The Nedd8-conjugated ROC1-CUL1 Core Ubiquitin Ligase Utilizes Nedd8 Charged Surface Residues for Efficient Polyubiquitin Chain Assembly Catalyzed by Cdc34*

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Lysine 48-linked polyubiquitin chains are the principal signal for targeting proteins for degradation by the 26 S proteasome. Here we report that the conjugation of Nedd8 to ROC1-CUL1, a subcomplex of the SCF-ROC1 E3 ubiquitin ligase, selectively stimulates Cdc34-catalyzed lysine 48-linked multiubiquitin chain assembly. We have further demonstrated that separate regions within the human Cdc34 C-terminal tail are responsible for multiubiquitin chain assembly and for physical interactions with the Nedd8-conjugated ROC1-CUL1 to assemble extensive ubiquitin polymers. Structural comparisons between Nedd8 and ubiquitin reveal that six charged residues (Lys4, Glu12, Glu14, Arg25, Glu28, and Glu31) are uniquely present on the surface of Nedd8. Replacement of each of the six residues with the corresponding amino acid in ubiquitin decreases the ability of Nedd8 to activate the ubiquitin ligase activity of ROC1-CUL1. Moreover, maintenance of the proper charges at amino acid positions 14 and 25 are necessary for retaining wild type levels of activity, whereas introduction of the opposite charges at these positions abolishes the Nedd8 activation function. These results suggest that Nedd8 charged surface residues mediate the activation of ROC1-CUL1 to specifically support Cdc34-catalyzed ubiquitin polymerization.

Nedd8 (or its orthologue Rub1) is a small ubiquitin (Ub)-like molecule that modifies all members of the Cullin/Cdc53 protein family (1, 2), resulting in the formation of an isopeptide bond linkage between the ε-amino group of a conserved Cullin lysine residue and the C-terminal carboxyl group of Nedd8 glycine 76. The conjugation is an ATP-dependent reaction that requires a residue and the C-terminal carboxyl group of Nedd8 glycine 76.

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Nedd8 Activates Cdc34-catalyzed Polyubiquitin Chain Assembly

CUL1 complex selectively stimulates Cdc34-catalyzed, but not Ubc4/5-catalyzed, Lys^48-linked multimer ubiquitin chain assembly. Furthermore, we present evidence that Nedd8 charged surface residues mediate the activation of ROC1-CUL1 in supporting ubiquitin polymerization.

EXPERIMENTAL PROCEDURES

Plasmids

**Construction of pET-His-HA-Cdc34 and Its C-terminally Truncated Derivatives—** To generate a plasmid for the expression of human Cdc34 in Escherichia coli, the full-length cDNA sequence (obtained from M. Pagano) was subcloned into the pET-3a vector (Novagen). Both the HA and six-histidine sequences were positioned at the N terminus of Cdc34. To construct various C-terminal Cdc34 truncations, stop codons were generated at the desired locations in the pET-His-HA-Cdc34 plasmid using the QuickChange™ site-directed mutagenesis kit (Stratagene) as per the manufacturer’s instructions. Primers used for PCR were as follows: Cdc34 (aa 1–208), GGCCTGTCGCTTCCGGATGATGTC (5’/H11032 CGAGGGC (5’); and Cdc34 (aa 1–194), GGCCTGTCGCTTCCGGATGATGTC (5’) and GCCGTTGCTGGCCGCTCGGCTTTCCGGTCAGCGTC (3’); and Cdc34 (aa 1–194), GGCCTGTCGCTTCCGGATGATGTC (5’) and GCCGTTGCTGGCCGCTCGGCTTTCCGGTCAGCGTC (3’). The presence of the desired mutation in each construct was verified by DNA sequencing.

Protein Expression and Isolation

**Preparation of His-HA-Cdc34 and Its C-terminally Truncated Derivatives—** pET-3a plasmids expressing the wild type and mutant Cdc34 proteins were transformed into the pY2 (Affinity)-containing BL21 (DE3) cells and grown in LB (0.5 liter) with 0.5 mg/ml ampicillin and 0.4% glucose in the presence of 0.4% isopropyl-D-thiogalactopyranoside at a final concentration of 0.8 g/ml antipain, and 0.2 mg/ml leupeptin. The harvested cell pellets were washed once with buffer A (25 ml) and twice with buffer D (50 ml Tris-HCl, pH 8.0, 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, 0.4 µg/ml antipain, and 0.2 µg/ml leupeptin). The washed pellet was then resuspended in 20 ml buffer E (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 5 mM imidazole) plus 8 mM urea, and the resulting mixture was incubated at room temperature for 30 min. The solubilized material (10 ml) was mixed with Ni^2+ nitrilotriacetic acid-agarose beads (3 ml for 2 h at 4 °C, followed by washing the beads with progressively decreasing concentrations of urea in buffer E). The bound protein was then eluted by imidazole and further purified using Q Sepharose and Sephadex-75 gel filtration chromatography as described above. A single peak of Cdc34 (aa 1–169) monomer was pooled and used in the studies described in this report.

