The Cdc6 Protein Is Ubiquitinated in Vivo for Proteolysis in Saccharomyces cerevisiae*

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The Saccharomyces cerevisiae Cdc6 protein is necessary for the formation of pre-replicative complexes that are required for firing DNA replication at origins at the beginning of S phase. Cdc6p protein levels oscillate during the cell cycle. In a normal cell cycle the presence of this protein is restricted to G1, partly because the CDC6 gene is transcribed only during G1 and partly because the Cdc6p protein is rapidly degraded at late G1/early S phase. We report here that the Cdc6p protein is degraded in a Cdc4-dependent manner, suggesting that phosphorylated Cdc6 is specifically recognized by the ubiquitin-mediated proteolysis machinery. Indeed, we have found that Cdc6 is ubiquitinated in vivo and degraded by a Cdc4-dependent mechanism. Our data, together with previous observations regarding Cdc6 stability, suggest that under physiological conditions budding yeast cells degrade ubiquitinated Cdc6 every cell cycle at the beginning of S phase.

In eukaryotes, duplication and segregation of the genome are strictly regulated processes that cells must alternate for successful cell division. Studies of yeast have suggested that this coordination is achieved by means of the Ser/Thr kinase activity of p34cdc2/cdc28*, given that activation of this kinase is required first for initiating genome replication and second for cells to enter mitosis (1). Nevertheless, initiation of DNA synthesis in replicating cells requires chromatin to be competent for the p34cdc2/cdc28-mediated activation. At the molecular level, competency means that a multisubunit protein complex, called pre-replicative complex (pre-RC),† must be formed at origins of replication (ARSs) previous to fire initiation of DNA replication. Association with chromatin is a hierarchical process in which ARSs first recruit the origin recognition complex, then Cdc6p, and finally the Mcm complex, consisting of Mm2–7 proteins (2, 3). In budding yeast Saccharomyces cerevisiae, formation of pre-replicative complexes is dependent upon Cdc6, because association of the Mcm proteins relies on the presence of a functional CDC6 gene product (3, 4). Furthermore, direct genetic evidence indicates that CDC6 is rate-limiting for the initiation of DNA replication by interacting with origin recognition complex at origins (5).

In S. cerevisiae the ubiquitin-mediated proteolysis may play a role in cell cycle (6). Proteolysis may trigger the initiation of S phase and the transition from metaphase to anaphase by two distinct ubiquitin-conjugation pathways, the former requiring CDC34 and the latter involving the anaphase-promoting complex (reviewed in Refs. 7 and 8). In G1 for example, Cln/Cdc28-phosphorylated Cln2p is targeted for degradation in a Cdc53p-dependent manner (9). Comparably, proteolysis of Sic1p relies on Cdc4p at the G1 to S phase transition (10). In fact, Sic1p must be Cdk-phosphorylated prior to its in vitro Cdc34-dependent ubiquitination (11). It is well known that the Cdc4p protein acts in concert with Cdc34p and Cdc53p to ubiquitinate proteins (12). Given the importance of some of these substrates, it is accepted that the Cdc4p-Cdc34p-Cdc53 complex controls the G1 to S phase transition (reviewed in Ref. 7). All these data support the notion that this complex may act to specifically target degradation of proteins implicated in cell cycle control in a CDC28-dependent phosphorylation manner (13–15). Cdc34p is an E2-type ubiquitin-conjugating enzyme that together with Cdc53p and Skp1p form the core complex of ubiquitination for degradation of G1 cyclins Cln1p and Cln2p and the Cdk inhibitor Sic1p. These ubiquitination processes require different F box proteins, whereas Grr1p participates in targeting G1 cyclins (15, 16), Cdc4p may interact with the Cdk inhibitor once it has been phosphorylated (10, 11). It has been argued that Cdc4p may physically interact both with phosphorylated substrates and Skp1p to form the ubiquitinating complex (reviewed in Ref. 12).

