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Characterization of a Dominant Negative Mutant of the Cell Cycle Ubiquitin-conjugating Enzyme Cdc34*

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The yeast *Saccharomyces cerevisiae CDC34* gene encodes a ubiquitin-conjugating enzyme that is required for the cell cycle G_1/S transition. We show here that a dominant negative Cdc34 protein is generated by simultaneously replacing both Cys^{95} and Leu^{99} with Ser residues. Cys^{95} is an essential catalytic residue that forms a transient thiol ester with ubiquitin during catalysis, and Leu^{99} is highly conserved among all known ubiquitin-conjugating enzymes. Mutants that encode either an alanine or a serine at one or both of these two positions are inactive. Of these eight mutants, overexpression of *CDC34-C95S,L99S* in wild type strains was found to block cell growth. Although cells overexpressing Cdc34-C95S,L99S do not exhibit the characteristic multibudded phenotype of *cdc34* temperature-sensitive or null mutants, this blockade is relieved by simultaneous overexpression of wild type Cdc34. Purified Cdc34-C95S,L99S protein can be shown to inhibit *in vitro* ubiquitination of the Cdc34-specific substrate, Cln2 protein. We suggest that Cdc34-C95S,L99S selectively sequesters a subset of Cdc34 substrates or regulators. These findings have implications for the structure/function relationships of ubiquitin-conjugating enzymes, and suggest a general method for identifying components and substrates of specific ubiquitination pathways of eukaryotes.

The ubiquitin-conjugating enzymes (*E*2)[1] constitute a family of conserved proteins that participate either in an intermediate or in the final step of substrate ubiquitination (Hershko and Ciechanover, 1992; Finley and Chau, 1991). These enzymes form a thiol ester adduct with ubiquitin (Ub) in the presence of ubiquitin-activating enzyme (*E*1) and ATP in the following reactions: 1) $E1_{SH} + Ub + ATP \leftrightarrow E1_{S\text{-}Ub} + AMP + PP_i$, and 2) $E1_{S\text{-}Ub} + E2_{SH} \leftrightarrow E1_{SH} + E2_{S\text{-}Ub}$.

Substrate proteins may be directly recognized by an individual *E*2 enzyme, resulting in the transfer of ubiquitin from an $E2_{S\text{-}Ub}$ to a lysine on substrate proteins. Alternatively, substrate recognition may require the presence of another group of proteins known as *E*3 or ubiquitin-protein ligases (Reiss and Hershko, 1990; Bartel *et al.*, 1990). A requirement for a specific *E*3 protein had been shown for the degradation of substrates in the N-end rule pathway (Bartel *et al.*, 1990) and for p53 (Scheffner *et al.*, 1993). In the N-end rule pathway, the Ubr1 (*E*3) protein contains separate sites for Rad6 (*E*2) and substrate bindings and confers specificity for one of the cellular Rad6-dependent ubiquitination pathways (Varshavsky, 1992). In the p53 degradation pathway, it has been further shown that ubiquitin from $E2_{S\text{-}Ub}$ is transferred to a cysteine in the *E*3 protein (Scheffner *et al.*, 1995), leading to the formation of an $E3_{S\text{-}Ub}$ thiol ester.

CDC34 is one of 10 known ubiquitin-conjugating enzyme-encoding genes in the yeast, *S. cerevisiae* (Goebl *et al.*, 1988; Jentsch, 1992). This gene was initially identified on the basis of its requirement for cells to undergo the cell cycle G_1 to S transition (Byers and Goetsch, 1973). Under nonpermissive conditions, temperature-sensitive mutants of *CDC34* develop numerous elongated buds, and the spindle pole body duplicates but fails to undergo the separation required for spindle formation (Byers and Goetsch, 1973). More recent studies have established a direct role of this ubiquitin-conjugating enzyme in targeting the degradation of specific regulators of the cell cycle. Known Cdc34-specific substrates in this category include the G_1 cyclins (Deshaies *et al.*, 1995; Yaglom *et al.*, 1995) and the Cdc28 kinase inhibitor Sic1 (Schwob *et al.*, 1994). In addition, mutations in the *CDC34* gene can lead indirectly to the abnormal accumulation of other cell cycle regulators such as the G_2-specific B-type cyclins (Amon *et al.*, 1994). Other than its cell cycle function, the Cdc34-encoded ubiquitin-conjugating enzyme has also been shown to target the degradation of the transcription factor GCN4 (Kornitzer *et al.*, 1994), and it is likely that other functions of this enzyme may be uncovered by the identification of additional substrate proteins.

The 295-residue Cdc34 protein contains a 170-residue N-terminal domain that is conserved among all *E*2 proteins. This conserved domain is apparently sufficient for $E2_{S\text{-}Ub}$ complex formation since the smallest *E*2 enzymes are comprised almost exclusively of this domain (Jentsch, 1992). In Cdc34 this conserved domain also contains an extra 12-residue sequence near the ubiquitin-accepting cysteine. This extra sequence is found only in one other yeast *E*2 protein, Ubc7 (Jungmann *et al.*, 1993), and in both cases, the function of this extra sequence segment remains undefined. In the present study, we report the effect of mutations at the ubiquitin-accepting cysteine as well as at Leu^{99}, a residue that is adjacent to this 12-residue segment. We show here that while both residues are essential for *CDC34* functions, a unique dominant negative allele of this gene, $CDC34^{DN}$, could be generated by simultaneously substituting these two residues with serines. In addition to its potential utility in genetic analysis, $CDC34^{DN}$ can be used to block Cdc34-dependent ubiquitination *in vitro*.

EXPERIMENTAL PROCEDURES

Materials—Ubiquitin was purchased from Sigma. The ^{125}I-labeled form of ubiquitin was obtained by radioiodination with the use of

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[1] The abbreviations used are: *E*2, ubiquitin-conjugating enzyme; *E*1, ubiquitin-activating enzyme; *E*3, ubiquitin protein ligase; PAGE, polyacrylamide gel electrophoresis; Ub, ubiquitin.

TABLE I
Bacterial phages and plasmids

Plasmid/phage	Genotype/description	Source/reference
M13mp19/M13mp18	Cloning vectors	Yanisch-Perron *et al.* (1985)
M13mp19*CDC34*	*CDC34* with *lac* promoter	Banerjee *et al.* (1993)
M13mp19*CDC34-C95S,L99S*	*CDC34-C95S,L99S* with *lac* promoter	This study
M13mp19*cdc34-L99S*	*cdc34-L99S* with *lac* promoter	This study
M13mp19*cdc34-L99A*	*cdc34-L99A* with *lac* promoter	This study
M13mp18*cdc34-C95S*	*cdc34-C95S* with *lac* promoter	This study, derivative of plasmid pYLC95S from M. Goebl
M13mp19*cdc34-C95S,L99A*	*cdc34-C95S,L99A* with *lac* promoter	This study
M13mp19*cdc34-C95A*	*cdc34-C95A* with *lac* promoter	This study
M13mp19*cdc34-C95A,L99S*	*cdc34-C95A,L99S* with *lac* promoter	This study
M13mp19*cdc34-C95A,L99A*	*cdc34-C95A,L99A* with *lac* promoter	This study
pNM*CDC34*	*CDC34* with λpL promoter	Banerjee *et al.* (1993)
pLλ*CDC34-C95S,L99S*	*CDC34-C95S,L99S* with λpL promoter	This study
pRD68	*CLN2* with T7 promoter	Deshaies *et al.* 1995

Iodogen (Pierce) according to the manufacturer's procedure. The specific activity of ^{125}I-ubiquitin was 5×10^5 cpm/μg. The ubiquitin-activating enzyme (*E*1) was purified to homogeneity from calf thymus as described previously (Ciechanover *et al.*, 1982) and was stored at −80 °C in 20% glycerol. *E*1 and Cdc34 concentrations were determined by first converting the proteins to $E1_{S\text{-}Ub}$ and $Cdc34_{S\text{-}Ub}$, and the concentrations of these thiol esters were estimated from the known specific radioactivity in [^{125}I]ubiquitin.

