Cyclin-dependent kinase and Cks/Suc1 interact with the proteasome in yeast to control proteolysis of M-phase targets

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Cell cycle-specific proteolysis is critical for proper execution of mitosis in all eukaryotes. Ubiquitination and subsequent proteolysis of the mitotic regulators Clb2 and Pds1 depend on the cyclosome/APC and the 26S proteasome. We report here that components of the cell cycle machinery in yeast, specifically the cell cycle regulatory cyclin-dependent kinase Cdc28 and a conserved associated protein Cks1/Suc1, interact genetically, physically, and functionally with components of the 26S proteasome. A mutation in Cdc28 (*cdc28-1N*) that interferes with Cks1 binding, or inactivation of Cks1 itself, confers stabilization of Clb2, the principal mitotic B-type cyclin in budding yeast. Surprisingly, Clb2-ubiquitination *in vivo* and *in vitro* is not affected by mutations in *cks1*, indicating that Cks1 is not essential for cyclosome/APC activity. However, mutant Cks1 proteins no longer physically interact with the proteasome, suggesting that Cks1 is required for some aspect of proteasome function during M-phase-specific proteolysis. We further provide evidence that Cks1 function is required for degradation of the anaphase inhibitor Pds1. Stabilization of Pds1 is partially responsible for the metaphase arrest phenotype of *cks1* mutants because deletion of *PDS1* partially releases the metaphase block in these mutants.

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The regulated turnover of many proteins in eukaryotes is accomplished by targeting them to the ubiquitin/proteasome pathway (Hochstrasser 1996). Proteins destined for destruction receive covalent repeats of a small polypeptide known as ubiquitin on specific acceptor lysines. The formation of ubiquitin conjugates requires the concerted activity of a series of enzymes that first activate ubiquitin (E1) and then recognize and transfer ubiquitin (E2 and E3) to proteins destined for turnover. Once such targeted proteins become polyubiquitinated, they are recognized and degraded by a particle known as the 26S proteasome. The 26S proteasome consists of a 20S protease core and 19S regulatory caps that recognize and access polyubiquitinated substrates. Although a number of subunits of the 19S proteasome cap have been identified, their precise functions in substrate recognition and presentation to the 20S core are not yet well understood.

Cell cycle-regulated proteolysis is of particular importance for entry into and exit from mitosis in all eukaryotes (King et al. 1996; Hershko 1997). Turnover of proteins that control chromatid adhesion is necessary for

cells to proceed from metaphase to anaphase (Holloway et al. 1993; Irniger et al. 1995; Cohen-Fix et al. 1996), whereas turnover of cyclin B is important for exit from mitosis (Ghiara et al. 1991; Gallant and Nigg 1992). Ubiquitination of cyclin B depends on a *cis*-acting peptide sequence, known as the mitotic destruction box (D-box) (Glutzer et al. 1991) and a multicomponent ubiquitinating complex known as the cyclosome/APC (anaphase promoting complex) (Irniger et al. 1995; King et al. 1995; Sudakin et al. 1995; Tugendreich et al. 1995). Similarly, in both budding yeast and fission yeast, proteins that need to be degraded for sister chromatid separation to occur (Pds1 in budding yeast and Cut2 in fission yeast) contain D-boxes that target them for cyclosome/APC-dependent ubiquitination. However, the precise molecular mechanism of how the cyclosome/APC is activated and induced to recognize specific proteins, as well as how these proteins are targeted to the proteasome, remains to be elucidated. Nevertheless, the timing of activation of M-phase-specific ubiquitination and proteolysis suggests that it is under the control of the mitotic cyclin-dependent kinase cyclin B/Cdk1. It has been shown in clam and frog egg extracts that activation of the cyclosome/APC is associated with phosphorylation of some of its subunits (Lahav et al. 1995; Peters et al. 1996). Recent experiments, however, suggest that bind-

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ing of cyclosome/APC activator proteins such as Cdc20 and Hct1/Cdh1 are primarily responsible for cyclosome/APC activation (Schwab et al. 1997; Visintin et al. 1997; Fang et al. 1998).

In budding yeast, several of the components of the cyclosome/APC were identified initially in a genetic screen for cell division cycle mutants. *cdc16*, *cdc23*, *cdc26*, and *cdc27* mutations all conferred a metaphase arrest, that is, with a short mitotic spindle, unseparated chromatids, and active Cdc28 kinase. Subsequently, these mutants were also shown to be defective in degradation of the principal *Saccharomyces cerevisiae* B-type cyclin Clb2 and the anaphase inhibitor Pds1, providing a link between the products of these genes and M phase-specific proteolysis (Irniger et al. 1995; Cohen-Fix et al. 1996; Zachariae et al. 1996). The identification of homologs of these proteins in cyclosome/APC complexes purified from clam and frog eggs provided a biochemical rationale for the genetic observations in yeast (King et al. 1995; Sudakin et al. 1995; Peters et al. 1996).

Mutations in the proteasome have also been shown to confer cell cycle phenotypes. *rpt1/cim5*, *rpt6/cim3*, and *rpn12/nin1*, all mutations in components of the 19S regulatory cap, confer a metaphase arrest similar to that described for mutational inactivation of the cyclosome/APC. This is not surprising, as polyubiquitinated targets need to be degraded by the proteasome. The metaphase arrest phenotype conferred by proteasome mutants indicates that cell cycle-related targets, specifically those involved in regulation of the metaphase–anaphase transition, are likely to be limiting substrates of the proteasome.

In budding yeast, the cyclin-dependent kinase Cdc28 combines with a number of different cyclins to promote the various cell cycle transitions. Genetic analyses indicate that the B-type cyclin Clb2 is the primary Cdc28 activator responsible for mitotic induction. Presumably, one of the mitotic functions of Clb2/Cdc28 is to promote the activation of the cyclosome/APC so that M-phase-specific substrates can be ubiquitinated and targeted for proteolysis.

To investigate mitotic functions of the Cdc28 kinase, we implemented a genetic screen for interactions with the *cdc28-1N* mutation. The results of that screen and subsequent analyses provide evidence for a direct physical and functional interaction between the Cdc28 kinase and the proteasome. We also provide evidence that a small evolutionarily conserved cyclin-dependent-kinase-interacting protein, Cks1, associates with the proteasome and is essential for proper proteasome function.

Results

A gene encoding a component of the 19S proteasome cap is a mutational enhancer of the cdc28-1N mutation

A genetic link between the cyclin-dependent kinase Cdc28, and Rpn3/Sun2, a subunit of the 19S proteasome,

was revealed by screening the *cdc28-1N* mutation for genetic interactors. Because the *cdc28-1N* mutation confers arrest of *S. cerevisiae* cells in mitosis (Piggott et al. 1982; Surana et al. 1991), we reasoned that interacting mutations might help elucidate the mitotic function of the Cdc28 kinase. Specifically, a screen was implemented to identify enhancer mutations of the *cdc28-1N* allele. Diploids homozygous for *cdc28-1N* were mutagenized and screened for heterozygous mutations that increased the temperature sensitivity of the strain. The rationale is that in a cell sensitized with compromised mitosis-specific Cdc28 function, that is, the *cdc28-1N* mutant, decreasing the dosages of wild-type gene products that have downstream functions would be expected to enhance the severity of the phenotype. Any interaction detected in this manner should be specific, because the cell still retains 50% of the normal level of the wild-type gene product. For further explanation of the mutational enhancer rationale see Marini et al. (1996).

One mutation that conferred an increase in temperature sensitivity of the *cdc28-1N* mutation showed haplolethal segregation when cells were induced through meiosis. The corresponding gene was cloned from a library of *S. cerevisiae* genomic DNA fragments by virtue of its ability to restore the characteristic restrictive temperature of the parental *cdc28-1N* strain and to rescue the spore lethality that cosegregated with this particular mutation. The rescuing fragment was subcloned and sequenced. Reference to the Yeast Genome Project Data Base indicated that the same ORF had already been designated *RPN3/SUN2* and had been identified on the basis of genetic interaction with a mutation in a gene (*RPN12/NIN1*) encoding a subunit of the 26S proteasome (Kawamura et al. 1996). Recently, Rpn3/Sun2 itself has been identified as being a subunit of the 19S regulatory cap of the 26S proteasome (Kominami et al. 1997; Glickman et al. 1998). A disruption of the *RPN3/SUN2* ORF was constructed and showed identical behavior (enhancement of *cdc28-1N* and haplolethal phenotype) to the original enhancer mutation (designated *rpn3-54*), consistent with this being a mutant allele of *RPN3* (Table 1).

