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Interaction between Yeast Cdc6 Protein and B-Type Cyclin/Cdc28 Kinases

Suzanne Elsasser,* Feng Lou,† Bin Wang,† Judith L. Campbell,*‡ and Ambrose Jong†‡

*Braun Laboratories, California Institute of Technology, Pasadena, California 91125; and †University of Southern California Medical School, Department of Pediatrics and Microbiology, Division of Hematology and Oncology, Children's Hospital of Los Angeles, Los Angeles, California 90027

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During purification of recombinant Cdc6 expressed in yeast, we found that Cdc6 interacts with the critical cell cycle, cyclin-dependent protein kinase Cdc28. Cdc6 and Cdc28 can be coimmunoprecipitated from extracts, Cdc6 is retained on the Cdc28-binding matrix p13-agarose, and Cdc28 is retained on an affinity column charged with bacterially produced Cdc6. Cdc6, which is a phosphoprotein in vivo, contains five Cdc28 consensus sites and is a substrate of the Cdc28 kinase in vitro. Cdc6 also inhibits Cdc28 histone H1 kinase activity. Strikingly, Cdc6 interacts preferentially with B-type cyclin/Cdc28 complexes and not Cln/Cdc28 in log-phase cells. However, Cdc6 does not associate with Cdc28 when cells are blocked at the restrictive temperature in a *cdc34* mutant, a point in the cell cycle when the B-type cyclin/Cdc28 inhibitor $p40^{Sic1}$ accumulates and purified $p40^{Sic1}$ inhibits the Cdc6/Cdc28 interaction. Deletion of the Cdc28 interaction domain from Cdc6 yields a protein that cannot support growth. However, when overproduced, the mutant protein can support growth. Furthermore, whereas overproduction of wild-type Cdc6 leads to growth inhibition and bud hyperpolarization, overproduction of the mutant protein supports growth at normal rates with normal morphology. Thus, the interaction may have a role in the essential function of Cdc6 in initiation and in restraining mitosis until replication is complete.

INTRODUCTION

The yeast Cdc6 protein seems to be essential for cell cycle-specific activation of DNA replication origins. Early studies showed that *cdc6* mutants arrest at the nonpermissive temperature with 1N DNA and one large bud (Hartwell, 1976). Reciprocal shifts suggested a block in initiation, because *cdc6* mutants remain sensitive to hydroxyurea, which inhibits elongation, after shift-down from the nonpermissive temperature and are insensitive to a temperature block after release from hydroxyurea arrest (Hartwell, 1976). Plasmids containing a single origin of DNA replication are unstable in *cdc6* mutants, but this plasmid-loss phenotype is suppressed by the presence of multiple origins on a single plasmid, suggesting a direct interaction between Cdc6 and yeast autonomously replicating sequences (ARS) (Hogan and Koshland, 1992). Furthermore, analysis of replication intermediates formed at the nonpermissive temperature in a *cdc6* mutant show that origin firing is defective (Liang *et al.*, 1995). The Cdc6 protein produced in baculovirus-infected insect cells has been shown to coimmunoprecipitate with yeast ORC, the six protein complex that binds specifically to ARSs and is required for the initiation of DNA replication (Bell and Stillman, 1992; Bell *et al.*, 1993; Liang *et al.*, 1995). In fact, Cdc6 is highly homologous to the largest subunit of ORC, ORC1 (Bell *et al.*, 1995). Recent evidence suggests that Cdc6 associates with ORC late in mitosis or early in G1 and remains associated with ORC during G1, but it is not associated with ORC after origin firing for the duration of S phase, G2, and early M (Diffley and Cocker, 1992; Diffley *et al.*, 1994; Rowley *et al.*, 1995; Cocker *et al.*,

‡ Corresponding authors.

1996). Expression of the *CDC6* gene is periodic, mRNA levels accumulating transiently in late G1 and then again in late M or early G1 (Zhou and Jong, 1990; Bueno and Russell, 1992; Piatti *et al.*, 1995). Unlike other replication proteins, the Cdc6 protein seems to be unstable, and its intracellular levels mirror mRNA accumulation (Piatti *et al.*, 1995). If expression of Cdc6 is turned off in growing cells, they fail to initiate DNA replication upon depletion of Cdc6 but undergo an aberrant mitosis, segregating unreplicated chromosomes (Piatti *et al.*, 1995). A homologue of *CDC6*, called cdc18^{+}, exists in *Schizosaccaromyces pombe* (Kelly *et al.*, 1993). *cdc18* mutants are defective in the initiation of DNA replication; furthermore, *cdc18* mutants, like *cdc6*, fail to arrest in mitosis but divide with unreplicated chromosomes. In summary, Cdc6 has the properties of a prime regulator of replication initiation, but molecular evidence for its precise mechanism of action is very limited.

An unexplained property of Cdc6 is that *CDC6* rescues mitotic catastrophe in *S. pombe cdc2 wee1* mutants, and overproduction under a *GAL* promoter leads to a G2 delay in *Saccaromyces cerevisiae* (Bueno and Russell, 1992). This observation has been used to suggest that Cdc6 is part of a signal that restrains mitosis until S phase is complete. It has recently been shown that the G2 delay observed in *S. cerevisiae* upon overexpression of Cdc6 requires active Clb5,6 and that constitutive overproduction of Clb5 leads to an identical G2 delay as overproduction of Cdc6 (Basco *et al.*, 1995). Clb5,6 is required for entry into S phase and, interestingly, also perhaps for preventing re-replication in the G2 phase of the cell cycle (Epstein and Cross, 1992; Richardson *et al.*, 1992; Kuhne and Linder, 1993; Schwob and Nasmyth, 1993; Dahmann *et al.*, 1995). Overproduction of *CLB5* also leads to constitutive overproduction of Cdc6 mRNA, which might account for the similar phenotypes. However, the phenotype also might reflect a direct interaction. Either explanation is consistent with the observation that *cdc6*^{ts} mutants are suppressed by overproduction of *CLB5* (Basco *et al.*, 1995).

Recently we have found that Cdc6 interacts with Cdc28, that it is specific for B-cyclin/Cdc28 complexes but not Cln/Cdc28 complexes, and that the association is cell cycle-regulated. Deletion of the Cdc28 interaction domain has multiple effects on the cell cycle. We propose that this association is important for the regulation, resulting in the correct ordering of DNA replication and mitosis in the cell cycle.

MATERIALS AND METHODS

Strains

Glutathione-*S*-transferase (GST) and fusion proteins GST-Cdc6 and GST-Cdc6/K114E were expressed in **BJ2168** (*a prc1-407 prb1-1122 pep4-3 leu2 trp1 ura 3-52*) and **RJD635** (*a ura3 leu2 trp1 pep4::TRP1 cdc28::CDC28-HA::TRP1 bar1::LEU2*), which contains hemagglutinin-tagged Cdc28. GST and GST-Cdc6 were also expressed in strain **RJD561** (*a cdc28-4 ura3 leu2 pep4::TRP1*).

Tagged cyclins for kinase and inhibition assays were prepared from **YMT263** (*a cln2::CLN2-HA-LEU2 ade2-1 trp1-1 leu2-3, 112 ura3 GAL+*), **K3819** (*a ura3::ADH-HA-CLB5-URA ade2-1 trp-1 leu2-3, 112 GAL^{+}*), and **YMG17** (*a leu2-3, 112::GAL-CLB2-HA-LEU2 ade2-1 trp1-1 ura3 GAL^{+}*) (Peter and Herskowitz, 1994).

Extracts containing tagged cyclins for binding experiments were prepared from **RJD934** (*a can1-100, leu2-3, 112, his3-11, 15, trp1-1, ura3-1, ade2-1, pep4::TRP1, leu2::GAL-CLN2-HA::LEU2::URA3*), **K3819**, and **JCY61**. Strain **JCY61** was prepared by crossing **K3819** with **RJD668** (*a leu2, trp1, ura3, pep::TRP1, cdc34-2*), sporulating, and selecting for segregants that were prototrophic for uracil and temperature-sensitive for growth. (All RJD strains were the gift of Ray Deshaies, California Institute of Technology, Pasadena, CA.)

Complementation experiments were performed with **K4055** (a *leu2-3, can1-100, ade2-1, GAL, CDC6::hisGURA3hisG, trp1-1::TRP1 MET-CDC6*) with *URA3* looped out (Piatti *et al.*, 1995), in which the only copy of *CDC6* is controlled by the methionine-repressible *MET3* promoter. Incubation of this strain in 2 mM methionine yields a conditional null.

Plasmids

Plasmids for the expression of GST fusion proteins in yeast were prepared as follows: YGp10, a yeast vector containing the *GAL1,10* promoter, was prepared by cloning an *Eco*RI fragment containing the entire *GAL1,10* promoter into the *Eco*RI site of YEp352 (Hill *et al.*, 1986) with the *Bam*HI site in the promoter cassette proximal to the polylinker. YGpKTU6, encoding GST-Cdc6, was generated in two steps. *CDC6* was first amplified from the *Pst*I site to the end of the gene (Zhou *et al.*, 1989), and the resulting *Pst*I-*Hin*dIII fragment was cloned into the same sites in YGp10, generating YGp10-PH6. The N terminus of the fusion protein was created by amplifying the bacterial GST fusion protein (described below) from the start codon through the *Pst*I site. The resulting *Sac*I-*Pst*I fragment was cloned into the same sites of YGp10-PH6, resulting in YGpKTU6. YGpME6, encoding GST-Cdc6/K114E, was generated by replacing a *Pst*I fragment of YGpKTU6, containing both gene and vector sequence, with a similar fragment in which lysine 114 had been mutated to glutamate. pRD56a contained GST alone, driven by the *GAL1,10* promoter, and was provided by Ray Deshaies.

