Conjugation of Nedd8 to CUL1 Enhances the Ability of the ROC1-CUL1 Complex to Promote Ubiquitin Polymerization*

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The SCF-ROC1 ubiquitin-protein isopeptide ligase (E3) ubiquitin ligase complex targets the ubiquitination and subsequent degradation of protein substrates required for the regulation of cell cycle progression and signal transduction pathways. We have previously shown that ROC1-CUL1 is a core subassembly within the SCF-ROC1 complex, capable of supporting the polymerization of ubiquitin. This report describes that the CUL1 subunit of the bacterially expressed, unmodified ROC1-CUL1 complex is conjugated with Nedd8 at Lys-720 by HeLa cell extracts or by a purified Nedd8 conjugation system (consisting of APP-BP1/Uba3, Ubc12, and Nedd8). This covalent linkage of Nedd8 to CUL1 is both necessary and sufficient to markedly enhance the ability of the ROC1-CUL1 complex to promote ubiquitin polymerization. A mutation of Lys-720 to arginine in CUL1 eliminates the Nedd8 modification, abolishes the activation of the ROC1-CUL1 ubiquitin ligase complex, and significantly reduces the ability of SCF-HOS/ß-TRCP-ROC1 to support the ubiquitination of phosphorylated IκBα. Thus, although regulation of the SCF-ROC1 action has been previously shown to preside at the level of recognition of a phosphorylated substrate, we demonstrate that Nedd8 is a novel regulator of the efficiency of polyubiquitin chain synthesis and, hence, promotes rapid turnover of protein substrates.

Degradation of a protein substrate by the ubiquitin (Ub)1-mediated proteasome pathway is dependent upon the coordinated action between the targeted substrate and components of the ubiquitination machinery that include the E1 activating enzyme, E2 conjugating enzymes, and E3 ligases (1). Recent studies have revealed an elegant mechanism by which the SCF-ROC1 E3 ligase complex specifically targets phosphorylated substrates for ubiquitination (2–8). However, little is known about regulation of the enzymatic mechanism through which a polyubiquitin chain is formed and attached onto a given substrate.

Although a RING finger plays in the mechanism of ubiquitination, its specific role remains to be established (13, 16). It has previously been shown that Nedd8 or its orthologue Rub1, small Ub-like proteins, modify several members of the cullin/Cdc53 family (18, 19). Studies with budding yeast have shown that deletion of RUB1 alone had no significant growth defect (20). However, when mutations in Cdc34, Cdc53, or Skp1 were combined with the RUB1 deletion, the growth and cell cycle defects of the former group were enhanced significantly (20). Recently, Osaka and co-workers (21) have shown that the Nedd8-modifying pathway is essential for cell viability and function of CUL1 in fission yeast (21). Furthermore, inactivation of the SMC1 (APP-BP1 homologue) gene, encoding for a

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subunit of the Nedd8 activating enzyme, caused cells to undergo multiple S phases without intervening mitosis (22). These results suggest that Nedd8 plays an important regulatory role in cell proliferation and raise an intriguing question as to whether the Nedd8 pathway exerts its regulatory function through its conjugation to cullin/Cdc53 proteins.

In this report, we have provided compelling evidence demonstrating that Nedd8 is conjugated to CUL1 at residue Lys-720 and that this modification markedly stimulates the ability of ROC1-CUL1 to support Ub polymerization. These studies illustrate that, although phosphorylation triggers substrate recognition by ROC1-CUL1, the role of Nedd8 conjugation to ROC1-CUL1 is to provide a means to up-regulate the Ub polymerization activity, leading to efficient degradation of the targeted protein substrates.

**Experimental Procedures**

**Plasmids**

Construction of pGEX-4T3/pcET-15b-(GST-HA-ROC1)-(His-Flag-CUL1(324–776))—To construct a plasmid allowing for the simultaneous expression of GST-fused, HA-tagged ROC1 and the C-terminal CUL1 fragment (aa 324–776) tagged with both a six-histidine and a Flag epitope, the following three cloning steps were used. First, the multi-restriction cloning site from the pcET-15b vector (Novagen) was inserted into the pGEX-4T3 vector (Amersham Pharmacia Biotech). For this purpose, a pair of primers (GACGTCGAC ATGCTATGCAACGTCGAG, which mutated the sequence coding Lys-720 to a stop codon. This was then confirmed using dGlu and NotI and inserted into pGEX-4T3 (digested with BamHI and NotI) to create pGEX-4T3-GST-UBC12.

