Anaphase initiation in *Saccharomyces cerevisiae* is controlled by the APC-dependent degradation of the anaphase inhibitor Pds1p

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Anaphase initiation has been postulated to be controlled through the ubiquitin-dependent proteolysis of an unknown inhibitor. This process involves the anaphase promoting complex (APC), a specific ubiquitin ligase that has been shown to be involved in mitotic cyclin degradation. Previous studies demonstrated that in *Saccharomyces cerevisiae*, Pds1 protein is an anaphase inhibitor and suggested that it may be an APC target. Here we show that in yeast cells and in mitotic *Xenopus* extracts Pds1p is degraded in an APC-dependent manner. In addition, Pds1p is directly ubiquitinated by the *Xenopus* APC. In budding yeast Pds1p is degraded at the time of anaphase initiation and nondegradable derivatives of Pds1p inhibit the onset of anaphase. We conclude that Pds1p is an anaphase inhibitor whose APC-dependent degradation is required for the initiation of anaphase.

[Key Words: Pds1; anaphase promoting complex; *Saccharomyces cerevisiae*; *Xenopus*; ubiquitin-dependent proteolysis]

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The metaphase to anaphase transition is an important step in the eukaryotic cell cycle. This process is believed to be regulated, at least in part, through sister chromatid pairing (for review, see Holm 1994; Miyazaki and Orr-Weaver 1994). Sister chromatid pairing is thought to be mediated by cohesion factors that act as a "molecular glue" in associating the sister chromatids along their entire length. These cohesion factors counteract the poleward forces exerted by spindle motors, thereby preventing precocious sister chromatid separation. At metaphase, the replicated and paired sister chromatids congress to the spindle midzone through their bipolar attachments to the spindle microtubules. At anaphase, the cohesion factors are inactivated and the sister chromatids separate synchronously and move to opposite poles by means of their spindle attachment. Misregulation of the cohesion factors could lead to precocious sister chromatid separation. If this occurs prior to the formation of a stable bipolar spindle attachment, the separated chromatids would not be identified as sisters, thereby diminishing the cell's ability to segregate the two sister chromatids to opposite poles. Anaphase initiation is also controlled by checkpoints that arrest the

cell cycle prior to anaphase in response to spindle or DNA damage, thus allowing time for repair (for review, see Hartwell and Kastan 1994). Following DNA or spindle damage, a premature initiation of anaphase caused by precocious sister chromatid separation would abolish this repair period, leading to chromosome missegregation and the inheritance of damaged DNA.

How the initiation of anaphase is regulated is not clear, but accumulating evidence points to the involvement of proteolysis via the ubiquitin pathway (Murray et al. 1989; Holloway et al. 1993; Irniger et al. 1995). In this process, a ubiquitin-conjugating activity (UBC or E2) together with a ubiquitin ligase activity (also known as E3) attach multiple ubiquitins onto a specific protein substrate, thereby targeting it for degradation (Ciechanover 1994; Hershko et al. 1994; Peters 1994; Murray 1995). The ubiquitin ligase that is thought to be involved in the metaphase to anaphase transition is a multisubunit complex called the anaphase promoting complex (APC) (King et al. 1995) or cyclosome (Sudakin et al. 1995). APC contains Cdc16p, Cdc23p, and Cdc27p (Lamb et al. 1994; Irniger et al. 1995; King et al. 1995), homologs of which have been found in several organisms, including *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Xenopus laevis*, *Drosophila melanogaster*, *Aspergillus nidulans* and human cells (Icho and Wickner 1987; Hirano et al. 1990; Sikorski et al. 1990; O'Donnell et al. 1991; Tu-

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gendreich et al. 1993). Inactivation of these three proteins in vivo leads to a preanaphase arrest (Culotti and Hartwell 1971; Hirano et al. 1988; Tugendreich et al. 1995). These observations suggest that APC-dependent proteolysis inactivates one or more proteins that serve as anaphase inhibitors.

Initially, the only proteins whose degradation was known to be required for passage through mitosis were the mitotic cyclins (Murray et al. 1989). Indeed, mitotic cyclins are largely degraded at the metaphase to anaphase transition, making them good candidates for being the anaphase inhibitor (Murray et al. 1989). The degradation of the mitotic cyclins depends on the presence of a degenerate nine-amino-acid motif, known as the destruction box (Glutzer et al. 1991). The destruction box is required for efficient ubiquitination by APC, and consequently in its absence, cyclins become resistant to APC-dependent degradation (Glutzer et al. 1991; Amon et al. 1994; King et al. 1995; Yu et al. 1996; Zachariae and Nasmyth 1996). Surprisingly, nondegradable cyclin derivatives inhibit the exit from mitosis, rather than anaphase onset (Holloway et al. 1993; Stueland et al. 1993; Surana et al. 1993; van der Velden and Lohka 1993). These findings suggest that the anaphase inhibitor is an APC substrate other than the mitotic cyclins. Indeed, precedence for a noncyclin substrate of APC, Cut2p, has recently been demonstrated in fission yeast (Funabiki et al. 1996b).

If anaphase is regulated by a single inhibitor, one would expect that in its absence, APC function would no longer be required for anaphase initiation. Recently, the product of the *S. cerevisiae* *PDS1* gene (for precocious dissociation of sister chromatids) was shown to fit this criterion: A significant fraction of *pds1 cdc16* and *pds1 cdc23* double mutant cells at the nonpermissive temperature arrested not prior to anaphase, as do the single *cdc* mutants, but rather at the end of mitosis (Yamamoto et al. 1996b). This suggests that Pds1p is an anaphase inhibitor whose degradation via APC is required for anaphase initiation. Hence, in the absence of Pds1p, the requirement for APC in anaphase onset is alleviated. Consistent with this is the observation that Pds1p is rapidly degraded at G_1 (Yamamoto et al. 1996a), a time when APC was shown to be involved in cyclin degradation (Amon et al. 1994). The failure to exit mitosis in the *pds1 cdc16* and *pds1 cdc23* double mutants probably results from the inability to degrade the mitotic cyclins due to APC inactivation.

