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

(Received for publication, June 14, 1995, and in revised form, August 22, 1995)

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The yeast Saccharomyces cerevisiae CDC34 gene encodes a ubiquitin-conjugating enzyme that is required for the cell cycle G1/S transition. We show here that a dominant negative Cdc34 protein is generated by simultaneously replacing both Cys95 and Leu99 with Ser residues. Cys95 is an essential catalytic residue that forms a transient thiol ester with ubiquitin during catalysis, and Leu99 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 (E2)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 (E1) and ATP in the following reactions: 1) E1SH + Ub + ATP ⇌ E1SUb + AMP + PPi, and 2) E1SUb + E2SH ⇌ E1SH + E2SUb. Substrate proteins may be directly recognized by an individual E2 enzyme, resulting in the transfer of ubiquitin from an E2SUb to a lysine on substrate proteins. Alternatively, substrate recognition may require the presence of another group of proteins known as E3 or ubiquitin-protein ligases (Reiss and Hershko, 1990; Bartel et al., 1990). A requirement for a specific E3 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 Ub1 (E3) protein contains separate sites for Rad6 (E2) 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 E2SUb is transferred to a cysteine in the E3 protein (Scheffner et al., 1995), leading to the formation of an E3SUb thiol ester.

Cdc34 is one of 10 known ubiquitin-conjugating enzyme-encoding genes in the yeast, S. cerevisiae (Goebi et al., 1988; Jentsch, 1992). This gene was initially identified on the basis of its requirement for cells to undergo the cell cycle G1 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 G1 cyclins (Deshaies et al., 1995; Yaglom et al., 1995) and the Cdc28 kinase inhibitor Sic1 (Schob 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 G2-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 E2 proteins. This conserved domain is apparently sufficient for E2SUb complex formation since the smallest E2 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 E2 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 Leu99, 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, Cdc34Miss, could be generated by simultaneously substituting these two residues with serines. In addition to its potential utility in genetic analysis, Cdc34Miss can be used to block Cdc34-dependent ubiquitination in vitro.

EXPERIMENTAL PROCEDURES

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

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*This research was supported by National Institutes of Health Grant GM 47604. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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iodogen (Pierce) according to the manufacturer's procedure. The specific activity of $^{125}$I-ubiquitin was $5 \times 10^5$ cpm/μg. The ubiquitin-activating enzyme (E1) was purified to homogeneity from calf thymus as described previously (Ciechanover et al., 1982) and was stored at −80 °C in 20% glycerol. E1 and Cdc34 concentrations were determined by first converting the proteins to E. coli and Cdc34, respectively. 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 Pgal promoter were constructed using the vectors YEplac195 and YIp211 (Gietz and Sugino, 1988) by isolating a 0.7-kilobase BamHI- EcoRI fragment of P12 that contained the Pgal promoter region (Slonim and Davis, 1984) and ligating the fragment into the high copy (2μ-based) plasmids YEplac195 and YEpplac181, and integrating the M13mid YIp211, yielding plasmid vectors YEplac195GAL, YEp181GAL, and YIp211GAL. Fragments of 0.9 kilobase encoding Cdc34 and its mutants (without the native promoter) bordered by SalI and SphI sites (Banerjee et al., 1993) were then ligated into the SalI- and SphI-cut YEplac195GAL and YIp211GAL, yielding various constructs used in this study. CDC34 was also cloned into the YEplac181GAL vector. Proper orientation was verified by colony hybridization and restriction mapping of the yeast strains.

Proteins were purified according to previously published protocols (Banerjee et al., 1993). Rad6 protein was purified from E. coli extracts that had the protein overexpressed from the RAD6 gene cloned in pK223-3 vector (Haas et al., 1991).

Expression of M13 mp19-CDC34 Mutant Genes—To express Cdc34 mutant proteins, individual mutant genes in M13 mp19 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 mL Tris (pH 7.5), and suspended in 100 μL of the same buffer and 0.5 mL 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 immuno blotting using the ECL (Amer sham Corp.) method of detection. Incubation times of 15 and 45 min were used in thiol ester and Ub-Cdc34 complex formation, respectively.

