The Bacterially Expressed Yeast CDC34 Gene Product Can Undergo Autoubiquitination to Form a Multiubiquitin Chain-linked Protein*

(Received for publication, May 7, 1992)

Amit Banerjee, Luisa Gregori, Yuping Xu, and Vincent Chau†

From the Department of Pharmacology, Wayne State University School of Medicine, Detroit, Michigan 48201

The CDC34 gene of Saccharomyces cerevisiae encodes a 285-residue ubiquitin-conjugating enzyme (E2). The function of this ubiquitin-conjugating activity remains to be defined as its in vivo substrates are presently unknown. The bacterially expressed and purified Cdc34 protein is shown here to catalyze its own ubiquitination via an intramolecular transfer of its thiol ester-linked ubiquitin to a lysine. In this process, multiple ubiquitin groups are added to Cdc34, and these ubiquitin groups were shown to be arranged predominantly in the form of a single Lys4'-specific multiubiquitin chain. Analysis of the hydroxylation-dependent cleavage of ubiquitin-Cdc34 conjugates at the single Asn-Gly sequence in Cdc34 placed the major ubiquitin linkage site within the C-terminal 215-295 residues of Cdc34. The 4 Lys residues (Lys73, Lys77, Lys79, and Lys84) in this region of Cdc34 were substituted by arginine either singly or in combination to produce a set of Cdc34 mutants. Analysis of these Cdc34 mutants for autoubiquitination revealed that the multibiquitin chain can be formed on any one of these 4 lysines although most Cdc34 conjugates contain a single multibiquitin chain. Since the presence of a Lys4'-specific multibiquitin chain in protein conjugates is known to target acceptor proteins for degradation in the ubiquitin-mediated proteolytic pathway, the present result raises the possibility that one function of the ubiquitin-conjugating activity in CDC34 may be used to target its own degradation.

Ubiquitin, a 76-residue eukaryotic protein, is found either free or joined to other cytoplasmic, nuclear, and membrane proteins by a post-translational modification process known as ubiquitination (1-3). In ubiquitin-protein conjugates, the C-terminal carboxyl group of ubiquitin is joined to a lysyl-amino group of acceptor proteins in an isopeptide bond. Additional ubiquitin groups may also be added to form conjugates in which the acceptor protein is linked with a multibiquitin chain (4, 5). In this chain, the Lys4'-amino group in ubiquitin provides the site that combines with the C-terminal carboxyl group of an adjoining ubiquitin to form the isopeptide bond (5-7). This Lys4'-specific multibiquitin chain has been found on proteins that are undergoing ubiquitin-mediated proteolysis in vivo and in vitro and has been proposed to serve the function of targeting conjugated proteins for proteolysis (4).

The ubiquitin-conjugating enzymes (E2)1 are a family of related proteins that are empirically defined by their ability to form a thiol ester adduct with ubiquitin (Ub) in the presence of ubiquitin-activating enzyme (E1) and ATP in the following reactions (7, 8).

\[
\begin{align*}
E_{1\text{SH}} &+ Ub + ATP \rightarrow E_{1\text{Ub}} + AMP + PP, \\
E_{1\text{Ub}} + E_{2\text{H}} &\rightarrow E_{1\text{Ub}} + E_{2\text{Ub}},
\end{align*}
\]

In the current model, ubiquitin in the E3-E2-Ub thiol ester is transferred to acceptor proteins either directly or by a reaction that may also require an additional substrate-binding protein known as E3 or the ubiquitin-protein ligase (9, 10). While a number of purified E2 enzymes can catalyze the linkage of ubiquitin to lysines on model substrates such as the histone proteins (11, 12), the ability to catalyze the Lys4'-dependent ubiquitin-ubiquitin linkage has been formally demonstrated only for an E2 enzyme that catalyzes the formation of a free multibiquitin chain (6). Nonetheless, a role for other E2 enzymes in multibiquitin chain formation can be inferred by their assigned function in various pathways of protein ubiquitination that result in this chain formation. For example, substrates in the "N-end rule" pathway are rapidly degraded when linked with the multibiquitin chain, and this pathway requires either the rabbit E2s.m, the Saccharomyces cerevisiae RAD6 gene product, or the human E2s.m (8, 13-16). The ubiquitin-galactosidase fusion protein undergoes ubiquitin-mediated proteolysis when the multibiquitin chain can be formed on Lys4'-residue of ubiquitin in the fusion protein, and this reaction requires the S. cerevisiae UBC4 gene (17).

