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TPR Proteins Required for Anaphase Progression Mediate Ubiquitination of Mitotic B-type Cyclins in Yeast

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The abundance of B-type cyclin-CDK complexes is determined by regulated synthesis and degradation of cyclin subunits. Cyclin proteolysis is required for the final exit from mitosis and for the initiation of a new cell cycle. In extracts from frog or clam eggs, degradation is accompanied by ubiquitination of cyclin. Three genes, *CDC16*, *CDC23*, and *CSE1* have recently been shown to be required specifically for cyclin B proteolysis in yeast. To test whether these genes are required for cyclin ubiquitination, we prepared extracts from G1-arrested yeast cells capable of conjugating ubiquitin to the B-type cyclin Clb2. The ubiquitination activity was cell cycle regulated, required Clb2's destruction box, and was low if not absent in *cdc16*, *cdc23*, *cdc27*, and *cse1* mutants. Furthermore all these mutants were also defective in ubiquitination of another mitotic B-type cyclin, Clb3. The Cdc16, Cdc23, and Cdc27 proteins all contain several copies of the tetratricopeptide repeat and are subunits of a complex that is required for the onset of anaphase. The finding that gene products that are required for ubiquitination of Clb2 and Clb3 are also required for cyclin proteolysis in vivo provides the best evidence so far that cyclin B is degraded via the ubiquitin pathway in living cells. *Xenopus* homologues of Cdc16 and Cdc27 have meanwhile been shown to be associated with a 20S particle that appears to function as a cell cycle-regulated ubiquitin-protein ligase.

INTRODUCTION

In eukaryotic cells, the timing and inter-dependence of DNA replication (S-phase) and mitosis (M-phase) are controlled by oscillations in the activities of cyclin-dependent kinases (Cdks). Higher eukaryotes have multiple Cdks whereas in yeasts, cell cycle progression requires a single Cdk known as Cdc2 in fission yeast and Cdc28 in budding yeast. Waves of kinase activities are determined to a large extent by cell cycle–dependent synthesis and degradation of Cdk's regulatory cyclin subunits. Entry into M-phase depends on the appearance of B-type cyclins whose associated kinase activity promotes formation of the mitotic spindle. In budding yeast two pairs of related B-type cyclins appearing during S-phase (Clb3,4) and G2 (Clb1,2) are involved in formation and elongation of the spindle (Fitch *et al.*, 1992). Most of the events that occur when cells exit from mitosis require degradation of mitotic B-type cyclins. Cyclin proteolysis causes a sudden drop in the Cdc2/Cdc28-dependent kinase activity at the onset of or during anaphase (Whitfield *et al.*, 1990; Pines and Hunter, 1991; Hunt *et al.*, 1992; Surana *et al.*, 1993). The introduction of nondegradable cyclin B variants prevents chromosome decondensation, cytokinesis, and disassembly of the mitotic spindle in frog eggs both in vivo and in extracts that undergo multiple cell cycles in vitro (Murray *et al.*, 1989). Similar results have been obtained in clam egg extracts (Luca *et al.*, 1991). Expression of nondegradable B-type cyclins causes yeast cells to arrest with elongated spindles and separated chromosomes in telophase (Surana *et al.*, 1993). Regulated cyclin proteolysis is therefore a key aspect of the cell cycle clock.

The mechanism of cyclin degradation has mainly been studied in vitro using extracts from frog or clam eggs. Exogenously added cyclin B remains stable in

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"interphase" extracts (i.e., during S- and G2-phases), whereas it is rapidly degraded in extracts arrested in a "late mitotic" state due to the presence of a nondegradable cyclin fragment (Murray *et al.*, 1989; Luca *et al.*, 1991). Cyclin B proteolysis depends on the destruction box, a degenerate nine-amino acid motif found in the N-termini of A- and B-type cyclins, and on adjacent lysine containing sequences (Glotzer *et al.*, 1991).

Cyclin B proteolysis in vitro was shown to be accompanied by the production of cyclin-ubiquitin conjugates (Glotzer *et al.*, 1991). Assembly of multi-ubiquitin chains on a short-lived protein is usually required for its efficient degradation by the 26S proteasome complex. The enzymatic reactions of the ubiquitin-dependent proteolytic pathway have thus far been characterized mainly using artificial substrates and only a limited number of natural substrates have been identified (see Ciechanover, 1994). In an ATP-dependent reaction the C-terminus of ubiquitin is first bound to an ubiquitin-activating enzyme (E1) forming a thioester intermediate. This "activated" ubiquitin is subsequently transferred to one member of a large family of ubiquitin-conjugating enzymes (E2s), forming a further thioester intermediate. By catalyzing the formation of an isopeptide bond, the E2 enzyme transfers ubiquitin to a lysine residue of the target protein. Repeated transfer of further ubiquitin molecules to particular lysine residues of conjugated ubiquitins gives rise to polyubiquitinated target proteins. The latter reaction requires a third activity called a ubiquitin-protein ligase (E3), which is thought to bind both the E2 enzyme and the target protein. The polyubiquitin chain serves as a recognition marker for the 26S proteasome, which degrades the target protein and releases free ubiquitin (Peters, 1994).

Efficient ubiquitination of cyclin B peptides is confined to "mitotic" extracts, suggesting that cell cycle control of cyclin proteolysis could be exerted at the level of ubiquitin conjugation. The finding that cyclin-ubiquitin conjugates generated in mitotic extracts can be degraded when transferred to interphase extracts suggests that the proteolytic activity of the 26S proteasome (at least toward B-type cyclins) might not be cell cycle regulated (Peters *et al.*, 1994). The ubiquitination activity of clam egg extracts can be fractionated into three components: a nonspecific E1, a cyclin-specific E2, and a large 20S complex that meets the criteria of a ubiquitin-protein ligase (Hershko *et al.*, 1994; Sudakin *et al.*, 1995). The activities of the E1 and the E2 fractions were not cell cycle regulated. The 20S ubiquitin-protein ligase was only active after isolation from the mitotic extract although it was also present in the interphase extract.

Few if any of the proteins involved in destruction box–mediated ubiquitination of cyclin B have been identified with certainty. Mutations in the *UBC9* gene, which encodes an E2 enzyme, and in genes that encode proteasome subunits (*CIM3/SUG1*, *CIM5*, and *mts2*$^{+}$), cause yeast cells to arrest in G2- or M-phase (Ghislain *et al.*, 1993; Gordon *et al.*, 1993; Seufert *et al.*, 1995). However, it is still unclear whether these cell cycle arrests are due to defects in cyclin proteolysis per se rather than some other proteolytic defect that prevents the mutants from reaching the cell cycle stage at which cyclin proteolysis is activated.

It has recently been discovered that cyclin proteolysis in yeast, although activated during anaphase, persists during the subsequent G1 period until re-activation of the Cdc28 kinase through the accumulation of Cln1 and Cln2 cyclins in late G1 (Amon *et al.*, 1994). By screening for mutations that permit accumulation of the Clb2 B-type cyclin in cells arrested in G1 by Cln-cyclin depletion, Irniger *et al.* (1995) identified three genes needed for cyclin B proteolysis: *CDC16*, *CDC23*, and *CSE1*. Subsequent work showed that *SRP1*, which encodes the nuclear import factor importin, is also required, suggesting that factors involved in nuclear transport have a role in cyclin B proteolysis (Loeb *et al.*, 1995).