Plasmids for the expression of *CDC6* and *cdc6ΔN*, a mutant missing the N-terminal 47 amino acids, from the *GAL1,10* promoter were generated as follows: YGp618, containing the *CDC6* gene, was generated by cloning *CDC6* from the *Ssp*I site upstream of the translational start site to the *Spe*I site downstream of the stop codon into YGp10. pSE19, containing *cdc6ΔN*, was generated by cutting YGp618 with *Bam*HI and *Pst*I, which cuts *CDC6* at residue 48, and replacing the fragment with an oligonucleotide cassette comprising SE27 and SE28, allowing for expression from residue 48 of *CDC6*.

Plasmids for the expression of *CDC6* and *cdc6ΔN* from the natural promoter were prepared as follows: the *CDC6* promoter from the *Xho*I site to the translation start site was amplified by polymerase chain reaction (PCR) with oligos SE23 and SE24. *Sac*I and *Bam*HI sites were introduced into the 5′ and 3′ ends, respectively. This promoter was cloned into pBluescript SK(−), generating pSE14. The full-length *CDC6* open reading frame was amplified by PCR with oligonucleotides SE35 and SE37, which introduced *Bam*HI and *Nde*I sites upstream of the coding sequence and a *Hin*dIII site downstream. This fragment was cloned into the *Bam*HI and *Hin*dIII sites of pSE14 to generate pSE20. For expression in yeast, pSE20 was cut with *Sac*I and *Hin*dIII, and the resulting fragment containing both promoter and coding region was cloned into the same sites in pRS315 (Sikorski and Hieter, 1989) to generate pSE26. *cdc6ΔN* was amplified by PCR with oligos SE36 and SE37, which introduced *Bam*HI and *Nde*I sites just upstream of the *Pst*I site and a *Hin*dIII site downstream of the stop codon. This fragment was cloned into the *Bam*HI and *Hin*dIII sites of pSE14 to generate pSE21. pSE21 was cut with *Sac*I and *Hin*dIII and cloned into pRS315 to generate pSE27.

Oligonucleotides

The following oligonucleotides were used in this work: SE23 GCGAAACCTAGAGGCTTTCTCGAGGCAAAAATTTTTCCAA, SE24 TCAATAGTACCATATACGTTCATGTCAGCTATACCAA, SE27 GATCCGGCATATGGGCGGCCTGCA, SE28 GGCCGCCCATATGCCG, SE35 GGAGGATCCGGAGGCCATATGTCAGCTATACCAATAACT, SE36 GGAGGATCCGGAGGCCATATGCTGCAGTTTGGCTCAC, and SE37 GGCCAAAGCTTCATTGTACCCCACCTC.

Expression and Purification of GST and Fusion Proteins from Yeast

Proteins were expressed in the yeast strains BJ2168 and RJD635 as follows: cells were grown to a density of 2×10^7 cells/ml in minimal medium containing raffinose, induced with 2% galactose, and harvested after 4 h. Extracts were prepared by glass bead lysis in buffer A (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.2% Nonidet P-40, 10% glycerol, 1.5 mM dithiothreitol [DTT], 2 mM EDTA, and 1 mM ethylene glycol bis(β-aminoethyl)ether-*N,N,N′,N′*-tetra-acetic acid [EGTA]) with protease inhibitors (60 μg/ml AEBSF [4-(2-aminoethyl)-benzenesulfonylfluoride, hydrochloride], 160 μg/ml benzamidine, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml antipain), and cleared by spinning at 35,000 rpm for 20 min at 4°C. Extract was incubated with glutathione-agarose beads at a ratio of 200 mg of extract per milliliter of matrix for 3 h at 4°C with gentle agitation. Beads were washed extensively with buffer A and then with buffer B (buffer A with 0.15 M salt in place of 0.5 M salt). Protein was eluted with 5 mM reduced glutathione in buffer B by incubating overnight at 4°C. Expression from RJD561 was as described above, except that cells were grown at 23°C.

Expression and Purification of bGST-Cdc6 Proteins and Charging of Beads with the Recombinant Proteins

All vectors for bacterial expression were based on pGEX-2T. bGST-CDC6 contains GST fused to the *CDC6* open reading frame. GST-NB contains GST fused to *CDC6* from the first ATG to the *Bgl*II site at residue 190. GST-NP contains GST fused to *CDC6* from the first ATG to the *Pst*I site at residue 48. GST-BE contains GST fused to a C-terminal fragment beginning at the *Bgl*II site at residue 190 and ending at the natural termination codon. Proteins were expressed by growing *Escherichia coli* DH5α cells containing these vectors to an OD_{600} of 0.6, inducing with 1 mM isopropyl thiogalactoside (IPTG) and shifting to 30°C, and harvesting after 4 h. Extracts were prepared by lysing cells in buffer C (50 mM Tris-HCl, pH 7.2, 50 mM NaCl, 10 mM DTT, 10% glycerol, 0.5% NP-40, and 0.5 mM EDTA) with protease inhibitors (as above). For each fusion protein, beads were prepared by incubating extract (3–5 mg of protein) of cells expressing fusion protein with 60 μl of glutathione-agarose beads for 1 h and then washing in lysis buffer.

Preparation of Hemagglutinin Epitope-tagged Cyclins

For preparation of extract for either kinase or binding assays, strains were grown as follows: RJD635, YMT263, and K3819 were grown in YPD to a density of 4×10^7 cells/ml and harvested. YMG17 cells were grown in YPRaffinose; expression of HA-Clb2 was induced with 2% galactose at a density of 2×10^7 cells/ml and harvested after 2h. RJD934 cells were grown to saturation in YPD, inoculated into YPGalactose at a dilution of 1:500, grown for 16 h, and harvested. Extracts were prepared by glass bead lysis in YEB (25 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid [HEPES], pH 7.4, 150 mM NaCl, 0.2% NP-40, 5% glycerol, 0.5 mM DTT, 2 mM EDTA, 5 mM NaF, and protease inhibitors, as described).

Preparation of Cdc6 Antibodies

Cdc6 antibody was generated to the C-terminal 330 amino acids of *CDC6* fused to *TrpE* and expressed in bacteria. Antibodies to TrpE were removed by passing serum over a TrpE affinity column.

Immunoprecipitations

12CA5 antibody was incubated for 1 h with extract prepared in YEB at a ratio of 1–5 μg of antibody per milligram of extract. One hundred microliters of 100 mg/ml protein A-Sepharose slurry was added and incubated for an additional hour. Beads were washed extensively with YEB before use in kinase assays.

Protein Kinase Assays

Reactions for immunoprecipitated kinases and kinases copurifying with GST-Cdc6 were performed in 50 mM Tris-HCl, pH 7.5, 2 mM DTT, 10 mM $MgCl_2$, 1 mM ATP, and 500 cpm/pmol [γ-^{32}P]ATP for 30 min at 37°C. Samples were electrophoresed on a 10% denaturing gel and autoradiographed. Protein kinase A (PKA) was ordered from New England Biolabs (Beverly, MA), and assays were performed per manufacturer's instructions.

Complementation of a MET3-CDC6 Strain by CDC6 and cdc6ΔN

Plasmids expressing *GAL-CDC6*, *GAL-cdc6ΔN*, *CDC6*, or *cdcΔN* were transformed into K4055 (see MATERIALS AND METHODS for description of plasmids and strain), and transformants were selected on medium lacking methionine and uracil (YEp352 series, *GAL1, 10* promoter) or lacking methionine and leucine (pRS315 series, *CDC6* promoter). Colonies were purified on the same medium and then tested for their ability to grow on solid medium containing 2 mM methionine. The *GAL1,10* promoter series was tested on medium containing glucose only, raffinose only, glucose and galactose, and raffinose and galactose as carbon sources. The *CDC6* promoter series was plated on medium containing glucose. All sugars were present at a concentration of 2%. Cells were grown on plates for 3 d, at which time both colonies and cells were photographed.

RESULTS

Cdc6 and Cdc28 Protein Interact

To study the molecular mechanisms by which Cdc6 participates in DNA replication, Cdc6 was purified from yeast as a GST-Cdc6 fusion protein (Figure 1A). The purified protein was assessed for biochemical activities that might be associated with a protein with the properties defined by genetic and molecular biological analysis of the *CDC6* gene. In particular, ATPase activity was investigated, but no ATPase activity could be demonstrated as intrinsic to the protein. Others have reported that an ATPase associates with bGST-Cdc6 purified from *E. coli* (Zwerschke *et al.*, 1994), and this discrepancy will be discussed elsewhere (Jong and Campbell, unpublished data). Given the pivotal role that Cdc6 is likely to play in the initiation of DNA replication, we next examined whether Cdc6 might interact with the Cdc28 protein kinase, the action of which is necessary for the firing of replication origins (Epstein and Cross, 1992; Richardson *et al.*, 1992; Kuhne and Linder, 1993; Schwob and

Nasmyth, 1993). As shown in the protein blot in Figure 1B, Cdc28 is indeed associated with the purified, recombinant Cdc6 protein. When glutathione-agarose charged with GST-Cdc6 is washed with buffer containing 1.5 M NaCl, bound Cdc28 can still be detected to the same degree (Jong and Campbell, unpublished data). Thus, the proteins seem to interact with very high affinity.