**Protein Expression and Isolation**

Preparation of GST-ROC1-CUL1324–776 and ROC1-CUL1324–776—The ROC1-CUL1324–776 complex was transformed into BL21 (DE3) and grown in LB (0.5 liter) with 0.4% glucose in the presence of ampicillin at 37 °C. Cultures were then cooled to room temperature when the optical density at 600 nm reached 0.5. Isopropyl-1-thio-β-D-galactopyranoside at a final concentration of 0.2 mM was added to induce the culture overnight (12–14 h) at 25 °C. Cells were pelleted at 5,000 × g for 15 min at 4 °C.

This pellet was resuspended in 1/25 culture volume of lysis buffer (50 mM Tris-HCl, pH 8.0, 1% Triton X-100, 0.5M NaCl, 10 mM EDTA, 10 mM EGTA, 10% glycerol, 2 mM phenylmethylsulfonyl fluoride, 0.4 μg/ml antipain, 0.2 μg/ml leupeptin, and 5 mM DTT). The resuspended material was then sonicated (four repetitive 20-s treatments) and centrifuged at 17,000 rpm in an SS-34 rotor for 30 min, 4 °C. The supernatant was saved. For glutathione affinity purification, indicated amounts of extracts were added to the beads (200 μl) and excess bacteria proteins were removed by washing the beads three times with lysis buffer followed by two washes with buffer A (25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.01% Nonidet P-40, 10% glycerol, 1 mM DTT, 0.2 μg/ml leupeptin, 0.2 μg/ml antipain, and 1 mM phenylmethylsulfonyl fluoride) plus 50 mM NaCl. Typically, 10 μl of GST-ROC1-CUL1324–776-containing extracts yielded approximately 5 pmol of the complex bound to the glutathione beads.

To generate the ROC1-CUL1324–776 complex, GST-ROC1-CUL1324–776-containing extracts (10 μl) were bound to glutathione beads (0.5 ml). Thrombin (Amersham Pharmacia Biotech) cleavage was carried out on beads with 35 units in phosphate-buffered saline for 2 h at 12 °C. More than 95% of GST-ROC1 was cleaved. This procedure yielded 0.6 mg of ROC1-CUL1324–776 with purity greater than 90% (Fig. 1A).

**Enzymes**—Human E1 was isolated from cytosolic extracts of HeLa cells using a Ua affinity column as described previously (4). Cdc34 was purified from extracts derived from Sf9 cells infected with mCdc34 baculovirus using a Ni2+-nitrilotriacetic acid-based affinity column (Qiagen) followed by a Q-Sepharose chromatographic step (4).

**Expression and Purification of GST-UCB12—**GST-UCB12 was transformed into BL21 (DE3) and grown in LB (100 ml) with 0.4% glucose in the presence of ampicillin at 37 °C. Cultures were induced for 3 h with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside added when the optical density at 600 nm reached 0.5. Extracts (4 ml) were made using the same method as for GST-ROC1-CUL1324–776, mixed with glutathione beads (0.5 ml, Amersham Pharmacia Biotech), pre-equilibrated with lysis buffer, and the mixture was rocked for 1 h at 4 °C. The slurry was then loaded onto a column and washed three times with 2 ml of lysis buffer, followed by washing twice with 2 ml of buffer A-50. GST-UCB12 was eluted with 20 mM glutathione in buffer A-50. 7.5 mg of GST-Ubc12 was obtained with purity greater than 95%.

