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G_1 cyclin-dependent activation of $p34^{CDC28}$ (Cdc28p) *in vitro*

(cdc2/cell cycle/yeast)

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ABSTRACT **In *Saccharomyces cerevisiae*, transient accumulation of G_1 cyclin/$p34^{CDC28}$ (Cdc28p) complexes induces cells to traverse the cell cycle Start checkpoint and commit to a round of cell division. To investigate posttranslational controls that modulate Cdc28p activity during the G_1 phase, we have reconstituted cyclin-dependent activation of Cdc28p in a cyclin-depleted G_1 extract. A glutathione *S*-transferase–G_1 cyclin chimera (GST–Cln2p) efficiently binds to and activates Cdc28p as a histone H1 kinase. Activation of Cdc28p by GST–Cln2p requires ATP, crude yeast cytosol, and the conserved Thr-169 residue that serves in other organisms as a substrate for phosphorylation by cyclin-dependent protein kinase-activating kinase. This assay may be useful for distinguishing genes that promote directly the posttranslational assembly of active Cln2p/Cdc28p kinase complexes from those that stimulate the accumulation of active complexes via a positive-feedback loop that governs synthesis of G_1 cyclins.**

Genetic analysis of progression through the G_1 phase of the cell cycle in *Saccharomyces cerevisiae* indicates that it is dependent upon *CDC28*, which encodes an *S. cerevisiae* homolog ($p34^{CDC28}$; Cdc28p) of the ubiquitous cyclin-dependent protein kinase (Cdk) family of protein kinases (1, 2), and the functionally redundant *CLN1*, *-2*, and *-3* genes (3) which encode cyclin-like proteins. Cln1p and Cln2p physically associate with active Cdc28p protein kinase, and their levels fluctuate during the cell cycle; both proteins accumulate as cells progress through the G_1 phase and abruptly disappear upon entry into the S/G_2 phase (4, 5). Besides *CDC28* and the *CLN* genes, several other genes are required for progression through cell cycle Start in the G_1 phase. Whereas several of these additional genes [*SIT4* (6) and *SWI4* and *-6* (7, 8)] are required for the efficient accumulation of G_1 cyclin mRNA, some of these genes, including *CKS1* (9) and *CDC37* (10), may influence the assembly or activity of the Start-promoting Clnp/Cdc28p protein kinase complex. At present, little is known about the posttranslational mechanisms governing the assembly or regulation of active cyclin/Cdc28p complexes in *S. cerevisiae.*

We wish to examine the regulation of Cdc28p protein kinase *in vitro* to determine whether G_1 cyclins are sufficient to activate Cdc28p and to achieve a molecular description of the posttranslational reactions that contribute to the transient accumulation of active G_1 cyclin/Cdc28p complexes during progression through the G_1/S transition. A biochemical dissection of Cdc28p regulation during this interval will help to reveal how diverse signaling pathways impinge on the molecular machinery that governs Start. In this paper, we employ an *in vitro* system to demonstrate that the G_1 cyclin Cln2p can activate Cdc28p protein kinase in extracts of G_1-arrested yeast cells. Dormant Cdc28p in G_1 extracts was readily activated in a reaction requiring G_1 cyclin, ATP, and crude yeast extract. Activation of Cdc28p by G_1 cyclin required the conserved Thr-169 residue that serves as a target for the Cdk-activating kinase (CAK) (13–15).

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MATERIALS AND METHODS

Reagents, Yeast Strains, and Plasmids. The genotypes of yeast strains used in this study are as follows: (*i*) RD205-3A is *cln1*Δ, *cln2*::*LEU2*, *cln3*Δ, $trp1^{am}$, *leu2*, *ura3*, *pep4*::*LEU2*, *MAT***a**, (*GAL-CLN3*, *URA3*, *CEN ARS*). (*ii*) pRD84/RD205-3A is a modification of RD205-3A containing $CDC28^{HA}$::*TRP1* integrated at the *CDC28* locus. (*iii*) RD240-3B is *cdc28-4*, *ura3*, *leu2*, *pep4*::*TRP1*, *bar1*::*LEU2*, *MAT***a**.

Expression of $Cdc28^{HA}$p in *Escherichia coli* was achieved by means of the following series of manipulations. The 5′ end of *CDC28* was altered by PCR mutagenesis to generate an *Nde* I site overlapping the initiator ATG codon, thereby yielding pRD47. DNA sequence analysis confirmed the correctness of the PCR-amplified segment of *CDC28* in pRD47. pRD47 was subsequently modified to incorporate the hemagglutinin (HA) epitope tag (16) at the 3′ end of the *CDC28* coding sequence, yielding pSF19 (17). $CDC28^{HA}$ was excised from pSF19 and inserted between the *Nde* I and *Eco*RI sites of pRK172 (18) to generate pRD88, which directs the expression of $CDC28^{HA}$ in *E. coli* by transcription from a T7 RNA polymerase promoter (19).