The putative anaphase inhibitor may also be involved in the preanaphase arrest conferred by the spindle and DNA damage checkpoints. If this were the case, cells lacking the inhibitor would become hypersensitive to spindle or DNA damaging agents, presumably as a result of the failure to implement a checkpoint arrest. Indeed, cells lacking *PDS1* were found to be hypersensitive to nocodazole, a microtubule depolymerizing drug that causes spindle disassembly, and to gamma irradiation, which causes primarily double-stranded breaks in the DNA (Yamamoto et al. 1996b). Furthermore, cells lacking Pds1p failed to maintain sister chromatid cohesion in

the presence of nocodazole (although DNA replication and rebudding remained inhibited), and failed to arrest altogether following gamma irradiation (Yamamoto et al. 1996b). These observations suggest that Pds1p is required for the preanaphase checkpoint arrest.

These results prompted us to examine the role of Pds1p in the metaphase to anaphase transition. Specifically, we set out to examine whether Pds1p is degraded in an APC-dependent manner, whether it is degraded at the time of anaphase initiation, and whether its degradation is important for its function as an anaphase inhibitor. To this end, we tested the dependence of Pds1p stability on APC function both in vivo and in vitro. Furthermore, Pds1p derivatives that were resistant to degradation were generated and their effect on cell cycle progression was examined. Based on the results presented here we suggest that Pds1p is indeed an anaphase inhibitor whose APC-dependent degradation is required for the metaphase to anaphase transition.

Results

Pds1p is degraded in vivo in an APC-dependent manner

S. cerevisiae strains defective for APC function arrest at the nonpermissive conditions prior to anaphase, presumably because of the inability to degrade the anaphase inhibitor. The ability of the *PDS1* deletion to bypass this arrest (Yamamoto et al. 1996b) suggested that perhaps Pds1p is an APC substrate. To examine whether APC is involved in Pds1p degradation, the stability of Pds1p was examined in G_1 -arrested cells, a phase of the cell cycle in which APC is active in Clb2p degradation (Amon et al. 1994). If Pds1p degradation requires APC function, one would expect that the stability of Pds1p would be greater in G_1 -arrested cells defective for APC function, that is, *cdc16* or *cdc23* cells, than in G_1 -arrested wild-type cells. For this purpose *PDS1* was placed under control of the *GAL1* promoter and introduced on a centromere-based plasmid into wild-type or *cdc16-123* cells. These cells were arrested in G_1 with α factor at 23°C and then shifted to 37°C to inactivate the *cdc16-123* gene product. Following the temperature shift, *PDS1* mRNA was expressed for a period of 30 min by the induction and subsequent repression of the expression from the *GAL1* promoter. The stability of Pds1p was monitored by examining the protein levels at 30 and 60 min after the repression of the *GAL1* promoter.

The results in Figure 1A show that Pds1p is unstable in G_1 -arrested wild-type cells, but is stable in G_1 -arrested *cdc16-123* cells. Both strains maintained a 1N DNA content as assayed by flow cytometry analysis (data not shown), excluding the possibility that Pds1p was stabilized as a result of an escape from the G_1 arrest. Furthermore, the *PDS1* mRNA was equally unstable in wild-type and mutant cells (Fig. 1B), suggesting that the presence of Pds1p in *cdc16-123* cells at 30 and 60 min following transcriptional repression truly reflects an increase in protein stability and not an alteration in gene

Figure 1. The degradation of Pds1p is dependent on CDC16p function. Wild-type, *cdc16-123*, and *cdc16-123* cells carrying *CDC16* on a plasmid (strains K1534, K4438, and K4438/pTK21, respectively) were transformed by a centromere-based plasmid carrying *GAL1-PDS1* (pOC30 for strains in A and B, POC29 for strains in panel C). All strains were grown in YEP + raffinose to early log phase at 23°C and were arrested in G_1 with α factor (final concentration 2×10^{-7} M). When >90% of the cells appeared morphologically to be arrested, the cultures were shifted to 37°C. After 30 min, the expression of *PDS1* from the *GAL1* promoter was induced for a period of 30 min by the addition of galactose (2% final concentration, at time -30) followed by the subsequent addition of glucose (2% final concentration, at time 0). Samples of equal cell densities were taken at the indicated time points (while maintaining the G_1 arrest at the elevated temperature), and assayed by immunoblotting for Pds1p (A and C) or by Northern analysis for *PDS1* and *ACT1* mRNA (B). Pds1p specific bands almost always appear as a doublet, with a minor form migrating slightly slower than the major form. The nature of these different forms is currently unknown.

expression or mRNA stability. The enhanced stability of Pds1p in the *cdc16* strain was directly related to the deficiency in Cdc16p activity because Pds1p was unstable in G_1 -arrested *cdc16-123* cells that carried wild-type *CDC16* on a centromere-based plasmid (Figure 1C). Similarly, Pds1p was stable in G_1 -arrested *cdc23-1* cells but not in the isogenic wild-type strain (data not shown). These results indicate that Pds1p is degraded in a Cdc16p

and Cdc23p-dependent manner, suggesting that APC is involved in Pds1p degradation.

Pds1p has a nine-amino-acid motif located near its amino-terminus that resembles the destruction-box motif of B-type cyclins (Yamamoto et al. 1996a; Figure 2A). To examine whether this sequence is a functional destruction box, two types of mutations were generated: *pds1-mdb*, in which the conserved RXXL was replaced by AXXA, and *pds1- Δ db*, in which the sequence coding for the putative destruction box was deleted and replaced by a *XhoI* restriction site, which encodes a leucine and a glutamic acid (Fig. 2A). To examine the stability of these Pds1p derivatives, the *pds1* mutant alleles and the wild-type *PDS1* gene were placed under control of a *GAL1* promoter on centromere-based plasmids and introduced individually into wild-type cells. Using a strategy similar

