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Intragenic Suppression among *CDC34* (*UBC3*) Mutations Defines a Class of Ubiquitin-Conjugating Catalytic Domains

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Ubiquitin-conjugating (E2) enzymes contain several regions within their catalytic domains that are highly conserved. However, within some of these conserved regions are several residues that may be used to define different classes of catalytic domains for the E2 enzymes. One class can be defined by the Ubc1 protein, which contains K-65, D-90, and D-120, while the corresponding positions within the Cdc34 (Ubc3) protein, which defines a second class of enzymes, contain S-73, S-97, and S-139, respectively. The presence of these differences within otherwise highly conserved regions of this family suggests that these residues may be critical for the specificity of Cdc34 function or regulation. Therefore, we have constructed a series of *cdc34* alleles encoding mutant proteins in which these serine residues have been changed to other amino acid residues, including alanine and aspartic acid. In vivo complementation studies showed that S-97, which lies near the active site C-95, is essential for Cdc34 function. The addition of a second mutation in *CDC34*, which now encoded both the S97D and S73K changes, restored partial function to the Cdc34 enzyme. Moreover, the deletion of residues 103 to 114 within Cdc34, which are not present in the Ubc1-like E2s, allowed the S73K/S97D mutant to function as efficiently as wild-type Cdc34 protein. Finally, the cloning and sequencing of the temperature-sensitive alleles of *CDC34* indicated that A-62 is also unique to the Cdc34 class of E2 enzymes and that mutations at this position can be detrimental to Cdc34 function. Our results suggest that several key residues within conserved regions of the E2 enzyme family genetically interact with each other and define a class of E2 catalytic domains.

Ubiquitin is a small 76-amino-acid-residue protein which can be covalently attached to other proteins (for a review, see reference 11). The ubiquitin-conjugating (or E2) enzymes catalyze isopeptide bond formation between ubiquitin and substrate proteins. Ubiquitin is first activated through the ATP-dependent formation of a thiolester linkage between ubiquitin and a ubiquitin-activating enzyme (E1). This ubiquitin is then transferred from the E1 enzyme to a member of the family of E2 enzymes, again through the formation of a thiolester. The E2 enzyme can then catalyze the formation of an isopeptide linkage between the COOH-terminal glycine of ubiquitin and the epsilon amine group of a lysine of the substrate protein. This reaction often requires the activity of a ubiquitin ligase (E3).

CDC34 (*UBC3*) encodes a 295-residue protein that belongs to this family of E2 enzymes (13). The Cdc34 protein has been shown to ubiquitinate histones in vitro (13) and itself both in vivo (12) and in vitro (1). This ubiquitination may regulate the turnover of substrate proteins, possibly including Cdc34 itself, via the ubiquitin-dependent proteolytic pathway (reviewed in references 11 and 15). To date, the genes encoding at least 10 distinct E2 enzymes have been identified in *Saccharomyces cerevisiae* (reviewed in reference 15). Their products are involved in a broad variety of biological functions. The Rad6 (Ubc2) protein serves several functions, affecting DNA repair, sporulation, induced mutagenesis, and proliferation. It also functions in the N-end rule pathway of selective proteolysis (8, 37, 40). Cdc34 is essential for the G_1-to-S phase transition (13). Ubc1, Ubc4, and Ubc5 play an important role in the stress response (35, 36). Ubc7 confers resistance to the heavy metal cadmium (17, 45). Pas2 (Ubc10) is necessary for peroxisome biogenesis (46), while Ubc9 is required for the G_2-to-M phase transition (34).

While the distinct E2 enzymes have in common a conserved catalytic domain that contains the active-site cysteine residue involved in ubiquitin transfer, these enzymes have been classified into two categories based on their primary structures (15). Class I enzymes consist solely of a catalytic domain, while class II enzymes bear a COOH-terminal extension in addition to the NH_2-terminal catalytic domain. Cdc34 consists of a 170-residue NH_2-terminal catalytic domain and a 125-amino-acid residue COOH-terminal extension which bears a polyacidic stretch (13). Rad6 bears a shorter acidic tail (28). Cdc34 and Rad6 are class II enzymes that require the presence of their COOH-terminal tails for efficient histone conjugation activity in vitro (13, 16, 41). It has recently been reported that a segment consisting of the first 74 residues of the COOH-terminal extension of Cdc34 is a major determinant of cell cycle function, although the NH_2-terminal catalytic domain is also essential for Cdc34 function (19, 27, 39).

Within the NH_2-terminal catalytic domain, several regions, including the active site, are highly conserved within all yeast E2 enzymes described to date. The active site can be identified by the presence of the ubiquitin-accepting cysteine (5, 6). This is often the only cysteine in an E2 enzyme, including Cdc34 (13). Intriguingly, several residues that reside within these highly conserved regions define two families of E2 catalytic domains. One family, of which Ubc1 is an example, can be defined by the presence of K-65 and D-90 (Fig. 1). On the other hand, the Cdc34 protein has serine residues at the corresponding positions, S-73 and S-97 (Fig. 1). In addition, within

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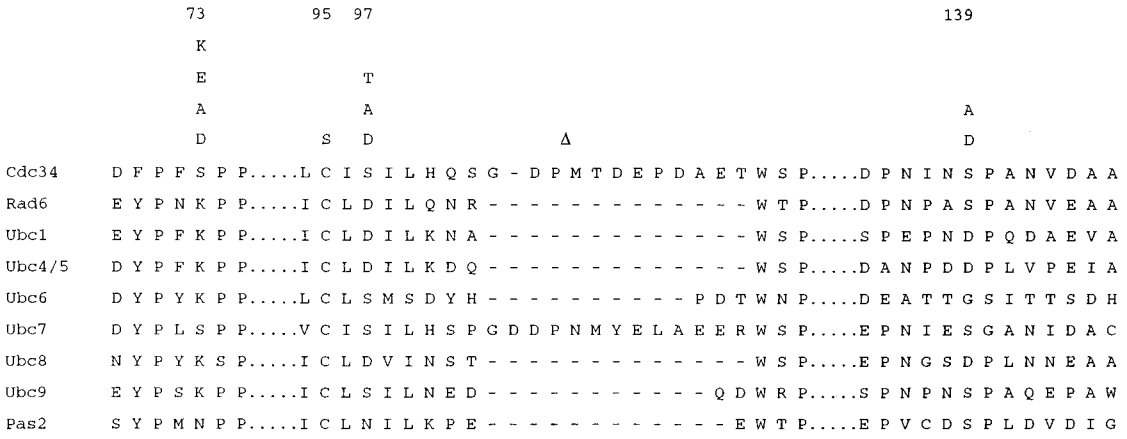

FIG. 1. Partial amino acid sequence alignment of yeast Ubc enzymes. Protein sequences were aligned by using the multiple alignment program MACAW (33). The amino acid residue numbers above the alignment indicate the positions of these residues within Cdc34. Periods indicate presence of amino acids not shown. Dashes indicate positions of gaps introduced to allow optimal alignment. Amino acids indicated above residues within the predicted amino acid sequences mark the substitutions created in Cdc34 at these positions.

Cdc34 there is an insertion of 12 amino acids from residues 103 to 114 that Ubc1 lacks. Among a wide variety of E2 enzymes, one finds serines at the positions corresponding to S-73 and S-97 and the insert of Cdc34 rather than lysine and aspartic acid residues, respectively, at the corresponding positions with no insert as in Ubc1. Most E2 enzymes fall into one of these categories, with few variations. We thus constructed a series of mutated *cdc34* genes encoding amino acid changes at these positions to evaluate the functional importance of these differences, including mutations that delete the insertion from both an otherwise wild-type Cdc34 protein and a Cdc34 S73K/S97D mutant protein. In addition, we have cloned and sequenced several temperature-sensitive alleles of *CDC34*. We report here the effects of these mutations on the function of the Cdc34 enzyme.