To test for specificity of the interaction with *cdc28-1N*, we produced a heterozygous *RPN3* disruption in another *cdc28* mutant that has been shown to be generally defective in cell cycle functions, *cdc28-4* (Reed 1980). No enhancement of temperature sensitivity was observed (Table 1). Another allele, *cdc28-13* confers arrest primarily in G₁ (Reed 1980) in contrast to the mitotic arrest conferred by *cdc28-1N*. However, a *cdc28-13 Δclb2* double mutant arrests in G₂ (E. Bailly and S.I. Reed, unpubl.). *CLB2* encodes the principal mitotic cyclin of *S. cerevisiae* and its loss presumably renders entry into and progression through mitosis the limiting function for the Cdc28-13 protein. Heterozygous disruption of *rpn3* in the *cdc28-13* or the *cdc28-13 clb2* mutant background, however, did not significantly enhance the temperature sensitivity of the strains (Table 1). Therefore, *rpn3* mutations interact strongly with *cdc28-1N* in an allele-specific manner.

Table 1. Enhancer test of various proteasome and APC subunit mutations in different *cdc28* and *cks1* genetic backgrounds

Genetic background ^a	Heterozygous mutation	Enhancement (°C) ^b
<i>cdc28-1N^c</i>	<i>rpn3-54</i>	yes (1°C)
	$\Delta rpn3::URA3$	yes (1°C)
	$\Delta cdc16::KAN^R$	no
	$\Delta cdc23::KAN^R$	no
<i>cdc28-4</i>	$\Delta rpn3::URA3$	no
<i>cdc28-13</i>	$\Delta rpn3::URA3$	no
<i>cdc28-13</i> $\Delta clb2$	$\Delta rpn3::URA3$	no
<i>cks1-38</i>	$\Delta rpn3::URA3$	yes (2°C)
<i>cks1-35</i>	$\Delta rpn3::URA3$	yes (1°C)
	$\Delta cdc16::KAN^R$	no
	$\Delta cdc23::KAN^R$	no

^aDiploid strain homozygous for the specific *cdc28* or *cks1* allele.^bRelative to the parental *cdc28* or *cks1* strain.^cThis strain harbors the *cdc28-4* allele at the *TRP1* locus, as it has been shown previously that *cdc28-1N* is epistatic over *cdc28-4*.*rpn3* is also a mutational enhancer of temperature-sensitive *cks1* mutations

The allele specificity of *cdc28-1N* in the genetic interactions described above suggested that the defect might not correspond to a simple loss of mitotic functions of the Cdc28 kinase. The *cdc28-1N* mutation, in fact, does not appear to confer a defect in Clb/Cdc28 kinase activity (Surana et al. 1991). During the course of this work, however, the three-dimensional structure of human Cdk2 (a Cdc28 homolog) bound to the human homolog of a small conserved protein, CksHs1, was determined crystallographically (Bourne et al. 1996). On the basis of this structure, and analysis of Cdk/Cks intermolecular contacts, the *cdc28-1N* mutation was predicted to interfere with binding of the yeast Cks homolog, Cks1. This prediction was borne out in a two-hybrid test (Bourne et al. 1996; Watson et al. 1996) and further confirmed by binding studies (Figs. 4A and 5B, below). Therefore, it is possible that the *cdc28-1N* phenotype results from a defect in Cdc28/Cks1 interactions, which is also consistent with the observation that temperature-sensitive *cks1* mutants confer a similar phenotype (Tang and Reed 1993).

Because Cdc28-1N is defective specifically in interaction with Cks1 (Bourne et al. 1996) rather than in catalytic functions, and because *rpn3* shows an allele-specific genetic interaction with *cdc28-1N*, it was possible that the enhancement of *cdc28-1N* by *rpn3* resulted from a defect in a Cks1-related function. Therefore, heterozygous *rpn3* disruptions were constructed in diploid strains homozygous for two different temperature-sensitive *cks1* mutations; *cks1-35* and *cks1-38* (Tang and Reed 1993). In each case, strong enhancement of the temperature-sensitive mutation was observed (Table 1). These genetic interactions are consistent with the idea that Cks1 function in concert with Cdc28 is important for some aspect of M-phase-specific proteolysis. It is notable

that despite their role in M-phase-specific proteolysis, there were no analogous genetic interactions detected between either *cks1* or *cdc28* mutations and heterozygous disruptions of genes *CDC16* and *CDC23*, which encode components of the cyclosome/APC (Irniger et al. 1995; Table 1).

cdc28-1N and *cks1* mutations confer a defect in proteolysis of Clb2

The genetic interactions between *cdc28-1N* and *cks1* mutations and gene dosage of *RPN3*, which encodes a component of the proteasome, suggested that Cdc28-1N and mutant Cks1 are defective in some aspect of M phase-specific proteolysis. To test this hypothesis, the stability of Clb2 was investigated in *cdc28-1N* and *cks1* mutants. Clb2, the principle mitotic cyclin of *S. cerevisiae* (Grandin and Reed 1993), is a key target of the ubiquitin/proteasome pathway in the context of M-phase regulation. Clb2 is stable during S phase and G₂ and becomes unstable during M phase and the subsequent G₁ interval, until the point known as START, when cells become committed to a new division cycle (Amon et al. 1994). To determine whether Cks1 function and the function defective in *cdc28-1N* mutants are required for ubiquitin-mediated turnover of Clb2, we measured the ability of Clb2 to accumulate in G₁-arrested *cks1* mutants as compared with wild-type cells. Cells were arrested in G₁ by treatment with the mating pheromone α -factor and tested for Clb2 accumulation. Synchronization of cells in G₁ maintains them at a point in the cell cycle in which M-phase proteolysis is active. Whereas Clb2 expressed from the strong *GAL1* promoter was undetectable in the wild-type strain, due to its rapid proteolysis, it accumulated in the *cks1* mutant (Fig. 1A). Furthermore, when synthesis of Clb2 was terminated by addition of dextrose in the *cks1* and *cdc28-1N* mutants, the remaining Clb2 protein turned over extremely slowly. (Fig. 1B). These effects were observed both at restrictive and permissive temperature (Fig. 1; data not shown). Thus, Cks1 function and the function defective in *cdc28-1N* mutants are required for efficient degradation of Clb2, consistent with roles in M-phase-specific proteolysis. The fact that stabilization of Clb2 was observed both at permissive and restrictive temperatures suggests that the defect in proteolysis of this target is constitutive and that this defect is not limiting for cell cycle progression (see Discussion).

Clb2 is a target of M-phase-specific proteolysis. However, many other proteins, including other Cdc28-associated proteins, are targets of the ubiquitin/proteasome pathway, but are not ubiquitinated in a cyclosome/APC-dependent fashion. To determine whether Cks1 function is required for proteolysis of non-M-phase targets of the ubiquitin/proteasome pathway, we determined the half-life of the G₁ cyclin Cln2 in *cks1* mutant and wild-type cells (Fig. 1C). The *cks1* mutation did not affect the half life of Cln2, indicating that Cks1 function is not generally required for proteolysis of targets of the ubiquitin/proteasome pathway or even of Cdc28-associated targets.