Because Cdc6 is overproduced in the experiments shown in Figure 1B, it was possible that the observed association was an artifact of overproduction. To show that Cdc6 interacts with Cdc28 at physiological concentrations and to confirm that endogenous Cdc6, as well as the yGST-Cdc6 fusion protein, binds to Cdc28, the association was investigated in wild-type yeast cells, that is, cells carrying neither tagged nor overproduced Cdc6 or Cdc28. Cdc28 was enriched on p13 agarose beads, and the Cdc28/p13 beads were assessed for bound Cdc6 by polyclonal Cdc6 antibody. As shown in Figure 2, Cdc6 is associated with Cdc28.

To demonstrate that copurification shown in Figure 1 was due to interaction between the two proteins, we coexpressed hemagglutinin epitope-tagged Cdc28 (HA-Cdc28) and yGST-Cdc6 in yeast. yGST-Cdc6 was found in immunoprecipitates of HA-Cdc28 prepared with the anti-epitope antibody 12CA5 (Figure 3A), but not in 12CA5 immunoprecipitates in which tagged Cdc28 was absent (Figure 3B). Similarly, HA-Cdc28 was present in glutathione-agarose–purified yGST-Cdc6, but not in yGST preparations (Figure 3C). In a separate set of experiments, we had observed that mutation of lysine 114 to glutamate generated a Cdc6 protein that failed to complement a *cdc6*Δ mutant (Jong and Campbell, unpublished data). We asked whether this defect in growth was correlated with a lack of Cdc28 binding and observed that the binding of Cdc28 to the mutant protein was equivalent to wild type (Figure 3A). Therefore, using diverse methodologies, we observed that the Cdc6 and Cdc28 proteins can form a stable and specific complex, although we estimate that only a small fraction of the total cellular Cdc28 is present in complexes with Cdc6.

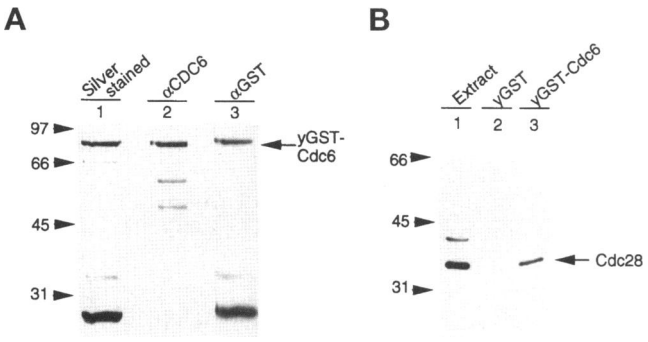

Figure 1. Cdc28 copurifies with Cdc6. (A) Electrophoretic analysis of yGST and yGST-Cdc6 expressed in yeast and affinity-purified on glutathione-agarose. Glutathione-*S*-transferase (GST) and GST-tagged Cdc6 were expressed in yeast and purified as described (see MATERIALS AND METHODS). Purified yGST-Cdc6 (100 ng) was electrophoresed, stained with silver (lane 1), and analyzed by immunoblotting with Cdc6 antibody (lane 2) and GST antibody (lane 3). Cdc6 antibody is described in MATERIALS AND METHODS. (B) Cdc28 copurifies with yGST-Cdc6. Purified yGST and yGST-Cdc6 protein were electrophoresed, blotted, and probed with Cdc28 antibody (gift of R. Deshaies, California Institute of Technology, Pasadena, CA). Lane 1, 100 μg of extract; lane 2, 100 ng of yGST; lane 3, 100 ng of yGST-Cdc6.

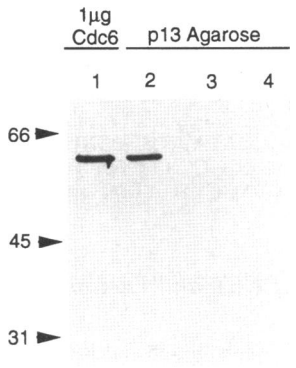

Figure 2. Association of endogenous Cdc6 with endogenous Cdc28. BJ2168 extract (10 mg) was prepared, and Cdc28 was precipitated by incubating extract with 20 μl of p13 agarose, followed by extensive washing. Protein was eluted, electrophoresed, and immunoblotted with Cdc6 antibody. Lane 1, 1 μg of conventionally purified, nontagged Cdc6 (to be described elsewhere); lanes 2–4, p13 affinity-purified Cdc28. Immunoblots were prepared as follows: lanes 1 and 2 were probed with Cdc6 antibody; lane 3 was probed with Cdc6 antibody, but secondary antibody was omitted; lane 4 was probed with nonspecific antiserum. Cdc6 was not present on control beads (no p13) incubated with extract (our unpublished observations).

The N-terminal 47 Amino Acids of Cdc6 Are Necessary and Sufficient for Cdc28 Binding

To identify the region of the Cdc6 protein that is important for interaction with Cdc28, we performed a deletion analysis. Full-length and truncated bGST-Cdc6 proteins were expressed in *E. coli* (Figure 4A), purified with glutathione-agarose beads, and incubated with yeast extracts in the presence or absence of ATP. After extensive washing, the bound proteins were eluted and analyzed by Western blotting. Cdc28 present in the yeast extract was clearly retained on the bGST-Cdc6 beads (Figure 4B, lane 1). This analysis revealed that the N-terminal 47 amino acids of Cdc6 are sufficient for interaction with Cdc28 (Figure 4B, lane 7). This region contains several Cdc28 recognition sites, two of which overlap a putative bipartite nuclear localization signal and one of which is adjacent to a potential PEST sequence (Zhou *et al.*, 1989). The 330 amino acid C-terminal domain did not form a complex with Cdc28 (Figure 4B, lane 3). Interestingly, Cdc28 associated with the bGST-Cdc6 only in the presence of ATP. This dependence does not seem to reflect a requirement for the Cdc6 nucleotide binding site (Zhou *et*

al., 1989), because fusions lacking the nucleotide binding site (NP) still bind Cdc28. Furthermore, proteins with a mutation (K114E) in the consensus nucleotide binding domain (Jong and Campbell, unpublished data) also interact with Cdc28 to the same extent as wild type. Thus, the role of the ATP binding site does not seem to be to promote the interaction with Cdc28.

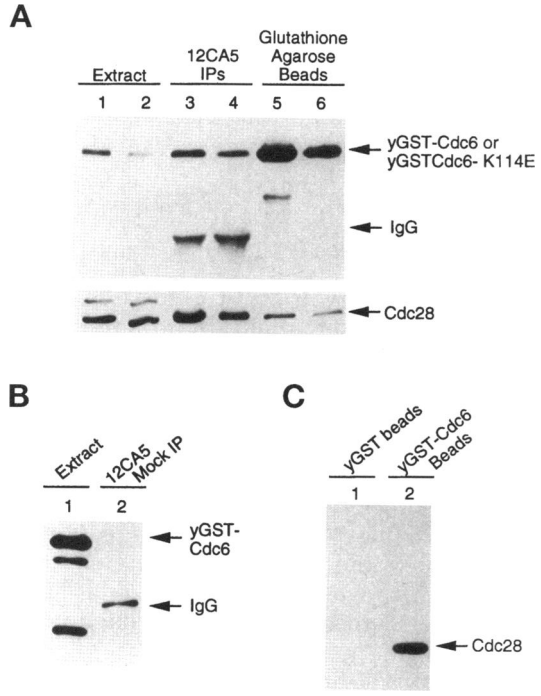

Figure 3. Association of Cdc28 with Cdc6 evaluated by coexpressing yGST-Cdc6 and HA-Cdc28 in yeast. yGST, yGST-Cdc6/K114E, and yGST-CDC6 were expressed in strain RJD635, containing hemagglutinin-tagged Cdc28. GST-Cdc6/K114E was generated by mutating lysine 114, which falls in the putative nucleotide-binding motif GTGKT, to glutamate. This mutant does not complement a deletion of cdc6 (Jong and Campbell, unpublished data). Extracts were prepared, and 1-mg samples were used for 12CA5 immunoprecipitations and glutathione-agarose purifications, omitting the elution step. (A) Cdc28 copurifies with yGST-Cdc6. Immunoprecipitates and glutathione-agarose–purified protein were run on 8 and 12% SDS-gels and transferred to Immobilon. The blot of the 8% gel was probed with GST antibody (top panel), and the blot of the 12% gel was probed with Cdc28 antibody (bottom panel). Top, lanes 1 and 2, 8% gel, 30 μg of extract; bottom, lanes 1 and 2, 12% gel, 3 μg of extract; lanes 3 and 4, 12CA5 immunoprecipitates; lanes 5 and 6, glutathione-agarose–bound protein. Odd-numbered lanes, yGST-Cdc6/K114E; even-numbered lanes, yGST-Cdc6. (B) Anti-GST immunoblot showing that GST-Cdc6 is not precipitated by 12CA5 when expressed in a strain lacking HA-tagged Cdc28. Lane 1, 25 μg of extract with GST-Cdc6 expressed in BJ2168; lane 2, 12CA5 immunoprecipitation from 2 mg of the same extract. (C) 12CA5 immunoblot showing that Cdc28 is only present in glutathione-agarose–purified yGST-Cdc6 and not in purified GST. Shown are, lane 1, purified yGST and, lane 2, purified yGST-Cdc6, as in Figure 3A, lane 6.