MATERIALS AND METHODS

Yeast strains. Standard methods were used for strain construction (30). YL10 (*MAT*α *cdc34-2 ura3-52 leu2Δ1 trp1Δ63 his3*Δ) is a meiotic product of a cross between FY24 (*MAT*α *ura3-52 leu2Δ1 trp1Δ63*; kindly provided by F. Winston) and MGG15 (*MAT***a** *cdc34-2 ura3-52 his3*Δ: this study) (*cdc34-2* is a temperature-sensitive allele). YL10-1 (*MAT***a** *cdc34-2 ura 3-52 leu2Δ1 trp1Δ63 his3*Δ) is a meiotic product of a cross between FY23 (*MAT***a** *ura3-52 leu2Δ1 trp1Δ63*; kindly provided by F. Winston) and YL10. Y382 (*MAT*α *ade2 ade3 ura3 leu2 trp1*) was a kind gift of A. Bender. YPH52 (*MAT***a** *ura3-52 lys2-801$_{am}$ ade2-101$_{oc}$ trp1Δ1 his3 Δ200*) and YPH54 (*MAT*α *ura3-52 lys2-801$_{am}$ ade2-101$_{oc}$ trp1Δ1 his3Δ200*) were kind gifts of P. Hieter.

YL18 (*MAT***a** *cdc34*::*HIS3 ura3-52 leu2Δ1 trp1Δ63 his3Δ200* pYL250) was constructed as follows. A heterozygous *cdc34* disruption strain, YL17 (*MAT***a**/α *cdc34*::*HIS3*/*CDC34 ura3-52*/*ura3-52 leu2Δ1*/*leu2Δ1 trp1Δ63*/*trp1Δ63 his3Δ200*/*his3Δ200*; this study), was constructed by using a strategy previously described by Goebl et al. (13). YL17 was then transformed with plasmid pYL250 (Table 1). In pYL250, the expression of *CDC34* is under the control of the *GAL7* promoter. Transformants were sporulated, and asci were dissected on YPGal medium. Ura$^+$ His$^+$ colonies were identified, one of which was YL18. This strain is viable only on galactose medium unless transformed with an additional *CDC34* gene. YNM101 was constructed by transforming Y382 with *Eco*RI-digested DNA from plasmid pNM101 (Table 1) and selecting for Ura$^+$ colonies. YNM101 contains a duplication of the *CDC34* locus with one wild-type and one mutated copy of the *CDC34* gene. A *rad6* disruption strain, Grad6-1 (*MAT***a** *rad6*::*URA3 ura3-52 leu2Δ1*), is the meiotic product of a cross between YPrad6 (*MAT*α *rad6*::*URA3 ura3-52 lys2-801$_{am}$ ade2-101$_{oc}$ trp1Δ1 his3Δ200*) and FY23. YPrad6 is the meiotic product of a diploid constructed by mating YPH52 and YPH54 and subsequently transformed with the plasmid pDEL21 as described previously (21). YL10-1, YL18, and Grad6-1 are all capable of growth on medium containing 2% galactose as the sole carbon source.

Media, growth conditions, and genetic assays. Standard rich (YPD) and defined minimal (SD) media were prepared as described previously (30). For galactose induction, rich YPGal and minimal S · Gal media, in which dextrose was replaced by galactose, were used. For plasmid selection, yeast cells were grown in defined minimal medium supplemented with the appropriate amino acids. 5-Fluoro-orotic acid (5-FOA; final concentration, 1 g/liter) was added where indicated to select against cells harboring a functional *URA3* gene (2).

Standard yeast genetic and transformation procedures were used as described previously (30). For complementation experiments, patches of single transformants were allowed to grow under permissive conditions: YL10-1 derivatives on SD-Leu at 23°C, and YL18 derivatives on S · Gal-Leu at 30°C. Then cell patches were replica plated to tester plates: YL10-1 derivatives to S · Gal-Leu and SD-Leu at 36°C, and YL18 derivatives to S · Gal+5-FOA and SD+5-FOA at 23 and 36°C.

To test for UV sensitivity, Grad6-1 transformants were grown in S · Gal-Leu liquid medium at 23°C to mid-exponential phase, diluted in sterile H_2O, sonicated briefly, spread onto YPGal plates, and irradiated with UV light at doses of between 0 and 40 J/m^2. Survival frequency was indicated as the number of colonies formed after irradiation divided by the number of colonies formed without irradiation.

Construction of *cdc34* mutations. Standard molecular biology techniques were performed as described by Sambrook et al. (32). To construct a series of *cdc34* mutations, a 1.0-kb *Sac*I-*Sph*I fragment containing the promoterless *CDC34* gene was cut from plasmid pYL110 (Table 1) and subcloned between the *Sac*I and *Sph*I sites of M13mp18 (m1). Site-directed mutagenesis (48) was performed on the m1 phage with primers that defined sequences encoding the desired amino acid substitutions (Table 2). For the double mutations encoding the changes S73K/S97A and S73K/S97D, a degenerate oligonucleotide encoding S97A and S97D substitutions was used with the S73K m1 derivative as the template for mutagenesis. For the deletion of the sequences encoding amino acids 103 to 114, a 32-mer oligonucleotide which lacks 36 nucleotides present in wild-type *CDC34* was used with the m1 phage as the template to generate *cdc34*-$\Delta_{103-114}$ and with the S73K/S97D or S73K/S97A m1 derivative as the template to generate S73K/S97D-$\Delta_{103-114}$ or S73K/S97A-$\Delta_{103-114}$, respectively. All changes were accomplished by using the Amersham oligonucleotide-directed in vitro mutagenesis kit as instructed by the manufacturer. The DNA sequences of both strands of each mutated *CDC34* were verified by the dideoxy-chain termination method (42), using Sequenase (U.S. Biochemical) as instructed by the manufacturer.

Construction of expression plasmids. DNA sequences containing the *cdc34* mutations constructed as described above were released from the corresponding M13 clones by digestion with *Sal*I and *Sph*I and subcloned between the *Sal*I and *Sph*I sites of pSJ101, a 2 μm-based *LEU2* expression plasmid which is a derivative of YEp51 (29) (kindly provided by S. Johnson). Expression of each mutated *cdc34* was thus under the control of the *GAL10* promoter, which lies in front of the polylinker region of pSJ101. A control plasmid (pYL150; Table 1) was also constructed by subcloning the wild-type *CDC34 Sal*I-*Sph*I fragment from the M13 clone into pSJ101. Plasmid pRAD6 (Table 1) was constructed by subcloning the 3.4-kb *Bam*HI fragment from pB2 (20) into the *Bam*HI site of pSJ101.

