A Novel Role for Cdc5p in DNA Replication

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DNA replication initiates from specific chromosomal sites called origins, and in the budding yeast Saccharomyces cerevisiae these sites are occupied by the origin recognition complex (ORC). Dbf4p is proposed to play a role in targeting the G1/S kinase Cdc7p to initiation complexes late in G1. We report that Dbf4p may also recruit Cdc5p to origin complexes. Cdc5p is a member of the Polo family of kinases that is required for the completion of mitosis. Cdc5p and Cdc7p each interact with a distinct domain of Dbf4p. cdc5-1 and orc2-1 double mutants are synthetically lethal. Levels of Cdc5p were found to be cell cycle regulated and peaked in G2/M. These results suggest a role for Cdc5p and possibly Polo-like kinases at origin complexes.

In order to maintain the integrity of its genome, a eukaryotic cell must not only replicate its DNA accurately but also limit replication to once per cell cycle. Initiation of DNA replication requires both cis- and trans-acting factors (58). The cis-acting element is a DNA sequence called an origin. In Saccharomyces cerevisiae, a DNA sequence which is capable of supporting autonomous replication both on plasmids and in a chromosomal context has been identified (8, 46). Linker substitution analysis of three such autonomously replicating sequence elements, ARS1, ARS3, and ARS307, shows that the ARS consists of several DNA elements, including an essential 11-bp consensus sequence (ACS) and a B region with at least two elements, B1 and B2 (8, 45, 52, 60). A complex of six proteins, the origin recognition complex (ORC), serves as a trans-acting factor. ORC binds to the B1 and ACS elements in a sequence-specific, ATP-dependent manner (3, 4, 16, 53, 55).

Analysis of replication origins via in vivo DNase I footprinting reveals that during the cell cycle, budding yeast origins are bound by at least two distinct complexes, termed the prereplicative and postreplicative complexes (17). The postreplicative complex, present from S phase through late M phase, resembles the in vitro footprint produced with purified ORC (17). A transition to the prereplicative complex occurs late in M and persists until late G1/early S phase. The prereplicative complex displays an extended region of nuclease protection which encompasses the ORC footprint. The binding of ORC to origins appears to be fundamental, but not sufficient, for establishing a competent initiation complex. ORC appears to be bound to origins throughout the cell cycle (55), and therefore additional trans-acting factors must play a role in establishing replication-competent origins either as prereplication factors or as modifiers of origin-bound ORC.

The formation of the prereplicative complex is proposed to be the first step in priming the origin for initiation of DNA replication (17). The final step is the activation of the prereplicative complexes, leading to the unwinding of the origin sequences and the eventual recruitment of the DNA replication elongation machinery (17). Two proteins required for activating initiation and for entry into S phase are the kinase Cdc7p (50) and its partner Dbf4p (35). Dbf4p is targeted to origins in a sequence-specific manner (18, 28). In addition, Dbf4p appears to regulate the kinase activity of Cdc7p (32), possibly in conjunction with Cdc28p (65), activating Cdc7p just prior to S phase. It has been suggested that Dbf4p targets Cdc7p to the prereplicative complex and that Cdc7p may phosphorylate ORC subunits or other proteins found at the origin (18). The transition from the postreplicative complex to the prereplicative complex late in M phase indicates that factors influencing the initiation of DNA replication are not temporally limited to S phase. Strikingly, this transition occurs concurrently with the cell’s requirement for Cdc5p (7, 51), an essential kinase whose function remains unknown (34). Strains deficient in CDC5 function arrest late in mitosis during anaphase/telophase with an extended spindle and a postreplication complex at origins (7, 17, 51). This paper reports in vitro evidence indicating a specific interaction between Cdc5p and Dbf4p. We also report in vivo data which in combination with the in vitro data suggest a novel role for Cdc5p in DNA replication.