Figure 1. Clb2 but not Cln2 is stabilized in *cks1* and *cdc28-1N* mutants. (A) Cells (PY148, PY346) were arrested with α -factor, Clb2(HA)3 expression induced with galactose, and extracts prepared at various times, as indicated. Clb2(HA)3 accumulation was monitored by Western blotting with a 12CA5 antibody directed against the (HA) epitope tag. Only the results of the experiment performed at 25°C with wild-type *CKS1* and the *cks1-35* mutant are shown (similar results were observed at 37°C or with the *cks1-38* mutant). (B) Cells of the indicated genotype (PY148, PY346, PY256) were arrested with α -factor. The flow cytometry analysis data for asynchronous cells presented in B corresponds to samples before addition of α -factor. (C) Cln2 degradation is not affected by *cks1* mutations. Strains (PY124 and PY274) were shifted to 35°C and Cln2(HA)3 expression was induced by addition of galactose (2%). After 1 hr, cells were transferred to prewarmed medium containing 2% dextrose to repress Cln2(HA)3 expression. Samples were taken in 10 min intervals and analyzed for Cln2(HA)3 levels by Western blotting as described above.

rested with α -factor in G₁ and Clb2(HA)3 expression was induced by addition of 2% galactose (final concentration). After 30 min, cells were transferred into fresh medium containing 2% dextrose to terminate Clb2(HA)3 expression (timepoint 0) and samples were taken after 30 and 60 min. As Clb2(HA)3 expression in G₁-arrested wild-type cells was not detectable because of its rapid degradation, we analyzed Clb2(HA)3 expression in asynchronously growing cultures to confirm expression of Clb2(HA)3 in these strains (3 hr induction with 2% galactose). The G₁ arrest of all strains during the course of the experiments shown in A and B was confirmed by determination of budding index (not shown) and cellular DNA content by flow cytometry analysis. The flow cytometry analysis data for asynchronous cells presented in B corresponds to samples before addition of α -factor. (C) Cln2 degradation is not affected by *cks1* mutations. Strains (PY124 and PY274) were shifted to 35°C and Cln2(HA)3 expression was induced by addition of galactose (2%). After 1 hr, cells were transferred to prewarmed medium containing 2% dextrose to repress Cln2(HA)3 expression. Samples were taken in 10 min intervals and analyzed for Cln2(HA)3 levels by Western blotting as described above.

cks1 extracts are not defective in ubiquitinating Clb2

Because impairment of M-phase-specific proteolysis could result from a defect in cyclosome/APC function, we tested whether *cks1* mutant extracts were capable of ubiquitinating Clb2. We compared cyclosome/APC activity in extracts prepared from G₁-arrested wild-type cells, *cks1* mutants and mutants known to be defective in APC function (*cdc23-1*). Extracts prepared from G₁-arrested cells were mixed with small amounts of extracts prepared from cells overexpressing (HA)3-epitope tagged Clb2 or Clb2 deleted for its destruction box (Clb2^{db-}). 6xHis-tagged ubiquitin was added to the reaction so that ubiquitin conjugates formed during the reaction could be specifically purified on Ni-NTA-agarose. Extracts prepared from wild-type or *cks1* mutant cells were equivalently active in ubiquitination of Clb2 in a D-box-dependent manner (Fig. 2A, cf. lanes 1 and 7), whereas APC-defective extracts prepared from *cdc23-1* mutant cells exhibit a dramatic reduction in ubiquitinated forms of Clb2 (Fig. 2A, lane 5). Cks1 did not play a role in substrate maturation or presentation, as Clb2 substrate prepared from *cks1* mutant cells functioned equivalently in the ubiquitination assay as compared with substrate prepared from wild-type cells (Fig. 2B). We therefore conclude that Cks1 is not required for APC activity in G₁-arrested cells, although Clb2 proteolysis is dependent on functional Cks1 (Fig. 1). These results are consistent with our genetic data (Table 1), which suggest that Cks1 activity is required for proteasome function rather than for cyclosome/APC-activity.

Figure 2. (A) *cks1* mutants do not exhibit a defect in Clb2-ubiquitination. Extracts prepared from wild-type (PY236), *cdc23-1* (PY228), and *cks1-35* (PY255) mutants arrested in G₁ with α -factor, were incubated for 5 min in the presence of an ATP-regenerating system, 6xHis-tagged or nontagged ubiquitin (lanes 3,10,12) and Clb2(HA)3 or Clb2^{db-}(HA)3 (prepared in strains PY253 and PY254, respectively), as indicated. Proteins covalently linked to 6xHis-ubiquitin during the reaction were purified on Ni-NTA-agarose. Clb2(HA)3 and Clb2^{db-}(HA)3 were detected by immunoblotting with the 12CA5 antibody directed against the (HA) epitope. (B) Identical results were obtained with Clb2(HA)3 substrate prepared from *cks1* mutants. Reactions were performed as in A, but Clb2(HA)3 substrate was produced in *cks1-35* mutants (PY272). (Arrows) Migration of Clb2(HA)3 and Clb2^{db-}(HA)3, respectively; (asterisks) degradation products of the substrate.

Clb2-ubiquitin conjugates accumulate in *cks1* mutants

To confirm that cyclosome/APC function is not defective in *cks1* mutants in vivo and to determine whether proteasome functions might be defective, we analyzed mutant and wild-type cell lysates for Clb2-ubiquitin conjugates. Although protein ubiquitin conjugates never accumulate to high levels in vivo, even when known proteasome functions are impaired, we set up a sensitive assay to detect any elevation over basal levels. As a positive control, we used a strain with a temperature-sensitive mutation in a 19S proteasome subunit (*rpn3-4*). *rpn3-4* mutants are defective in Clb2 degradation in G₁-arrested cells (E. Bailly and S.I. Reed, in prep.). *cks1* mutants, wild-type cells, and *rpn3* mutants were arrested in G₁ by addition of α -factor and expression of HA-tagged Clb2 from the *GAL1* promoter was induced. Clb2(HA)3 was immunoprecipitated, separated by SDS-PAGE, and Clb2-ubiquitin conjugates detected by Western blotting with antibodies directed against polyubiquitin conjugates (Fig. 3). Although Clb2-ubiquitination is fully active in G₁, only a small proportion of Clb2 exists in polyubiquitinated forms. This can be most likely attributed to strong deubiquitinating activities, because even when proteasome function was impaired by a mutation in *RPN3*, Clb2-ubiquitin conjugates represented only a very small fraction of the total amount of Clb2. Nevertheless, as expected for mutations in proteasome subunits, *rpn3* mutants show a significant accumulation of Clb2-ubiquitin conjugates (Fig. 3). Likewise, we reproducibly detected a modestly increased level of Clb2-ubiquitin conjugates in *cks1* mutants as compared with wild-type cells, although the increase was lower than that observed in *rpn3* mutants (Fig. 3). In agreement with the D-box of Clb2 being required for its polyubiquitination, no ubiquitin conjugates of the D-box mutant of Clb2 were detected (Fig. 3). *rpn3* mutants are defective in degradation of a broad spectrum of proteins and therefore generally accumulate proteins with attached polyubiquitin chains. This was apparent from total protein immunoblots by use of anti-polyubiquitin antibodies (Fig. 3, right). This general increase in polyubiquitinated proteins in *rpn3* mutants presumably stabilizes polyubiquitin chains because substrate competition is likely to inhibit deubiquitination. In contrast, genetic and biochemical analyses of *cks1* mutants suggest that any associated proteasome defect is likely to be much more target specific. This may account for the less dramatic accumulation of Clb2-ubiquitin conjugates observed, because a general increase in polyubiquitinated proteins does not occur (Fig. 3, right). These results are consistent with the *cks1* mutation conferring a defect in proteasome function, as opposed to cyclosome/APC function.