Strains of Bacteria and Yeast, Plasmid Vectors, and Genetic Techniques—The bacterial plasmids and phage strains used in this work are listed in Table I. The *S. cerevisiae* strains are listed in Table II. All DNA manipulations were done according to Sambrook *et al.* (1989). Plasmid vectors that expressed Cdc34 from the galactose-inducible P_{GAL1} promoter were constructed using the vectors YEplac195 and YIplac211 (Gietz and Sugino, 1988) by isolating a ~0.7-kilobase *Bam*HI-*Eco*RI fragment of pG12 that contained the $P_{GAL1/GAL10}$ promoter region (Johnston and Davis, 1984) and ligating the fragment into the high copy (2μ-based) plasmids YEplac195 and YEplac181, and integrating plasmid YIplac211, yielding plasmid vectors YEp195GAL, YEp181GAL, and YIp211GAL. Fragments of ~0.9 kilobase encoding *CDC34* and its mutants (without the native promoter) bordered by *Sal*I and *Sph*I sites (Banerjee *et al.*, 1993) were then ligated into the *Sal*I- and *Sph*I-cut YEp195GAL and YIp211GAL, yielding various constructs used in this study. *CDC34* was also cloned into the YEp181GAL vector. Proper induction by galactose of Cdc34 and mutant proteins for each of the yeast strains integrated/transformed with these plasmids was checked by immunoblotting with anti-Cdc34 polyclonal antibodies.

Growth and Induction of Yeast Strains—The *S. cerevisiae* strains used in this work were grown at 30 °C (332 derivatives) or 23 °C (MGG15 derivatives) in rich (YPD) or synthetic media (Sherman *et al.*, 1986), with the latter containing 2% dextrose (SD medium), 2% raffinose (SR medium), or 2% galactose (SG medium). Transformation/integration of *S. cerevisiae* was carried out by the methods of Sherman *et al.* (1986) or Shiestel and Gietz (1989). For galactose induction of yeast strains, cells were first grown to A_{600} of ~0.1 in SR medium lacking uracil (and relevant amino acids when necessary), and galactose was added to a final concentration of 2%. Samples were withdrawn at time points described under "Results" and processed.

Construction of CDC34 Mutants—The *CDC34* mutants were constructed by site-directed mutagenesis of the *CDC34* gene in M13 mp19 (Banerjee *et al.*, 1993). The Cys^{95} TGT codon was changed to either a TCT for Ser or a GCT for Ala. The Leu^{99} TTA codon was changed to either a TCA for Ser or a GCA for Ala. These changes were made by the procedure of site-specific mutagenesis (Taylor *et al.*, 1985) with an Amersham mutagenesis kit. DNA sequencing by the chain termination method (Sanger *et al.*, 1977) was used to verify all constructs. The *CDC34-C95S,L99S* sequence was also inserted into the λP_L-promoter-based plasmid as described previously for the wild type *CDC34* gene (Banerjee *et al.*, 1993), resulting in the plasmid pLλ*CDC34-C95S,L99S*.

Expression of Cdc34, Cdc34-C95S,L99S, and Rad6 Proteins in Escherichia coli and Their Purification—The plasmids pNM*CDC34* (Banerjee *et al.*, 1993) and pLλ*CDC34-C95S,L99S* were transformed into *E. coli* host AR58, which contains a temperature-sensitive λ repressor. These cells, harboring pNM*CDC34* and pLλ*CDC34-C95S,L99S* plasmids, were grown at 30 °C to a density of 1 absorbance unit at 600 nm. The culture (2 liters) was shifted to 42 °C for 2 h and incubated for an additional 3 h at 39 °C. An SDS gel analysis of proteins in crude *E. coli* extracts revealed the induction of Cdc34 as a major protein. The proteins were purified according to previously published protocols (Banerjee *et al.*, 1993). Rad6 was purified from *E. coli* extracts that had the protein overexpressed from the *RAD6* gene cloned in pKK223–3 vector (Haas *et al.*, 1991).

Expression of M13 mp19-CDC34 Mutant Genes—To express Cdc34 mutant proteins, individual mutant genes in M13 mp19/18 were used to infect a 1:500 dilution of an overnight culture of *E. coli* host TG1 in LB to obtain a multiplicity of infection of 30–40. After 3 h of growth at 37 °C, the medium was adjusted to contain 1 mM isopropyl-1-thio-β-D-galactopyranoside, and incubation was continued for an additional 2 h. Cells from 3-ml cultures were harvested by centrifugation, washed with 50 mM Tris (pH 7.5), and suspended in 100 μl of the same buffer and 0.5 mM dithiothreitol. Cells were lysed by sonication for 30 s at 30 watts, and after 5 min of centrifugation at 12,000 rpm in an Eppendorf centrifuge, the supernatant was used to assay for ubiquitin thiol ester and conjugate formation in a final reaction volume of 20 μl using standard reaction conditions. The amounts of Cdc34 and mutant proteins were determined and normalized by immunoblotting using the ECL (Amersham Corp.) method of detection. Incubation times of 15 and 45 min were used in thiol ester and Ub-Cdc34 complex formation, respectively.

$Cdc34_{S\text{-}Ub}$ and Ub-Cdc34 Complex Formation—Reactions were carried out in 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 2 mM ATP, 50 μM dithiothreitol, and 5 μM ubiquitin at 30 °C. Unless stated otherwise, 50 nM of *E*1 (from calf thymus) and 100 nM of Cdc34 or its mutant proteins were used in the reactions. The amounts of Cdc34 and mutant Cdc34 proteins in the assay were determined by quantitation with Cdc34-specific antibodies using the Amersham ECL detection method and purified Cdc34 standards. Reactions were stopped by withdrawing aliquots of the reaction mixture into SDS-sample buffer in which β-mercaptoethanol had been omitted. When Ub-Cdc34 complexes were assayed, protein samples were adjusted to contain 5% β-mercaptoethanol, and samples were heated in a boiling water bath for 3 min. Protein samples were subjected to electrophoresis in a 14% SDS-polyacrylamide gel, and autoradiography was used to visualize radiolabeled bands.