MATERIALS AND METHODS

Strain growth conditions and preparation of yeast extracts. Yeast strains used in this study are listed in Table 1. YCH1-35 was constructed by transforming a W303 MATa strain with Cdc5p from CenHIS3 (an integrating vector containing a 3' fragment of CDC5 fused to three tandem hemagglutinin [HA] epitopes in ARS3006) (57). Ura+ transformants were screened by PCR for appropriate integration and by Western blot (immunoblot) analysis for expression of Cdc5p-HA. Yeast strains without plasmids were grown in YPD. Yeast strains bearing plasmids were grown in selective synthetic medium (SC) with 2% sugar (galactose or raffinose as indicated). Strains with plasmids to be induced with galactose were grown on medium containing 10% glycerol, 25 mM Tris-HCl (pH 7.5), 15 mM EGTA, 15 mM MgCl2, 1 mM dithiothreitol, 0.1% Triton X-100, 1 mM NaN3, 150 mM NaCl, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 5 mg of leupeptin per ml, 2 mM pepstatin A, 50 mM NaF, 10 mM sodium pyrophosphate, and 0.1 mM sodium metavanadate. Cells were lysed by adding 0.5 ml of acid-washed glass beads and vortexing in pulses until 90% lysis was achieved. The lysate was separated from the beads, and the beads were washed with 0.3 ml of fresh buffer. This wash was combined with the lysate and spun for 10 min at 3,000 rpm. The supernatant was aliquoted and frozen in liquid nitrogen. Pellets were resuspended in 0.5 ml of lysis buffer (L buffer) containing 10% glycerol, 25 mM Tris-HCl (pH 7.5), 15 mM EGTA, 15 mM MgCl2, 1 mM dithiothreitol, 0.1% Triton X-100, 1 mM NaN3, 150 mM NaCl, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 5 mg of leupeptin per ml, 2 mM pepstatin A, 50 mM NaF, 10 mM sodium pyrophosphate, and 0.1 mM sodium metavanadate. Cells were lysed by adding 0.5 ml of acid-washed glass beads and vortexing in pulses until 90% lysis was achieved. The lysate was separated from the beads, and the beads were washed with 0.3 ml of fresh buffer. This wash was combined with the lysate and spun for 10 min at 3,000 rpm. The supernatant was aliquoted and frozen in liquid nitrogen. Protein concentrations were determined with the Bio-Rad protein assay.

Copurification of yeast proteins. Four hundred milligrams of lysate was incubated with 0.1 ml of glutathione-agarose (75 mg/ml in L buffer, Sigma) at room temperature for 1.5 h. Glutathione precipitates were pelleted by centrifugation and washed with 10 volumes of L buffer. Samples were resuspended in an equal volume of sodium dodecyl sulfate (SDS)-gel loading buffer, incubated for 5 min at 100°C, and resolved by electrophoresis on an SDS–7.5% polyacrylamide gel.

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Proteins were electrophoretically transferred to nitrocellulose membranes. Blots were incubated for 1 h at room temperature with primary antibody (either 12CA5 tissue culture supernatant or rabbit polyclonal anti-glutathione S-transferase [GST]) in 10 mM Tris-Cl (pH 7.5)-150 mM NaCl-0.05% Tween 20-MgCl2-5 mM MnCl2-1 mM phenylmethylsulfonylfluoride for Cdc5p and Dbf4p. Immunoprecipitates were then washed four times with 0.25 ml of l buffer containing 23°C. DNA was transformed into yeast by the lithium acetate method as described in Escherichia coli was exposed to film at room temperature overnight.

DNA manipulations and plasmids. Cloning and plasmid DNA isolation from Saccharomyces cerevisiae was performed essentially as described previously (56). Plasmid DNA was transformed into yeast by the lithium acetate method as described previously (31). Plasmids used in this study are listed in Table 2. For the CDC5 constructs, two oligonucleotides (Cdc5-1 [5’ TAAAGATATCGGGCTTCCTC 3’] and Cdc5-2 [5’ TAAGATATCGGGCTTCCTC 3’]) were used in the PCR to create a BamHI restriction fragment of the CDC5 open reading frame for insertion into the indicated vector backbones. The inactive kinase (pCH797) was made by using PCR with site-directed oligonucleotides, Cdc5-3 (5’ TAAAGATATCGGGCTTCCTC 3’) and Cdc5-4 (5’ TACGAAAGATATCGGGCTTCCTC 3’). These oligonucleotides changed conserved amino acid N-209 to an A. Creating the mutations required several temperature-sensitive cell cycle mutants, including dbf4-1 (which arrests at the G1/S transition), dbf2-1, cdc5-1, and cdc20-1 (which affect passage through mitosis) (34). To determine if any of these interactions represent specific protein-protein interactions, the yeast two-hybrid system