Cdc28 and Cks1 bind to the proteasome

The genetic and biochemical results described above, specifically dosage-sensitive interactions between *cks1*/*cdc28-1N* mutants and a regulatory subunit of the pro-

Figure 3. Clb2 ubiquitin conjugates accumulate in *cks1* mutants in vivo. Wild-type (strain PY334, lane 3), *cks1-35* (strain PY332, lane 2), and *rpn3-4* (strain PY364, lane 5) expressing Clb2(HA)3 and *cks1-35* expressing Clb2^{db}(HA)3 (strain PY351, lane 4) under control of the *GAL1* promoter were arrested in G₁ with α -factor. After cells were arrested (>90% unbudded cells), cultures were shifted to 36°C for 60 min before expression of Clb2(HA)3 and Clb2^{db}(HA)3 was induced for 10 min by addition of galactose, and samples were taken to confirm the G₁ arrest by flow cytometry analysis. To obtain a control sample lacking Clb2(HA)3 expression, glucose instead of galactose was added to one of the cultures (strain PY332, lane 1) to repress expression from the *GAL1* promoter. Clb2(HA)3 and Clb2^{db}(HA)3 were immunoprecipitated with 12CA5 antibodies (directed against the HA epitope) and Clb2(HA)3-ubiquitin conjugates were detected by Western blotting with monoclonal antibodies directed against polyubiquitin (*left*). Cell lysates used for the immunoprecipitation were separated by SDS-PAGE and analyzed with the polyubiquitin antibody to detect total cellular polyubiquitin conjugates (*right*). Expression of Clb2(HA)3 and Clb2^{db}(HA)3 was confirmed by Western blotting with the 12CA5 antibody (data not shown).

teasome but not cyclosome/APC components, and the lack of a defect in cyclosome/APC activity in *cks1* extracts, suggested that Cks1 and/or Cdc28 might interact directly with the proteasome to regulate M-phase-specific proteolysis. To test this idea, Cdc28 complexes im-

munoprecipitated from yeast cell lysates were analyzed by Western blotting for components of the 19S regulatory subunit of the proteasome, including Rpn3. As shown in Figure 4A, Cdc28 immunoprecipitates contained significant amounts of Rpn3 and Rpt1, both components of the 19S proteasome (Glickman et al. 1998). It should be pointed out that only a small fraction of the Rpn3/Rpt1 pool was found in immunocomplexes with Cdc28. Cdc28-1N immunoprecipitates also contained Rpn3 and Rpt1, (Fig. 4A), indicating that Cdc28 association with the proteasome is not mediated via Cks1 because Cdc28-1N is defective in Cks1 interaction (Figs. 4A and 5B; Bourne et al. 1996). Longer exposures of Western blots revealed small amounts of Cks1 in immunocomplexes with Cdc28-1N (data not shown). This reflects either residual affinity of Cks1 for Cdc28-1N or could indicate Cdc28-independent binding of Cks1 to the proteasome (see below). Interestingly, when the amounts of Rpn3 and Rpt1 found in immunocomplexes were normalized to the amount of Cdc28 or Cdc28-1N, respectively, we repeatedly detected twofold more Rpn3/Rpt1 associated with Cdc28-1N (Fig. 4A). To determine whether proteasome association with Cdc28 might be mediated via polyubiquitinated Clb2, immunoprecipitates of Clb2 and a mutant Clb2 deleted for the D-box, were analyzed for proteasome components. Both Clb2 and Clb2^{db-} were associated with roughly equivalent

amounts of Rpn3 and Rpt1 (Fig. 4B). Thus, the ubiquitination state of Clb2 does not influence the association between bound Cdc28 and the proteasome. In contrast, and consistent with genetic data described above, immunoprecipitates of cyclosome/APC components Cdc16 and Cdc23 contained no detectable Cdc28 or Cks1 even when Western blots were exposed to film for extremely long intervals (data not shown).

To confirm that Cdc28 complexes are associated with functional proteasomes, proteasomes were partially purified from yeast cell lysates. A significant amount of Cdc28 cochromatographed with functional 26S proteasomes over several column purification steps, indicating a tight association (Fig. 4C). Significantly, Cks1 also cochromatographed with active proteasomes (Fig. 4C), suggesting the possibility of a direct interaction between Cks1 and the proteasome. The majority of the Cdc28 and Cks1 protein was lost at the first purification step, so only a small proportion of the Cdc28 and Cks1 pool copurified with the 26S proteasome.

To confirm that the interaction between the proteasome and Cks1 was not mediated by Cdc28, proteasomes were partially purified from a *cdc28-1N* mutant strain and analyzed for Cks1 protein. As with the wild-type *CDC28* strain, Cks1 cochromatographed with active proteasomes (Fig. 4D), indicating that Cks1 binding to the proteasome is independent of Cdc28 binding. Con-

Figure 4. Cdc28 and Cks1 interact with the proteasome. (A) Immunoprecipitation of Cdc28 and Cdc28-1N. Extracts were prepared from cells expressing RGS6H epitope-tagged Rpn3 and either Cdc28(HA)1 (PY137) or Cdc28-1N(HA)1 (PY136). To avoid any cell cycle position effects, all strains harbored untagged wild-type Cdc28. Extracts were subjected to immunoprecipitation with the 12CA5 antibody, and immunoblotted with the antibodies to RGS6H (Rpn3), Cim5/Rpt1, PSTAIRE (Cdc28), and Cks1, respectively. (B) Immunoprecipitation of Clb2 and Clb2^{db-}. Extracts from cells expressing a galactose inducible (HA)3-tagged Clb2 (PY166) or Clb2^{db-}(PY161) were prepared 40 min after cells were shifted to galactose-based medium. Clb2(HA)3 and Clb2^{db-}(HA)3 were immunoprecipitated with the 12CA5 antibody and associated proteins were detected by immunoblotting with the same antibodies as in A. (C,D) Proteasomes were partially purified on DEAE-Affigel blue followed by ion exchange chromatography on Q-Sepharose from a wild-type strain with RGS6H epitope-tagged Rpn3 and Pre1 (PY169) (C) or a strain disrupted for *CDC28* and kept alive by the *cdc28-1N* allele (PY139) (D). Fractions were eluted from Q-Sepharose with increasing concentrations of NaCl, tested for peptidase activity against Suc-LLVY-AMC, and analyzed by immunoblotting. Antibodies to the proteins indicated were used except for Rpn3 and Pre1, which were detected with antibodies to the RGS6H epitope and Cdc28, which was detected with an antibody to the PSTAIRE epitope.

sistent with the results from the immunoprecipitation experiments, Cdc28-1N also cochromatographs with active 26S proteasomes. We don't know the significance of the separate population of Cdc28-1N that is not clearly cochromatographing with Rpt1 and Rpt6 (Fig. 4D).

To further test for an interaction between the proteasome and Cks1, yeast extracts were incubated with recombinant Cks1 proteins coupled to agarose beads (Cks1 beads). Specific retention of Rpn3 indicated that 19S proteasomes bound to wild-type Cks1 beads but not mutant Cks1 beads [Cks1-35 (Tang and Reed 1993) and Cks1E94Q (M.H. Watson and S.I. Reed, unpubl.)] (Fig. 5A). Cks1-35 and Cks1E94Q beads were prepared with recombinant Cks1 protein harboring mutations that eliminate Cks1 function in vivo. Cks1, and its homologs, coupled to a solid matrix have long been used as an affinity reagent for immobilizing cyclin-dependent kinases. As expected, on the basis of the position of the *cdc28-1N* mutation in the three-dimensional structure of a complex between mammalian Cks1 and Cdc28 homologs and previous binding studies (Bourne et al. 1996), Cdc28-1N does not bind to Cks1 beads (Fig. 5B). However, 19S proteasome component Rpt1 from yeast lysates prepared from wild-type and *cdc28-1N* cells is retained on Cks1 beads but not BSA control beads (Fig. 5B). Thus, proteasome regulatory subunits bind directly to Cks1 protein independently of binding to Cdc28 and this interaction is eliminated by mutations in Cks1 that abolish its biological function. Taken together, these data suggest that the 19S proteasome is capable of binding separately to Cdc28 and Cks1. There is no evidence for

an association between the proteasome and Cdc28/Cks1 complexes, although such an association cannot be excluded on the basis of the current data.