CDC34 and Ub-Cdc34 Complex Formation—Reactions were carried out in 50 mL Tris (pH 7.5), 10 mM MgCl2, 2 mM ATP, 50 μM dithiothreitol, and 5 μM ubiquitin at 30 °C. Unless stated otherwise, 50 mL of E1 (from calf thymus) and 100 mL 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 radio labeled 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 mL Tris-HCl buffer, pH 7.5, 1 mM dithiothreitol, and resuspended in 200 μL of breakage buffer (50 mL 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 100-μl aliquot of the supernatant was assayed for Cdc34 protein by Western blotting, 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 chemiluminescence (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 to: 

| Plasmid/phage | Genotype/description | Source/reference |
|--------------|----------------------|-----------------|
| M13mp19/M13mp18 | Cloning vectors | Yanisch-Perron et al. (1985) |
| M13mp19CDC34 | CDC34 with lac promoter | Banerjee et al. (1993) |
| M13mp19CDC34-C95S.L95S | CDC34 with lac promoter | This study |
| M13mp19cdc34-L99S | CDC34 with lac promoter | This study |
| M13mp19cdc34-L99A | CDC43 with lac promoter | This study |
| pLCDC34-C95S.L95S | CDC34 with lac promoter | Deshaies et al. 1995 |
| pLCDC34-C95S.L95A | CDC34 with lac promoter | This study |
| pLCDC34-C95A | CDC34 with lac promoter | This study |
| pLCDC34-C95A.L95S | CDC34 with lac promoter | This study |
| pLCDC34-C95A.L95K | CDC34 with lac promoter | This study |
| pRD68 | CLN2 with T7 promoter | This study |

Expression of Cdc34 and mutant proteins in E. coli: 1. Cdc34 was expressed in E. coli using the pUC19 vector. 2. Mutant Cdc34 was expressed in E. coli using the pUC19 vector. 3. Cdc34 and mutant proteins were purified by Ni-NTA chromatography. 4. Proteins were subjected to SDS-PAGE and Coomassie Blue staining. 5. Proteins were assayed for ubiquitin thiol ester and conjugate formation in a final reaction volume of 20 μL using standard reaction conditions. 6. 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. 7. Reactions were stopped by withdrawing aliquots of the reaction mixture into SDS-sample buffer in which β-mercaptoethanol had been omitted. 8. 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. 9. Protein samples were subjected to electrophoresis in a 14% SDS-polyacrylamide gel, and autoradiography was used to visualize radiolabeled bands. 10. CDC34 and Ub-Cdc34 Complex Formation—Reactions were carried out in 50 mL Tris (pH 7.5), 10 mM MgCl2, 2 mM ATP, 50 μM dithiothreitol, and 5 μM ubiquitin at 30 °C. Unless stated otherwise, 50 mL of E1 (from calf thymus) and 100 mL of Cdc34 or its mutant proteins were used in the reactions.
TABLE II
S. cerevisiae strains

| Name        | Relevant markers/plasmids | Source/Comment       |
|-------------|---------------------------|----------------------|
| 332         | Mata ura3–52 leu2 his3    | R. Needelman         |
| ABY200      | Mata URA3::GAL CDC34 leu2 his3 | This study, derivative of 322 |
| ABY100      | Mata URA3::GAL CDC34-C95S-L99S leu2 his3 | This study, derivative of ABY100 |
| ABY102      | Mata URA3::GAL CDC34-C95S-L99S leu2 his3 + YEp181GAL CDC34 | This study, derivative of 322 |
| ABY110      | Mata URA3::GAL CDC34-L99S leu2 his3 | This study, derivative of 322 |
| ABY120      | Mata URA3::GAL CDC34-L99A leu2 his3 | This study, derivative of 322 |
| ABY130      | Mata URA3::GAL CDC34-C95S leu2 his3 | This study, derivative of 322 and plasmid pYL C95S from M. Goebl |
| ABY140      | Mata URA3::GAL CDC34-C95S-L99A leu2 his3 | This study, derivative of 322 |
| ABY150      | Mata URA3::GAL CDC34-C95A leu2 his3 | This study, derivative of 322 |
| ABY160      | Mata URA3::GAL CDC34-C95A-L99S leu2 his3 | This study, derivative of 322 |
| ABY170      | Mata URA3::GAL CDC34-C95A-L99S leu2 his3 | This study, derivative of 322 |
| MGG15       | Mata cdc34–2 ura3 his3 | M. Goebl |
| ABY210      | Mata cdc34–2 ura3 his3 + YEp195GAL CDC34 | This study, derivative of MGG15 |
| ABY212      | Mata cdc34–2 ura3 his3 + YEp195GAL CDC34-C95S-L99S | This study, derivative of MGG15 |
| ABY214      | Mata cdc34–2 ura3 his3 + YEp195GAL CDC34-L99S | This study, derivative of MGG15 |
| ABY216      | Mata cdc34–2 ura3 his3 + YEp195GAL CDC34-C95S | This study, derivative of MGG15 |
| pRD84/RD205–3A | dna1Δ, dna2::LEU2, dna3Δ, trp1Δ, leu2Δ, ura3Δ, pep4::LEU2 + [GAL-CLN3, URA3, CEN, ARS] | Deshaies et al. (1995) |

Analysis of Cin2 Multiubiquitination—Expression of Cin2 in vitro was achieved by inserting a BamHI fragment containing the CLN2 gene into the BamHI site of pGEM2, yielding pRD68, where the CLN2 gene could be transcribed by the T7 promoter (Deshaies et al., 1995). This DNA was used for in vitro translation by the rabbit reticulocyte lysate system. Reagents for the in vitro translation were obtained from Promega Biotech. [35S]Cln2 synthesized in vitro translation by the rabbit reticulocyte lysate system.

