The Stability of the Cdc6 Protein Is Regulated by Cyclin-dependent Kinase/Cyclin B Complexes in *Saccharomyces cerevisiae*<sup>*</sup>

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The *Saccharomyces cerevisiae* Cdc6 protein is necessary for the formation of prereplicative complexes that are a prerequisite for firing origins during DNA replication in the S phase. In budding yeast, the presence of Cdc6 protein is normally restricted to the G<sub>1</sub> phase of the cell cycle, at least partly because of its proteolytic degradation in the late G<sub>1</sub>/early S phase. Here we show that a Cdc28-dependent mechanism targets p57<sub>CDC6</sub> for degradation in mitotic-arrested budding yeast cells. Consistent with this observation, Cdc6–7 and Cdc6–8 proteins, mutants lacking Cdc28 phosphorylation sites, are stabilized relative to wild-type Cdc6. Our data also suggest a correlation between the absence of Cdc28/Cib kinase activity and Cdc6 protein stabilization, because a drop in Cdc28/Cib-associated kinase activity allows mitotic-arrested cells to accumulate Cdc6 protein. Finally, we also show that cdc28 temperature-sensitive G<sub>1</sub> mutants accumulate Cdc6 protein because of a post-transcriptional mechanism. Our data suggest that budding yeast cells target Cdc6 for degradation through a Cdc28-dependent mechanism in each cell cycle.

Recent work aimed at elucidating the mechanisms that underlie the control of DNA replication in eukaryotes has afforded a two-step model (1–4). This model first proposes the establishment of a state of competence for replication at origins, as soon as cells exit from mitosis, and then its activation for the initiation of DNA synthesis, during the G<sub>1</sub>-to-S phase transition. At the same time, these steps must somehow be linked to the precise control of cell cycle progression to prevent reduplication of the genome before passage through mitosis. This latter type of control appears to be integrated through modulation by cyclin-dependent kinase (CDK)<sup>1</sup> activity (5–7).

The state of competence means that a multisubunit protein complex must be formed at the origins of replication, the discrete sites at which DNA synthesis is initiated. In *Saccharomyces cerevisiae*, the sequence elements making up these origins have been well characterized (8). These origins are bound throughout the cell cycle by a complex of six proteins called the origin recognition complex (ORC), which is required for the initiation of DNA replication (9–11). Although ORCs at origins are essential, the binding of additional factors is needed for the initiation of genome duplication, as shown by *in vivo* footprint pattern changes during the cell cycle (11). In *S. cerevisiae* a number of gene products have been identified that interact with some of the components of the ORC, participating in the assembly of the initiation complex, or prereplicative complex (pre-RC). These include Mcm proteins, Cdc45, Cdc7, and Dbf4, as well as Cdc6 (12–15).

The formation of prereplicative complexes in budding yeast is dependent on Cdc6 (16). Genetic evidence has indicated that Cdc6 is rate-limiting for the initiation of DNA replication through its interaction with ORC (13). The closest homolog of CDC6 in fission yeast, *cdc18*<sup>+</sup>, is also required for DNA replication (17). Cdc18 protein levels vary during the cell cycle, peaking at G<sub>1</sub>/S like Cdc6 (18, 19). Additionally, the overexpression of *cde18*<sup>+</sup> induces continuous DNA synthesis (18, 20), suggesting that this gene plays a key role in initiating DNA replication. In fission yeast a link between the cell cycle machinery and pre-RCs has emerged through the demonstration that Cdc18 interacts *in vivo* with Cdc2 (the fission yeast homolog of budding yeast Cdc28) and Orp2 (the fission yeast homolog of budding yeast Orc2) (21). Also, it has been shown that in budding yeast the Cdc6 protein when overexpressed interacts with Cdc28 (22, 23).

The inactivation of Cdc28/B-cyclin kinase seems to be required for both the exit from mitosis and the resetting of chromatin for the subsequent S phase (6, 7). This resetting involves Mcm binding to chromatin in a hypophosphorylated state (24 and references therein) and Cdc6 being recruited to origins, an essential step for the formation of pre-RCs (15, 16). At the same time, reformation of the prereplicative complex is actively inhibited by Cdc28/B-cyclins (7). Because B cyclins are required for both entry into the S phase and the inhibition of nuclear resetting, another round of replication cannot occur until B-cyclin/Cdc28 kinases have been destroyed in the subsequent mitosis (6, 7). Consistent with this hypothesis, it has been suggested that a Cdc2-mediated mechanism regulates Cdc18 degradation in *Schizosaccharomyces pombe*, preventing this fission yeast from entering a second round of DNA replication within the same cell cycle (25, 26).