CDC16, *CDC23*, and the related *CDC27* gene encode essential proteins that are required for the metaphase to anaphase transition. All three proteins contain several copies of a 34-amino acid motif called the tetratricopeptide repeat (TPR) (Goebl and Yanagida, 1991) and have been shown to be subunits of a complex (Lamb *et al.*, 1994). The metaphase arrest of *cdc16* and *cdc23* mutants cannot be explained by their failure to degrade known mitotic cyclins because their hyperaccumulation does not inhibit the onset of anaphase (Surana *et al.*, 1993). It has therefore been proposed that these TPR proteins might also be involved in the degradation of proteins that inhibit sister chromatid separation. Studies using frog egg extracts have also suggested that ubiquitin conjugation might be involved in sister chromatid separation (Holloway *et al.*, 1993).

To address whether *CDC16*, *CDC23*, and *CSE1* are needed for cyclin B ubiquitination or for some other step in cyclin proteolysis, we prepared cell free extracts from yeast that are capable of cell cycle–regulated and destruction box–dependent ubiquitination of Clb2. We show here that extracts from G1-arrested *cdc16*, *cdc23*, *cdc27*, and *cse1* mutants are defective in ubiquitination of Clb2 and another mitotic B-type cyclin, Clb3. The finding that genes required for Clb2 and Clb3 ubiquitination in vitro are essential for cyclin proteolysis in vivo provides compelling evidence that cyclin B is degraded by the ubiquitin pathway in living cells.

In the course of our studies, antibodies against human homologues of Cdc16 and Cdc27 were shown to recognize two subunits of a 20S particle that functions as an ubiquitin-protein ligase and promotes destruction box–dependent cyclin B ubiquitination in frac-

tionated extracts from *Xenopus* eggs (King *et al.*, 1995). Furthermore, antibodies specific for Cdc27 were shown to block anaphase in human cells (Tugendreich *et al.*, 1995). The activity of the TPR protein–containing particle called the anaphase-promoting complex (APC) or cyclosome therefore seems to be conserved from yeast to humans.

MATERIALS AND METHODS

Media, Growth Conditions, and Cell Cycle Arrest

Yeast strains were grown in rich medium (1% yeast extract, 2% bactopeptone, 50 mg/l adenine) containing 50 mM sodium phosphate, pH 6.5, and 2% of either glucose (YEPP_iD) or raffinose (YEP$P_i$raff). To induce expression from the *GAL* promoter, 2% galactose was added to a culture pregrown in YEP$P_i$raff. Cells were grown to exponential phase by diluting overnight cultures into fresh medium to an optical density at 600 nm (OD_{600}) of 0.2 and growing them to an OD_{600} of 0.5. *bar1* cells were arrested in G1 by addition of α-factor pheromone to 0.4 μg/ml. *BAR1* strains (K5526, K5527, and K5934) were grown at 25°C from OD_{600} 0.2 to 0.5 and α-factor was added to 5 μg/ml followed by a second addition to 5 μg/ml after 1.5 h. Cells were harvested 1.5 h later. Cells were arrested in M-phase by addition of nocodazole to 15 μg/ml. To arrest cells using a *cdc* mutation, cultures pregrown at 25°C were transferred to a water bath set at 37°C. Cultures were arrested for 2.5–3.5 h and the arrest was confirmed by microscopic examination and flow cytometric analysis. The DNA content of cells stained with propidium iodide was measured on a Becton Dickinson FACScan (San Jose, CA) as described by Epstein and Cross (1992).

Yeast Strains and Plasmid Constructions

The genotypes of yeast strains used in this study are shown in Table 1. Except K5526, K5527, and K5934 all strains are derivatives of W303 (K699). K5526, K5527, and K5934 were derived from SUB328. In this strain, all four *UBI* genes are deleted and the parts encoding ribosomal proteins are expressed separately. Viability depends on a *URA3* marked plasmid expressing wild-type ubiquitin (pUB146) from the *GAL* promoter (Finley *et al.*, 1994; Spence *et al.*, 1995). pUB146 was replaced by the 2 μ plasmids YEp96, YEp105 (Ellison and Hochstrasser, 1991), or pJD421 (Dohmen *et al.*, 1995), which direct expression of wild-type ubiquitin, a myc-epitope–tagged variant, or a hexahistidine-tagged variant, respectively, from the *CUP1* promoter (strains kindly provided by J. Dohmen). K5526, K5527, and K5934 were obtained after disruption of the *PEP4* gene using pTS15. All *pep4* derivatives were obtained by genetic crosses or by one-step gene disruption. Strains were transformed with pTS15 (*pep4::URA3*) cut with *Eco*RI–*Xho*I or pTS17 (*pep4::LEU2*) cut with *Bam*HI. *pep4* disruption was verified by a color assay as described by Jones (1977). To express Clb2ha_3, Clb2ΔDBha_3, or Clb3ha_3 from the *GAL10* promoter, several copies of pWZV1, pWZV4, or c3190, respectively, were integrated at the *ura3* locus. The plasmids were cut with *Apa*I in the *URA3* sequence and transformed into K1534 giving strains K5515, K5516, or K5935. Strains K5515 and K5516 were selected to express Clb2ha_3 and Clb2ΔDBha_3, respectively, to equivalent levels. pWZV1 consists of the *GAL10* promoter on a *Bam*HI–*Eco*RI fragment fused to an *Eco*RI–*Asp*718 fragment containing *CLB2* sequences from the start codon to position +1803 (ATG = +1) in the *URA3*-based integrative vector YIplac211 (Gietz and Sugino, 1988). The *CLB2* sequence contains a triple hemagglutinin epitope tag (ha_3) (Tyers *et al.*, 1993) inserted into a *Not*I site in front of the stop codon (Lin and Arndt, 1995). pWZV4 is a derivative of pWZV1 containing a deletion of the destruction box (Surana *et al.*, 1993). In c3190 the *GAL10* promoter is fused to an *Eco*RI–*Sal*I fragment containing *CLB3* sequences from the start codon to position +1415 (ATG = +1; Fitch *et al.*, 1992) with a triple ha tag inserted into a *Not*I site in front of the stop codon. The *GAL-CLB3*ha_3 fusion was cloned into YIplac211.