Cks1 function is required for efficient M-phase-specific proteolysis of Pds1

Although *cks1* mutants were shown to be defective in proteolysis of Clb2, this cannot explain the cell cycle phenotype conferred by these mutations. Whereas *cks1* mutants arrest in metaphase, overexpression of mutationally stabilized Clb2 confers an anaphase block (Surana et al. 1993). On the other hand, degradation of Pds1 via the ubiquitin/proteasome pathway has been shown to be required for the metaphase-to-anaphase transition (Cohen-Fix et al. 1996). To determine whether *cks1* mutation confers stabilization of Pds1, which might account for the metaphase arrest phenotype, *cks1* mutant and wild-type cells were G₁-arrested by treatment with the mating pheromone α -factor. Then Pds1 expression from a *GAL1:PDS1* allele was induced for a short period. Pds1 synthesis was terminated by addition of dextrose and the rate of turnover of Pds1 determined. Pds1 was modestly stabilized by the *cks1* mutation. The half-life of Pds1 in the wild-type G₁-arrested strain was ~10 min, whereas it was ~20 min in the *cks1* mutant (data not shown). Because it has not been demonstrated that Pds1 is equally unstable in G₁ as it is during mitosis, we sought to compare its turnover in wild-type and *cks1* mutant cells arrested in metaphase when Pds1 degradation is physiologically relevant (Fig. 6). Cells expressing endogenous (HA)3-epitope-tagged Pds1 and harboring a galactose inducible construct that allows overexpression of a D-box mutant of Pds1 (Pds1^{db-}) were G₁-arrested by treatment with the mating pheromone α -factor. Cells were subsequently released from the G₁ arrest and when progressing into S phase (after 75 min), cells were shifted to a semipermissive temperature for *cks1-35* mutants and expression of Pds1^{db-} was induced by addition of galactose. Overexpression of the stabilized form of Pds1, Pds1^{db-}, results in a metaphase arrest at a point where cyclosome/APC is active and Pds1 is readily degraded (Cohen-Fix et al. 1996). Samples were collected throughout the time course and analyzed by flow cytometry to determine progression through the cell cycle and for levels of endogenous Pds1 protein. Whereas in wild-type cells Pds1 is rapidly degraded as cells reach metaphase, Pds1 is stable in *cks1* mutants (Fig. 6). Parallel RNA blots revealed no differences in *PDS1* mRNA levels that might account for these effects (data not shown).

The effect of the *cks1* mutation on Pds1 stability led us ask whether stabilization of Pds1 is responsible for the metaphase arrest in *cks1* mutants. To this end, we compared the cell cycle progression of *cks1* mutants and *cks1 pds1* double mutants. The mutants were synchronized in S phase with hydroxyurea (HU) at the permissive temperature (20°C), shifted to 37°C for 60 min to inactivate the temperature-sensitive *cks1-35* allele and shifted to fresh medium without inhibitor to allow exit from the HU block at 37°C. Samples were taken every 30

Figure 5. Interaction between Cks1 and the proteasome in vitro. (A) The proteasome interacts with wild-type but not mutant Cks1 protein. An extract prepared from cells expressing RGS6H-tagged Rpn3 (PY170) was incubated with Affi-Gel beads bound to either wild-type, Cks1-35, Cks1-E94Q, or BSA. After several washing steps, bound proteins were eluted with SDS and fractions analyzed for Rpn3 by Western blotting with an antibody directed against the RGS6H epitope. (B) Binding of the proteasome to Cks1 beads is independent of Cdc28. Extracts prepared from *CDC28* wild-type cells (lanes 1–3) or *cdc28-1N* mutants (PY139) (lanes 4–6) were incubated with Cks1- or BSA beads. Bound proteins were eluted and analyzed for Rpt1 and Cdc28 as in A.

Figure 6. Pds1 is stabilized in *cks1* mutants in metaphase. Wild-type cells (DCY1361) and *cks1-35* mutants (DCY1601) expressing endogenous Pds1(HA)3 and harboring a D-box mutant Pds1 (Pds1^{db-}) under the control of the *GAL1* promoter were synchronized in G₁ with α -factor. When the majority of the cells were arrested (>90% unbudded cells), cells were released from the arrest in fresh prewarmed YEP-raffinose at 30°C. Cells were monitored for passage through START microscopically, and when most of the cells started budding (~75 min), expression of Pds1^{db-} was induced by addition of galactose. Samples were collected at 15 min intervals and analyzed for cell cycle position by flow cytometry and Pds1(HA)3 levels by Western blotting with the 12CA5 antibody. Cdc28 was detected with PSTAIRE antibodies.

min and analyzed by flow cytometry and DNA staining. The majority of cells of both mutant strains finished DNA replication after 60 min as indicated by a 2N DNA content (data not shown). Two hours after the release from the HU block, 82% of the *cks1* mutants had a single undivided nucleus and 12% of the cells had undergone anaphase, as evidenced by separation of chromatin into two masses. In contrast, at the same time point, 55% of the *cks1 pds1* double mutants had undergone anaphase, although with unequal separation of chromatin in many cases, and only 45% of the cells were arrested with undivided nuclei. These results are comparable with those obtained in a similar experiment analyzing mutants defective in cyclosome/APC function in combination with a deletion of *PDS1* (Yamamoto et al. 1996).

Thus, the *cks1* mutation has a profound effect on the stability of Pds1 during mitosis when its destruction is important, partially accounting for the *cks1*-associated metaphase arrest.

Discussion

Interactions between Cks proteins and the cyclosome/APC

We report here that Cks1 in yeast interacts both geneti-

cally and physically with the proteasome, potentially explaining the defect in M-phase-specific proteolysis in *cks1* mutants. We were not able to detect strong genetic or physical interactions with the cyclosome/APC. This is in apparent conflict with previous reports. Patra and Dunphy (1998) found that a small fraction of p9, a *Xenopus* homolog of Cks1, can be detected in immunocomplexes with Cdc27, a component of cyclosome/APC. It hasn't been demonstrated, however, whether p9 is associated with cyclosome/APC complexes or monomeric Cdc27. Furthermore, phosphorylation of recombinant-purified Cdc27 by cycB/Cdc2 complexes is stimulated by p9. This correlates with the observation that p9 immunodepletion prevents Cdc27 modification in *Xenopus* egg extracts (Patra and Dunphy 1998). In agreement with these results, Sudakin et al. (1997) reported that active phosphorylated cyclosome/APC complexes from clam eggs bind to p13^{suc1} beads. p13^{suc1} is the fission yeast homolog of Cks1. However, no effect of p13 addition on in vitro activation or function of the cyclosome/APC was detected. Taken together, these results from studies in *Xenopus* and clam eggs suggest that Cks proteins are involved in activation or modulation of the cyclosome/APC. However, a direct requirement of Cks protein function for cyclosome/APC activation has not been demonstrated.

Although the strongest evidence so far for Cks proteins playing a role in cyclosome/APC activation is the effect of p9 on Cdc27 phosphorylation (Patra and Dunphy 1998), the biological role of Cdc27 phosphorylation is not clear. Several observations argue against a crucial role of Cdc27 phosphorylation for cyclosome/APC activity. (1) Cdc27 in G₁-arrested HeLa cells is unphosphorylated, yet cyclosome/APC is fully active (Kramer et al. 1998). (2) Budding yeast Cdc27 doesn't appear to be phosphorylated (Zachariae et al. 1996, 1998), suggesting that other mechanisms lead to cyclosome/APC activation. This is consistent with the observation that cyclosome/APC in budding yeast can be activated regardless of cell cycle position by overexpression of Cdc20 or Hct1/Cdh1 (Schwab et al. 1997; Visintin et al. 1997). (3) Recent experiments with immunopurified cyclosome/APC from *Xenopus* eggs strongly support this idea. It has been demonstrated that Cdc20 binding to cyclosome/APC rather than phosphorylation of cyclosome/APC is the critical activation step. Inactive cyclosome/APC purified from interphase extracts can be fully activated by incubation with immunopurified Cdc20 without addition of cyclin B/Cdc2 (Fang et al. 1998). Thus, binding of Cks proteins to cyclosome/APC and Cdc27 phosphorylation are not likely to be essential for activation of cyclosome/APC, although a modulatory role cannot be ruled out.