Our work here addresses the mechanism regulating the degradation of the key DNA replication initiator Cdc6 protein in *S. cerevisiae* at the G<sub>1</sub>-to-S phase boundary. We have previously shown that Cdc6 is ubiquitinataed *in vivo* for degradation (27). In accordance with published data (28), our results indicate that Cdc4 and Cdc34 participate in Cdc6 proteolysis, suggesting that the initiator protein is phosphorylated for degradation through the proteasome. Consistent with this hypothesis, here we report data suggesting that the phosphorylation of Cdc6 by Cib/Cdc28 kinases stimulates Cdc6 proteolysis and therefore restricts the formation of pre-RCs to G<sub>1</sub>. In light of these results, we propose not only that Cdc6 acts to initiate DNA...
replication at the onset of the S phase but also that its degrada-
tion is one of the multiple CDK-mediated events that pre-
vent the reinitiation of DNA replication within the same cell
cycle, because in the absence of Cdc6 no pre-RCs are formed.  

**EXPERIMENTAL PROCEDURES**

**Strains and Plasmids—** All *S. cerevisiae* strains were derived from 15Dau, a *MATα* derivative of BF264-15D (29). The temperature-sensi-
tive and deletion mutants used were cdc6-1 leu2 ura3; cdc6-URA3 G11-10: CDC6(LEU2) ura3 leu2 cdc6-23; cdc6-13 bar1 ura3; cdc6-8 bar1 ura3; cdc6-18 N1-1 leu2 ura3 his3. All cdc6-1 13GAL1-10::CDC1; cdc6-1 13XGAL1-10::SIC1, cdc6-13 13XGAL1-10::SIC1, GAL1-10::CDC6, GAL1-10::cdc6-7, GAL1-10::cdc6-8, and cdc6-KANMX bar1 ura3 strain transformed with pH70CDC6, pH70cdc6-7, or pH70cdc6-8.

CDC6 mutants were constructed with an *in vitro* mutagenesis kit from Bio-Rad, using a 5.5-kb BamHI-HindIII CDC6 genomic fragment cloned into a pTZ18R as template. cdc6-7 was constructed by mutating threonines 23, 39, 135, and 368 and serines 43, 354, and 372 to alanines. A total of 16 different mutants were constructed by also mutating threonine 7 to alanine. The mutagenic oligonucleotides used were: 5′-ATACCAATAGCTCAGAATCCG-3′ (A7), 5′-GGCTCCAGGGGGCGGGCCCG-3′ (A23), 5′-ACAGATGTTGCTACAGAATTG-3′ (A32), 5′-CCAGCTATGCTACAGAATTG-3′ (A42), 5′-GCTCTCGGCGGGCCCGGG-3′ (A135), 5′-GGTCTTACAGGGGTACAGAATTG-3′ (A368), 5′-CCAGCTATGCTACAGAATTG-3′ (A372), and 5′-GGAGCCGCTGCGAGAGG-3′ (E114). The underlined nucleotides are the mutated ones. pH70CDC6, pH70cdc6-7, and pH70cdc6-8 plasmids were constructed by inserting the 5.5-kb BamHI-HindIII fragments from the pTZ19Rcdc6, pTZ19Rcdc6-7, and pTZ19Rcdc6-8 into pH70, a centromeric vector. To construct the integrative vectors YIpG3, the 28-mer 3′ oligonucleotide was amplified using *in vitro* mutagenesis described previously (30), using pTZ19Rcdc6-7 as template and a high-fidelity polymerase (Pwo polymerase from Roche Molecular Biochemicals). All plasmids were sequenced using the dideoxy nucleotide method of Hutter and Eipel (36), by staining them with propidium iodide.

**Nocodazole-induced Cell Cycle Arrest—** Cultures were incubated with shaking in the presence of nocodazole (15 μg/ml). Cells were further incubated and collected for fluorescence-activated cell sorting and microscopical analysis.

**Protein Extract Preparation—** Soluble protein extracts were prepared as described previously (37). Cells were collected, washed, and broken in 30 μl of histone buffer using glass beads. The HB buffer contained 60 mM β-glycerophosphate, 15 mM p-nitrophenylphosphate, 25 mM 4-morpholinepropanesulfonic acid (pH 7.2), 15 mM MgCl2, 15 mM EGTA, 1 mM diithiothreitol, 0.1 mM sodium orthovanadate, 1 mM phenylmethylsulfo-

**RESULTS**

**Cdc6 Interacts In Vivo with Cdc28 in *S. cerevisiae* Cells—** A direct interaction between recombinant Cdc6 and ORC has been described (13). More recent biochemical evidence suggests that, when overexpressed, Cdc18, a Cdc6-like protein in fission yeast, interacts *in vivo* with p34CDC28 and Orp2, a putative component of the *S. pombe* ORC (21). In fact, also in budding yeast, Cdc6 interacts *in vivo* with Cdc28, at least under overexpressing conditions (22, 23).