Figure 2. Pds1p contains a destruction box motif that is involved in Pds1p degradation by APC. (A) The amino acid sequence (single letter amino acid code) of the Pds1p destruction box starting at position 85 of the protein (full-length Pds1p consists of 373 amino acids) (Yamamoto et al. 1996a). The consensus sequence of the destruction box was derived from Glotzer et al. (1991). Two destruction-box mutant derivatives of *PDS1* were generated as described in the text. (B) Wild-type cells (K1534) containing *PDS1*, *pds1-mdb*, and *pds1- Δ db* under a *GAL1* promoter on centromere-based plasmids (pOC56, pOC57, and pOC58, respectively) were grown at 30°C to early log-phase in YEP + raffinose and arrested in G_1 with α factor. When >90% of the cells appeared morphologically to be arrested, the expression from the *GAL1* promoter was induced for 30 min as described for Fig. 1. Protein samples were taken every 10 min and the levels of the Pds1p derivatives were examined by Western blot analysis.

to the one described above, these cells were arrested in G_1 with α factor and the expression of either *PDS1* or its mutant derivatives from the *GAL1* promoter was induced for 30 min. Samples were taken every 10 min following the transcriptional shut-off and Pds1p levels were monitored by Western blot analysis. As can be seen in Figure 2B, wild-type Pds1p levels dropped significantly after 20 min, whereas no significant change in the levels of the destruction box mutant gene products was observed throughout the course of the experiment (>70 min). These results demonstrate that Pds1p degradation requires an intact destruction box, suggesting that Pds1p is a direct substrate of APC.

Pds1p ubiquitination by APC

To test whether Pds1p is an APC substrate, the ability of purified APC to promote Pds1p ubiquitination in vitro was examined. Because APC has so far only been purified from *Xenopus*, we initially tested whether Pds1p is degraded in the crude *Xenopus* egg extracts, an in vitro system that faithfully reproduces the cell cycle regulation of mitotic cyclin degradation (Murray and Kirschner 1989; Murray et al. 1989; Glotzer et al. 1991; Holloway et al. 1993). Figure 3A shows that 35 S-labeled Pds1p was stable in an interphase extract but was rapidly degraded in a mitotic extract, resembling the behavior of cyclin B in these extracts (Glotzer et al. 1991). The mitotic instability of Pds1p was APC-dependent because Pds1p was not degraded in mitotic extracts immuno-depleted of APC with anti-Cdc27p antibodies, whereas depletion with nonspecific control antibodies had no significant effect (Fig. 3B, cf. the level of Pds1p at 60 mins and 10 min). Moreover, high molecular mass forms of Pds1p appear following incubation with the untreated and control-depleted extracts, but not following incubation with the APC-depleted extract (Fig. 3B). As will be described below, these forms are likely to be ubiquitinated forms of Pds1p, and their absence in the APC-depleted reaction further suggests that Pds1p is an APC substrate. Mitotic degradation of Pds1p in *Xenopus* extracts was also dependent on an intact destruction box, as the Pds1p destruction box mutant derivatives were significantly more stable than the wild-type protein (Fig. 3C). We noted that the efficiency of Pds1p degradation varied from one extract preparation to another (Figs. 3A–C). Nonetheless, the average half-life of Pds1p was >90 min in interphase extracts, ~12 min in mitotic extracts, and >60 min in APC-depleted mitotic extracts. The half-lives of both destruction-box Pds1p mutants in mitotic extracts was >60 min. These results are consistent with the involvement of APC in Pds1p degradation.

To test whether the degradation of Pds1p was a result of ubiquitination by APC, 35 S-labeled Pds1p was incubated in reaction mixtures containing immuno-purified *Xenopus* APC and either one of two E2's, UBC4 or UBCx. The ubiquitin ligase activity of APC can be detected by the appearance of a characteristic ladder of increasingly higher molecular mass products that represent conjugates of the radiolabeled substrate protein

Figure 3. Pds1p is degraded in mitotic *Xenopus* egg extracts in an APC-dependent manner. (A) 35 S-labeled Pds1p was incubated in reaction mixtures containing either interphase or mitotic *Xenopus* egg extracts, as described under Materials and Methods. Samples were removed at the indicated time points and analyzed by SDS-PAGE and PhosphorImaging. (B) 35 S-labeled Pds1p was incubated in reaction mixtures that contained mitotic *Xenopus* egg extracts that were either left untreated, depleted with preimmune antibodies (control-depleted) or immuno-depleted with anti-Cdc27p antibodies (APC-depleted). Samples were taken at the indicated time points and processed as described in A. Occasionally in these extracts Pds1p was initially converted to a slower migrating form, the nature of which is currently unknown (see also panel C). (C) 35 S-labeled wild-type Pds1p (from plasmid pOC59) and its destruction-box mutant derivatives R85A,L88A (the protein product of *pds1-mdb* from plasmid pOC60) and ΔDB (the protein product of *pds1-Δdb* from plasmid pOC61) were incubated in reaction mixtures containing mitotic *Xenopus* egg extracts and analyzed as described in A.

with different numbers of ubiquitin residues (King et al. 1995). As can be seen in Figure 4A, the reconstituted system promoted the formation of high molecular mass conjugates of Pds1p in an APC-dependent reaction that resembled the ubiquitination of an amino-terminal fragment of cyclin B. The molecular masses of modified Pds1p in the ladder (Fig. 4A) differed by an average of 9-kD increments, in agreement with a modification that

Figure 4. Pds1p is ubiquitinated by APC. (A) Immunopurified APC coupled to Cdc27p antibody beads (APC) and preimmune control beads (C) were assayed for the ability to ubiquitinate ^{35}S -labeled Pds1p or a cyclin B-protein A fusion (13–91–prA) in the presence of purified ubiquitin, E1 and either UBC4 or UBCx. The reaction products were analyzed as described in Materials and Methods. The molecular masses of the modified ^{35}S -labeled Pds1p bands that can be individually distinguished are indicated. (B) ^{35}S -labeled Pds1p wild-type (lanes 1–6) and destruction-box mutant (R85A, L88A) (lanes 7–10) were analyzed in APC ubiquitination assays as in A. (C) ^{35}S -labeled Pds1p was analyzed in APC ubiquitination assays containing methylated ubiquitin. Note that only conjugates of lower molecular mass are formed as compared with the conjugates generated in reactions containing unmodified ubiquitin only (reactions in B and C are from the same experiment).

is a result of the conjugation of one to several ubiquitins. The processivity of the reaction, which is determined by the number of ubiquitins conjugated per substrate molecule, was greater with UBC4 than with UBCx, as can be seen from the distribution of the modified ^{35}S -labeled Pds1p bands (Fig. 4A, note the smear at the top of the UBC4 + APC lane). When these reactions were carried out in the presence of methylated ubiquitin, an inhibitor of ubiquitin chain elongation, Pds1p forms of much lower molecular mass were generated (Fig. 4C), confirming that the slow migrating Pds1p forms represent ubiquitinated Pds1p.