**Expression and Purification of Nedd8 Mutants—** The various Nedd8 mutant proteins were purified identically as the wild type protein using the procedure as previously described (21). The proteins were concentrated to ~0.3 mg/ml.

**Other Reagents—** APP-BP1/UBA3 was affinity-purified as described previously (21). For preparation of Ubc12, GST-fused Ubc12 was expressed and isolated on glutathione beads as previously described (21).

**Asays—** Glutathione-Sepharose 4B (Amersham Biosciences) was mixed with glutathione-Sepharose 4B (Amersham Biosciences) for 1 h at 4 °C. After centrifugation, the supernatant containing both Ubc12 and thiolom was mixed with streptavidin beads (Amersham Biosciences; 5 µl of beads/unit of thiolom), and the resulting suspension was rocked for 1 h at 4 °C to allow the absorption of the biotinylated thiolom to the beads. Ubc12, free of GST, and thiolom was obtained by centrifugation.

**Human Ub E1 was prepared as described (5).** The expression and purification of Ub56e were carried out as described by Ohta et al. (6).

**Nedd8-dependent Ub Ligase Activation Assay—** To conjugate Nedd8 or its mutant form to ROC1-CUL1, E. coli extract containing GST-ROC1-CUL1 (aa 324–776) (21) were mixed with glutathione-Sepharose 4B (Amersham Biosciences) for 1 h at 4 °C. Bacterial proteins were removed by washing the beads three times with 0.5 ml of buffer F (50 mM Tris-HCl, pH 8.0, 1% Triton X-100, 0.5 mM NaCl, 10 mM EDTA, 10 mM EGTA, 10% glycerol, 2 mM phenylmethylsulfonyl fluoride, 0.4 µg/ml antipain, and 0.2 µg/ml leupeptin) and twice with buffer C plus 50 mM NaCl. Glutathione beads containing ~1 µg of GST-ROC1-CUL1 (aa 324–776) were used in the assay. For Nedd8 conjugation, the immobilized GST-ROC1-CUL1 (aa 324–776) was incubated with 50 mM Tris-HCl, pH 7.5, 5 mM MgCl_2, 2 mM ATP, 0.6 mM DTT, 0.1 mg/ml bovine serum albumin, APP-BP1/UBA3 (25 ng), Nedd8 (0.5 µg), and Ubc12 (1 µg) at 37 °C for 60 min. Excess Nedd8 modification agents were removed by washing the beads three times with 0.5 ml of buffer F and twice with buffer C plus 50 mM NaCl.

**Glutathione beads containing ~1 µg of GST-ROC1-CUL1 (aa 324–776) were used in the assay.** For Nedd8 conjugation, the immobilized GST-ROC1-CUL1 (aa 324–776) was incubated with 50 mM Tris-HCl, pH 7.5, 5 mM MgCl_2, 2 mM ATP, 0.6 mM DTT, 0.1 mg/ml bovine serum albumin, APP-BP1/UBA3 (25 ng), Nedd8 (0.5 µg), and Ubc12 (1 µg) at 37 °C for 60 min. Excess Nedd8 modification agents were removed by washing the beads three times with 0.5 ml of buffer F and twice with buffer C plus 50 mM NaCl. The GST-ROC1-CUL1 (aa 324–776) complex, conjugated with the wild type or mutant Nedd8 protein, was then incubated in a reaction mixture (30 µl) that contained 50 mM Tris-HCl, pH 7.5, 5 mM MgCl_2, 2 mM NaF, 10 mM sodium dodecyl sulfate, 2 mM ATP, 0.6 mM DTT, 0.1 mg/ml bovine serum albumin, APP-BP1/UBA3 (25 ng), Nedd8 (0.5 µg), and Ubc12 (1 µg) at 37 °C for 30 min. This treatment was sufficient to abolish the majority of the DTT-sensitive Ub-linked thiol-esters, resulting in an identical pattern of Ub ligation products as observed when the DTT concentration was increased to 0.1 mM (data not shown). Thus, the Ub-conjugates,
visualized by autoradiography following 10% SDS-PAGE, were predominantly those linked via isopeptide bonds.

Ub Conjugation Assay—The reaction mixture (20 μl) contained 50 mM Tris-HCl, pH 7.4, 5 mM MgCl2, 0.2 mM DTT, 2 mM ATP, 3 μg of 32P-Ub, E1 (0.6 pmol), and 0.5 μg of the E2 protein was used. C, the effect of Nedd8 was analyzed using subsaturating levels of Ubc5c (amounts indicated). Aliquots of each reaction were separated by 10% SDS-PAGE followed by autoradiography. The high molecular weight Ub polymers (greater than 100 kDa) were quantitated using a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA). The results are presented in graphs.

