The Cdc4/34/53 pathway targets Cdc6p for proteolysis in budding yeast

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The budding yeast Cdc6 protein (Cdc6p) is essential for formation of pre-replicative complexes (pre-RCs) at origins of DNA replication. Regulation of pre-RC assembly plays a key role in making initiation of DNA synthesis dependent upon passage through mitosis and in limiting DNA replication to once per cell cycle. Cdc6p is normally only present at high levels during the G₁ phase of the cell cycle. This is partly because the *CDC6* gene is only transcribed during G₁. In this article we show that rapid degradation of Cdc6p also contributes to this periodicity. Cdc6p degradation rates are regulated during the cell cycle, reaching a peak during late G₁/early S phase. Removal of a 47-amino-acid domain near the N-terminus of Cdc6p prevents degradation of Cdc6p. Likewise, mutations in the Cdc4/34/53 pathway involved in ubiquitin-mediated degradation block proteolysis and genetic evidence is presented indicating that the N-terminus of Cdc6p interacts with the Cdc4/34/53 pathway, probably through Cdc4p. A stable Cdc6p mutant which is no longer degraded by the Cdc4/34/53 pathway is, none the less, fully functional. Constitutive overexpression of either wild-type or stable Cdc6p does not induce re-replication and does not induce assembly of pre-replicative complexes after DNA replication is complete.

Keywords: cell cycle/DNA replication/proteolysis

Introduction

The *Saccharomyces cerevisiae* Cdc6 protein and its *Schizosaccharomyces pombe* homologue cdc18⁺ play central roles in DNA replication (Diffley, 1996; Nasmyth, 1996; Stillman, 1996; Wuarin and Nurse, 1996). These proteins are essential for initiating DNA replication and must be synthesized during the G₁ phase of each cell cycle. In the absence of *de novo* synthesis of Cdc6p or cdc18⁺ during G₁, cells are unable to enter S phase and, instead, rapidly lose viability and undergo chromosome segregation without replication (Kelly *et al.*, 1993; Piatti *et al.*, 1995).

Budding yeast replication origins exist in two chromatin states during the cell cycle, both on high copy plasmids and in their normal chromosomal location (Brown *et al.*, 1991; Diffley *et al.*, 1994; Santocanale and Diffley, 1996). After replication origins fire they are in the 'post-

replicative state', which is characterized primarily by binding of the six subunit origin recognition complex (ORC) (Bell and Stillman, 1992; Diffley and Cocker, 1992; Rao and Stillman, 1995; Rowley *et al.*, 1995; Santocanale and Diffley, 1996). At the end of mitosis a 'pre-replicative complex' (pre-RC) assembles, which is characterized by an additional region of protection adjacent to the ORC binding site (Diffley *et al.*, 1994). This is approximately the time when Cdc6p first appears (Piatti *et al.*, 1995). In the absence of *de novo* Cdc6p synthesis, pre-RCs do not form at replication origins (Cocker *et al.*, 1996) and DNA replication cannot occur (Piatti *et al.*, 1995; Detweiler and Li, 1997). In addition, Cdc6p is bound to chromatin during G₁ (Donovan *et al.*, 1997) and pre-RCs are thermolabile *in vivo* in a *cdc6* temperature-sensitive mutant (Cocker *et al.*, 1996; Detweiler and Li, 1997). *CDC6* interacts genetically with the origin recognition complex (ORC) (Li and Herskowitz, 1993; Liang *et al.*, 1995; Loo *et al.*, 1995) and recombinant Cdc6p can interact in cell extracts with ORC (Liang *et al.*, 1995). Cdc6p is required, at least in part, to load the Mcm proteins onto pre-replicative chromatin, a reaction which has been conserved from yeast to *Xenopus* (Coleman *et al.*, 1996; Donovan *et al.*, 1997). Together these results indicate that Cdc6p plays a direct role as a component of functional pre-initiation complexes at replication origins in budding yeast.

In both fission and budding yeasts cyclin-dependent protein kinases (cdks) play a key role in blocking re-replication (Broek *et al.*, 1991; Hayles *et al.*, 1994; Moreno and Nurse, 1994; Dahmann *et al.*, 1995; Piatti *et al.*, 1996). This occurs in budding yeast, at least in part, because Cdc28p (cdk1), together with the B-type cyclins, blocks formation of pre-RCs. Expression of Cdc6p during G₁ can promote efficient formation of pre-RCs and subsequent DNA replication. However, Cdc6p expressed after a 'point of no return' in late G₁ can no longer promote pre-RC formation and DNA replication (Piatti *et al.*, 1996). This point of no return occurs concomitantly with the activation of Cdc28p kinase activity by the S phase-promoting B-type cyclins Clb5 and Clb6 and can be delayed by deletion of the *CLB5* and *CLB6* genes. Furthermore, overexpression of the Clb kinase inhibitor Sic1p in G₂/M is sufficient to drive pre-RC formation (Dahmann *et al.*, 1995). Because entry into S phase requires the presence of at least one Clb (Schwob *et al.*, 1994) and because Clb kinases are present from the beginning of S phase until the end of mitosis, new pre-RCs cannot assemble until after mitosis. Therefore, inhibition of pre-RC formation by Clb kinase appears to play a critical role in limiting DNA replication to once per cell cycle and in making entry into S phase dependent upon passage through mitosis. Understanding how Clb kinases block Cdc6p-dependent pre-RC formation is an important goal in understanding how replication is regulated.