**Expression and Purification of Nedd8—**The pET3a-Nedd8 plasmid (kindly provided by C. Pickart) was transformed into BL21 (DE3). It was then expressed and harvested using the same method as GST-UCB12 except the protein was found in the pellet. The protein was solubilized and purified using a published procedure (23) with modifications. The inclusion body pellet was resuspended and washed three times with lysis buffer, and two times with centrifugation buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 2% sucrose, 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, 0.4 μg/ml antipain, 0.2 μg/ml leupeptin). The pellet was solubilized in urea buffer (50 mM Tris-HCl, pH 7.5, 8 M urea, and 2 mM EDTA) by rocking at room temperature for 30 min. Solubilized material was then clarified using the ultracentrifuge at 100,000 × g for 1 h, 16 °C. The clarified material was thoroughly dialyzed against 1 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.01% NaN3, 0.5% glycerol, and 50 mM NaCl. The Nedd8 solution was then concentrated using an M, 5,000 cutoff filter (Millipore) and passed through Q- and SP-Sepharose (Amersham Pharmacia Biotech) columns, pre-equilibrated with dialysis buffer, consecutively. The flow-through from the SP column was then further purified on a Superdex-75 column using a fast protein liquid chromatograph. 3 mg of pure Nedd8 was obtained per 0.5 liter of induced culture.
Purification of APP-BP1/UBA3 from HeLa Extract—Human APP-BP1/UBA3 was purified from extracts of HeLa cells using a Nedd8 affinity column. To pack a Nedd8 affinity column, affinity chromatography beads were washed twice with buffer B and then twice with buffer A-50. The washed beads were then assayed for Ub ligation as described above.

Activation of the Ub Ligase Activity of ROC1-CUL1 Complex by HeLa Extracts—We have shown recently that the RING-H2 finger protein ROC1 specifically binds to the C terminus of CUL1 (spanning amino acid residues 324–776) in transfected 293T cells and that the resulting ROC1-CUL1 324–776 complex is fully active in supporting Ub ligation (9). To identify factors that may regulate the Ub ligase activity of the ROC1-CUL1 complex, we co-expressed GST-ROC1 with CUL1 324–776 in E. coli. As shown in Fig. 1A, the two co-expressed proteins formed a complex that was co-purified from a glutathione affinity column (lane 2). The co-eluted ~33 kDa polypeptides were probably proteolyzed or prematurely terminated products of GST-ROC1 since they reacted with anti-GST antibodies (data not shown). Furthermore, the GST moiety can be removed by thrombin digestion, yielding the ROC1-CUL1 324–776 complex (lane 3).

The Ub ligase activity of GST-ROC1-CUL1 324–776 was measured by the previously established [32P]Ub incorporation assay (4, 5, 9, 13). In this system, the bacterial-assembled GST-ROC1-CUL1 324–776 complex was coupled to glutathione-beads and the resulting matrix was incubated with E1, Cdc34, and [32P]Ub. The results showed that GST-ROC1-CUL1 324–776 converted monomeric Ub molecules into high molecular mass Ub polymers in a concentration-dependent manner (Fig. 1B, lanes 1–5). This reaction depended on the presence of Cdc34 as its omission abolished the reaction (lane 6). This result demonstrates that ROC1 and the CUL1 324–776 fragment contain all of the essential elements that constitute a Ub polymerization activity.

To explore whether the unmodified GST-ROC1-CUL1 324–776 could be activated, the glutathione beads coupled with the GST-ROC1-CUL1 324–776 complex were incubated with HeLa cell extracts in the presence of an ATP regenerating system. Following a brief incubation, excess HeLa proteins were removed by extensively washing the beads. The treated-complex was then incubated with ubiquitination agents to initiate the Ub polymerization reaction. The results showed that the treated complex was significantly more active than the un-treated complex in supporting Ub ligation (Fig. 1B, compare lanes 2–5 with lanes 7–10). As shown, the Ub ligase activity of the GST-ROC1-CUL1 324–776 complex was increased up to 10-fold when low levels of the complex were used (Fig. 1B, bottom panel). Of note, at the highest levels of GST-ROC1-CUL1 324–776 used, the majority of monomeric Ub had been consumed (lanes 9 and 10), thus limiting the production of high molecular weight Ub conjugates. The depletion of the Ub substrate may have significantly resulted in the underestimation of the degree of activation in the presence of high levels of GST-ROC1-CUL1 324–776. Additionally, the results from kinetic analysis indicated that the HeLa extract-treated GST-ROC1-CUL1 324–776 promoted the polymerization of Ub molecules more rapidly than the mock-treated complex (data not shown).