To test *in vivo* interactions between Cdc6 and Cdc28, we con-
structed a series of *S. cerevisiae* strains carrying a single copy of Ha-tagged Cdc6 (see “Experimental Procedures”). Gene re-
placement experiments revealed that Cdc6Ha was fully func-
tional (data not shown). Immunoprecipitation from Cdc6Ha-
expressing cells with Ha monoclonal antibody coprecipitated the H1 kinase activity (Fig. 1A). A cdc28-13 Cdc6Ha double mu-
 tant strain was also constructed. The kinase activity co-immu-
noprecipitated from this double mutant was thermosensitive in an *in vitro* assay, indicating that Cdc28 was physically inter-
acting with Cdc6Ha (Fig. 1A). Furthermore, we detected p34CDC28 in Cdc6Ha immunoprecipitates from those strains by Western analysis (Fig. 1A). These results suggest that Cdc6 interacts *in vivo* with Cdc28 in *S. cerevisiae* cells under physi-
ological conditions.

**Cdc6 Protein Is Degraded in Mitotic-arrested Cells by a Cdc28-
dependent Mechanism—** The above biochemical interaction suggested that Cdc6 could be an *in vivo* substrate for Cdc28 kinase, indicating the possibility that Cdc6 function or stability may be regulated by Cdc28 phosphorylation. Consistent with this hypothesis, it has been shown that Cdc6 is an *in vivo* phosphoprotein (22) and that bacterially produced Cdc6 is an *in vitro* substrate for Clb-associated Cdc28 activity (Refs. 22, 23, Downloaded from http://www.jbc.org/ by guest on July 23, 2018
this work, and Fig. 1B). We therefore evaluated Cdc6 stabilization in cells deficient in p34CDC28-associated kinase activity by Western analysis. Using a previously characterized rabbit polyclonal antibody (27) we examined the rate of Cdc6 disappearance in promoter shut off experiments in a cdc28-13 GAL1-10: CDC6 strain. CDC6 expression was induced in nocodazole-arrested cells by incubating them in galactose for 60 min at 37 °C. Transcription was repressed by the addition of glucose, and Cdc6 stability was estimated by Western blotting at both 25 or 37 °C (permissive temperature) and 37 °C (restrictive temperature) (Fig. 2). Whereas Cdc6 became rapidly unstable at the restrictive temperature or in a wild-type control at both temperatures, its levels remained constant up to 45 min at the restrictive temperature for cdc28-13. Comparable results were obtained when cycloheximide was added to inhibit protein synthesis at the same time that transcription was repressed with glucose (data not shown); nevertheless, Cdc6 protein was barely detectable after releasing the cells at the permissive temperature, as shown for Cdc28 wild-type cells (Fig. 3C, upper panel). These results strongly suggest that cells blocked in mitosis require Cdc28 to degrade ectopically produced Cdc6.

Cdc6-7 and Cdc6-8 Proteins Lacking Cdc28 Putative Phosphorylation Sites Are Stabilized Relative to Wild-type Cdc6—We reasoned that if mitotic-arrested cells required a functional Cdc28 kinase to degrade Cdc6, it is conceivable that p57CDC6 may be targeted for degradation by p34CDC28 phosphorylation. If this were the case, it could be predicted that mutant forms of Cdc6 protein lacking its phosphorylation sites would be more stable than wild-type protein. Cdc6 contains eight serines or threonines at putative Cdc28 phosphorylation sites. Two mutant forms; one of them, named cdc6-7, carries alanine substitutions of Thr-23, Thr-39, Ser-43, Thr-135, Ser-354, Thr-368, and Ser-372. In the other we changed all phosphorylatable

FIG. 1. Cdc6 co-immunoprecipitates with Cdc28. A, Cdc6 associates with Cdc28-dependent H1 kinase activity in S. cerevisiae cells. Protein extracts were prepared from cdc28-13 CDC6HA (cdc28ts) or CDC28 CDC6HA (wt) growing exponentially at 25 °C. Anti-Ha immunoprecipitates obtained from 3 mg of cdc28-13 Cdc6HaHa or CDC28 Cdc6Ha cell extracts were split into four parts. Two aliquots were tested for their ability to phosphorylate H1 in vitro at 25 or 37 °C, and the other two were used for the detection of p34CDC28 by immunoblotting with α-Cdc28 antibody (lower bands). An untagged Cdc6 control is also shown (control). B, recombinant Cdc6 protein is an in vitro substrate for Cdc28-associated kinase activity. Cdc6 was expressed in Escherichia coli, purified to homogeneity, and used as an exogenous substrate for Cdc28. The kinase was immunoprecipitated from S. cerevisiae 15Dau protein extracts with specific antibodies and assayed for its ability to phosphorylate different samples of either Cdc6 or histone H1 or both, as indicated.