As mentioned above, the stability of Pds1p in the mitotic *Xenopus* egg extracts could be enhanced by mutating its destruction box. To test whether this effect resulted from inefficient ubiquitination by APC, the ubiquitination of ^{35}S -labeled wild-type Pds1p and the Pds1p destruction-box mutant R85A, L88A was examined in reconstituted APC reaction mixtures (Fig. 4B). In reactions containing APC and UBC4, the ubiquitination of the Pds1p destruction box mutant was significantly less than that of wild-type Pds1p (cf. lanes 3 and 7, note the absence of the multiple ubiquitin conjugated forms in lane 7). In these reactions the amount of modified wild-type Pds1p was five-fold greater than the amount of modified Pds1p destruction-box mutant, as determined by densitometry. Similarly, in reactions containing UBCx, the amount of modified wild-type Pds1p was three-fold higher than the amount of modified Pds1p destruction box mutant (cf. lanes 5 and 9). Mutation of the Pds1p destruction box did not result in the complete loss of

ubiquitination, as has been observed previously with similar destruction box mutants of cyclin B (Yu et al. 1996), but rather led to a significant reduction in the number of ubiquitins that were conjugated per protein. This suggests that mutations in the destruction box reduce the affinity between APC and its substrates. Proteins with shorter ubiquitin chains are not efficiently recognized by the 26S proteasome (Chau et al. 1989), resulting in their stabilization. Taken together, these results strongly suggest that Pds1p is a substrate of APC and that efficient recognition of Pds1p by APC is mediated by the Pds1p destruction box.

Pds1p is degraded prior to anaphase initiation

Because Pds1p appeared to be a substrate of APC, it was of interest to determine when in the cell cycle Pds1p is degraded, and whether its degradation correlates with the stages of the cell cycle at which APC is active, namely, from early mitosis to the G_1/S boundary (Amon et al. 1994). As endogenous levels of Pds1p could not be detected with the anti-Pds1p antibodies available, the wild-type *PDS1* gene was replaced with a construct encoding a hemagglutinin epitope-tagged version of *PDS1*, *PDS1::HA*. The *PDS1::HA* allele was found to fully complement the known *pds1Δ* phenotypes (data not shown). To determine when during the cell cycle Pds1p is present, *PDS1::HA* under the control of wild-type *PDS1* promoter was introduced into a *cdc15* strain, which arrests late in mitosis when grown at 37°C. The levels of Pds1p–HA were then examined in these cells when ar-

rested in G₁ (by treatment with α factor), M (by treatment with nocodazole), or in late M (by growth at 37°C). Pds1p-HA was present in M phase-arrested cells, but not in G₁ cells, and the level of Pds1p-HA in late M was greatly reduced (Fig. 5A). The reduced level of Pds1p in the *cdc15* cells arrested at 37°C was not a result of a general instability of the protein at the elevated temperature because Pds1p-HA was stable in wild-type, *cdc16*, and *cdc13* cells that were incubated for 3 hr at 37°C (data not shown).

To determine the pattern of Pds1p expression with greater precision, *PDS1::HA* cells were released synchronously from a hydroxyurea (HU) arrest, in which cells are blocked in early S phase as large budded cells with a single nucleus. Cell morphology, as well as Pds1p-HA and β -tubulin levels were determined at various time points following the release. As can be seen in Figure 5B, the level of Pds1p-HA decreased at 75 min following the release and rose again at ~30 min later. When comparing this pattern with the cellular morphology (Fig. 5C), one can observe a strong correlation between the decrease in the level of Pds1p-HA and the decrease in the fraction of cells with a preanaphase morphology (large-budded cells with a single nucleus). This suggests that Pds1p is degraded as cells enter anaphase. The level of Pds1p-HA increases prior to the increase in the fraction of preanaphase cells in the subsequent cell cycle, in agreement with the finding that Pds1p is present in small-budded, late G₁ cells (Yamamoto et al. 1996a; see below).

Fusing the HA epitope to Pds1p also enabled us to use indirect immunofluorescence to visualize the endogenous levels of Pds1p in unperturbed cycling cells. An asynchronous population of wild-type diploid cells, homozygous for *PDS1::HA*, was fixed and immunostained for Pds1p-HA. These cells were then examined for bud morphology, DNA distribution, and positive staining for the HA epitope, thus correlating the cell cycle stage with the presence of Pds1p-HA. In cells that were stained for the antigen, a general nuclear staining could be observed (Fig. 6). Presently, we cannot determine whether this signal is distributed throughout the nucleoplasm or is confined to the chromosomes. No signal was observed in the control *PDS1* cells, demonstrating that this staining was specific to the HA antigen. Strikingly, the HA-specific staining was restricted to cells that were in between the late G₁ (small budded cells) and preanaphase (large budded cells with a single nucleus) stages of the cell cycle. HA-specific staining was not observed in anaphase or telophase cells and only rarely detected in unbudded cells. These results suggest that in normal cycling cells, Pds1p is degraded shortly before or at the onset of anaphase, and reappears at the time of bud emergence, as was suggested from our analysis of staged cells (Fig. 5).

