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Novel *CDC34* (*UBC3*) Ubiquitin-Conjugating Enzyme Mutants Obtained by Charge-to-Alanine Scanning Mutagenesis

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***CDC34* (*UBC3*) encodes a ubiquitin-conjugating (E2) enzyme required for transition from the G_1 phase to the S phase of the budding yeast cell cycle. *CDC34* consists of a 170-residue catalytic N-terminal domain onto which is appended an acidic C-terminal domain. A portable determinant of cell cycle function resides in the C-terminal domain, but determinants for specific function must reside in the N-terminal domain as well. We have explored the utility of "charge-to-alanine" scanning mutagenesis to identify novel N-terminal domain mutants of *CDC34* that are enzymatically competent with respect to unfacilitated (E3-independent) ubiquitination but that nevertheless are defective with respect to its cell cycle function. Such mutants may reveal determinants of specific in vivo function, such as those required for interaction with substrates or *trans*-acting regulators of activity and substrate selectivity. Three of 18 "single-scan" mutants (in which small clusters of charged residues were mutated to alanine) were compromised with respect to in vivo function. One mutant (*cdc34-109*, *111*, *113A*) targeted a 12-residue segment of the Cdc34 protein not found in most other E2s and was unable to complement a *cdc34* null mutant at low copy numbers but could complement a null mutant when overexpressed from an induced *GAL1* promoter. Combining adjacent pairs of single-scan mutants to produce "double-scan" mutants yielded four additional mutants, two of which showed heat and cold sensitivity conditional defects. Most of the mutant proteins expressed in *Escherichia coli* displayed unfacilitated (E3-independent) ubiquitin-conjugating activity, but two mutants differed from wild-type and other mutant Cdc34 proteins in the extent of multiubiquitination they catalyzed during an autoubiquitination reaction. Our results validate the use of clustered charge-to-alanine scanning mutagenesis for exploring ubiquitin-conjugating enzyme function and have identified additional mutant alleles of *CDC34* that will be valuable in further genetic and biochemical studies of Cdc34-dependent ubiquitination.**

Covalent attachment of the highly conserved protein ubiquitin to other eucaryotic proteins is required for many cellular functions, including stress resistance, selective proteolysis of most normal and abnormal short-lived proteins, cell cycle progression, and DNA repair (14, 27, 31). Ubiquitination of proteins is catalyzed by an elaborate multienzyme conjugation pathway whose components are conserved in eucaryotes (31). The first step in the ubiquitin ligation pathway is the ATP-dependent activation of ubiquitin via the formation of a thiol ester between the ubiquitin C terminus and a cysteine residue in the ubiquitin-activating (E1) enzyme. Ubiquitin is then transferred from the E1 to one of several ubiquitin-conjugating (E2) enzymes, also via a thiol ester formed with a conserved Cys residue. Finally, ubiquitin is transferred from the E2 to a protein substrate in a reaction that often requires a third factor called a ubiquitin protein ligase or E3 (14, 27, 31).

The yeast *Saccharomyces cerevisiae* has provided a genetically tractable system for studies of ubiquitin-dependent pathways (21, 32). *CDC34* (also called *UBC3*) was first identified as encoding an E2 on the basis of its homology to yeast *RAD6* (*UBC2*) (20). Two temperature-sensitive alleles of *CDC34* have been described; at their nonpermissive temperature both cause yeast cells to arrest their growth at the G_1-S-phase boundary of the cell cycle just prior to initiating DNA synthesis, with duplicated but unseparated spindle pole bodies (13, 20, 34). In vitro, the *CDC34*-encoded ubiquitin-conjugating enzyme synthesizes the formation of lysine 48-linked multiubiquitin chains (24). Since lysine 48-linked chains are required for the ubiquitin-dependent degradation of many substrates of the ubiquitin system (6, 22), it has been widely supposed that the function of the Cdc34 protein is to target for destruction proteins whose elimination is necessary for progression through the cell cycle. Two regulators of the yeast cell cycle, Far1 (5) and Sic1 (41), are plausible targets for Cdc34 activity (11, 38, 41). Far1 is required for mating factor-induced arrest of the cell cycle (5), while Sic1 appears to govern progression into S phase in the normal yeast vegetative cell cycle (11, 41). Both appear to act by inhibiting cyclin-dependent Cdc28 protein kinase activity through direct interaction with the cyclin-Cdc28 complex (5, 39, 40, 42, 43, 49).

Relatively little is known about how E2 ubiquitin-conjugating activity is targeted to specific substrates. E3 proteins (when they are required) appear to govern substrate selectivity through formation of a ternary complex with the E2 and its substrate (26, 28, 29, 45). Thus, the *UBR1*-encoded yeast E3 that governs substrate selection by the *RAD6*-dependent N-end rule proteolytic pathway forms a stable complex with the Rad6 protein and simultaneously binds target proteins that have a destabilizing amino-terminal residue (4, 10, 37, 48). Associated factors may play other roles in regulating E2 activity and E2-dependent functions. *CDC4* (44, 51) and *CDC53* (18a, 33) are two yeast genes that appear to participate with

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CDC34 in execution of the G_1-to-S-phase cell cycle transition. They do not resemble any previously described member of ubiquitination pathways and may act to govern Cdc34 enzyme activity directly rather than help to define its substrate selectivity (33).

Because it provides an essential function in *S. cerevisiae* and yields a clear and specific cell cycle phenotype when perturbed and because it can catalyze unfacilitated (E3-independent) ubiquitination reactions in vitro, Cdc34 protein is an attractive subject for structure function studies of ubiquitin-conjugating enzymes. Differences in E2 function necessarily reflect differences in E2 structure; the most striking differences are displayed by the class II E2s, which bear a carboxyl-terminal domain appended onto a conserved catalytic amino-terminal domain (21, 31). The C-terminal domains are dissimilar and clearly distinguish the class II E2s from one another and from the class I E2s, which lack a C-terminal extension. A 45-residue portion of the Cdc34 protein C-terminal domain (total length, 125 residues) contains a portable determinant that can confer Cdc34 function onto the N-terminal domain of the Rad6 (Ubc2) protein (34, 47, 48a). However, class I E2s also serve in specific cellular functions, indicating that they contain determinants of functional specificity within their N-terminal (catalytic) domains (31); it seems unlikely that class II E2s (like Cdc34) in contrast would concede all functional determinants to their C-terminal extensions. Moreover, the catalytic domains of Cdc34 and Rad6 can partially restore cell cycle function to a *cdc34 ts* strain, whereas Ubc4 does not (47), and a chimeric E2 formed by fusion of the Cdc34 C-terminal domain onto either yeast Ubc1 or yeast Ubc4 failed to provide Cdc34 cell cycle function (34). While not conclusive, the above observations suggest that there might also exist determinants of functional specificity within the N-terminal domain of Cdc34 as well.

We describe here the use of "charge-to alanine" scanning mutagenesis to search for mutations within the N-terminal domain of the Cdc34 protein that perturb its in vivo function. Specifically, our aim has been to identify mutants that are competent for unfacilitated (E3-independent) ubiquitin-conjugating activity but that are nevertheless deficient with respect to Cdc34-dependent cell cycle function. We expect that such mutations will affect function by perturbing determinants governing the regulation of its activity or substrate selectivity, for instance, by perturbing interactions with regulatory or specificity factors such as E3s. These efforts have yielded several mutants with novel in vivo and in vitro characteristics.