Construction of pNM101. Klenow fragment was used to flush the ends of the 1.2-kb *Hin*dIII fragment from pKK34-1 (13). This fragment was then inserted into the *Sma*I site of pTSV31A (Table 1) (kindly provided by A. Bender), generating plasmid pADE31P-1. A *Sac*I-*Xba*I fragment containing the *CDC34* gene was then released from pADE31P-1 and inserted into the *Sac*I-*Xba*I frag-

TABLE 1. Plasmids and phages used in this study

Plasmid or phage	Description	Source or reference
pGEM5Zf(+)	*ori* f1 *ori* Amp^r *lacZ*	Promega
pGEM7Zf(+)	*ori* f1 *ori* Amp^r *lacZ*	Promega
pYL100	1.0-kb *CDC34* promoterless PCR product amplified from the ATG codon to the *Fnu*DII site into pGEM5Zf(+) at *Eco*RV	This study
pYL110	pYL100 with *Spe*I-*Sal*I fragment of the polylinker removed	This study
pRS304	*ori* Amp^r *TRP1*	P. Hieter (38)
pRS306	*ori* Amp^r *URA3*	P. Hieter (38)
M13mp18	*ori lacI lacZ*	BRL
m1	M13mp18 + 1.0-kb *Sac*I-*Sph*I *CDC34* fragment from pYL110	This study
pSJ101	2μm, *LEU2* Amp^r, *GAL10* promoter	S. Johnson
pTSV31A	2μm, *ADE3 URA3* Amp^r	A. Bender
pYL150	pSJ101 + 1.0-kb *Sal*I-*Sph*I *CDC34* fragment from m1	This study
pBM756	*ARS1 CEN4 URA3* Amp^r, *GAL7* promoter	M. Johnston
pYL250	pBM756 + 1.0-kb *Sal*I-*Hin*dIII *CDC34* fragment from m1	This study
pGEM 34.B/S.Bam::HIS3	pGEM-1 + *cdc34*Δ::*HIS3*	13
pYL11	pYL150 with S73A mutation in *CDC34*	This study
pYL12	pYL150 with S73D mutation in *CDC34*	This study
pYL13	pYL150 with S73E mutation in *CDC34*	This study
pYL14	pYL150 with S73K mutation in *CDC34*	This study
pYL17	pYL150 with C95S mutation in *CDC34*	This study
pYL18	pYL150 with S97A mutation in *CDC34*	This study
pYL19	pYL150 with S97D mutation in *CDC34*	This study
pYL20	pYL150 with S97T mutation in *CDC34*	This study
pYL23	pYL150 with S139A mutation in *CDC34*	This study
pYL24	pYL150 with S139D mutation in *CDC34*	This study
pYL25	pYL150 with S73K and S97A mutations in *CDC34*	This study
pYL26	pYL150 with S73K and S97D mutations in *CDC34*	This study
pYL27	pYL150 with 12-residue deletion of G-103 to T-114 in *CDC34*	This study
pYL28	pYL150 with 12-residue deletion of G-103 to T-114 and S73K and S97A mutations in *CDC34*	This study
pYL29	pYL150 with 12-residue deletion of G-103 to T-114 and S73K and S97D mutations in *CDC34*	This study
pYLB2	pYL150 with G58R change in *CDC34*	This study
pYLD2	pYL150 with A62V change in *CDC34*	This study
pDEL21	*ori* Amp^r *URA3 rad6*Δ	M. Kupiec (21)
pRAD6	pSJ101 + 3.4-kb *Bam*HI fragment containing *RAD6* from plasmid pB2 (21)	This study
pADE31P-1	Blunt-ended 1.2-kb *Hin*dIII fragment containing *CDC34* from pKK34-1 (13), inserted into *Sma*I site of pTSV31A	This study
pGEM7-34(1)	1.2-kb *Sac*I-*Xba*I fragment of pADE31P-1 containing *CDC34* and *Sac*I-*Xba*I fragment of pGEM7Zf(+)	This study
pGEM7-34(2)	1.0-kb *Sph*I-*Xho*I fragment of pYL29 containing *CDC34* and *Sph*I-*Xho*I fragment of pGEM7Zf(+)	This study
pGEM7-34(3)	2.3-kb *Asp* 700 fragment of pGEM7-34(1) and 1.9-kb fragment of pGEM7-34(2)	This study
pRS304-2	1.2-kb *Apa*I-*Sac*I fragment of pGEM7-34(3) containing mutated *CDC34* and *Apa*I-*Sac*I fragment of pRS304	This study
pNM101	pRS306 + 1.2-kb *Kpn*I-*Sac*I fragment of pRS304-34 with 12-residue deletion of G-103 to T-114 and S73K and S97D mutations in *CDC34*	This study

ment of pGEM7Zf(+) (Table 1), generating plasmid pGEM7-34(1). A *Sph*I-*Xho*I fragment containing the mutated *CDC34* gene was also released from plasmid pYL29 (Table 1) and inserted into plasmid pGEM7Zf(+), generating pGEM7-34(2). An *Asp* 700 fragment containing the 3′ region of the *CDC34* gene was released from pGEM7-34(1) and replaced with the equivalent fragment from pGEM7-34(2) now containing the mutated region of *CDC34*, generating pGEM7-34(3). Plasmid pGEM7-34(3) now consists of the mutated *CDC34* coding region and the promoter region from the wild-type *CDC34* gene. A *Sac*I-*Apa*I fragment containing *CDC34* was released from pGEM7-34(3) and inserted into the *Sac*I-*Apa*I fragment of pRS304 (Table 1) (kindly provided by P. Hieter), generating pRS304-34. Finally, a *Kpn*I-*Sac*I fragment of pRS304-34 containing the *CDC34* gene was inserted into the *Kpn*I-*Sac*I fragment of pRS306 to generate pNM101.

Southern blotting. Yeast genomic DNA was isolated (30), digested with the appropriate restriction enzymes, separated by agarose gel electrophoresis, and transferred to nylon membrane as described previously (13). A PCR-generated fragment containing the coding region of the *CDC34* gene was used as a hybridization probe. Hybridization and visualization were performed by using a DNA labeling and detection kit (Boehringer Mannheim) as instructed by the manufacturer.

Western blotting (immunoblotting). YL10-1 and YL18 transformants were grown in S · Gal-Leu liquid medium at 23°C to mid-exponential phase. Cell lysates were prepared by the glass bead method described by Bram and Kornberg (3) in the presence of the following protease inhibitors: leupeptin (2 μg/ml), aprotinin (0.75 μg/ml), phenylmethylsulfonyl fluoride (0.75 μM), and benzamidine (1.5 mM). Cell lysate containing approximately 20 μg of protein was loaded per gel lane for sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis. Gel electrophoresis and transfer were performed as described by Burnett (4) except that a polyvinylidene difluoride instead of nitrocellulose membrane was used. Western immunoblot analysis was performed by standard methods (32). Affinity-purified anti-Cdc34 antiserum (13) (dilution of 1:1,000) was used as the primary antibody. The primary antibody was detected by the addition of the secondary antibody (anti-rabbit antibody conjugated to horseradish peroxidase), which was then visualized by enhanced chemiluminescence (ECL kit; Amersham) as instructed by the manufacturer.

PCR analysis. Yeast genomic DNA was isolated by standard techniques (30) and used for PCR analysis. PCR analysis was performed as described previously (23). The PCR products generated from strains MGG11 and ts171 were formed by using primers which annealed to the 5′ end (5′-TATGAGTAGTCGCAA AAGCACCG-3′) and the 3′ end (5′-CGCGTATGTATAAATTGATTCATGT TTTGC-3′) of the *CDC34* gene and cloned into the *Sma*I site of M13mp18 (Table 1). The PCR products generated from strains YNM102, MGG15, ts124, and ts391 were formed by using primers which annealed to the 5′ end (5′-ATT AACCATGGATATGAGTAGTCGCAAAAGCACC-3′) and the 3′ end (5′-CC GGGTCGACTATTTTCTTTGAAACTCTTTCTAC-3′) of the *CDC34* gene. The use of these primers generated PCR products which contained an *Nco*I site at the 5′ end of the *CDC34* gene and a *Sal*I site at the 3′ end. These sites were then utilized to clone these fragments between the *Nco*I and *Sal*I sites of pGEM5Zf(+) (Table 1).