Both *CDC6* and *cdc18⁺* are transcribed during a narrow window in the G₁ phase of the cell cycle (Zhou and Jong, 1990; Kelly *et al.*, 1993; Zwierschke *et al.*, 1994; Piatti *et al.*, 1995) and the levels of Cdc6 and *cdc18⁺* proteins closely mirror the levels of transcript, indicating that they are unstable proteins (Nishitani and Nurse, 1995; Piatti *et al.*, 1995; Muzi-Falconi *et al.*, 1996). An important issue at present is whether regulation of levels and stability of these proteins is important for orderly cell cycle regulation. Overexpression of either *cdc18⁺* or the cdk inhibitor *rum1⁺* causes re-replication in fission yeast. Overexpression of *rum1⁺* leads to accumulation of *cdc18⁺* and it has been suggested that *rum1⁺*-induced endoreduplication might occur by stabilization of *cdc18⁺* (Jallepalli and Kelly, 1996). On the other hand, *cdc4* mutants (see below) stabilize Cdc6 protein and allow its accumulation in G₂/M nuclei without causing re-replication, suggesting that proteolysis of Cdc6p is not the only mechanism by which re-replication is blocked in budding yeast (Piatti *et al.*, 1996). In neither case, however, is it possible to rule out secondary effects; *rum1⁺* overproduction might have an effect unrelated to *cdc18⁺* stability which is responsible for inducing re-replication, while the *cdc4* mutant might also be defective in degradation of an additional factor, such as Sic1p, which might prevent re-replication. Therefore, a better understanding of the mechanisms regulating degradation of these proteins is required.

In this paper we examine degradation of Cdc6p during the cell cycle. We show that a 47-amino-acid domain in the N-terminus of Cdc6p is essential for targeting it for degradation via the Cdc4/34/53 pathway. We also show that stable mutants of Cdc6p support normal, regulated formation of pre-replicative complexes and do not exhibit any evidence of re-replication. These results are discussed with respect to regulation of pre-replicative complex formation in budding and fission yeast.

Results

Identification of Cdc4p as a Cdc6p-interacting protein

In order to better understand the function and regulation of Cdc6p, we searched for Cdc6p-interacting proteins. We have employed a two-hybrid screen (Fields and Song, 1989) using the entire Cdc6p fused to the LexA DNA binding domain as 'bait' and a library of yeast cDNAs fused to the Gal4 transcriptional activation domain (Durfee *et al.*, 1993). A single positive clone that interacted with the Cdc6p-LexA fusion but not LexA alone was identified in this analysis. Sequencing revealed that this clone contained a truncated copy of the *CDC4* gene fused in-frame to the Gal4p activation domain. Genetic experiments indicate that *CDC4* interacts with *CDC34*, *CDC53* and *SKP1* (Mathias *et al.*, 1996). Cdc34p is an E2-type ubiquitin conjugating enzyme (Goebel *et al.*, 1988) and, together, Cdc4p, Cdc34p, Cdc53p and Skp1p target the Cdk inhibitor Sic1p for degradation at the end of the G₁ phase (Schwob *et al.*, 1994). The Cdc4 protein plays an unknown but essential role in this complex. It is composed of an 'F box', required for interaction with Skp1p (Bai *et al.*, 1996), and a series of 'WD-40' repeats (Fong *et al.*, 1986). All of the WD-40 repeats of Cdc4p are present in

Fig. 1. Two-hybrid interaction between Cdc6p and Cdc4p.

(A) Features of the truncated Cdc4p clone isolated in a two-hybrid screen with LexA-CDC6. The relative positions of the F box and the seven WD40 repeats in full-length Cdc4p (amino acids 1–779) are shown above. Below is an indication of the Cdc4p sequences found in the rescued clone (amino acids 339–779), which contains the WD-40 region but not the F box. (B) Cells containing the indicated Gal4 activation domain (GAD) and LexA two-hybrid vectors were assayed for β -galactosidase activity. The column on the left shows cells expressing GAD alone. The column on the right shows cells expressing the GAD-Cdc4 fusion protein. Cells also expressed LexA fused to the indicated regions of Cdc6p.

the clone we have isolated, while the F-box is absent (Figure 1A).

We next used this *CDC4*-containing clone to identify regions of Cdc6p that are necessary for these interactions in the two-hybrid system (Figure 1B). Initial analysis indicated that the first 47 amino acids of Cdc6p were sufficient to interact with Cdc4p (Figure 1B), while the C-terminal 466 amino acids did not (data not shown). Subdivision of the N-terminal 47-amino-acid domain showed that Cdc4p can interact with amino acids 1–18 as well as amino acids 17–47, but the second element (17–47) induced considerably more β -galactosidase activity. This may reflect a higher affinity of Cdc4p for this region, although other explanations are also possible.

The Cdc4p-interacting domain of Cdc6p is required for its degradation

In Figure 2A wild-type (W303-1a) cells were first synchronized in G₁ with α -factor mating pheromone and then released. Levels of Cdc6p, expressed from its own promoter, were then examined by immunoblotting with a

Fig. 2. Removal of the Cdc4p interaction domain stabilizes Cdc6p. (A) A culture of W303-1a cells was grown in YPD until the cells reached a density of 1×10^7 cells/ml, whereupon they were blocked with α -factor. After the population was uniformly blocked (~ 3 h), α -factor was removed by centrifugation and washing. Cells were resuspended in fresh YPD at 1×10^7 cells/ml and returned to 30°C . At the indicated times samples were taken to assess the budding index of the cells and for determination of protein levels by immunoblotting. (B) YLD24 (GAL1-10-*CDC6*) and YLD25 (GAL1-10-*CDC6* Δ NT) were grown in YP-GAL plus 2 mM methionine until they reached a density of 5×10^6 cells/ml, whereupon they were blocked with nocodazole or α -factor. When the cells were blocked glucose was added ($T = 0$ min) to repress the GAL1-10 promoter. Samples were processed and immunoblotted to detect both Cdc6p and Cdc6 Δ NTp.

monoclonal antibody to Cdc6p. This experiment demonstrates that Cdc6p levels are sharply periodic, appearing in the second and third cycles at the end of mitosis and disappearing as buds emerge, which is approximately the time that DNA replication begins. This confirms previous results reported by Piatti *et al.* (1995) and demonstrates that the epitope tag used by these investigators did not significantly alter Cdc6p degradation.