MATERIALS AND METHODS

Construction of *CDC34* yeast expression plasmids. All *CDC34* single-scan mutants were produced by unique-site elimination mutagenesis (9), except for G, K, and P, which were produced by PCR-directed mutagenesis (30). The double-scan mutants BC, LM, NO, and PQ were also produced by PCR-directed mutagenesis. Descriptions of oligonucleotides used for mutagenesis are available on request. The sequences of mutant genes were verified by double-stranded dideoxysequencing. Mutant and wild-type *CDC34*s encoded in YCplac111 (18) consisted of the 1.2-kb fragment containing a functional *CDC34* promoter and its coding sequence (the same described by Kolman et al. [34]) subcloned between the *Sal*I and *Sph*I sites of the vector. Mutant and wild-type *CDC34*s encoded in YCplac33 (18) and pMW29 contained a nine-residue hemagglutinin (HA) epitope tag appended to their carboxyl termini (35) and were also positioned between the *Sal*I and *Sph*I sites of the vector. A subset of mutants was put under control of the GAL1 promoter by subcloning the *Bsp*EII-*Hin*dIII fragment from the mutant into the large *Bsp*EII-*Hin*dIII fragment of YEplac112GAL-CDC34 (34). These constructs contained a unique *Nde*I site at the start of the *CDC34* coding sequence. To obtain integrating vectors encoding *CDC34* and its mutants, YCplac111 with a *CDC34* insert was cut with *Spe*I and *Bgl*II and then blunted and recircularized; this removed the CEN sequence and autonomously replicating sequence normally found in YCplac111. pMW29 is a *URA3*-marked CEN vector that contains a multiple cloning site positioned between the *GAL1* promoter and a *CYC* terminator and the *ADE3* gene (obtained from M. Solomon and originally constructed by M. Wahlberg). pMW29-CDC34 was made by subcloning a fragment that encoded epitope-tagged Cdc34-HA between the *Sal*I and *Sph*I sites of its multiple cloning site. The glutathione *S*-transferase (GST)-Cdc34 fusion protein expression vector pGST-CDC34 was made by subcloning the small *Xba*I-*Hin*dIII fragment of YEplac112GAL-CDC34 (containing the *CDC34* coding sequence) between the *Xba*I and *Hin*dIII sites of pGEX-KG (23), obtained from S. Roeder.

Construction of *CDC34* bacterial expression plasmids. *Escherichia coli* T7 expression vectors encoding wild-type *CDC34* and scan mutants I, J, and K were made by subcloning the *Nde*I-*Hin*dIII fragment encoding the entire *CDC34* scan mutants coding sequence (obtained from the appropriate YEplac112GAL expression construct) into pET17xb (Novagen) between its *Nde*I and *Hin*dIII sites. T7 expression vectors encoding *CDC34* double-scan mutants LM, NO, and PQ were constructed by subcloning the 0.8-kb *Nae*I-*Hin*dIII fragment from the *CDC34* mutants into pET17xb-CDC34 between its unique *Nae*I and *Hin*dIII sites, thereby swapping in the mutant sequences in place of wild-type sequences. T7 expression vectors encoding *CDC34* double-scan mutant BC were constructed by amplifying the *CDC34* coding region of YCplac111-CDC34-BC with oligonucleotides CDC34-5-2 and CDC34-3 (34) and then by subcloning between the *Nde*I and *Hin*dIII sites of pET17xb.

Yeast strains, growth, transformation, and microscopy. *S. cerevisiae* was propagated on yeast extract-peptone-dextrose (YPD) or synthetic complete (SC) medium with the appropriate selective omissions (2). Lithium acetate transformation of *S. cerevisiae* was performed by the method of Schiestl and Gietz (46). For photomicroscopy, cells were fixed in 5% formaldehyde for at least 3 h. Photomicrographs were taken with a Zeiss Axioscope through a 63 × 1.4 NA objective in DIC mode. The strains used throughout this study were made from YMW1 (*MATα ade2-1 ade3-22 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*; obtained from M. Solomon and originally constructed by M. Wahlberg). To construct the *cdc34* null tester strain ZY16, YMW1 was first transformed with pMW29-CDC34. The *Sca*I-*Ase*I fragment of pZ63 (containing a *HIS3* disruption of *CDC34* where the *HIS3* cassette was subcloned between the *Bam*HI and *Eco*RI sites of *CDC34*) was then transformed into this strain. His^+ transformants were selected (this was possible despite the *ade3* mutation of YMW1 because of the wild-type *ADE3* gene on pMW29) and the presence of a genomic *HIS3* disruption of *CDC34* was verified by Southern analysis. For some experiments, YCplac22GAL-CDC34 (which is a *TRP1*-marked plasmid based on YCplac22 [18] that encodes *CDC34* under the control of the *GAL1* promoter) was transformed into ZY16, and the pMW29-CDC34 plasmid was subsequently evicted by growth on 5-fluoroorotic acid (5-FOA) to yield ZY38 containing YCplac22GAL-CDC34. Yeast strains made by stably integrating a *CDC34* variant at the *leu2* locus of ZY16 are referred to as ZY38-X, where X denotes the letter designation given to the *CDC34* variant (see Table 1). To construct the ZY38-X strains, integrating vector constructs containing the *CDC34* scan mutants (see above) were digested with *Xcm*I and transformed into *S. cerevisiae*. Integration into the genomic *LEU2* site was confirmed by Southern analysis.

Assay for Cdc34-dependent cell cycle function. In the absence of any other plasmid, the viability of strain ZY16 was dependent on galactose-dependent expression of *CDC34* from pMW29-CDC34; ZY16 was inviable when grown on either glucose-containing plates (glucose represses expression of *CDC34* from pMW29-CDC34) or on 5-FOA-containing media (5-FOA prevents growth as long as the presence of the *URA3*-marked pMW29-CDC34 is required). To test for the ability of a *LEU2*-marked plasmid to complement essential *CDC34* function in vivo, the plasmid was transformed into the tester strain ZY16 and then grown to saturation in SC−Ura−Leu+galactose. Cultures were diluted to an optical density at 600 nm of 1.0 and stamped onto plates of SC−Ura−Leu+galactose, SC−Ura−Leu, or SC supplemented with 5-FOA (SC+FOA) with a 48-pin inoculating manifold (a "frogger"). The plates were then incubated at the indicated temperatures. Growth on either 5-FOA- or glucose-containing media indicates that the *LEU2*-marked plasmid could supply essential *CDC34* function without the presence of pMW29-CDC34 or expression of its Cdc34-encoding insert. Results obtained with either 5-FOA- or glucose-containing test media were identical. To assay single-scan mutant P encoded in YCplac33, strain ZY16 was first transformed with (*TRP1*-marked) YCplac22GAL-CDC34. The *URA3*-marked pMW29-CDC34 was then evicted by growth on SC+FOA medium containing galactose rather than glucose. This yielded the tester strain ZY31 (containing YCplac22GAL-CDC34), which like ZY16 was inviable on glucose-containing media unless transformed with a second plasmid that had *CDC34*-complementing activity. The *URA3*-marked plasmid YCplac33-CDC34-P was then transformed into ZY31 and plated onto glucose to assay for its complementing activity.

Preparation of Cdc34 polyclonal antisera. BL21 cells containing pET17xb-CDC34-HA were grown and harvested according to Novagen's protocols. Cdc34 protein was purified by the method of Chau et al. (3), except that a Resource Q column (Pharmacia) was used instead of a Mono-Q column. As the final step in the purification, Cdc34 protein was subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and the 45-kDa (apparent molecular mass) band was excised from the gels following a brief staining with Coomassie blue. The gel bands were neutralized by being washed in water and macerated by passage through a 22-gauge needle five times. GST-Cdc34 protein was purified from *E. coli* transformed with pGST-CDC34 according to standard

methods (2). New Zealand White rabbits were injected with 200 μg of Cdc34 protein. The rabbits were boosted at 4-week intervals once with 200 μg of wild-type protein and twice with 1 mg of GST fusion protein. Anti-Cdc34 immunoglobulin G antibodies were purified from serum on a T-gel (Pierce Chemical Co.) according to the manufacturer's instructions and then on an Affigel-15 GST-Cdc34 affinity column (Bio-Rad) according to standard protocols (25).