TABLE 2. DNA templates, oligonucleotides, and the generated amino acid changes in *CDC34*

Template (phage single-stranded DNA)	Oligonucleotide	Oligonucleotide sequence[a]	Amino acid change(s)
m1	34-S73[b]	5′-GAAACTGTGGTGGATTAAAGGGAAAGTCT-3′ GGC C T	S73A, S73D, S73E, S73K
m1	34-C95S	5′-ATAGAAATAGAAAGCCTG-3′	C95S
m1	34-S97[b]	5′-CTTTGGTGTAAAATAGCAATACAAAGCCTG-3′ TT	S97A
S73K	34-S97	5′-CTTTGGTGTAAAAATAGCAATACAAAGCCTG-3′	S73K, S97A
m1	34-S97T	5′-GTAAAATAGTAATACAAAG-3′	S97T
m1	34-S97D	5′-GTGTAAAATATCAATACAAAG-3′	S97D
S73K	34-S97D	5′-GTGTAAAATATCAATACAAAG-3′	S73K, S97D
m1	34-S139[b]	5′-TCGACATTTGCTGGGGCGTTGATATTGGGG-3′ TT	S139A
m1	34-S139D	5′-TTTGCTGGGTCGTTGATAT-3′	S139D
m1	34-DEL	5′-CTGCACGGGGGACCA/ACTTTGGTGTAAAATAG-3′[c]	cdc34-$\Delta_{103–114}$
S73K/S97A	34-DEL	5′-CTGCACGGGGGACCA/ACTTTGGTGTAAAATAG-3′[c]	S73K/S97A-$\Delta_{103–114}$
S73K/S97D	KD-DEL	5′-CTGCACGGGGGACCA/ACTTTGGTGTAAAATATC-3′[c]	S73K/S97D-$\Delta_{103–114}$

[a] Altered amino acid codons are underlined.
[b] Degenerate oligonucleotides were random mixtures potentially containing each base listed in each column at the underlined position.
[c] The slash indicates the position of the internal deletion.

RESULTS

Construction of *cdc34* point mutations. A partial sequence comparison of the NH_2-terminal catalytic domains of the known yeast E2 enzymes is shown in Fig. 1. Three conserved regions of particular interest, including the region bearing the active site cysteine 95 involved in ubiquitin transfer, are shown. Within these regions are several residues that define distinct classes of E2 catalytic domains. Cdc34 and Ubc7 have a serine at position 73 (as determined in Cdc34]), while other E2 enzymes (including Ubc1, Rad6, Ubc4, Ubc5, and Ubc8) contain a lysine residue at the corresponding positions. The positions corresponding to S-97 and S-139 within Cdc34 (the corresponding positions are also serines in Ubc7) are aspartic acid residues in many other E2 enzymes. While Ubc6, Ubc9, and Pas2 are somewhat distinct, each of these enzymes contains these conserved domains with distinct variations at the positions corresponding to those described above. The variation at these positions within otherwise conserved regions may account for some of the genetic and biochemical specificity of Cdc34 as well as the other E2 enzymes.

To determine the importance of these serine residues in Cdc34 function, we have constructed a series of point mutations (Fig. 1 and Table 2) within the *CDC34* coding sequence (see Materials and Methods). Each serine was changed to either alanine or aspartic acid (as well as other amino acid residues for S-73 and S-97). Alanine was chosen to replace the Cdc34 unique serines since it lacks only a hydroxyl group (compared with serine) and so represents a relatively conservative change (7). In addition, alanine is a very common amino acid in both the interior and the solvent-exposed surface of globular proteins. Thus, it is likely that alanine substitutions will have a minimal effect on the tertiary folding of the catalytic domain. Aspartic acid replacements were constructed because aspartic acid is the amino acid often found at the positions corresponding to S-97 and S-139 of Cdc34 within many other E2 enzymes. Serine 73 was also changed to lysine because this is the amino acid typically found at this position in many other E2 enzymes. This position was also changed to glutamic acid because an E2 enzyme encoded by the African swine fever virus (which resembles the Cdc34 class of catalytic domains) contains a glutamic acid residue at the corresponding position (14). We also made a serine substitution at cysteine 95, which is a component of the active site (5, 6).

Complementation of a *cdc34* temperature-sensitive mutation by mutated alleles. When the wild-type *CDC34* gene is placed into the expression vector pSJ101 (creating pYL150; see Materials and Methods and Table 1) and YL10-1 cells containing this plasmid are grown on galactose medium, pYL150 can complement the temperature-sensitive (Ts^-) defect of *cdc34-2* (Fig. 2, colony 1A; Table 3, S73A) and the Cdc34 protein is overproduced (Fig. 3; compare lanes 1 and 2). Plasmid pYL150 can also complement the Ts^- defect of YL10-1 when cells harboring this plasmid are grown on dextrose medium (Fig. 2, colony 1A; Table 3, *CDC34*). However, no overproduction of the Cdc34 protein can be observed under these conditions (22). We therefore used the conditions of growth on dextrose or galactose medium to allow us to observe the effects of our various *CDC34* mutated alleles when they are expressed at low or high levels, respectively.

To assess the functions of the mutant proteins encoded by the *CDC34* mutated alleles described above, a series of plasmids containing the various *cdc34* mutations (Table 1) was transformed into YL10-1. Isolated transformants were transferred onto medium lacking leucine, which selected for the presence of the plasmid, and then incubated at 23°C (the permissive temperature for YL10-1). The ability of each mutated *CDC34* allele to rescue the Ts^- phenotype of YL10-1 was assessed by the growth of its corresponding replica-plated colony patch at 36°C.

The results are summarized in Fig. 2 and Table 3. All of the mutations encoding changes at S-73 that we examined rescued the Ts^- defect of YL10-1 on either galactose or dextrose medium (Table 3, S73A, S73E, and S73K), indicating that S-73 is not critical to Cdc34 function. On the contrary, neither S97A nor S97D mutant Cdc34 protein was capable of supporting YL10-1 growth at 36°C, even when the cells were grown on galactose medium (Table 3). Introduction of the very conservative S97T substitution into Cdc34 allowed YL10-1 cells to grow well on galactose medium at 36°C, although growth was poor on dextrose medium at 36°C (Table 3). Mutated alleles

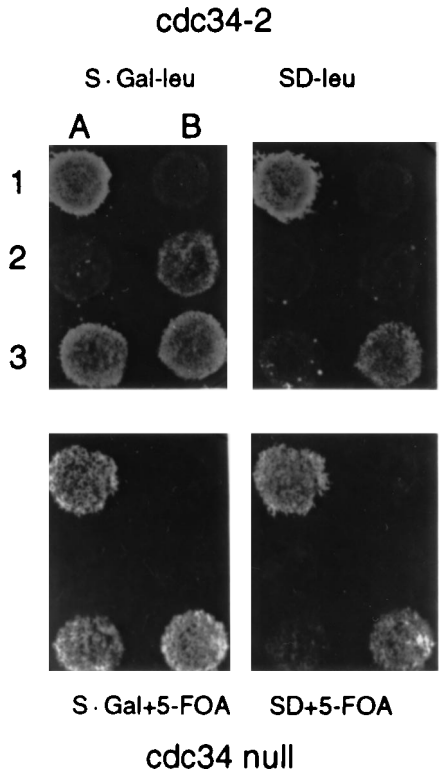

FIG. 2. Functional analysis of wild-type and mutated *CDC34* alleles. YL10-1 (*cdc34-2*) and YL18 (*cdc34* null) were transformed with plasmids expressing the wild-type allele or mutated alleles of *CDC34* containing either the $\Delta_{103-114}$ deletion or combinations of mutations. Cells were replica plated to the indicated medium at 36°C. Results were essentially the same for YL18 containing the various plasmids and grown at 23°C (22). 1A, wild-type *CDC34*; 1B, vector alone; 2A, S73K/S97A; 2B, S73K/S97D; 3A, $\Delta_{103-114}$; 3B, S73K/S97D-$\Delta_{103-114}$.

encoding the chanegs S139A and S139D were both capable of rescuing the Ts$^-$ growth defect of YL10-1 when cells were grown on galactose medium, but only the allele encoding the S139A change rescued growth at 36°C on dextrose medium (Table 3). The allele encoding the C95S mutant protein failed to complement, as expected (Table 3), confirming that the ubiquitin-conjugating activity of Cdc34 is essential for cell cycle progression.