Preparation of Polyclonal Anti-Cdc34 Antibodies and Western Blot Analysis—Anti-Cdc34 antiserum was prepared in New Zealand White rabbits using the recombinant Cdc34 protein produced and purified by the method of Banerjee *et al.* (1993). Immunization and antibody processing techniques were done according to the protocols of Harlow and Lane (1988). For visualization and determination of the amount of Cdc34 protein, yeast cells were harvested by centrifugation, washed once with 50 mM Tris-HCl buffer, pH 7.5, 1 mM dithiothreitol, and resuspended in 200 μl of breakage buffer (50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 2 μg/ml pepstatin A). Cells were broken by vortexing with glass beads. A 100-μl aliquot of 3 × PAGE sample buffer (without β-mercaptoethanol) was added, and the mixture was immediately boiled for 3 min. The glass beads and cell debris were removed by centrifugation. A 10-μl aliquot was removed for protein assay by Pierce BCA reagent, and the remainder of the sample was adjusted to contain 5% β-mercaptoethanol. Cell extracts containing equal amounts of protein (300 μg) were electrophoresed on a 15% SDS-PAGE gel (Laemmli, 1970). Proteins were transferred to polyvinylidene difluoride membrane and visualized by reaction with anti-Cdc34 polyclonal antibodies by protocols described by Harlow and Lane (1988). The enhanced chemiluminiscence (ECL) kit was purchased from Amersham Corp. and used according to their instructions. Alkaline phosphatase-conjugated anti-rabbit IgG was purchased from Sigma, and color reaction was done according

TABLE II
S. cerevisiae strains

Name	Relevant markers/plasmids	Source/Comment
332	*Mat***a** *ura3–52 leu2 his3*	R. Needleman
ABY200	*Mat***a** *URA3*::GAL*CDC34 leu2 his3*	This study, derivative of 322
ABY100	*Mat***a** *URA3*::GAL*CDC34-C95S,L99S leu2 his3*	This study, derivative of 322
ABY102	*Mat***a** *URA3*::GAL*CDC34-C95S,L99S leu2 his3* + YEp181GAL*CDC34*	This study derivative of ABY100
ABY110	*Mat***a** *URA3*::GAL*cdc34-L99S leu2 his3*	This study, derivative of 322
ABY120	*Mat***a** *URA3*::GAL*cdc34-L99A leu2 his3*	This study, derivative of 322
ABY130	*Mat***a** *URA3*::GAL*cdc34-C95S leu2 his3*	This study, derivative of 332 and plasmid pYLC95S from M. Goebl
ABY140	*Mat***a** *URA3*::GAL*cdc34-C95S,L99A leu2 his3*	This study, derivative of 322
ABY150	*Mat***a** *URA3*::GAL*cdc34-C95A leu2 his3*	This study, derivative of 322
ABY160	*Mat***a** *URA3*::GAL*cdc34-C95A,L99S leu2 his3*	This study, derivative of 322
ABY170	*Mat***a** *URA3*::GAL*cdc34-C95A,L99A leu2 his3*	This study, derivative of 322
MGG15	*Mat***a** *cdc34–2 ura3 his3*	M. Goebl
ABY210	*Mat***a** *cdc34–2 ura3 his3* + YEp195GAL*CDC34*	This study, derivative of MGG15
ABY212	*Mat***a** *cdc34–2 ura3 his3* + YEp195GAL*CDC34-C95S,L99S*	This study, derivative of MGG15
ABY214	*Mat***a** *cdc34–2 ura3 his3* + YEp195GAL*cdc34-L99S*	This study, derivative of MGG15
ABY216	*Mat***a** *cdc34–2 ura3 his3* + YEp195GAL*cdc34-C95S*	This study, derivative of MGG15
pRD84/RD205–3A	*cln1*Δ*, cln2::LEU2, cln3*Δ*, trp1, leu2, ura3, pep4::LEU2 + [GAL-CLN3, URA3, CEN, ARS]*	Deshaies *et al.* (1995)

to Blake *et al.* (1984).

Analysis of Cln2 Multiubiquitination—Expression of Cln2 *in vitro* was achieved by inserting a *Bam*HI fragment containing the *CLN2* gene into the *Bam*HI site of pGEM2, yielding pRD68, where the *CLN2* gene could be transcribed by the T7 promoter (Deshaies *et al.*, 1995). This RNA was used for *in vitro* translation by the rabbit reticulocyte lysate system. Reagents for the *in vitro* translation were obtained from Promega Biotech. [^{35}S]Cln2 synthesized *in vitro* was incubated in 15 mg/ml pRD84/RD205–3A extract. Reactions were supplemented with ~1 μM Cdc28 (Deshaies *et al.*, 1995) and the indicated amounts of Cdc34 or Cdc34-C95S,L99S, all purified from *E. coli*. Reactions were directly evaluated by SDS-PAGE and fluorography.

RESULTS

Overexpression of Cdc34-C95S,L99S Inhibits Cell Growth—The *S. cerevisiae CDC34* gene encodes a ubiquitin-conjugating enzyme that is required for the cell cycle G_1/S transition (Byers and Goetsch, 1973). The yeast strain MGG15 contains a temperature-sensitive *cdc34–2* allele, and these cells are inviable at nonpermissive temperatures (Goebl *et al.*, 1988). In the course of using MGG15 cells to determine the functional competence of several *CDC34* site-specific mutants, we noted that MGG15 cells failed to form colonies even at permissive temperatures when these cells are also expressing a *CDC34-C95S,L99S* mutant via a galactose-inducible promoter in a 2μ plasmid (Fig. 1*A*). In contrast, similar expression of the two singly substituted mutants, *cdc34-C95S* and *cdc34-L99S*, did not affect the viability of MGG15 cells. *CDC34-C95S,L99S* is likely to encode an inactive protein since the singly substituted mutants, *cdc34-C95S* and *cdc34-L99S*, each failed to complement the temperature-sensitive growth defect of MGG15 cells (data not shown).

To test whether the dominant effect of *CDC34-C95S,L99S* mutant is uniquely dependent on the *cdc34–2* allele, we also introduced this mutant gene into a yeast strain that carries a wild type *CDC34* allele. The ABY100 strain contains an integrated copy of *CDC34-C95S,L99S* whose expression is regulated by the P_{GAL1} promoter (Table II). These cells also failed to grow on galactose medium (Fig. 1*B*) and became inviable (data not shown). These results indicate that the dominant effect of *CDC34-C95S,L99S* on cell growth and viability is not restricted to the *cdc34–2* strain. Similar expression of the singly substituted mutants, *cdc34-C95S* and *cdc34-L99S*, did not alter cell viability (data not shown).