Detection of Cdc34 protein from bacterial and yeast lysates by Western blotting (immunoblotting). Lysates for Western blot analysis were prepared as follows. Bacterial lysates were made by harvesting 1 ml of a bacterial culture grown in Luria broth (LB) by centrifugation, washing the sample with 1 ml of phosphate-buffered saline, resuspending it in 50 μl of 10 mM Tris-HCl (pH 7.5)–1 mM EDTA (TE) and 50 μl of 2× SDS loading dyes, and boiling the mixture for 5 min. Yeast lysates were made by resuspending 0.1 g of yeast cells in 0.5 ml of ice-cold lysis buffer [0.4 M $(NH)_4SO_4$ (pH 7.5), 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM EDTA, 1% Nonidet P-40, 0.1% SDS], adding the cells to 0.5 ml of glass beads (0.5 mm diameter) in a 2-ml O-ring-cap centrifuge tube, and grinding them in a Mini-BeadBeater-8 (Biospec Products) set on homogenize for two 2-min cycles with 2 min on ice in between. Tubes were spun at 14,000 × *g* in a microcentrifuge, and protein concentrations were determined by Bradford assays. Protease inhibitor cocktails (made up as two 1,000× stock solutions in water or dimethyl sulfoxide) were designed after those described elsewhere (1) and added just prior to lysis. PIC-D (1,000×) contained 88 mg of phenylmethylsulfonyl fluoride (0.5 M) per ml, 5 mg of pepstatin A per ml, 1 mg of chymostatin per ml, 1.1 mg of phosphoramidon per ml, and 7.2 mg of E-64 per ml in dimethyl sulfoxide; 1,000× PIC-W contained 208 mg of benzamidine (1 M) per ml, 131 mg of aminocaproic acid (1 M) per ml, 5 mg of aprotinin per ml, 1 mg of leupeptin per ml, and 190 mg of sodium metabisulfite (1 M) per ml in water. Also, *N*-ethylmaleimide (Aldrich) was added to a final concentration of 20 mM as indicated. Total protein extracted ranged from 10 to 50%, on the basis of using the Bradford assay and 10% wet weight as indices of total protein in the yeast sample. Cdc34 protein was more reproducibly detected in samples with 25% or more total protein recovered.

For Western blotting, up to 100 μg of protein was resolved by SDS-PAGE on a 12% (37.5:1) gel with a Bio-Rad Mini-Protean II electrophoresis apparatus. Proteins were transferred to Zeta-probe filters at 7 V/cm for 90 min at 4°C in Tris-glycine buffer. Zeta-probe filters were blocked in 5% milk blocker (Bio-Rad) in TBS (0.5 M NaCl, 20 mM Tris-HCl [pH 7.5]) for 3 h at 22°C, incubated with 120 ng of affinity-purified anti-Cdc34 antibody per ml in BLOTTO-TBS for 4 h at 22°C, washed three times for 10 min (each wash) in 50 ml of TBS plus 0.1% Tween 20, incubated with affinity-purified goat anti-rabbit alkaline phosphatase antibody conjugate (Bio-Rad) in BLOTTO-TBS for 45 to 60 min, washed three times for 10 min (each wash) in 50 ml of TBS plus 0.1% Tween 20, washed once with TBS for 5 min, and developed with the Immun-Lite chemiluminescence system according to the manufacturer's (Bio-Rad) instructions.

Metabolic labeling and immunoprecipitation of Cdc34 protein from *S. cerevisiae*. For [^{35}S]methionine labeling, cells were grown to 1×10^7 to 2×10^7/ml, collected by centrifugation, and washed twice in SC-Met+galactose. Cells were resuspended in 10 mM KP_i (pH 7.4)–2% (wt/vol) galactose with 200 μCi of Tran^{35}S-label (ICN) and incubated for 5 min at 30°C (or 23°C for scan mutant I). Cells were collected by centrifugation and resuspended in 100 μl of 2% galactose and added to 1 ml of ice-cold lysis buffer and 0.8 g of glass beads and lysed and cleared as described above. To preclear the lysate, 50 μl of immunoprecipitin (Bethesda Research Laboratories; washed according to the manufacturer's instructions) was added to 800 μl of the supernatant, incubated for 15 min on ice, and removed by centrifugation. A 3-μl aliquot of the supernatant was removed and analyzed for total incorporated counts by trichloroacetic acid precipitation onto filters. The supernatant was mixed with 1 μg of affinity-purified anti-Cdc34 antibodies and incubated on ice for 1 h. A total of 50 μl of immunoprecipitin (blocked by storage in BLOTTO for at least 24 h at 4°C) was added, and the samples were rocked for 30 min at 4°C. Immune complexes were collected by centrifugation for 2 min at 3,000 × *g*, and samples were washed five times for 2 min (each wash) with radioimmunoprecipitation assay buffer (0.15 M NaCl, 50 mM Tris [pH 8], 1% Nonidet P-40, 0.5% deoxycholate, 0.1% [wt/vol] SDS), using a Mini-BeadBeater-8 set on high, and then they were collected with centrifugation at 3,000 × *g* for 2 min. Before the final pelletting, the samples were transferred to a fresh microcentrifuge tube. Pellets were resuspended in 2× SDS loading dyes and boiled for 5 min. The amount of the samples loaded per lane was normalized to the total amount of counts incorporated into acid-insoluble material on the basis of trichloroacetic acid precipitation. Gels were fixed in methanol-acetic acid for 15 min, washed in 10% (vol/vol) glycerol for 15 min, and soaked in Autofluor (National Diagnostics) for 30 min before being dried under vacuum and autoradiography.

Bacterial expression of Cdc34 wild-type and mutant protein. *E. coli* BL21 transformed with pET17xb-CDC34 was grown in LB+ampicillin overnight, and 50 μl of the culture was inoculated into 5 ml of LB+ampicillin and grown for 2 h at 37°C. After removal of 1 ml of culture to assay for uninduced protein expression by SDS-PAGE, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the remaining 4 ml to a final concentration of 1 mM to induce Cdc34 production, and growth continued at 37°C for 2 h more. After removal of 1 ml from the induced culture to assay for Cdc34 production, the remaining 3 ml of cell culture was pelleted, resuspended in 200 μl of 50 mM Tris-HCl (pH 7.5) containing 1× PIC-D and PIC-W (see above), and sonicated to lyse the cells (a total of five pulses of 5 s each). The lysate was centrifuged at 4°C for 30 minutes at 16,000 × *g*, and equal amounts of total protein from the supernatant were used for assay of E2 activity. Protein concentrations of bacterial lysates were measured by the Bradford assay; typical lysate concentration was about 20 mg/ml. Analysis of Coomassie-stained SDS-polyacrylamide gels of bacterial lysates showed that approximately equal amounts of Cdc34 wild-type and scan mutant proteins were recovered in extracts.

TABLE 1. *CDC34* scan mutants

CDC34 mutant designation	Mutation(s)[a]
A	R4,K5A
B	R14,R17,E18A
C	D21,K23,K24A
D	E32,E34,D35,D36A
E	E51,D52A
F	K61,R65A
G	E68,D69A
H	R78A
I	R90,D91,R93A
J	D104,D108A
K	E109,D111,E113A
L	E122A
M	E133,D134A
N	D144,D148A
O	R150,K151,E154A
P	K157,R159,K161A
Q	E163,E165,R166A
R	K168,D170A
BC	R14,R17,E18A,D21,K23,K24A
LM	E122A,E133,D134A
NO	D144,D148A,R150,K151,E154A
PQ	K157,R159,K161A,E163,E165,R166A

[a] Given as, e.g., R4,K5A, signifying that R-4 and K-5 were changed to A.

In vitro assay for ubiquitin conjugation and thiol ester formation by Cdc34 protein. To assay for ubiquitin conjugation, reaction mixtures (20 μl) contained a final concentration of 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 2 mM ATP, 50 mM KCl, 0.2 mM dithiothreitol, and (when present) 500 ng of purified yeast E1, 85 μg of *E. coli* extract, 10 μg of histone H2B, and 550 ng of ^{125}I-ubiquitin (labeled by the chloramine T method). Ubiquitin-activating (E1) enzyme was purified from *S. cerevisiae* essentially as described above, except that yeast lysate was prepared from dried, 90% active, viable *S. cerevisiae* type II (Sigma; catalog number YSC-2) by a procedure to be described elsewhere. The reaction mixtures were incubated at the indicated temperature for 30 min, and then 10 μl of stop buffer (200 mM Tris-HCl [pH 6.8], 6% [wt/vol] SDS, 30% [vol/vol] glycerol, 15% [vol/vol] 2-mercaptoethanol, 0.06% [wt/vol] bromophenol blue) was added and incubated at 100°C for 3 min. Samples were electrophoresed by SDS–12.5% PAGE and then autoradiographed with Amersham HR film. Thiol ester bond formation between ubiquitin and E2 was detected in identical reaction mixtures lacking histone H2B; the stop buffer in this case did not contain 2-mercaptoethanol, and the samples were electrophoresed without prior boiling.

RESULTS

Mutagenesis strategy. We wished to identify novel N-terminal domain mutants of Cdc34 that were competent for unfacilitated E2 function but that were nevertheless unable to perform the Cdc34-dependent function required for the G_1-S-phase transition of the cell cycle. Because it seemed likely that the majority of randomly obtained mutations would simply catalytically inactivate the E2 enzyme (for example, by causing the protein to misfold), we examined the feasibility of using a directed mutagenesis strategy that was more likely to reveal mutants with the desired properties.