Pds1p degradation is required for the initiation of anaphase

Our results suggest that Pds1p is degraded via APC ubiquitination at the time of anaphase initiation. It was therefore of interest to determine whether this degrada-

Figure 5. Pds1p is present at G₂/M but not in late M or G₁. (A) A *cdc15-2* strain containing *PDS1::HA* (OCF1515-7D) was grown to early log phase at 23°C and then divided into four separate cultures. One culture was left untreated and the remaining three were arrested in either G₁ with α factor (final concentration 2×10^{-7} M), in M with nocodazole (final concentration 15 μ g/ml) or in late M (telophase) by temperature shifting to 37°C. The cultures were grown under these conditions for 4 hr at which time the percentage of cells that appeared arrested at the expected cell cycle stage was 93% for the α factor treated culture, 91% for the nocodazole treated culture, and 90% for the temperature shifted culture. Samples of equal cell densities were harvested and analyzed for Pds1p-HA (upper panel) or β -tubulin (lower panel) by Western blot analysis. Duplicate samples from identical conditions were fractionated side by side. (B,C) Wild-type cells harboring *PDS1::HA* (OCF1521.0) were grown at 30°C and arrested in early S phase with HU. When >90% of the cells appeared morphologically to be arrested, the cells were released from the arrest by washing out the drug. Samples were taken at the indicated time points and analyzed for Pds1p-HA and β -tubulin by Western blot analysis, and for cell morphology by DAPI staining. In panel C, the percentage of preanaphase cells [large-budded with a single nucleus (□)] and the relative amount of Pds1p-HA [normalized to the amount of β -tubulin (●)] is presented for each time point.

Figure 6. Pds1p is undetectable in anaphase, telophase, and unbudded cells. A *PDS1::HA* homozygous diploid strain (OCF1507) and a wild-type *PDS1* diploid strain (OCF1511) were grown to early log phase at 30°C and processed for immunofluorescence as described in Materials and Methods. It should be noted that the distribution of cell types as seen in the *PDS1::HA* strain panels does not reflect the cell type distribution in this culture (which was identical to that of the wild type *PDS1* culture). Rather, these particular fields were chosen in order to demonstrate the type of signal seen in cells of different stages of the cell cycle.

tion is important for the transition from metaphase to anaphase. If this were the case, one would expect that the expression of nondegradable Pds1p derivatives would be deleterious to the cell, as the cells would not be able to initiate anaphase properly. Indeed, attempts to transform wild-type cells with centromere-based plasmids carrying *pds1-mdb* or *pds1-Δdb* expressed from the wild-type *PDS1* promoter did not yield viable transformants (data not shown), suggesting that even low levels of the destruction box-mutated Pds1p were lethal. To overcome the toxicity of the Pds1p destruction-box mutants, the expression of the *pds1* destruction-box mutants, as well as that of *PDS1*, was placed under the control of the *GAL1* promoter. As expected, under constitutive inducing conditions the expression of the destruction box *pds1* mutants, as well as that of the wild-type *PDS1*, was found to be lethal (data not shown). To examine the effect of the destruction box Pds1p mutants on the cell cycle, wild-type cells and cells carrying one of the galactose-inducible constructs described above were arrested in early S phase with HU and the expression from the *GAL1* promoter was induced for 1 hr. Following the transcriptional repression, the HU was removed and the cells were released from the S-phase block. Cell cycle progression and Pds1p levels were monitored.

The rate of recovery from the HU block was initially

the same for the wild-type cells and cells that had transiently overexpressed either *PDS1*, *pds1-mdb* or *pds1-Δdb*, as all cultures gave rise to a similar fraction of cells with a 2N DNA content within 40 min (Fig. 7A).

The cellular morphology, as determined by 4',6'-diamidino-2-phenylindole (DAPI) staining (Fig. 7B), revealed that the wild-type cells proceeded through the cell cycle, such that 60 min following the release from the HU arrest ~60% of the cells were in either anaphase or telophase. However, cells that had transiently overexpressed either form of the Pds1p destruction box mutant failed to initiate anaphase (Fig. 7B), and remained arrested at the preanaphase stage for at least 1 hr after the wild-type strain had initiated anaphase. This was evident by their dumbbell shape, their 2N DNA content, their undivided nucleus with a short spindle, and the lack of sister chromatid separation (Fig. 7A,D,E). It is noteworthy that when cells continuously overexpressed the Pds1p destruction box mutants for >6 hr, virtually no anaphase or telophase cells were observed, although with time a small population of cells that appeared to be aploid accumulated, as if following an initial arrest some cells underwent cytokinesis in the absence of nuclear division (data not shown).

Cells that had transiently overexpressed wild-type Pds1p while arrested in early S phase exhibited a delay in initiating anaphase, as seen by the delay relative to the wild-type cells in the decline in the fraction of G₂/M figures, in the appearance of anaphase/telophase cells, and in the appearance of cells with 1N DNA content (Fig. 7A,B). In these cells, the appearance of a significant fraction of cells with anaphase/telophase morphology (~80 min following the release from the HU block) correlated with a significant decrease in the level of Pds1p (Fig. 7C), further supporting the notion that Pds1p degradation is required for the initiation of anaphase. Taken together, these results demonstrate that nondegradable Pds1p derivatives block anaphase initiation and suggest that the degradation of Pds1p via APC-dependent ubiquitination is required for the initiation of anaphase.

Could the inhibitory affect of the nondegradable Pds1p derivatives be the result of a nonspecific inhibition of APC function? To address this question we wanted to test the stability of an APC substrate in the presence of the Pds1p destruction box mutants. Because we had shown that Pds1p is an APC substrate, we examined whether wild-type Pds1p is degraded at the preanaphase arrest induced by a Pds1p destruction-box mutant. For this purpose, the *GAL1-pds1-mdb* construct was introduced into cells carrying *PDS1::HA*. These cells were arrested in early S phase with HU and the expression from the *GAL1* promoter was induced for 1 hr, as described above. Following the repression of expression from the *GAL1* promoter, the cells were released from the HU block, and the Pds1p-HA and β-tubulin levels as well as cell morphology were monitored (Fig. 8B). As controls, *PDS1::HA* cells lacking the *GAL1* inducible construct were also arrested in HU, treated with galactose, and released in the presence of α factor (Fig. 8A) or in the presence of nocodazole (Fig. 8C). As can be seen in

Figure 7. (See facing page for legend.)

amount of Pds1p-HA seen at the late time points in A probably resulted from cells that had escaped the α factor arrest, as was evident by cell morphology (data not shown).