Binding of Cdc6 to Different Cyclin/Cdc28 Complexes

In *S. cerevisiae*, the major cell cycle transitions are accompanied by accumulation of different classes of cyclins: *CLN1-3*, non-B-type cyclins present in G1 and involved in commitment to the cell cycle and budding; *CLB5* and *6*, B-type cyclins active at G1/S through G2 and involved in the initiation of DNA replication and perhaps in prevention of re-replication in S, G2, and M; and *CLB1,2*, B-type cyclins active at G2/M and required for passage through mitosis. Additional B-type cyclins, *CLB3,4*, are expressed during S, G2, and M. Because Cdc6 is required for specific cell cycle transitions and not continuously during the cell cycle, one might then expect a specific cyclin class to be present in the Cdc28/Cdc6 complexes. To test this, we immobilized yGST-Cdc6 on glutathione beads and mixed it with extracts expressing either HA-Cln2 or HA-Clb5. HA-Clb5 was retained on the beads, whereas HA-Cln2 was not (Figure 5). HA-Clb2, like HA-Clb5, was also found to interact with yGST-Cdc6 in cells coexpressing the two tagged proteins (our unpublished observations). Therefore, there is specific formation of B-type cyclin/Cdc28/Cdc6 complexes, suggesting that there is cell cycle-specific binding of Cdc6 to Cdc28 and not simply nonspecific binding.

Cdc6 Is an In Vitro Substrate of Cdc28 Kinases

Although the stable association does not necessarily indicate that Cdc6 is a substrate of the kinase, the fact that there are three potential Cdc28 phosphorylation sites within the 47 N-terminal amino acids of Cdc6 suggested that Cdc6 may be phosphorylated by Cdc28 (Zhou *et al.*, 1989). As shown in Figure 3A, when affinity-purified yGST-Cdc6 containing Cdc28 was incubated with [γ-^{32}P]ATP, yGST-Cdc6, but not yGST, was efficiently phosphorylated. To confirm that the kinase activity was due to Cdc28, we expressed yGST-Cdc6 and yGST in the temperature-sensitive mutant *cdc28-4*. As shown in Figure 6A, phosphorylation of yGST-Cdc6 purified from the strain was highly thermolabile. The levels of cdc28-4 protein in the Cdc6 preparation were equal to or greater than the levels of Cdc28 present when Cdc6 was purified from wild-type cells. To confirm that the phosphorylated protein in Figure 6A was indeed Cdc6, we tested bGST-Cdc6 as a substrate of hemagglutinin epitope (HA)-tagged Cdc28. HA-Cdc28 was immunoprecipitated from asynchronously growing yeast and incubated with bGST or bGST-Cdc6 and [γ-^{32}P]ATP. bGST-Cdc6 was phosphorylated, but not bGST (Figure 6B). As a preliminary assessment of the possible physiological significance of Cdc6 phosphorylation, cells were labeled with $^{32}P_i$, and Cdc6 was shown to be a phosphoprotein in vivo (Figure 6C). Phosphoamino acid determi-

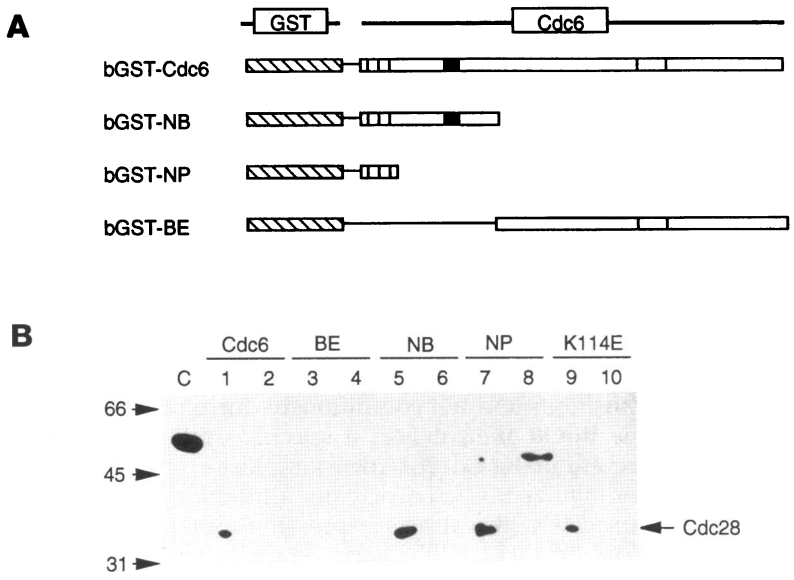

Figure 4. Mapping of the region of Cdc6 required for binding Cdc28. (A) Schematic of the *CDC6* truncations and summary of binding. Cross-hatched region, GST tag; vertical black lines, putative Cdc28 phosphorylation sites; black box, nucleotide binding site. Constructs: bGST-Cdc6, GST fused to full-length *CDC6*; bGST-NB, GST fused to residues 1–190 of *CDC6*; bGST-NP, GST fused to residues 1–47 of *CDC6*; bGST-BE, GST fused to residues 191–513 of *CDC6*. (B) Binding of bGST-Cdc6 derivatives to Cdc28. bGST-Cdc6 and mutant proteins were expressed in bacteria and beads bearing 10–20 μg of fusion proteins were prepared as described in MATERIALS AND METHODS. Charged beads were incubated with 11.25 mg of yeast extract in the presence (odd-numbered lanes) or absence (even-numbered lanes) of 1 mM ATP. After extensive washing of beads, protein was eluted and electrophoresed in SDS, and the presence of Cdc28 was evaluated by immunoblotting with Cdc28 antibody. Lane C, 1 μg of purified GST-Cdc28 (60 kDa) as control for Cdc28 antibody; lanes 1 and 2, bGST-Cdc6; lanes 3 and 4, bGST-BE; lanes 5 and 6, bGST-NB; lanes 7 and 8, bGST-NP; lanes 9 and 10, bGST-Cdc6/K114E.

nation showed the presence of phosphoserine (our unpublished observations).

Because yGST-Cdc6 binds preferentially to B-type cyclin/Cdc28 complexes, it was of interest to test the specificity of the Cdc28 kinase phosphorylating Cdc6. 12CA5 immunoprecipitates were prepared from strains carrying HA-tagged Cln2, Clb5, or Clb2 protein and tested for their ability to phosphorylate bGST-Cdc6. Because three putative Cdc28 phosphorylation sites and the Cdc28 interaction domain are located within the first 47 amino acids, bGST-NP, described in Figure 3, was tested first as a substrate with normalized levels of Cln and Clb kinases. It is clear that bGST-NP is preferentially phosphorylated by B-type cyclin/Cdc28 kinases (Figure 6D). The phosphorylated bands, labeled bGST-NP, were shown to cross-react with GST antibody. Because the Cdc6 antibody was prepared with a fusion protein missing the N-terminal 190 amino acids, the presence of Cdc6 amino acids could not be tested directly. Two additional potential Cdc28 phosphorylation sites are located near the N terminus of the protein, and therefore bGST-BE (see Figure 3A) was also tested as a substrate. bGST-BE is not detectably phosphorylated by any of

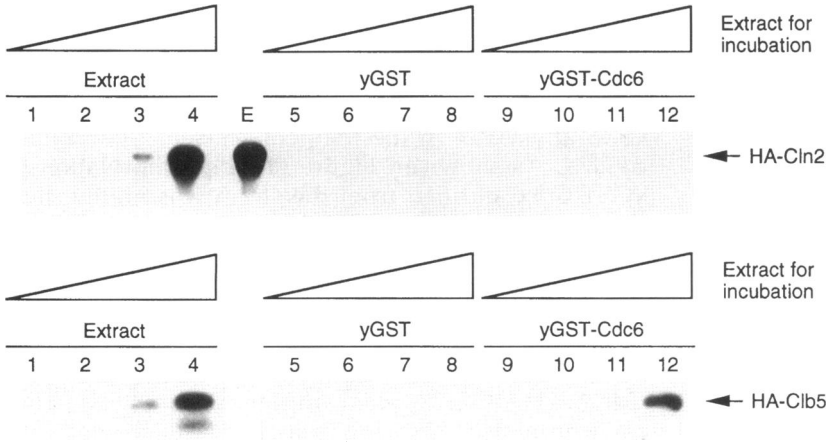

Figure 5. Binding of yGST-Cdc6 to Clb5/Cdc28 and not to Cln2/Cdc28. yGST and yGST-Cdc6 were expressed in yeast as described in MATERIALS AND METHODS and shown in Figure 1. Cdc6-containing beads were prepared by incubating extract with glutathione-agarose for 2 h, followed by washing, as described for purification of fusion proteins from yeast. Beads equivalent to 2 mg of extract were then incubated with extracts of cells expressing HA-tagged Clb5 or HA-tagged Cln2 and 2 mM ATP (Peter and Herskowitz, 1994). Strain RJD934 was used as a source of tagged Cln2. As shown by the 12CA5 Westerns on the left, the amount of extract of cells expressing equivalent amounts of HA-Cln2 or HA-Clb5 was determined, and the amount of extract incubated with beads was adjusted to contain approximately equal levels of HA-Cln2 (top panel) or HA-Clb5 (bottom panel). For HA-Cln2, four concentrations ranging from 0.08 to 10 mg protein/ml were used for binding; for HA-Clb5, four concentrations ranging from 0.02 to 2.0 mg protein/ml were used. yGST or yGST-Cdc6 beads were incubated in the diluted extracts and then washed, and the amount of cyclin retained was determined by immunoblotting. Lanes 1–4 (top and bottom panels), 10 μl of extracts at increasing concentration. Lane E (top panel only) shows an extract identical to lane 4 that was used as positive control for the HA-Cln2 Western after binding the Cdc6-beads. Lanes 5–8 (top and bottom panels) show tagged cyclin retained by yGST beads incubated in increasingly concentrated extract; lanes 9–12 (top and bottom panels) show tagged cyclin retained by yGST-Cdc6 beads incubated in increasingly concentrated extract.