Since the unique effect of the *CDC34-C95S,L99S* mutant may be due to its higher level of accumulation than those of the

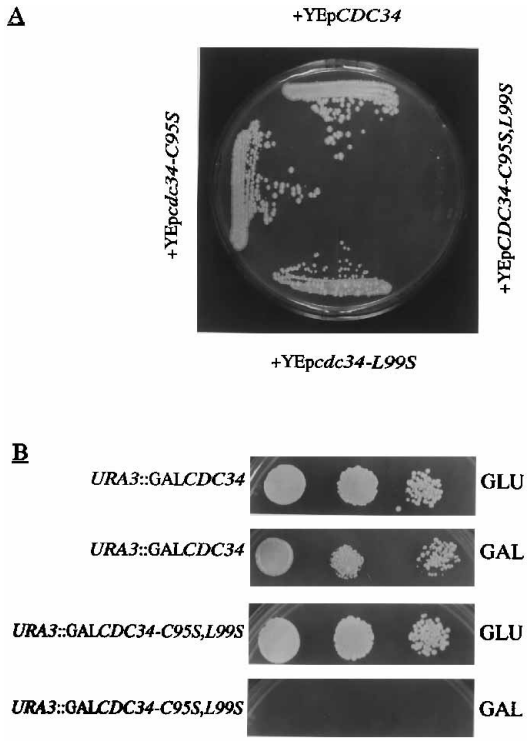

FIG. 1. **Effect of CDC34-C95S,L99S expression in yeast cells.** *Panel A*, effect of *CDC34-C95S,L99S* expression in MGG15 (Table II) cells carrying *ts-cdc34–2* at the permissive temperature of 23 °C. ABY210 (*cdc34–2* + YEpGAL*CDC34*), ABY214 (*cdc34–2* + YEpGAL-*cdc34-L99S*), ABY216 (*cdc34–2* + YEpGAL*cdc34-C95S*), and ABY212 (*cdc34–2* + YEp GAL*CDC34-C95S,L99S* were grown to an A_{600} of 0.2 in SR medium at 23 °C and streaked in duplicate onto medium containing galactose. *Panel B*, the GAL1-*CDC34-C95S,L99S* mutant was integrated into wild type yeast strain 332 (ABY100). Similarly integrated GAL1-*CDC34* resulted in ABY200 (Table II). Spot assays were done to compare the viability of ABY100 and ABY200 cells upon galactose-induced expression of *CDC34* and *CDC34-C95S,L99S* alleles. ~25,000 cells (grown in SR medium to an A_{600} of 0.2, and counted by hemocytometer) were spotted in the first spot on each *panel*. The next two spots in each panel are two successive 10-fold dilutions. Plates were incubated at 30 °C. ABY200 (*URA3*::GAL*CDC34*) spots in galactose medium show no deleterious effect or loss in viability, whereas ABY100 (*URA3*::GAL*CDC34-C95S,L99S*) spots in galactose medium show lethal effect on wild type yeast cells by expression of the *CDC34-C95S,L99S* allele.

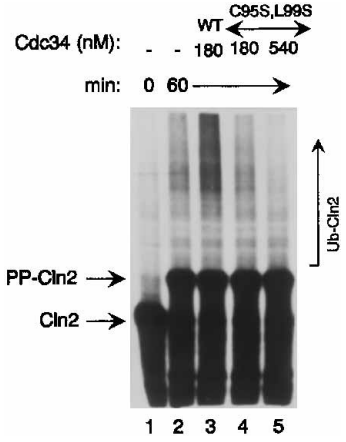

FIG. 2. **Inhibition of Cln2 multiubiquitination by Cdc34-C95S,L99S.** [^{35}S]Cln2 was synthesized *in vitro* in the reticulocyte lysate system and was incubated in 15 mg/ml yeast extract for 0 (*lane 1*) or 60 (remaining *lanes*) min. at 24 °C in the presence of the indicated amounts of Cdc34 or Cdc34-C95S,L99S (see "Experimental Procedures"). *Lane 2* has the reaction incubated without exogenous Cdc34. *Lane 3* has the reaction supplemented with 180 nM of wild type Cdc34. The reaction in *lane 4* had 180 nM, and that in *lane 5* had 540 nM of Cdc34-C95S,L99S added. *Cln2*, unphosphorylated Cln2 with a molecular mass of ~66 kDa; *PP-Cln2*, hyperphosphorylated Cln2 with a molecular mass of ~84 kDa; *Ub-Cln2*, multiubiquitinated Cln2.

singly substituted mutants, *cdc34-C95S* and *cdc34-L99S*, we also used immunoblotting to assess the level of mutant proteins in cells expressing these various mutant genes. Immunoblots of Cdc34 proteins from cells before and after 2 or 8 h of induction in galactose indicated that the Cdc34-C95S,L99S protein did not accumulate to a higher level than the other mutants (data not shown). Thus, the unique effect of Cdc34-C95S,L99S on cell viability is not due simply to the overexpression of an inactive Cdc34 protein.

CDC34-C95S,L99S Acts on the Same Pathway as CDC34—Immunoblots, probed with ubiquitin-specific antibodies, showed that the expression of *CDC34-C95S,L99S*, *cdc34-C95S*, and *cdc34-L99S* mutants did not cause detectable changes in the overall level of ubiquitin-protein conjugates (data not shown). However, two lines of evidence indicated that *CDC34-C95S,L99S* affects cell viability by disruption of *CDC34*-dependent protein ubiquitination. First, the effect of *CDC34-C95S,L99S* in ABY100 cells was suppressed by the concomitant expression of wild type *CDC34* via either its natural promoter or a galactose-inducible promoter in 2μ plasmids (data not shown). Similar plasmids carrying either the singly substituted *cdc34-C95S* or *cdc34-L99S* mutant did not restore growth of ABY100 cells on galactose medium (data not shown). Second, purified Cdc34-C95S,L99S protein inhibited the *in vitro* ubiquitination of the G_1 cyclin Cln2, a reaction that has recently been shown to require Cdc34 (Deshaies *et al.*, 1995). Yeast extract derived from wild type *CDC34* cells catalyzes the ubiquitination of Cln2 obtained by *in vitro* translation in reticulocyte lysate (Deshaies *et al.*, 1995; Fig. 2, *lane 2*). This reaction could be further stimulated by the addition of purified Cdc34 protein (Fig. 2, *lane 3*). Ubiquitination of this substrate was inhibited by additions of purified Cdc34-C95S,L99S protein in a dose-dependent manner (Fig. 2, *lanes 4* and *5*).

The inhibition of Cln2 protein ubiquitination is not due to general inactivation of ubiquitin conjugation pathways since the overexpression of this mutant protein did not affect ubiquitin conjugation to other endogenous proteins (Fig. 3*A*). We have also assayed the effect of Cdc34-C95S,L99S on purified ubiquitin-activating enzyme by monitoring the catalytic transfer of ubiquitin from the ubiquitin-activating enzyme to another yeast ubiquitin-conjugating enzyme, Rad6 (Fig. 3*B*). As