The levels of these mutant Cdc34 proteins were detected by Western immunoblotting (see Materials and Methods); in galactose medium, the mutant proteins were overproduced to about the same level as the wild-type protein (Fig. 3). Thus, differences in the abilities of the various constructs to complement the *cdc34-2* allele could not be attributed to differences in the levels of production of the different mutant proteins.

Complementation of a *cdc34* disruption mutation. The results presented above indicated that except for amino acid residues C-95 and S-97, which are essential, changes at the amino acid residues that we examined do not completely abolish Cdc34 function. However, this complementation might have required the defective but partially functional Ts$^-$ protein. To test this possibility, we examined the ability of each mutated *cdc34* allele to rescue the growth defect of a *cdc34* disruption strain (see Materials and Methods). *CDC34* function in this strain is maintained by plasmid pYL250, which contains a wild-type *CDC34* gene (under the control of the *GAL7* promoter) and the *URA3* selective marker (Table 1). Although YL18 cells are viable when grown on galactose medium, these cells rapidly die and fail to form a colony when shifted to dextrose medium (22) or to medium containing 5-FOA (Fig. 2, colony 1B).

Each plasmid containing a mutated *cdc34* allele was transformed into YL18, and individual transformants were maintained on S · Gal-Leu medium. Complementation was assessed by the ability of each plasmid to allow colony formation after selection against pYL250, using 5-FOA on both galactose and dextrose media at 23°C. These results essentially paralleled those obtained with the Ts$^-$ strain YL10-1 (Table 3). Thus, most of the mutated *cdc34* alleles have complementing ability independent of the presence of the *cdc34-2* allele.

One difference between the results seen with the Ts$^-$ strain and disruption strain concerned the S97T mutant protein. Although this protein supported growth of the disruption strain on galactose or dextrose medium, it supported the growth of the Ts$^-$ strain only poorly on dextrose medium (Table 3). It is unclear whether this difference results from genetic background differences between the strains or from interference by the defective Ts$^-$ protein present in YL10-1 with the function of the S97T mutant protein.

Construction and analysis of the *cdc34*-$\Delta_{103-114}$ deletion mutation. In addition to the distinctive serine residues present at positions 73, 97, and 139, the Cdc34 protein also contains an insertion of 12 amino acids (positions 103 to 114) that is not found within the Ubc1 class of enzymes (Fig. 1). As this domain has been found in E2 enzymes capable of catenating ubiquitin to itself, Cook et al. (5) have suggested that this domain may be directly involved in this reaction. To determine the importance of this domain for Cdc34 function, we constructed a deletion allele which encoded a mutant protein lacking this region (see Materials and Methods) and performed complementation analysis as described above. Unexpectedly, *cdc34*-$\Delta_{103-114}$ not only restored growth to the Ts$^-$ strain YL10-1 at 36°C when grown on galactose medium but also allowed the disruption strain YL18 to grow on galactose

TABLE 3. Complementation of *cdc34* mutations by mutated *CDC34* alleles

Plasmid	Growth[a]			
	cdc34 Ts$^-$ mutant (36°C)		*cdc34* disruption mutant (23°C)	
	S · Gal-Leu	SD-Leu	S · Gal-5-FOA	SD + 5-FOA
Vector only	−	−	−	−
CDC34	+++	+++	+++	+++
S73A	+++	+++	+++	+++
S73D	+++	+++	+++	+++
S73E	+++	+++	+++	+++
S73K	+++	+++	+++	+++
C95S	−	−	−	−
S97A	−	−	−	−
S97D	−	−	−	−
S97T	+++	+	+++	+++
S139A	+++	++	+++	+++
S139D	+++	−	+++	−
S73K/S97A	−	−	−	−
S73K/S97D	+	−	+	−
$\Delta_{103-114}$	+++	−	++	−
S73K/S97A-$\Delta_{103-114}$	−	−	−	−
S73K/S97D-$\Delta_{103-114}$	+++	+++	+++	+++

[a] Degree of growth of cell patches after 3 days of incubation at the indicated temperature. +++, growth comparable to that of wild-type cells; ++, growth noticeably slower than of wild-type cells; +, growth barely detectable; −, no growth.

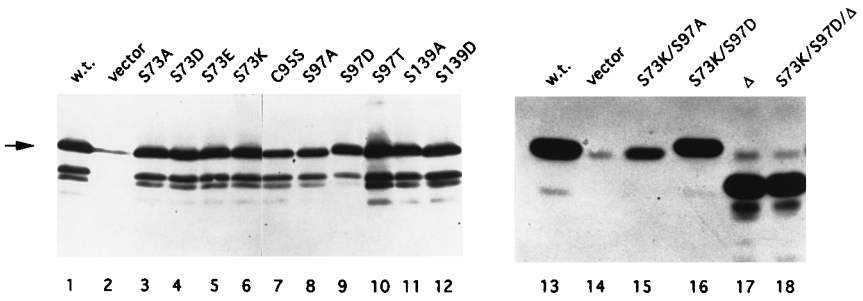

FIG. 3. Western blot analysis of the levels of mutant Cdc34 proteins. The vector in each case is pSJ101. The *cdc34* allele expressed from each plasmid in YL10-1 is indicated above each lane. The arrow indicates the position of full-length Cdc34 protein. w.t., wild type.

medium containing 5-FOA (Table 3; Fig. 2, colony 3A), indicating that this domain is dispensable for function. However, the *cdc34*-$\Delta_{103–114}$ allele could not complement either the *cdc34-2* allele or the *cdc34* disruption allele when cells were grown on dextrose medium (Table 3; Fig. 2, colony 3A). Thus, the *cdc34*-$\Delta_{103–114}$ allele can replace the wild-type *CDC34* gene only when overexpressed (Fig. 3, lane 17).

Intragenic suppression of *cdc34* mutations. A chimeric gene encoding a fusion protein consisting of the Rad6 catalytic domain and the COOH-terminal extension of Cdc34 is capable of restoring growth to yeast strains containing either *cdc34-2* or a disruption allele of *CDC34* (19, 39). Thus, the Rad6 catalytic domain is capable of replacing the Cdc34 catalytic domain. However, paradoxically, several Cdc34 enzymes containing changes (S97D or $\Delta_{103–114}$) to amino acids present in Rad6 do not function as well as wild-type Cdc34. One possibility is that Cdc34 and Rad6 utilize two distinct mechanisms for ubiquitin attachment and that these positions represent key residues that are important in these distinctive reactions. If this is the case, then while single changes may disrupt catalytic functions, creating these changes together may reconstruct a functional protein.

To test this possibility, we constructed a *CDC34* gene encoding both the S73K and S97D changes and examined whether the encoded mutant protein could rescue the Ts^- and disruption strains in the absence of wild-type Cdc34 as described above. This gene only marginally complemented the *cdc34-2* allele (Table 3, S73K/S97A; Fig. 2, colony 2B) and did so only when the protein was overproduced (Fig. 3, lane 16), as rescue was observed only on galactose medium and not on dextrose medium (Table 3; Fig. 2, colony 2B). Rescue of the disruption strain was even more difficult to detect, as extended incubation was required for a cell patch to show growth (22). In contrast, the mutant S73K/S97A protein was incapable of rescuing either Ts^- or disruption strains, even when overproduced on galactose medium (Table 3; Fig. 2, colony 2A). Thus, although the S73K/S97D double mutant is capable of only marginal Cdc34 function, it is clearly more functional than either the S97D or S73K/S97A mutant.