Table 1. Yeast strains used in this study

Name	Relevant genotype
K1534	*MAT***a** *bar1::hisG*
K1719	*MAT***a** *cdc28-4 pep4::URA3*
K1728	*MAT***a** *cdc15-2 pep4::URA3*
K1736	*MAT*α *cdc13-1 pep4::URA3*
K1771	*MAT***a** *pep4::URA3 bar1::LEU2*
K5515	*MAT***a** *GAL-CLB2*ha_3*/URA3* (~18 copies) *bar1::hisG*
K5516	*MAT***a** *GAL-CLB2* Δ*DB*ha_3*/URA3* (several copies) *bar1::hisG*
K5517	*MAT***a** *pep4::URA3 bar1::hisG*
K5518	*MAT***a** *pep4::LEU2 bar1::hisG*
K5519	*MAT***a** *cdc16-123 pep4::URA3 bar1::hisG*
K5520	*MAT***a** *cdc23-1 pep4::URA3 bar1::LEU2, TRP1*
K5521	*MAT***a** *cse1-22 pep4::LEU2 bar1::URA3*
K5522	*MAT***a** *cdc27-1 pep4::LEU2 bar1::hisG*
K5526*	*MAT***a** *ubi1-Δ1::TRP1 ubi2-Δ2::ura3 ubi3-Δub-2 ubi4-Δ2::LEU2 pep4::URA3* (pUB100) (YEp96)
K5527*	*MAT***a** *ubi1-Δ1::TRP1 ubi2-Δ2::ura3 ubi3-Δub-2 ubi4-Δ2::LEU2 pep4::URA3* (pUB100) (YEp105)
K5575	*MAT***a** *cdc20-1 pep4::LEU2 bar1::hisG*
K5934*	*MAT***a** *ubi1-Δ1::TRP1 ubi2-Δ2::ura3 ubi3-Δub-2 ubi4-Δ2::LEU2 pep4::URA3* (pUB100) (pJD421)
K5935	*MAT***a** *GAL-CLB3*ha_3*/URA3* (several copies) *bar1::hisG*

Unless marked with an asterisk all strains were isogenic derivatives of W303 (also called K699) whose full genotype is *MAT***a** *ho ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 ssd1*. Strains marked with an asterisk were derived from SUB328 (Spence *et al.*, 1995) whose full genotype is *MAT***a** *lys2-801 leu2-3,112 ura3-52 his3-Δ200 trp1-1 ubi1-Δ1::TRP1 ubi2-Δ2::ura3 ubi3-Δub-2 ubi4-Δ2::LEU2* (pUB146) (pUB100).

Preparation of Extracts

Protein extracts for the ubiquitination assay were prepared from 350 ml cultures grown to an OD_{600} of 0.5 and arrested for 2.5–3.5 h. Cells were collected on membrane filters and resuspended in 30 ml of 0.1 M Tris-H_2SO_4, pH 9.5, 10 mM dithiothreitol. After 3 min cells were washed with 30 ml spheroplast medium (50 mM Tris-HCl, pH 7.5, 1.2 M sorbitol, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.3 × YEP, 0.5% carbon source) and resuspended in 15 ml of the same medium containing 0.5 mg lyticase (Sigma L5763; St. Louis, MO). Carbon source and incubation temperature were chosen according to the conditions used to arrest the cells. For cells arrested with pheromone or nocodazole the spheroplast medium was supplemented with α-factor (0.5 μg/ml for *bar1*; 5 μg/ml for *BAR1*) or nocodazole (15 μg/ml). Usually more than 90% of cells were converted to spheroplasts after 10–20 min as judged by microscopic examination of samples containing 1% SDS. The following steps were carried out at 0–4°C. The spheroplast suspension was diluted to 50 ml with ice cold 1.2 M sorbitol. Spheroplasts were collected by centrifugation (500 × *g*, 2 min), washed once in 50 ml sorbitol, and collected again (1700 × *g*, 2 min). The spheroplasts were resuspended in 0.3 volumes of buffer A (150 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES]-KOH, pH 7.3, 180 mM sodium acetate, 15 mM magnesium acetate, 30% glycerol, 3 mM dithiothreitol, 0.3 mM phenylmethylsulfonyl fluoride, 3 μg/ml pepstatin) and disrupted by shaking with 0.3 ml of glass beads (0.5 mm) for 4 min. The lysate was cleared by centrifugation for 5 min in an Eppendorf micro-centrifuge and the supernatant was used for the ubiquitination assay. Extracts from

strains overexpressing epitope-tagged Clb2 or Clb3 were frozen in liquid nitrogen and stored at −80°C. Protein concentrations were determined with the Bio-Rad protein assay (Richmond, CA) using bovine serum albumin as a standard. The protein concentration of extracts was 30–40 mg/ml.

Ubiquitination Assay and Western Analysis

Extracts were normalized for protein concentration before the assay. Reactions containing 32 μl extract, 4 μl 10× ATP regenerating system (10 mM ATP, 600 mM creatine phosphate, 10 mM magnesium acetate, 1.5 mg/ml creatine kinase in 0.3 × buffer A) and 2 μl Clb2ha$_3$, Clb2ΔDBha$_3$, or Clb3ha$_3$ extract in a total volume of 40 μl were incubated for 5 min at the temperatures indicated. Some reactions contained 2 μl of yeast ubiquitin (Sigma U2129, 20 mg/ml in 0.3 × buffer A). Reactions were diluted 1:5 into hot SDS sample buffer and incubated for 5 min at 95°C. Samples of 25 μl were separated by electrophoresis on 8% SDS polyacrylamide gels and proteins were transferred to polyvinylidene difluoride membranes (Immobilon P, Millipore, Bedford, MA). Uniform transfer of proteins was confirmed by staining the membranes with 0.2% Ponceau S in 0.3% trichloroacetic acid. After blocking in phosphate-buffered saline containing 0.1% Tween 20 and 4% nonfat dry milk, membranes were incubated with the 12CA5 monoclonal antibody directed against the hemagglutinin epitope (Field *et al.*, 1988). A horseradish peroxidase-conjugated sheep anti-mouse IgG served as a secondary antibody. Epitope-tagged cyclin and cyclin-ubiquitin conjugates were visualized using the enhanced chemoluminescence detection system purchased from Amersham (Buckinghamshire, United Kingdom). A short exposure (10 s) was used to confirm that each reaction received an equal amount of epitope-tagged cyclin. For detection of cyclin-ubiquitin conjugates blots were exposed for 5–15 min to autoradiography films.

Purification of Clb2-Ubiquitin Conjugates

Extracts were prepared from α-factor–arrested cells of K5934 and K5526, which express 6-histidine-ubiquitin or the wild-type protein, respectively. Thirty-two microliters of extract were incubated with the ATP regenerating system and 2 μl of Clb2ha$_3$-extract in the presence or absence of exogenous ubiquitin (1 mg/ml) in a total volume of 40 μl for 5 min at 25°C. To check the formation of Clb2ha$_3$-ubiquitin conjugates, 5-μl samples were removed, mixed with SDS sample buffer, and incubated at 95°C. Meanwhile 665 μl buffer 1 (6 M guanidinium-HCl, 0.1 M sodium phosphate, 10 mM Tris, adjusted to pH 8.0 with NaOH) containing 5 mM *N*-ethylmaleimide were added to the remaining 35 μl of each reaction followed by 120 μl of 50% nickel-nitrilotriacetic acid (NTA)-agarose (Qiagen, Hilden, Germany) in buffer 1. Imidazole was added to a final concentration of 2 mM and the suspensions were incubated with end-over-end rotation at 4°C overnight. The resins were applied to Bio-Rad Econo-columns and successively washed with the following buffers: 1.2 ml buffer 1, 1.2 ml buffer 2 (buffer 1 adjusted to pH 6.0), 1.2 ml buffer 3 (buffer 1 containing 5 M urea instead of guanidinium-HCl), 0.6 ml buffer 3 plus 10 mM imidazole, and 0.6 ml buffer 3 plus 50 mM imidazole. Bound proteins were eluted with 0.6 ml buffer 3 containing 0.5 M imidazole. The latter fractions were concentrated to 50 μl using Centricon-10 filtration devices (Amicon, Beverly, MA). Samples were mixed with SDS sample buffer, incubated at 37°C for 5 min, and loaded onto a SDS polyacrylamide gel. Purification of conjugates consisting of Clb2ha$_3$ and 6His-ubiquitin was analyzed by Western blotting using the 12CA5 antibody.