RESULTS
Conjugation of Nedd8 to CUL1 Activates Cdc34-catalyzed, but Not Ubc5c-catalyzed, Polyubiquitin Chain Assembly—We have recently demonstrated that Nedd8 modification activates the ability of ROC1-CUL1 to support Ub polymerization in the presence of Cdc34 (21). Previous studies have shown that both Ubc4 and -5 are also capable of mediating ROC1-dependent polyubiquitin chain assembly (6). To determine whether Nedd8 activation was E2-specific, we compared Cdc34 and Ubc5c in their ability to catalyze Ub polymerization in the presence of the Nedd8-conjugated ROC1-CUL1 complex. For this purpose, the unmodified GST-ROC1-CUL1 (aa 324–776) complex, expressed and assembled in E. coli, was immobilized on glutathione-Sepharose 4B. To conjugate Nedd8 to the CUL1 (aa 324–
Cdc34 (Fig. 1) or Ubc5c (Fig. 1). In all cases, the Ub ligase A included free Ub chains (5, 10) and autoubiquitinated E2 (7, 8). The high molecular weight mass Ub conjugates formed were complex and likely conjugates derived from the monomeric Ub. The high molecular weight mass Ub conjugates formed were complex and likely included free Ub chains (5, 10) and autoubiquitinated E2 (7, 8).

Consistent with our previous observation (21), Nedd8 modification activated the Cdc34-catalyzed assembly of 32P-Ub chains by 19-fold within 9 min of incubation (Fig. 1A). Surprisingly, Ubc5c-catalyzed Ub polymerization was not significantly affected by Nedd8 modification at all of the time points tested (Fig. 1B). Furthermore, Nedd8 activation was not observed with Ubc5c that was present in sub saturating levels (Fig. 1C). This excludes the possibility that the apparent absence of activation by Nedd8 with Ubc5c was due to the use of saturating levels of the E2 protein. Similarly, no Nedd8 activation was detected when Ubc4, which shares a high degree of sequence homology with Ubc5, was used as the E2 in the reaction (data not shown). These results demonstrate that the conjugation of Nedd8 to CUL1 selectively activates Cdc34-catalyzed, but not Ubc4/5c-catalyzed, polyubiquitin chain formation.

We next explored the difference between Cdc34 and Ubc5c in the utilization of Ub lysine receptor residue(s) for the assembly of polyubiquitin chains. Consistent with our previous observation (5), when UbK48R was used in place of the wild type Ub, Cdc34-catalyzed polyubiquitin chain formation was almost completely inhibited (Fig. 2A), confirming that Cdc34 assembles Lys48-linked Ub chains. In contrast, when Ubc5c was used as the E2, polyubiquitin chains were still assembled efficiently (Fig. 2B, lanes 2–5). Conjugation of Nedd8 to CUL1 had no significant effect on the Ubc5c-catalyzed synthesis of non-Lys48-linked (most likely Lys63-linked; Refs. 22 and 23) Ub chains (lanes 6–9). Taken together, these results show that Nedd8 specifically activates the Cdc34 catalyzed synthesis of Lys48-linked multi-Ub chains.

The C Terminus of Human Cdc34 Is Required for Mediating the Nedd8-stimulated and ROC1-CUL1-dependent Assembly of Polyubiquitin Chains—The above studies suggest a specific cooperation between Nedd8 and Cdc34 that promotes the Ub polymerization reaction. Previous studies have demonstrated that the C terminus of S. cerevisiae Cdc34 is required for cell cycle control (24, 25) and that residues 171–209 constitute a minimal motif both necessary and sufficient for binding to the SCF components (26). These findings prompted us to examine the role of the C-terminal tail of human Cdc34 in mediating the Nedd8 stimulated and ROC1-CUL1-dependent Ub polymerization by deletion analysis. Based on sequence alignment to its S. cerevisiae counterpart, the human Cdc34 contains a C-terminal tail spanning residues 171–236. Both the wild type and the three C-terminally truncated Cdc34 proteins (Cdc34 residues 1–169, 1–194, and 1–208) were expressed in bacteria and purified to homogeneity as judged by Coomassie staining analysis (Fig. 3A).

Purified Cdc34 protein possesses a number of biochemical activities. These include the ability of Cdc34 to conjugate Ub in the presence of E1, to catalyze autoubiquitination (27), to directly interact with ROC1-CUL1 for assembling Lys48-linked multi-Ub chains (11), and to cooperate with Nedd8 for the activated synthesis of Ub polymers (Ref. 21; Fig. 1). In keeping with the notion that the Ubc domain (spanning amino acid residues 1–170 in human Cdc34; Ref. 28) is responsible for Ub conjugation activity, all three truncated Cdc34 proteins retained their ability to conjugate Ub in the presence of E1 (Fig. 3B). The observed DTT-insensitive Cdc34-Ub conjugates are monoubiquitinated species (see below).