It remains to be demonstrated whether the ubiquitin-conjugating activity in Rad6 and Ubc4 is sufficient to catalyze the Lys4'-specific multibiquitin chain formation on their respective substrates. In contrast, ubiquitination of model substrates by a number of purified E2 enzymes does not involve the formation of a Lys4'-specific multibiquitin chain (20). Thus, the ability to catalyze this Lys4'-specific multibiquitin chain is not shared by all E2 enzymes.

CDC34 is one of eight known E2-encoding genes in the yeast S. cerevisiae (2). This E2-encoding gene is apparently required for cells to undergo G2 to S transition in the cell cycle (18). Under nonpermissive conditions, temperature-sensitive mutants of CDC34 develop numerous elongated buds, and the spindle pole body duplicates but fails to undergo the separation required for spindle formation (19). The bacterially expressed Cdc34 protein has been shown to form the active E2-ubiquitin thiol ester intermediate and to catalyze the

*This research was supported by National Institutes of Health Grant GM 35803. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†To whom correspondence should be addressed: Dept. of Pharmacology, Wayne State University School of Medicine, 540 E. Canfield, Detroit, MI 48201. Tel: 313-577-0841; Fax: 313-577-6799.

1The abbreviations used are: E2, ubiquitin-conjugating enzyme; E1, ubiquitin-activating enzyme; E3, ubiquitin-protein ligase; Ub, ubiquitin; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; PCR, polymerase chain reaction.
ubiquitination of model substrate proteins (20). This E2 enzyme is shown here to undergo autoubiquitination. Because the ubiquitin groups in Cdc34 conjugates are arranged in the form of a Lys-specific mult ubiquitin chain, the result raises the possibility that the ubiquitin-conjugating activity in E2 may function in part to target its own degradation.

EXPERIMENTAL PROCEDURES

Materials—Ubiquitin was purchased from Sigma. Ub-R48 is a ubiquitin mutant in which Lys76 was replaced by an arginine. The expression of Ub-R48 in Escherichia coli and its purification have been described elsewhere (4). The [35S]labeled form of ubiquitin and ubiquitin mutant in which Lys6 was replaced by an arginine. The specific activity of [35S]ubiquitin and [35S]Ub-R48 were 6.4 × 106 and 1.5 × 106 cpm/μg, respectively. The ubiquitin-activating enzyme (E1) was purified to homogeneity from rabbit reticulocytes as described previously (21) and was stored at −70 °C in 20% glycerol. The CDC34-encoding gene in a pGEM3 plasmid (pGEM3 B/S) and Cdc34-specific antibodies were generous gifts from Mark Goebel, Indiana University. E1 and Cdc34 concentrations were determined by first converting the proteins to E1Hb and Cdc34Hb, and the specific activities of these thiol esters were estimated from the known specific radioactivity in [35S]-ubiquitin. Alkaline phosphatase-conjugated antirabbit IgG was purchased from Sigma.

Expression of Cdc34 in E. Coli and Protein Purification—The CDC34-encoding gene in the pGEM3 B/S plasmid was used as the template for introduction of an engineered Ndel site at the initiating methionine codon by PCR amplification. The 5′ end of the coding sequence was obtained with the 35-nucleotide PCR primer, 5′-GAGCGTTATATTTCGTTATGAGATCTGATCCTCGCACAAGCTAATAA-3′. The underlined ATG residues indicate the initiating methionine codon, and the sequences AAGCTT, GTCGAC, and CATATG correspond to the recognition sequence of restriction endonucleases HindIII, SalI, and Ndel, respectively. The 27-nucleotide sequence, 5′-GGAGACTTATATTTCGTTATGAGATCTGATCCTCGCACAAGCTAATAA-3′, was used as the PCR primer of the second strand synthesis. The underlined TTA residues indicate the complement of the termination codon, and the AAGCTT sequence corresponds to the recognition sequence of HindIII endonuclease. After synthesis, the approximate 1-kilobase PCR product was recovered by mutagenesis with the oligonucleotides, 5′-GTTGTTTTCATTATCATGC, 5′-CATCTTCAGCCTTTGG, and 5′-GCTTATACTTGTGAAACTC, respectively. In each case, the underlined T nucleotide encodes the necessary mutation at the specified position to restore the wild-type CDC34 sequence from CDC34-4R.