RESULTS

Ubiquitination of Clb2 in a Cell-free Extract

To reconstitute cyclin ubiquitination in vitro, we used as a substrate a full-length Clb2 protein synthesized in yeast, because previous work had suggested that truncated Clb2 proteins lacking C-terminal sequences are not properly degraded in vivo (Irniger and Nasmyth, personal communication). A version of Clb2 carrying three copies of the hemagglutinin epitope tag at its C-terminus (Clb2ha$_3$) was expressed in yeast under the control of the galactose-inducible *GAL1–10* promoter. Induction with galactose of a strain carrying approximately 18 copies of *pGAL-CLB2ha$_3$* caused mitotic arrest and led to the accumulation of large amounts of soluble Clb2ha$_3$ protein. A small aliquot of an extract prepared from these cells was incubated with an excess of an extract made from cells arrested in G1, which also contained an ATP regenerating system. Clb2ha$_3$ protein and derivatives of higher molecular weight produced by conjugation to ubiquitin were detected by Western blotting using an anti-ha monoclonal antibody. Ubiquitination of Clb2 was inefficient in extracts prepared from whole cells, possibly because a strong ATPase activity associated with yeast cell walls leads to rapid ATP depletion (Deshaies and Kirschner, 1995). Protein extracts were therefore prepared from spheroplasts generated by treating cells with lyticase. To reduce nonspecific protein degradation during incubation of extracts, we found it necessary to use strains lacking the *PEP4* gene.

Clb2ha$_3$ was added to an extract prepared from wild-type cells previously arrested in G1 with pheromone. In the presence of an ATP regenerating system, a small fraction of Clb2ha$_3$ was converted to a ladder of high molecular weight derivatives (Figure 1A, lane 2). These products were not formed when the ATP regenerating system (Figure 1A, lane 3), the G1 extract (Figure 1A, lane 1), or the Clb2ha$_3$-containing extract (Figure 1A, lane 5) were omitted from the reaction. Few high molecular weight products were formed when an extract prepared from cells overexpressing (to equivalent levels as Clb2ha$_3$) a version of Clb2ha$_3$ lacking its destruction box was used as a source of substrate (Figure 1A, lane 4). However, conversion of Clb2ha$_3$ into high molecular weight products was not influenced by exogenously added ubiquitin (Figure 1B).

To test whether the high molecular weight products consist of cyclin-ubiquitin conjugates, we used yeast strains containing deletions in all four ubiquitin genes, whose survival is sustained by individual ubiquitin genes present on plasmids (Finley *et al.*, 1994; Spence *et al.*, 1995). Extracts were prepared from G1-arrested cells of strains expressing ubiquitin fused to a myc-epitope tag or the wild-type protein. The high molecular weight derivatives of Clb2ha$_3$ produced in the extract from the myc-ubiquitin strain showed a different pattern than those generated in the extract from the control strain (Figure 1B, lanes 2 and 4). The altered pattern was restored to the wild-type pattern by the addition of yeast ubiquitin to the myc-ubiquitin–

containing extract (Figure 1B, lanes 4 and 5). This result indicates that the two ubiquitin versions compete for incorporation into the high molecular weight derivatives of Clb2ha$_3$.

In the experiment shown in Figure 1C, Clb2ha$_3$ was added to extracts from strains expressing a hexahistidine-tagged version of ubiquitin or the wild-type protein. Proteins containing a stretch of histidines can be purified by nickel-chelate chromatography on NTA-agarose (Hochuli *et al.*, 1987, 1988). Also, the 6His-ubiquitin caused an alteration in the pattern of high molecular weight Clb2ha$_3$ derivatives (Figure 1C, lanes 2 and 4). In the presence of exogenously added ubiquitin, high molecular weight products similar to those produced by the control extract were generated (Figure 1C, lanes 4 and 5). Reactions were diluted with a buffer containing 6 M guanidinium hydrochloride and incubated with nickel-NTA-agarose. These denaturing conditions prevent degradation or deubiquitination of ubiquitin-protein conjugates as well as any noncovalent protein-protein interaction but do not interfere with binding of the hexahistidine tag to the affinity resin. After washing, bound proteins were eluted with imidazole and analyzed by Western blotting. As shown in Figure 1C, Clb2ha$_3$ containing conjugates could be isolated from the 6His-ubiquitin extract (Figure 1C, lanes 4 and 9) but not from the control reaction (Figure 1C, lanes 2 and 7). In the presence of exogenously added ubiquitin, the 6His-ubiquitin extract produced conjugates that did not bind efficiently to nickel-NTA-agarose (Figure 1C, lanes 5 and 10). Similar results were obtained with a ha$_3$-tagged version of Clb3 (our unpublished results). We conclude that the high molecular weight products produced in the in vitro reaction consist of cyclin-ubiquitin conjugates.

Ubiquitination of Clb2 In Vitro Is Cell Cycle Regulated

Clb2 protein is rapidly degraded from late anaphase until the end of the subsequent G1-phase but stable