FIG. 3. Cdc6-7 and Cdc6-8 mutant proteins are more stable than wild-type Cdc6 protein. A, schematic drawing of the strategy used in the experiment shown in C. B, immunoblot detection of the Cdc6, Cdc6-7, and Cdc6-8 proteins. Protein extracts prepared from cells deleted for CDC6 (lane 1) or asynchronous cultures of cells overexpressing, from the GAL1-10 promoter, wild-type CDC6 (lane 2), cdc6-7 (lane 3), or cdc6-8 (lane 4) were analyzed by immunoblotting with affinity-purified goat antiserum to Cdc6 (yN-19) or rabbit antiserum to Cdc28 for loading control. C, Analysis of the relative stability of Cdc6 phosphorylation site mutants. Immunoblot analysis of cell extracts from GAL1-10/CDC6, GAL1-10/cdc6-7, and GAL1-10/cdc6-8 strains is shown. The three mutant strains were blocked with nocodazole at 30 °C (NZ), and galactose was added for CDC6 induction during 60 min. Afterwards, GAL1-10 expression was repressed by addition of glucose (CH) as well as protein synthesis inhibited by adding cycloheximide (CH; 20 μg/ml). Samples were taken at indicated intervals, processed for Western blotting, and probed with affinity-purified goat antiserum to Cdc6 or rabbit antiserum to Cdc28.
cating that the purine nucleotide-binding motif is essential for CDC6 function in vivo. Because the rescue of the cdc6-1 temperature-sensitive (ts) allele does not necessarily mean that Cdc6-7 or Cdc6-8 are functional because, for instance, they could serve to stabilize the ts protein, we set up a complementation test of a null allele of cdc6 with these cdc6 phospho-rylation site mutants (Fig. 4). A cdc6 deletion strain (cdc6::KANMX4), carrying a GAL1-10:CDC6 allele at the URA3 locus that supports growth in galactose-based media, was transformed with high multiplicity plasmids (YEp13) bearing wild-type CDC6, cdc6-7, or cdc6-8 under the control of the CDC6 wild-type promoter. To test the ability of these plasmids to complement the null mutation, GAL1-10:CDC6 cells were changed to glucose medium to repress GAL1-10:CDC6 expression. All mutants rescued the deletion of CDC6. Nevertheless, it is remarkable that, in consonance with a delay in G2, YEp13cdc6-7 transformants formed colonies of elongated cells. These results indicate that both Cdc6-7 and Cdc6-8 proteins are functional.

A Drop in Cdc28-associated Kinase Activity Allows Mitotic-arrested Cells to Accumulate Cdc6 Protein—It is already known that de novo synthesis of Cdc6 protein is required for cells to enter the S phase (19, 23). On the other hand, it has been shown that transient inhibition of Cdc28-Clb kinases by expression of the Cdc28 inhibitor p40SIC1 causes mitotic-arrested cells to undergo an extra round of DNA replication (7). To test whether CDC6 function is needed for this rereplication phenotype, p40SIC1-conditionally expressing wild-type CDC6 and cdc6-1 mutant strains were constructed. The rereplication ability in both strains was analyzed at either 25 or 37 °C. After 40 min of induction, p40SIC1 expression was repressed, and the DNA content was measured in samples taken at regular intervals from cultures incubated at the permissive or restrictive temperatures. As shown in Fig. 5A, rereplication in nocodazole-arrested cells caused by transient inhibition of Cdc28-Clb kinases was prevented in cdc6-1 mutant cells at the restrictive temperature. These data suggest that the accumulation or presence of a fully functional Cdc6 protein is required for rereplication induced by a drop in Cdc28-associated kinase activity.

To understand whether this Sic1-dependent inhibition of p34CDC28 would cause the accumulation of p57CDC in nocom-azole-arrested cells, we monitored the Cdc6 protein content by Western blot using antiserum to Cdc6. After induction of p40SIC1, samples were taken and divided into two aliquots for Western and p34 kinase assays. As shown in Fig. 5B, Cdc6 protein accumulated as soon as the kinase activity dropped, suggesting a correlation between the lack of p34CDC28 activity and p57CDC stabilization in mitotic-arrested cells.

Rereplication in Nocodazole-arrested Cdc6-7-expressing Cells Is Dependent on a Fall in Cdc28 Kinase Activity—The evidence presented above suggests that it might be important for mitotic cells to degrade the Cdc6 protein to prevent and extra round of DNA replication before cell division. If degradation of Cdc6 during mitosis is the only condition for preventing rereplication, it could be predicted that a stable form of the inhibitor protein, Cdc6-7 or Cdc6-8, would induce an extra round of DNA synthesis independently of a Sic1-dependent drop in Cdc28/Clb-associated kinase activity in nocodazole-arrested cells. To address this possibility we repeated the experiment described in Fig. 5 in a cdc6-1 GAL1-10:SIC1 strain transformed with a plasmid expressing cdc6-7 from the wild-type CDC6 promoter (pHR7cdc6-7), with the idea of testing DNA re-replication ability of the cdc6-7 allele at the restrictive temperature for cdc6-1. SIC1 expression was induced in cells previously blocked in nocodazole, as checked by fluorescence-activated cell sorting.
Cdc28 Regulates Cdc6 Proteolysis

We were interested also in understanding whether G1 CDK-deficient mutant cells accumulate the Cdc6 protein by means of a post-transcriptional mechanism. To evaluate Cdc6 stabilization and morphological analysis. After 40 min of induction, GAL1-10-regulated expression was repressed with glucose, and cell DNA content was measured in samples taken at regular intervals from cultures incubated at 25 or 37 °C, permissive and restrictive temperatures for cdc6-1, respectively. As shown in Fig. 6, rereplication was dependent upon the SIC1-induced drop in Cdc28 kinase activity, suggesting that stabilization of Cdc6 does not interfere with regular controls limiting DNA re-replication during mitosis. A similar result was obtained with cdc6-1 GAL1-10:SIC1 pHR70CDC6 cells used as wild type control.