If the two-hybrid interactions described above are of biological significance, we can make two predictions. Firstly, deletion of the N-terminal 47 amino acids should render Cdc6p more stable *in vivo* and, secondly, Cdc6p should be rendered more stable in *cdc4* mutants.

To test the first prediction we used a strain containing a single copy of the intact *CDC6* gene under the control of the *MET3* promoter (Piatti *et al.*, 1995, 1996; Cocker *et al.*, 1996; Donovan *et al.*, 1997). In the presence of

methionine, which represses this promoter (Cherest *et al.*, 1987), this strain rapidly ceases to proliferate (Piatti *et al.*, 1995). Into this strain we have integrated single copies of either full-length *CDC6* or a 5' deletion removing amino acids 2–47 (Cdc6 Δ NT), each under the control of the GAL1-10 promoter. In the presence of methionine and galactose only *CDC6* genes under the control of the GAL1-10 promoter are expressed. In Figure 2B cells were first blocked in either G_2/M with nocodazole or in G_1 with α -factor. The full-length and truncated *CDC6* genes were then switched off by addition of glucose and the rate of Cdc6p or Cdc6 Δ NTp disappearance was followed by immunoblotting. Lanes 1–10 indicate that the full-length protein disappears rapidly after transcriptional repression in both G_1 and G_2/M blocked cells ($t_{1/2} = 13.5$ and 14 min respectively). Figure 2B, lanes 11–20 demonstrate that, unlike the full-length protein, truncated Cdc6 Δ NTp disappears only very slowly after transcriptional repression in both G_1 and G_2/M ($t_{1/2} = 76$ and 46 min respectively). Therefore, in both G_1 and G_2/M the rate of Cdc6p degradation is significantly enhanced by the presence of amino acids 2–47.

Cdc6p degradation requires the *CDC4*, *CDC34* and *CDC53* gene products

Stabilization of Cdc6p in *cdc4* mutants has previously been shown in cells blocked in G_2/M with the microtubule inhibitor nocodazole (Piatti *et al.*, 1996). We have extended this to examine whether two other members of this epistasis group, Cdc34p and Cdc53p, were also required for Cdc6p destruction during G_2/M . To address this we have constructed strains in which the full-length *CDC6* gene was placed under the control of the GAL1-10 promoter. The rate of Cdc6p disappearance in wild-type cells as well as *cdc4*, *cdc34* and *cdc53* mutant cells was examined as follows. After blocking in G_2/M with nocodazole, cultures were shifted to the restrictive temperature (37°C) in galactose-containing medium. After 30 min transcription was repressed by addition of glucose at the restrictive temperature and the rate of Cdc6p disappearance was measured by immunoblotting. In the parental wild-type strain Cdc6p rapidly disappears (Figure 3A, lanes 1–4), so that by 15 min $>50\%$ of the protein is gone. In the *cdc4* (lanes 5–8), *cdc34* (lanes 9–12) and *cdc53* (lanes 13–16) mutants, however, Cdc6p levels remain constant for 60 min. Therefore, Cdc6p destruction requires all three gene products in cells blocked in G_2/M . When similar experiments were performed on strains harbouring mutations in genes encoding components of the anaphase-promoting complex (APC) (Hershko *et al.*, 1994; Lamb *et al.*, 1994; Irniger *et al.*, 1995; King *et al.*, 1995, 1996; Sudakin *et al.*, 1995; Tugendreich *et al.*, 1995; Nasmyth, 1996; Peters *et al.*, 1996; Zachariae *et al.*, 1996) (*cdc16*, Figure 3B, lanes 1–5; *cdc23*, Figure 3B, lanes 6–10) Cdc6p was not stabilized, suggesting that the APC is probably not required for Cdc6p proteolysis during G_2/M .

Cdc6p is not normally present in cells during G_2/M however (Piatti *et al.*, 1995) and, moreover, the experiments described above involve expression of Cdc6p from the strong GAL1-10 promoter. We therefore sought to examine the genetic requirements for Cdc6p degradation under more physiological conditions. More specifically, we sought to determine if Cdc4p is essential for degradation of

Fig. 3. Cdc6p is stabilized in *cdc4/34/53* temperature-sensitive mutant cells. (A) Logarithmically growing cultures of YLD15 (wild-type), YLD16 (*cdc4*), YLD17 (*cdc34*) and YLD18 (*cdc53*) were blocked with nocodazole. When 90% of the cells were blocked the cultures were resuspended in YP-GAL containing nocodazole at 37°C to induce the GAL1-10 promoter. At time $T = 0$ min GAL1-10-*CDC6* expression was repressed by addition of glucose. Samples were taken and processed for immunodetection of Cdc6p. (B) An experiment similar to that shown in (A) was performed using YLD13 (*cdc16*) and YLD14 (*cdc23*), which also harbour an integrated copy of GAL1-10-*CDC6*. (C) and (D) MTY668 and its congenic parental strain were blocked in G_2 with nocodazole. Cells were released from the block into YPD containing α -factor at 37°C. Samples were taken every 10 min to assess budding index (D) and to monitor the levels of endogenous Cdc6p (C) as described in Materials and methods.

Cdc6p when Cdc6p is expressed from its own promoter during G_1 . To address this we arrested wild-type or *cdc4* mutant cells in nocodazole at 25°C and then released them into α -factor mating pheromone at the *cdc4* restrictive temperature. Consistent with what happens during a normal cell cycle, in wild-type cells Cdc6p first accumulates after mitosis and is then largely destroyed during G_1 (Figure 3C, lanes 1–10).