Banerjee et al. (27) have previously shown that the purified S. cerevisiae Cdc34 protein catalyzes its own ubiquitination to assemble a multi-Ub chain on a lysine residue within its C terminus. As shown, human Cdc34 alone produced an array of Ub conjugates (Fig. 3C, lane 4). While the predominant reaction product was the monoubiquitinated Cdc34 that migrated as a doublet of ~47 kDa, other Ub conjugates included the E2 protein linked with chains composed of up to five Ub moieties. Cdc34 (aa 1–208) was more active than the wild type protein in catalyzing autoubiquitination, producing Ub conjugates with molecular masses up to 200 kDa (lane 6). Cdc34 (aa 1–194) catalyzed autoubiquitination with an efficiency comparable to that observed with the wild type protein (compare lanes 4 and 8). Interestingly, while Cdc34 (aa 1–169) was still capable of mono- and diubiquitination, it did not form multi-Ub chains (lane 10), suggesting a processivity deficiency in polymerizing...
FIG. 3. **Multiple biochemical activities are associated with the C terminus of human Cdc34 protein.** A, Coomassie staining analysis of the wild type and C-terminally truncated Cdc34 proteins. Bacterially expressed and purified Cdc34 (lane 1), Cdc34 (aa 1–208) (lane 2), Cdc34 (aa 1–194) (lane 3), and Cdc34 (aa 1–169) (lane 4) (1 μg of protein each) were electrophoresed on 12.5% SDS-PAGE followed by Coomassie staining. B, Ub conjugation activity of the wild type and C-terminally truncated Cdc34 proteins. The Ub conjugation assay was carried out as described under “Experimental Procedures” in the presence or absence of the indicated components. When samples were treated with 0.1 M DTT (lanes 3,
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In the presence of ROC1-CUL1, but in the absence of Nedd8 modification, Cdc34 (aa 1-208) supported multi-Ub chain assembly, albeit with about a 100-fold lower efficiency compared with the wild type (Fig. 3D, compare lanes 4 and 5 and lanes 10 and 11). Under these conditions, both the wild type and Cdc34 (aa 1-208) assembled Ub chains with molecular mass greater than 200 kDa, demonstrating a role for ROC1-CUL1 in promoting the assembly of extensive polyubiquitin chains. Cdc34 (aa 1-194) was only barely stimulated by ROC1-CUL1 (compare lanes 15 and 17). Furthermore, in contrast to both the wild type and Cdc34 (aa 1-208), Ub polymers formed by Cdc34 (aa 1-194) and ROC1-CUL1 were predominantly those of limited lengths that migrated in the range of 70-100 kDa. Finally, the addition of ROC1-CUL1 could not activate Cdc34 (aa 1-169) to assemble multi-Ub chains (Fig. 3D, lanes 20-23). These results demonstrate that the extreme C terminus of human Cdc34 (residues 209-236) is required for its maximal activity in catalyzing ROC1-CUL1-dependent synthesis of Ub polymers. Furthermore, assembly of extensive polyubiquitin chains by ROC1-CUL1 requires Cdc34 residues 195-208. This suggests that Cdc34 (aa 195-208) may constitute a motif that interacts with the ROC1-CUL1 complex.

Similar to that observed with the wild type Cdc34 (Fig. 3D, lanes 4-7; see 2-h exposure), Cdc34 (aa 1-208)-catalyzed Ub polymerization was stimulated up to 10-fold when the ROC1-CUL1 complex was conjugated with Nedd8 (compare lanes 11 and 13). Production of Ub conjugates by Cdc34 (aa 1-194) was only slightly increased by Nedd8 modification (compare lanes 16 and 18). Moreover, Nedd8 did not increase the mass of Ub polymers produced by Cdc34 (aa 1-194) from 100 to 200 kDa. No effect by Nedd8 was observed with Cdc34 (aa 1-169) (Fig. 3D, lanes 22-25). These data are consistent with the notion that Nedd8 acts to increase the efficiency with which Cdc34 and ROC1-CUL1 polymerize Ub. Furthermore, the observation that Nedd8 activated the wild type and Cdc34 (aa 1-208) to a similar extent (maximally 10-20-fold) suggests that Cdc34 residues 208-236 are not critically involved in mediating the interaction with Nedd8. Of note, like the wild type protein, both Cdc34 (aa 1-208) and Cdc34 (aa 1-194) assembled Lys48-linked polyubiquitin chains (data not shown).