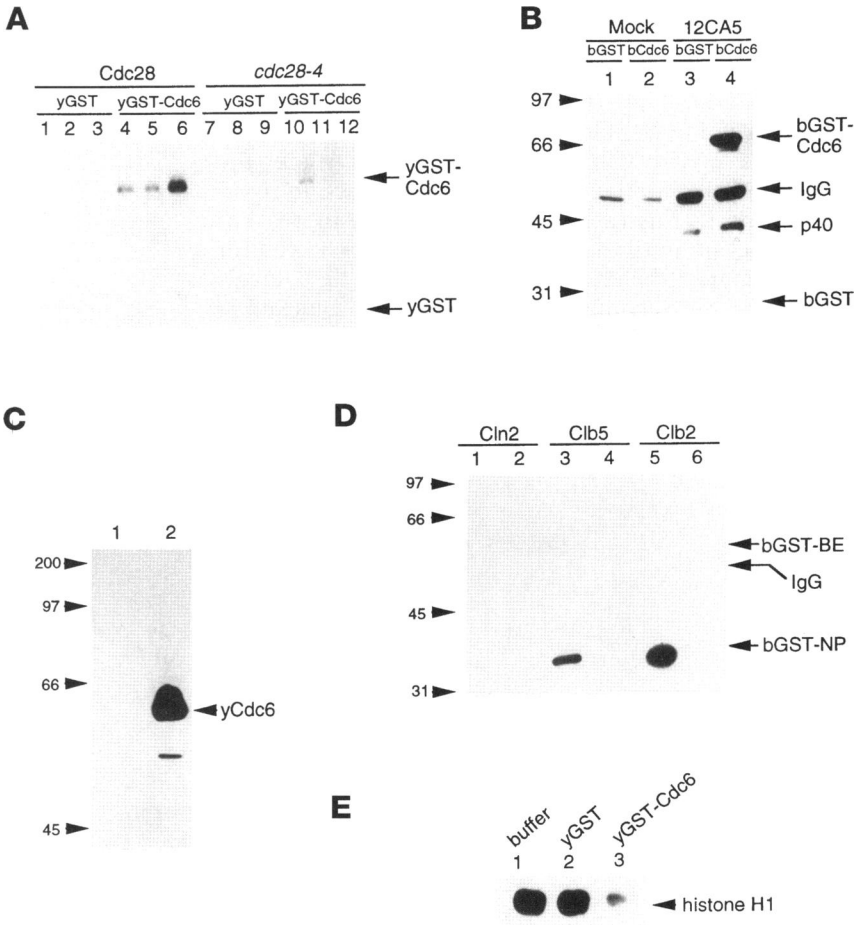

Figure 6. Cdc6 is a substrate for Cdc28 kinase and a phosphoprotein in vivo. (A) Cdc28 purifies with and phosphorylates Cdc6. yGST and yGST-Cdc6 were expressed as before and purified from wild-type (BJ2168) and *cdc28-4* (RJD561) strains. Purified proteins were assayed for associated kinase activity at 30, 37, and 42°C (see MATERIALS AND METHODS). The products of the kinase assay were run on a 10% polyacrylamide gel containing SDS and autoradiographed. Lanes 1–6 show protein purified from wild-type cells; lanes 7–12 show protein purified from *cdc28-4* cells. Lanes 1–3 and 7–9, 200 ng of yGST; lanes 4–6 and 10–12, 100 ng of yGST-Cdc6. Lanes 1, 4, 7, and 10 were assayed at 30°C; lanes 2, 5, 8, and 11 were assayed at 37°C; lanes 3, 6, 9, and 12 were assayed at 42°C. (B) Bacterially produced Cdc6 is a substrate for Cdc28. bGST and bGST-Cdc6 were expressed in bacteria and purified. Kinase assays were performed with hemagglutinin epitope-tagged Cdc28 immunoprecipitated from RJD635 extracts with 12CA5 antibody. Mock immunoprecipitates were prepared with extract lacking epitope-tagged Cdc28. Lanes 1 and 2 show mock immunoprecipitates prepared with 5 mg of extract; lanes 3 and 4 show 12CA5 immunoprecipitates prepared with 5 mg of extract. Lanes 1 and 3, 500 ng of bGST; lanes 2 and 4, 500 ng of bGST-Cdc6. The band labeled p40 has not been verified as $p40^{Sic1}$. (C) Cdc6 is phosphorylated in vivo. Log-phase cells were labeled with inorganic $^{32}P_i$ (Yoon and Campbell, 1991). Cdc6 immunoprecipitates were prepared from cell extract. Proteins were eluted, electrophoresed, and autoradiographed. Lane 1 shows mock immunoprecipitate with nonimmune rabbit serum; lane 2 shows Cdc6 immunoprecipitate. Potato acid phosphatase decreased the label, showing it was due to phosphorylation (our unpublished observation). (D) The major region phosphorylated in Cdc6 is within the N-terminal 47 amino acids. The fusion proteins bGST-NP and bGST-BE were purified from bacteria, as from yeast, as described in MATERIALS AND METHODS. The purity of the proteins was equivalent to that shown in Figure 1. Lanes 1, 3, and 5 indicate bGST-NP as substrate for the indicated kinases. Lanes 2, 4, and 6 indicate bGST-BE (see Figure 3) as substrate for the indicated kinases. (E) Cdc6 inhibits HA-Clb2/Cdc28 histone H1 kinase activity. yGST-Cdc6 and yGST were prepared as above. HA-Clb2/Cdc28 was immunoprecipitated from 50 μg of extract and assayed for histone H1 kinase in the presence of buffer (lane 1), 300 ng of yGST (lane 2), or 300 ng of yGST-Cdc6 (lane 3).

the kinases in our assays (Figure 6D), suggesting that the major cyclin B/Cdc28 phosphorylation sites are at the N terminus of Cdc6.

Cdc6 Protein Inhibits HA-Clb2/Cdc28 Kinase

Constitutive expression of *CDC6* has been shown to delay cells in G2 and to cause bud hyperpolarization (Bueno and Russell, 1992), phenomena that also occur when the mitotic cyclins are downregulated in G2 (Lew and Reed, 1993). Cdc6 may therefore directly or indirectly inhibit the mitotic Cdc28 kinases. We observed that in vitro Cdc6 directly inhibited phosphorylation of histone H1 by Cdc28 kinase prepared with either p13-agarose (our unpublished observations) or HA-Clb2 immunoprecipitates (Figure 6E). Because Cdc6 is also a substrate of B-type cyclin/Cdc28, the inhibition seen here may be competitive and therefore only of physiological significance when the Cdc6 protein is present in excess (see DISCUSSION). However, as shown below (Figure 8), the levels of Cdc6 used to inhibit the HA-Clb2 immunoprecipitates (Figure 6E) are only slightly higher than the levels of $p40^{Sic1}$ required to inhibit HA-Clb5 immunoprecipitates under the conditions of our assays.

$p40^{Sic1}$ Inhibits the Association of Clb5,6/Cdc28 and Cdc6 In Vivo and In Vitro

Because both Cdc6 and Clb5,6 are required for the initiation of replication (Epstein and Cross, 1992; Richardson *et al.*, 1992; Kuhne and Linder, 1993; Schwob and Nasmyth, 1993), we have investigated their association in greater detail. B-type cyclin-dependent ki-

nases are inhibited by $p40^{Sic1}$, which is expressed in late mitosis and early G1 (Schwob *et al.*, 1994). In late G1, $p40^{Sic1}$ is degraded by the *CDC34*-dependent pathway (Schwob *et al.*, 1994), leading to activation of Clb5/Cdc28 kinase. One possible role for the interaction of Cdc6 with Clb5/Cdc28 may be to trigger the conversion of the inactive origin into an active replication fork. In this case, $p40^{Sic1}$ may serve to prevent the interaction until the cell is completely prepared for S phase. To test the effect of intracellular $p40^{Sic1}$ levels on Cdc6/Cdc28 complex formation, we blocked a *cdc34* strain carrying the HA-tagged *CLB5* gene at the restrictive temperature, and the presence of Clb5 and its ability to associate with Cdc6 were assessed. Although HA-Clb5 present in asynchronous cultures