Cdc34-4R and Ub-Cdc34 Conjugate Formation—Reactions were carried out in 50 mm Tris (pH 7.5), 10 mm MgCl2, 2 mM ATP, 50 μM DTT, and 5 μM ubiquitin (or Ub-R48) at 30 °C. Unless stated otherwise, 40 nm E1 and 1.3 μM Cdc34 were used in the reaction. Reactions were stopped by withdrawing aliquots of the reaction mixture into SDS sample buffer in which β-mercaptoethanol had been omitted. When Ub-Cdc34 conjugates were assayed, protein samples were adjusted to contain 5% β-mercaptoethanol, and samples were heated in a boiling water bath for 3 min. Protein samples were subjected to electrophoresis in a 12% polyacrylamide SDS gel, and autoradiography was used to visualize radio labeled bands. For kinetic analyses of Cdc34-4R and Ub-Cdc34 conjugates, gel bands were quantitated directly by scanning in an Ambis radioimaging system. The first-order rate constant was calculated as (C1/C0) × 100. The time-dependent values for the percent maximal change were calculated as (C1/C0) × 100.

Expression of M13mp19-CDC34 Mutants—To express Cdc34 mutant proteins, individual mutant clones in M13mp19 were used to infect a 1/200 dilution of an overnight culture of E. coli host TG1 in LB to obtain a multiplicity of infection of 30-40. After 3 h of growth at 37 °C, the bacteria were transferred to 0.5% isopropyl-1-thio-β-D-galactopyranoside, and incubation was continued for an additional 2 h. Cells from 1 ml cultures were harvested by centrifugation, washed with 50 mm Tris (pH 7.5), and suspended in 0.5 ml of reaction buffer. 10 μl of the supernatant, after 5 min centrifugation at 12,000 × g in an Eppendorf centrifuge, were used to assay for ubiquitin thiol ester and conjugate formation in a final reaction volume of 20 μl using standard reaction conditions. Incubation times of 9 and 30 min were tolerated in the presence of the indicated concentrations of E1 and Cdc34, respectively.

Hydroxylation Cleavage of Ub-Cdc34 Conjugates—Cdc34 conjugates with either [35S]- ubiquitin or [35S]-Ub-R48 were formed under standard reaction conditions for 2 h. A 12% polyacrylamide gel-SDS
was used to resolve conjugates of different mobilities. A gel lane containing the resolved proteins was excised and placed in a solution at room temperature. At the end of the reaction, the gel slice was equilibrated sequentially with 0.5 M Tris (pH 6.8) and with the SDS sample buffer (4). The gel slice was then subjected to a second-dimension electrophoresis in a SDS-14% polyacrylamide gel, and autoradiography was used to visualize radiolabeled gel spots.

RESULTS

Formation of Both Thiol-sensitive and -insensitive Ubiquitin-cdc34 Adducts—The expression of the yeast *S. cerevisiae* CDC34 gene in *E. coli* under the control of a λP1 promoter and the purification of the Cdc34 protein have been described under "Experimental Procedures." When the purified Cdc34 protein was assayed for its ability to form a Cdc34-ubiquitin thiol ester with 125I-ubiquitin, we noted that a number of additional radiolabeled adducts were also formed at longer reaction times. Fig. 2 shows analyses of these reaction products by SDS-PAGE and autoradiography. Electrophoreses were carried out either with (Fig. 2B) or without (Fig. 2A) pretreating the protein samples with β-mercaptoethanol. The rapidly formed 42-kDa adduct in Fig. 2A was assigned to be the Cdc34-ubiquitin thiol ester (Cdc34s.m) on the basis of its sensitivity to the addition of β-mercaptoethanol, i.e. present in Fig. 2A but not in Fig. 2B. The apparent molecular weight of this adduct is also consistent with the linkage of ubiquitin to the single cysteine in Cdc34. In addition to this expected 42-kDa adduct, a number of other radiolabeled adducts were also detected in these SDS gels. At least a subset of these adducts were not eliminated by pretreating the protein samples with β-mercaptoethanol prior to electrophoresis (Fig. 2B). These thiol-insensitive adducts were derived from Cdc34 since they were also reactive to Cdc34-specific antibodies on immunoblots (Fig. 3). The presence of these thiol-insensitive Ub-Cdc34 adducts in the reaction products indicates that multiple ubiquitin groups may also link to lysine(s) in Cdc34 to form Ub-Cdc34 conjugates. These Ub-Cdc34 conjugates were heterogeneous and migrated with different mobilities on SDS gels to produce a "ladder" of bands (Figs. 2B and 3). This form of heterogeneity has also been shown for other ubiquitin-protein conjugates and has been attributed to varying numbers of ubiquitin in the conjugates. The observed time-dependent change in the apparent molecular weight distribution of Ub-Cdc34 conjugates is consistent with progressively more ubiquitin groups being added to Cdc34 with increasing reaction times.