A residual population of Cdc6p remains for an extended period in these α -factor-blocked cells (lanes 7–10). A significant fraction of the Cdc6p present in α -factor-blocked cells is found bound to chromatin (Donovan *et al.*, 1997) which, taken with the result described above, suggests that Cdc6p in pre-RCs may be more stable than free Cdc6p. Note that expression of Cdc6p from the GAL1-10 promoter, even for short periods of time (e.g. Figure 2B), results in levels of Cdc6p which are very much higher than is normally seen during the cell cycle (data not shown). Thus the fraction of Cdc6p in pre-RCs is likely to be exceedingly small. Longer exposures of the immunoblot in Figure 2B, lanes 1–10 show that significant amounts of Cdc6p are still present after 60 min (data not shown).

In the *cdc4* mutant (Figure 3C, lanes 11–20) Cdc6p accumulates to significantly higher levels and is not destroyed during the course of the experiment. Therefore, the dominant pathway for Cdc6p degradation when

expressed at normal levels from its own promoter involves the Cdc4/34/53 pathway. Previous experiments have indicated that Cdc4p has an additional role late in the cell cycle (Schwob *et al.*, 1994). In the experiment shown in Figure 3C and D *cdc4* mutant cells proceed through mitosis and enter G_1 with normal kinetics (Figure 3D), arguing that Cdc4p is not required after the nocodazole block.

Finally, additional evidence for interaction between the N-terminus of Cdc6p and the Cdc4/34/53 pathway is shown in Figure 4. Overproduction of Cdc6p does not alter the morphology of wild-type W303-1a cells (Figure 4A). Overproduction of Cdc6p does not block cell growth but does cause a dramatic change in cell morphology at the permissive temperature in a congenic strain harbouring the *cdc4* mutant (Figure 4C). Similar results are seen when full-length Cdc6p is overproduced in *cdc34* and *cdc53* mutants (data not shown). This phenotype is very similar to the terminal phenotype these mutants exhibit at the restrictive temperature; in each case the cells become multiply budded and elongated. The N-terminus of Cdc6p is critical for generating this phenotype, since overproduction of Cdc6 Δ Ntp did not effect cell morphology (Figure 4D). Taken together, these results indicate that the N-terminal 47 amino acids of Cdc6p target it for destruction via the Cdc4/34/53 pathway, probably via interactions with Cdc4p.

Fig. 4. Genetic evidence for interaction between the N-terminus of Cdc6p and Cdc4p. W303-1a cells overexpressing Cdc6p or Cdc6 Δ NTp from the GAL1-10 promoter at 25°C do not exhibit any change in cell morphology (**A** and **B** respectively). Cdc6p overproduction in a congenic *cdc4* mutant growing at 25°C causes the cells to become elongated and multiply budded (**C**). This effect is mediated by the N-terminus of Cdc6p, since overproduction of Cdc6 Δ NTp in the *cdc4* strain (**D**) had no observable effect.

Post-transcriptional regulation of Cdc6p levels in the cell cycle

Since the *CDC6* gene is periodically transcribed roughly coincident with the appearance of Cdc6p (Zhou and Jong, 1990; Zwierschke *et al.*, 1994; Piatti *et al.*, 1995), the periodic appearance of Cdc6p might simply be explained by periodic transcription followed by constitutive rapid degradation. Alternatively, the stability of Cdc6p may also vary during the cell cycle. To begin to address this, logarithmically growing cells expressing Cdc6p constitutively from the GAL1-10 promoter were synchronized by arrest in G₁ with α -factor followed by release from the block. Cdc6p levels were monitored after release from the block and throughout the following cell cycles. As can be seen in Figure 5A, even with constitutive *CDC6* expression, levels of Cdc6p oscillate dramatically during the cell cycle. The rapid drop in Cdc6p level begins with the emergence of buds (30 min, lane 4) and by the time all of the cells are budded, Cdc6p is barely detectable (40 and 50 min, lanes 5 and 6). Cdc6p levels start to rise again later in the cell cycle, well before cell division (lanes 7–11), and reach a maximum again during the next G₁ period (lanes 12–15). A second period of Cdc6p disappearance can be seen in the next cell cycle, again concomitant with bud emergence (lanes 16–17). This result suggests that Cdc6p is unable to accumulate during late G₁ and/or early S phase. Additional block and release

Fig. 5. Post-transcriptional regulation of Cdc6p levels during the cell cycle. (**A**) A culture of YLD23 (GAL1-10-*CDC6*) grown in YP-GAL was first synchronized with α -factor and then released into fresh medium. After release from the cell cycle block samples were taken at 10 min intervals for 180 min. The budding index was determined to assess synchrony and immunoblotting was performed to observe levels of Cdc6p at each time point. Lanes 1–18 in the immunoblot correspond to the time points above each lane. (**B**) YLD29 (*cdc7*, GAL1-10-*CDC6*) was grown in YP-Raf at 25°C to mid-log phase and synchronized with α -factor. Once a 90% block was achieved the cells were harvested, washed and resuspended in YP-GAL at 37°C. After bud emergence (80 min later) expression of *CDC6* was repressed by addition of glucose. Samples were taken and processed for immunoblotting to detect Cdc6p. (**C**) YLD15 and YLD16 were grown in YP-Raf at 25°C to mid-log phase and then blocked with HU (0.2 M). The cells were left to block for ~5 h, at which time >95% cells were arrested with large buds. The cells were harvested and resuspended in YP-GAL plus HU. After 30 min glucose was added at 2% to repress *CDC6* expression from the GAL1-10 promoter. Samples were taken at the times indicated and processed for immunoblotting.

experiments have indicated that the inability to accumulate Cdc6p during this period requires the N-terminal 47 amino acids of Cdc6p (data not shown).