Cdc6 Protein Becomes Stabilized on G1 CDK-deficient Mutant Yeast Strains Because of a Post-transcriptional Mechanism—Based on nocodazole-arrested cells, to here, our results clearly suggest that there is an inverse correlation between Cdc6 protein levels and CDK kinase activity during mitosis. We were interested also in understanding whether G1 CDK-deficient mutant cells accumulate the Cdc6 protein by means of a post-transcriptional mechanism. To evaluate Cdc6 stabilization in cells deficient in p34^CDC28^-associated kinase activity, we took advantage of cdc6-4 and cdc6-13 thermosensitive alleles. Cells bearing the temperature-sensitive cdc6-13 allele were incubated at the permissive (25 °C) or the restrictive (38 °C) temperature for 60 or 90 min, and protein extracts were analyzed with antiserum to Cdc6 (Fig. 7A, right panel). Cdc6 wild-type protein accumulated at the restrictive temperature as a single band of 57 kDa. We also analyzed the accumulation of Cdc6 in a cdc6-4 mutant background (Fig. 7A, left panel). It has previously been described that this particular mutation of the kinase leads to an efficient pre-START block of S. cerevisiae cells (40). In fact, p34 immunoprecipitates from these mutant cells have low kinase activity (Fig. 8A), and we therefore expected to find a significant stabilization of p57^CDC28 in this background. An exponentially growing culture at the permissive temperature (25 °C) was shifted to the restrictive temperature (37 °C), and samples were taken at 1-h intervals for fluorescence-activated cell sorting analysis and protein detection. Flow cytometry and microscopic analysis demonstrated that cells were arrested in G1 (data not shown). Western analysis of the cell extracts revealed that Cdc6 accumulated as a single band that was not present either in cells deleted for CDC6 or in asynchronous cells (Fig. 7A, left panel). The accumulation of the Cdc6 protein in Cdk-deficient yeast strains cdc6-28-4 and cdc6-28-13 was attributable to a post-transcriptional mechanism, given that CDC6 mRNA did not accumulate when both mutant strains were blocked at the restrictive temperature (Fig. 7B), whereas the CLN1 mRNA already accumulated as described previously (41). These findings suggest that Cdc6 protein is stabilized in S. cerevisiae cells devoid of a functional p34^CDC28 kinase.

A Transient Loss of Cdc28 Function in S. cerevisiae cdc6-28-4 or cdc6-28-13 Mutant Cells Correlates the Absence of Kinase Activity with Cdc6 Protein Stabilization—An advantage of using these two G1-deficient alleles of CDC28 is that they show different levels of kinase activity under permissive or restrictive conditions (Fig. 8A). In fact, on measuring the in vitro kinase activity immunoprecipitable from cdc6-28-4 or cdc6-28-13...
Asyn, cells overexpressing arrested at 37 °C. Total RNA was prepared from asynchronous wild appears in the Western analysis that is also detectable in a B, GAL1::CDC6 cdc28-4 for of the CLN1 as probes (the latter for a loading control). Note the accumulation agarose gels and analyzed by Northern blot using of total RNA from each sample were separated on 1% formaldehyde-
the restrictive temperature at the indicated intervals. Ten micrograms growing either asynchronously at 25 °C (cdc28-4) or after arrest by shift to OH cdc moter (cdc28-13 mutants defective in Cdc28-kinase activity (40, 42). Neverthe-
cdc28-4 strain bearing the temperature-sensitive mutant allele had to be incubated at 38 °C (Fig. 7). We
therefore sought to determine whether a pulse of limited du-
ratation at 37 °C (a generation time) would cause a different
degree of accumulation of Cdc6 protein in cdc28-4 compared with cdc28-13 S. cerevisiae mutant cells and whether Cdc6 would be more stable on a return to 25 °C in cdc28-4 mutant cells, given that this allele shows lower p34\textsuperscript{CDC28}-associated kinase activity. At this temperature cdc28-13 mutant cells accumulated less Cdc6 protein than did cdc28-4 (Fig. 8B), consistent with the leakiness of the former mutant allele (42). After release at 25 °C, most p57\textsuperscript{CDC6} had disappeared by ~120 min in cdc28-13 cells (Fig. 8B, upper panel). The behavior of the strain bearing the cdc28-4 temperature-sensitive mutant allele was quite different. At the restrictive temperature Cdc6 accumulated at higher levels, and after release the protein became stabilized for up to 180 min and was still, although barely, detectable after 260 min (Fig. 8B, lower panel). Again the accumulation of the Cdc6 protein was attributable to a post-
transcriptional mechanism of stabilization, because CDC6 mRNA did not accumulate during the experiment, as checked by Northern analysis (data not shown). These experiments show that cdc28-4 cells accumulate more p57\textsuperscript{CDC6} protein than
cdc28-13 cells and suggest a correlation between low kinase activity and Cdc6 protein stabilization in G\textsubscript{1}.