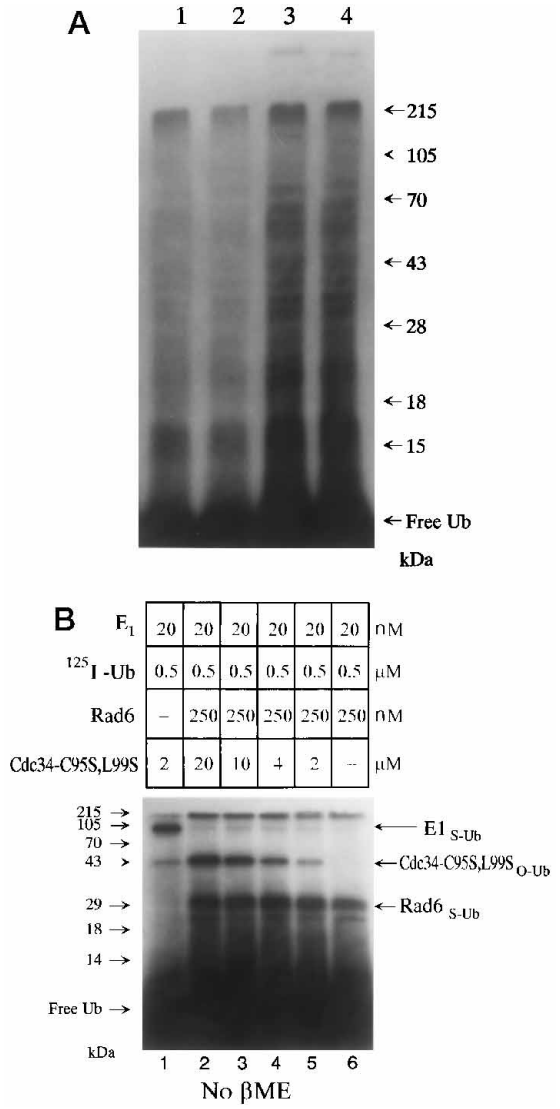

FIG. 3. **Ubiquitin-activating enzyme is not affected by overexpression of the CDC34-C95S,L99S.** *Panel A*, extracts were prepared from 10-h galactose-induced ABY200 (*URA3*::GAL*CDC34*), and ABY100 (*URA3*::GAL*CDC34-C95S,L99S*) in breakage buffer; ~300 μg of total protein in 60 μl volume was supplemented with 200 ng of ^{125}I-Ub and incubated at 30 °C for 30 min. 30 μl of 3 × SDS-PAGE sample buffer was added, samples were boiled for 3 min, and the reactions were analyzed by 14% Laemmli gels. *Lanes 1* and *3*, ABY100 reaction at 10 and 30 min; *lanes 2* and *4*, ABY200 reaction at the same time points. The radioactive protein ladder indicating ubiquitination of yeast proteins is similar in intensity and pattern for extracts from both of these cell types. The positions of Life Technologies, Inc. prestained high molecular mass markers (myosin, 215 kDa; phosphorylase B, 105 kDa; bovine serum albumin, 70 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 29 kDa; β-lactoglobulin, 18 kDa; and lysozyme, 14 kDa) are marked on the *left*. *Panel B*, formation of $Rad6_{S\text{-}Ub}$ thiol ester complexes was assayed by incubation with 250 nM purified Rad6 protein and indicated amounts of Cdc34-C95S,L99S protein with ubiquitin-activating enzyme (*E*1), magnesium, ATP, and ^{125}I-ubiquitin for 20 min at 30 °C. The reaction products were electrophoresed in a 14% SDS gel in the absence of thiol-reducing agents, and radiolabeled proteins were visualized by autoradiography.

shown in Fig. 3*B*, the level of ubiquitin-Rad6 thiol ester was not detectably affected by 2–20 μM of Cdc34-C95S,L99S. These results, taken together, indicate that Cdc34-C95S,L99S inhibits Cln2 ubiquitination via specific inhibition of the Cdc34-dependent pathway.

While the above results indicated that *CDC34-C95S,L99S* exerts its effect by interfering with an essential *CDC34*-dependent process, cells overexpressing this mutant did not ex-

hibit the morphological phenotype of previously characterized loss-of-function mutants. The *cdc34* null mutant strain, as well as the temperature-sensitive *cdc34–1* and *cdc34–2* strains arrest as multibudded cells (Goebl *et al.*, 1988). This morphology is absent in ABY100 cells that assume aberrant morphology after switching cells to a galactose-containing medium. A majority of these aberrant cells were found to have a single elongated bud (data not shown). The absence of multibudded cells is not due to a strain difference since MGG15 cells that are overexpressing Cdc34-C95S,L99S also took on, at both permissive and nonpermissive temperatures for *cdc34–2*, morphologies similar to those of ABY100 cells in galactose (data not shown). This difference in morphology is consistent with the notion that Cdc34-C95S,L99S does not simply inactivate endogenous wild type Cdc34 protein. Possible mechanisms of Cdc34-C95S,L99S action are described further under "Discussion."

The Role of Cys^{95} *and Leu*^{99} *in Cdc34-C99S,L99S*—Since neither *cdc34-C95S* nor *cdc34-L99S* affects cell viability, it appears that both mutations must be present to confer the dominant negative phenotype. Cys^{95} is expected to be an essential residue since it is the only cysteine in the Cdc34 protein, and the presence of this amino acid is required for all ubiquitin-conjugating enzymes to form a thiol ester complex with ubiquitin. Leu^{99} is a highly conserved residue among ubiquitin-conjugating enzymes, and the inability of *cdc34-L99S* to complement *cdc34–2* suggests that this residue is also essential. We have also generated a set of mutants in which Cys^{95} and Leu^{99} were replaced by alanines or by alanine/serine pairs. None of these mutants was able to complement *cdc34–2* (data not shown), suggesting that these two residues are indeed essential. We also constructed yeast strains with wild type *CDC34* that had an integrated copy of these mutant genes under the control of a P_{GAL1} promoter (ABY110, ABY120, ABY130, ABY140, ABY150, ABY160, and ABY170 in Table II) and tested their effect on cell growth. Unlike *CDC34-C95S,L99S*, none of these mutants was found to block cell growth (data not shown). These results suggest that the dominant negative effect may have a stringent requirement for serines at these two residue positions.

The requirement for a serine at the Cys^{95} residue may be due to the unique ability of a serine to form a more stable linkage with ubiquitin (Sung *et al.*, 1991). In a normal ubiquitin-conjugating enzyme, the active site cysteine forms a thiol ester linkage with the C-terminal carboxyl of ubiquitin. This thiol ester-linked ubiquitin is subsequently transferred either to a *E*3 protein or to substrates directly. Substitution of the active site cysteine by a serine has been shown to inactivate other ubiquitin-conjugating enzymes (Sung *et al.*, 1991; Seufert *et al.*, 1995) presumably because the more stable oxygen ester-linked ubiquitin is not further transferred. In the case of Cdc34, we have previously shown that the thiol ester-linked ubiquitin can also be transferred to a lysine within this enzyme in an intramolecular reaction to form a Lys^{48}-specific multiubiquitin chain (Banerjee *et al.*, 1993). This autoubiquitination reaction was used here to test the effect of Cys^{95} substitution. For these experiments, the *cdc34* mutants were expressed in *E. coli* and assayed for their ability to accept [^{125}I]ubiquitin in the presence of added ubiquitin-activating enzyme (Fig. 4). Under our assay conditions, the reaction with wild type Cdc34 leads to products that migrated as a set of discrete bands on SDS gels (Fig. 4*A*, *lane 1*). These discrete bands are due to the linkage of multiple ubiquitin groups, in the form of a ubiquitin chain, to a lysine residue in Cdc34 (Banerjee *et al.*, 1993). In contrast, only a single ubiquitin-Cdc34 adduct was detected with the mutants Cdc34-C95S, Cdc34-C95S,L99A, and Cdc34-C95S,L99S. This