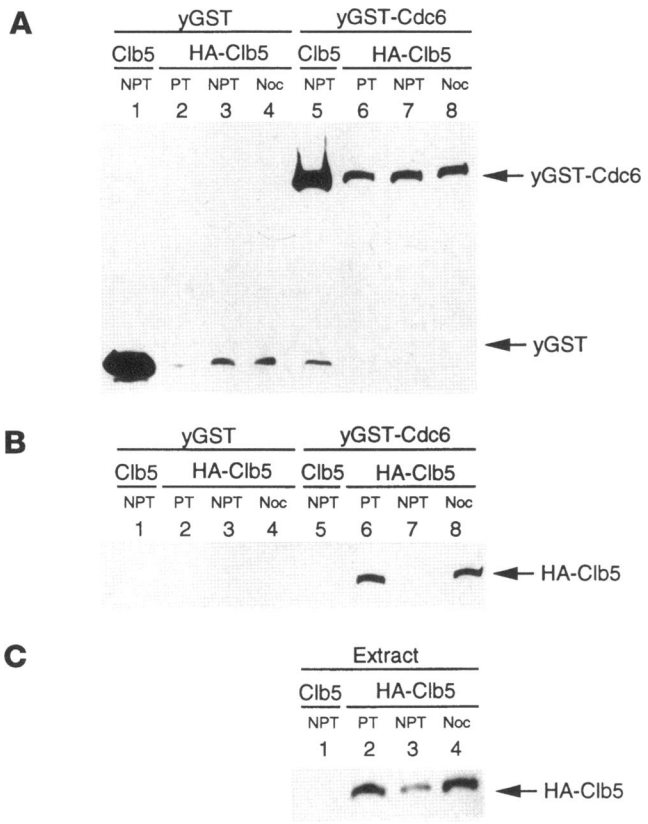

Figure 7. Clb5/Cdc28 binds to Cdc6 only in the absence of $p40^{Sic1}$. The experimental conditions were as in Figure 5. Beads charged with yGST or yGST-Cdc6 were incubated with extracts expressing HA-Clb5 and blocked at either the *cdc34* step or the Nocodazole step in the cell cycle. Beads were washed and analyzed by SDS gel analysis and immunoblotting. (A and C are controls, indicating that nearly equivalent amounts of material were used in these experiments in B.) (A) Anti-GST immunoblot of yGST protein beads (lanes 1–4) or yGST-Cdc6 beads (lanes 5–8) incubated in various extracts. Lanes 1 and 5 show extracts of RJD668 (*cdc34-2*) incubated for 4 h at the nonpermissive temperature; lanes 2 and 6 show extracts of JCY61 (*cdc34-2, ADH-CLB5-HA*; see MATERIALS AND METHODS) cycling at the permissive temperature; lanes 3 and 7 show extracts of JCY61 incubated for 4 h at the nonpermissive temperature; lanes 4 and 8 show strain JCY61 arrested with Nocodazole at the permissive temperature. (B) 12CA5 immunoblot of HA-Clb5 protein bound to bGST and bGST-Cdc6 beads (lanes are the same as in A). (C) Control 12CA5 immunoblot showing constitutive expression of HA-Clb5 in all JCY61 extracts.

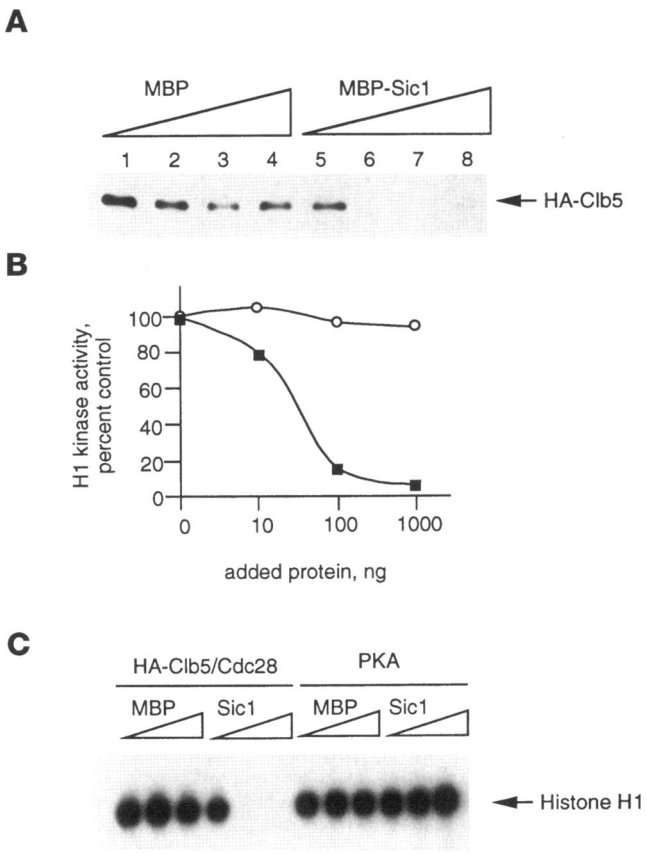

Figure 8. $p40^{Sic1}$ inhibits association of HA-Clb5 and Cdc6. Maltose binding protein (MBP) was fused to $p40^{Sic1}$. MBP and MBP-$p40^{Sic1}$ were expressed in *E. coli* and purified to homogeneity. The protein concentration was determined by Bradford analysis. The purified fusion protein was the gift of Dr. R. Deshaies, California Institute of Technology. (A) HA-Clb5 extract (0.25 mg) was incubated with increasing amounts of MBP or MBP-$p40^{Sic1}$ (0, 80 ng, 400 ng, and 2 μg). Preincubated extracts were then further incubated with bGST-Cdc6 beads in the presence of 2 mM ATP for 2 h. Beads were washed and analyzed for HA-Clb5 binding by protein blotting with 12CA5 antibody. (B) Inhibition of Clb5/Cdc28 activity by MBP-$p40^{Sic1}$. HA-Clb5 extract was incubated with MBP and MBP-$p40^{Sic1}$ as in A. HA-Clb5 was immunoprecipitated and assayed for histone H1 kinase activity. Open symbols, MBP; closed symbols, MBP-$p40^{Sic1}$. Protein concentration was determined by Bradford assay. (C) Inhibition of Clb5/Cdc28 by MBP-$p40^{Sic1}$ is specific. Histone H1 kinase activity of HA-Clb5 immunoprecipitated from 0.2 mg of extract was assayed in the presence of increasing amounts of MBP (ranging from 0 to 2 μg, first set of three lanes) and increasing amounts of MBP-$p40^{Sic1}$ (ranging from 0 to 2 μg, second set of three lanes). Histone H1 kinase activity of protein kinase A was assayed in the presence of increasing amounts of MBP (ranging from 0 to 2 μg, third set of three lanes) and increasing amounts of MBP-$p40^{Sic1}$ (ranging from 0 to 2 μg, fourth set of three lanes). Products were electrophoresed and autoradiographed. Phosphorylated histone H1 is indicated.

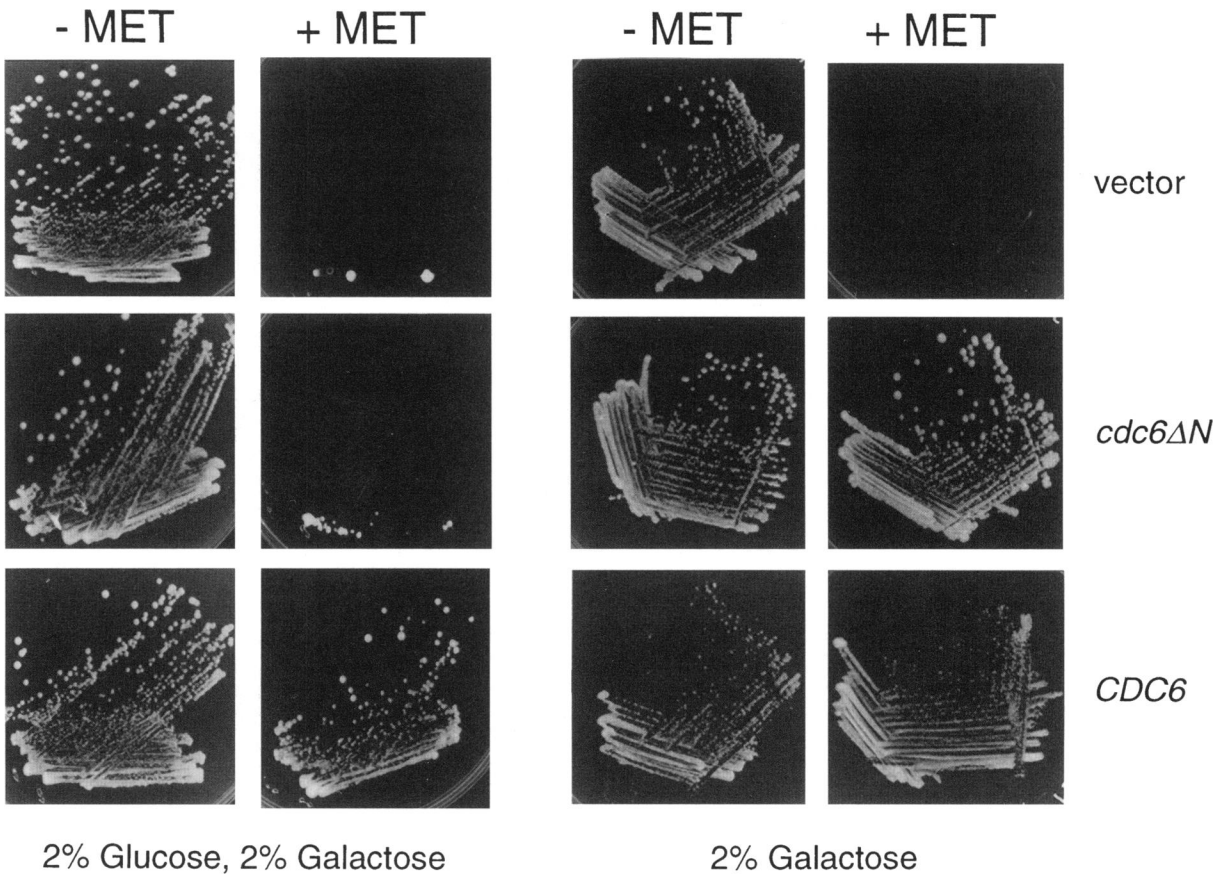

Figure 9. *Cdc6ΔN* complements a *cdc6* null only at elevated levels. Strain K4055 was transformed with plasmids YGp618 and pSE19, expressing *GAL-CDC6* and *GAL-cdc6ΔN* protein, respectively. The plasmid YGp10, carrying the *GAL1,10* promoter only, was used as a control. Transformants were streaked onto medium either lacking or containing 2 mM methionine and containing 2% dextrose and 2% galactose (*GAL*-repressing conditions) or 2% raffinose and 2% galactose (*GAL*-inducing conditions). Plates were incubated at 30°C for 3 d and then photographed.