**DISCUSSION**

Control Regulating Cdc6 Proteolysis in *S. cerevisiae*—Bio-
chemical and genetic studies have strongly suggested that the Cdc6 DNA replication protein is ubiquitinated for degradation in each cell cycle at the beginning of the S phase by a Cdc4/Cdc34/Cdc53-dependent mechanism (23, 27, 28). Although Cdc28-mediated phosphorylation of key regulatory proteins is known to play a role in regulating their Cdc4/Cdc34/Cdc53 ubiquitination, as is the case for Sic1 and Cln2 (39, 43, 44), to our knowledge until now the role of Cdc28/Cib-mediated Cdc6 phosphorylation in *S. cerevisiae* remains to be elucidated.

In this report we have described a regulatory mechanism controlling Cdc6 protein degradation. Several of the lines of evidence presented here suggest that one consequence of the rise in Cdc6 activity during the G\textsubscript{1}-to-S phase transition is the phosphorylation of Cdc6 protein for targeting it for proteolysis. In this regard, we have shown that under restrictive condi-
tions, in which the Cdc28 kinase is thermolabile, p57\textsuperscript{CDC6} accumulates in cdc28-4 and cdc28-13 ts alleles (Figs. 7A and 8), mutants defective in Cdc28-kinase activity (40, 42). Nevertheless, the accumulation of a protein could be achieved by regul-
ating its turnover or its synthesis. Northern analysis of the CDC6 mRNA abundance (Fig. 7B) rules out a role for the accumulation of the CDC6 transcript in the regulation of the Cdc6 protein abundance. These lines of evidence suggest that Cdc6 is stabilized in *S. cerevisiae* cells devoid of a functional p34\textsuperscript{CDC28} kinase because of a post-transcriptional mechanism. Because cdc28-4 accumulates more Cdc6 protein than cdc28-13 (Fig. 8), our experiments with these two cdc28 ts alleles also...
suggest a direct correlation between the lack of Cdc28 kinase and Cdc6 protein stabilization (Figs. 7 and 8). Nevertheless, the accumulation of a given protein could be an indirect consequence of the cell cycle defect of the mutant being analyzed. In fact, wild-type Cdc6 protein accumulates in cdc28-13 and cdc28-4 mutant strains of S. cerevisiae, cdc28 ts alleles that block cell cycle progression in G1 (45, 46), but not in cdc28-1N (data not shown), a G1-defective allele (40, 47). Nonetheless, our data regarding Cdc6 accumulation in mitotic-arrested cells by inducing a reduction in Cdc28 kinase activity (Fig. 5) suggest that the initiator protein stabilizes because of a genuine defect in p34 kinase. Moreover, our approach to examining whether Cdc28 affects or controls Cdc6 stability by analyzing the rate of its disappearance in promoter shut off experiments showed that nocodazole-arrested cells require Cdc28 to degrade Cdc6 protein (Fig. 2). Taken together, these data and the biochemical evidence described above suggest that a Cdc28-dependent mechanism targets Cdc6 for degradation. Consistent with this hypothesis, the mutation of seven of eight or all Cdc28 consensus phosphorylation sites results in Cdc6 protein stabilization (Fig. 3). In fact, certain data not included in this report suggest that Cdc6 has two different “hot” Cdc28 consensus phosphorylation locations, each involving two phosphorylatable residues and hence playing a role in protein stability, because double mutations in residues T39A and S43A or T368A and S372A result in mutant forms of Cdc6 protein that are considerably more stable than wild-type Cdc6 (to be described elsewhere).

Finally, it is worth mentioning that p57CDC6 is ubiquitinated for degradation through the Cdc4/34/53 pathway (27, 28), because it has been argued that this complex may act to specifically target the degradation of proteins involved in cell cycle control in a Cdc28-dependent manner of phosphorylation (48). Our work, together with the above observations regarding Cdc6 proteolysis (27, 28), indicates that Cdc28 phosphorylates Cdc6 and, on doing so, targets it for degradation through the ubiquitin-mediated proteolysis pathway. Yet some important details regarding Cdc6 protein phosphorylation remain to be elucidated. For example, at present it is unknown whether the CDK consensus phosphorylation sites present in Cdc6 are in vivo substrates for Cdc28. However, given the interactions between Cdc6p and Cdc28 (Ref. 22 and Fig. LA) and the results presented here with Cdc6-7 and Cdc6-8 mutant proteins (Fig. 3), we think this is most likely to be the case.