This paper addresses the mechanism degrading the key DNA replication initiator Cdc6p protein at the G1 to S phase boundary. Cdc6p is accumulated in ubiquitin-mediated proteolysis mutants, cdc4-1 and cdc34-2. In accordance with recently published data (17), our results indicate that Cdc4p and Cdc34p participate in Cdc6p proteolysis, suggesting that the initiator protein is phosphorylated for degradation through the proteasome. Consistent with this hypothesis, we present data showing that the Cdc6p protein is ubiquitinated in vivo in budding yeast before it is degraded.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, Culture Conditions, and General Techniques—All S. cerevisiae strains were derived from 15Dau unless indicated. 15Dau is a MATa derivative of BF264-15D (18). Temperature-sensitive and deletion mutants used were as follows: cdc5-1 leu2 ura3; cdc4-1 GAL1: CDC6(URA3) leu2 ura3; cdc34-1 sic1::URA3 leu2 ura3; cdc34-2 ura3 his3; cdc34-2 sic1::URA3 leu2 ura3; and cdc6::kanMX4 GAL1::CDC6 (URA3).

Cells were grown in YEPD (1% yeast extract, 2% Bactopeptone, 2% glucose), YEPR (1% yeast extract, 2% Bactopeptone, 2% raffinose), or YEPGal (1% yeast extract, 2% Bactopeptone, 2.5% galactose), except when selecting for plasmids. In this case, cultures were grown in minimal media with supplemented amino acids. The strain deleted for CDC6 conditionally grows on galactose media thanks to the GAL1:

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† The abbreviations used are: pre-RC, pre-replicative complex; MOPS, 4-morpholinepropanesulfonic acid; Cdk, cyclin-dependent kinase; Ha, hemagglutinin; Ub, ubiquitin; FACS, fluorescence-activated cell sorter; ARS, autonomously replicating sequence.

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**CDC6 allele inserted at the **ura3** locus, as checked by Southern blots. Repressing with glucose results in cell cycle block due to the lack of any functional Cdc6 product, as described previously (19). Induction of genes from the CUP1 promoter was accomplished by growing cells in YEPD supplemented with 25 μM CuSO4 according to Ecker et al. (20).**

General molecular techniques were performed as described (21, 22).

The DNA content of individual cells was measured using a Becton Dickinson FACScan. Cells were prepared for flow cytometry by staining them with propidium iodide following the method of Hutter and Eipel (23).

### Preparation of Cdc6 Antibodies—**An NdeI-BamHI Cdc6 containing fragment was cloned into bacterial expression vector pT7-7. *Escherichia coli* BL21-DE3 cells transformed with this plasmid were induced to express the fusion protein by isopropyl-1-thio-

**E. coli** fragment was cloned into bacterial expression vector pT7-7. Cdc6 protein was purified to homogeneity from inclusion bodies by solubilization in 6 mM NaH2PO4, 4 mM Na2HPO4, 2% SDS, pH 6.8 buffer. Solubilized protein was loaded into a 11% SDS-polyacrylamide electrophoresis gel and purified by electrosolution. SDS was removed and fusion protein partially refolded dialyzing against 25 mM Tris, 192 mM glycine, pH 8.3 buffer. Protein concentration was estimated by comparing with bovine serum albumin standards. Rabbits were immunized with 25 μg of full-length Cdc6. After the third injection, antibody-containing serum was collected and titrated by immunoblot.

The antibodies were affinity purified on nitrocellulose filter prefixed with Cdc6 fusion protein purified to homogeneity. This antiserum was incubated with the filter, washed with phosphate-buffered saline solution, and eluted by 100 mM glycine HCl, pH 2.5. The Cdc6 affinity purified antibody was also characterized by immunoblot.

Mouse polyclonal antibodies were obtained as described for rabbit antibodies with some modifications as follows: 12 μg of purified and refolded Cdc6264 protein per dosage were intraperitoneally injected into mice with Freund's complete adjuvant. 10 days after the third injection, ascites fluid was collected, centrifuged to discard cell debris, and the antibody-containing supernatant titrated and characterized by immunoblot (Fig. 3) and used in the indicated dilutions.