was able to bind to Cdc6, HA-Clb5 present in *cdc34*-arrested cells, which cannot degrade $p40^{Sic1}$ and therefore contain high levels of $p40^{Sic1}$, did not bind (Figure 7). In contrast, in cells arrested in G2 with Nocodazole, where $p40^{Sic1}$ is absent, HA-Clb5/Cdc28 did associate with Cdc6 (Figure 7). This suggested that $p40^{Sic1}$ not only inhibits the kinase activity of Clb5/Cdc28 but also inhibits the association of Clb5/Cdc28 with Cdc6.

To establish that the lack of binding in the *cdc34*-arrested cells was due to $p40^{Sic1}$, we purified a maltose binding protein (MBP)-$p40^{Sic1}$ fusion protein (Figure 8, legend). As shown in Figure 8B, the fusion protein was active in inhibiting Clb5/Cdc28 histone H1 kinase activity and also inhibited the association of Clb5/Cdc28 with the yGST-Cdc6 beads (Figure 8A). As a control for the specificity of the inhibition, protein kinase A was shown to be resistant to inhibition by the MBP-$p40^{Sic1}$ fusion protein (Figure 8C). Therefore, Clb5/Cdc28 does not bind to Cdc6 in the presence of $p40^{Sic1}$, and binding in vivo, therefore, probably occurs after degradation of $p40^{Sic1}$.

Effect of Deletion of the N-Terminal 47 Amino Acids from CDC6

We showed above that the 47 N-terminal amino acids of *CDC6* are sufficient for interaction between Cdc6 and Cdc28. To evaluate the specific role of the Cdc28/Cdc6 interaction in vivo, we deleted the first 47 amino acids of *CDC6*. The phenotype of cells expressing only this *cdc6ΔN* mutant was evaluated by a *MET3-CDC6::TRP1cdc6Δ* strain. In the presence of methionine, this strain will grow only if a functional copy of *CDC6* is provided on a plasmid. Because the levels of Cdc6 protein are regulated dramatically in the cell cycle (Piatti *et al.*, 1995) and because the N-terminal 47 amino acids contain a PEST sequence that could be important for such regulation, we examined the ability of *cdc6ΔN* to complement the methionine-repressed

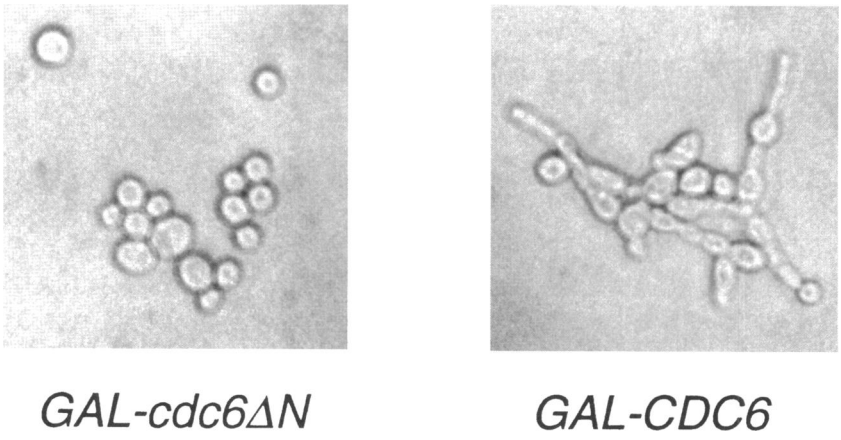

Figure 10. Overproduction of full-length, but not cdc6ΔN, protein perturbs cell growth and morphology. Colonies were picked from plates pictured in Figure 9, and cells were photographed under a light microscope. In left panel, K4055 transformed with pSE19, *GAL-cdc6ΔN*, and grown on medium with 2 mM methionine, 2% raffinose, and 2% galactose. In right panel, K4055 transformed with YGp618, *GAL-CDC6*, and was grown on the same medium.

host not only under the control of the native promoter but also under several different expression regimes. When expressed from the *GAL* promoter on medium containing glucose plus galactose, the wild-type *GAL-CDC6* gene supported growth of the *MET-CDC6* strain in the presence of methionine (Figure 9). In contrast, the *GAL-cdc6ΔN* mutant did not support growth under these growth conditions. *GAL-cdc6ΔN* also failed to support growth on the nonrepressing carbon source raffinose (our unpublished observations), whereas the wild type supported growth at normal rates. Assuming that both mutant and wild-type proteins are transcribed and translated at the same level, a reasonable assumption given our experience with the GST-tagged constructs, these experiments suggest that the *cdc6ΔN* mutant is defective in some essential function of Cdc6.

A different result was obtained after induction of *GAL-CDC6* and *GAL-cdc6ΔN* with galactose. The overproduced cdc6ΔN protein is capable of supporting growth, suggesting that cdc6ΔN is less efficient than Cdc6 at promoting DNA replication but can do so when present in excess. What is interesting in addition is that overproduction of wild-type *CDC6* leads to slow growth and bud hyperpolarization, as originally reported by others (Bueno and Russell, 1992), but overproduction of *cdc6ΔN* results in growth at normal rates, and the cells show normal morphology (Figure 10). It has been proposed that overproduction of wild-type Cdc6 leads to restraint of mitosis, and the results shown in Figure 10 suggest that the Cdc6/Cdc28 interaction is important for this inhibition of mitosis.

When *CDC6* and *cdc6ΔN* were expressed under control of the *CDC6* promoter, *cdc6ΔN* supported growth at the same rate as wild type. Sufficient *cdc6ΔN* must be produced under these expression conditions so that initiation can occur. It may be the case that *cdc6ΔN* is present at elevated levels because of the lack of the complete putative PEST sequence.

DISCUSSION

The yeast replication cycle model proposed recently on the basis of genetic and physiological experiments posits that Clb5/Cdc28 is required both for activation of replication origins in G1 and for inhibition of reactivation of origins in S, G2, and M (Dahmann *et al.*, 1995; Piatti *et al.*, 1996). We have now shown that Cdc6 has all the right properties to be a critical target of Clb5/Cdc28 regulation. Cdc6 associates stably with Cdc28 in vivo. Cdc6 binds preferentially to Clb rather than Cln/Cdc28 complexes (see Figure 5), and the two proteins are not associated before the *CDC34*-dependent step is completed in G1, presumably because of inhibition by $p40^{Sic1}$ (see Figure 8). That the association of the Cdc6 and Cdc28 kinase is physiologically important is demonstrated by the effect of deleting the Cdc28-interacting domain from the Cdc6 protein. At low levels of expression, where wild-type Cdc6 supports cellular growth, the mutant fails to form colonies. At high levels of expression, the mutant can support growth. Thus, the mutant protein is less efficient than wild-type Cdc6 in promoting cell growth. In addition, the inhibition of growth and bud hyperpolarization seen when wild type is overexpressed is not observed, suggesting that the Cdc28 interaction domain has an important role restraining mitosis and, thus, in ordering DNA replication and mitosis.