Figure 8A, the level of Pds1p-HA in otherwise wild-type cells decreased when cells exited the preanaphase state. In the presence of the destruction-box Pds1p mutant, the cells did not initiate anaphase, but nonetheless the levels of Pds1p-HA decreased at a rate comparable to that observed in wild-type cells undergoing anaphase (cf. Fig. 8A,B). The degradation of Pds1p-HA in the presence of the Pds1p destruction box mutant was not a result of the prolonged preanaphase arrest because the Pds1p-HA levels remain high in cells arrested prior to anaphase by treatment with nocodazole (Fig. 8C). These results indicate that APC is active in the presence of the Pds1p destruction-box mutants. Moreover, this strongly suggests that the inability to initiate anaphase is directly

caused by the inability to degrade the Pds1p destruction-box mutants.

Discussion

Several lines of evidence implicate APC in Pds1p degradation: First, Pds1p, which is normally not detected in extracts of G_1 -arrested wild-type cells, is stabilized in G_1 -arrested cells that are impaired in APC function. The APC-dependent degradation of Pds1p in G_1 is similar to that observed for Clb2p, an established APC substrate (Amon et al. 1994). Second, mutations in the putative destruction-box motif of Pds1p enhanced protein stabil-

Figure 7. The presence of Pds1p coincides with the inability to initiate anaphase. A wild-type strain (OCF1517.0) and strains containing *GAL1-PDS1* (OCF1517.1), *GAL1-pds1-mdb* (OCF1517.2), or *GAL1-pds1- Δdb* (OCF1517.3) were grown to early log phase at 30°C in YEP + raffinose and were arrested at early S phase with HU (final concentration 0.1 M). When >85% of the cells appeared morphologically to be arrested (~3.5 hr following the addition of HU), the expression from the *GAL1* promoter was induced for 1 hr by the addition of galactose (2% final concentration). The cells were then harvested, washed twice in YEP + glucose, and then resuspended YEP + glucose in the original volume. Samples were taken every 20 min and processed for FACS analysis, DAPI staining, Western blot analysis, immunofluorescence, and FISH as described in Materials and Methods. (A) FACS analysis of the DNA content in wild-type, *GAL1-PDS1*, and *GAL1-pds1-mdb* strains from samples taken at the indicated time points. (B) The cell-type distribution of wild-type ($\square$), *GAL1-PDS1* ($\diamond$), *GAL1-pds1-mdb* ($\bullet$), and *GAL1-pds1- Δdb* ($\blacktriangle$) at the HU arrest and following the release and *GAL1*-dependent transcription shut off (time 0). Only the fraction of large-budded cells with a single nucleus (preanaphase cells, *top*) and large-budded cells with separated DNA masses (anaphase and telophase cells, *bottom*) are shown. (C) The protein levels of Pds1p (*top*) or Pds1p(R85A L88A) [the product of *pds1-mdb*, *bottom*] at the indicated time points as detected by Western blot analysis using anti-Pds1p antibodies. (D) DAPI staining and indirect immunofluorescence using anti- α -tubulin antibodies of *GAL1-pds1-mdb* cells taken 100 min after HU release and treated as described in Materials and Methods. (E) FISH analysis of the *GAL1-pds1-mdb* cells at the time of the HU arrest (open bars) and 2 hr following the *GAL1*-transcription shut-off and release from the HU block (solid bars). Each FISH signal corresponds to one sister chromatid or two sister chromatids that have not separated (Guacci et al. 1994). Had the sister chromatids separated, an increase in the number of nuclei with two FISH signals would have been expected (Guacci et al. 1994; Yamamoto et al. 1996b). The probe used was of a centromere proximal probe of chromosome 16. Similar results were obtained when a centromere distal probe of chromosome 16 was used (data not shown). The total number of nuclei counted was 300 (for the HU-arrested cells) and 600 (for the 2 hr time point).

ity even in APC-proficient strains, consistent with the requirement for a destruction box in the APC-dependent degradation of Clb2p (Surana et al. 1993). Third, Pds1p is degraded in *Xenopus* egg extracts in an APC-dependent manner. Finally, in a reconstituted system, the *Xenopus* APC was capable of promoting Pds1p ubiquitination in a manner that was dependent on the presence of a functional destruction box. Based on these results we conclude that Pds1p is a direct target of APC.

Using both immunofluorescence and immunoblotting analysis we were able to establish that Pds1p is present in cells from late G₁ and until the point of anaphase initiation, and that the time of its degradation correlates with the time of anaphase onset. Moreover, we have shown that not only is Pds1p degraded in an APC-dependent manner, but that its degradation is important for its function as an anaphase inhibitor. Indeed, when Pds1p was rendered nondegradable by inactivating its destruction box, anaphase did not occur. Furthermore, when Pds1p was transiently overexpressed, there was a correlation between the delay in anaphase initiation and the extent of Pds1p degradation. We conclude that the degradation of Pds1p via APC ubiquitination is an integral part of its biological function as an anaphase inhibitor.

An alternative explanation for the inhibitory effect of the nondegradable Pds1p derivative is that anaphase was inhibited not because of the inability to degrade Pds1p per se, but because of competition of the Pds1p destruction-box mutant with other putative APC substrates. Although we cannot eliminate this possibility, it is unlikely. We demonstrated that in the presence of the Pds1p destruction-box mutants APC was active, as seen by the degradation of endogenous Pds1p-HA while the cells were maintained in the preanaphase arrest (this study). This is similar to the case of the cyclin B destruction box mutants, where it has been shown that wild-type cyclin B was degraded in the presence of stable cyclin B derivatives (Glotzer et al. 1991; Luca et al. 1991). The reduced levels of ubiquitination of the destruction box mutants observed in vitro suggest that they have lower affinity for APC and, hence, are likely to be poor competitors for APC function. Another indication that Pds1p itself needs to be degraded at the metaphase to anaphase transition is the ability of a *pds1Δ* to bypass the *cdc16* or *cdc23* preanaphase arrest (Yamamoto et al. 1996b). Taken together, we suggest that the inability to initiate anaphase in the presence of the Pds1p destruction box mutants was not the result of competition for APC activity, but resulted directly from the inability to degrade this form of Pds1p.