A GST-based pull-down assay was employed to test the ability of the wild type and C-terminally truncated Cdc34 proteins to interact with glutathione bead-immobilized GST-ROC1-

6, 9, and 12) following the conjugation reaction, the mixture was boiled for 3 min prior to 12.5% SDS-PAGE. C, Cdc34 residues 170-194 are required for multi-Ub chain assembly. The autoubiquitination of the wild type and C-terminally truncated Cdc34 proteins was assayed by incubating 1 μg of the E2 protein in a solution containing 50 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 2 mM NaF, 10 mM okadaic acid, 2 mM ATP, 0.6 mM DTT, and 5 μg of 32P-Ub, in the presence (lanes 4, 8, 9, and 10) or absence (lanes 3, 5, 7, and 9) of E1 (0.6 pmol). The reaction was incubated at 37 °C for 30 min, and products were separated by 10% SDS-PAGE, D, the ROC1-CUL1-dependent polyubiquitin chain assembly by Cdc34 requires a minimal motif spanning residues 195-208 within the E2 protein. The Nedd8-stimulated and ROC1-CUL1-dependent Ub polymerization by Cdc34 was carried out as described in the legend to Fig. 1. The incubation time was 30 min. Two levels of the wild type and mutant Cdc34 proteins, 0.25 μg (lanes 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24) and 0.5 μg (lanes 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, and 25), were used. The autoradiogram containing reactions 1-13 is shown with both long (48-h) and short (2-h) exposures for better comparison of the effects of Nedd8 modification.
CUL1 (aa 324–776). As shown in Fig. 4A, the wild type Cdc34 was specifically bound to the complex (lanes 3 and 4), in keeping with our previous observation (11). It was estimated that under the conditions used, ~8% of the Cdc34 input was retained by the GST-ROC1-CUL1 (aa 324–776) complex. While removal of residues 209–236 reduced the binding by 60% (compare lanes 4 and 7), further deletions eliminated the interaction (lanes 9, 10, 12, and 13). For unknown reasons, Cdc34 (aa 1–169) was found to interact with GST substantially, whereas the other Cdc34 derivatives did not (lane 12). Since the replacement of GST by GST-ROC1-CUL1 (aa 324–776) did not further increase the binding of Cdc34 (aa 1–169) (compare lanes 12 and 13), the observed interaction between Cdc34 (aa 1–169) and GST-ROC1-CUL1 (aa 324–776) was due to an affinity of the truncated E2 protein for GST, but not ROC1-CUL1 (aa 324–776). Taken together, these results suggest the presence of ROC1-CUL1-interacting residues within the region spanning amino acids 195–208 of Cdc34. However, the extreme C-terminal portion (residues 209–236) plays a significant role in enhancing the interaction. This is entirely consistent with the observation that while Cdc34 residues 195–208 were essential for the ROC1-CUL1-dependent synthesis of Ub polymers, the maximal activity requires residues 209–236 within the E2 protein (Fig. 3D).

A data base search identified putative Cdc34 orthologs from Drosophila and C. elegans. Sequence analysis reveals an expected conservation in the catalytic Ubc domain among human, Drosophila, C. elegans, and S. cerevisiae Cdc34 proteins (Fig. 5). Interestingly, an additional homologous region, corresponding to the human Cdc34 residues 170–208, was found among these four Cdc34 orthologs (29% similarity at amino acid levels). This suggests that Cdc34 may utilize this evolutionary conserved region for interacting with the Nedd8-conjugated ROC1-CUL1 core Ub ligase to assemble multi-Ub chains.

**Substitution of Nedd8 Charged Surface Amino Acids with the Corresponding Ub Residues Inhibits Nedd8 Activity**—To further understand the role of Nedd8 in activating the ROC1-CUL1 mediated, Cdc34-catalyzed Ub polymerization, we sought to identify Nedd8 amino acid residue(s) that are required for this activity. Comparison of the crystal structures between Nedd8 and Ub identified unique amino acids within Nedd8 that may contribute to its function. Like Ub, Nedd8 displays an asymmetric distribution of charged residues that are organized to form “acidic” and “basic” faces (29). Most noticeably, some of these charged Nedd8 residues are conserved among the various Nedd8 orthologs but differ from Ub at the corresponding positions. As illustrated, on helix 1 of Nedd8, Glu28 and Glu31 form an electronegative surface that is not present on Ub (Fig. 6). There are also Glu12 and Glu14 on strand 2 that form another electronegative surface. Other notable differences between Ub and Nedd8 include an Asn to Arg substitution on residue 25 and a Phe to Lys substitution on residue 4. Intriguingly, these six charged Nedd8-specific residues (Lys4, Glu12, Glu14, Arg25, Glu28, and Glu31) are arranged in two surface patches that lie along each side of the Nedd8 molecule (Fig. 6), suggesting a possible role for these residues involved in electrostatic interactions with other proteins.

