} that allowed it to bind cyclins and drive the cell cycle in spite of the T169E mutation. Our results

suggest that Thr-169 phosphorylation is required for tight binding of Cln2p to Cdc28p. It appears that the T169E mutation is a weak mimic of constitutive phosphorylation and that it may induce structural changes that affect cyclin binding.

Several lines of evidence suggest that, in other eukaryotes, Cdks are activated by a pathway in which they first form complexes with cyclins and are subsequently phosphorylated by CAK. Many Cdks do not require activating phosphorylation to bind to cyclins in vitro or in vivo. Unphosphorylated Cdk2 forms stable complexes with cyclins A and E in vitro (Desai *et al.*, 1995). Human Cdc2 does not require activating phosphorylation to bind to cyclin B in vitro (Desai *et al.*, 1995), and mutation of the activating threonine of *Xenopus* Cdc2 does not impair its ability to form complexes with cyclin B in egg extract (Solomon *et al.*, 1992). Conversely, cyclin binding stimulates the activating phosphorylation of Cdks. Recombinant Cdc2 is efficiently phosphorylated by endogenous CAK in insect cell lysate only in the presence of cyclin B (Desai *et al.*, 1992), and the addition of cyclin A stimulates the phosphorylation of Cdk2 by purified human CAK (Fisher and Morgan, 1994; Kaldis *et al.*, 1998b). Cdc2 is not phosphorylated in *Xenopus* egg extracts that lack cyclin but quickly becomes phosphorylated upon cyclin addition (Solomon *et al.*, 1992). Unlike budding yeast Cdc28p, Cdc2 in other organisms may be dephosphorylated after cyclin degradation. *Xenopus* Cdc2 shifts to a lower-mobility form that is consistent with dephosphorylation of its activating threonine, Thr-161, at the end of mitosis (Lorca *et al.*, 1992). Cyclin-bound Cdc2 is not dephosphorylated, thereby restricting the onset of dephosphorylation until after anaphase, when cyclins are degraded (Lorca *et al.*, 1992). Fission yeast Cdc2 is unphosphorylated when cells are arrested in G1 after nitrogen starvation (Simanis and Nurse, 1986). Genetic data further suggest that fission yeast cells that cannot dephosphorylate the activating threonine are defective in mitotic exit. A mutant form of Cdc2 in which its activating threonine was changed to glutamate (T167E) allowed cells to complete the G1/S and G2/M transitions, but they had multiple septa and nuclei as a result of failure to complete late mitotic events (Gould *et al.*, 1991).

The strikingly different activation pathways in budding yeast and other eukaryotes are consistent with the properties of their different CAKs. Unlike monomeric Cak1p, CAKs in other organisms consist of a catalytic subunit (Cdk7), a regulatory subunit (cyclin H), and, in some cases, an assembly factor (Mat1; reviewed by Kaldis, 1999). The two types of CAKs also differ in their functional properties in ways that suggest that they may be specialized for different Cdk activation pathways. For example, Cdk/cyclin complexes are excellent substrates for Cdk7/cyclin H, whereas monomeric Cdks are phosphorylated very poorly by this enzyme (Fisher and Morgan, 1994; Kaldis *et al.*, 1998b). Cak1p, on the other hand, efficiently phosphorylates monomeric Cdks but not Cdk/cyclin complexes (Kaldis *et al.*, 1998b). In keeping with these different substrate preferences, unphosphorylated Cdk2 binds strongly to cyclin A, stimulating phosphorylation by Cdk7/cyclin H (Kaldis *et al.*, 1998b). In contrast, the weak binding of cyclin to unphosphorylated Cdc28p that we observed results in a large steady-state pool of free Cdc28p, the preferred form for phosphorylation by Cak1p. Cak1p and Cdk7/cyclin H also have strikingly different subcellular localizations: Cdk7/cyclin H is nuclear, whereas Cak1p is predominantly cytoplasmic (Tassan *et al.*, 1994; Jordan *et al.*, 1997; Kaldis *et al.*, 1998a). Many cyclins are nuclear proteins or at least translocate to the nucleus during the period of the cell cycle when they function (reviewed by Pines, 1995). If cyclin binding promotes the nuclear localization of Cdks, the timing of cyclin binding relative to CAK phosphorylation would dictate the appropriate localization of the CAK. In budding yeast, a cytoplasmic CAK would be required to phosphorylate cytoplasmic Cdc28p monomers (Wittenberg *et al.*, 1987). In other organisms, cyclin binding would lead to the nuclear import of unphosphorylated Cdks, so a nuclear CAK would be required to activate them. In summary, budding yeast Cak1p and Cdk7/cyclin H may be very different because their substrates—monomeric Cdks and Cdk/cyclin complexes, respectively—are very different.

It is unclear why Cdc28p would possess a site of activating phosphorylation if it is constitutively phosphorylated and never used in a regulatory manner. Similar questions had been asked about the inhibitory phosphorylation sites of Cdc28p. Although other organisms use these sites as a way of restraining mitosis during every cell cycle or as part of the response to DNA damage, they are not required for these processes in budding yeast (Amon *et al.*, 1992; Sorger and Murray, 1992; Coleman and Dunphy, 1994). Then it was discovered that inhibitory phosphorylation of Cdc28p was required for a checkpoint that monitors the synchrony of the budding and nuclear division cycles and a second checkpoint that monitors cytokinesis (Lew and Reed, 1995; Sia *et al.*, 1996; Barral *et al.*, 1999). Although we found in this work that activation of two well-characterized checkpoints—the unreplicated DNA checkpoint by HU and the spindle assembly checkpoint by benomyl—did not influence Thr-169 phosphorylation, perhaps there is another checkpoint pathway that affects the activating phosphorylation site.

Although these experiments have highlighted differences between Cdk activation in budding yeast and other organisms, they do not offer any clue regarding why the components of the cell cycle machinery, which are highly conserved, are not used in the same order in all organisms. Neither situation seems to have an advantage from a regulatory point of view. Both Cak1p and Cdk7/cyclin H are active throughout the cell cycle (reviewed by Kaldis, 1999), and each can act as soon as its substrate (monomeric Cdc28p in budding yeast and Cdk/cyclin complexes in other organisms) becomes available. Therefore, activating phosphorylation does not appear to be rate limiting for Cdk activation in either case. It would be interesting to know whether the different Cdk activation pathways used by budding yeast and other organisms offer their respective organisms any benefits or if they are simply two equivalent solutions to the problem of activating cell cycle regulatory kinases.

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