### Protein Extract Preparation—**Soluble protein extracts were prepared as described previously (24). Cells were collected, washed, and broken in 30 μl of HB buffer by glass beads. HB buffer contains 60 mM β-glycerophosphate, 15 mM N-phenethylphosphate, 25 mM MOPS, pH 7.2, 15 mM MgCl2, 15 mM EGTA, 1 mM dithiothreitol, 0.1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 20 μg/ml leupeptin and aprotinin. Glass beads were washed with 500 μl of HB buffer, and the supernatant was recovered. Protein concentration was measured using the BCA assay kit (Pierce).

### Western Blotting—**Protein extracts and immunoprecipitates were electrophoresed using 10 or 8–16% gradient SDS-polyacrylamide gels (25). For Western blots, 40 μg of total protein extracts from each sample were blotted to nitrocellulose, and proteins were detected using anti-Cdc6 affinity purified polyclonal mouse antibody (1:3000), or rabbit (1:100), or the anti-Cdc6 polyclonal antibody (9) (1:5000). Horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies and the ECL kit (Amersham Pharmacia Biotech) were used.

### Results

#### Detection of Cdc6 Gene Product—**The Cdc6 gene encodes a polypeptide of predicted molecular mass of 56 kDa. In order to study the Cdc6 protein in budding yeast, α-Cdc6 polyclonal antisera were produced by injecting rabbits with the full-length protein or mouse with a truncated Cdc6 polypeptide (named Cdc6264) both made in *E. coli* (see “Experimental Procedures”). This 30.6-kDa polypeptide lacks the carboxyl-terminal 249 amino acids downstream of the region interacting with Cdc28 and Cdc4 (17, 26). Cdc6 protein was only detected in immunoblots of protein extracts of cdc28Δ strains,2 cdc4-1, or cdc34-2 mutants blocked at the restrictive temperature. Neither with rabbit α-Cdc6 nor with mouse α-Cdc6264 antibodies did we detect Cdc6 protein in immunoblots of extracts from asynchronous cultures of wild type cells, indicating that it is not an abundant protein.

Affinity purified rabbit antibodies detected two bands by Western blot, not detected by preimmune serum, of approximately 57 and 65 kDa in cdc4-1 cells blocked in G1 (Fig. 1). The fastest species was absent from protein extracts of strains deleted for Cdc6, suggesting that could be Cdc6. Moreover, cells overexpressing Cdc6 accumulated the same molecular weight band (Fig. 1), so we concluded that this antibody detected specifically endogenous p57CDC6. Affinity purified mouse polyclonal α-Cdc6264 antibodies identified three bands of approximately 53, 57, and 70 kDa present in lysates from cdc4-1 cells blocked in G1. The p57 protein was absent in cell lysates of a strain deleted for Cdc6 and prominent in extracts from cells overexpressing that gene (Fig. 3), indicating that this single band is the endogenous Cdc6.

**Cdc6 Protein Becomes Stabilized on Ubiquitin-mediated Proteolysis-deficient Mutant Yeast Strains—**Cdc6p and Sic1p proteins become unstable late in G1. Given that Sic1p is degraded through the ubiquitin-mediated proteolysis pathway (10, 11), we were interested in studying whether stabilization of Cdc6p is regulated in a similar way. For this reason, we monitored the Cdc6 protein content by Western blot with antisera to Cdc6 in cdc4-1 and cdc34-2 mutants incubated at the restrictive temperature. Samples were taken every hour as shown in Fig. 2. Microscopic and flow cytometry analysis confirmed that the cdc4-1 and cdc34-2 mutant strains arrested with the characteristic multibudded phenotype and that cells had a 1C DNA content. In both mutant backgrounds p57CDC6 accumulated as a single band (Fig. 2a for cdc4-1 and data not shown for cdc34-2). Accumulation of a given protein could be an indirect consequence of the cell cycle defect of the mutant under analysis. A first approach to understand whether or not Cdc6 affects Cdc6 stability was to examine the rate of Cdc6 disappearance in promoter shut-off experiments in a cdc4-1 GAL1-10:CDC6 strain. Expression of CDC6 was induced in nucodazole-arrested cells by incubating them on galactose for 60 min at 37 °C. Transcription was repressed by addition of glucose and Cdc6 stability estimated by Western blotting either at 25 °C (permis-