### RESULTS

Cdc5p interacts with Dbf4p. Overexpression of Cdc5p suppresses several temperature-sensitive cell cycle mutants, including dbf4-1 (which arrests at the G1/S transition), dbf2-1, cdc5-1, and cdc20-1 (which affect passage through mitosis) (34). RESULTS

**TABLE 1. Strains**

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<th>Strain</th>
<th>Genotype</th>
<th>Source or reference</th>
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<td>MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 lys2</td>
<td>R. Rothstein</td>
</tr>
<tr>
<td>W303-1B</td>
<td>MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 lys2</td>
<td>R. Rothstein</td>
</tr>
<tr>
<td>L40</td>
<td>MATa his3 a200 sup1-901 leu2-3,112 ade2 lys25: [LexAop] pHS3 URA3: [LexAop] lacZ</td>
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<td>This study</td>
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<td>MATa cdc-5-1 his7 ura1</td>
<td>B. Gavin and L. Hartwell</td>
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<td>YCHII-50</td>
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<td>Segregant from YCHII-51 × LI28-2D transformed with pRS316-CDC5 (= pCH622)</td>
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**FIGURE 1. Determination of plasmid loss rates.** Transformants were grown into early log phase in liquid SC minus leucine at 23°C before inoculation into liquid YPD and growth at 38°C for approximately one division. The loss rate, L, is equal to 1 – 10m, and m is calculated by the formula m = log(L0) - log(Lt)/number of divisions. F1 and F2 are, respectively, the fraction of cells which initially have the plasmid and the fraction of cells which have the plasmid after short growth in nonselective medium at 38°C (42). The fractions were determined by plating dilutions of the culture onto plates containing SC either with or without leucine. The strains used in this study were YCHII-50 (cdc5-1) and W303-1A (CDC5). The plasmids used were pDK243 (CEN ARS LEU2 ADE5) and pH367-7 (CEN ARS LEU2 ADE5 ARS-H4).
was used (22). Cdc5p fused to LexBD specifically interacts with Dbf4p fused to GAD (Fig. 1). Neither LexBD-Cdc5p or GAD-Dbf4p alone nor GAD-Dbf4p plus either HAepitope-Orc2p or LexBD-Orc2p activated $\beta$-galactosidase transcription (28). Furthermore, Cdc5p did not interact in the two-hybrid system with Dbp2p, Cdc15p, Cdc20p, or Cdc7p (28).

To confirm the Cdc5p-Dbf4p interaction, copurification experiments with yeast cell lysates were conducted. A functional GST-Dbf4p fusion or GST alone was expressed in a strain bearing a chromosomally integrated HA-tagged copy of CDC5 (YCHII-35). Extracts from these strains were incubated with glutathione-agarose. Following extensive washing of the glutathione-agarose, purified proteins were analyzed by immunoblotting. Cdc5p-HA copurified with GST-Dbf4p (Fig. 2a) but not with GST alone (Fig. 2b).

Cdc5p interacts with the amino-terminal region of Dbf4p. Dbf4p has previously been shown to interact with Cdc7p, a protein kinase required for DNA replication, and Dbf4p is also targeted to replication origins (18, 32). These two interactions are separable, with the origin-interacting domain located at the amino terminus of Dbf4p and the Cdc7p-interacting domain located more carboxy terminal (18). To determine if Cdc5p interacts with a unique region of Dbf4p, distinct from the origin-binding and Cdc7p-interacting domains, both two-hybrid and one-hybrid systems were used (22, 40). Several aminoa- and carboxy-terminal truncations of GAD-Dbf4p were tested for the ability to interact with LexBD-Cdc5p or with LexBD-Cdc7p. These GAD-Dbf4p truncations were also tested for interaction with replication origins in a one-hybrid assay (40). The one-hybrid assay was performed with a strain (YJL363) carrying the lacZ reporter downstream of a multimer of the ACS element, and activation by the GAD-Dbf4p fusions was monitored. Deletions of the amino terminus of Dbf4p eli-
nated its interaction with Cdc5p, whereas the first 147 amino acids of Dbf4p were sufficient to interact with Cdc5p (Fig. 1). It is noteworthy that these first 147 amino acids did not target Dbf4p to the origin, nor did they interact with Cdc7p. The domains of Dbf4p which interacted with Cdc7p and Cdc5p are clearly separable (constructs G<sub>AD</sub>-Dbf4<sub>1-147</sub>, G<sub>AD</sub>-Dbf4<sub>241-410</sub>, and G<sub>AD</sub>-Dbf4<sub>537-605</sub> in Fig. 1). It is interesting that the C-terminal region contained in G<sub>AD</sub>-Dbf4<sub>537-605</sub> is essential (35). Neither Cdc5p nor Cdc7p is active in the one-hybrid assay (18, 28); thus, Dbf4p is likely targeted to origins independent of its interaction with either Cdc5p or Cdc7p. Taken together, the data suggest that Dbf4p has at least three separable domains which interact with Cdc7p, Cdc5p, and replication origins, as diagrammed in the lower portion of Fig. 1.