To determine whether residues Lys4, Glu12, Glu14, Arg25, Glu28, and Glu31 are required for Nedd8 function, point mutants were generated by site-directed mutagenesis, replacing charged amino acids with the corresponding Ub residues, designated as K4F, E12T, E14T, R25N, E28A, and E31Q. Mutant...
proteins were expressed in bacteria, and the purified Nedd8 variants were compared with the wild type protein for their ability to conjugate to CUL1 and for their capacity to activate the Ub ligase activity of ROC1-CUL1. Immunoblot analysis revealed that all six purified mutant Nedd8 proteins were conjugated to CUL1 (aa 324–776) with similar efficiencies compared with the wild type protein (Fig. 7A, compare lanes 3 and lanes 4–9). Consistent with our previous observation (21), the conjugation reaction converted ~50% of CUL1 (aa 324–776) into two slow migrating species conjugated with one or two Nedd8 moieties. Of note, no conjugate was formed between CUL1 (aa 324–776) and two Nedd8E31Q moieties (Fig. 7A, lane 9). It is presently unclear whether the ROC1-CUL1 complex conjugated with two Nedd8 moieties is more efficient than that with one Nedd8 molecule in activating Ub polymerization. It should be noted that only single Nedd8 conjugates of CUL1 are detected in cells.

Next, the mutant Nedd8 variants were compared with the wild type protein for their ability to activate the ROC1-CUL1 Ub ligase. As shown in Fig. 7B, all of the mutants were less effective than the wild type, albeit at varying degrees, in activating ROC1-CUL1. While the effects of Nedd8K14T and Nedd8E14D were the most dramatic, reducing the Nedd8 activation function by 4-fold, Nedd8E12T and Nedd8E31Q appeared to possess modest effects, decreasing the Nedd8 activity by less than 25%. These results suggest that Nedd8 charged surface residues are critical in activating the ability of ROC1-CUL1 to promote Ub polymerization.

Maintenance of Proper Electrostatic Potential at Positions 14 and 25 Is Critical for Nedd8 Activity—We next determined whether the Nedd8 activity was critically dependent on the type of electrostatic potential possessed by the charged surface amino acid residues. For this purpose, Nedd8 residues Glu14 and Arg25 were each replaced by a residue of the same charge group, or a residue with the opposite electrostatic potential. Purified Nedd8 mutant proteins were compared with the wild type for their ability to conjugate to CUL1 as well as to activate the Ub ligase activity of ROC1-CUL1. As shown (Fig. 8A), Nedd8 variants containing aspartate (lane 5) or arginine (lane 6) in place of glutamate at position 14 were conjugated to CUL1 (aa 324–776) with approximately equal efficiency as the wild type (lane 3) or Nedd8E14T (lane 4). When examined for their ability to activate the Ub ligase activity of ROC1-CUL1, Nedd8E14T or Nedd8K14T reduced the level of activation by 5- or 30-fold, respectively, in comparison with the wild type protein (Fig. 8B, compare lane 3 with lanes 4 and 6). In contrast, Nedd8E14D, maintaining the same charge at position 14, had a minimal effect, reducing the Nedd8 activity by 1.5-fold (lane 5). These results suggest that the presence of an acidic residue and, presumably, its negative charge at position 14 is critical for the Nedd8-mediated activation of the ROC1-CUL1 ubiquitin ligase.

When Nedd8 residue Arg25 was mutated to lysine or glutamate, immunoblot analysis revealed that neither substitution affected the conjugation of Nedd8 to CUL1 (aa 324–776) (Fig. 9A, lanes 3–6). While Nedd8E25K, retaining the same charge as the wild type, was as active as the wild type protein (Fig. 9B, compare lanes 3 and 5), Nedd8E25R, containing the opposite charge at position 25, reduced the level of activation by 10-fold (Fig. 9B, compare lanes 3 and 6). These results strongly support the notion that specific types of electrostatic potential at proper positions on the surface of Nedd8 are involved in the activation of ROC1-CUL1 to support Ub polymerization.

**DISCUSSION**

*Distinct Role of Cdc34 in the Assembly of Nedd8-stimulated Polyubiquitin Chains—* In this report, we have shown that Nedd8 selectively activates the Cdc34-catalyzed synthesis of Lys48-linked Ub polymers and that this effect is mediated by Nedd8 charged surface residues.

Cdc34 is a member of the class II E2 ubiquitin-conjugating enzymes, which are characterized by the presence of a C-terminal extension in addition to the N-terminally located conserved catalytic domain (Ubc domain) (28). The budding yeast Cdc34 is an essential gene product that primarily acts at the G1 to S-phase transition by mediating the ubiquitin-dependent degradation of the cyclin-dependent kinase inhibitor Sic1 in a process that requires the participation of the SCP3-E1-ROC1/Rbx1 E3 ubiquitin ligase (30–33).