Inspired by these observations, we constructed an allele combining the S73K/S97D and $\Delta_{103–114}$ changes and performed complementation analysis as described above. Remarkably, the S73K/S97D-$\Delta_{103–114}$ mutant protein rescued both the Ts^- and disruption strains on both galactose and dextrose media (Table 3; Fig. 2, colony 3B; reference 22). In contrast, the gene encoding the S73K/S97A-$\Delta_{103–114}$ protein failed to rescue either strain (Table 3). Immunoblot analysis showed that the levels of the $\Delta_{103–114}$, S73/S97D-$\Delta_{103–114}$, and S73/S97A-$\Delta_{103–114}$ proteins on galactose were similar to that of wild-type protein (Fig. 3, lanes 17 and 18; reference 22). Thus, the S73K/S97D-$\Delta_{103–114}$ mutant protein functions much more efficiently than proteins containing either the S73K and S97D changes or the $\Delta_{113–114}$ change alone.

The *cdc34* S73K/S97D-$\Delta_{103–114}$ deletion mutation can replace the genomic copy of *CDC34*. To assess more fully the ability of the *cdc34* S73K/S97D-$\Delta_{103–114}$ mutation to replace the wild-type allele of *CDC34*, we set out to generate a strain in which the coding sequence of the *cdc34* S73K/S97D-$\Delta_{103–114}$ allele was substituted for the coding sequence of the genomic wild-type allele of *CDC34*. First, strain YNM101 was generated by inserting plasmid pNM101 (Table 1) at the *CDC34* locus (see Materials and Methods). We then attempted to generate a strain in which the wild-type copy of *CDC34* was removed and only the *cdc34* S73K/S97D-$\Delta_{103–114}$ allele remained. This was done by plating YNM101 onto SD-complete medium containing 5-FOA and selecting for resistant colonies. Since YNM101 contains a *URA3* gene (within pNM101) integrated at the *CDC34* locus, only those cells which have excised the vector sequences of pNM101 by homologous recombination will be able to grow on the selective medium (31). Since excision of the vector will leave one copy of *CDC34* which could be either the wild-type allele or the *cdc34* S73K/S97D-$\Delta_{103–114}$ allele, genomic DNA was isolated from 21 5-FOA-resistant colonies and subjected to PCR amplification using a pair of oligonucleotide primers which bracket the coding region of *CDC34* (see Materials and Methods). The amplified fragments were then digested with *Bam*HI, which will cleave the wild-type *CDC34* gene (13); however, this *Bam*HI site is absent from the mutated gene. A strain (YNM102) which apparently contained the mutated gene was subjected to further analysis. As can be seen in Fig. 4, Western immunoblot analysis indicated that YNM102 produced only the mutant protein. Furthermore, when the genomic DNA from YNM102 is cleaved with *Bam*HI and subjected to Southern analysis using the coding region of *CDC34* as a probe, a 5.0-kb band diagnostic for the presence of the wild-type allele of *CDC34* is missing (24). Finally, the PCR product generated from YNM102 genomic DNA was cloned into pGEM5Zf(+) and subjected to DNA sequence analysis, which again indicated that YNM102 contained the mutated allele of *CDC34* (24). These results indicate that expression of the *cdc34* S73K/S97D-$\Delta_{103–114}$ mutated gene can provide *CDC34* function when present in the genome as a precise replacement of the wild-type gene and expressed from the native *CDC34* promoter.

Molecular analysis of *cdc34* temperature-sensitive alleles. To identify additional residues critical for the function of the Cdc34 enzyme, we have also characterized several temperature-sensitive alleles of *CDC34*. The mutation responsible for the temperature sensitivity of *cdc34-1* has previously been de-

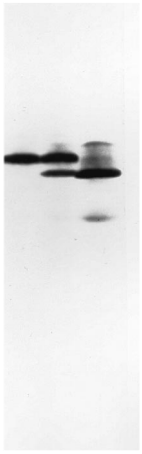

FIG. 4. Western immunoblot analysis of the levels of Cdc34 protein from yeast cells containing an integrated copy of the *CDC34* gene. Lane 1, Y382 (wild-type *CDC34*); lane 2, YNM101 (wild-type *CDC34* and *cdc34*-S73K/S97D-$\Delta_{103-114}$); lane 3, YNM102 (*cdc34*-S73K/S97D-$\Delta_{103-114}$).

scribed and creates a P71S change in the mutant protein (10). Two temperature-sensitive strains that failed to complement a *cdc34-1* strain were determined to be defective in DNA replication by Dumas et al. (9). They termed this complementation group *DNA6*. The *CDC34*/*DNA6* temperature-sensitive mutations within strains JS58 and JL210 were backcrossed several times to strains YPH52 and YPH54 (these strains are isogenic to each other) to generate strains MGG15 and MGG11, respectively. Strains ts171, ts214, and ts391, which also fail to complement a *cdc34-1* strain, were obtained from M. Winey. The *CDC34* gene from each strain was cloned as described in Materials and Methods and subjected to DNA sequence analysis. The results of this analysis are summarized in Table 4. The DNA sequence of each of these temperature-sensitive alleles of *CDC34* created amino acid changes at four different positions, including a mutation identical to that found in *cdc34-1* (10). All four mutations caused amino acid changes within a localized region of the Cdc34 protein. Three of these mutations cause changes in amino acid residues (G-58, P-71, and P-75) that are very highly conserved within all E2 enzymes to date. However, the other mutation creates a change at amino acid residue A-62. Close inspection of the sequences of the various E2 enzymes reveals that only enzymes that can be placed into the Cdc34 class of catalytic domains by the criteria described above have an alanine at this position (e.g., see Fig. 6). The fact that a mutation creating a change at amino acid residue A-62, which is unique to the Cdc34 family of enzymes, can generate a temperature-sensitive phenotype in the host cells indicates that this distinctive amino acid residue is important for *CDC34* function.

TABLE 4. DNA sequences changes in temperature-sensitive alleles

Strain	Allele	Codon change	Amino acid change
MGG15	*cdc34-2*[a]	^{172}GGG to AGG	G-58 to R
MGG11	*cdc34-2*	^{172}GGG to AGG	G-58 to R
ts171	*cdc34-4*	G^{185}CT to GTT	A-62 to V
ts214	*cdc34-5*	^{223}CCA to TCA	P-75 to S
ts391	*cdc34-1*	^{211}CCC to TCC	P-71 to S

[a] This result has also been described by Prendergast et al. (26).

TABLE 5. Temperature-sensitive complementation of *cdc34* null by cloned *cdc34-2* and *cdc34-4*

CDC34 allele	Growth[a]			
	23°C		36°C	
	SD+5-FOA	S · Gal+5-FOA	SD+5-FOA	S · Gal+5-FOA
Vector alone	−	−	−	−
CDC34	+++	+++	+++	+++
cdc34-2	+++	+++	−	+++
cdc34-1	+++	+++	−	+++

[a] Degree of growth of cell patches after 3 days of incubation at the indicated temperature. +++, growth comparable to that of wild-type cells; −, no growth.

Temperature-dependent complementation of a *cdc34* null mutation by *cdc34-2* and *cdc34-4*. To verify that the PCR DNA fragments cloned above encoded temperature-sensitive alleles of *CDC34*, DNA fragments encoding *cdc34-2* and *cdc34-4* were cloned into pSJ101 as described for the other *CDC34* mutated genes, generating plasmids pYLB2 and pYLD2, respectively (see Materials and Methods). These plasmids were transformed into YL18, and the abilities of these plasmids to complement a *cdc34* null allele were examined at 23 and 36°C when the expression levels of the genes were low (growth on dextrose) or high (growth on galactose) as described above. Under conditions of low expression, each allele can complement the *cdc34* null allele at 23°C but fails to complement at 36°C (Table 5). Thus, both cloned temperature-sensitive alleles can complement a *cdc34* null allele in a temperature-dependent manner under these conditions. However, both alleles can complement the *cdc34* null allele at both temperatures when overexpressed (Table 5), indicating that the temperature sensitivity of these alleles is influenced by the expression level of *CDC34*.