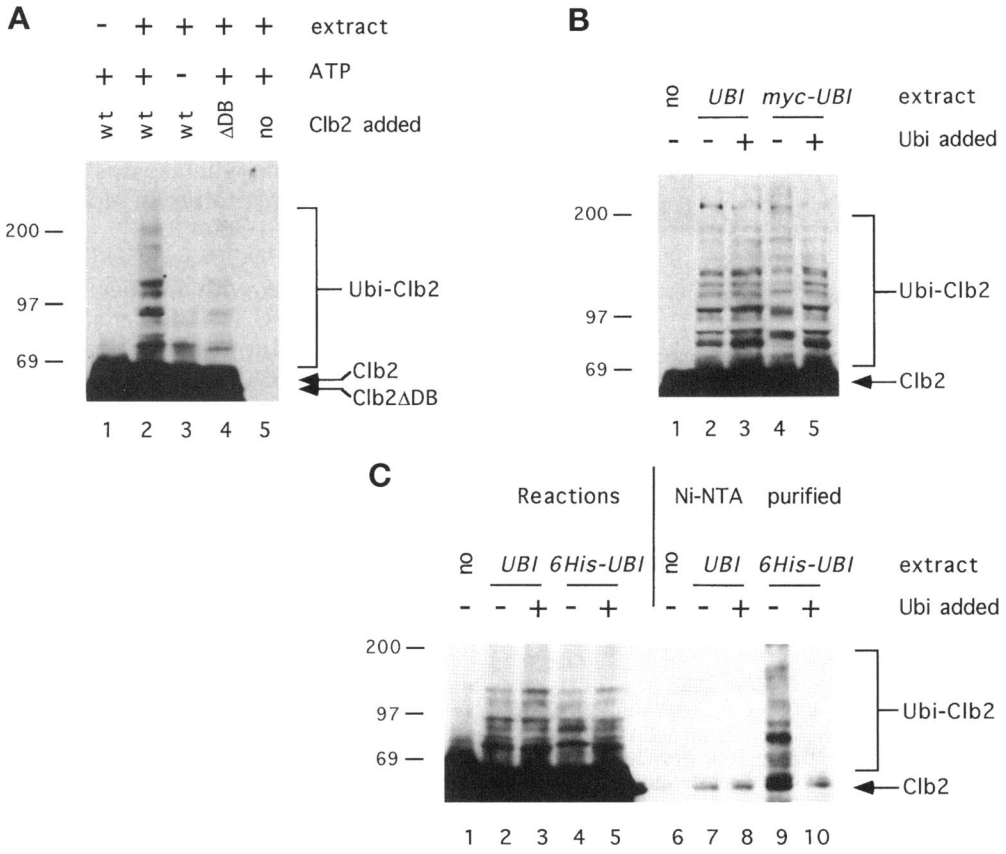

Figure 1. Ubiquitination of Clb2 in a protein extract from cells arrested in G1. (A) Extracts (40 mg/ml) from strains overexpressing either Clb2ha$_3$ (labeled wt) or Clb2ΔDBha$_3$ (ΔDB) were incubated with a 16-fold excess of an extract (40 mg/ml) from a wild-type strain (K1771) that had been arrested in G1 with α-factor. Reactions were incubated at 25°C for 5 min in the presence (+) or absence (−) of an ATP regenerating system and subjected to denaturing gel electrophoresis. Clb2ha$_3$ was detected by Western blotting using a monoclonal antibody directed against the hemagglutinin epitope tag. Only part of the free cyclin band is shown. The positions of molecular weight markers are indicated (kDa). (B) A Clb2ha$_3$ containing extract (35 mg/ml) was incubated with extracts (44 mg/ml) from G1-arrested cells of strains expressing either wild-type ubiquitin (K5526, *UBI* extract) or a myc-epitope–tagged version (K5527, *myc-UBI* extract) as described in panel A. When indicated, extracts contained exogenously added yeast ubiquitin at a final concentration of 1 mg/ml. Clb2ha$_3$ and high molecular weight derivatives were detected by Western blotting. (C) A Clb2ha$_3$ containing extract (45 mg/ml) was incubated with extracts (50 mg/ml) from G1-arrested cells of strains expressing either wild-type ubiquitin (K5526, *UBI* extract) or a hexahistidine-tagged variant (K5934, *6His-UBI* extract) in the presence or absence of yeast ubiquitin as described in panel B. Five microliters of each reaction were diluted into SDS sample buffer (lanes 1–5). The remaining 35 μl of each reaction were incubated with Ni-NTA-agarose in the presence of 6 M guanidinium-HCl. After washing with 0.05 M imidazole, bound proteins were eluted with 0.5 M imidazole (lanes 6–10). Clb2ha$_3$ and Clb2ha$_3$-ubiquitin conjugates were detected by Western blotting.

during S, G2, and early stages of mitosis (Amon *et al.*, 1994). To check whether ubiquitination of Clb2 in vitro was cell cycle regulated, we prepared extracts from cells arrested at different stages of the cell cycle. $Clb2ha_3$ ubiquitination was also high in extracts from cells arrested in G1 by the *cdc28–4* mutation, which is consistent with the finding that Cdc28 function is not required for Clb2 proteolysis during G1 (Amon *et al.*, 1994). In contrast, extracts prepared from cells arrested in G2 (*cdc13–1*) or M-phase (nocodazole) had little or no activity (Figure 2A). In addition, extracts from a *cdc15–2* mutant arrested in late anaphase did not show efficient ubiquitination of $Clb2ha_3$ (Figure 2B), which is consistent with the finding that *cdc15–2* mutants arrest with an elevated concentration of Clb2 protein and Clb2-associated kinase activity (Surana *et al.*, 1993). The cell cycle dependence of $Clb2ha_3$ ubiquitination in vitro is therefore similar to that of Clb2 proteolysis in vivo.

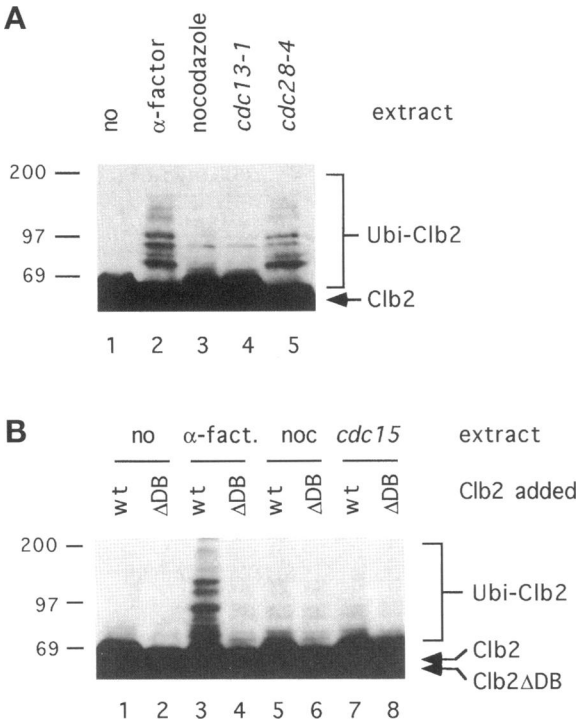

Figure 2. Ubiquitination of Clb2 in vitro is cell cycle regulated. Extracts (30 mg/ml) were prepared from strains arrested at different stages of the cell cycle and incubated with $Clb2ha_3$ or $Clb2\Delta DBha_3$ containing extracts (30 mg/ml). Formation of Clb2-ubiquitin conjugates was analyzed by Western blotting as described in the legend to Figure 1A. (A) Extracts were prepared from a wild-type strain (K1771) arrested in G1 with α-factor or in M-phase with nocodazole. A *cdc13–1* strain (K1736) and a *cdc28–4* strain (K1719) were grown at 25°C and arrested in G2 or in G1, respectively, by shifting the cultures to 37°C. (B) Extracts were prepared from a wild-type strain arrested with α-factor or nocodazole and from a *cdc15–2* mutant (K1728) grown at 25°C and arrested in late anaphase by shifting the culture to 37°C.