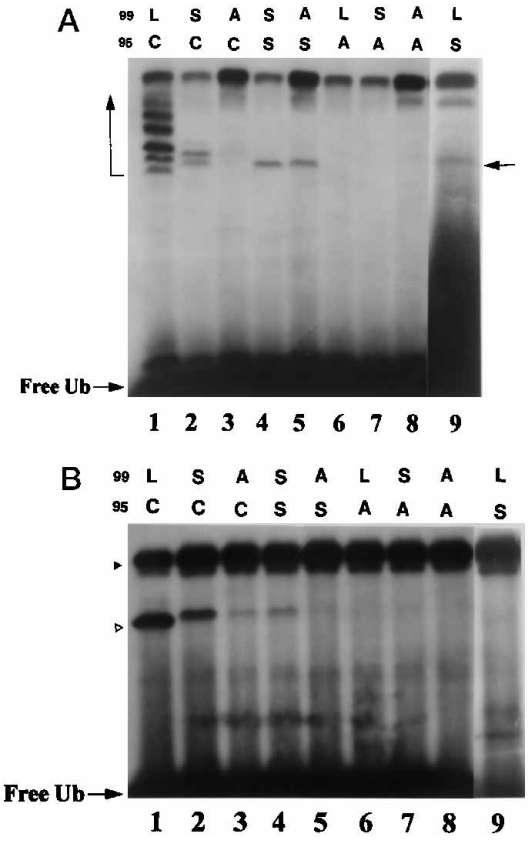

FIG. 4. **Formation of Ub-Cdc34 complexes by the mutant Cdc34 proteins.** *Panel A*, Cdc34 or its mutants were expressed in *E. coli* harboring the appropriate M13 mp19/18-*CDC34* constructs (see "Experimental Procedures"). Amounts of the recombinant protein in the bacterial extracts were normalized for the assays. Thiol-insensitive Ub-Cdc34 complexes were assayed by incubating *E. coli* extracts with ~100 nM overexpressed Cdc34 or its mutant proteins with ubiquitin-activating enzyme, magnesium, ATP, and ^{125}I-ubiquitin for 45 min. Samples were adjusted to contain 5% β-mercaptoethanol and heated at 90 °C for 3 min prior to SDS-PAGE. The uppermost band corresponds to ubiquitin linked to a lysine on *E*1. The ladder bands with the wild type Cdc34 are due to the linkage of a multiubiquitin chain to a lysine on Cdc34 (Banerjee *et al.*, 1993). The single band with the C95S mutants is presumably due to the formation of a Ub-Cdc34 oxygen-ester at Cdc34-Ser^{95} (position indicated by *arrow* on the *right*). *Panel B*, formation of $Cdc34_{S\text{-}Ub}$ thiol ester complexes was assayed by incubating *E. coli* extracts with ~100 nM overexpressed Cdc34 or its mutant proteins with ubiquitin-activating enzyme, magnesium, ATP, and [^{125}I]ubiquitin as in *panel A*, except the reaction time was reduced to 15 min. The reaction products were electrophoresed in a 14% SDS gel in the absence of thiol-reducing agents, and radiolabeled proteins were visualized by autoradiography. *Open arrow* indicates Ub-Cdc34 complex; *closed arrow* indicates $E1_{S\text{-}Ub}$ complex.

single adduct was not present in any of the C95A-substituted mutants, consistent with the notion that this single adduct is due to the linkage of ubiquitin to the substituted serine at position 95. The absence of additional adducts of lower electrophoretic mobilities in these C95S-substituted mutants indicates that autoubiquitination did not occur with these mutants. These results indicate that the C95S-substituted *cdc34* mutants can indeed form a more stable oxygen ester with ubiquitin and raise the possibility that the effect of *CDC34-C95S,L99S* on cell viability may require the linkage of ubiquitin to Ser^{95}.

We have also monitored the formation of ubiquitin-Cdc34 thiol ester with shorter reaction time (15 min), using a nonreducing SDS gel (Fig. 4*B*, *lanes 1–3*). Both Cdc34-L99S and Cdc34-L99A were found to retain partial activity as indicated by the presence of a 42-kDa radiolabeled band that corresponds to ubiquitin-Cdc34 thiol ester. This result indicated that unlike

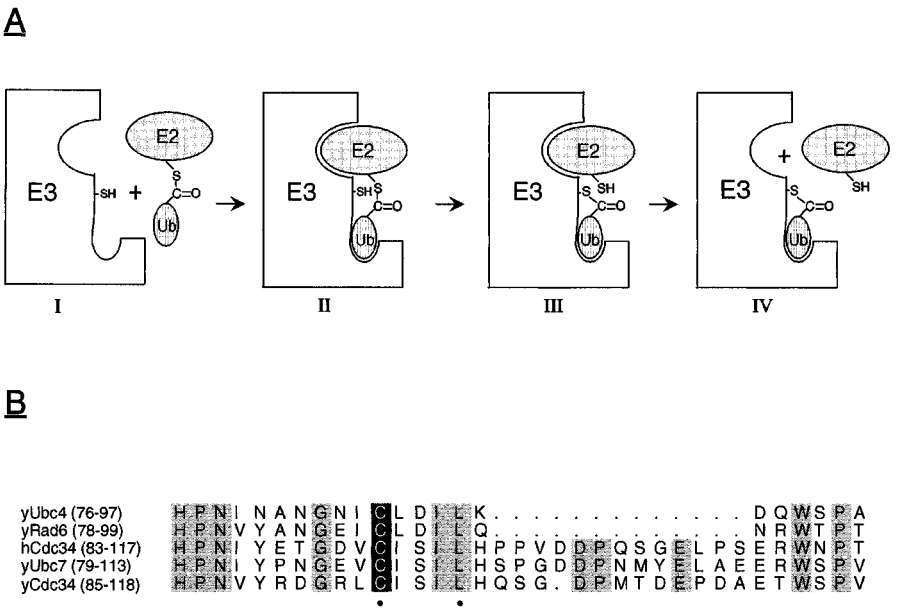

FIG. 5. *Panel A*, model of the ternary complex formation between *E*3 and $E2_{S\text{-}Ub}$ ubiquitin-*E*2 thiol ester complex docks on an *E*3 by noncovalent interactions to form II. Both ubiquitin and *E*2 contribute to the stability of II. Transthiolation (Scheffner *et al.*, 1995) leads to the attachment of ubiquitin to a cysteine in *E*3, and ubiquitin no longer contributes to the retention of *E*2 in the ternary complex III. In the case of Ub-Cdc34-C95S,L99S oxygen ester, ubiquitin is not transferred to *E*3, leading to the sequestration of *E*3 in an inactive complex. *Panel B*, alignment of the Cdc34 catalytic site sequence with other ubiquitin-conjugating enzyme sequences. The yeast Cdc34 (Goebl *et al.*, 1988) sequence is aligned with those of human Cdc34 (Plon *et al.*, 1993), yeast Ubc7 (Jungmann *et al.*, 1993), Rad6 (Jentsch *et al.*, 1987), and Ubc4 (Seufert and Jentsch, 1990) to show the positioning of the 12/13-residue segment in Cdc34 and Ubc7. Positions of mutant residues in Cdc34-C95S,L99S are indicated *below* by *closed circles*. The starting and end residue numbers for each sequence in the alignment are given in *parentheses*.