In a previous study of *PDS1* (Yamamoto et al. 1996a) *GAL1-PDS1* expression was induced in nocodazole-arrested cells from which the drug was removed subsequently. Under these conditions Pds1p was detected in telophase cells, but was absent from G₁ cells. This result differs from the finding presented here that Pds1p-HA was not detected by immunofluorescence in telophase cells. This difference may be the result of the excess amounts of Pds1p caused by its overproduction. Alternatively, Pds1p produced in nocodazole-arrested cells

may be partially exempt from degradation. We suggest that prior to anaphase initiation, Pds1p is present in a complex, and that this complexed form is specifically targeted for APC-dependent degradation at the time of anaphase onset. In nocodazole-arrested cells, ectopically expressed Pds1p may be excluded from this previously assembled complex and spared from degradation. This exclusion is consistent with a preliminary result that expression of a Pds1p destruction box mutant in nocodazole-arrested cells does not inhibit anaphase initiation upon removal of the drug (O. Cohen-Fix and D. Koshland, unpubl.). It is, therefore, possible that during mitosis, APC recognizes its targets as part of larger complexes, a feature that may also contribute to the specificity of APC-dependent degradation. Upon exiting mitosis, both complexed and noncomplexed Pds1p are degraded, perhaps to set the stage for a new cell cycle.

Is Pds1p the only negative regulator of anaphase? Three observations argue against this possibility. First, assuming that anaphase onset must be regulated in every cell cycle, *pds1Δ* strains are still viable, albeit very sick (Yamamoto et al. 1996a). Second, in the *pds1Δ cdc16* or *pds1Δ cdc23* double mutants only ~50% of the cells can bypass the preanaphase block of the single *cdc16* or *cdc23* strains (Yamamoto et al. 1996b). Finally, in the absence of Pds1p only ~50% of the cells show premature sister chromatid separation when arrested in nocodazole (Yamamoto et al. 1996b). These results suggest that perhaps there is another anaphase inhibitor that is also an APC substrate and that partially complements Pds1p activity in inhibiting anaphase. No homologs of Pds1p were identified in *S. cerevisiae* and so far none have been found in other organisms. However, the *Cut2* gene of *S. pombe* has been shown recently to have properties similar to those of Pds1p: Cut2p contains two destruction box motifs, and its degradation requires a functional Cut9p, a homolog of Cdc16p and, therefore, likely a subunit of fission yeast APC (Funabiki et al. 1996b). In addition, Cut2p derivatives that lack the destruction box inhibit the onset of anaphase (Funabiki et al. 1996). Interestingly, Cut2p associates with Cut1p (Funabiki et al. 1996a), a homolog of the *S. cerevisiae* gene *ESP1* (Uzawa et al. 1990; McGrew et al. 1992) with which Pds1p may have genetic interactions (V. Guacci, O. Cohen-Fix, and D. Koshland, unpubl.). The distribution of the charged amino acids in Cut2p resembles that of Pds1p, in that there is clustering of acidic and basic residues (Uzawa et al. 1990; Yamamoto et al. 1996a). However, there is no extensive sequence homology between the two proteins. The localization of Cut2p and Pds1p is different: Cut2p is associated with the spindle (Funabiki et al. 1996b), whereas Pds1p shows a general nuclear localization. Moreover, Cut2p is essential whereas Pds1p is not. Some phenotypes of *pds1* mutant strains have yet to be examined in *cut2* mutants. These include the examination of whether *cut2* mutants have a defect in maintaining sister chromatid cohesion when arrested in metaphase, whether the absence of Cut2p alleviates the requirement for APC function at the metaphase to anaphase transition, and whether *cut2* mutants are sensitive to ionizing

radiation. We are currently investigating whether Cut2p is functionally homologous to Pds1p.

Our results demonstrate that APC can ubiquitinate a protein other than the mitotic cyclins. Genetic data imply the existence of additional substrates, such as Cut2p (Funabiki et al. 1996b) and Ase1p (David Pellman, pers. comm.). These findings suggest that APC is not involved in the degradation of a single regulator that in turn activates a cascade of events. Rather, APC is involved in the degradation of multiple regulators at different stages of mitosis (i.e., anaphase initiation by the degradation of Pds1p and the exit of mitosis by the degradation of Clb2p), as predicted by Holloway et al. (1993). This raises the interesting question of how APC recognizes different substrates, and what provides the temporal regulation where different substrates are degraded at different stages of the cell cycle. In this regard, it will be interesting to examine mitotic cyclin degradation in cells arrested prior to anaphase by the Pds1p destruction-box mutant.

Although these studies provide significant insights into Pds1p regulation, we know little about how Pds1p acts as an anaphase inhibitor. Pds1p may be a structural component of sister chromatid cohesion and the inhibition of sister chromatid separation may in itself be sufficient to inhibit the onset of anaphase. Alternatively, Pds1p may regulate cohesion or other cellular functions that in turn lead to a preanaphase arrest. If this is the case, the requirement for Pds1p degradation in the initiation of anaphase raises the possibility that the cohesion factor itself is not degraded at the onset of anaphase and is inactivated by a different mechanism. Pds1p is also essential for exerting a preanaphase DNA damage checkpoint arrest (Yamamoto et al. 1996b), and it will be of interest to determine whether Pds1p inhibits anaphase in the same manner in the case of the DNA damage checkpoint as it does under normal growth conditions. The identification of cellular components with which Pds1p interacts should help in elucidating the activity of Pds1p at the molecular level.

Materials and methods

Plasmids and strains

Plasmid: pTK21 was a generous gift from R. Wickner (National Institutes of Health, Bethesda, MD) (Icho et al. 1987). pOC29 and pOC30 carry the *PDS1* open reading frame plus 80 bp of 5' flanking sequence ligated into the *SmaI* site of p415GAL1 and p416GAL1, respectively (Mumberg et al. 1994). pOC52 is a pUC19-based plasmid carrying *PDS1::HA* and *URA3*, which was integrated at an *XbaI* site located 3' of the *PDS1* open reading frame. *PDS1::HA* is an in frame fusion of the triple HA repeat at the *BglIII* site of *PDS1* (position 520 relative to the first open reading frame base). pOC56, pOC57, and pOC58 carry *PDS1*, *pds1-mdb*, and *pds1-Δdb*, respectively ligated into the *SmaI* site of p416GAL1 (Mumberg et al. 1994). pOC59, pOC60, and pOC61 were constructed by ligating *PDS1*, *pds1-mdb*, and *pds1-Δdb*, respectively, into the *SmaI* site of pCS2⁺ (Turner and Weintraub 1994). Strains K1534 [*MATa ade2 trp1 can1 leu2 his3 ura3 bar*] and K4438 [as K1534 but *cdc16-123*] were a generous gift from K. Nasmyth (Forschung Institut für Molekulare