Complementation of a *rad6* disruption strain. Kolman et al. (19) and Silver et al. (39) have shown that when the COOH-terminal extension of Cdc34 is fused to the catalytic domain of Rad6, the resulting chimeric enzyme can rescue the loss of either wild-type Rad6 or Cdc34 function. Because the catalytic domain of the S73K/S97D-$\Delta_{103-114}$ protein resembles that of Rad6, we determined whether the S73K/S97D-$\Delta_{103-114}$ protein could rescue the growth or UV sensitivity defects of a *rad6* mutant. A *rad6* disruption strain, Grad6-1, was transformed with plasmids pRAD6 (*RAD6*), pYL150 (*CDC34*), pSJ101 (vector), pYL25 (*cdc34* S73K/S97A), pYL26 (*cdc34* S73K/S97D), pYL27 (*cdc34*-$\Delta_{103-114}$), and pYL29 (*cdc34* S73K/S97D-$\Delta_{103-114}$). Single transformants were patched onto SD-Leu medium and then replica plated to S · Gal-Leu medium at 23°C to allow expression of the constructs. The *RAD6* gene is not essential, but *rad6* mutants have a slower growth rate than wild-type strains (21). Only *RAD6* itself conferred a faster growth rate on Grad6-1 (22), indicating that none of the *cdc34* genes rescued the growth deficiency of the *rad6* disruption strain. In addition, only *RAD6* itself could rescue the UV sensitivity of the *rad6* disruption strain (Fig. 5). Thus, although the 12-residue internal deletion restores the function of the S73K/S97D double-mutant protein essentially to that of wild-type Cdc34, the mutant protein does not gain Rad6 functions that affect either proliferation or UV resistance.

DISCUSSION

Within the family of E2 enzymes, there are several regions common to all members characterized to date. An intriguing

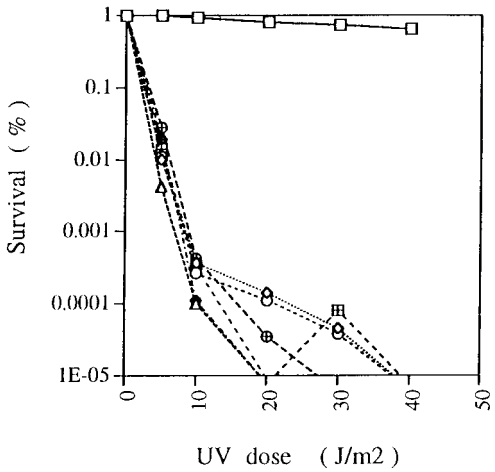

FIG. 5. UV sensitivity of a *rad6* disruption strain harboring various mutated *cdc34* genes. Mid-exponential-phase cell suspensions of Grad6-1 (*rad6* disruption) transformed with the indicated constructs were plated on S · Gal medium and irradiated with UV light at the indicated doses. The survival fraction was determined relative to unirradiated control cells. Symbols: □, pRAD6; ◇, pYL150; ○, pSJ101; △, S73K/S97A; ⊞, S73K/S97D; ◆, cdc34-$\Delta_{103–114}$; ⊕, S73K/S97D-$\Delta_{103–114}$.

feature of the Cdc34 protein is the presence of several distinct amino acid residues within conserved regions in the NH_2-terminal catalytic domain that distinguish it from Ubc1 and a number of other E2 enzymes. To ascertain the importance of these amino acid residues to Cdc34 function or regulation, we have mutated the *CDC34* gene to create changes at these positions. Our results demonstrate that amino acid residue S-73 can be altered to a variety of other amino acids without affecting the function of Cdc34; however, S-97 is essential for the in vivo cell cycle function of Cdc34. Removal of a 12-residue insert absent from Ubc1-like enzymes results in a protein that clearly functions more poorly than the wild-type enzyme. Not surprisingly, changing the catalytic cysteine at position 95 to a serine causes Cdc34 to become nonfunctional.

Kolman et al. (19) and Silver et al. (39) have demonstrated that when the catalytic domain of Rad6 is fused to the COOH-terminal extension of Cdc34, the resulting chimeric protein retains the functions of both Rad6 and Cdc34. Paradoxically, several changes (S97D; loss of the 12-residue insert) make Cdc34 more closely resemble the Rad6 protein; yet these mutant proteins function more poorly than the wild-type enzyme. We reasoned that while chimeric proteins might lose function, making these changes in concert might create a functional enzyme. Our results have demonstrated that this is indeed the case. A Cdc34 enzyme that contains both the S73K and S97D changes displays a subtle complementing effect on both *cdc34-2* and a disruption allele. The combination of these changes with deletion of the 12-amino-acid insert results in a Cdc34 enzyme that is functionally indistinguishable from wild-type Cdc34. This finding is further supported by the fact that integrative replacement of the wild-type genomic copy of the gene with the mutated allele leads to a viable haploid yeast strain with no obvious phenotypic differences from a wild-type strain. These results provide a compelling genetic argument for a functional interaction between these positions. However, while this triple-mutant protein now resembles the Rad6 protein at these positions, it is not capable of replacing Rad6 function.

The genetic interaction seen between these residues is made more compelling by comparing several of the various subfamilies of E2 enzymes to each other (Fig. 6). The Cdc34/Ubc7 family also contains two other proteins, a human homolog of Cdc34 (25) and the Ubc7 protein of wheat (44). Both of these proteins have the features diagnostic of the Cdc34 family. Highly related subfamilies have also been described for enzymes related to Ubc4 (43, 47) and Rad6 (18). A comparison of the consensus sequences of these families demonstrates that only a small number of the amino acid residues that are absolutely conserved among the four members of the Cdc34 family are also unique to this family: A-62, S-73, I-96, S-97, and the insert from 103 to 112 (Fig. 6). Our results clearly indicate the importance of the interaction between S-97 and the insert at 103 to 112. Cloning and sequencing of temperature-sensitive alleles of *CDC34* have shown that one of these alleles (*cdc34-4*) encodes a change from alanine to valine at position 62. Thus, the unique identity of the catalytic domain of Cdc34 seems limited to these residues, several of which we have shown to be critical positions in the function or regulation of Cdc34.