Our data do not absolutely exclude a role of Cks proteins in cyclosome/APC activation, although several lines of evidence suggest that this aspect of Cks1 function has a minor role in *S. cerevisiae*. (1) Despite various attempts, we were unable to detect a physical association between Cks1 or Cdc28 and cyclosome/APC in budding yeast. Our experimental approaches included coimmunoprecipitation from asynchronous and syn-

chronized cells (using HA epitope-tagged Cdc16 or Cdc23), efforts to bind APC from whole yeast cell lysates or partially purified APC to Cks1 beads, and finally glycerol-gradient centrifugation using both total cell extracts or partially purified APC in combination with recombinant Cks1. (2) Although Clb2 is stabilized in G₁-arrested *cks1* mutants (Fig. 1), G₁-arrested *cks1* mutants showed no defect in cyclosome/APC ubiquitination activity in vitro (Fig. 2). Furthermore, Clb2-ubiquitin conjugates accumulated in G₁-arrested *cks1* mutants, indicating active cyclosome/APC in *cks1* mutants in vivo (Fig. 3). (3) Our genetic data demonstrate a strong genetic interaction of *cks1* and *cdc28-1N* mutants with reduction of dosage of the 19S proteasome subunit *RPN3*, but no equivalent interactions with dosage of the cyclosome/APC genes *CDC23* or *CDC16*. Furthermore, a *cdc28-13 Δclb2* double mutant that arrests in G₂, presumably due to a defect in cyclosome/APC activation, did not genetically interact in the above-described manner with gene-dosage reduction of *RPN3*, indicating that the genetic interaction of *RPN3* gene dosage and *cks1/cdc28-1N* mutants is not a result of compromised cyclosome/APC activation or reduced Clb2 kinase activity.

Taken together, the results from studies in *Xenopus* and clam eggs and budding yeast, it can be concluded that Cks proteins play an essential role in M-phase-specific proteolysis. In budding yeast, this seems to be exerted at the level of the proteasome, whereas in the in vitro cell cycle generated from egg extracts some evidence points to a role of Cks proteins in activation or modulation of substrate specificity of cyclosome/APC. These differences may reflect the fact that different components of the cell cycle and proteolysis machinery are likely to be limiting in somatic cells and eggs.

Clb2 is not a limiting proteolysis target for mitosis

In this report, we demonstrate that *cks1* and *cdc28-1N* mutants are defective in proteolysis of Clb2 at both the permissive and restrictive temperature. Yet, cell cycle progression is not grossly impaired at the permissive temperature. This observation suggests that efficient Clb2 proteolysis is not necessary for progression through and exit from mitosis when Clb2 is expressed at normal levels. Anaphase arrest occurs only when grossly overexpressed Clb2 (or Clb1) is shielded from proteolysis, for example, when Clb1 or Clb2 with mutated D-boxes are expressed from the *GAL1* promoter (Ghiara et al. 1991; Surana et al. 1993). When Clb2 destruction is prevented at normal levels of expression, no cell cycle arrest occurs, as shown by mutation of *hct1/cdh1*, which encodes a cofactor essential for Clb2-ubiquitination/degradation or by mutation of the D-box of the endogenously expressed Clb2 (Schwab et al. 1997; Visintin et al. 1997). On the other hand, when Pds1 proteolysis is significantly impaired, as in a *cdc20* mutant, wild-type levels of Pds1 expression are sufficient to confer a metaphase arrest (Guacci et al. 1994; Shirayama et al. 1998). This and the observation that *cks1* and *cdc28-1N* mutants exhibit a metaphase (rather than anaphase) arrest is con-

sistent with Pds1 being the limiting target of M-phase proteolysis and not Clb2. Presumably then, it is the impairment of Pds1 proteolysis that blocks *cdc28-1N* and *cks1* mutant cells in metaphase. Consistent with this idea, we isolated *CDC20*, a cofactor for Pds1 degradation, as a multicopy suppressor of *cks1* mutants (M.H. Watson and S.I. Reed, unpubl.) and *CDC20* has also been described as a high-copy suppressor of the *cdc28-1N* mutation (Lim and Surana 1996). However, Pds1 degradation in G₁-arrested cells was only moderately affected by *cks1* mutations (data not shown). This could reflect a difference in Pds1 degradation during the G₁ phase of the cell cycle as compared with its degradation at the metaphase/anaphase transition when Pds1 degradation is biologically most important. In this respect, it is interesting that Cdc20, an important component for Pds1 ubiquitination is not present in the G₁ interval at substantial levels (Prinz et al. 1998; Shirayama et al. 1998). We have found Pds1 to be stabilized in *cks1* mutant cells during mitosis, when Cdc20 is reportedly present. Genetic evidence supports the idea that the defect of *cks1* mutants to degrade Pds1 at the metaphase/anaphase transition is (partially) responsible for their metaphase arrest phenotype. As with mutants defective in cyclosome/APC function (Yamamoto et al. 1996), deletion of *PDS1* allows ~50% of *cks1* mutant cells to undergo nuclear division. Similar to *cdc20 pds1*, *cdc16 pds1*, and *cdc23 pds1* double mutants, a significant proportion (≥50%) still arrests with undivided nuclei (Yamamoto et al. 1996; this report), suggesting the existence of an additional essential step at the metaphase/anaphase transition other than degradation of Pds1.

Function of the Cks1/proteasome interaction

The specific genetic interaction of *cks1* and *cdc28-1N* mutants with mutations in the 19S subunit Rpn3 and the physical interactions between Cks1, Cdc28, and the proteasome strongly suggest regulation of the proteasome by the cell cycle machinery. However, the data presented here provide little insight concerning the precise mechanism by which Cks1 affects proteasome function during M-phase-specific proteolysis. Nevertheless, mutations in Cks1 that abolish its function in vivo interfere with its ability to bind to the proteasome. The affinity of Cks1 for Cdc28 suggests that Cks1 might function as a docking factor for Cdc28 in the context of its interaction with the proteasome. However, the ability of Cdc28-1N to bind to the proteasome despite its defect in association with Cks1 argues against this hypothesis, unless the Cdc28-1N/proteasome interaction is different from the Cdc28-Cks1/proteasome association. We cannot rule out that some of the Cdc28/proteasome and Cks1/proteasome interactions we detected in our experiments are mediated by polyubiquitinated proteins bound to Cdc28 or Cks1. However, several experiments suggest that the interactions are not mediated by proteasome substrates: (1) The binding of Clb2/Cdc28 to proteasome subunits is proportional to Clb2 levels even when the D-box is deleted (Fig. 4B); and (2) the protea-

some purification steps were carried out under conditions that were permissive for 26S proteasome activity so that ubiquitinated substrates were most likely degraded.

Perhaps a clue to Cks1 function lies in our observation that more cdc28-1N is found in immunocomplexes with proteasome subunits than wild-type Cdc28. Similar observations have also been made in in vitro experiments in which portions of the 19S proteasome cap have been reconstituted (data not shown). Taking into account both differential binding of Cdc28-1N and Cdc28 to the proteasome and the independent association of Cks1 with the proteasome, we suggest that Cks1 may function as a recycling factor for Cdc28. After proteolysis of Clb2, Cks1 binding to Cdc28 would release the kinase subunit from a putative receptor on the proteasome and allow new Cdc28/Clb2 complexes to bind. A defect in Cdc28/Cks1 complex formation would disrupt the recycling cycle and result in a blocked receptor leading to defects in Clb2 proteolysis. Consistent with this idea, Cdc28-1N confers a partial dominant-negative phenotype when overexpressed (data not shown). The model suggested above is largely hypothetical and a more direct mechanism of regulation of proteasome activity by Cdc28/Cks1 is equally plausible.

Further genetic and biochemical investigation will be required to determine the molecular details of how such interactions might be involved in regulation of protea-

some function in the context of M-phase-specific proteolysis.