Pathologie, Vienna, Austria). OCF1511: *Mata/Mata bar1/bar1 ura3/ura3 leu2/LEU2 TRP1/trp1*. OCF1507: as OCF1511 except also *PDS1::HA URA3/PDS1::HA URA3*. OCF1517.0, OCF1517.1, OCF1517.2, and OCF1517.3 are as K1534 except that *LEU2*, *PDS1 LEU2*, *pds1-mdb LEU2*, or *pds1-Δdb LEU2* were integrated at *leu2* of K1534, respectively. OCF1515-7D: *MATa bar1 cdc15-2 PDS1::HA URA3 (ura3)*. OCF1521.0: *Mata PDS1::HA URA3 (ura3) leu2::LEU2 his3 trp1 ade2*. OCF1521.2: as OCF1521.0 except *leu2::GAL1-pds1-mdb LEU2*. The *pds1* destruction-box mutants were constructed using the Transformer Mutagenesis kit by Clontech (Palo Alto, CA) according to manufacturer's instructions.

Media and reagents

All media for yeast cultures were prepared as described previously (Rose et al. 1990). YEP media contained 1% yeast extract, 2% bactopectone, and 2.5 mg/l adenine. Carbon sources (glucose, raffinose, or galactose) were used at 2% final concentration. αfactor, HU, and 4 DAPI were purchased from Sigma Chem. Co. (St. Louis, MO). Nocodazole was purchased from Aldrich Chem. Co. (Milwaukee, WI).

In vitro degradation and ubiquitination assay

[³⁵S]methionine labeled proteins were generated from pOC59, pOC60, and pOC61, using a coupled transcription and translation rabbit reticulocyte lysate system containing SP6 RNA polymerase (Promega). For degradation assays, 4 μl translation mixture were incubated at room temperature with 16 μl interphase or mitotic *Xenopus* egg extracts (King et al. 1995), containing 100 μg/ml cycloheximide, 1.25 mg/ml bovine ubiquitin (Sigma), energy mix (7.5 mM creatine phosphate, 1 mM ATP, 1 mM MgCl₂, 0.1 mM EGTA), and, in the case of the mitotic extract, a bacterially expressed non-degradable 890 fragment of sea urchin cyclin B (Glotzer et al. 1991). Samples were removed at various time points, mixed with SDS sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and an ImageQuant analysis using a Molecular Dynamics PhosphorImager. Immunodepletions of extracts with Cdc27p antibodies (Tugendreich et al. 1995) or preimmune antibodies were done as described (King et al. 1995). For ubiquitination assays, 2 μl of the translation mixture were incubated for 20 min at room temperature in a 10-μl reaction mixture containing 5 μl of immunopurified mitotic *Xenopus* APC bound to CDC27 antibody beads (King et al. 1995), 1.25 mg/ml bovine ubiquitin bacterially expressed and purified, wheat E1 and either *Xenopus* UBC4 or UBCx (50 μg/ml each) (Yu et al. 1996), and 10 mM Tris-HCl (pH 7.7), 100 mM KCl, 1 mM MgCl₂ and energy mix. Reactions were stopped by addition of SDS sample buffer and analyzed as above. Methylated ubiquitin was the kind gift of Renee Yew (Harvard Medical School, Cambridge, MA) and was used at a concentration of 3 mg/ml. An in vitro translated fusion protein consisting of the amino-terminal residues 13–92 of sea urchin cyclin B fused to protein A (13–91-prA; Glotzer et al. 1991) was used as a positive control in degradation and ubiquitination assays. Quantitation of the distribution of the radiolabeled bands was done by using the ImageQuant program version 1.1 from Molecular Dynamics (Sunnyvale, CA).

Other methods

Isolation of RNA and Northern blot analysis were carried out as described (Megee et al. 1995). *PDS1* mRNA was detected using a radiolabeled DNA fragment containing the *PDS1* open reading frame. *ACT1* mRNA was detected using a radiolabeled *EcoRI*–*BamHI* fragment containing an intronless *ACT1* gene from plasmid KCe44 (Chapman and Boeke 1991) [a generous gift from J.

Boeke, Johns Hopkins University Medical School, Baltimore, MD). Labeling was done using the Random Primer DNA labeling Kit (Boehringer Mannheim).

For Western blot analysis, protein extracts were prepared as described (Payne et al. 1987) and electro blotting was done onto a Protran membrane (Schleicher and Schuell, Keene, NH). The primary antibodies used were rabbit polyclonal anti-Pds1p (Yamamoto et al. 1996a), 12CA5 monoclonal antibody against the hemagglutinin epitope (BAbCO, Richmond, CA); and rabbit polyclonal 206-1 raised against the last 12 carboxy-terminal amino acids of yeast β -tubulin (a generous gift from Frank Solomon, MIT, Cambridge, MA). The secondary antibodies used were horseradish peroxidase-conjugated goat antirabbit or goat antimouse antibodies (Bio-Rad, Hercules, CA). The protein bands were detected by the Renaissance Chemiluminescence reagent (DuPont NEN, Boston, MA). To quantify the chemiluminescent signal, the autoradiographs were scanned and analyzed using ImageQuant. Several different exposures of each gel were analyzed to confirm that the measurements were within the linear signal range. Indirect immunofluorescence was carried out as described (Kilmartin and Adams 1984). When Pds1p-HA was visualized, cells were fixed for 20–25 min only (longer fixation times resulted in loss of HA-dependent immunofluorescence). The primary antibodies used were 12CA5 monoclonal antibody against hemagglutinin antigen (BAbCO, Richmond CA), and YOL1/34 monoclonal antibody against α -tubulin (Serotec, Indianapolis, IN). Fluorescent in situ hybridization (FISH) was carried out as described (Guacci et al. 1994).

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O Cohen-Fix, J M Peters, M W Kirschner, et al.

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