Fig. 3A also shows that only approximately half of the purified Cdc34 could be converted to the Cdc34s.m thiol ester. This ratio was not altered by increasing the E1 concentration in the reaction (data not shown), indicating that it is not due to insufficient E1. It is unlikely that a portion of Cdc34 is present in an inactive form since purified Cdc34 could be completely retained by a ubiquitin-affinity column in the presence of E1 and ATP (data not shown). As noted in Fig. 1, Cdc34 may exist as a homodimer, and it is possible that
jugates with wild type ubiquitin are formed by the incorporation of a solution of affinity-purified Cdc34-specific rabbit antibodies. To form Ub-Cdc34 conjugates product.

As shown in Fig. 4B, the formation of higher order Cdc34 conjugates was indeed blocked by replacing ubiquitin with the "1-Ub-R48. Only the major reaction product in each panel has been labeled, and the nature of the various reaction products is discussed in the text.

only one of the two subunits in a homodimer could be converted into the thiol ester form. This possibility is under investigation.

Lys4-dependent Ub-Ub Linkages in Ub-Cdc34 Conjugates—Multiple ubiquitin groups in Ub-Cdc34 conjugates may be formed by the linkage of ubiquitin to several lysines in Cdc34. Alternatively, they may be incorporated into the conjugates in the form of a Lys4-specific multiubiquitin chain that is linked to one or a limited number of lysines in Cdc34. These two modes of ubiquitin linkage can be distinguished by replacing ubiquitin in the reaction with the mutant Ub-R48 which only blocks the formation of mult ubiquitin chain but does not affect ubiquitin linkage to lysines on an acceptor protein (4).

As shown in Fig. 4B, the formation of higher order Cdc34 conjugates was indeed blocked by replacing ubiquitin with the mutant Ub-R48, indicating that the majority of Cdc34 conjugates with wild type ubiquitin are formed by the incorporation of ubiquitin groups into a Lys4-specific multiubiquitin chain. The predominant reaction product with Ub-R48 was a 43-kDa conjugate which would be consistent with the linkage of one Ub-R48 to Cdc34. Two minor conjugates of 41 and 47 kDa could also be detected. Results to be described below indicated that the 41- and the 43-kDa conjugates were formed by the linkage of Ub-R48 to two different lysines in Cdc34, and two ubiquitin groups are present in the 47-kDa conjugate.

In the absence of added β-mercaptoethanol (Fig. 4A), the reaction products with Ub-R48 consisted primarily of three adducts that migrated at 6 and 43 kDa (data not shown). This result indicated that the 46-kDa adduct was a Cdc34 conjugate. The 43-kDa Ub-Cdc34 conjugate.

Reaction Pathway of Ub-Cdc34 Conjugate Formation—Ub-Cdc34 conjugates can be formed by the transfer of the thiol ester-linked ubiquitin, from either E1s.m or Cdc34s.m, to a lysine in Cdc34 as shown below.