Figure 5B shows that the inability to accumulate Cdc6p is due, at least in part, to an increase in the rate of Cdc6p degradation at the end of G₁. Cells were blocked at the G₁/S boundary using a *cdc7* temperature-sensitive mutant and Cdc6p degradation rates were measured as above. In this experiment the degradation of Cdc6p was so rapid that we were unable to estimate its half-life, since the protein had largely disappeared by the first time point after transcriptional repression ($t_{1/2} < 5$ min). In longer

Fig. 6. *Cdc6ΔNTp* supports normal growth and regulated pre-replicative complex formation and does not induce re-replication. (A) YLD23 (vector alone), YLD24 (GAL1-10-*CDC6*) and YLD25 (GAL1-10-*CDC6ΔNT*) were streaked out on agar plates to test whether *Cdc6ΔNTp* could support growth. (Panel i) plates contained 2% glucose and 2% galactose as well as 2 mM methionine (repressing both the GAL1-10 and MET3 promoters). (Panel ii) plates contained 2% glucose and 2% galactose but without methionine (repressing the GAL1-10 promoter while inducing the MET3 promoter). (Panel iii) plates contained 2% galactose plus methionine (inducing the GAL1-10 promoter, repressing the MET3 promoter). (Panel iv) plates contained galactose, without methionine (inducing both the GAL1-10 and MET3 promoters). (B) YLD24 (GAL1-10-*CDC6*) and YLD25 (GAL1-10-*CDC6ΔNT*) were grown in YP-GAL to mid-log phase and blocked with either nocodazole or α -factor. Samples were then processed for footprinting of the 2 μ origin of DNA replication. Two DNase I concentrations are shown for each sample. The position of the ORC-induced hypersensitive site is marked with an asterisk in *G₂* blocked cells. The extended region of protection corresponding to the pre-RC is marked with a bracket. (C) Cultures of W303-1a, YLD24 (*CDC6*), YLD25 (*CDC6ΔNT*) and YLD26 (*CDC6NLSΔNT*) were grown overnight in YP-GAL, diluted the next morning and grown to mid-log phase in YP-GAL. Samples were taken and prepared for FACS.

exposures of this immunoblot (data not shown) a low residual level of Cdc6p which does not disappear can be seen, consistent with the idea that Cdc6p in pre-RCs is more stable than free Cdc6p. These experiments show that Cdc6p degradation rates are not constant over the cell cycle but, instead, peak as cells pass from *G₁* into S phase.

Figure 5C shows that wild-type cells blocked in S phase with the ribonucleotide reductase inhibitor hydroxyurea (HU) are unable to accumulate high levels of Cdc6p. *cdc4* mutant cells, however, are capable of accumulating high levels of Cdc6p when blocked in S phase with HU. Moreover, this Cdc6p is completely stable after transcriptional repression. From these experiments we conclude that Cdc6p becomes hyper-unstable during late *G₁* and S phase and is degraded during this period by a Cdc4-dependent pathway.

Stable Cdc6p supports normal cell cycle progression and regulated pre-RC assembly and does not induce re-replication

The experiments described so far reveal rich post-transcriptional regulation of Cdc6p levels. Because of this and because of the effects of *cdc18⁺* overexpression in fission yeast, it was of particular interest to investigate the effects of stabilization of Cdc6p. Identification of the Cdc4p-interacting domain and generation of the stable *Cdc6ΔNT*

mutant has allowed us to do this. Strains containing both MET3-*CDC6* and either GAL1-10-*CDC6* or GAL1-10-*CDC6ΔNT* were first plated under conditions where Met-*CDC6* was repressed and either GAL1-10-*CDC6* or GAL1-10-*CDC6ΔNT* were expressed. As shown in Figure 6A, both full-length and truncated, stable Cdc6p are capable of supporting growth to a similar extent.

Recently, Elsasser *et al.* (1996) have tested a very similar N-terminal Cdc6p deletion and have concluded that their truncation was less functional than the full-length protein. This conclusion was based on the fact that while both full-length and truncated Cdc6p could support growth on galactose, only the full-length protein could support growth on medium containing both glucose and galactose, conditions that should severely repress this promoter. Although we agree that truncated Cdc6p does not support growth on glucose and galactose, we also find that even the full-length protein is unable to support growth under these conditions (Figure 6A), thus we see no differences between the full-length and truncated Cdc6p. Although the reasons for this discrepancy are unresolved, one possibility is that the full-length construct used by Elsasser *et al.* contained 52 bp of Cdc6 promoter sequence absent from their truncation and absent from both of our constructs. It is possible that the growth supported by full-length Cdc6p is due to residual basal

transcription from the remaining Cdc6 promoter. In our hands both full-length and truncated Cdc6p support very similar growth rates on galactose and lose ARS-containing plasmids at similar rates (data not shown) under these conditions. Although the two proteins support very similar levels of growth and DNA replication, we cannot conclude unequivocally that Cdc6p and Cdc6 Δ NTp are equally functional, since comparison is inevitably complicated by the fact that Cdc6 Δ NTp is more stable and, therefore, always more abundant than full-length Cdc6p.

The N-terminal 47 amino acids contain four of the eight $S_{T/P}$ motifs present in Cdc6p, which represent relaxed consensus sequences for cdk1 kinases (Langan *et al.*, 1989; Shenoy *et al.*, 1989). Furthermore, Elsasser *et al.* have shown that Cdc28–Clb kinase interacts strongly with Cdc6p through the N-terminal 47 amino acids (Elsasser *et al.*, 1996). Since Clb kinases block formation of Cdc6p-dependent pre-RCs (Dahmann *et al.*, 1995), it was of considerable interest to examine whether Cdc6 Δ NTp-dependent pre-RC formation was still under cell cycle regulation. To address this we used genomic footprinting to examine the 2 μ origin in α -factor- (Figure 6B, lanes 1, 2, 5 and 6) and nocodazole-arrested (Figure 6B, lanes 3, 4, 7 and 8) cells expressing either full-length (lanes 1–4) or truncated (lanes 5–8) Cdc6p constitutively from the GAL1-10 promoter. In both strains the ORC-induced hypersensitive site (asterisk) is clearly visible in nocodazole-arrested cells. In G_1 blocked cells, a strong pre-RC is seen. This is manifested as suppression of the ORC-induced hypersensitive site. Additionally, DNase I cleavage sites within the bracketed region are relatively less intense in the pre-RC. From this we conclude that the truncated Cdc6 protein supports efficient pre-RC formation during G_1 . Moreover, neither the full-length nor the stable Cdc6p support any detectable pre-RC formation when overproduced during G_2 .