To replace chromosomal *CDC28* with $CDC28^{HA}$, we constructed pRD84 as follows: pSF19 (17) was digested with *Sac* I, and a fragment containing both the 3′ end of $CDC28^{HA}$ and transcription termination sequences from the *ACT1* gene was blunt-ended and inserted into the blunt-ended *Kpn* I and *Sac* I sites of pRS304 (20). pRD84 linearized within the *CDC28* coding sequences by digestion with *Kpn* I was used to transform yeast strain RD205-3A. This yields two tandem copies of *CDC28* in the chromosome: a 5′-truncated copy with a normal 3′ end and a full-length copy with the HA epitope appended at the 3′ end. Transformants were assayed by immunoblotting with anti-HA (α-HA) and anti-Cdc28p (α-Cdc28p) antibodies to confirm that recombination between pRD84 and chromosomal sequences resulted in the expression of $CDC28^{HA}$ but not *CDC28.*

$CDC28^{HA}$ clones with point mutations at either Thr-169 or Lys-40 and Lys-41 were generated by PCR mutagenesis, and the mutant sequences were substituted for *CDC28* sequences in pRD88. *E. coli* extracts prepared from four independent isolates of each mutant were analyzed in the *in vitro* $cdc28^{ts}$ complementation assay to compensate for the possible introduction of mutations during PCR.

A plasmid (pRD71) that directs synthesis of a glutathione *S*-transferase (GST)–Cln2p fusion protein in *E. coli* was derived by inserting a *Bam*HI fragment encoding *CLN2* [from

Abbreviations: Cdk, cyclin-dependent kinase; CAK, Cdk-activating kinase; GST, glutathione *S*-transferase; HA, hemagglutinin.

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YCpG2CLN2 (4)] into the *Bam*HI site of pGEX-2T (21). pTB5, which encodes a GST–Cln2p fusion protein lacking amino acids 377–545 of Cln2p, was created by deleting an internal *Bam*HI fragment from pRD71. pTB6, which encodes a GST–Cln2p fusion protein lacking amino acids 90–163 within the cyclin box homology domain of Cln2p, was created by deleting an internal *Xho* I–*Spe* I fragment of pRD71.

Expression and Purification of Recombinant Proteins. GST fusion proteins were expressed in *E. coli* and purified essentially as described by Solomon *et al.* (22), except that induction was allowed to proceed for 150 min at 25°C. Amounts of GST–Cln2p were estimated by comparison with known amounts of bovine serum albumin on Coomassie blue-stained SDS/polyacrylamide gels.

Various derivatives of $Cdc28^{HA}p$ were expressed in *E. coli* strain BL21(DE3)/pLysS as described (19), except that $Cdc28^{HA}p$ synthesis was induced for 5 h at 24°C. Following induction, 100 ml of cells was harvested, washed with 50 mM Tris·HCl, pH 7.6/100 mM NaCl, pelleted (Sorvall SS-34 rotor; 5 min at 7000 rpm), washed with 1 ml of cold yeast extract buffer (YEB; 125 mM potassium acetate/30 mM Hepes·KOH, pH 7.2/3 mM EDTA/3 mM EGTA/2 mM dithiothreitol/0.5 mM phenylmethylsulfonyl fluoride and 10 μg each of pepstatin and leupeptin per ml), resuspended in 1 ml of cold YEB, and frozen in liquid nitrogen. Frozen cell suspensions were thawed, sonicated two times for 20 sec each, and then centrifuged first for 10 min at 14,000 × *g* and then 15 min at 50,000 rpm (Beckman TLA100.3 rotor). $Cdc28^{HA}p$ accounted for ≈0.25% of soluble extract protein (≈60 ng of $Cdc28^{HA}p$ per μl of an *E. coli* extract containing 25 μg of protein per μl of extract).

Preparation of Concentrated Yeast Extracts. Yeast cells were grown overnight at 30°C in 2 liters of 1% yeast extract/2% peptone/2% galactose supplemented with 50 mM sodium phosphate, pH 6.5 to a density of ≈0.5–1.5 × 10^7 cells per ml. *CLN3* expression was terminated by adding 30 g of glucose per liter of culture, and cells were incubated for an additional 195 min to allow imposition of a G_1 cell cycle arrest. Cultures were harvested (Sorvall H-6000A rotor; 5 min at 5000 rpm), and cell pellets were washed two times with ice-cold water. The final cell pellet was resuspended in 100 ml of 0.1 M Tris·H_2SO_4, pH 9.2/10 mM dithiothreitol, incubated 15 min at 24°C, harvested by centrifugation (Sorvall SS-34 rotor; 4 min at 5000 rpm), washed with 100 ml of ice-cold water, and recentrifuged. Cell walls were digested by resuspending the cell pellet in 100 ml of 1 M sorbitol/50 mM Tris·HCl, pH 7.65/1 mM $CaCl_2$/1 mM $MgCl_2$ and adding 1.1 mg of lyticase (Sigma L8137) per g (wet weight) of yeast cells. After ≈20–30 min of incubation at 30°C, spheroplasts were pelleted by centrifugation (Sorvall SS-34 rotor; 6 min at 6000 rpm), washed once with 100 ml of 1 M sorbitol, and recentrifuged. The washed spheroplast pellet was resuspended in 1 M sorbitol, spheroplasts were sedimented through a 1.9 M sorbitol cushion (Sorvall HB-4 rotor; 8 min at 8000 rpm), and the pellet was resuspended carefully in 0.5 vol of 2.5× YEB, yielding a thick slurry of spheroplasts. This slurry was dripped into liquid nitrogen, and the frozen spheroplast kernels were ground in five 40-sec intervals in a mortar and pestle prechilled with liquid nitrogen. In between each interval of grinding, liquid nitrogen was added to the mortar and allowed to evaporate. The resulting frozen extract was then thawed, cleared by centrifugation for 20 min at 65,000 rpm (Beckman TLA100.3 rotor), desalted by spin-column gel filtration on Sephadex G25 equilibrated in 1× YEB, and recentrifuged for 10 min at 55,000 rpm (Beckman TLA100.3 rotor). Clarified extracts (45–75 mg of protein per ml) were frozen in liquid nitrogen, and stored at −80°C. Protein determinations were performed with the Bradford reagent with bovine gamma globulin as the standard.