Specifically, we constructed a set of mutations within the N-terminal domain of Cdc34 protein by charge-to-alanine scanning mutagenesis (Table 1 and Fig. 1). In this approach, clusters of charged residues (which are likely to lie on the solvent-accessible surface of the protein) are mutated to alanine (12, 17, 50). Such mutations may disrupt protein-protein interactions while causing only a minor perturbation in protein

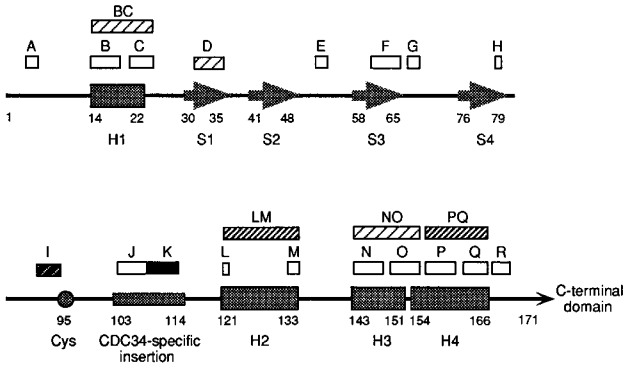

FIG. 1. Location of Cdc34 scan mutants relative to putative Cdc34 protein secondary structure. The assignment of secondary structural elements to the Cdc34 protein is based on a comparison of the *CDC34* and *A. thaliana UBC1* sequences and the X-ray crystallographic structure of the Ubc1 protein (7). The four putative alpha-helices (H1 through H4) are denoted by rectangles; the four putative beta-strands (S1 through S4) are denoted by large arrows. The active-site cysteine at residue 95 and the 12-residue Cdc34-specific insertion are also indicated. The residue numbers at the start and end of the various structural elements are noted just below their representations. The regions of Cdc34 spanned by the single-scan mutants (A through R) and the double-scan mutants (BC, LM, NO, and PQ) are indicated by the rectangles positioned over the representation of CDC34 putative secondary structure. The density of shading within the rectangles reflects the severity of the in vivo defect shown by the scan mutants, ranging from wild-type behavior (no shading) to severely dysfunctional (solid). See text for details regarding the specifics of the in vivo phenotypes displayed by the mutants.

structure; a priori, such mutations are more likely to produce an E2 that is competent for unfacilitated activity and yet unable to perform a facilitated (E3-dependent) in vivo function (because of failure to interact with an E3, for example).

We refer to each mutant by the letter designation shown in Table 1. Single-scan mutants (A through R) consisted of mutations of two or more charged residues within a window of five, except for mutants H and L, in which only one charged residue was mutated. After preliminary characterization of the single-scan mutants, we made double-scan mutants (BC, LM, NO, and PQ) which combined pairs of adjacent single-scan mutants such that they span and eliminate the charges that lie on each of four putative amphipathic alpha-helices of the Cdc34 protein (Fig. 1).

Complementation of essential Cdc34 in vivo function by *CDC34* scan mutants. YCplac111 plasmids containing either wild-type or mutant *CDC34* inserts were transformed into the *cdc34*Δ::*HIS3* tester strain ZY16 and assayed for their abilities to complement essential cell cycle function as described in Materials and Methods (Fig. 2 and 3). Identical results regarding their abilities to complement a null mutation were obtained when the single-scan mutants were stably integrated into the *leu2* locus of ZY16 (data not shown). Most of the single-scan mutants had wild-type *CDC34* complementing activity as judged by growth on plates, either as patches (Fig. 2) or struck out as single colonies (data not shown), but three mutants displayed clearly aberrant phenotypes. *CDC34* single-scan mutant D showed a partial temperature sensitivity that was reflected in poorer growth on plates and in liquid media at 37°C (data not shown), as well as in an aberrant morphology (Fig. 4). Mutant I was strongly temperature sensitive, showing an aberrant morphology and little growth on plates at 37°C (Fig. 2 and 4), and its growth and morphology were perturbed at 30°C as well (data not shown). Mutant K showed the most severe phenotype of all the single-scan mutants: it was all but unable to provide essential Cdc34 function at any temperature,

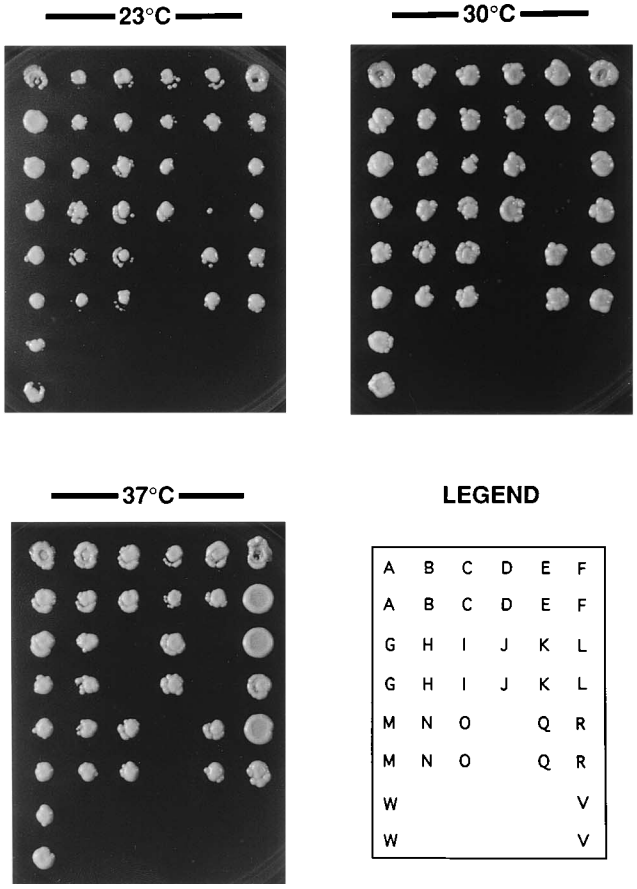

FIG. 2. Assay for in vivo activity of *CDC34* single-scan mutants. The tester strain ZY16 was transformed with *CDC34* single-scan mutants or controls encoded on YCplac111, grown in SC−Ura−Leu+galactose, and stamped onto SC−Ura−Leu plates with a 48-pin inoculating manifold as described in Materials and Methods. Plates were then incubated at the indicated temperature for several days. Letters A to R in the legend indicate the single-scan mutant encoded in the YCplac111 vector. W refers to ZY16 transformed with YCplac111-CDC34 (wild type); V refers to ZY16 transformed with YCplac111 (vector only).

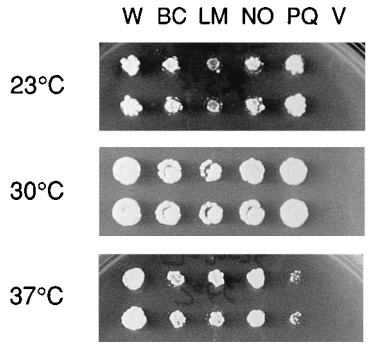

FIG. 3. Assay for in vivo activity of *CDC34* double-scan mutants. The tester strain ZY16 was transformed with *CDC34* double-scan mutants or controls encoded on YCplac111, grown in SC−Ura−Leu+galactose, and inoculated onto SC−Ura−Leu for growth at the indicated temperature as described in Materials and Methods. BC, LM, NO, and PQ indicate the double-scan mutants encoded in the YCplac111 vector. W refers to ZY16 transformed with YCplac111-CDC34 (wild type); V refers to ZY16 transformed with YCplac111 vector only.