Finally, we have tested whether overexpression of stable or unstable Cdc6p could cause re-replication. To address this we examined the DNA content of cells overproducing either full-length or stable Cdc6p by fluorescence-activated cell sorting (FACS) analysis. The N-terminal 47 amino acids of Cdc6p contain a sequence which resembles a nuclear localization sequence and which can promote nuclear localization when fused to β -galactosidase (Jong *et al.*, 1996). Therefore, to rule out effects caused by differences in subcellular localization, we have also tested a construct in which this N-terminal domain of Cdc6p was replaced with the nuclear localization signal sequence from SV40 large T antigen. This protein, Cdc6 Δ NT–NLSp, is considerably more stable than the full-length protein and is not degraded by the Cdc4/34/53 pathway (data not shown). Cdc6p, Cdc6 Δ NTp or Cdc6 Δ NT–NLSp were expressed for 48 h from the GAL1-10 promoter and DNA content was analysed by FACS analysis. As a control for 1C and 2C DNA contents, W303-1a cells were treated in an identical manner and subjected to FACS analysis. As shown in Figure 6C, overexpression of full-length Cdc6p, Cdc6 Δ NTp or Cdc6 Δ NT–NLSp does not result in accumulation of significant numbers of cells with a DNA content measurably greater than 2C. Thus even high levels of overexpression of stable Cdc6 protein do not induce endoreduplication in budding yeast.

Discussion

Mechanism of Cdc6p degradation

The results described in this paper indicate that a short sequence in the N-terminus of Cdc6p targets it for rapid degradation via the Cdc4/34/53 pathway. This sequence interacts with Cdc4p in a two-hybrid assay, suggesting that Cdc4p may play a role in substrate recognition. This interaction may be direct or may occur through some other component of the pathway that in turn interacts with Cdc4p. The Cdc4p-containing clone we isolated does not contain the 'F box' and, therefore, cannot interact with Skp1 (Bai *et al.*, 1996). Consequently, if the Cdc4p–Cdc6p interaction is indirect, it is unlikely to occur through Skp1. Further biochemical analysis is required to analyse these interactions and the availability of a short Cdc4p-interacting sequence should facilitate this analysis.

PEST sequences have been postulated to play a role in directing unstable proteins for degradation (Rechsteiner and Rogers, 1996) and a single PEST sequence (score 5.15, where scores >5 are considered significant) is found between amino acids 32 and 46. Consistent with the idea that the PEST sequence may be important for interaction, a deletion that removes it (compare Figure 1, amino acids 17–47–17–33) has the consequence that the protein no longer interacts with Cdc4p. While this PEST sequence may be important for Cdc4p interaction, we note that amino acids 1–18, which interact weakly with Cdc4p, do not contain a near match to the PEST sequence.

Two lines of evidence suggest that additional sequences within Cdc6p are also essential for targeting it for degradation. Firstly, the N-terminus of Cdc6p does not confer instability when fused to a heterologous protein, β -galactosidase (data not shown). Since this domain is sufficient for Cdc4p interaction in the two-hybrid system (see Figure 1), this suggests that Cdc4p interaction, though essential, is not sufficient to target a protein for degradation. Secondly, we have recently identified a point mutation within the C-terminal half of Cdc6p which results in significant Cdc6p stabilization (data not shown). Further experiments are underway to characterize this second domain of Cdc6p.

Many important details regarding the mechanism of Cdc6p degradation remain to be worked out. For example, the requirement of Cdc34p for degradation of Cdc6p suggests that degradation might occur via ubiquitination of Cdc6p, however, at present we do not know if Cdc6p is ubiquitinated *in vivo* or *in vitro* by Cdc34p. In addition, we do not know if proteolysis is catalysed by the proteasome. However, given the interactions between Cdc6p and the Cdc4/34/53 pathway, we think this is most likely to be the mechanism.

Regulation of Cdc6p degradation

Phosphorylation of substrate proteins is thought to play a critical role in targeting them for degradation via the Cdc4/34/53 pathway. Thus Sic1p is stable during G_1 but becomes destabilized upon activation of G_1 cyclins (Schwob *et al.*, 1994). Likewise, Cdc53p has been shown to bind to Cln2 only when Cln2 has been phosphorylated (Willems *et al.*, 1996). At present we do not know if Cdc6p phosphorylation plays a role in regulating its degradation, however, the fact that the rate of Cdc6p