Genes Needed for Ubiquitination of Mitotic Cyclins

Irniger *et al.* (1995) have identified three genes, *CDC16*, *CDC23*, and *CSE1*, which are required for Clb2 proteolysis during G1. Ectopically expressed Clb2 accumulates in G1-arrested cells even at the permissive temperature in *cdc23–1* and *cse1–22* mutants but only at the restrictive temperature in *cdc16–123* mutants. To address whether the defective Clb2 proteolysis in *cdc16*, *cdc23*, and *cse1* mutants could be due to defective ubiquitination, we prepared extracts from mutant and wild-type cells after pheromone-induced G1 arrest. Cells were grown and subsequently treated with pheromone at 25°C, the mutant's permissive temperature. Each arrest was checked by flow cytometric analysis (Figure 3D). Destruction box–dependent ubiquitination of $Clb2ha_3$ was greatly reduced in extracts prepared from *cdc16–123*, *cdc23–1*, and *cse1–22* mutants, even when the reactions were performed at 25°C (Figure 3, A–C). The *cdc23–1* and *cse1–22* mutations caused a stronger defect than the *cdc16–123* mutation when extracts were prepared from G1-arrested cells kept at 25°C. The residual activity of the *cdc16–123* extract was reduced to background levels by a short incubation (30 min) of the arrested cells at 37°C. Flow cytometric analysis confirmed that cells remained arrested in G1 (our unpublished results). These data are consistent with the finding that Clb2 proteolysis is temperature sensitive in *cdc16–123* mutants whereas it is already impaired at the permissive temperature in *cdc23–1* and *cse1–22* mutants (Irniger *et al.*, 1995).

CDC16 and *CDC23* encode proteins that form a complex with another TPR containing protein encoded by *CDC27* (Lamb *et al.*, 1994). The *cdc27–1* mutation also reduced ubiquitination of $Clb2ha_3$ but to a lesser extent than the other mutations (Figure 4). Ubiquitination in vitro is more severely affected by the *cdc27–1* mutation than Clb2 proteolysis in vivo, which is barely if at all reduced. The residual ubiquitination activity in a *cdc27–1* mutant might be sufficient to prevent accumulation of Clb2 to detectable levels. These data are consistent with the notion that Clb2 proteolysis depends on destruction box–dependent ubiquitination of Clb2 mediated by the the Cdc16–23-27 complex.

With respect to primary sequence and expression pattern the four mitotic cyclins of budding yeast form two pairs: Clb1,2 and Clb3,4. Clb3,4 appear earlier in the cell cycle than Clb1,2 and all disappear at the end of mitosis (Grandin and Reed, 1993). As shown in Figure 5, a version of Clb3 containing a triple ha tag at its C-terminus is strongly ubiquitinated by an extract from G1-arrested wild-type cells. Extracts from G1-arrested *cdc16–123*, *cdc23–1*, *cdc27–1*, and *cse1–22* mutants were clearly defective in ubiquitination of $Clb3ha_3$. These data are consistent with the notion that

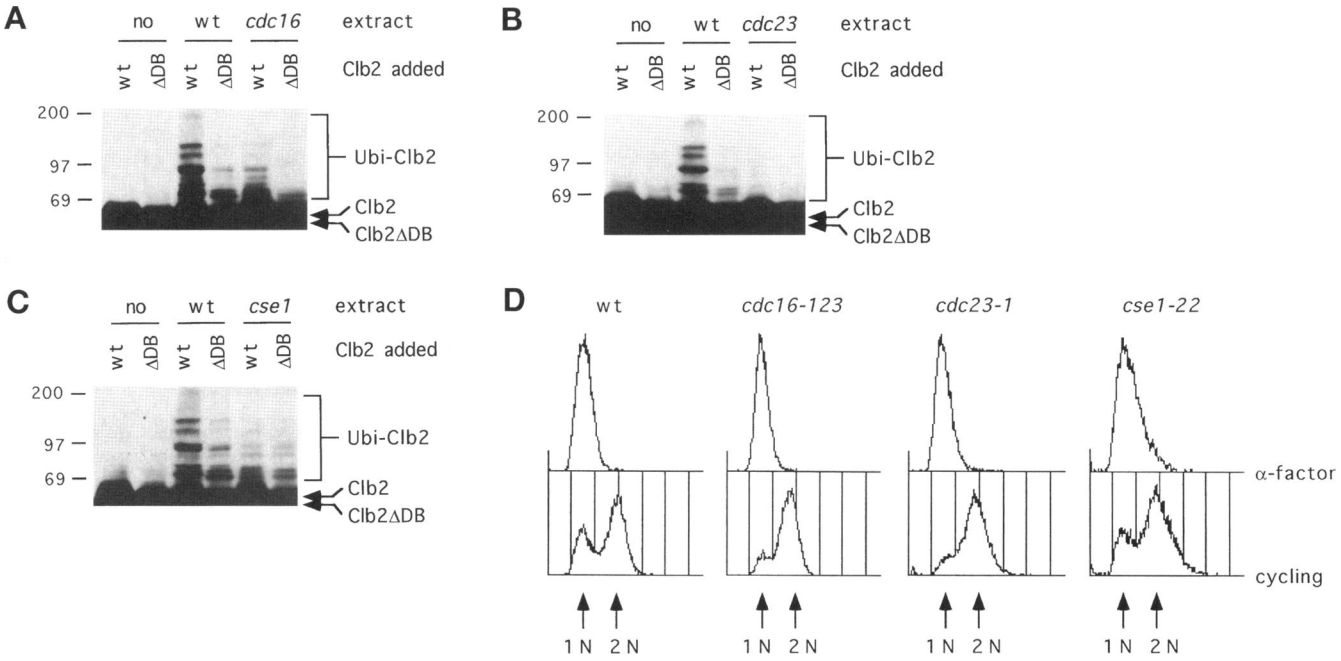

Figure 3. Mutations that lead to accumulation of Clb2 in G1 cells cause a defect in ubiquitination of Clb2 in vitro. Wild-type and mutant strains were grown at 25°C in YEPP$_i$raff medium and arrested in G1 with α-factor. Extracts (35 mg/ml) were prepared and incubated with Clb2ha$_3$ (wt) or Clb2ΔDBha$_3$ (ΔDB) containing extracts (35 mg/ml) as described in the legend to Figure 1A. Western analysis of reactions incubated at room temperature are shown. Reactions incubated at 30°C or 37°C gave similar results. Extracts from the following strains were compared: (A) wild-type (K5517) and *cdc16–123* mutant (K5519); (B) wild-type (K1771) and *cdc23–1* mutant (K5520); and (C) wild-type (K1771) and *cse1–22* mutant (K5521). (D) DNA content was measured by flow cytometric analysis of samples taken before addition of pheromone (cycling) and before harvesting the cells for extract preparation (α-factor). Histograms of fluorescence intensity versus cell number are shown.

ubiquitination and subsequent proteolysis of all four mitotic cyclins depend on the Cdc16–23-27 complex.