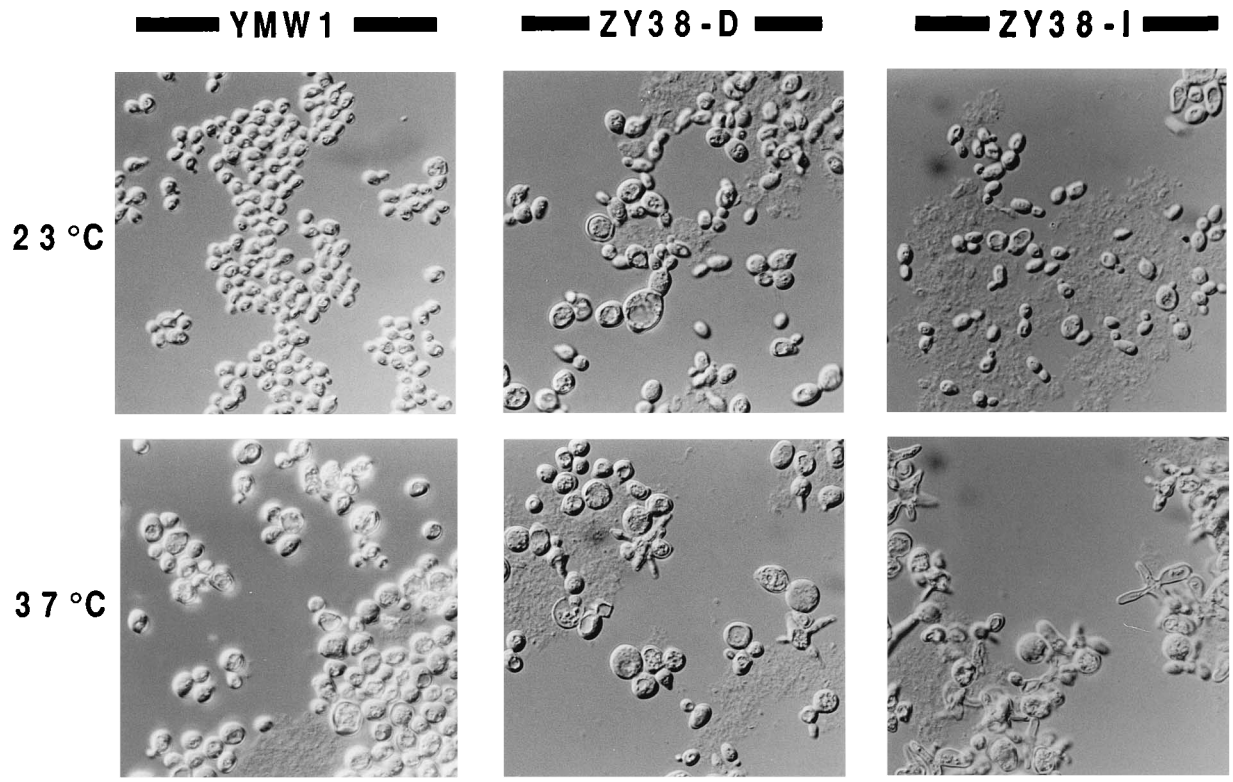

FIG. 4. Photomicrographs of wild-type *S. cerevisiae* and alanine scanning mutants D and I grown at the indicated temperatures. The ZY38 strain contains a *cdc34*Δ::*HIS3* disruption and the indicated alanine scanning mutant integrated into the *leu2* locus. ZY38 cells containing functional *CDC34* alleles integrated at the *leu2* locus were indistinguishable from those of the parental YMW1 strain (data not shown).

whether on a plasmid or integrated into the genome (after extended incubation, some slight growth was observed, whereas a control strain containing the YCplac111 failed to show any growth [Fig. 2 and data not shown]). However, mutant K complemented a *cdc34*Δ::*HIS3* disruption when it was overexpressed from a GAL1 promoter on a high-copy-number plasmid, and this complementation was not temperature sensitive (Fig. 5 and data not shown).

Unlike the other single-scan mutants, *CDC34* mutant P was assayed when expressed from a YCplac33 (*URA3*-marked) plasmid by transforming it into ZY16 after the (*URA3*-marked) pMW29-CDC34 had been shuffled out and replaced with a (*TRP1*-marked) YCplac22GAL-CDC34 construct. Like the majority of other single-scan mutants, mutant P complemented the *cdc34*Δ::*HIS3* at all temperatures examined (data not shown).

We sought to produce stronger defects in Cdc34 function by combining pairs of single-scan mutants to produce four double-scan mutants (Table 1; Fig. 1). YCplac111 plasmids encoding the double-scan mutants were transformed into ZY16 and assayed for Cdc34 function as was done for the single-scan mutants. The *cdc34*Δ::*HIS3* tester strain transformed with double-scan mutants indeed showed growth defects under assay conditions (growth on glucose-containing media [Fig. 3 and 5]). As judged by growth on plates, mutants BC and NO were slightly cold sensitive, but mutant LM was more strongly cold sensitive, showing inhibited growth at 23°C [Fig. 3] and little or no growth at 16°C (data not shown). Mutants BC and LM also showed partial temperature sensitivity when assayed for growth at 37°C, but mutant PQ showed significant temperature sensitivity (Fig. 3). However, *S. cerevisiae* containing any of the four double-scan mutants was viable at 30°C despite displaying some aberrant morphology (Fig. 3 and 5); thus, none was as severely compromised in cell cycle function as a tester strain containing single-scan mutant K (Fig. 2 and 4). As shown in Fig. 1, the double-scan mutants span (and eliminate) the charges that lie on each of four putative amphipathic alpha-helices. Their abilities to provide Cdc34 function under even a restricted range of temperatures, especially given the extensive amino acid changes made in them, stand in contrast to the severe defect of single-scan mutant K.

Immunological detection of mutant Cdc34 protein in vivo. We wished to confirm the production of Cdc34 mutant K protein in *S. cerevisiae* because of the severity of the mutant K phenotype. We used affinity-purified polyclonal antibodies raised against *E. coli*-produced Cdc34 protein to assay for expression of wild-type and mutant Cdc34 proteins. PAGE of immunoprecipitants from ^{35}S-labeled wild-type YMW1 extracts detected a band with an apparent molecular mass of 45 kDa. A band with slightly altered (lower) mobility was detected in PAGE of immunoprecipitants from extracts of ZY16, which produced only an epitope (HA)-tagged Cdc34-HA protein (Fig. 6B). The same differences in mobility were detected in Western blotting experiments of both bacterial and yeast extracts producing either Cdc34 or epitope-tagged Cdc34-HA protein (Fig. 6A). The relative mobilities of these bands, along with previous reports showing that Cdc34 protein runs anomalously at an apparent molecular mass of about 45 kDa on SDS-polyacrylamide gels (3, 19), clearly identify them as untagged and HA-tagged Cdc34, respectively.

When wild-type Cdc34 protein was produced from YCplac111-CDC34 in ZY16, both the untagged and the epitope-tagged