Lysis of yeast by grinding frozen cell pellets was originally described for the preparation of extracts from cells with an intact cell wall (23). Extracts prepared from intact yeast cells by using the growth regimen and buffers described above consumed ATP rapidly; an ATP-regenerating system with 1 mM ATP, 15 mM creatine phosphate, and 50 μg of creatine kinase per ml was exhausted within 7 min at 24°C when the extract was at 25 mg of protein per ml. The lifetime of the ATP-regenerating system was increased severalfold when cell walls were removed enzymatically prior to lysis. Under the reaction conditions described herein, ATP levels were sustained for at least 45 min in extracts containing 25 mg of protein per ml at 24°C.

Activation Reactions, Immunoprecipitations, and Protein Kinase Assays. Each 10-μl activation reaction mixture contained yeast extract plus YEB to a final volume of 5 μl, 1 μl of 10× ATP mix (10 mM ATP/350 mM creatine phosphate/20 mM Hepes, pH 7.2/10 mM magnesium acetate/500 μg of creatine kinase per ml), 1 μl of 10× reaction buffer (50 mM magnesium acetate/10 mM dithiothreitol/5 mM phenylmethylsulfonyl fluoride and 100 μg each of leupeptin and pepstatin per ml), and cyclin storage buffer (100 mM potassium acetate/20 mM Hepes, pH 7.2/2 mM dithiothreitol) plus GST–cyclin to a total volume of 3 μl. Reactions were incubated for 0–60 min at 24°C and terminated by dilution into ice-cold IPB (3 μl of reaction mixture in 200 μl of IPB; IPB contains 50 mM glycerol 2-phosphate, 100 mM sodium chloride, 5 mM EDTA, 0.2% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, and 1 μg each of pepstatin and leupeptin per ml at pH 7.5). $Cdc28^{HA}p$ was recovered for protein kinase assays by immunoprecipitation with 0.15 μl of α-HA ascites fluid containing 5 μg of protein per μl of fluid and 5 μl of packed protein A–Sepharose beads. After incubation for 90 min on a rotating wheel at 4°C, immune complexes were washed three times with cold IPB and two times with cold kinase assay buffer (KAB; 10 mM Hepes, pH 7.2/10 mM $MgCl_2$/50 mM NaCl/2 mM EDTA/1 mM dithiothreitol, 0.02% Triton X-100). Washed immune complexes were mixed with 10 μl of a cocktail containing 1.5 μCi of [γ-^{32}P] ATP (1 Ci = 37 GBq), 68 μM ATP, and 150 μg of histone H1 per ml in KAB and incubated for 15 min at 24°C. The kinase reactions were terminated by the addition of 7 μl of 3× SDS/PAGE sample buffer. The samples were heated for 3 min at 95°C and applied to SDS/15% polyacrylamide gels. Following electrophoresis, gels were stained with Coomassie blue R-250, dried, and exposed to autoradiography film. Kinase reactions were quantitated by excising histone H1 bands from the gel and counting Cerenkov radiation.

RESULTS

To investigate the activation of *S. cerevisiae* Cdc28p by G_1 cyclins, we devised the following strategy: *cln1*, *-2*, and *-3*Δ cells, sustained by a *GAL-CLN3* allele (24) and bearing a copy of *CDC28* tagged with the HA epitope (*$CDC28^{HA}$*; ref. 16), were depleted of Cln3p by incubation in glucose, resulting in G_1 arrest. Concentrated cell extracts prepared by grinding cells in liquid nitrogen (23) were supplemented with an ATP-regenerating system and a recombinant GST–G_1 cyclin fusion protein (GST–Cln2p) purified from *E. coli*. After incubation, HA-tagged Cdc28p ($Cdc28^{HA}p$) was recovered by immunoprecipitation with α-HA, and protein kinase activity was assayed by using histone H1 as a substrate. Kinase activity was quantitated by SDS/PAGE and scintillation counting of excised gel slices containing histone H1. Kinase activity in α-HA precipitates was attributable to Cdc28p; activation reactions supplemented with GST–Cln2p yielded high levels of α-HA-precipitable histone H1 kinase (Fig. 1), but no histone kinase activity was detected either when the HA peptide antigen was included during immunoisolation or when activation reactions were conducted with an extract prepared from a strain expressing untagged Cdc28p. Since $Cdc28^{HA}p$ kinase activity was measured after immunoprecipitation, various treatments