mutations at Cys^{95}, mutants of Leu^{99} are inactive at a step subsequent to ubiquitin-thiol ester formation. Thus, mutations at Leu^{99} appear to interfere with Cdc34 interactions with either substrate or *E*3 protein(s). Interestingly, a higher activity was found for the L99S-substituted mutant than the L99A mutant. Similarly, the shorter reaction time also revealed a faster ubiquitin linkage to Ser^{95} in Cdc34-C95S,L99S as compared with Cdc34-C95S and Cdc34-C95S,L99A (Fig. 4*B*, *lanes 4*, *9*, and *5*, respectively). Whether this difference in reactivity could account for the stringent requirement for the L99S substitution remains to be determined.

DISCUSSION

The ability of Cdc34-C95S,L99S to inhibit the cell cycle function of *CDC34* is indicated by its *in vivo* effect on cell viability and by its *in vitro* effect on Cln2 ubiquitination. Proteins that are known to be targeted by Cdc34 for ubiquitin-mediated proteolysis include G_1 cyclins (Deshaies *et al.*, 1995, Yaglom *et al.*, 1995), the yeast transcription factor GCN4 (Kornitzer *et al.*, 1994) and the Cdc28 kinase inhibitor Sic1 (Schwob *et al.*, 1994). Since overexpression of Cln2 does not lead to cell inviability (Lew and Reed, 1993), it is likely that Cdc34-C95S,L99S also inhibits the degradation of other Cdc34-dependent substrates. One likely candidate is Sic1, which is normally degraded prior to cell entry into the S phase (Schwob *et al.*, 1994), and a moderate overexpression of this protein has previously been shown to produce cellular morphology (Nugroho and Mendenhall, 1994) similar to those found for cells overexpressing Cdc34-C95S,L99S. Consistent with this notion is the recent demonstration that the human homolog of Cdc34-C95S,L99S could also inhibit Cdc34-dependent degradation of the cyclin-dependent kinase inhibitor, p27 (Pagano *et al.*, 1995). The ability of Cdc34-C95S,L99S to inhibit the *in vitro* ubiquitination and/or degradation of two dissimilar substrates in two different species raises the possibility that this mutant may be used in analogous manner to establish the identity of additional Cdc34-specific substrates.

Cells expressing Cdc34-C95S,L99S exhibit a morphology that differs significantly from the multibudded morphology of previously characterized loss-of-function *cdc34* mutants. A significant proportion of these cells contain a single, elongated bud, while multibudded cells are conspicuously absent. As the mechanism for multibudding in the *cdc34* null mutant has not been defined, the morphological difference here could not be readily addressed. Nonetheless, this difference suggests that the effect of Cdc34-C95S,L99S is not equivalent to a straightforward loss of *CDC34* functions. Previous studies have indicated that Cdc34 is capable of self association, and this process requires a region in the sequence that is apparently essential for its cell cycle function (Ptak *et al.*, 1994). Conceivably, Cdc34-C95S,L99S could exert its effect by sequestering endogenous Cdc34. However, this mechanism is incompatible with the absence of multibudded cells. Furthermore, the effect of Cdc34-C95S,L99S could not be obtained with the other seven inactive *cdc34* mutants that contain the same determinant for self-association. In addition, we have obtained preliminary results indicating that purified Cdc34-C95S,L99S does not inhibit a previously characterized *in vitro* autoubiquitination of Cdc34 (Banerjee *et al.*, 1993) or the conjugation of ubiquitin to histone proteins (Haas *et al.*, 1991). Thus, it is unlikely that the effect of Cdc34-C95S,L99S is due to the sequestration of endogenous Cdc34.

Since Cdc34-C95S,L99S encodes an inactive ubiquitin-conjugating enzyme, the mechanism of inhibition is likely to reside in a binding step where this mutant could compete effectively with Cdc34. A key unanswered question here is whether substrate recognition in this pathway also requires *E*3 proteins. A requirement for *E*3 has been shown for several other ubiquitination pathways. For example, the Ubr1 protein is required in the N terminus rule pathway (Bartel *et al.*, 1990), and a protein known as E6AP is required for the ubiquitination of p53 (Scheffner *et al.*, 1993). The ubiquitination of mitotic cyclins appears to require a large protein complex consisting of several distinct proteins (Sudakin *et al.* (1995); reviewed in Murray (1995)). Thus, the effect of Cdc34-C95S,L99S could result from the sequestration of Cdc34-specific substrates or the required *E*3 protein(s).

Cys^{95} and Leu^{99} are located within a sequence region that is highly conserved among ubiquitin-conjugating enzymes. This conserved sequence region has been termed the catalytic core domain and is conserved in tertiary folding as shown by the crystal structures of *Arabidopsis thaliana* Ubc1 and *Saccharomyces cerevisiae* Ubc4 (Cook *et al.*, 1993). A structural model of the Cdc34 catalytic core could be constructed by aligning residues 10–100 of Cdc34 with the N-terminal 91 residues of Ubc4.

Both Cys^{95} and Leu^{99} could be placed within this structural model at positions that are occupied by identical amino acids in Ubc4. In this model, substitution of Cys^{95} by either alanine or serine would not introduce other structural perturbations. Thus, the difference between Cdc34-C95S,L99S and Cdc34-C95A,L99S is unlikely to be structural but rather in the ability of Ser^{95} to form a stable oxygen ester with ubiquitin. This suggests that the inhibitory effect of Cdc34-C95S,L99S may require prior formation of the ubiquitin-Cdc34-C95S,L99S ester.

A model that could account for the inhibitory effect of Cdc34-C95S,L99S is depicted in Fig. 5*A*. In this model, ubiquitin contributes partly to the energetics of the ternary complex formation between the ubiquitin-*E*2 thiol ester and *E*3. Once ubiquitin has been transferred to *E*3, the ubiquitin-conjugating enzyme would presumably bind less tightly since it is no longer linked to ubiquitin. The reduced affinity may then facilitate the dissociation of the ubiquitin-conjugating enzyme, which could be recharged with ubiquitin by the ubiquitin-activating enzyme (*E*1). The existence of a ubiquitin binding site on *E*3 is supported by studies on a reticulocyte *E*3 in the N-end rule pathway (Reiss and Hershko, 1990). This model makes the prediction that *E*2 mutants containing a stably linked ubiquitin would be better inhibitors than inactive enzymes that cannot be linked with ubiquitin and explains the unique requirement for the C95S mutation. The requirement for the L99S mutation could be explained by the observation that this mutation causes ubiquitin to be linked to Ser^{95} at a faster rate (Fig. 4). A structural basis of this effect could not be readily assessed using the two known structures of *E*2 enzymes since Cdc34 contains an extra 12-residue segment beginning at residue 101, and this extra segment could not be accommodated in a structural model. A similar sequence segment is also present in the yeast Ubc7 protein (Fig. 5*B*), and the crystal structure of this ubiquitin-conjugating enzyme has recently been determined,[2] and work is in progress to determine the mutational effect of the corresponding leucine residue in this enzyme.