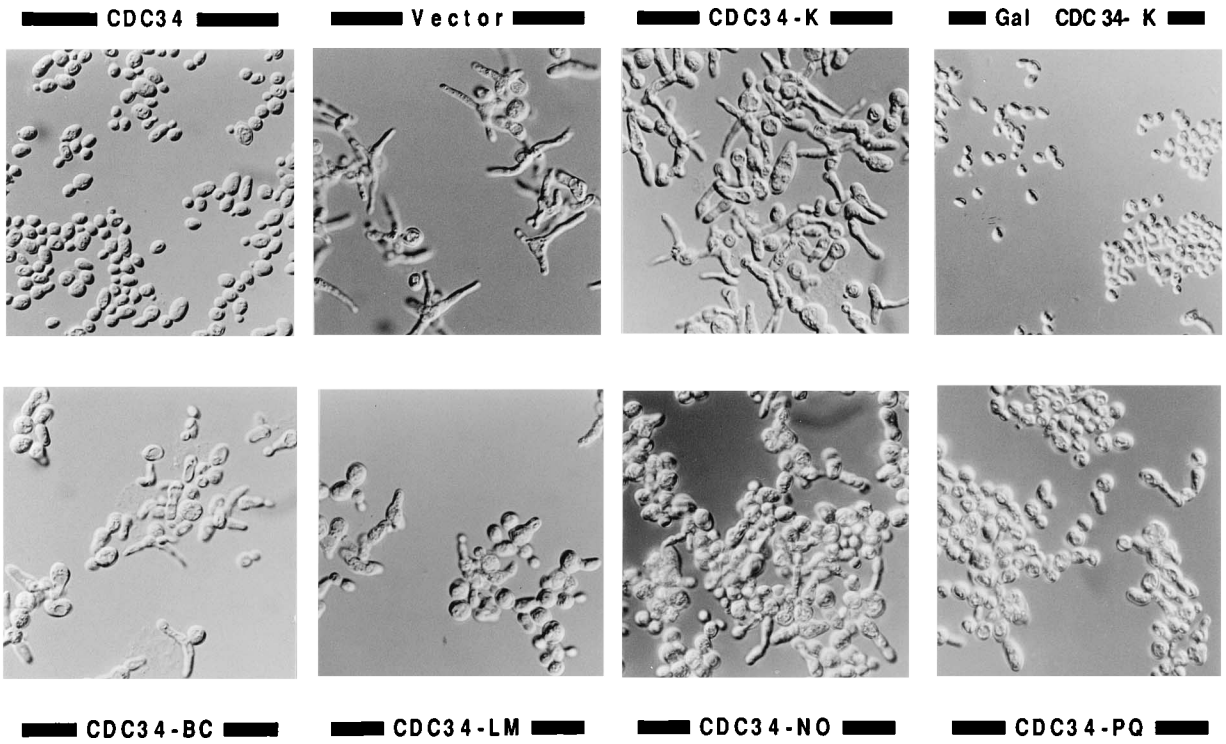

FIG. 5. Photomicrographs of ZY16 cells grown on SC−Ura−Leu at 30°C containing the indicated *CDC34* alleles carried on YCplac111, except for Gal-CDC34-K, in which the mutant K insert was cloned in YEplac112GAL and grown in SC−Ura−Leu+galactose at 30°C.

Cdc34 proteins (the latter produced from the pMW29-CDC34 plasmid contained in ZY16) were visible (Fig. 6B, lane 6). Identical patterns were also seen when single-scan mutants I and K were expressed in ZY16, indicating that both mutant proteins were expressed (Fig. 6B, lanes 4 and 5). However, the steady-state level of mutant I protein was consistently less than that of wild-type Cdc34 and mutant K proteins, as judged by immunoprecipitation of labeled protein (Fig. 6B) as well as by Western blotting from cells overproducing wild-type and mutant Cdc34s (data not shown). Similarly, we also detected protein from each of the double-scan mutants in the *S. cerevisiae* tester strain grown at 30°C, when all of the double-scan mutant-expressing tester strains were viable (Fig. 6C, lanes 3 through 8). Curiously, Cdc34 double-scan mutant LM displayed a slightly altered mobility relative to those of other Cdc34 scan mutants, possibly because of the elimination of several charged residues from the protein; in any case, two independently obtained mutant LM constructs behaved identically with respect to their in vivo phenotypes (data not shown).

Wild-type Cdc34 protein is long lived as judged by immunofluorescence observation of protein in yeast cells (19) and by pulse-chase measurement of its half-life (44a). Since we observed comparable levels of the various Cdc34 mutant proteins, we conclude that none of them is grossly unfolded and destabilized relative to the wild-type protein. In particular, we conclude that the severe phenotype of mutant K cannot be ascribed to either low level of production or instability of the mutant K protein.

In vitro activity of Cdc34 scan mutant proteins. The amino acid changes in mutant K lie in a 12-residue segment of Cdc34 that has no corresponding match in most other yeast E2s and that is within a few residues of the Cdc34 active site. Given the proximity of its amino acid changes to the active site, a trivial explanation for mutant K's inability to complement a *cdc34* null mutant (in low copy numbers) is that the active site of the mutant K protein is locally misfolded, yielding an E2 with strongly depressed enzymatic activity. Expressing mutant K from an induced galactose promoter might then complement a null mutant simply by increasing the levels of Cdc34 enzyme activity above that required for viability. To address this possibility, we investigated whether mutant K was significantly impaired with respect to unfacilitated (E3-independent) E2 activity relative to that of wild-type Cdc34 in vitro.

The two most dysfunctional Cdc34 single-scan mutants, I and K, as well as wild-type Cdc34, mutant I, and all four double-scan mutants, were produced in *E. coli* and tested for (i) thiol ester formation with ubiquitin, (ii) ubiquitination of histones, and (iii) autoubiquitination, as described in Materials and Methods. Cdc34 activity was measured directly in the bacterial extract without purification; previous reports as well as our results below show that E2 activity can be readily assayed in bacterial extracts and that the E2 conjugates little if any ubiquitin to bacterial proteins.

All three activities were easily detected in extracts from bacteria producing wild-type Cdc34 protein upon the addition of ubiquitin-activating (E1) enzyme, ^{125}I-labeled ubiquitin, ATP plus an ATP-regenerating system, and suitable reaction buffers (Fig. 7 and 8). Wild-type Cdc34 protein readily ubiquitinated both histone H2B and Cdc34 itself (Fig. 7, lane W; histone conjugates are identified as those ubiquitinated bands that are present only in the reaction mixtures which have added histone H2B). We confirmed that the higher-molecular-weight ubiquitin conjugates formed in these reactions do in fact correspond to autoubiquitinated Cdc34 by showing that they were immunoprecipitable by anti-Cdc34 antibody (data

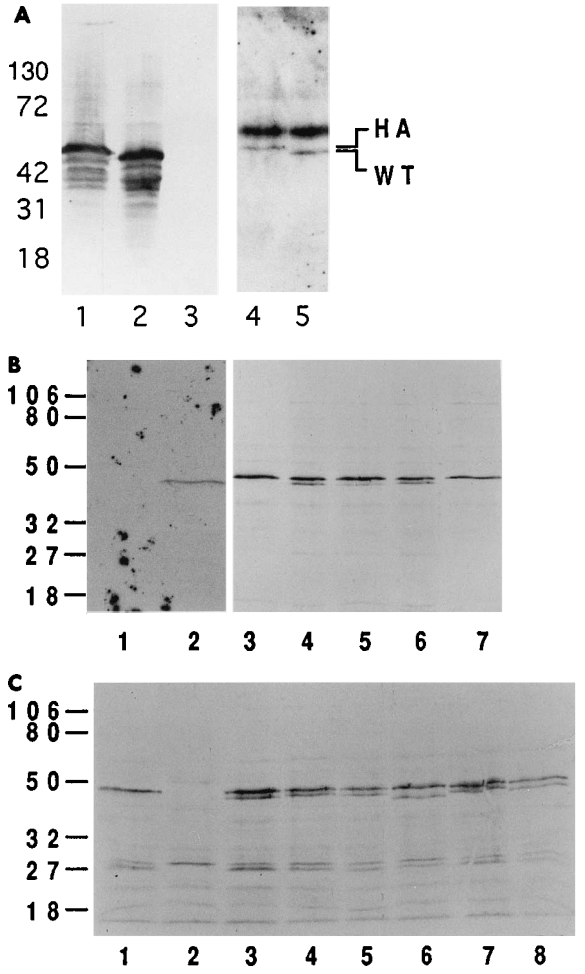

FIG. 6. Immunological detection of Cdc34 protein in bacterial and yeast lysates. (A) Detection of Cdc34 protein by Western blotting of cell lysates. Extracts were prepared as described in Materials and Methods from the indicated bacterial or yeast strain. Lane 1, *E. coli* BL21 expressing epitope-tagged Cdc34-HA from pET17xb; lane 2, BL21 expressing untagged Cdc34 from pET17xb; lane 3, BL21 containing pET17xb with no insert; lane 4, yeast ZY16 expressing epitope-tagged CDC34-HA from pMW29-CDC34; lane 5, ZY16 producing untagged Cdc34 from YCplac111-CDC34 after eviction of pMW29-CDC34. The positions of the wild-type Cdc34 (WT) and epitope-tagged Cdc34-HA (HA) are indicated on the right. The prominent bands in lanes 4 and 5 just above the bands identified as Cdc34 are from the immunoprecipitated heavy chain of the rabbit anti-Cdc34 antibody. (B) Detection by immunoprecipitation of [^{35}S]methionine-labeled Cdc34 in yeast lysates from ZY16 yeast cells transformed with YCplac111-CDC34 wild-type or single-scan mutant plasmids. Immunoprecipitations were performed on extracts made from the indicated *S. cerevisiae* strain. Lane 1, mock immunoprecipitation of extract from strain YMW1 (no anti-Cdc34 used in the reaction mixture); lane 2, YMW1 (expressing the endogenous *CDC34*); lane 3, ZY16 containing pMW29-CDC34-HA and a discarded candidate of YCplac111-CDC34-BC which contained an inactivating mutation; lane 4, ZY16 containing pMW29-CDC34-HA and YCplac111-CDC34-K; lane 5, ZY16 containing pMW29-CDC34-HA and YCplac111-CDC34-I; lane 6, ZY16 containing pMW29-CDC34-HA and YCplac111-CDC34 (wild type); lane 7, ZY16 containing pMW29-CDC34-HA and YCplac111 (no insert). (C) Detection by immunoprecipitation of [^{35}S]methionine-labeled Cdc34 in yeast lysates from ZY16 yeast cells transformed with YCplac111-CDC34 wild-type or double-scan mutant plasmids. Immunoprecipitations were performed on extracts made from the indicated *S. cerevisiae* strain. Lane 1, ZY16 containing pMW29-CDC34-HA and YCplac111; lane 2, mock immunoprecipitation of extract from ZY16 containing pMW29-CDC34-HA (no anti-Cdc34 added to the reaction mixture); lane 3, ZY16 containing pMW29-CDC34-HA and YCplac111-CDC34-BC; lane 4, ZY16 containing pMW29-CDC34-HA and YCplac111-CDC34 (wild type); lane 5, ZY16 containing pMW29-CDC34-HA and YCplac111-CDC34-BC; lane 6, ZY16 containing pMW29-CDC34-HA and YCplac111-CDC34-LM; lane 7, ZY16 containing pMW29-CDC34-HA and YCplac111-CDC34-NO; lane 8, ZY16 containing pMW29-CDC34-HA and YCplac111-CDC34-PQ. The numbers at the left of the panels are sizes in kilodaltons.