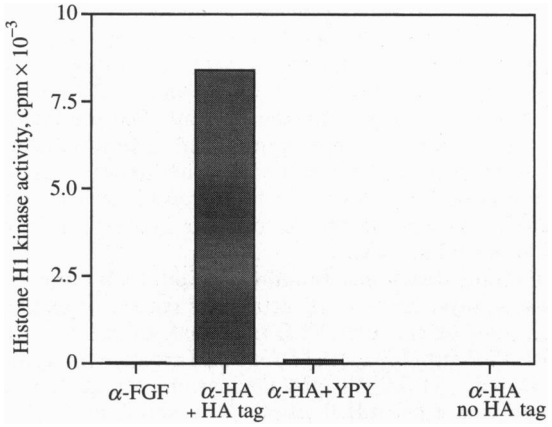

FIG. 1. α-HA antibody specifically precipitates Cdc28^{HA}p-associated protein kinase activity. Activation reaction mixtures containing cell extract prepared from strain pRD84/RD205-3A (relevant genotype: *cln1*, *-2*, and *-3Δ*, *GAL-CLN3*, *CDC28^{HA}*; + HA tag) were supplemented with 75 nM GST–Cln2p, incubated for 20 min at 24°C, and then subjected to immunoprecipitation with 6.5 μg of affinity-purified antifibroblast growth factor antibodies (α-FGF), 0.09 μl of α-HA ascites fluid (α-HA), or 0.09 μl α-HA ascites fluid plus 1 μg of the HA peptide YPYDVPDYA (α-HA + YPY) prior to kinase assay. An activation reaction containing extract prepared from strain RD205-3A (relevant genotype: *cln1*, *-2*, and *-3Δ*, *GAL-CLN3*, *CDC28*; no HA tag) was conducted exactly as described above, and the mixture was subjected to immunoprecipitation with 0.18 μl of α-HA ascites fluid. Activation of Cdc28p in this extract was confirmed by immunoprecipitation with affinity-purified anti-GST antibodies (α-GST) prior to the kinase assay (see Fig. 3, column 6).

could be evaluated for their effects on the assembly of an active Cln2p/Cdc28^{HA}p complex, independent of their effects on Cdc28^{HA}p activity *per se*. All treatments described herein were imposed during the first (activation) stage and not during the actual protein kinase assay.

Cln3p-depleted G_1 extracts contained a very low level of endogenous Cdc28^{HA}p-associated histone H1 kinase activity (Fig. 2*A*). Addition of increasing amounts of GST–Cln2p (from 0 to 70 nM) provoked a linear increase in the amount of Cdc28^{HA}p activity. (In some experiments, GST–Cln2p achieved saturation at concentrations as low as 35 nM.) As Cdc28^{HA}p is present at ≈250 nM in these extracts, some other factor may become limiting for Cdc28^{HA}p activation when GST–Cln2p exceeds 70 nM. Cln2p activity required sequences conserved among members of the cyclin family, as a Cln2p mutant lacking a segment of the cyclin box failed to activate Cdc28^{HA}p (Fig. 2*A*, GST–Cln2Δbox). Deletion of the 3′ third of *CLN2* produces a dominant allele (*CLN2-1*) that perturbs regulation of the G_1 phase (3). This C-terminally truncated form of Cln2p (GST–Cln2ΔCTD) activated Cdc28^{HA}p less effectively than full-length Cln2p. Therefore, deletion of the C terminus of Cln2p does not increase its potential to activate Cdc28^{HA}p; the dominant phenotype of *CLN2-1* may arise instead from loss of some form of negative regulation that is not readily detectable *in vitro*. In the experiments described above, Cdc28^{HA}p activation was allowed to proceed for 30 min. Time-course experiments indicated that the activation of Cdc28^{HA}p by added GST–Cln2p, as determined by increases in histone H1 kinase activity, increased linearly for 15 min and then remained constant for up to 60 min (Fig. 2*B*).

Activation of Cdc28^{HA}p protein kinase activity by GST–Cln2p was accompanied by the formation of a tight complex between the two polypeptides (Fig. 3). Histone H1 kinase activity was precipitated by either α-HA (column 1) or α-GST (column 4) antibodies. Approximately 33% of the activity precipitable with α-HA was also retrieved on glutathione–agarose beads (unpublished data). Adsorption of GST–cyclin/