HeLa Extracts Catalyze the Conjugation of Nedd8 to the CUL1 324–776 Fragment—Subsequent immunoblot analysis revealed that a fraction of CUL1 324–776 was conjugated with NEDD8 following incubation with HeLa extracts (Fig. 2, upper panel). In contrast, no detectable mobility changes were observed with GST-ROC1 (Fig. 2, bottom panel). Collectively, these results demonstrate that the HeLa extracts catalyze the conjugation of NEDD8 to the CUL1 324–776 subunit of the GST-ROC1-CUL1 324–776 complex and, concomitantly, enhance the ability of the complex to support Ub ligation.

Nedd8 Is Conjugated to CUL1 at Lys-720, and This Modification Is Required for the Activation of the Ub Ligase Activity of the ROC1-CUL1 Complex by HeLa Extracts—To determine whether Nedd8 modification was responsible for the activation of the ROC1-CUL1 Ub ligase, a Lys → Arg mutation was introduced into CUL1 324–776 at residue 720. This residue has...
been implicated as the receptor site for NEDD8 (25, 26). GST-ROC1-CUL1324–776 harboring this mutation exhibited similar Ub ligase activity as observed with the wild type complex that was not incubated with HeLa cell extracts (Fig. 3A, compare lanes 2 and 3 with lanes 4 and 5). However, in contrast to the wild type complex, the mutant complex was no longer activated following incubation with HeLa extracts (Fig. 3A, compare lanes 8 and 9 with lanes 10 and 11). Consistent with this, a complex formed by GST-ROC1 and a CUL1324–719 deletion mutant, that lacked the Lys-720 NEDD8 receptor residue, was not significantly activated by HeLa extracts (Fig. 3A, lanes 6, 7, 12, and 13). Immunoblot analysis indicated that neither CUL1(K720R)324–776 nor CUL1324–719 was conjugated with NEDD8 following incubation with HeLa extracts (Fig. 3A, lanes 6, 7, 12, and 13). This suggests that the activation of ROC1-CUL1 by HeLa extracts is quantitatively dependent on Nedd8 conjugation.

It should be noted that the immunoblot analysis of the anti-Flag immunoprecipitates of GST-ROC1-CUL1324–776 detected a ~45-kDa polypeptide (Fig. 3B, lanes 2–5 and 8–11). This anti-Flag recognized protein was not detected in the reaction in which GST-ROC1-CUL1324–776 was omitted (lane 1), suggesting that it is a proteolyzed or prematurely terminated product of CUL1324–776. The presence of this polypeptide should not significantly influence Nedd8 modification analysis presented in this study. First, it was present in much less quantity than the CUL1324–776 fragment. Second, this species migrated almost identically to, or slightly faster than, the CUL1324–719 fragment (compare lanes 2–5 with lanes 6 and 7), suggesting that it may lack the Lys-720 residue as well.

We examined whether conjugation of NEDD8 to CUL1 affects the ability of CUL1 to support the ubiquitination of IκBα by introducing a K720R substitution into the full-length Flag-tagged wild type CUL1. In keeping with a recent observation by Read and co-workers (26), our co-transfection experiments revealed that, whereas the wild type SCFHOs/β-TRCP-ROC1 supported the ubiquitination of the IκBα-phosphorylated GST-IκBα(1–54), the CUL1K720R-containing complex was significantly less efficient in the ubiquitination reaction (data not shown). Immunoblot analysis indicated that both Flag-CUL1 and CUL1K720R were able to form SCFHOs/β-TRCP-ROC1 complexes with comparable efficiencies (data not shown). We, therefore, conclude that NEDD8 conjugation to CUL1 enhances the ability of SCFHOs/β-TRCP-ROC1 to support the ubiquitination of IκBα. This is most likely due to Nedd8 conjugation-mediated activation of the ROC1-CUL1 core Ub polymerization complex.
activity within the holo-E3 complex based on results presented in Figs. 1 and 3.