**Cdc5p immunocomplex phosphorylates Dbf4p.** Given that Cdc5p is a kinase (34) and that it interacted with Dbf4p in vitro, Dbf4p was tested as a potential substrate for Cdc5p. Extracts from cells overexpressing both HA-Cdc5p and GST-Dbf4p were immunoprecipitated with the anti-HA 12CA5 monoclonal antibody and Protein A-Sepharose. Parallel experiments with cells overexpressing HA-Cdc7p were conducted to test whether Cdc7p from yeast cells also phosphorylates Dbf4p. By immunoblotting, GST-Dbf4p was present in all the cell lysates (Fig. 3a) and in the immunoprecipitation complexes with either HA-Cdc5p or HA-Cdc7p (Fig. 3c). However, in both cases, the association may not be entirely specific. Under identical conditions (Fig. 3c, lane 1) and with repeated washing in high salt (28), low levels of GST-Dbf4p were also precipitated from a strain not expressing an HA-tagged protein. Regardless, the GST-Dbf4p present in the immunocomplexes served as a substrate in kinase assays conducted directly on the washed protein A-Sepharose. In the kinase reactions, as shown by the autoradiograph in Fig. 3d, GST-Dbf4p was phosphorylated by HA-Cdc5p (Fig. 3d, lane 2) and HA-Cdc7p (Fig. 3d, lane 4) immunocomplexes.

The requirement for Cdc5p and Cdc7p to phosphorylate GST-Dbf4p was tested with kinase-dead proteins. Asn-209 in Cdc5p and Asn-168 in Cdc7p were each replaced with alanine. This Asn residue is conserved in all protein kinases (26). The crystal structure of protein kinases indicates that this invariant asparagine residue is in the catalytic loop, interacts directly with the catalytic base, and therefore is essential to catalytic activity (14, 36, 37). In experiments using extracts derived from...
Cdc7p kinases were overexpressed from plasmids carrying a CDC5 cassette with either HA-Cdc5p or HA-Cdc7p immunocomplexes. Analysis of kinase activity was conducted with immunoprecipitates, using 12CA5 and crude extracts derived from yeast cells overexpressing either HA-Cdc7p (lanes 1 and 2) or HA-Cdc5p (lane 3 to 5). Bacterially derived GST-Dbf4p was added to the HA-Cdc7p and HA-Cdc5p (lanes 2 and 4) immunocomplexes and phosphorylated, as detected by autoradiography. Bacterially derived GST was added to the HA-Cdc7p and HA-Cdc5p immunocomplexes, and no phosphorylation was detected by autoradiography (lanes 1 and 3). A signal was also not detected in lane 5, in which no substrate was added to the HA-Cdc5p immunocomplex. (b) Immunoblotting with anti-GST antibody of the GST-Dbf4p (lane 2) and GST (lane 1) used as the substrate in panel a. GST-Dbf4p and GST were purified from E. coli transformed with pC7H73 and pGEX-3X, respectively. Extensive proteolysis of GST-Dbf4p occurred, and the full-length product is designated. The HA-Cdc5p and HA-Cdc7p kinases were overexpressed from plasmids carrying a pCH740 and pGEX-3X, respectively. Extensive proteolysis of GST-Dbf4p was not attributable to Cdc5p and that another kinase(s) in either the Cdc5p or Cdc7p immunoprecipitate is probably dependent on Cdc5p. Furthermore, the assay conditions for Cdc5p and Cdc7p kinase activities are markedly distinct (see Materials and Methods). When Cdc5p immunoprecipitates were assayed under Cdc7p conditions, no kinase activity was detected. This result indicates that the residual activity in the cdc7N168A-Dbf4p immunocomplex was not attributable to Cdc5p and that the kinase activity observed in the Cdc5p immunoprecipitate was that of Cdc7p or vice versa. Several pieces of evidence argue against this possibility. First, there was no kinase activity in complexes immunoprecipitated from cells expressing cdc5N209A-Dbf4p, which we presume to be catalytically inactive. This suggests that the kinase activity observed in the Cdc5p-Dbf4p immunoprecipitate is probably dependent on Cdc5p. YCH11-35 expressing HA-Cdc5p was synchronized in G1 with α-factor. After release into fresh media, samples were harvested every 10 min and synchrony was monitored by assessing the morphology of the cells (Fig. 5a). The levels of Cdc5p and actin from each time point were determined by immunoblotting (Fig. 5b). The quantity of Cdc5p clearly showed fluctuation through the cell cycle compared to actin (Fig. 5b). Cdc5p started to appear 50 min after release, corresponding to a time when 70% of the cells had medium-size buds. Cdc5p persisted for 30 more min, before its levels declined. This increase and subsequent decrease in Cdc5p corresponded to the transition from medium budded cells (50 to 70 min) to large budded cells (80 min) and finally to unbudded cells (90 to 100 min). This result suggests that Cdc5p is present late in the cell cycle probably in both G2 and M, in agreement with the reported CDC5 transcript levels (34).