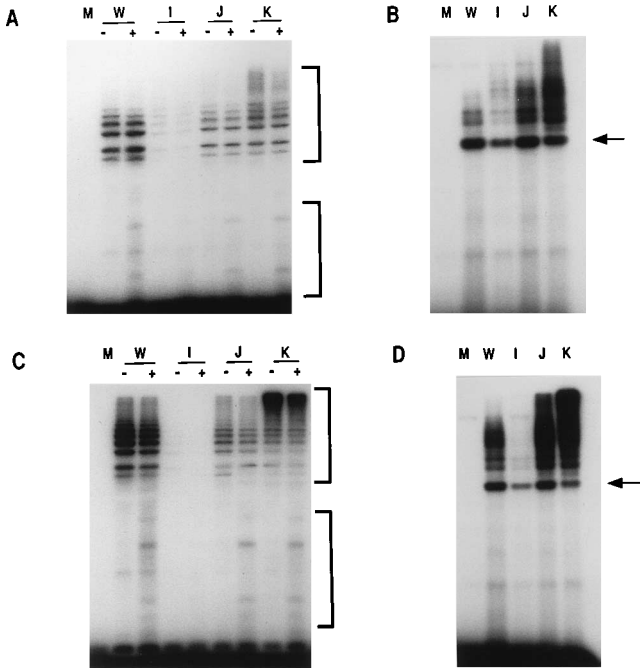

FIG. 7. Ubiquitin conjugation and thiol ester-forming activity of wild-type and single-scan mutant Cdc34 proteins produced in *E. coli*. The proteins tested were those encoded by wild-type Cdc34 (lanes W) and single-scan mutants I, J, and K (lanes I, J, and K, respectively). (A and C) Autoradiograms of conjugation reactions done either in the absence (−) or in the presence (+) of histones; (B and D) autoradiograms of thiol ester formation assays; (A and B) reactions performed at 23°C; (C and D) reactions performed at 37°C. Lanes M, mock reactions done in the absence of Cdc34. (A and C) The upper brackets indicate Cdc34-ubiquitin conjugates, and the lower brackets indicate histone-ubiquitin conjugates. The arrows in panels B and D indicate the position of the Cdc34-ubiquitin thiol ester adduct. The patterns obtained from the mock reaction lanes were identical to the patterns found in all of the other control reaction mixtures which omitted either ATP or ubiquitin-activating enzyme, except for the presence of the high-molecular-weight E1-ubiquitin thiol ester under nonreducing conditions.

not shown). As previously shown by Banerjee et al. (3), electrophoresis under nonreducing conditions yielded a more diffuse pattern of higher-molecular-weight forms (corresponding to Cdc34-ubiquitin conjugates) plus an additional, more rapidly migrating species (indicated by the arrows in Fig. 7 and 8) which corresponds to a Cdc34-ubiquitin thiol ester on the basis of its apparent sensitivity to reduction (3).

Despite its failure to provide essential in vivo function, Cdc34 protein expressed by mutant K also appeared competent for histone conjugation and autoubiquitination at all temperatures (Fig. 7A and C, lanes K). Interestingly, however, more higher-molecular-weight Cdc34-ubiquitin conjugates were formed by mutant K relative to other Cdc34 reactions, especially at 37°C (Fig. 7C and A, compare lanes K). By comparison, even though the total level of multiubiquitin conjugates formed by mutant J was less than that formed by wild-type Cdc34, the distribution of ubiquitin-Cdc34 conjugates formed by mutant J (whose mutation lies immediately adjacent to that of mutant K) was much more similar to the distribution formed by wild-type Cdc34 than it was to the distribution formed by mutant K (Fig. 7A and C, lanes J). Specifically, mutant J failed to form the very-high-molecular-weight forms that mutant K synthesized (Fig. 7C, compare lanes J and K).

For further comparison, we also examined the in vitro activities of several other mutant proteins. Cdc34 mutant I activity was strongly depressed with respect to autoubiquitina-

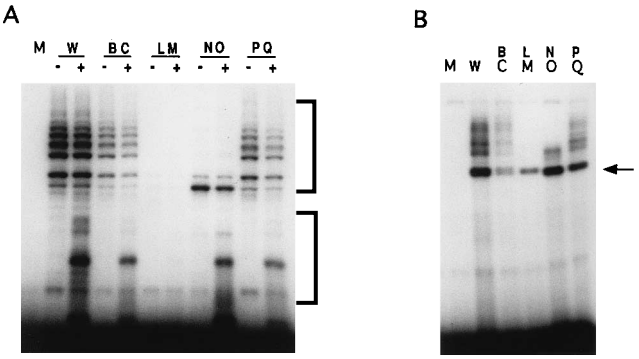

FIG. 8. Ubiquitin conjugation and thiol ester-forming activity of wild-type and double-scan mutant Cdc34 proteins produced in *E. coli*. The proteins tested were those encoded by wild-type Cdc34 (lanes W) and double-scan mutants BC, LM, NO, and PQ (lanes BC, LM, NO, and PQ, respectively). (A) Autoradiogram of conjugation reactions done either in the absence (−) or in the presence (+) of histones. The upper bracket indicates the position of the Cdc34-ubiquitin conjugates, and the lower bracket indicates the position of the histone-ubiquitin conjugates. (B) Autoradiogram from thiol ester formation assays. The arrow indicates the position of the Cdc34-ubiquitin thiol ester adduct. The reactions shown were performed at 30°C; reactions performed at either 23 or 37°C were qualitatively the same. Lane M, mock reaction done in the absence of Cdc34. The pattern obtained from the mock reaction lane was identical to the pattern found in all of the other control reaction mixtures which omitted either ATP or ubiquitin-activating enzyme, except for the presence of the high-molecular-weight E1-ubiquitin thiol ester under nonreducing conditions.

tion; even at temperatures permissive for in vivo function (23°C), its autoubiquitinating activity appeared to be much less than that of the wild-type protein (Fig. 7A through D, lanes I). Under the conditions for our assays, the histone-conjugating activity of mutant I was only slightly less than that of wild-type Cdc34 at 23°C, but was significantly depressed at 37°C (Fig. 7). However, significant formation of Cdc34-ubiquitin thiol ester still occurred even at 37°C when both histone and autoconjugation activity was low (Fig. 7B and D, lanes I). Thus, the enzymatic defect in mutant I appears to reside more in transfer of ubiquitin from the thiol ester to substrate than in the initial formation of a ubiquitin thiol ester. More importantly, mutant I protein consistently displayed much lower E2 activity than did mutant K, even at 23°C, at which temperature mutant I could complement a *cdc34* null mutant but mutant K could not.