Table I. Strain list

Strain	Genotype	Background	Source
4142	<i>cdc15-2, cdc6::hisG URA3 hisG, trp1::TRP1 MET-CDC6, leu2</i>	W3031a	Cocker <i>et al.</i> (1996)
MTY668	<i>cdc4-1</i>	W303-1a	M.Tyers
MTY670	<i>cdc34-2</i>	W303-1a	Willems <i>et al.</i> (1996)
MTY871	<i>cdc53</i>	W303-1a	M.Tyers
5600	<i>MATα, cdc16-123</i>	W303-1a	K.Nasmyth
5087	<i>MATα, cdc23-1</i>	W303 1a	K.Nasmyth
YMIG02	<i>cdc16-123</i>	5600	this study
YMIG03	<i>cdc23-1</i>	5087	this study
2032	<i>cdc7-1, TRP</i>	W303-1a	Piatti <i>et al.</i> (1996)
YLD1	<i>cdc6::hisG</i>	4142	this study
YLD13	<i>cdc16-123, URA3, GAL1-10-CDC6</i>	YMIG02	this study
YLD14	<i>cdc23-1, URA3, GAL1-10-CDC6</i>	YMIG03	this study
YLD15	<i>HIS3, GAL1-10-CDC6</i>	W303-1a	this study
YLD16	<i>cdc4, HIS3, GAL1-10-CDC6</i>	MTY668	this study
YLD17	<i>cdc34-2, HIS3, GAL1-10-CDC6</i>	MTY670	this study
YLD18	<i>cdc53, HIS3, GAL1-10-CDC6</i>	MTY871	this study
YLD23	<i>HIS3</i>	YLD1	this study
YLD24	<i>HIS3, GAL1-10-CDC6</i>	YLD1	this study
YLD25	<i>HIS3, GAL1-10-CDC6ΔNT</i>	YLD1	this study
YLD26	<i>HIS3, GAL1-10-CDC6ΔNTNLS</i>	YLD1	this study
YLD29	<i>cdc7-1, URA3, GAL1-10-CDC6</i>	2032	this study
YLD31	<i>GAL1-10-CDC6ΔNTNLS</i>	W303-1a	this study
YLD32	<i>GAL1-10-CDC6ΔNTNLS</i>	MTY668	this study
L40	<i>URA3::(<i>lexAop</i>)₈-lacZ, LYS2::(<i>lexAop</i>)₄-HIS3</i>	AMR69	Hollenberg <i>et al.</i> (1995)

All strains are MAT α unless otherwise stated.

degradation varies during the cell cycle (Figure 5) suggests that this may be the case. Interestingly, both Cdc6p and Sic1p become unstable late in G₁, suggesting that they may be regulated in a similar manner. Cdc6p and Sic1p differ, however, in the fact that Sic1 is extremely stable in α -factor-blocked cells while Cdc6p is unstable.

Why is Cdc6p an unstable protein?

The experiments presented in this paper demonstrate that free Cdc6p is rapidly degraded, especially around the time that DNA replication initiates. Yet deletion of the Cdc4p interaction domain, which greatly stabilizes the protein without affecting its ability to promote DNA synthesis, causes no obvious phenotype. We estimate that, because of the increased stability of Cdc6 Δ NTp and its expression from the strong GAL1-10 promoter, this protein accumulates to levels during G₂ that are at least 1000-fold higher than normal (data not shown). Despite this vast overexpression, re-replication does not occur and pre-RCs are not formed during G₂. Thus it is reasonable to ask 'why is Cdc6p unstable?'. We suggest two possibilities.

First, we note that the results described here are in sharp contrast to those obtained in fission yeast, where more modest overexpression of *cdc18*⁺ is sufficient to induce rampant re-replication (Nishitani and Nurse, 1995; Muzi-Falconi *et al.*, 1996). Thus one possibility is that in budding yeast redundant mechanisms may act to ensure once per cell cycle regulation of DNA replication, while in fission yeast regulation of *cdc18*⁺ proteolysis may be the primary mechanism by which re-replication is blocked. Since inhibition of Clb–Cdc28 kinase activity is sufficient to promote pre-RC formation in G₂ (Dahmann *et al.*, 1995), any additional mechanisms blocking pre-RC formation during G₂ must also be blocked by Clb–Cdc28 kinase. Candidates for such additional targets might include the Mcm proteins, which are components of Cdc6p-dependent

pre-replicative chromatin (Coleman *et al.*, 1996; Donovan *et al.*, 1997). In budding yeast the Mcm proteins are only present in the nucleus during G₁ (Hennessy *et al.*, 1990; Yan *et al.*, 1993; Dalton and Whitbread, 1995; Whitbread and Dalton, 1995), while in fission yeast at least one of the Mcm proteins (*cdc21*⁺) is constitutively nuclear (Maiorano *et al.*, 1996). Thus perhaps nuclear localization of Mcm proteins in budding yeast is inhibited by Clb kinase, as is the case for nuclear localization of the Swi5p transcription factor (Moll *et al.*, 1991), and this pathway may not exist in fission yeast.

A second possible reason for Cdc6p degradation might be that the N-terminus of Cdc6p has dual functions: it may target Cdc6p for degradation and it may have an additional inhibitory affect on cell cycle progression. Elsasser *et al.* (1996) have shown that this region of Cdc6p interacts very tightly with Clb–Cdc28 and can inhibit its kinase activity. Thus degradation of full-length Cdc6p may be important to allow full activation of Clb–Cdc28 kinase. This might help to explain why overexpression of full-length Cdc6p in certain strain backgrounds causes a G₂/M delay (Bueno and Russell, 1992; Elsasser *et al.*, 1996; data not shown). Why the N-terminus of Cdc6p should interact so tightly with Clb–Cdc28 kinase is unclear since, as shown here, it is not required for blocking pre-RC formation or for firing of replication origins.

Materials and methods

Strains and media

Strains (listed in Table I) were grown in YP containing either glucose (YPD), raffinose (YP-Raf) or galactose (YP-Gal) at 2% unless otherwise stated (Rose *et al.*, 1989).