(I) E1s.m + Cdc34 → E1s.m + Ub-Cdc34

(II) Cdc34s.m + Cdc34 → Cdc34s.m + Cdc34s.m

(III) Cdc34s.m + Ub-Cdc34s.m

These three reaction pathways (I–III) could be distinguished by kinetic analyses on the reaction at different reactant concentrations. The reaction can be carried out under the condition that the formation of Cdc34s.m is no longer rate-limiting. Under this condition, only pathway I (Eq. 5) is expected to exhibit a dependence on E1 concentrations. Pathway II (Eq. 6) is expected to exhibit a dependence on Cdc34 concentrations, whereas pathway III (Eq. 7) is expected to follow first-order kinetics with a rate constant that is independent of E1 and Cdc34 concentrations.

To test for E1 dependence, conjugation of Ub-R48 to Cdc34 (400 nM) was carried out with either 40 or 80 nM of E1. Fig. 5 shows time-dependent changes in the 42-kDa Cdc34 adduct thiol ester and in the Cdc34 conjugates. At these reaction conditions, Cdc34 was quantitatively and rapidly converted to the thiol ester species in both reactions as judged by a similar radioactivity in the 42-kDa band at the 5 min time point. Analysis of the time-dependent changes for Cdc34s.m (Ub-R48) and (Ub-R48)-Cdc34 indicated that the reaction could be adequately described by first-order kinetics with a similar rate constant for both E1 concentrations (Fig. 5, A and B). These results are, therefore, compatible with pathway I. To distinguish between pathways II and III, reactions were also carried out with either 400 or 130 nM Cdc34 at 40 nM of E1 (Fig. 5, A and C). As shown in Fig. 5, time-dependent changes in Cdc34s.m (Ub-R48) and in (Ub-R48)-Cdc34 conjugates were also adequately described by first-order kinetics, and a similar rate constant was estimated for both reactions.

These results, the first-order reaction kinetics and the independence on E1 and Cdc34 concentrations, are most consistent with a model in which Ub-Cdc34 conjugates are formed via an intramolecular transfer of ubiquitin in the Cdc34s.m thiol ester to a lysine on Cdc34. The first-order rate constant for this reaction was estimated to be 0.025 ± 0.005 min⁻¹ by averaging three independent experiments. It should be noted that Cdc34 may exist as a homodimer as suggested by its behavior on gel exclusion chromatography (Fig. 1C), and if this is the case, our kinetic analysis does not rule out the possibility that ubiquitin is transferred from the thiol ester to a lysine on a different polypeptide chain within a dimer.

Localization of the Major Ubiquitination Site to a C-terminal 80-Residue Fragment—To obtain a preliminary indication on the site of ubiquitin linkage, we analyzed the hydroxylamine cleavage fragments of Cdc34 that were conjugated with either ubiquitin or Ub-R48. Hydroxylamine is expected to cleave the single Asn214-Gly215 sequence in the 295-residue Cdc34 protein. This cleavage site is not present in ubiquitin. Fig. 6 shows a two-dimensional SDS gel analysis of (Ub-R48)-Cdc34 conjugates before (Fig. 6B) and after cleavage (Fig. 6A). In this SDS gel system, conjugates were first separated horizontally (first dimension) prior to hydroxylamine-dependent cleavages. Cleavage fragments were then separated vertically from each uncleaved conjugate by electrophoresis.

**FIG. 3.** Immunostaining of Cdc34s.m thiol ester and Ub-Cdc34 conjugates by Cdc34-specific antibodies. Reactions were carried out for 5 min to form the Cdc34s.m thiol ester (A) and 30 min to form Ub-Cdc34 conjugates (B) as described under "Experimental Procedures." The reaction products were subjected to SDS-PAGE, transferred onto a polyvinylidene fluoride membrane, and incubated with a solution of affinity-purified Cdc34-specific rabbit antibodies. Immunoreactivity was detected by the use of alkaline phosphatase-conjugated goat antibodies against rabbit IgG according to the procedure of Blake et al. (31). Lane 1, uncleaved Cdc34; lane 2, reaction product.