Recently, the crystal structures for both the Ubc1 enzyme of *Arabidopsis thaliana* and the Ubc4 enzyme of *S. cerevisiae* have been solved (5, 6). These two enzymes have strikingly conserved secondary and tertiary structures, suggesting that these proteins illustrate a common overall structural theme for the

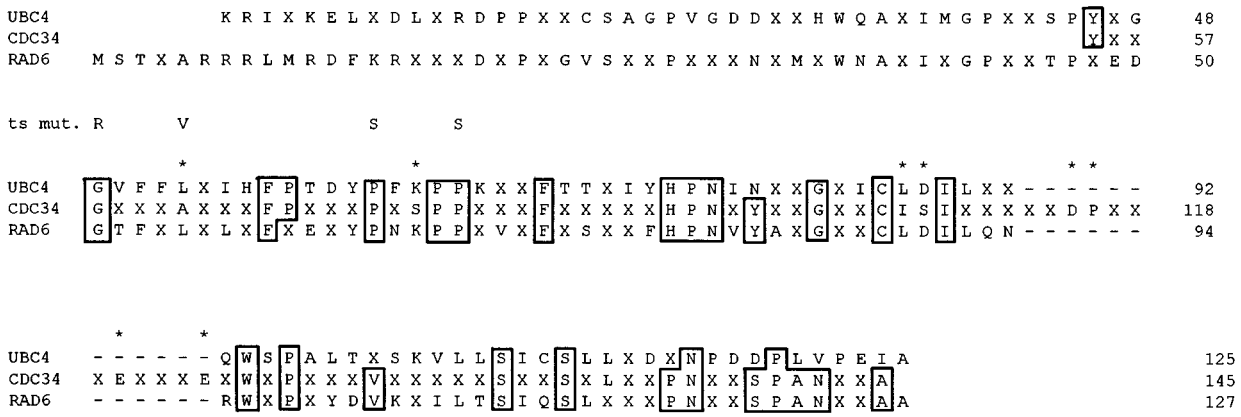

FIG. 6. Alignment of the Ubc4, Cdc34, and Rad6 subfamily consensus amino acid sequences. An amino acid indicated at a specific position means that that amino acid is absolutely conserved in the family members identified to date, whereas an X represents a variable position. Residues common to the Cdc34 and the Ubc4 or Rad6 subfamilies are boxed. Conserved residues that are unique to the Cdc34 subfamily are marked by asterisks.

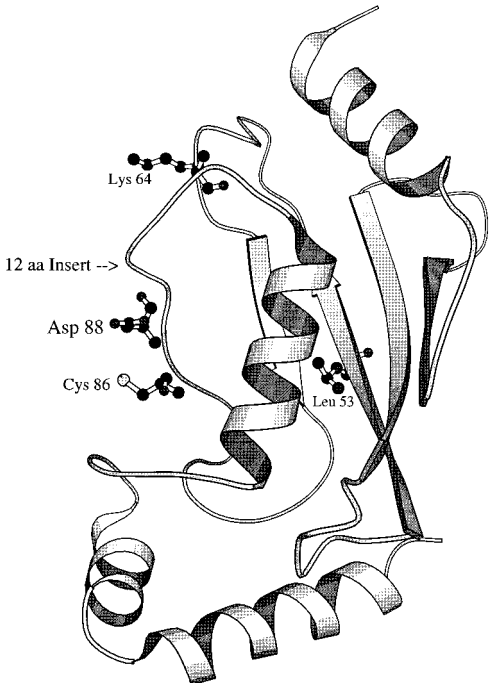

FIG. 7. Crystal structure of Ubc4 from *S. cerevisiae* (6) generated from MOLSCRIPT (20). Residues K-64, L-53,and D-88 are indicated. The corresponding positions in Cdc34 are S-73, A-62, and S-97. The arrow represents the predicted position of the Cdc34 unique insert. aa, amino acid.

family of E2 enzymes, including Cdc34. Analysis of the positions within these two structures equivalent to S-73 and S-97 in Cdc34 shows that their positions relative to the catalytically active cysteine are conserved (Fig. 7). The S-97-equivalent residue (aspartic acid in both structures; Fig. 7) is quite close to the catalytic cysteine and immediately adjacent to the loop of alpha helix that is created by the remainder of the conserved catalytic domain. It is within this loop, just three to five residues COOH terminal from S-97, that the Cdc34 unique insert is located. The S-73-equivalent residues (lysine in both structures) are also quite proximal to this region (Fig. 7). They are located in an extended loop between the second and third beta strands. This location brings the lysine (S-73 equivalent) of each enzyme into a position just on the other side of the catalytic alpha helix from the S-97 residue. Thus, the S-97 and S-73-equivalent residues bracket the region occupied by the Cdc34 unique insert, which is unanticipated from the primary sequence. Our genetic evidence suggests that these three regions interact to create a functional catalytic domain and is thus consistent with the crystal structure. Surprisingly, the Cdc34 mutant protein containing S73K, S97D, and a deletion of the unique insert, which presumably restructures this region to resemble the crystalline structures, yields a biologically active protein. This observation suggests that the S-73/insert/S-97 domain structure may not be directly involved in substrate selection but perhaps has a more subtle effect on regulation of the activity.

Another of the amino acid residues distinct to the Cdc34 class of enzymes is A-62. This position is usually occupied by the bulky hydrophobic residue leucine in other E2 enzymes (valine in Ubc1). Interestingly, we have found that changing A-62 to valine causes Cdc34 to become temperature sensitive. Close examination of this region in the crystal structure of Ubc4 indicates that L53 (A-62 equivalent) is located in the middle of a beta strand and projects into the heart of the globular protein. L-53 interacts with I-38, I-55, I-68, F-70, L-104, and I-107 to form a hydrophobic core within the protein. Most of these hydrophobic residues are conserved among the other E2 proteins. The potential role of A-62 in the function or regulation of Cdc34 is less clear. It is presumably located in approximately the same environment as the corresponding L-53 of Ubc4. However, changing the alanine to a valine (which one would predict to stabilize the molecule) causes a loss of Cdc34 function at higher temperatures. This paradox suggests that the role of A-62 is interacting with domains of Cdc34 which are distinct from their Ubc4 counterparts. A-62 may interact with the large acidic COOH terminus of Cdc34, the other amino acid residues distinctive for Cdc34, or cofactor molecules. The solution of the crystal structures of Cdc34 itself will be necessary to define precisely the role of A-62.

We have also assessed the importance of S-139 in the function of the Cdc34 enzyme. This position is intriguing because while it is an aspartic acid residue at the corresponding position in Ubc4 and most of the other Ubc1-like enzymes, it is a serine residue in Rad6 (Fig. 1 and 6). While the catalytic domain of Rad6 can replace that of wild-type Cdc34 enzyme, the catalytic domain of Ubc4 fused to the COOH-terminal extension of Cdc34 has no detectable Cdc34 activity (19, 39). Consistent with this finding is the fact that Cdc34 protein containing the S139D change replaces the wild-type Cdc34 enzyme only when it is overproduced. Close inspection of the primary sequences indicates that the region surrounding the S-139-equivalent position of Rad6 is more closely related to Cdc34, while the rest of the catalytic domain more closely resembles the Ubc1 and Ubc4 enzymes (Fig. 1 and 6). These results suggest the possibility that this region of Cdc34 is involved in substrate recognition rather than regulation of enzymatic activity. Evaluation of the crystal structures available reinforces the importance of S-139. A serine residue is located in the equivalent position of the *A. thaliana* Ubc1 protein (5). The conserved domain about this residue forms a loop structure between two alpha helices. This position is very well conserved between the two structures and serves to bring the S-139-equivalent residue into close proximity to the catalytic cysteine. It is thus not surprising that this serine would serve as an important determinant in Cdc34 function. On the other hand, at least one other mutation, that creating S139A, can be tolerated without detectable loss of Cdc34 function.

Combining the genetic and crystallographic evidence leads to some interesting observations. The fact that S-73, insert, and S-97 interact genetically is not surprising given their proximity to each other in the crystal structures. The exact positioning of the insert and its interaction with these residues are not known, but that they could act together in either catalysis or cofactor recruitment is certainly plausible. In addition, Cdc34 appears to have a rather hydrophilic catalytic site, with serines in the important 97 and 139 positions. However, it does not tolerate a negative charge at these positions, since S97D is nonfunctional and S139D requires marked overproduction for function. This finding suggests that the substrate(s) for Cdc34 may contain recognition sites that have a markedly anionic character created by the presence of acidic amino acid residues or perhaps by the regulated presence of a phosphate group. The evidence presented here describes several key features of the Cdc34 catalytic pocket, and the mutants generated in this work may prove useful tools for the identification and characterization of regulatory proteins and substrates of the enzyme.

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