Plasmid constructs

pRS303G is an integrating vector with the GAL1-10 promoter. It was constructed by inserting an 813 bp fragment containing the GAL1-10

Table II. Oligonucleotide primers

Primer	Sequence 5'→3'
49362	CGCGGATCCGTTGCGTATATCATTGCATC
49627	AAAAAGGATCCGATGTCAGCTATACCAATAACTCCA
49628	AAAAAGGATCCGATGTCAGCTATACCAAT
67393	AAAAAGTTCGACCTACTGCAGTTTTCTGGGGA
67394	AAAAAGTTCGACCTAGTCAAATAGATTTCCTCT
67395	AAAAAGGATCCATGCAGTTTGGCTCACAGTCT
67396	AAAAAGGATCCATTTGACGATGCTCCA
70588	AAAAAGGATCCAGTTTACAGATGTTACAC
70589	TTTTTGTGCGACCTACAACCTTTTCTTTTCA
74507	GATCTGACCTCCGCTTCTTCTTGGGCATTC
74508	GATCGAATGCCCAAGAAGAAGCGGAAGGTCA

promoter between the *EcoRI* and *BamHI* sites of the pRS303 polylinker (Sikorski and Hieter, 1989). pLD3 and pLD4 have full-length *CDC6* and truncated *CDC6* (termed *CDCΔNT*, encoding amino acids 48–513) respectively subcloned into the *BamHI* site of pRS303G so that the 5'-end of the gene is at the promoter-proximal side. In pLD5 the *BamHI*–*SpeI* fragment from pLD3, containing the intact *CDC6* gene, was inserted between the *BamHI* and *SpeI* sites of pRS303G, thus removing the *BamHI* site at the 3'-end of the gene. The N-terminal 139 nt fragment (corresponding to amino acids 1–47) was replaced with a double-stranded oligonucleotide coding for the SV40 nuclear localization signal (oligonucleotide numbers 74507 and 74508 in Table II). pLD6 and 7 are based on pRS306 (Sikorski and Hieter, 1989). pLD6 contains the entire GAL1–10–*CDC6* sequence subcloned from pLD3 using *Clal* and *SacI*, and pLD7 has the entire GAL1–10–*CDC6ΔNT* fragment subcloned from pLD4 in a similar manner.

Oligonucleotide primers

The oligonucleotide primers are listed in Table II.

To construct a LexA–Cdc6p fusion in pBTM116, *CDC6* was generated by PCR amplification using primer 49628 for the 5'-end of *CDC6* and primer 49362, which anneals to a sequence 200 bp 3' of the *CDC6* stop codon. Cdc4p interactions with the N-terminus of Cdc6p were investigated using PCR-generated fragments cloned into the *BamHI* and *SalI* sites of pBTM116. The following pairs of primers were used (5' and 3' respectively); 49628 and 67393 for amino acids 1–47, 49628 and 67394 for amino acids 1–18, 67396 and 67393 for amino acids 17–47, 67396 and 70589 for amino acids 17–33, and 70588 and 67393 for amino acids 34–47.

The *CDC6* gene in pLD3 was amplified by PCR from W303-1a genomic DNA using the 5' primer 49627 and the 3' primer 49362. pLD4 contains a copy of *CDC6ΔNT* generated by PCR using the 5' primer 67395 and the 3' primer 49362.

Two-hybrid experiments

A two-hybrid screen was performed with pBTM116 containing a full-length *CDC6* gene. A yeast cDNA library fused to the Gal4 transcriptional activation domain (a gift from Steve Elledge) was transformed into strain L40 containing the LexA–Cdc6 plasmid. Transformants were first selected for histidine prototrophy and then assayed for induction of β-galactosidase activity. Plasmids from positive clones were rescued into *Escherichia coli* and re-transformed into L40 containing either the LexA–Cdc6 plasmid or LexA alone (pBTM116).

Initial experiments to define the Cdc4p-interacting region of Cdc6p utilized three restriction digest fragments from *CDC6* sub-cloned into BTM116 in-frame with *lexA*. The first fragment derived from a *PstI* digest of the BTM116/*CDC6* plasmid followed by re-ligation of the plasmid. The resulting plasmid contains the first 139 nt of the *CDC6* coding sequence. The second clone contains the first 700 nt of the *CDC6* coding sequence and was also derived from the BTM116/*CDC6* plasmid. This plasmid was generated by a *BglII/BamHI* double digest and re-ligation of the compatible cohesive ends. The third construct was made by removing the *CDC6* gene from the BTM116 plasmid using *HincII* (nt 205 in *CDC6*) and *SalI* (in the polylinker downstream of *CDC6*) and subcloning this fragment into the *SmaI* and *SalI* sites of pBTM116.

Cell cycle experiments and footprinting

Cell cycle synchronization was performed as previously described (Diffley *et al.*, 1994). α-factor block and release or nocodazole block and release experiments entailed washing the blocked cells with 2×2

vol. fresh medium and returning them to fresh medium at the same cell density.

To analyse protein binding at the 2 μ origin, DNase footprinting was performed as described previously (Diffley *et al.*, 1994; Santocanale and Diffley, 1996).

Cells extracts and immunoblotting

Samples of 10 ml (10⁷ cells/ml) were harvested and resuspended in 200 μl ice-cold lysis buffer (100 mM NaCl, 25 mM Tris, pH 7.6, 10% w/v glycerol, 0.1% w/v Tween) containing protease inhibitors (1 μg/ml pepstatin A, 10 mM benzamidine, 2 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride). An equal volume of acid-washed glass beads was added and the samples were vortexed to achieve lysis. One half volume of 3× Laemmli SDS sample buffer was added to the lysates and they were boiled for 3 min. Samples were run on 10% SDS–polyacrylamide gels, immunoblotted and detected using ECL (Amersham) reagents as per the manufacturer's instructions. Detection of Cdc6p was performed using monoclonal antibody 9H85 (Donovan *et al.*, 1997).

Estimation of protein degradation rates

Autoradiographs were scanned using a Molecular Dynamics densitometer and amounts of Cdc6p were determined using ImageQuant software. Plots of time against ln[Cdc6p] were used to estimate half-lives using the formula $t_{1/2} = -0.69/m$, where m is the slope of the line.

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