**FIG. 4.** Formation of Cdc34s.m thiol ester and Ub-Cdc34 conjugates with "1-Ub-R48. Conditions were identical to those reported for Fig. 2 except "1-ubiquitin in the reaction was replaced by 2-1-ubiquitin R48. Only the major reaction product in each panel has been labeled, and the nature of the various reaction products is discussed in the text.
in a second dimension. As shown in Fig. 6A, the radiolabeled ubiquitin from the 43-kDa conjugate was found to be retained in an 18-kDa cleavage fragment. This fragment size is consistent with assigning the ubiquitin linkage site in the 43-kDa conjugate to a lysine within the C-terminal 80 residues of Cdc34. This cleavage fragment was designated as Ub-H2 in Fig. 6A. In contrast, cleavage of the 41-kDa conjugate yielded a 33-kDa radiolabeled fragment, indicating that the minor 41-kDa conjugate was formed by the linkage of ubiquitin to a site within the N-terminal 214 residues of Cdc34, and this fragment was designated as Ub-H1. Thus, the 41- and 43-kDa (Ub-R48)-Cdc34 conjugates were formed by the linkage of ubiquitin to distinctly different lysines in Cdc34. Although it is reasonable to expect a difference in the SDS gel mobility for two conjugates that differ in the position of ubiquitin linkage, the result shown here has provided the first experimental support.

Because the 43-kDa conjugate is significantly more abundant than the 41-kDa conjugate, it would appear that the linkage site for the 43-kDa conjugate is kinetically preferred. Further support for a preferred ubiquitin linkage site within the C-terminal 80 residues of Cdc34 was provided by analysis of the hydroxyamine-dependent cleavage of [125I]Ub-Cdc34 conjugates (Fig. 6, C and D). As expected, cleavage of the 43- and 41-kDa conjugates yielded the respective 18- and 33-kDa fragments. The major radiolabeled cleavage fragment from each higher order Ub-Cdc34 conjugate migrated at an apparent molecular mass that was 22–25 kDa lower than their uncleaved counterparts. This reduction in apparent molecular weight for each of these conjugates is consistent with the removal of the N-terminal 214-residue fragment by the hydroxyamine-dependent cleavage. Thus, the higher order conjugates were likely formed by the linkage of multiple ubiquitin groups to the C-terminal 80-residue region of Cdc34. These fragments were marked as Ub-H2 in Fig. 6C and were the predominant cleavage products. Longer autoradiography also revealed minor radiolabeled fragments with mobilities that were consistent with the removal of an 80-residue fragment from the radiolabeled conjugates although the possibility of nonspecific cleavages cannot be excluded in the interpretation of these minor cleavage products.

Assignment of Ubiquitin Linkage Site by Site-specific Cdc34 Mutants—Of the 15 lysine residues in Cdc34, only 4 are located within the C-terminal 80 residues of Cdc34. These lysines are clustered within a 22-residue sequence and are located at residue positions 273, 277, 293, and 294 (Fig. 6). To provide further confirmation that the kinetically preferred ubiquitin linkage site is located within this region of Cdc34, we constructed a Cdc34 mutant (Cdc34-4R) in which all 4 lysines were replaced by arginines (Fig. 7A). The Cdc34-4R protein was found to retain the ability to form thiol ester-linked ubiquitin adduct (Fig. 7B). However, only the previ-
**Fig. 6. Hydroxylamine cleavages of Cdc34 conjugates.** Cdc34 conjugates were formed with $^{125}$I-Ub-R48 (A and B) or with $^{125}$I-ubiquitin (C and D) for 2 h under standard reaction conditions. Conjugates were resolved in a 12% polyacrylamide gel-SDS in the first dimension, subjected to hydroxylamine cleavage (A and C), and the fragments were analyzed by a second-dimension electrophoresis in a SDS-14% polyacrylamide gel as described under “Experimental Procedures.” Conjugates that have not been subjected to hydroxylamine cleavages are shown in B and D. The schematic depicts the location of the 15 lysine residues in Cdc34 and the site of hydroxylamine cleavage.

Previously minor 41-kDa conjugate was formed with Cdc34-4R (Fig. 7C). This result is in agreement with the above assignment of a kinetically preferred ubiquitin linkage site within the C-terminal 80 residues of Cdc34 and a minor ubiquitin linkage site within the N-terminal 214 residues of Cdc34.