A model incorporating our results and those of others is shown in Figure 11. In vivo footprinting of ARSs has suggested that yeast replication origins alternate between at least two different states (Diffley *et al.*, 1994). During late mitosis and early G1, origins are in a prereplicative state primed for initiation. Upon origin activation at G1/S, ARSs are converted to a postreplicative state, which is refractory to re-initiation and persists through S, G2, and part of M. The postreplicative complex (post-RC) consists of ORC and ABF1 (Diffley *et al.*, 1994). Resetting to the prereplicative complex (pre-RC) occurs late in mitosis coincident

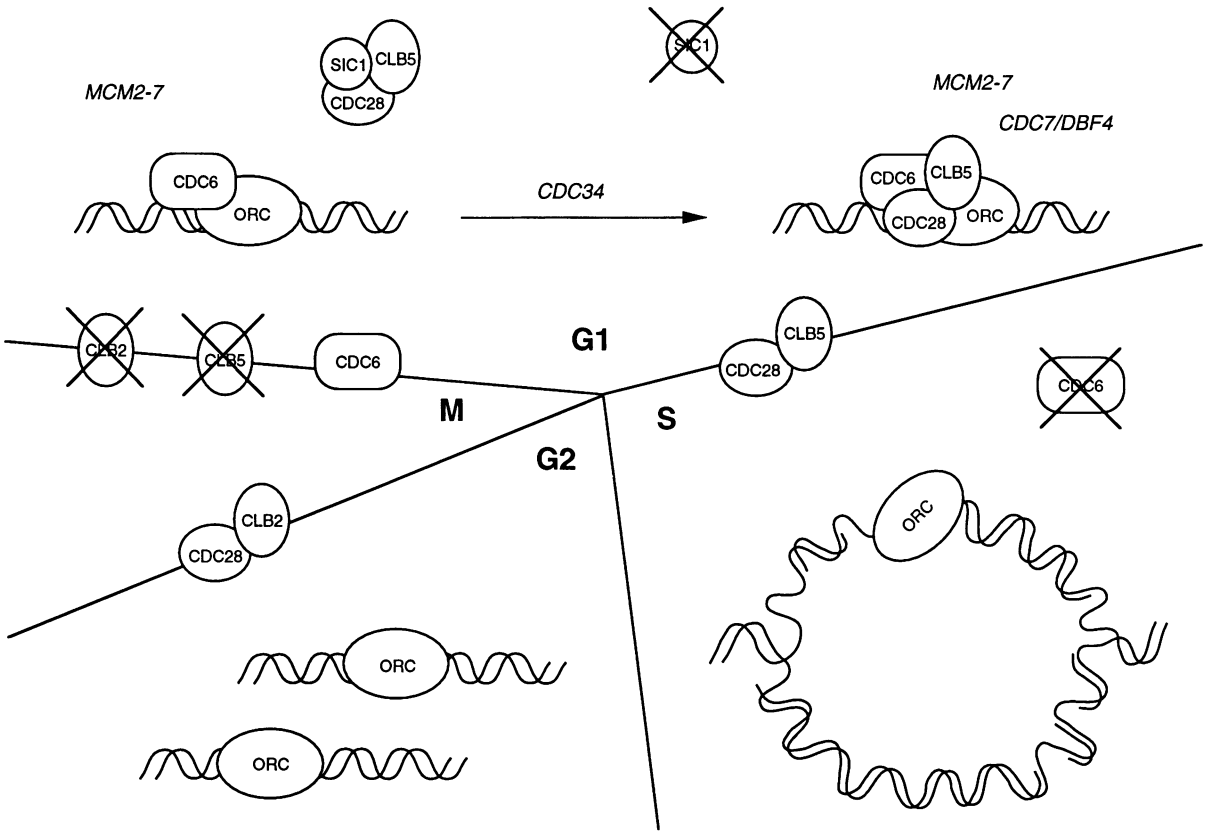

Figure 11. The Cdc6 cycle. After the mitotic cyclins are degraded in late mitosis, Cdc6 binds to ORC at replication origins in a step required to recruit additional replication proteins, such as MCMs, to the origins for initiation (formation of the pre-RC; Diffley *et al.*, 1994). In G1, $p40^{Sic1}$ binds to Clb5/Cdc28 (Schwob *et al.*, 1994), preventing the kinase from binding to Cdc6. When $p40^{Sic1}$ is degraded by the *CDC34*-dependent pathway in late G1, Clb5/Cdc28 associates with Cdc6 in the pre-RC and perhaps with other components as well. This leads to origin activation, dissociation of Cdc6, and conversion of pre-RCs to post-RCs. (Post-RCs are found at this earliest portion of S phase.) At this time and until mitosis, Cdc6 is prevented from reassociating with the origin through interaction with B-type cyclin/Cdc28. This interaction may lead to degradation, sequestration, or inactivation of an activity of Cdc6 (Nishitani and Nurse, 1995; Piatti *et al.*, 1995). Cdc6/cyclin B/Cdc28 might also form a signal for preventing mitosis In late mitosis, cyclins are degraded, and *CDC6* mRNA and protein accumulate; Cdc6 binds to ORC to form the pre-RC and primes the origin for the next round of replication.

with the time of cyclin B degradation, $p40^{Sic1}$ expression, and Cdc6 expression (Diffley *et al.*, 1994; Donovan *et al.*, 1994; Piatti *et al.*, 1995). It is likely that a key step in the transition involves recruitment of Cdc6 into the ORC/ABF1 complex, because formation of pre-RCs requires Cdc6, because pre-RCs are thermolabile in $cdc6^{ts}$ mutants, and because Cdc6 both shows genetic interactions with ORC and interacts stably with ORC in vitro (Li and Herskowitz, 1993; see Figure 5, Diffley *et al.*, 1994; Liang *et al.*, 1995; Cocker *et al.*, 1996). Cdc6 may, in turn, recruit other replication proteins, such as the MCM family (Yan *et al.*, 1993). Three key observations suggest that the putative recruitment of Cdc6 into the pre-RC is regulated by cyclin B/Cdc28. First, formation of the pre-RC can be brought about by premature expression in G2 of the Clb5/Cdc28 inhibitor $p40^{Sic1}$. Second, in *sim1* and *sim2* mutants, which undergo a second round of replication when arrested in G2, cyclin B/Cdc28 kinase levels are very low, and overproduction of Clb5, rather than stimulating initiation, actually prevents re-replication (Dahmann *et al.*, 1995). Third, there exists a "point of no return" in G1, after which Cdc6 cannot be recruited into the pre-RC; this point of no return coincides temporally with the degradation of $p40^{Sic1}$ and activation of Clb5/Cdc28 (Piatti *et al.*, 1996). Our demonstration that Cdc6 interacts biochemically with Clb5/Cdc28 and that the interaction is inhibited by $p40^{Sic1}$, which has now also in part been shown by others (Piatti *et al.*, 1996), suggests that Cdc6 is a target of Clb5/Cdc28 and that Clb5/Cdc28 inhibits Cdc6 recruitment into the pre-RC. The *cdc6ΔN* mutant we describe (see Figure 9) may be able to function when present in excess, but not at lower levels, because there is a longer window for formation of the pre-RC, continuing even after activation of Clb5/Cdc28 and providing time for a less efficient

activator to function. Only after cyclin B degradation and $p40^{Sic1}$ expression late in mitosis can the pre-RC form. Our results taken together argue for a post-translational role for the kinase/Cdc6 interaction rather than for indirect effects on Cdc6 mRNA expression. A prediction of this model is that Cdc6 binding to Clb5/Cdc28 should prevent binding of Cdc6 to the ORC/ARS complex.

The proteins in the pre-RC are poised for origin firing, but initiation requires further activation in G1 by S-phase–promoting factors. Both Cdc7/Dbf4 kinase (Rowley *et al.*, 1995) and Clb5,6/Cdc28 kinase (Schwob and Nasmyth, 1993) are required and probably phosphorylate proteins in the pre-RC, such as ORC (Leatherwood *et al.*, 1996). The interaction of Clb5/Cdc28 with Cdc6 in late G1 may also be a key step in origin activation, because deletion of the Cdc28 interaction domain leads to defects in growth. A sudden conversion of the pre-RC into the post-RC (which seems to contain ORC but not Cdc6) is observed at the beginning of S phase and is thought to coincide with origin activation. Interaction of Cdc6 with Clb5/Cdc28, which occurs only after $p40^{Sic1}$ degradation, might lead to the transition. Phosphorylation may or may not be required. Clb5/Cdc28 might extract Cdc6 from the complex with ORC and sequester the Cdc6 or target it for degradation. The latter model is attractive, because Cdc6 protein has a short half-life. However, degradation of Cdc6 has not yet been shown to be essential for entry into S phase (Piatti *et al.*, 1996). In this way, activation of Clb5/Cdc28 might lead not only to firing of primed origins, perhaps by relieving some inhibitory function of Cdc6, but also to suppression of de novo pre-RC formation both immediately after origin duplication and until cyclin B destruction at mitosis. Interaction of Cdc6 with Clb5/Cdc28 could therefore account for once and only once replication and preservation of the order of replication and segregation of chromosomes, i.e., prevention of re-replication without an intervening mitosis.

When constitutively expressed at high levels, Cdc6 can transiently inhibit mitosis (Bueno and Russell, 1992) and slow the growth of yeast (this work). Our demonstration that high levels of Cdc6 inhibit Clb2/Cdc28 (see Figure 6) in vitro could account for the G2 delay observed when Cdc6 is overproduced and therefore present in excess or for a long time. Levels of Cdc6 we observe in vivo after 4 h of expression are comparable to concentrations used in our assays. During normal cell cycles, Cdc6 is not likely to regulate the G2/M transition by such direct inhibition, primarily because Cdc6 levels are either quite low or absent at this phase of the cell cycle. Other observations, however, suggest that Cdc6 at normal concentrations may play a more indirect role in restraining mitosis. Rao and Johnson (1970) showed that, when a G2 cell is fused to an S-phase cell, the G2 nucleus does not enter mitosis until the S-phase nucleus has also completed DNA synthesis and traversed G2 (Rao and Johnson, 1970). It has been proposed that cells in S phase generate a signal that restrains mitosis. The Cdc6/cyclin B/Cdc28 complex might constitute such a signal. The difference between the phenotype of cells overexpressing wild-type *CDC6* and the *cdc6ΔN* truncation mutant that does not interact with Cdc28 supports this idea (see Figure 10). Therefore, destruction of Cdc6 may be required for entry into mitosis.

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