Synthetic lethality between cdc5-1 and orc2-1. The interaction between Cdc5p and Dbf4p suggests that Cdc5p may have a role in DNA replication. We tested whether CDC5 exhibited any genetic interactions with genes encoding other factors known to act at the origin. Synthetic lethality is a genetic indicator that proteins might perform a related function. For example, double mutants with orc2-1 and either cdc7-1, orc3-1, or orc5-1 (28, 42), all of which contain mutations which cause defects in replication initiation, are inviable. We found that double mutants with orc2-1 and cdc5-1 were not viable (Fig. 6). This lethality was rescued by a plasmid bearing the CDC5 gene on a URA3 CEN plasmid. In contrast, cdc5-1 was not synthetically lethal with dbf4-1, cdc7-1, or orc5-1.

Plasmids containing extra origins are maintained preferentially in cdc5-1. Mutations in genes encoding DNA replication factors often cause an enhanced rate of chromosome and plasmid loss (29, 42). If this increased loss rate is due to inefficient initiation of replication by limiting amounts of an essential initiation factor, the addition of multiple potential origins to a plasmid might suppress the loss (30). The case is for strains carrying mutations in the known replication initiation genes ORC2, ORC3, ORC5, and CDC6 (27, 30, 42). In contrast, strains carrying mutations in genes whose products are required for the elongation step of DNA replication, Cdc9p (DNA ligase) and Cdc1p (catalytic subunit of DNA polymerase α), display a plasmid maintenance defect that is not suppressed by the addition of multiple origins (30). The cdc5-1 mutant exhibits high frequencies of recombination and also high frequencies of chromosome loss (29). In a direct plasmid loss assay, after growth at the nonpermissive temperature of 38°C for 3 h, we found that cdc5-1 cells lost a plasmid containing one origin (ARS1) (pDK243) at a 46-fold-greater rate than an isogenic wild-type strain (loss rates, 0.46 and 0.01, respectively). In contrast, when the transformed plasmid (pDK368-7) contained an insert of seven tandemly repeated copies of the origin ARS-H4, it was lost from cdc5-1 cells at a rate ninefold lower than that for cells carrying the single ARS plasmid (loss rates, 0.05 and 0.46, respectively) and only fivefold greater than that for wild-type cells (loss rates, 0.05 and 0.01, respectively). This suppression of the cdc5-1 plasmid maintenance defect by multiple origins suggests a role for the Cdc5p kinase in regulating DNA replication.