Materials and methods

Yeast strains and methods

The relevant genotypes of the yeast strains used in this study are listed in Table 2. All strains are isogenic to 15DaubΔ, a *bar1Δ ura3Δns* derivative of BF264-15D (Reed et al. 1985). All strains were grown in standard culture medium and standard yeast genetic methods were used (Guthrie and Fink 1991). The *rpn3-54* mutation was initially isolated after UV-induced mutagenesis and screening for mutational enhancers of a diploid homozygous *cdc28-1N* strain as it rendered the mutant cells unable to form colonies at the new restrictive temperature of 35°C. Sporulation of the diploid and tetrad dissection revealed that the mutational enhancer segregated (2:2) with lethality, causing the nonviable spores to arrest as large budded cells after one or two divisions. However, the lethality could be rescued by spore cell-mating experiments in which each dissected spore was placed next to a *cdc28-1N* haploid cell to permit conjugation in the case of compatible mating types. This strategy allowed us to verify unambiguously that the increased temperature sensitivity observed in the *cdc28-1N* diploid background consistently segregated with the *rpn3-54* mutation. Cloning of the corresponding gene was attempted by screening *S. cerevisiae* genomic libraries for centromeric plasmids that were able to rescue the increased temperature sensitivity of the enhancer-containing strain. Among those rescuing plasmids, only those

Table 2. Yeast strains used in this study

Strain	Relevant genotype	Source
15Daub	<i>a bar1Δ ura3Δns ade1 his2 leu2-3, 112 trp1-1^a</i>	Reed et al. (1985)
PY124	<i>a GAL1-CLN2(HA)3::LEU2</i>	this study
PY136	<i>a < cdc28-1N(HA)1::TRP1 > CEN RPN3(RGS6H)::HIS2</i>	this study
PY137	<i>a < CDC28(HA)1::TRP1 > CEN RPN3(RGS6H)::HIS2</i>	this study
PY139	<i>α < cdc28-1N(HA)1::TRP1 > CEN cdc28::LEU2</i>	this study
PY148	<i>a GAL1-CLB2(HA)3::LEU2::KAN^R</i>	G. Mondesert (unpubl.)
PY161	<i>a GAL1-CLB2CLB2^{db}(HA)3::LEU2</i> <i>RPN3(RGS6H)::HIS2</i>	this study
PY166	<i>a GAL1-CLB2(HA)3::LEU2 RPN3(RGS6H)::HIS2</i>	this study
PY169	<i>a GAP2-PRE1(RGS6H)::HIS2 pre1::KAN^R</i> <i>RPN3(RGS6H)::HIS2</i>	this study
PY170	<i>a RPN3(RGS6H)::HIS2</i>	this study
PY228	<i>a bar1 cdc23-1 pep4::URA3</i>	this study
PY236	<i>a bar1 pep4::URA3</i>	this study
PY253	<i>a bar1 pep4::URA3 2μGAL1-CLB2(HA)3::TRP1</i>	this study
PY254	<i>a bar1 pep4::URA3 2μGAL1-CLB2^{db}(HA)3::TRP1</i>	this study
PY255	<i>a bar1 cks1-35::TRP1 pep4::URA3</i>	this study
PY256	<i>a bar1 cdc28-1N::TRP1 cdc28::LEU2</i> <i>GAL1-CLB2(HA)3::LEU2::KAN^R</i>	this study
PY272	<i>a bar1 cks1-35::TRP1 pep4::URA3</i> <i>2μGAL1-CLB2(HA)3::LEU2</i>	this study
PY274	<i>a cks1-35::TRP1 GAL1-CLN2(HA)3::LEU2</i>	this study
PY332	<i>a bar1 cks1-35::URA3 2μGAL1-CLB2(HA)3::LEU2</i>	this study
PY332	<i>a bar1 2μGAL1-CLB2(HA)3::LEU2</i>	this study
PY346	<i>a bar1 cks1-35::TRP1 GAL1-CLB2(HA)3::LEU2::KAN^R</i>	this study
PY351	<i>a bar1 cks1-35::URA3 2μGAL1-CLB2^{db}(HA)3::TRP1</i>	this study
PY364	<i>a bar1 rpn3-4::LEU2 2μGAL1-CLB2(HA)3::TRP1</i>	this study
DCY1361	<i>a bar1 PDS1(HA)3::KAN^R GAL1::PDS1^{db}::LEU2</i>	this study
DCY1601	<i>a bar1 cks1-35::TRP1 PDS1(HA)3::KAN^R</i> <i>GAL1-PDS1^{db}::LEU2</i>	this study

capable of complementing the haplolethal phenotype of the *rpn3-54* mutation were kept for further analyses. Drop-out DNA manipulations were used to narrow down the complementing insert fragment that was ultimately subjected to DNA sequencing. Disruption of the wild-type *RPN3* gene was done in the different *cdc28* and *cks1^{ts}* genetic backgrounds by single-step gene replacement with a construct in which most of the *RPN3* ORF (the internal 1-kb *Bgl*III fragment) had been deleted and replaced by the *URA3* arker. Genomic disruptants were checked by PCR with site-specific primers.

Analysis of Clb2 and Cln2 turnover

CKS1 and *cks1-35* in a *bar1* background containing an integrated *GAL1::CLB2(HA)3* allele (PY148 and PY346) were grown in YEP-raffinose at 25°C to an $OD_{600} = 0.3$ and arrested in G_1 by addition of 200 ng/ml α -factor. After the cells arrested, as determined by budding index (>90% unbudded cells), galactose was added to a final concentration of 2% to induce Clb2(HA)3 expression. Half of the culture was grown at 25°C and half was shifted to 37°C. Extracts were prepared from aliquots after 0, 10, 30, 60, and 120 min and analyzed for the amount of Clb2(HA)3 by Western blotting with 12CA5 antibodies directed against the HA epitope. In the case of the experiment shown in Figure 1B, cells were grown and induced as described above, but expression of Clb2(HA)3 was terminated by collecting the cells and shifting them to medium containing 2% dextrose and 200 ng/ml α -factor. Samples were collected at 0, 30, and 60 min after repression of Clb2(HA)3 expression. In the case of the asynchronous samples, cells were grown in YEP-Gal for 3 hr prior to sample preparation. Cln2 turnover was assayed in *CKS1* and *cks1-35* cells harboring an integrated *GAL1::CLN2(HA)3* allele (PY124 and PY274). Cells were grown in YEP-raffinose to an $OD_{600} = 0.3$ at 25°C, shifted to 35°C, and Cln2(HA)3 expression was induced by addition of galactose to a final concentration of 2%. After 1 hr cells were collected on filters and shifted to prewarmed medium containing 2% dextrose to repress Cln2(HA)3 expression. Samples were taken in 10 min intervals and analyzed for Cln2(HA)3 levels by Western blotting as described above.

Clb2 in vitro ubiquitination activity

Preparation of ubiquitination-competent extracts and assay conditions were as described (Zachariae and Nasmyth 1996) with the following modifications: Cells were grown in YEPD and depleted for their ATP pool by addition of 0.02% azide (final concentration) during the 15 min incubation with lyticase. Each reaction contained 50 mM HEPES-KOH (pH 7.3), 60 mM sodium acetate, 5 mM magnesium acetate, 10% glycerol, 1 mM DTT, 1 μ g/ml aprotinin, leupeptin, and pepstatin A, 0.1 mM PMSF, 1 mM ATP, 60 mM creatine phosphate, 150 μ g/ml creatine phosphokinase, 1 μ M okadaic acid, 50 μ M N-acetyl-leu-leu-norleucinal, 1 mg/ml 6 $\times$ HIS ubiquitin or untagged ubiquitin, 36 mg/ml ubiquitination extract and 2.25 mg/ml extracts prepared from cells overexpressing Clb2(HA)3 or Clb2^{db}(HA)3 from a high-copy plasmid under the control of the *GAL1* promoter. Reactions were incubated at 30°C for 5 min and terminated by addition of 1 ml of buffer C (8 M urea, 50 mM sodium phosphate buffer at pH 8.0, 10 mM Tris-HCl at pH 8.0, 300 mM NaCl) containing 5 mM N-ethylmaleimide. 50 μ l of Ni-NTA-agarose and imidazole (final concentration 10 mM) were added and the suspension was mixed overnight at 4°C. The beads were successively washed with 1 ml each of buffer C + 10 mM imidazole, buffer C adjusted to pH 6.0, buffer C plus 20 mM imidazole, and buffer C without urea. Bound proteins were eluted in 200 μ l 2 $\times$

SDS sample buffer and analyzed by Western blotting with the 12CA5 antibody.