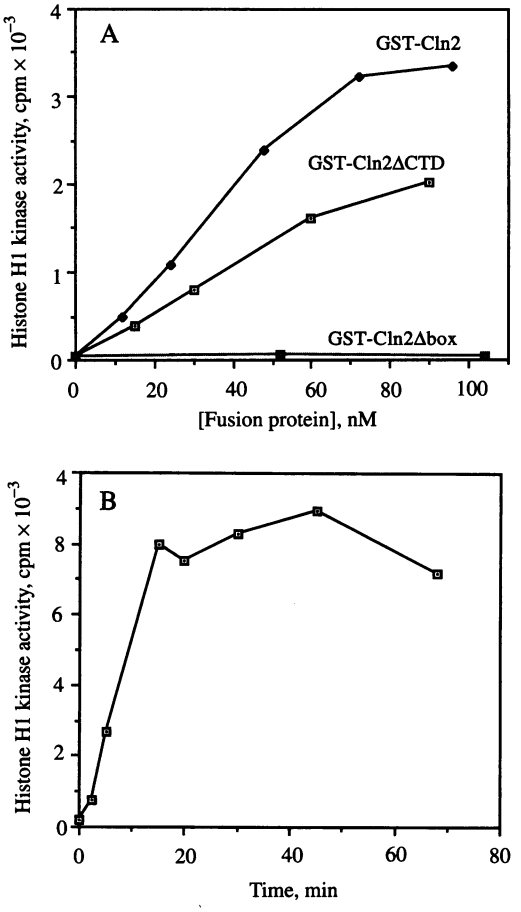

FIG. 2. Activation of Cdc28^{HA}p by GST–Cln2p is linear with respect to both time and cyclin concentration. (*A*) Indicated amounts of various GST–Cln2p fusion proteins were added to pRD84/RD205-3A cell extract (200 μg) and incubated for 30 min at 24°C prior to immunoprecipitation and measurement of histone H1 kinase activity. GST–Cln2 (◆), wild-type Cln2p; GST–Cln2ΔCTD (⊡), Cln2p lacking amino acids 377–545 (3); GST–Cln2Δbox (■), Cln2p lacking amino acids 90–163. This deletion removes a portion of the Cln2p cyclin box homology domain, which extends from amino acid 23 to 203 (25). (*B*) pRD84/RD205-3A extract (200 μg) supplemented with 35 nM GST–Cln2p was incubated for the indicated amounts of time at 24°C prior to immunoisolation of Cdc28^{HA}p and quantitation of histone H1 kinase activity.

Cdc28^{HA}p complexes to glutathione–agarose provides a convenient method for preparing active Cdc28p assembled with specific cyclins (18).

To gain more insight into the action of GST–Cln2p, we tested whether ATP and cytosolic factors were required in addition to GST–Cln2p to achieve Cdc28^{HA}p activation. Full activation of Cdc28^{HA}p required inclusion of both ATP and an ATP-regenerating system (Fig. 4*A*). As shown in Fig. 2*A*, Cdc28^{HA}p immunoisolated from untreated extract possessed low protein kinase activity (Fig. 4*B*, column 1). Subsequent incubation with either cell extract (Fig. 4*B*, column 2) or GST–Cln2p (column 3) failed to stimulate Cdc28^{HA}p activity further, whereas incubation with both extract and GST–Cln2p provoked a dramatic increase in Cdc28^{HA}p activity (Fig. 4*B*, column 4).

Activation of *Xenopus* p34^{cdc2} by GST–cyclin B in *Xenopus* extract requires phosphorylation of p34^{cdc2} on Thr-161 by a protein kinase referred to as CAK (13–15). A similar requirement for a CAK-like activity might contribute to the ATP and cytosol dependence we observed for GST–Cln2p-dependent activation of Cdc28^{HA}p. To test whether the analogous Thr-169 residue of Cdc28^{HA}p was required for the assembly of

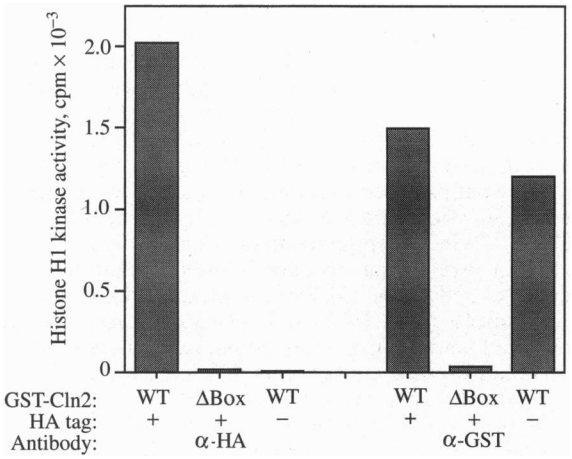

FIG. 3. Activated Cdc28^{HA}p protein kinase is associated with GST–Cln2p. Wild-type GST-Cln2p (WT) or GST–Cln2ΔBox (ΔBox) (at 35 μM) was incubated for 30 min at 24°C in 200 μg of yeast extract containing either Cdc28p (−HA tag, prepared from RD205-3A) or Cdc28^{HA}p (+ HA tag, prepared from pRD84/RD205-3A), as indicated. After quenching, reaction products were immunoprecipitated with either 0.09 μl of α-HA ascites fluid or 10 μg of α-GST antibodies, and precipitates were assayed for histone H1 kinase activity.