**Covalent Linkage of NEDD8 to Lys-720 of CUL1 Is Sufficient to Activate the Ub Ligase of the ROC1-CUL1 Complex**—To determine whether the covalent linkage of NEDD8 to Lys-720 of CUL1 is sufficient to activate the Ub ligase, we reconstituted the NEDD8 modification on CUL1 with purified components. For this purpose, both recombinant human Nedd8 and the Ubc12 E2 conjugating enzyme were expressed and purified from bacteria, with the latter fused with GST. The human Nedd8 activating enzyme is a heterodimer of APP-BP1 and Uba3 (19, 27, 28) and was purified from HeLa cells using a...
Nedd8 affinity column (see “Experimental Procedures”). To conjugate NEDD8 to CUL1342–776, ROC1-(Flag-CUL1342–776), immobilized on anti-Flag antibody-linked protein A beads, was incubated with purified Uba3/(APP-BP1), Ubc12, and NEDD8. After extensive washing to remove excess NEDD8 modification agents, the resulting beads were added with ubiquitination components to initiate the Ub polymerization reactions. The results showed that the addition of Nedd8 conjugation agents resulted in more than a 6-fold stimulation of the complex’s Ub polymerization activity (Fig. 4A, compare lanes 1 and 2). Omission of APP-BP1/Ubα3 (lane 3), GST-Ubc12 (lane 5), or Nedd8 (lane 6) abolished the activation, demonstrating that each of the Nedd8 conjugation components is essential for activating the Ub polymerization activity of ROC1-CUL1342–776. In addition, reducing the amount of GST-Ubc12 from 100 to 30 ng resulted in a slight decrease in the extent of activation (compare lanes 2 and 4).

Immunoblot analysis showed that approximately 50% of the CUL1324–776 were converted to Nedd8-conjugated forms (Fig. 4B). As shown, a small percentage of the CUL1324–776 was conjugated with two NEDD8 molecules. It is presently unclear as to whether the second Nedd8 conjugation was due to an additional site other than Lys-720. It is also possible that, under the conditions used, the carboxyl-terminal glycine of the
second Nedd8 forms an isopeptide bond with one of the lysine residues of the Lys-720-conjugated Nedd8, in a manner similar to the formation of Ub chains. The Nedd8 conjugation required APP-BP1/Uba3 (lane 3), GST-Ubc12 (lane 5), and Nedd8 (lane 6). A marginal, but detectable, decrease in the level of Nedd8 modification on CUL1324–776 was noted when the amount of GST-Ubc12 was reduced from 100 to 30 ng (compare lanes 2 and 4).

To confirm that Lys-720 is the primary site for Nedd8 conjugation in the purified reconstitution system, GST-ROC1-CUL1(K720R)324–776, immobilized on glutathione beads, was tested for Nedd8 conjugation in the presence of APP-BP1/Uba3, GST-Ubc12, and Nedd8. No Nedd8 conjugation was observed (data not shown). These results suggest that the purified system modifies CUL1324–776 at Lys-720, in keeping with the observations made with the HeLa extracts (Fig. 3B).

To rule out the presence of a human protein(s) co-purified with APP-BP1/Uba3 that may contribute to the activation of the ROC1-CUL1 Ub ligase activity, the APP-BP1/Uba3 preparation was fractionated by glycerol gradient sedimentation. Silver staining analysis revealed that APP-BP1/Uba3 migrated as a doublet (55 and 65 kDa in size) that peaked at fraction 19 (Fig. 4C, upper panel, lane 7). A minor contaminating polypeptide of ~45 kDa peaked at fraction 25 (lane 9).

To assess the capacity of the glycerol gradient fractions to activate the ROC1-CUL1 Ub ligase, aliquots of the fractions were incubated with M2 bead-bound ROC1-CUL1342–776 in the presence of bacterially expressed Nedd8 and GST-Ubc12. Following washing, the treated beads were then assayed for Ub ligation activity. The results showed that fraction 19, the peak of the APP-BP1/Uba3 doublet, contained the highest level of activity that stimulated the Ub ligase activity of the ROC1-CUL1342–776 complex (Fig. 4C, bottom panel, lane 9). Taken together, these results unequivocally demonstrate that the conjugation of Nedd8 to CUL1 at Lys-720 by the combined action of Nedd8, APP-BP1/Uba3, and Ubc12 is sufficient to activate the Ub ligase activity of the ROC1-CUL1 complex.