Detection of Clb2-ubiquitin conjugates in vivo

cks1, *rpn3* mutants and wild-type strains expressing Clb2(HA)3 or Clb2^{db}(HA)3 from a high-copy plasmid under the control of the *GAL1* promoter (strains PY332, PY364, PY334, and PY351) were grown overnight at 25°C in selection medium containing 2% raffinose. Cells were diluted to $OD_{600} = 0.25$ in YEP raffinose and grown for 2 hr at 30°C. A total of 200 ng/ml α -factor was added to arrest cells in G_1 . When >90% of the cells were arrested in G_1 as monitored by microscopic observation (~2.5 hr at 30°C), an additional 100 ng/ml α -factor was added and cells were shifted to 36°C to inactivate the temperature-sensitive alleles. After 60 min, galactose (or dextrose) was added to a final concentration of 2% to induce (or repress) expression of Clb2(HA)3 or Clb2^{db}(HA)3, respectively, and incubation at 36°C was continued for 10 min. Cells were harvested and washed once in ice-cold water and pellets flash-frozen in liquid nitrogen and stored at -80°C. Cells were broken in RIPA/NEM-buffer (1% deoxycholic acid, 1% Tween 20, 1% NP-40, 0.1% SDS, 250 mM NaCl, 50 mM Tris-HCl at pH 7.5, 5 mM N-ethylmaleimide) with glass beads and cell debris was spun down twice for 15 min at 13000 rpm. One milligram of protein extract was mixed with anti-HA antibodies covalently coupled to protein A beads and incubated in a total volume of 1.5 ml for 2 hr at 4°C. The beads were washed five times in 1 ml of RIPA buffer and immunoprecipitated proteins eluted by boiling the beads in 2 $\times$ SDS sample buffer for 5 min. Eluted proteins were separated by SDS-PAGE on a 7.5% gel and probed for the presence of polyubiquitin conjugates with antibodies directed against polyubiquitin (kindly provided by E. Wayner, A. Kahana, and D. Gottschling, Fred Hutchinson Cancer Center). Monoclonal antibodies were raised against glutaraldehyde cross-linked ubiquitin according to the procedure described in Wayner and Carter (1987). Antibodies were partially purified from hybridoma supernatant by two precipitations with ammonium sulfate.

Immunoprecipitation and purification of the 26S proteasome

Extracts for immunoprecipitation were prepared as described (Kaiser et al. 1998). Cells were broken in buffer B (50 mM sodium phosphate buffer at pH 7.6, 150 mM NaCl, 10% glycerol, 10 mM sodium pyrophosphate, 5 mM EDTA, 5 mM EGTA, 0.1 mM orthovanadate, 1 mM PMSF, 2 μ g/ml aprotinin, leupeptin, and pepstatin A). A total of 500 μ g of extract was incubated with 12CA5 antibodies cross-linked to protein A-Sepharose and immunocomplexes were washed five times with 1 ml of buffer B. Bound proteins were eluted by boiling in 2 $\times$ SDS sample buffer for 5 min, separated by SDS-PAGE and analyzed by immunoblotting with anti-HA (12CA5, BabCO), anti-RGS6H (Qiagen), anti-Rpt1/Cim5, and anti-PSTAIR antibodies. Rpn3 was epitope tagged by inserting the RGS6H epitope in front of the stop codon at the chromosomal locus. For expression of Clb2^{db}(HA)3, the *CLB2* ORF truncated for 47 amino-terminal amino acids was expressed under the control of the *GAL1* promoter. The 26S proteasomes were partially purified as described (Rubin et al. 1996) except that a sodium phosphate-based buffer (buffer A: 50 mM sodium phosphate buffer at pH 7.6, 5 mM MgCl₂, 0.5 mM EDTA, 2 mM ATP, 1 mM DTT, 10% glycerol) was used. Briefly, 1 liter of yeast culture ($OD_{600} = 1$) was harvested, washed in buffer A, and lysed with a French press. The lysate was cleared by centrifugation at 10,000g for 10 min, 200,000g for 30 min, and filtration through a 0.45 μ m filter. After restoration of the pH, the extract was applied to a DEAE-

Affigel Blue column (5 ml bed volume) (Bio-Rad), and washed extensively. Protein was eluted with 150 mM NaCl in buffer A. The eluate was applied to a Q-Sepharose column (Bio-Rad) and eluted with a gradient of NaCl in buffer A. Fractions were tested for their peptidase activity in buffer A containing 100 μ M Suc-LLVY-AMC (Sigma). Fluorescence was read with a fluorometer (excitation 380 nm, emission 440 nm) after the reaction was terminated by addition of 2 volumes of 10% SDS. RGS6H-tagged Pre1 was expressed under the control of the GAP promoter and integrated at the *HIS2* locus in a strain disrupted for *PRE1*. All epitope-tagged strains grew normally, indicating that the epitope-tagged proteins are fully functional.

Proteasome binding to Cks1 beads

Recombinant Cks1, Cks1-35, and Cks1-E94Q were expressed in *Escherichia coli* and purified as described (Tang and Reed 1993). The recombinant-purified proteins and BSA were coupled to Affigel 15 beads (Bio-Rad), as recommended by the manufacturer. For binding studies, beads were incubated for 2 hr at 4°C with 500 μ g of extract in a total volume of 500 μ l buffer B. Beads were washed sequentially with 1 ml of buffer B, 200 μ l of buffer B containing 0.15 M, 0.3 M, or 1 M of KCl and 200 μ l of 2% SDS. Fractions were analyzed by Western blotting with antibodies directed against the RGS6H epitope (Rpn3), PSTAIRE (Cdc28) Rpt6, or Rpt1 (Ghislain et al. 1993) as indicated.

Pds1 stability in metaphase

Wild-type and *cks1* mutant cells expressing endogenous (HA)3-epitope-tagged Pds1 and a nontagged D-box mutant Pds1 (Pds1^{db-}) under the control of the *GAL1* promoter (strains DCY1361 and DCY1601) were grown in YEP-*raffinose* at 30°C to an OD₆₀₀ = 0.25. α -Factor (200 ng/ml) was added, and incubation at 30°C was continued. When cells were arrested in G₁ (>90% unbudded cells), α -factor was removed by collecting the cells on filters and washing them once in medium. Cells were then transferred to prewarmed (30°C) YEP-*raffinose* and incubated at 30°C. After the majority of the cells passed START as indicated by small buds on most of the cells (~75 min), the cultures were shifted to 35°C and galactose was added to a final concentration of 2% to induce expression of *PDS1*^{db-}. Cells were incubated at 35°C and samples were collected at 15-min intervals for flow cytometry, protein, and RNA analysis. The levels of endogenous Pds1(HA)3 were analyzed by Western blotting with anti-HA antibodies (12CA5, BabCo). For detection of mRNA expression levels of *PDS1*(HA)3, Northern blots were analyzed with a DNA probe detecting the (HA)-epitope sequence. The D-box of Pds1 was mutated by site-directed mutagenesis (Clontech Mutagenesis Kit, mutagenesis primer: AT-GCGCAACAACAAGGACTCGAGAATAGATCGAAAAGCTT). The procedure changed the D-box sequence of Pds1 (RLP-LAAKDN) into (LE). The endogenous *PDS1* was epitope tagged by inserting the sequence encoding a triple HA epitope tag in front of the stop codon.

Analysis of *cks1 pds1* double mutants

cks1 mutants (strain PY259) and *pds1 cks1* double mutants (strain DCY1736) were grown in YEPD at 20°C to an OD₆₀₀ = 0.2, arrested in S phase with 0.4 M HU for 3 hr at 20°C and shifted to 37°C for 1 hr in the presence of HU. Arrested cells were collected on filters, washed with prewarmed medium (37°C), and resuspended in fresh prewarmed YEPD medium. Incubation was continued at 37°C and samples were taken every 30 min for FACS analysis and DNA staining. DNA was

stained with Sytox (Molecular Probes) and nuclear separation was analyzed microscopically. Two-hundred cells were scored for each mutant.

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Cyclin-dependent kinase and Cks/Suc1 interact with the proteasome in yeast to control proteolysis of M-phase targets

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