active Cln2p/Cdc28^{HA}p complexes, we produced recombinant, mutated forms of Cdc28^{HA}p for analysis *in vitro*. Cdc28^{HA}p produced in *E. coli* was inactive when assayed as is (Fig. 5*A*, lane 1) or after exposure to either GST–Cln2p (lane 2) or yeast cell extract (lane 3; yeast cell extract contained nontagged Cdc28p). Recombinant Cdc28^{HA}p was potently activated, however, when simultaneously incubated with GST–Cln2p and yeast cell extract (Fig. 5*A*, lane 4). Activation of *E. coli*-produced Cdc28^{HA}p did not require wild-type Cdc28p resident in yeast cell extract. Extract prepared from a *cdc28^{ts}* (as compared to *cln1*, *-2*, and *-3*Δ) strain failed to yield p13^{suc1}– or glutathione–agarose-precipitable histone H1 kinase following exposure to GST–Cln2p (compare Fig. 5*A*, lanes 9 and 10 with lanes 7 and 8). The activation defect of *cdc28^{ts}* extract was fully rescued by the addition of *E. coli*-produced Cdc28^{HA}p (compare Fig. 5*A*, lanes 5 and 6 with lanes 7 and 8). *E. coli* extracts containing comparable amounts of either "wild-type" Cdc28^{HA}p (Fig. 5*B*, lane 2) or Cdc28^{HA}p-T169A (lanes 3 and 4) were mixed with *cdc28^{ts}* extract and supplemented with GST–Cln2p. Whereas wild-type Cdc28^{HA}p efficiently complemented *cdc28^{ts}* extract, Cdc28^{HA}p-T169A was inactive. Likewise, Cdc28^{HA}p mutated at a conserved lysine located in the active site of the homologous Cdk2 protein kinase (26) was also inactive (Fig. 5*B*, lanes 5 and 6).

DISCUSSION

We report the activation of epitope-tagged Cdc28p (Cdc28^{HA}p) by a G_1 cyclin in extracts of G_1-arrested yeast cells. A GST–Cln2p fusion protein purified from *E. coli* bound to and activated the histone H1 kinase activity of Cdc28^{HA}p in a crude extract. In addition to GST–Cln2p, activation of Cdc28^{HA}p required ATP and yeast extract protein. These requirements may reflect an essential role for a CAK homolog in yeast extract to phosphorylate Cdc28^{HA}p on Thr-169, as substitution of this conserved residue with alanine blocked GST–Cln2p-mediated activation of Cdc28^{HA}p.

Previous work has implicated the Cln proteins in the formation of an activated Cdc28p protein kinase during the G_1 phase (4, 5, 27, 28). Since activation of Cdc28p by G_1 cyclins is thought to trigger a positive-feedback loop resulting in elevated expression of a set of proteins including G_1 cyclins (8, 29, 30), it is difficult to determine whether the G_1 cyclins are the only proteins whose elevated synthesis is required for

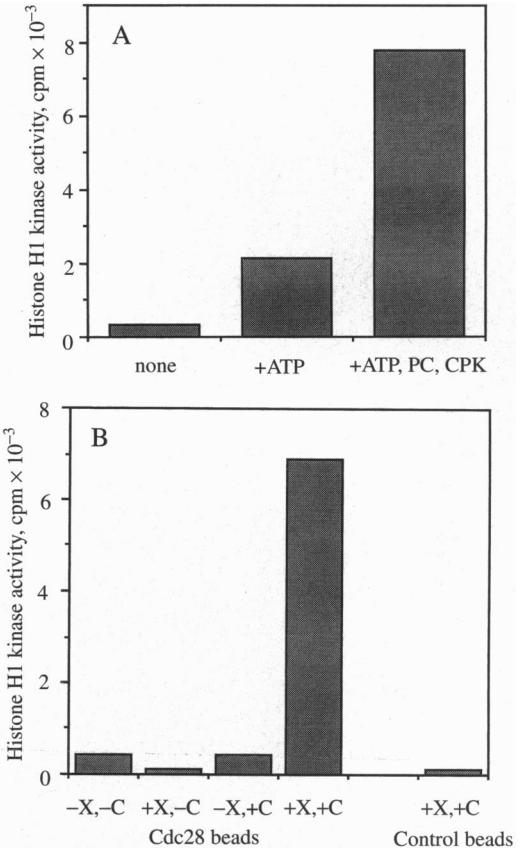

FIG. 4. Activation of Cdc28^{HA}p by GST–Cln2p requires ATP and cytosolic factors. (*A*) Activation reactions were conducted with no ATP regenerating system (none), 1 mM ATP alone (+ATP), or a complete ATP regenerating system [+ATP, PC (creatine phosphate), CPK (creatine kinase)]. Reaction mixtures were programmed with 35 nM GST–Cln2p and were incubated for 20 min at 24°C prior to immunoprecipitation of Cdc28^{HA}p and quantitation of histone H1 kinase. (*B*) Cdc28^{HA}p was immunoprecipitated with α-HA from 60 μg of cell extract prepared from *CDC28^{HA}* cells, and washed protein A–Sepharose beads (Cdc28 beads) containing immobilized immune complexes were incubated in the presence (+) or absence (−) of 150 μg of yeast extract (X) or 35 nM GST–Cln2p (C) for 30 min at 24°C. Control beads, same as above, except Cdc28p was immunoprecipitated with α-HA from 60 μg of cell extract prepared from *CDC28* cells. Following incubation, Cdc28 and control beads were washed and assayed for histone H1 kinase activity.

maximal Cdc28p activation. Experiments presented here indicate that Cln2p is sufficient to activate Cdc28^{HA}p in a cyclin-depleted G_1 extract, indicating that any components required for Cdc28^{HA}p activation besides Cln proteins are already present before the positive-feedback loop is mobilized. Although addition of GST–Cln2p to G_1 extract was sufficient to activate endogenous Cdc28^{HA}p, incubation of purified GST–Cln2p with purified Cdc28^{HA}p did not yield active kinase. In addition to GST–Cln2p, ATP and yeast cytosol were required for Cdc28^{HA}p activation. Since a mutant of Cdc28^{HA}p lacking the conserved Thr-169 target of CAK was inactive, it is likely that yeast extract supplied a CAK-like entity required for Cdc28^{HA}p activation. In agreement with the results we have obtained *in vitro*, the presence of a threonine at residue 169 appears crucial for *CDC28* function during the G_1 phase *in vivo* (P. Sorger, personal communication).