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2 M. Sánchez, A. Calzada, and A. Bueno, unpublished results.
been described that temperature-sensitive mutants. Nevertheless, it has formally blocked in G1 (for showed that after 3 h, at the restrictive temperature cells were univ- and microscopic examination on samples taken at indicated intervals

37 °C. Samples were taken at indicated intervals, processed for Western-glycose (26); furthermore, Cdc6p accumulates in CDC6 and galactose was added for Afterward, -Cdc6 or -Cdc6 polyclonal antisera. This affinity purified anti-

vivo substrate for this kinase as well as an in vitro phospho-

protein (26); furthermore, Cdc6p accumulates in temperature-sensitive mutants. Then, it is formally possible that Cdc6p stabilizes in cdc4 mutants as a direct consequence of the drop in CDC28-associated kinase activity due to the accumulation of Sic1p. Alternatively, this accumulation might be the consequence of a defect in CDC4 function related to Cdc6p degradation. To distinguish between these two possibilities we studied Cdc6p accumulation in two different mutant backgrounds, cdc4-1 single and cdc4-1 Δsic1 double mutants. The latter strain, although incapable of performing the Cdc4 function at the restrictive temperature (37 °C) and in contrast with the single mutant, arrests with a 2C DNA content (Fig. 2b) consistent with a G2-like arrest as previously shown by others (10). We therefore analyzed by Western blot the content of Cdc6 protein in a cdc4-1 sic1::URA3 double mutant arrested at 37 °C by taking samples at regular intervals. As shown in Fig. 2, Cdc6 also accumulated rapidly under these conditions, and the kinetics of stabilization was comparable to the cdc4-1 single mutant. Similar results were obtained for cdc34-2 and cdc34-2 sic1::URA3 strains. We conclude that Cdc6p is degraded by the CDC4-CDC34 ubiquitin-mediated pathway because these data indicate that it is due to a genuine defect on CDC4-CDC34 function.

Cdc6 Is Ubiquitinated in Vivo—The Cdc4p protein then plays an unknown but essential role in the pathway of degradation of the Cdc6p protein (Ref. 17 and this work). It has been argued that Cdc4p may physically interact both with phosphorylated substrates and Skp1p to form the ubiquitinating complex (12). If this is true it should be possible to block cdc4-1 mutant cells, accumulating Cdc6p in a phosphorylated but non-ubiquitinated form, release them from the block, and detect ubiquitinated-Cdc6p protein prior to degradation. With the purpose of examining the kinetic of accumulation and degradation of ubiquitinated forms of the Cdc6p protein, an indirect strategy was designed. A cdc4-1 yeast strain was transformed with a plasmid carrying the ubiquitin gene tagged with the Ha epitope (Ub-Ha) under the control of the CUP1 promoter (see “Experimental Procedures”). Upon Cu2+ induction at 25 °C, permissive conditions for the cdc4 allele, Ub-Ha efficiently labeled many polypeptides, as shown by Western blot with α-Ha monoclonal antibody (Fig. 1, lower panel). By using this efficient labeling, we wished to see if Cdc6p was one of the Ub-Ha-tagged proteins. A block and release experiment was carried out in which samples were taken at regular intervals for immunoprecipitation and DNA content analysis. Either cdc4-1 or cdc4-1 CUP1:UBHA strains were incubated at 25 °C in the presence of Cu2+ for 2 h before shifting to 37 °C. Cells were incubated for an additional 2 h at this temperature to allow them to accumulate Cdc6p and then were released at 25 °C. After releasing, samples were taken at 0, 5, 20, 35, and 65 min; protein extracts were quantitated, immunoprecipitated with α-Ha monoclonal antibody, and immunoblotted using mouse α-Cdc6 polyclonal antisera. This affinity purified antibody recognizes a single Cdc6-related band in cells overexpressing CDC6 or in cdc4-1-blocked cultures (Fig. 3a, left panel) but reacts with multiple high molecular weight bands as well as low molecular weight bands in cdc4-1 CUP1:UBHA (Fig. 3a, right panel); these bands were absent in the cdc4-1 control strain grown under identical conditions (Fig. 3a, right panel), suggesting that they could correspond to Cdc6p ubiquitin conjugates. To investigate this possibility a second block and release experiment was done in which samples were immunoprecipitated with crude antiserum to Cdc6 and immunoblotted with α-Ha antibody obtaining comparable results (Fig. 4a, left panel). Given that after releasing cultures from the block the proteolytic machinery is re-activated, we interpret that low molecular weight bands are Cdc6p degradation products. These analyses revealed that Cdc6p protein was ubiquitinated in vivo. Ubiquitin conjugates of Cdc6p reached a maximum at 5
Immunoblot analysis with affinity purified mouse antibody that specifically detects the Cdc6 protein in yeast cell extracts. Completed S phase.