Does Cdc6 Proteolysis Play Any Role in Genome Ploidy Maintenance?—The experiments presented in this paper strongly suggest that Cdc6 is degraded by a Cdc28-dependent mechanism, given that elimination of the CDK consensus phosphorylation sites of Cdc6 results in the stabilization of the initiator protein (Fig. 3). On top of that, the mutants lacking these phosphorylation sites are fully functional (Figs. 4 and 6), because they complement the deletion of Cdc6 (Fig. 4), and fully stable. Our results are consistent with those presented previously regarding a truncated form of Cdc6 in which the Cdc4 interaction domain has been deleted (28), resulting in a stable protein also able to promote DNA synthesis. Thus, it is reasonable to ask why the Cdc6 protein is unstable. Cyclin B-CDK complexes prevent recombination during G2 and M phases by inhibiting reformation of pre-RCs at replication origins (7). At the same time, Cdc6 is an absolute requirement for DNA replication, because in the absence of this initiator protein no pre-RCs are formed (16). Furthermore, normal S. cerevisiae cells degrade Cdc6 every cell cycle at the beginning of S phase (19, 28), most probably in a Cdc28-dependent manner (this work). Taken together these lines of evidence suggest that budding yeast Cdc28-controlled Cdc4/34/53-mediated Cdc6 proteolysis is part of a redundant CDK-mediated mechanism that prevents the reinitiation of DNA replication within the same cell cycle.

Role of CDK-mediated Phosphorylation of the Cdc6/Cdc18 Class of Proteins in Eukaryotes—The closest homolog of Cdc6 in fission yeast is the cdc18+ gene-encoded product. Both in fission and budding yeast, gene expression of cdc18+ and CDC6 are transcriptionally regulated, and both Cdc18 and Cdc6 proteins levels vary during the cell cycle, peaking at the G1-to-S phase transition (18, 19, 49, 50). In these unicellular eukaryotes, de novo Cdc18 and Cdc6 protein synthesis is critical for the formation of pre-RCs required for the initiation of DNA replication. Cdc18 phosphorylation by CDKs has recently been shown to play a role in targeting the initiator protein for degradation at the G1-to-S phase transition (25, 26). Thus, from the biochemical evidence presented here, and given that Cdc18 and Cdc6 are ubiquitinated for proteolysis in fission and budding yeast (27, 51), the Cdc28/2-mediated mechanism by which these simple eukaryotes control Cdc18/Cdc6 stability appears to be conserved. Although human Cdc6 is also a substrate for CDKs, the consequences of such phosphorylation seem to be quite different. It has recently been reported that CDK phosphorylation of human Cdc6 regulates its subcellular localization throughout the cell cycle (52). Unphosphorylated protein is nuclear during G1, whereas CDK-phosphorylated Cdc6 localizes at the cytoplasm along the rest of the cell cycle.

Acknowledgments—We thank Francisco Antequera, Manolo Arelano, Jose Castano, Karim Labib, Sergio Moreno, and the Avelino Sergio Paco laboratory for helpful discussions and Francisco Antequera, Karim Labib, and Sergio Moreno for critically reading the manuscript. We are grateful to Ricardo Basco, Daniel Lew, and Curt Wittenberg for yeast strains and antibodies. We also thank N. Skinner for correcting the English text.