DISCUSSION

In this report, we show by both genetic and biochemical assays that the kinase Cdc5p interacts with Dbf4p. Furthermore, phosphorylation of Dbf4p in a Cdc5p immunoprecipitation complex is dependent on Cdc5p kinase activity. These in vitro data suggest that Cdc5p, by virtue of its interaction with...
Dbf4p, may act at the origin. The argument for a role for Cdc5p in replication initiation is strengthened by two pieces of in vivo data. Analogous to numerous genes involved in initiation, including CDC6 (30) and ORC2, ORC3, and ORC5 (27, 42), cells carrying the cdc5-1 mutation have a plasmid maintenance defect which is rescued by the addition to the plasmid of multiple potential origins. In addition, a strain carrying mutations in both CDC5 and ORC2 is not viable.

Does Cdc5p have multiple roles in mitosis? Putative CDC5 homologs include Drosophila POLO (39), Schizosaccharomyces pombe PLO1 (49), and human PLK1 (25). The kinase activities of both POLO (21) and PLK1 (24) are cell cycle regulated and peak during mitosis. Based on the phenotypes of cells carrying mutations in CDC5, POLO, and PLK1, a mitotic role in regulating microtubules has been suggested (21, 24, 49). Yet direct evidence for the involvement of Cdc5p in regulating microtubule behavior is limited to the unusual response of cdc5-1 mutants in the presence of methyl-benzimidazole-2-ylcarbamate, a drug which binds tubulin and depolymerizes microtubules (63). When held at a nonpermissive temperature in the presence of this drug, cdc5-1 mutants will divide several times before arresting, whereas wild-type cells and other cdc mutants are unable to divide. POLO and POLO do not complement the cdc5-1 temperature-sensitive mutant and also do not interact with Dbf4p in the two-hybrid system (28). This finding suggests that the primary structural homology between Cdc5p and the Polo-like kinases may not be functionally significant. The evidence in this report does not further support a role for Cdc5p in tubulin regulation. However, we cannot rule out such a potential function for Cdc5p.

Roles for Cdc5p in DNA replication. How does a mitotic kinase (Cdc5p) play a role in regulating initiation of DNA

FIG. 5. Cdc5p is expressed late in the cell cycle. Cells expressing an integrated HA-tagged copy of Cdc5p were arrested in G1 with α-factor. After release to fresh medium, samples were harvested every 10 min. (a) Cell morphology was determined. (b) The levels of HA-Cdc5p (top) and actin (bottom) were determined by immunoblotting with anti-HA and antiactin antibodies, respectively.

FIG. 6. cdc5-1 orc2-1 double mutants are inviable. Haploid strains harboring a CDC5 URA3 CEN plasmid and chromosomal alleles of both cdc5-1 and either cdc7-1, dbf4-1, orc2-1, or orc5-1 were tested for growth. All grew at 23°C on rich medium (YPD; left); after replica plating to medium containing 5-fluoroorotic acid (5-FOA), which selects against the URA3 CEN plasmid, the cdc5-1/orc2-1 cells were dead (right).
replication late in G1? Cdc5p might repress the function of factors that inhibit initiation of replication. Alternatively, Cdc5p might stimulate the action of factors that promote the transition from the pre- to postreplicative origin state. Several lines of evidence suggest not only that B-type cyclin–cyclin-dependent kinase (Cdk) complexes, specifically Cib5p and Cib6p-Cdk complexes, play a role in activating replication during late G1 but also that during G2 they play a role in preventing reinitiation prior to completion of mitosis (64). Cdk5s are thought to inhibit inappropriate initiation during G2 by blocking the assembly of preinitiation complexes during G1 (15). Cdc5p could play a role in activating the complex involved in the degradation of the Cib-Cdk kinase activity that is implicated in the exit from mitosis (1, 47, 59). Such activated proteolysis of the Cib cyclins would then allow the timely transition during anaphase/telophase, between the post- and prereplication complexes. Regulated changes of state at origins may allow the cell to efficiently mark the origins which will become competent to activate initiation of replication later in G1. Perhaps factors which act directly at the origin might also be affected by the activation of the protein degradation complex. This could explain the multiple ARS suppression of the plasmid maintenance defect in cdc5-1 mutants. The regulation of the mitotic proteolysis machinery cannot be the only function of Cdc5p, however, because defects in this machinery cause cells to arrest prior to a time in the cell cycle, telophase, when Cdc5p is required (47).