As for Fig. 1, the CDC6 deletion control sample was obtained by repressing on glucose CDC6 expression in the strain cdc6::URA3 GAL1: CDC6; nevertheless it was able to grow on galactose-based media. Right panel, immunodetection of ubiquitinated forms of Cdc6 in S. cerevisiae cells. cdc4-1 or cdc4-1 Ub-Ha mutant cells were blocked for 2 h at 37 °C and then released to 25 °C (permissive temperature). Ubiquitin-Ha (Ub-Ha) was expressed from the copper metallothionein (CUP1) promoter by previously incubating cells in YEPD with 25 μM Cu²⁺ for 2 h and then shifting them to 37 °C in the same media to start the experiment as described above. Samples were taken at indicated intervals for immunoprecipitation (Ip). Protein extracts were immunoprecipitated with Ha monoclonal antibody, blotted, and probed with affinity purified mouse a-Cdc6 antibody. b, flow cytometry analysis of DNA content of cdc4-1 and cdc4-1 Ub-Ha cells. Aliquots of samples described in a (right panel) were analyzed by FACS. Note that 65 min after releasing at the permissive temperature (25 °C) most cells have synchronously completed S phase.

and 20 min after releasing cells at 25 °C and then disappeared, that is to say coincident with S phase initiation as analyzed by flow cytometry (Fig. 3b). Ubiquitin ted Cdc6p forms were not further detected. Our results, together with previous observations demonstrating that the Cdc6 protein is degraded early in S phase (17, 19), suggest that p57cdc6 is polyubiquitinated and degraded as soon as budding yeast cells initiate genome replication.

Cdc6 Protein Is Ubiquitinated by a Cdc4-dependent Mechanism—To investigate if the Cdc6 protein was polyubiquitinated in a Cdc4-dependent manner as suggested by experiments described above, we studied Cdc6 ubiquitination in block and release experiments in cdc4-1 and cdc4-1 sic1Δ mutant strains transformed with a plasmid containing the CUP1:UBHA allele. As discussed earlier even though both strains are defective for Cdc4 function at 37 °C, the latter double mutant mimics a G2 block due to the lack of sic1 function (10). Cells expressing Ub-Ha were blocked at 37 °C to accumulate Cdc6. After releasing at 25 °C samples were immunoprecipitated with crude anti-
A distinguishing feature of the Cdc6/Cdc18 class of proteins is their oscillatory pattern of appearance each cell cycle during G1 due in part to its periodic destruction as cells enter S phase (17, 19). Recently, it has been reported that the Cdc6p protein interacts with Cdc4p and that this interaction is required for its degradation (17). To address details regarding the mechanism and the timing of Cdc6p degradation further, we have combined genetic analysis and yeast biochemistry in studying these questions. The major conclusion of this report is that Cdc6p is ubiquitinated each cell cycle in a Cdc4p-dependent manner.