Mutant protein from BC and PQ also appeared competent for autoubiquitination and ubiquitin-histone conjugation (Fig. 8). The size distribution of ubiquitin-Cdc34 conjugates formed by BC and PQ was comparable to that formed by wild-type Cdc34 even though the overall level of conjugates formed was somewhat less. Strikingly, mutant NO synthesized predominantly only a single ubiquitinated species, suggesting that it was competent for initiating a ubiquitin chain but could not efficiently elongate the chain via multiubiquitination (Fig. 8A, lanes NO). Although mutant NO was clearly able to ubiquitinate histones, assays with purified protein are required to test whether mutant NO is deficient in multiubiquitination of histones. Mutant LM was the most severely handicapped. It appeared to form some thiol ester with ubiquitin, but we detected only very low levels of autoubiquitination, even though LM supported essential function in vivo (Fig. 8, lanes LM).

DISCUSSION

Alanine-scanning mutagenesis has been used as a strategy to obtain conditional alleles of genes and to survey the surfaces of proteins that may be involved in interactions with regulators or substrates (12, 17, 50). In mutagenesis studies with actin (50), this strategy yielded a significant percentage of mutants having one of several phenotypes (temperature sensitivity or cold sensitivity, either dominant or inactive). However, Cdc34 cell cycle function proved surprisingly resistant to mutational perturbation by this strategy. In retrospect, part of the explanation of why the single-scan mutants did not more severely affect Cdc34 function may be that in vivo levels of Cdc34 protein, while purportedly low (20), are apparently in excess of the minimum required for viability, unlike those of structural proteins such as actin and tubulin. For example, *S. cerevisiae* containing only the single-scan mutant I grew at nearly wild-type rates at 23°C, and yet its in vitro activity at this temperature appears much less than that of the wild type.

Since combining single-scan mutants to form double-scan mutants yielded more severe phenotypes, we suspect that making more such combinations may yield additional Cdc34 mutant proteins with perturbed function. It is worth noting, however, that the striking resistance of Cdc34 function to perturbation by scanning mutagenesis is not necessarily shared by other E2 enzymes. In a parallel effort to identify novel mutants of the *RAD6*-encoded conjugating enzyme by charge-to-alanine scanning mutagenesis, we found that five of nine Rad6 scan mutant proteins were defective with respect to at least some Rad6-dependent functions, and even the most severely affected displayed unfacilitated (E3-independent) activity in vitro (37a). This approach is likely to have general utility for generating novel mutant alleles in other E2s as well.

We obtained one single-scan mutant (mutant K) which was catalytically active with respect to unfacilitated (E3-independent) ubiquitination but unable to provide essential in vivo function at any temperature when expressed from a low-copy-number vector. To our knowledge, this is the first demonstration of a *CDC34* mutant whose product is competent for unfacilitated ubiquitination in vitro but not cell cycle function in vivo. Intriguingly, this mutation lies in a 12-residue segment whose presence in Cdc34 distinguishes the *CDC34*-encoded E2 from most other ones in *S. cerevisiae*. Like the diverged C-terminal domains of E2s, this unusual structural feature is an attractive candidate for a regulatory determinant of Cdc34, especially given its proximity to the Cdc34 active site, and the phenotype of mutant K here provides further weight to that speculation.

Without an experimentally determined Cdc34 protein crystal structure, it is unclear how the 12-residue insertion is to be accommodated within its N-terminal domain, even given the homology between Cdc34 and two other E2s whose crystal structures are known (7, 8). Cook et al. (7) have suggested that the segment may extrude in a loop that lies adjacent to the E2 active site. This hypothetical loop would probably extend from the E2 polypeptide at or near the elongated and narrow face of the enzyme where the active site is located (7, 8), where it could provide a unique and easily accessible determinant for recognition by *trans*-acting factors required for Cdc34 function. Two candidates for such regulatory factors are those encoded by *CDC4*, which encodes a 779-residue protein distinguished by the presence of β-transducin motifs (16, 51), and *CDC53*, which encodes a protein with no resemblance to any other protein in the databases (18a). At nonpermissive temperatures, the phenotypes of *cdc4* and *cdc53 ts* mutants are identical to that of *cdc34* mutants, and both display synthetic lethality when combined with a *cdc34 ts* mutant (33). This and other genetic evidence strongly imply that close (if not direct) interactions between these proteins are required for progression into S phase.

It is also possible that this hypothetical loop plays a more

intimate role in governing Cdc34 activity than just providing a binding site for a *trans*-acting regulator. If this loop were conformationally flexible, it might form a "flap" that modulates enzyme activity by interacting with residues at or near the active site. The modulation of Cdc34 activity of the loop could be controlled by interaction with *trans*-acting factors (such as those encoded by *CDC4* or *CDC53*) or by reversible modifications of the Cdc34 polypeptide (such as phosphorylation [19, 36]) which could switch the loop from one conformation to another. It may be significant that the extent of autoubiquitination of Cdc34 was noticeably greater for mutant K than for the wild-type protein, suggesting that the interaction of the C-terminal tail with the body of the E2 might be altered in mutant K. Clearly, further work is required to define the role of this insertion and to determine if the altered extent of multiubiquitination in mutant K is related to its severe in vivo defect.

Several other novel alleles of *CDC34* were identified in our study. Mutant I is a strong temperature sensitivity allele; the proximity of the amino acid changes to the active site and the relatively poor activity of *E. coli*-produced protein suggest that this mutant is compromised in terms of its intrinsic (unfacilitated) enzymatic activity. Mutant PQ showed its most severe defect at high temperatures; however, since the mutant PQ protein showed unfacilitated (E3-independent) activity in vitro at 37°C, this mutation may also target specific determinants of E2 function. Mutant PQ targets residues within a portion of Cdc34 predicted to form an amphipathic alpha-helix (specifically, helix 4 [7]), and at least in terms of linear sequence distance, these residues lie near the region of the C-terminal domain containing a portable positive determinant of Cdc34 activity (34, 47). We therefore speculate that sequences in helix 4 may compose part of the same C-terminal determinant. Mutant LM targets residues that lie along an alpha-helix that resides near an area of the E2 suggested to interact with either ubiquitin or the E1-activating enzyme (8). The severe defect in unfacilitated Cdc34 activity in vitro shown by mutant LM protein is consistent with this supposition.

Mutant NO was arguably the least compromised with respect to in vivo activity at various temperatures but showed a curious behavior with respect to in vitro activity. It was able to conjugate a single ubiquitin to itself (and, with much less efficiency, a second ubiquitin moiety), but it did not catalyze the formation of an extensive multiubiquitin chain. Examination of the crystal structures of the *Arabidopsis thaliana* Ubc1 protein (7) and the *S. cerevisiae* Ubc4 protein (8) shows that the changes in mutant NO are expected to lie on the same narrow, elongated face of Cdc34 as the active site. One explanation for the multiubiquitination defect of mutant NO is that the C-terminal domain of Cdc34 is normally in contact with the accessible face of the alpha-helix targeted by NO (helix 3) and that mutant NO disrupts interactions that stabilize its conformation. Mutant NO might then inhibit formation of a multiubiquitin chain on Cdc34's C-terminal lysine residues by permitting greater conformational flexibility of the tail. Another explanation is based on the suggestion that E2 dimer formation is necessary for multiubiquitin chain formation (47). If this is so, then mutant NO could be deficient for multiubiquitination because it is deficient in forming stable homodimers. This might be true if the face of helix 3 makes up part of a dimerization interface. Additional quantitative studies on the activity of purified mutant proteins are needed to address these speculations. We expect further study of mutant NO (as well as mutant K) to yield insights into the mechanistic and structural requirements for multiubiquitin chain formation by an E2. Such studies address questions of direct biological relevance, since key ubiquitin-dependent processes (such as proteolysis) require and are governed by the formation of a multiubiquitin chain (6, 15, 22).

To summarize, we have identified a set of novel alleles of the *CDC34* ubiquitin-conjugating enzyme by charge-to-alanine scanning mutagenesis. One allele in particular, mutant K, is striking in its lack of complementing activity when expressed from a low-copy-number vector, despite its expression of ubiquitin-conjugating activity in vitro. Mutant K and another mutant, NO, both show altered extents of multiubiquitin chain formation in an in vitro autoubiquitination reaction. Another mutant, called LM, displayed cold-sensitive function in vivo, while two other mutants (I and PQ) were temperature sensitive. Our results validate the use of clustered charge-to-alanine scanning mutagenesis for exploring ubiquitin-conjugating enzyme function. More detailed genetic, biochemical, and structural study of these mutants should provide useful insight into the regulation of Cdc34 activity in vitro and in vivo.

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