One candidate for a prereplication factor is Cdc6p, a protein required for the formation and maintenance of the prereplication complex (12). In earlier studies, Cdc6p was shown to interact with ORC biochemically and genetically and to play a role in the determining the frequency of initiation of DNA replication in the genome (41). Other prereplication candidates include the evolutionarily conserved Mcm proteins. In budding yeast, the Mcm proteins appear to play a role during G1 because cells depleted of Mcm function arrest just prior to S phase with a single complement of DNA (61). Recent biochemical studies using Xenopus extracts show that a complex containing the Xenopus homologs of the yeast Mcm2, Mcm3, and Mcm5/Cdc46 proteins are bound to chromatin exclusively during G1, at which time they play a role in licensing the replication of the Xenopus genome (11, 38, 43). An unidentified kinase is essential for the activation of the Xenopus replication licensing activity (5). Interestingly, the activity of this kinase is needed after metaphase at about the same point in the cell cycle that Cdc5p is required (5). Cdc5p may activate the targeting of Cdc6p to origin complexes and/or the licensing activity of the Mcm complex. In regard to possible Cdc5p phosphorylation of Mcm proteins, it should be noted that studies in mammalian and Xenopus cells show that although phosphorylation levels of Mcm proteins increase as cells enter S phase and again in G2 and early mitosis, they plummet in late mitosis (33, 54). Therefore, in regard to activation of Mcm licensing function, Cdc5 might play a role, later in mitosis, in regulating the factor which loads the Mcm complex on to chromatin (11, 44) rather than the Mcm proteins directly.

Is Dbf4p a scaffold for Cdc5p and Cdc7p? An emerging model for regulating multiple kinases is that of scaffold and anchoring proteins (20). An example of a scaffold protein is Ste5p from the yeast pheromone response pathway. Ste5p is believed to tether simultaneously the multiple protein kinases in the mitogen-activated protein kinase cascade (10). The Ste5p template on which these kinases bind probably positions them in favorable orientations relative to their downstream partners. Such an arrangement may contribute to the fidelity of this pathway (10). Anchoring proteins serve to localize a kinase to a particular target locus (20). Anchoring proteins would likely possess at least two domains, a kinase-binding domain and a target-binding domain. Examples of anchoring proteins include A-kinase anchoring proteins (9, 48), which have been shown to target a number of different kinases to distinct subcellular locations (13, 19). Based on the evidence reported, Dbf4p may act as an anchoring protein. It possesses an origin-targeting domain and two separable kinase-binding domains for Cdc5p and Cdc7p. Dbf4p could anchor the two kinases, perhaps sequentially, to the origin, where each would potentially modify origin-bound substrates.

Alternatively, Cdc5p could phosphorylate Dbf4p late in M, targeting Dbf4p to the prereplicative complex, where it could subsequently recruit Cdc7p. It is not clear from the GAD-Dbf4p activation of the ACS-lacZ reporter in the one-hybrid experiments when, during the cell cycle, Dbf4p is part of the origin complex (18). It is possible that Dbf4p is present transiently at the surface of the origin complex, which might allow a GAD-Dbf4p fusion to participate in transcriptional activation. In this model, Dbf4p might play a role not as an anchor but in chaperoning various kinases to their specific substrates.

Cdc5p is not unique among cellular proteins whose actions during mitosis appear to affect the initiation of DNA replication late in G1. Cdc14p, a putative tyrosine phosphatase (62), is also required for the completion of mitosis, and cdc14 mutants produce an ARS plasmid maintenance defect which is suppressible by multiple ARS elements (30). In addition, mutations in CDC14 like those in CDC5 are synthetically lethal with mutations in ORC2.

Strikingly, bob1-1 mutants bypass the requirement for both Cdc7p and Dbf4p (32). However, they do not bypass the requirement for Cdc5p (28). This finding suggests that Cdc5p may function independent of Dbf4p, at least in the bob1-1 background. An understanding of the nature of the bob1 mutation is therefore crucial to the further characterization of the roles that Dbf4p, Cdc7p, and Cdc5p play at the origin.

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