We have shown that in the presence of ROC1-CUL1, Ubc4/5 is capable of assembling Ub polymers in a reaction that is not stimulated by Nedd8 modification. The Ubc4/5 family of proteins, required for stress response, belongs to the class I subtype E2 conjugating enzymes, which are primarily composed of the Ubc core catalytic domain without a C-terminal tail (28). Cdc34 and Ubc4/5 differ in that the former enzyme catalyzes Lys48-linked Ub polymer formation, the latter assembles both Lys48- and Lys29-linked multi-Ub chains (5, 22, 23, 27). While the precise role of Lys29-linked chains remains to be...
determined, it is evident, however, that uniform Lys29-linked chains cannot be broadly used in proteolytic targeting. This is because yeast cells expressing UbK29R as the sole source of Ub exhibit unaltered proteolytic competence (35). In contrast, yeast cells induced to express UbK48R are arrested in late G2 or M phase of the cell cycle and are defective in the turnover of short-lived proteins, demonstrating that the Lys48-linked chains are the principal signal for targeting proteins for degradation by the 26S proteasome (reviewed in Ref. 34). It remains to be determined whether Cdc34 functions as the predominant E2 conjugating enzyme for SCF-ROC1 in vivo to assemble Lys48-linked polyubiquitin chains for targeting substrate degradation.

Our data is consistent with the hypothesis that Nedd8 specifically up-regulates the Cdc34-dependent proteolytic pathway. We demonstrate that Nedd8 acts to increase the rate and efficiency with which Cdc34 and ROC1-CUL1 polymerize Ub (Fig. 1). This is in keeping with the observed effects of Nedd8 on the Cdc34-catalyzed ubiquitination of IκBα in vitro. Reed et al. (18) have shown that the SCFp-TRCP complex containing a CUL1K720R mutant subunit exhibited a decreased efficiency of the ubiquitination of IκBα with both Cdc34 and UbcH5a present. However, the overall pattern of polyubiquitin chains produced by this non-Nedd8-modified E3 complex resembles those formed by the Nedd8-conjugated complex. Consistent with this, we have observed that in the presence of Cdc34, while SCFp-TRCP-ROC1 conjugated with Nedd8 promotes the ubiquitination of IκBα more efficiently than the mutant SCFp-TRCP-ROC1 complex containing CUL1K720R, the polyubiquitin chains generated by both complexes are similar (data not shown). These data suggest that in the Cdc34-catalyzed ubiquitination reactions, Nedd8 functions to increase the efficiency of polyubiquitin chain synthesis.

Role of the C-terminal Tail of Human Cdc34 in Polyubiquitin Chain Assembly—The distinct function of S. cerevisiae Cdc34 in cell cycle control has been attributed to its C-terminal tail (amino acids 170–295). Both the Ellison (24) and Gonda (25) laboratories have shown that a chimeric Ubc2-Cdc34 protein, containing Ubc2 residues 1–151 (the Ubc domain) and Cdc34 residues 171–244, possesses both Ubc2 and Cdc34 activities in vivo. In subsequently published studies, the Ellison (36) and Goebl (26) groups have further defined residues 171–209 as a minimal motif (called CCD) that is required for Cdc34 function in vivo and for binding to the SCF components. These findings suggest a unique role for the C terminus of S. cerevisiae Cdc34 in mediating its cell cycle function by interacting with the SCF.

Previous studies have shown that the human Cdc34 cDNA can functionally substitute for the S. cerevisiae cdc34 gene (37), demonstrating a functional conservation between the two orthologs. In this study, we presented evidence implicating that the C terminus of human Cdc34 contains multiple biochemical activities (summarized in Fig. 4B). Based on results with both yeast (27) and human (this study) Cdc34, it is evident that this E2 enzyme possesses an intrinsic ability to assemble Ub

FIG. 7. Substitution of Nedd8 charged surface residues with the corresponding Ub residues inhibits Nedd8 activity. (GST-ROC1)-FLAG-CUL1 (aa 324–776), immobilized on glutathione-Sepharose 4B, was modified by purified Nedd8 wild type protein or mutant variants as indicated (0.6 μg of protein used in each case) using the procedure described under “Experimental Procedures.” The resulting beads were incubated with the ubiquitination components including 0.5 μg of Cdc34, 20 ng of E1, 3 μg of 32P-Ub, and other components as described under “Experimental Procedures.” Aliquots of the reaction products were separated by 10% SDS-PAGE followed by immunoblot analysis using anti-FLAG antibodies (A) or by direct autoradiography (B). Production of high molecular mass Ub polymers (>100 kDa) were quantitated using a PhosphorImager and shown in bar graphs. The activity shown in lane 2 was considered as the basal value that had been subtracted when the wild type and mutant Nedd8 were compared for their ability to activate the Ub ligase activity of ROC1-CUL1 (see “Results”).

![Diagram](http://www.jbc.org/bibgraphics/fig7a.png)
chains. In humans, this activity appears to require Cdc34 amino acid residues 170–194. The Ellison group has previously proposed a Cdc34 dimerization model to account for its ability to catalyze the Ub ligation reaction (36). It was suggested that CCD (residues 171–209) directly contacts the catalytic domain of the other monomer, bringing two conjugated Ub molecules into proximity for ligation. In support of this, cross-linking analysis indicates a critical role of CCD for the yeast Cdc34 oligomerization reaction (36). Whether the human Cdc34 residues 170–194 are directly involved in an intermolecular interaction with the catalytic domain of another Cdc34 monomer remains to be determined.