In addition to the Cdc16–23-27 complex, entry into anaphase also requires the function of the *CDC20* gene product (Byers and Goetsch, 1974). At the permissive temperature the distribution of DNA content in *cdc20–1* cells is comparable to that of *cdc23–1* cells, indicating a defect in cell cycle progression through the G2/M-phase (Figure 6B). In contrast to the *cdc23–1* mutant the level of Clb2 ubiquitination in extracts from G1-arrested *cdc20–1* cells was similar to that found in wild-type extracts (Figure 6A). Clb2 ubiquitination in G1 extracts was also not affected by a mutation in the *CDC15* gene (*cdc15–2*), which encodes a protein kinase (Schweitzer and Philippsen, 1991) required for dismantling mitotic spindles and reentry into a new cell cycle (our unpublished results).

Recombinant human Ubc4 can ubiquitinate cyclin B in an in vitro system prepared from frog eggs (King *et al.*, 1995). We tested extracts from G1-arrested cells containing a deletion of the *UBC4* or the related *UBC5* gene (Seufert and Jentsch, 1990) and found no effect on Clb2 ubiquitination. Likewise Clb2 ubiquitination was not impaired in extracts from a temperature-sensitive *ubc9* mutant arrested in G1 and shifted to 37°C for 3.5 h (our unpublished results).

DISCUSSION

Until recently, the argument that B-type cyclin proteolysis depended on ubiquitination rested mainly on the observation that both processes were similarly reduced by deletion or mutation of cyclin destruction box sequences. Inhibition of proteolysis by high levels of methylated ubiquitin (Hershko *et al.*, 1991) could be explained by indirect effects. Recent genetic analysis has identified three genes, *CDC16*, *CDC23*, and *CSE1*, which are specifically required for proteolysis of the Clb2 B-type cyclin in yeast. Cdc16 and Cdc23 are composed of TPR motifs and form a complex with a third TPR-containing protein, the CDC27 gene product. All three proteins are required for the metaphase to anaphase transition. To test whether these genes are involved in ubiquitination, we prepared cell-free extracts from yeast, which are capable of ubiquitinating exogenously added Clb2 protein. Ubiquitin-conjugating activity dependent on Clb2's destruction box was cell cycle regulated, being high in extracts from G1-

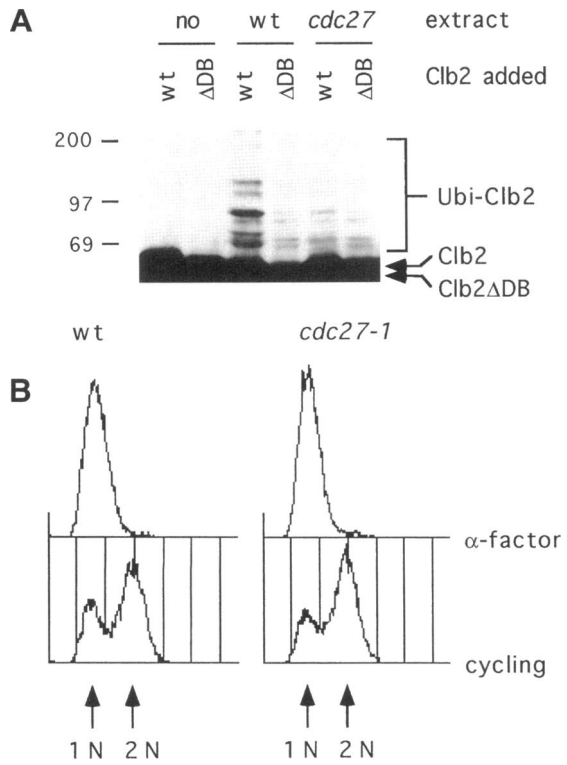

Figure 4. Ubiquitination of Clb2 is reduced in a G1 extract from a *cdc27–1* mutant. A wild-type strain (K5518) and a congenic *cdc27–1* mutant (K5522) were grown at 25°C in YEPP$_i$raff medium and arrested in G1 with α-factor. (A) Extracts (45 mg/ml) were prepared and incubated with Clb2ha$_3$ (wt) or Clb2ΔDBha$_3$ (ΔDB) containing extracts (40 mg/ml) as described in Figure 1A. The Western analysis of reactions incubated at room temperature is shown. (B) Flow cytometric analysis of cells taken before pheromone addition (cycling) and before harvesting (α-factor).

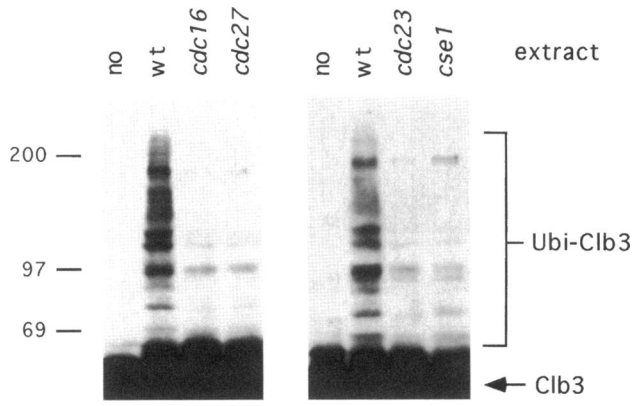

Figure 5. Ubiquitination of Clb3 in vitro requires Cdc16, Cdc23, Cdc27, and Cse1. Wild-type and mutant strains were grown at 24°C in YEPP$_i$raff medium and arrested in G1 with α-factor. G1 arrest was confirmed by flow cytometric analysis (not shown but similar to Figure 3D and 4B). Extracts (45 mg/ml) were prepared and incubated with a small amount of an extract (45 mg/ml) from a strain overexpressing Clb3ha$_3$. Ubiquitination of Clb3 was analyzed by Western blotting as described in legend to Figure 1A. In the experiment shown in the left panel extracts from wild type (K1771), *cdc16–123* (K5519), and *cdc27–1* (K5522) were compared. The right panel shows a comparison of extracts from wild type (K1771), *cdc23–1* (K5520), and *cse1–22* (5521).

arrested cells and low in extracts from G2- or M-phase–arrested cells. Furthermore, it was reduced, in some cases severely, in extracts prepared from G1-arrested *cdc16*, *cdc23*, *cdc27*, and *cse1* mutants. G1 extracts from these mutants were also defective in ubiquitination of the mitotic cyclin Clb3. Thus, all three genes, identified solely on the basis of mutants having Clb2 proteolysis defects, are also required for Clb2 and Clb3 ubiquitination. These data possibly constitute the strongest evidence yet that cyclin B proteolysis in vivo depends on ubiquitination.