The histone H1 kinase activity of Cdc28p in synchronized yeast cultures is very low during the G_1 phase and increases gradually thereafter, reaching a maximum in mitosis (11, 12). Immunoprecipitation experiments suggest that only a very small fraction of Cdc28^{HA}p histone H1 kinase activity is

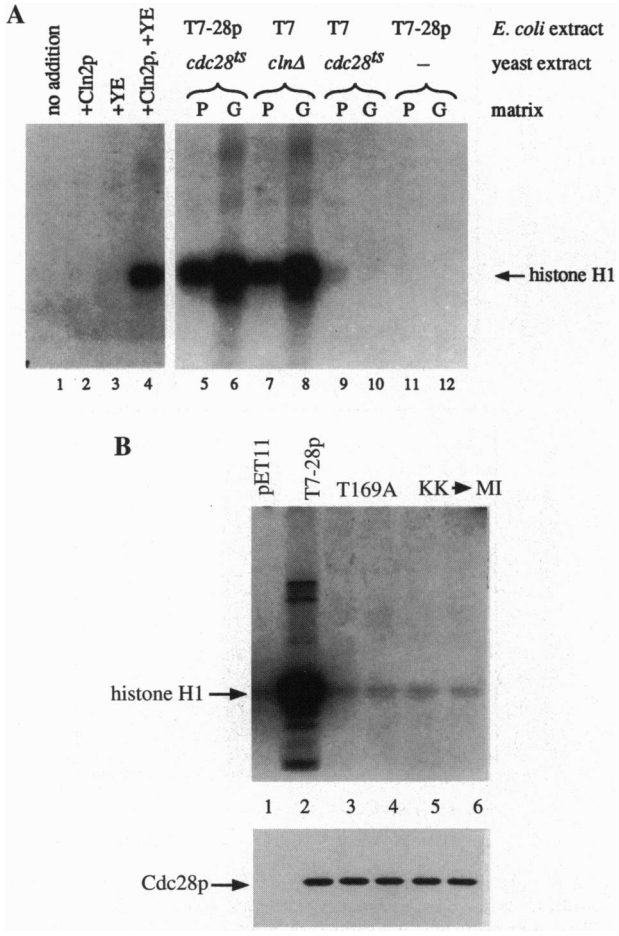

FIG. 5. Stimulation of $Cdc28^{HA}p$ protein kinase by GST–Cln2p appears to require a CAK-like activity. (*A*) *E. coli* extract (25 μg, containing ≈100 ng of $Cdc28^{HA}p$) was incubated under standard conditions for 35 min with no additions (lane 1), 25 nM GST–Cln2p (+Cln2p; lane 2), 175 μg of yeast cell extract (YE; lane 3), or 25 nM GST–Cln2p plus 175 μg of yeast extract (lane 4), followed by immunoprecipitation of $Cdc28^{HA}p$ and detection of phosphorylated histone H1. *E. coli* extract (25 μg) either containing (T7-28p) or lacking (T7) $Cdc28^{HA}p$ was incubated with 180 μg of cell extract prepared from either *cdc28ts* cells or 205-3A (*clnΔ*) cells for 30 min as indicated. $Cdc28^{HA}p$ complexes were retrieved for kinase assay by adsorption to either $p13^{suc1}$–Sepharose beads (P, lanes 5, 7, 9, and 11) or glutathione–agarose (G, lanes 6, 8, 10, and 12). (*B Upper*) Histone H1 kinase assay of $Cdc28^{HA}p$ immunoprecipitates from 45-min activation reactions conducted in 180 μg of extract prepared from *cdc28ts* cells and supplemented with 50 nM GST–Cln2p plus 15 μg of extract prepared from *E. coli* cells expressing the indicated $CDC28^{HA}$ alleles. pET11, T7 expression vector alone; T7-28p, "wild-type" $CDC28^{HA}$; T169A, $CDC28^{HA}$ with alanine substituted for Thr-169; KK → MI, $CDC28^{HA}$ with methionine and isoleucine substituted for lysines 40 and 41, respectively. Two independent clones of both the T169A and KK → MI mutants were assayed. (*B Lower*) α-Cdc28p immunoblot of samples from the same reactions depicted in *Upper*.

associated with G_1 cyclins in lysates of asynchronous cells (4). Whereas the low level of Cdc28p activity observed in G_1 cells may reflect a low specific activity for G_1 cyclin/Cdc28p complexes as histone H1 kinases, we found that, at low doses, Cln2p was a potent activator of $Cdc28^{HA}p$ histone H1 kinase. Interestingly, GST–Cln2p became saturated for $Cdc28^{HA}p$ activation at relatively low concentrations. One possible explanation for this result is that only a fraction of Cdc28p is competent to assemble productively with Cln2p. The fraction of Cdc28p available to Cln2p could be governed by either posttranslational modification or Cdc28p-associated proteins.