In order to locate the kinetically preferred ubiquitin linkage site to one of these 4 lysine residues, we constructed a set of Cdc34 mutants that contained a combination of lysine to arginine substitutions (Fig. 7A). To reduce the number of permutations, we considered the two adjacent Lys293 and Lys294 to be a single site, and this pair of lysines were either altered or left intact as a unit. All these mutants were found to retain the ability to form ubiquitin thiol ester (Fig. 7B). While there may be quantitative differences between the 4 lysines to accept ubiquitin, the data shown in Fig. 7C indicated the presence of either Lys237, Lys277, or the Lys293/294 was sufficient to provide a site for the formation of multiple ubiquitin-Cdc34 conjugates. These results indicated that all four lysines at this region of Cdc34 can be linked with ubiquitin. Since the majority of (Ub-R48)-Cdc34 conjugates were linked with a single Ub-R48, it is reasonable to conclude that while the linkage of the first ubiquitin can be at any one of these 4 lysines, additional linkages at more than 1 lysine can only occur at much slower rates. This mode of ubiquitin linkages has also been found with ubiquitin-$\beta$-galactosidase conjugates in which a single Lys48-specific multiubiquitin chain is linked to either one of 2 vicinal lysines in the $\beta$-galactosidase (4).

**DISCUSSION**

The *S. cerevisiae* CDC34 gene has previously been shown to encode a ubiquitin-conjugating enzyme (18). The function of this ubiquitin-conjugating activity, however, remains to be defined since Cdc34-dependent ubiquitination of cellular proteins has yet to be described. In this report, we demonstrated an in vitro autoubiquitination activity with bacterially expressed Cdc34.

The formation of Ub-Cdc34 conjugates in autoubiquitination involves the addition of multiple ubiquitin groups to Cdc34 in a highly specific manner. The addition of the first ubiquitin involves the transfer of the thiol ester-linked ubiquitin to one of 4 specific lysines that are clustered at the C-terminus of Cdc34. Additional ubiquitin groups are then added sequentially to the Lys48 residue of a preattached ubiquitin to form a multiubiquitin chain. The highly specific nature of linkages in Cdc34 autoubiquitination suggests that this activity is not an artifact from heterologous expression but is an inherent catalytic activity in Cdc34. The ability to catalyze the formation of specific ubiquitin-ubiquitin linkage at Lys48 is not shared by many of the presently purified E2 enzymes. In fact, this activity has only been formally demonstrated for an E2 enzyme that is able to catalyze the formation of a free Lys48-specific multiubiquitin chain (6). We discuss below possible physiological relevances of autoubiquitination in Cdc34 cell function.

The Lys48-specific multiubiquitin chain has only been found
ubiquitin with variants in which the Lys48 residue is either replaced or is reductively methylated. These results have led to the suggestion that the proteolytic targeting signal in ubiquitin-mediated proteolysis is served by the mult ubiquitin chain rather than by the simple linkage of a ubiquitin to an acceptor protein (4). A recent study on the in vivo degradation of a ubiquitin-Pro-ß-galactosidase fusion protein in S. cerevisiae provided a dramatic contrast on the effectiveness of a single ubiquitin versus the Lys48-specific mult ubiquitin chain to act as targeting signals in ubiquitin-mediated proteolysis (17). For this model substrate protein, a Lys48-specific multi ubiquitin chain is formed with the ubiquitin moiety in the fusion protein acting as the first ubiquitin unit. The formation of the mult ubiquitin chain can be blocked by either replacing Lys48 in the fused ubiquitin or by deleting the UBC4 gene, and under these conditions the in vivo half-life of this fusion protein is extended from 5 min to greater than 1 h. Given this functional correlation, it is reasonable to suggest that the Lys48-specific mult ubiquitin chain on Cdc34 can perform the same function to target Cdc34 for rapid degradation. There is evidence that Ub-Cdc34 conjugates are indeed formed in vivo. These conjugates have been detected in immunoprecipitates of yeast extracts with Cdc34-specific antibodies (2, 4). The demonstration of Cdc34 autoubiquitination here raises the possibility that the ubiquitin-conjugating activity in Cdc34 may function in part in the regulation of its own half-life, and the regulated degradation of Cdc34 may be an integral part of this protein’s function in the G1 to S transition in the cell cycle. Our present study utilized Cdc34 that was expressed in E. coli, and it remains an open question whether Cdc34 autoubiquitination is a conditional event in yeast cells because of additional post-translational modification(s) or by virtue of its interactions with other cellular proteins. While these interesting questions remain to be addressed, the present characterization on the site of autoubiquitination should provide an important means for future studies to perturb or to block this reaction in vivo. These studies are currently being pursued.