What might be the ubiquitination function of the Cdc16–23-27 TPR proteins, which presumably act as a complex? Sudakin *et al.* (1995) have recently characterized a ubiquitin-protein ligase activity associated with a 20S particle they called the "cyclosome," which in conjunction with a ubiquitin-activating enzyme and a ubiquitin-conjugating enzyme, promotes ubiquitination of cyclin A and B in clam oocyte extracts. While our work was in progress, King *et al.* (1995) reported the isolation from mitotic frog egg extracts of a 20S particle with a similar activity to the cyclosome, which contains *Xenopus* homologues of the Cdc16 and Cdc27 proteins. This particle has been called the APC to take in account the role of Cdc16 and Cdc27 in promoting the metaphase to anaphase transition. Unlike the E1 and E2 enzymes, the activities of both the clam cyclosome and the frog APC are cell cycle regulated. They are inactive when isolated from interphase extracts but active when isolated from extracts arrested in a late stage of mitosis by nondegradable B-type cyclins. It is likely, but not yet proven, that the cyclosome, the frog APC, and the Cdc16–23-27 complex from yeast are equivalent or homologous complexes, which are necessary for the division of all eukaryotic cells. Preliminary characterization of the APC from frogs suggests that there may be several other components besides the TPR containing Cdc16 and Cdc27 proteins. Our data along with that from Lamb *et al.* (1994) suggest that Cdc23 is also a component. E3 enzymes are thought to provide specificity by binding both E2 enzyme and its target protein. Destruction boxes might be necessary for the recognition of cyclins by the E3-like APC.

Whether Cse1 is also a component of the APC or required for its correct assembly or just needed for its activation are important questions for the future. A cold-sensitive *cse1–1* mutant is suppressed by multiple copies of the *SRP1* gene that encodes a yeast homologue of the vertebrate nuclear import receptor called importin (Xiao *et al.*, 1994; Adam, 1995). A *srp1–31* mutant was shown to be defective in Clb2

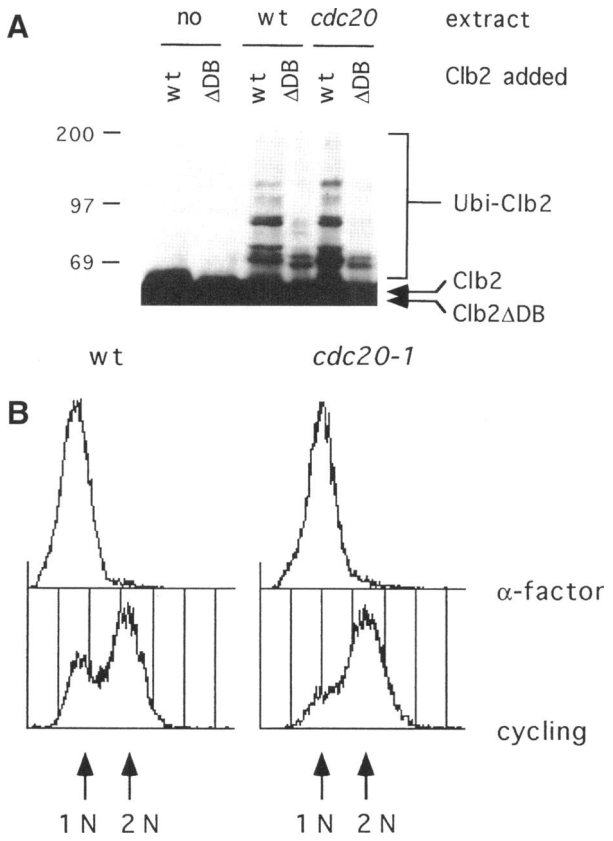

Figure 6. Clb2 ubiquitination in a G1 extract is not affected by the *cdc20–1* mutation. A wild-type strain (K1771) and a congenic *cdc20–1* mutant (K5575) were grown at 24°C in $YEPP_{i}raff$ medium and arrested in G1 with α-factor. (A) Extracts (33 mg/ml) prepared from these cells were incubated with $Clb2ha_3$ (wt) or $Clb2\Delta DBha_3$ (ΔDB) containing extracts (35 mg/ml) as described (see Figure 1A). The Western analysis of reactions incubated at 25°C is shown; similar results were obtained with reactions incubated at 30°C. (B) Flow cytometric analysis of cells taken before pheromone addition (cycling) and before harvesting (α-factor).

proteolysis in G1 cells (Loeb *et al.*, 1995). However, Clb2 (and Clb3) ubiquitination in extracts from G1-arrested *srp1–31* mutant cells shifted to 37°C was not impaired (our unpublished results). Srp1 might be required for a step in Clb2 proteolysis different from ubiquitination. Alternatively, Srp1 function might be dispensable for APC activity in a cell-free extract.

It is presently unclear which E2 enzyme is responsible for conjugating ubiquitin to cyclin. In frog egg extracts two E2s were found, one of which reacts with antibodies to the human homologue of yeast Ubc4 (King *et al.*, 1995). This is consistent with the finding that deletion of *UBC4* is lethal in *cdc23–1* mutants grown at their permissive temperature (Irniger *et al.*, 1995). On the other hand, neither deletion of *UBC4* nor deletion of the closely related *UBC5* gene had any effect on Clb2 ubiquitination in G1 extracts. It is possible that either gene product is sufficient. We could not test the phenotype of double mutants because they are lethal in our strain background (W303) or grow so slowly in others that it might not be possible to obtain reliable results. Conditional alleles will probably be required to address this issue. In clam extracts, a cyclin-specific E2 enzyme called E2-C was found that cannot be replaced by recombinant Ubc4 (Hershko *et al.*, 1994; Sudakin *et al.*, 1995). A mutation in the *UBC9* gene leading to a growth defect at 37°C did not affect Clb2 ubiquitination in extracts from G1 cells.

The anaphase arrest of *cdc16*, *cdc23*, and *cdc27* mutants cannot be easily explained by defects in the proteolysis of cyclins alone. For example, expression of nondegradable Clb2 in yeast inhibits not anaphase but the subsequent disassembly of the mitotic spindle. It has therefore been proposed that the APC promotes ubiquitination not only of cyclins but also that of other proteins whose destruction is needed for the onset of anaphase. TPR proteins have been implicated in specific protein-protein interactions (Tzamarias and Struhl, 1995) and it is possible that different substrates are recognized via different TPR motifs.

Activation of the APC, at least toward proteins (yet to be discovered) that inhibit sister chromatid separation, could be a key step in the onset of anaphase. How this process is controlled is therefore an important question. Several pieces of evidence suggest that prior activation of cyclin B-Cdk1 kinases might be a key step (Felix *et al.*, 1990), but this is neither necessary for maintaining APC activity during G1 in yeast (Amon *et al.*, 1994; Irniger *et al.*, 1995) nor is it sufficient for activating the APC when the mitotic spindle is damaged. Most cells use surveillance mechanisms called checkpoint controls to prevent the onset of anaphase when the mitotic spindle has not been properly assembled. Microtubule depolymerizing agents like nocodazole cause cells to arrest in a state with stable B-type cyclins and high Cdc2/Cdc28 kinase activity. Several genes have recently been identified that are required for this cell cycle arrest (Hoyt *et al.*, 1991; Li and Murray, 1991). It is tempting to speculate that the mitotic checkpoint genes or even those that register DNA damage, function at least partly by preventing activation of the APC. This new major cell cycle player may turn out not only to be a highly conserved component of eukaryotic cells but also to have many key roles in regulating their cell cycles.

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