However, in the absence of ROC1-CUL1, the Ub chains assembled by the human Cdc34 are both inefficient and of limited lengths. Evidence provided by this study and previous works strongly suggests that ROC1-CUL1 contacts Cdc34 at residues 195–208 and that this interaction is critical for the assembly of extensive polyubiquitin chains. First, while human Cdc34 (aa 1–194) catalyzed autoubiquitination with an efficiency comparable with the wild type and Cdc34 (aa 1–208) (Fig. 3C), it was not activated by ROC1-CUL1 to form Ub chains of extensive lengths (Fig. 3D). Second, results from a GST-based pull-down experiment indicated that human Cdc34 residues 195–208 were required for a stable association between ROC1-CUL1 and the E2 protein (Fig. 4A). Third, physical analysis has shown that the CCD domain is proteolytically accessible and structurally distinct from the C-terminal portion of the tail of yeast Cdc34 (36), suggesting an availability of CCD for interactions with other protein(s), such as the ROC1-CUL1 complex. Last, a significant evolutionary conservation is found in the region corresponding to the human Cdc34 residues 170–208 (Fig. 5).

Based on data presented in this report, while the human Cdc34 residues 209–236 played no role in autoubiquitination, they were required for the efficient synthesis of Ub polymers in the presence of ROC1-CUL1. Intriguingly, while Cdc34 (aa 1–208) retained nearly 40% of the capacity of the wild type protein to bind to ROC1-CUL1 (Fig. 4A), it only possessed 1% of the wild type level of activity in promoting ROC1-CUL1-de-
Nedd8 Activates Cdc34-catalyzed Polynubiquitin Chain Assembly

We have previously demonstrated that Nedd8 helps recruit Ubc4 to ROC1-SCF/H9252. To determine whether Nedd8 exhibits different modes of action to activate ubiquitination reactions catalyzed by divergent E2 conjugating enzymes, we examined whether Nedd8 functions in Cdc34-catalyzed polyubiquitin chain assembly (Fig. 1). The presence of a Nedd8 moiety, conjugated to CUL1 at Lys^220, may significantly alter the kinetics of the interaction between the ROC1 RING finger and Cdc34. In support of this, we have observed that the Nedd8-conjugated ROC1-CUL1 promoted the Cdc34-catalyzed Ub polymerization more rapidly than the untreated complex, whereas this modification had no effect on the Ubc5c-mediated multi-Ub chain assembly (Fig. 1). It is therefore conceivable that Nedd8 may utilize its charged surface residues, such as Glu^14 and Arg^25, to bind to the C-terminal tail of Cdc34, presumably at a region spanning amino acids 170–208. However, despite repeated attempts using GST pull-down assays, we have been unsuccessful in detecting any significant effect of Nedd8 modification on the interaction between ROC1-CUL1 and Cdc34 either in its free or Ub-conjugated forms (the latter was produced by incubating Cdc34 with ATP, Ub, and E1). In light of these findings, we propose that Nedd8 may mediate an electrostatic interaction with Cdc34 that transiently stabilizes the association between the RING “groove” and the E2 protein. Alternatively, Nedd8 charged surface residues might facilitate the Ub transfer reaction in a mechanism yet to be determined. We favor the former model, since the Nedd8 effect is critically dependent on the concentrations of both ROC1-CUL1 and Cdc34. Elevated levels of ROC1-CUL1 (21) or Cdc34 (data not shown) can obviate the requirement of Nedd8 for the efficient synthesis of Ub polymers in vitro. These observations can be explained by postulating that high concentrations of ROC1-CUL1 or Cdc34 promote a more efficient interaction between the two components, thus leading to Nedd8-independent synthesis of Ub polymers. It may prove to be informative to analyze the effects of Nedd8 modification on the interaction between ROC1-CUL1 and Cdc34 under equilibrium conditions using techniques such as BIACore.

While this study has demonstrated a role for Nedd8 in increasing the efficiency of polynubiquitin chain assembly in Cdc34-catalyzed reactions, Kawakami et al. (39) have recently shown that Nedd8 helps recruit Ubc4 to ROC1-SCF/H9252 for the efficient ubiquitination of IxBa. We have previously demonstrated that Cdc34 and Ubc4/5 produce distinct Ub conjugate patterns in the ubiquitination of IxBa (10), suggesting that these two different classes of E2 act differently in assembling polynubiquitin chains. Further studies are required to determine whether Nedd8 exhibits different modes of action to activate ubiquitination reactions catalyzed by divergent E2 conjugating enzymes.

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The Nedd8-conjugated ROC1-CUL1 Core Ubiquitin Ligase Utilizes Nedd8 Charged Surface Residues for Efficient Polyubiquitin Chain Assembly Catalyzed by Cdc34
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