Dominant negative mutants in other yeast genes have proved useful for the identification of interacting proteins via suppresser analyses. If the action of Cdc34-C95S,L99S depends on its interaction with *E*3 proteins, one class of suppressers is expected to be comprised of these proteins. The ability of purified Cdc34-C95S,L99S to block the *in vitro* ubiquitination of Cln2 suggests a further utility of this mutant for the identification of additional Cdc34-specific substrates. Although substrates in a specific ubiquitin-dependent pathway could usually be identified in yeast by showing their increased stability in a specific *ubc* mutant, this approach may be insufficient to provide unambiguous identification of *CDC34*-specific substrates. For example, while the G_2-specific B-type cyclins are stabilized in *CDC34* mutants (Amon *et al.*, 1994), degradation of these cyclins is apparently mediated by *UBC9* (Seufert *et al.*, 1995), and the effect of *cdc34* in this case could be attributed to the abnormal accumulation of Cln proteins in *cdc34* mutants. Thus, an unambiguous identification of a *CDC34*-specific substrate may also require the use of *in vitro* approaches to demonstrate a direct requirement of this ubiquitin-conjugating enzyme. One such approach may be by using the Cdc34-C99S,L99S mutant protein to inhibit the ubiquitination or the degradation of a candidate substrate protein in a cell-free system. This approach has been used recently to help in establishing a role of Cdc34 in degradation of the human cyclin-dependent kinase inhibitor, p27. Dominant negative mutants of yeast *UBC* genes could be readily identified by genetic screens. The creation of an analogous dominant negative mutant of human Cdc34 with mutations identified in the yeast enzyme points to the important possibility that other dominant negative mutants of mammalian ubiquitin-conjugating enzymes could be obtained by a similar approach. Such mutants could then be used for exploring substrates and/or regulators of protein ubiquitination in mammalian systems.

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[2] P. Martin, R. Yamazaki, W. Cook, B. Edwards, and V. Chau, unpublished result.

REFERENCES

Amon, A., Irniger, S., and Nasmyth, K. (1994) *Cell* **77,** 1037–1050
Banerjee, A., Gregori, L., Xu, Y., and Chau, V. (1993) *J. Biol. Chem.* **268,** 5668–5675
Bartel, B., Wünning, I., and Varshavsky, A. (1990) *EMBO J.* **9,** 3179–3189
Blake, M. S., Johnston, K. H., Russell-Jones, G. J., and Gotschlich, E. C. (1984) *Anal. Biochem* **136,** 175–179
Byers, B., and Goetsch, L. (1973) *Cold Spring Harbor Symp. Quant. Biol.* **38,** 123–131
Ciechanover, A., Elias, S., Heller, H., and Hershko, A. (1982) *J. Biol. Chem.* **257,** 2537–2542
Cook, W. J., Jeffrey, L. C., Xu, Y., and Chau, V. (1993) *Biochemistry* **32,** 13809–13817
Deshaies, R. J., Chau, V., and Kirschner, M. W. (1995) *EMBO J.* **14,** 303–312
Finley, D., and Chau, V. (1991) *Annu. Rev. Cell Biol.* **7,** 25–69
Gietz, R. A., and Sugino, A. (1988) *Gene* (*Amst.*) **74,** 527–534
Goebl, M. G., Yochem, J., Jentsch, S., McGrath, J. P., Varshavsky, A., and Byers, B. (1988) *Science* **241,** 1331–1335
Haas, A. L., Reback, P. B., and Chau, V. (1991) *J. Biol. Chem.* **266,** 5104–5112
Harlow, E., and Lane, D. (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
Hershko, A., and Ciechanover, A. (1992) *Annu. Rev. Biochem.* **61,** 761–807
Jentsch, S., McGrath, J. P., and Varshavsky, A. (1987) *Nature* **329,** 131–134
Jentsch, S. (1992) *Annu. Rev. Genet.* **26,** 179–207
Johnston, M., and Davis, R. W. (1984) *Mol. Cell. Biol.* **4,** 1440–1448
Jungmann, J., Reins, H.-A., Schobert, C., and Jentsch, S. (1993) *Nature* **361,** 369–371
Kornitzer, D., Raboy, B., Kulka, R. G., and Fink, G. R. (1994) *EMBO J.* **13,** 6021–6030
Laemmli, U. K. (1970) *Nature* **227,** 680–685
Lew, D. J., and Reed, S. I. (1993) *J. Cell Biol.* **120,** 1305–1320
Murray, A. (1995) *Cell* **81,** 149–152
Nugroho, T. T., and Mendenhall, M. D. (1994) *Mol. Cell. Biol.* **14,** 3320–3328
Pagano, M., Tam, S. W., Theodora, A. M., DelSal, G., Yew, P. R., Chau, V., Draetta, G. F., and Rolfe, M. (1995) *Science* **269,** 682–685
Plon, S. E., Leppig, K. A., Do, H. N., and Groudine, M. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 10484–10488
Ptak, C., Prendergast, J. A., Hodgins, R., Kay, C. M., Chau, V., and Ellison, M. J. (1994) *J. Biol. Chem.* **269,** 26539–26545
Reiss, Y., and Hershko, A. (1990) *J. Biol. Chem.* **265,** 3685–3690
Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
Sanger, F., Niklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 5463–5467
Scheffner, M., Huibregtse, J. M., Vierstra, R. D., and Howley, P. (1993) *Cell* **75,** 495–505
Scheffner, M., Nuber, U., and Huibregtse, J. M. (1995) *Nature* **373,** 81–83
Schwob, E., Bohm, T., Mendenhall, M. D., and Nasmyth, K. (1994) *Cell* **79,** 233–244
Seufert, W., and Jentsch, S. (1990) *EMBO J.* **9,** 543–550
Seufert, W., Futcher, B., and Jentsch, S. (1995) *Nature* **373,** 78–81
Sherman, F., Fink, G. R., and Hicks, J. B. (1986) *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
Shiestel, R., and Gietz, R. D. (1989) *Curr. Genet.* **16,** 339–346
Sudakin, V., Ganoth, A., Dahan, H., Hershko, J., Luca, F. C., Ruderman, J. V., and Hershko, A. (1995) *Mol. Biol. Cell* **6,** 185–198
Sung, P., Prakash, S., and Prakash, L. (1991) *J. Mol. Biol.* **221,** 745–749
Taylor, J. W., Ott, J., and Eckstein, F. (1985) *Nucleic Acids Res.* **13,** 8764–8785
Varshavsky, A. (1992) *Cell* **69,** 725–735
Yaglom, J., Linskens, M. H. K., Sadis, S., Rubin, D. M., Futcher, B., and Finley, D. (1995) *Mol. Cell. Biol.* **15,** 731–741
Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) *Gene* (*Amst.*) **33,** 103–119