Several important details regarding the Cdc4-Cdc34-mediated degradation of Cdc6 are reported in this paper. First, although Drury et al. (17) reported that cdc4ts, cdc34ts, and cdc53ts mutants do not degrade Cdc6p, their experiments were made ectopically expressing CDC6 from the GAL1-10 promoter. Corroborating their results, we report here that Cdc6p accumulates at the block point of cdc4ts and cdc34ts mutants (Fig. 2). Second, the Cdc4-Cdc34-Cdc53 complex controls the G1 to S phase transition ubiquitinating for degradation the potent Cdk inhibitor Sic1 (10, 11) (reviewed in Refs. 7 and 12). As previously discussed (Fig. 2), our results regarding the accumulation of Cdc6p in cdc4-1 sic1 double mutants indicate that the inhibitor protein stabilizes due to a genuine defect in the ubiquitination pathway rather than a drop in kinase activity. Third, the requirement of Cdc4 and Cdc34 suggests that Cdc6 degradation may involve ubiquitination of the inhibitor protein; however, it does not prove it. Nevertheless, we show here cdc4-1 block and release experiments in which Cdc6p is detected forming ubiquitin conjugates. Our data, together with previous observations regarding Cdc6 stability (17, 19), show that ubiquitination and proteolysis are consecutive steps in Cdc6p degradation that occur soon after the transition of G1 to S phase is initiated. Moreover, by arresting cells in different points of the cell cycle, we have shown that Cdc6 is ubiquitinated in a Cdc4-dependent manner (Fig. 4). Although these data suggest that proteolysis of Cdc6 might be catalyzed by the proteasome, at present we do not know if this is the case. Further experiments need to be done in order to show that the proteasome is indeed involved in Cdc6 proteolysis even though it is likely to be the mechanism.

The closest homologue of CDC6 in fission yeast, cdc18ts is also required for DNA replication (28). Cdc18 protein levels oscillate during the cell cycle, peaking at G1 as Cdc6 does (19, 29). Although at present it is unknown if the Cdc18 protein is degraded at the same time that DNA replication is initiated, given that the overexpression of cdc18ts induces continuous DNA synthesis (29, 30), it has been suggested that proteolysis of this protein might play a key role in limiting DNA replication to once per cell cycle. On the other hand, it has been shown that Cdc18 interacts in vivo with Pop1 and Sud1, Cdc4-related proteins in fission yeast, and that Cdc18 protein accumulates in pop1 and sud1 mutants as well as in proteasome-deficient mutants of the fission yeast, in the latter mutants possibly forming ubiquitin conjugates (31, 32). These data and the results presented here show that degradation of Cdc6/Cdc18 class of inhibitor proteins is conserved at least in simple eukaryotes such as yeast. Despite these similarities, the degree of competence of this degradation mechanism in controlling genome ploidy appears to be slightly different in fission and budding yeast. In Schizosaccharomyces pombe, deletion of either pop1ts or sud1ts results in cells becoming polyploids, suggesting that Cdk-regulated proteolysis may be critical to ensure a single round of DNA replication (31–33). Ploidy control in budding yeast appears to be a Cdk-controlled redundant...
mechanism in which some of the pre-RCs components may be degraded, as is the case for Cdc6 (Refs. 17, 19, and this work), whereas association and/or localization of others may be regulated by Cdk phosphorylation (34–37). Consistent with this notion, cdc4ts mutants do not polyplidize under restrictive conditions (10), or cells expressing a stable form of Cdc6 do not re-replicate (17). In any case, budding yeast Cdc4-regulated Cdc6p proteolysis is part of the mechanism restricting genome duplication to S phase, because in the absence of Cdc6p no pre-RCs are formed (4). Multicellular eukaryotes apparently have developed a slightly different mechanism in which nuclear non-phosphorylated Cdc6 associates with chromatin; meanwhile, Cdk-phosphorylated Cdc6 is probably membrane-bound or cytosolic and therefore unable to interact with chromatin. This has been suggested for *Xenopus* and *Homo sapiens* Cdc6 protein homologues (38–39). Whether or not degradation of nuclear Cdc6 plays a role in maintenance of genome ploidy in higher eukaryotes remains to be elucidated.

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