REFERENCES

1. Chong, J. P. J., Thomés, P., and Blow, J. (1996) Trends Biochem. Sci. 21, 102–106
2. Kearsey, S. E., Labib, K., and Maiorano, D. (1996) Curr. Opin. Genet. Dev. 6, 208–214
3. Wurin, J., and Nurse P. (1996) Cell 85, 785–787
4. Dutta, A., and Bell, S. P. (1997) Annu. Rev. Cell Dev. Biol. 13, 293–332
5. Brock, D., Bartlett, R., Crawford, K., and Nurse, P. (1991) Nature 349, 328–332
6. Haylès, J., Fisher, D., Woolard, A., and Nurse, P. (1994) Cell 78, 813–822
7. Dahmann, C., Difley, J. F. X., and Nasmyth, K. A. (1995) Curr. Biol. 5, 1257–1260
8. Newton, C. S., and Theis, J. F. (1993) Curr. Opin. Genet. Dev. 3, 752–758
9. Bell, S. P., and Stillman, B. (1992) Nature 357, 128–134
10. Difley, J. F. X., and Cockier, J. H. (1992) Nature 357, 169–172
11. Difley, J. F. X., Cockier, J. H., Bowl, S. J., and Rowley, A. (1994) Cell 78, 353–361
12. Dowell, S. J., Romanowski, P., and Difley, J. F. X. (1994) Science 265, 1243–1246
13. Liang, C., Weinreich, and Stillman, B. (1995) Cell 81, 667–676
14. Apricoro, O. M., Weinstein, D. M., and Bell, S. P. (1997) Cell 91, 59–69
15. Tanaka, T., Knapp, D., and Nasmyth, K. (1997) Cell 90, 649–660
16. Cocker, J. H., Piatti, S., Santocanale, C., Nasmyth, K., and Difley, J. F. X. (1996) Nature 379, 180–182
17. Kelly, T. J., Martin, G. S., Forsburg, S. L., Stephen, R. J., Russo, A., and Nurse, P. (1995) Cell 74, 371–382
18. Nishihata, H., and Nurse, P. (1995) Cell 83, 397–405
19. Piatti, S., Lengauer, C., and Nasmyth, K. (1995) EMBO J. 14, 3788–3799
20. Musi-Falconi, M., Brown, G. W., and Kelly, T. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1566–1570
21. Leatherwood, J., Lopez-Girona, A., and Russell, P. (1996) Nature 379, 360–363
22. Elssasser, S., Lou, F., Wang, B., Campbell, J. L., and Jang, A. (1996) Mol. Biol. Cell 7, 1723–1735
23. Piatti, S., Böhm, T., Cockier, J., Difley, J. F. X., and Nasmyth, K. (1996) Genes Dev. 10, 1516–1531
24. Couté, M., Kearsey, S., and Mechali, M. (1996) EMBO J. 15, 1085–1097
25. Jalliappan, P. V., Brown, G. W., Musi-Falconi, M., Tierny, D., and Kelly, T. J. (1997) Genes Dev. 11, 2767–2779
26. López-Girona, A., Mondesert, O., Leatherwood, J., and Russell, P. (1998) Mol. Biol. Cell 9, 63–73
27. Sánchez, M. M., Calazans, A., and Bueno, A. (1999) J. Biol. Chem. 274, 9092–9098
28. Drury, L. S., Perkins, G., and Difley, J. F. X. (1997) EMBO J. 16, 5966–5976
29. Cole, G. M., Stone, D. E., and Reed, S. I. (1996) Mol. Cell. Biol. 16, 510–517
30. Bueno, A., and Russell, P. (1992) EMBO J. 11, 2167–2176
31. Sanger, F., Nicklen, S., and Coulson, A. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5469
32. Sadhu, K., Reed, S. I., Richardson, H., and Russell, P. (1990). Proc. Natl. Acad. Sci. U. S. A. 87, 5139–5143
33. Millar, J., McGowan, C., Lenaers, G., Jones, R., and Russell, P. (1991). EMBO J. 10, 4301–4309
34. Sambrook, J., Fritsch, E., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
35. Guthrie, C., and Fink, G. R. (1991). Methods Enzymol. 194, 1–933
36. Hutter, K. J., and Eipel, H. E. (1979). J. Gen. Microbiol. 113, 369–375
37. Moreno, S., Klar, A., and Nurse, P. (1991). Methods Enzymol. 194, 795–723
38. Laemmli, U. K. (1970). Nature 227, 680–685
39. Lanker, S., Valdivieso, M. H., and Wittenberg, C. (1996). Science 271, 1597–1601
40. Surana, U., Robitsch, H., Price, C., Schuster, T., Fitch, I., Futcher, A. B., and Nasmyth, K. (1991). Cell 65, 145–161
41. Wittenberg, C., Sugimoto, K., and Reed, S. I. (1990). Cell 62, 225–237
42. Reed, S. I., and Wittenberg, C. (1990). Proc. Natl. Acad. Sci. U. S. A. 87, 5697–5701
43. Schwob, E., Böhm, T., Mendenhall M., and Nasmyth K. (1994). Cell 79, 233–244
44. Willems, A. R., Lanker, S., Patton, E. E., Craig, K. L., Nason, T. F., Kobayashi, K., Wittenberg, C., and Tyers, M. (1996). Cell 86, 453–463
45. Hartwell, L., Culotti, J., Pringle, J., and Reed, B. (1974). Science 183, 46–51
46. Reed, S. (1980). Genetics 95, 566–577
47. Piggott, J. A., Rai, R., and Carter, B. L. A. (1982). Nature 298, 391–394
48. Deshaies, R. J. (1995). Curr. Opin. Cell Biol. 7, 781–789
49. Zwerschke, W., Rottjakob, H. W., and Kuntzel, H. (1994). J. Biol. Chem. 269, 23351–23356
50. Muzi-Falconi, M., Brown, G. W., and Kelly, T. (1996). Curr. Biol. 6, 229–233
51. Kominami, K., and Toda, V. (1997). Genes Dev. 11, 1548–1560
52. Petersen, B. O., Lukas, J., Sørensen, C. S., Bartek, J., and Helin, K. (1999). EMBO J. 18, 396–410
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J. Biol. Chem. 2000, 275:9734-9741. doi: 10.1074/jbc.275.13.9734

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