The 295-residue Cdc34 protein contains 15 lysine residues. It is shown here that the preferential ubiquitination site is provided by one of 4 lysines (Lys273, Lys277, Lys283, and Lys294) that are clustered near the Cdc34 C terminus. This region of Cdc34 is dispensable for Cdc34 to accept ubiquitin from E1 in vivo since a truncated protein with the first 170 residues of Cdc34 is sufficient to retain this activity (18). Thus, the active site Cys80 and ubiquitin-accepting lysines are likely to be located on separate structural domains. The preferential selection of these lysines, however, suggests that these two domains are either located in proximity to each other or may interact transiently because of motional freedom in the polypeptide chain as suggested by Bachmair and Varshavsky (30). There is also a second consideration which suggests that these two domains are not locked in a rigid structure. After the addition of the first ubiquitin, the acceptor lysine must be moved so that Lys48 in ubiquitin can occupy the site that accepts the transfer of a second ubiquitin from the active site. Each subsequent ubiquitin linkage should also require a translocation step to position Lys48 in the end ubiquitin group for a further ubiquitin transfer. Thus, a considerable degree of freedom is likely required.

Our kinetic analysis indicated that Cdc34 autoubiquitination proceeds via an intramolecular transfer of the Cys80-linked ubiquitin to a lysine in Cdc34 with a first-order rate constant of 0.025 min⁻¹ (t₁/₂ ≈ 28 min). This value is similar

2 M. Goebl, personal communication.
3 A. Banerjee, L. Gregori, Y. Xu, and V. Chau, unpublished results.

**FIG. 7. Formation of Cdc34-Lys and Ub-Cdc34 conjugates with site-specific Cdc34 mutant proteins.** A, a schematic depicting the specific Lys→Arg substitutions in the various Cdc34 mutants. Mutant CDC34 clones in M13mp19 were used to infect E. coli TG1 cells, and Cdc34 mutant proteins were induced to express by isopropyl-1-thio-ß-D-galactopyranoside as described under “Experimental Procedures.” Cdc34 mutant proteins in crude E. coli extracts and 125I-ubiquitin were used to assay for the formation of Cdc34-Lys-thiol ester (B) and Ub-Cdc34 conjugates (C). In B, the E1L3 and Cdc34L3 thiol esters are indicated by open and closed arrows, respectively. In C, the bracket indicates Ub-Cdc34 adducts. Because of variation in the expression level of different Cdc34 mutants, autoradiography exposure times were adjusted to obtain a uniform Cdc34-L3 level for the purpose of comparison.

previously on proteins that are undergoing ubiquitin-mediated proteolysis (4, 5, 17, 25-29). In these cases, the degradative rates of substrate proteins are severely reduced when the formation of mult ubiquitin chains is blocked by replacing

Retrieval from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3331522/
to those reported for the \( E2_{26,16} \)-catalyzed formation of ubiquitin dimer (0.006 min\(^{-1} \)) and the transfer of ubiquitin from other purified \( E2 \) enzymes to model substrate proteins (6, 11, 12). Subsequent additions of ubiquitin groups to form the mult ubiquitin chain appears to follow processive kinetics as higher order conjugates were formed with rates much faster than those that could be predicted from simple distributive kinetics. We also noted that free ubiquitin can compete efficiently with autoubiquitination at concentrations of ubiquitin > 0.1 mM, resulting in the formation of a Lys\(^{48} \)-linked ubiquitin dimer. This is not unexpected given that Lys\(^{48} \) in ubiquitin is a recognition determinant in mult ubiquitin chain formation, and ubiquitin, albeit with low affinity, may serve as an alternate substrate.

Acknowledgments—We thank Dr. Russell Yamazaki for helpful discussion and comments on the manuscript, Dr. Mark Goebl for providing Cdc34-specific antibodies and unpublished results. We gratefully acknowledge help from Dr. David Gonda in the use of PCR to obtain a Cdc34-4R sequence.

REFERENCES