Positive feedback on cyclin expression during the G_1 phase (8, 29, 30) complicates the interpretation of cell division cycle mutations or physiological treatments that result in G_1 arrest. One expects that all nonpermissive conditions will result in G_1 arrest with low levels of G_1 cyclin protein and Cdc28p kinase activity regardless of which step in the pathway leading to mobilization of the positive-feedback loop is blocked, and this is in fact what has been observed by other investigators (4, 5). In contrast, measuring the GST–Cln2p-dependent activation of $Cdc28^{HA}p$ in mutant extracts provides a direct measure of whether a given gene product is directly required for the assembly of active Cln/Cdc28p complexes. Preliminary experiments indicate that *CDC25* and *SIT4* are not required for the assembly of active Cln/Cdc28p complexes, whereas *CDC37* is (R.J.D. and Monica Gerber, unpublished data).

The extraordinary success of genetic analysis in the investigation of cell cycle control in both *S. cerevisiae* and *S. pombe* has resulted in the identification of about 100 genes that impinge on the cell division cycle in some manner. We are hopeful that the union of cell cycle genetics with biochemical exploration of cell cycle control proteins in yeast will lead to a better understanding of the enzymology of cell cycle control.

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1. Lorincz, A. T. & Reed, S. I. (1984) *Nature (London)* **307**, 183–185.
2. Meyerson, M., Enders, G. H., Wu, C. L., Su, L. K., Gorka, C., Nelson, C., Harlow, E. & Tsai, L. H. (1992) *EMBO J.* **11**, 2909–2917.
3. Hadwiger, J. A., Wittenberg, C., Richardson, H. E., de Barros-Lopes, M. & Reed, S. I. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 6255–6259.
4. Wittenberg, C., Sugimoto, K. & Reed, S. I. (1990) *Cell* **62**, 225–237.
5. Tyers, M., Tokiwa, G. & Futcher, B. (1993) *EMBO J.* **12**, 1955–1968.
6. Fernandez-Sarabia, M. J., Sutton, A., Zhong, T. & Arndt, K. T. (1992) *Genes Dev.* **6**, 2417–2428.
7. Nasmyth, K. & Dirick, L. (1991) *Cell* **66**, 995–1013.
8. Ogas, J., Andrews, B. J. & Herskowitz, I. (1991) *Cell* **66**, 1015–1026.
9. Tang, Y. & Reed, S. I. (1993) *Genes Dev.* **7**, 822–832.
10. Reed, S. I. (1980) *Genetics* **95**, 561–577.
11. Reed, S. I. & Wittenberg, C. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 5697–5701.
12. Surana, U., Robitsch, H., Price, C., Schuster, T., Fitch, I., Futcher, A. B. & Nasmyth, K. (1991) *Cell* **65**, 145–161.
13. Fesquet, D., Labbe, J., Derancourt, J., Capony, J. P. & Galas, S. (1993) *EMBO J.* **12**, 3111–3121.
14. Poon, R. Y. C., Yamashita, K., Adamczewski, J. P., Hunt, T. & Shuttleworth, J. (1993) *EMBO J.* **12**, 3123–3132.
15. Solomon, M. J., Harper, J. W. & Shuttleworth, J. (1993) *EMBO J.* **12**, 3133–3142.
16. Field, J., Nikawa, J., Broek, D., MacDonald, B., Rodgers, L., Wilson, I., Lerner, R. A. & Wigler, M. (1988) *Mol. Cell Biol.* **8**, 2159–2165.
17. Sorger, P. & Murray, A. W. (1992) *Nature (London)* **355**, 365–368.
18. Booher, R. N., Deshaies, R. J. & Kirschner, M. (1993) *EMBO J.* **12**, 3417–3426.
19. Studier, F. W., Rosenberg, A. H., Dunn, J. J. & Dubendorff, J. W. (1990) *Methods Enzymol.* **185**, 60–89.
20. Sikorski, R. S. & Hieter, P. (1989) *Genetics* **122**, 19–27.
21. Smith, D. B. & Johnson, K. S. (1988) *Gene* **67**, 31–40.
22. Solomon, M. J., Glotzer, M., Lee, T., Philippe, M. & Kirschner, M. W. (1990) *Cell* **63**, 1013–1024.
23. Dunn, B. & Wobbe, C. R. (1993) in *Current Protocols in Molecular Biology*, eds. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (Greene and Wiley-Interscience, New York), Vol. 2, pp. 13.13.5–13.13.9.
24. Cross, F. R. (1990) *Mol. Cell. Biol.* **10**, 6482–6490.
25. Xiong, Y., Connolly, T., Futcher, B. & Beach, D. (1991) *Cell* **65**, 691–699.
26. Debondt, H. L., Rosenblatt, J., Jancarik, J., Jones, H. D. & Morgan, D. O. (1993) *Nature (London)* **363**, 595–602.
27. Tyers, M., Tokiwa, G., Nash, R. & Futcher, B. (1992) *EMBO J.* **11**, 1773–1784.
28. Cross, F. R. & Blake, C. M. (1993) *Mol. Cell. Biol.* **13**, 3266–3271.
29. Cross, F. R. & Tinkelenberg, A. H. (1991) *Cell* **65**, 875–883.
30. Dirick, L. & Nasmyth, K. (1991) *Nature (London)* **351**, 754–757.