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 G1/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., 1973). The yeast strain MGG15 contains a temperature-sensitive cdc34–2 allele, and these cells are inviable at nonpermissive temperatures (Goebl et al., 1973). The yeast strain MGG15 contains a temperature-sensitive cdc34–2 allele, and these cells are inviable at nonpermissive temperatures (Goebl et al., 1973). The yeast strain MGG15 contains a temperature-sensitive cdc34–2 allele, and these cells are inviable at nonpermissive temperatures (Goebl et al., 1973).

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 Pgal promoter (Table II). These cells also failed to grow on galactose medium (Fig. 1B) 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
concomitant expression of wild type pendent protein ubiquitination. First, the effect of C95S,L99S affects cell viability by disruption of shown). However, two lines of evidence indicated that the overall level of ubiquitin-protein conjugates (data not shown). Therefore, two lines of evidence indicated that 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 G1 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. 3A). 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. 3B). As shown in Fig. 3B, the level of ubiquitin-Rad6 thiolester 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 arrested as multibudded cells (Goehring 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—since neither cdc34-C95S nor cdc34-L99S affects cell viability, it appears that both mutations must confer the dominant negative phenotype. Cys95 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, as shown in Figs. 4 and 5 (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 Cys95 and Leu99 in Cdc34-C95S, L99S—Since neither cdc34-C95S nor cdc34-L99S affects cell viability, it appears that both mutations must confer the dominant negative phenotype. Cys95 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, as shown in Figs. 4 and 5 (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 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 125I-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 E1. The ladder bands with the wild type Cdc34 are due to the linkage of a multibiquitin 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-Ser95 (position indicated by arrow on the right). Panel B, formation of Cdc34-C95S, L99S 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 125I-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 UB complex.
FIG. 5. Panel A, model of the ternary complex formation between E3 and E2s, ubiquitin-E2 thiol ester complex docks on an E3 by noncovalent interactions to form II. Both ubiquitin and E2 contribute to the stability of II. Translocation (Scheffner et al., 1995) leads to the attachment of ubiquitin to a cysteine in E3, and ubiquitin no longer contributes to the retention of E2 in the ternary complex III. In the case of Ub-Cdc34-C95S,L99S oxygen ester, ubiquitin is not transferred to E3, leading to the sequestration of E3 in an inactive complex. Panel B, alignment of the Cdc34 catalytic site sequence with other ubiquitin-conjugating enzyme sequences. The yeast Cdc34 (Goeb et al., 1988) sequence is aligned with those of human Cdc34 (Pion 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 by closed circles. The starting and end residue numbers for each sequence in the alignment are given in parentheses.

DISCUSSION

The ability of Cdc34-C95S,L99S to inhibit cell cycle function of Cdc34 is indicated by its in vivo effect on cell viability and its in vitro effect on Cln2 ubiquitination. Proteins that are known to be targeted by Cdc34 for ubiquitin-mediated proteolysis include G1 cyclins (Deshai et al., 1995, Yaglom et al., 1995), the yeast transactivation factor GCN4 (Kornitzer et al., 1995), and the Cdc28 kinase inhibitor Sic1 (Schwob et al., 1994). Although 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 E3 proteins. A requirement for E3 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 E3 protein(s).

Cys95 and Leu99 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 Cys95 and Leu99 could be placed within this structural model at positions that are occupied by identical amino acids in Ubc4. In this model, substitution of Cys95 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 Ser95 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. 5A. In this model, ubiquitin contributes partly to the energetics of the ternary complex formation between the ubiquitin-E2 thiol ester and E3. Once ubiquitin has been transferred to E3, 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 (E1). The existence of a ubiquitin binding site on E3 is supported by studies on a reticulocyte E3 in the N-end rule pathway (Reiss and Hershko, 1990). This model makes the prediction that E2 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 Ser95 at a faster rate (Fig. 4). A structural basis of this effect could not be readily assessed using the two known structures of E2 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 structural model. A similar sequence segment is also present in the

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