**DISCUSSION**

The recent discovery of the ROC/APC11 RING-H2 finger protein family has helped uncover a superfamily of ROC/cullin-based Ub E3 ligases. These members each contain a common dimeric core element formed by combinatorial interactions between the ROC/APC11 and CUL/APC2 family proteins. The three best characterized of these, SCF-ROC1, anaphase-promoting complex, and the von Hippel-Lindau tumor suppressor complex, function to regulate the abundance of substrate proteins required for the control of the progression of the cell cycle, the activation of signal transduction pathways, and the execution of tumor suppressor activities (4–8). Within the multi-subunit E3 complex, the ROC/APC11-CUL1/APC2 subassembly is likely to carry out a common ligase function that recruits a cognate E2 and facilitates the transfer of activated Ub from the E2 to the targeted substrates.

In this study, we provide unequivocal biochemical evidence demonstrating that the conjugation of Nedd8 to CUL1 at Lys-720 activates the ability of the ROC1-CUL1 complex to support Ub polymerization. First, incubation of HeLa extracts with the bacterially expressed, unmodified ROC1-CUL1 complex led to both the activation of the ROC1-CUL1-mediated Ub ligase activity and the conjugation of Nedd8 to the CUL1 protein (Figs. 1 and 2). Second, replacement of Lys-720 with arginine in CUL1 eliminated Nedd8 modification, confirming that Lys-720 is the primary site for Nedd8 conjugation (Fig. 3B). Third, in contrast to the wild type ROC1-CUL1 complex, ROC1-CUL1 harboring the K720R mutation was no longer activated by HeLa extracts (Fig. 3A). Fourth, the SCF-ROC1 complex containing CUL1K720R was substantially less active than the wild type complex in supporting the ubiquitination of IκBα (data not shown; see Ref. 26). Finally, reconstitution of Nedd8 conjugation to the CUL1 subunit of the ROC1-CUL1 complex with purified components markedly stimulated the Ub ligase activity (Fig. 4).

Previous studies have shown that ROC1 binds five members of the cullin family (CUL1, CUL2, CUL3, CUL4A, and CUL4B), while ROC2 interacts with CUL5 (5). In addition to ROC1-CUL1, ROC1-CUL2 (29, 30) and APC11-APC2 (5) contain Ub ligase activity as well, implicating that the ROC-CUL complex commonly possesses an intrinsic ability to support Ub ligation. In light of the observation by Hori et al. (31), who have shown that all members of the human cullin family of proteins are modified by Nedd8, Nedd8 conjugation to CUL may be a general mechanism to activate the ROC-CUL-based core Ub ligase.

There is a body of evidence suggesting that Nedd8 modification plays a regulatory role in cell proliferation and development. In Saccharomyces cerevisiae, the Rub1 pathway is critical to cell growth when the function of the SCF is compromised by mutations in CDC34, CDC4, CDC53, or SKP1 (20). In fission yeast, the Nedd8-modifying pathway is essential for cell viability (21). The expression of Nedd8 is down-regulated during embryonic development, and, in adult mice, Nedd8 mRNA levels are high in heart and skeletal muscle (32). A dramatic cell cycle effect was observed in the ts41 hamster cell line where the Nedd8 pathway is defective (22). This cell harbors a temperature-sensitive allele of SMC1, a homologue of human APP-BP1. At a non-permissive temperature, the ts41 cell underwent multiple rounds of DNA replication without intervening mitoses. This suggests that, under this condition, a lack of Nedd8 conjugation leads to the limited degradation of a substrate(s), perhaps Cdc18 (33, 34), by the SCF-ROC1 pathway, whose sustained high levels initiate re-replication. Furthermore, in Arabidopsis thaliana, recessive mutations in the AXR1 gene (a homologue of APP-BP1) resulted in a decreased response to auxin, a hormone that regulates diverse developmental processes by promoting changes in cell division and elongation (35).

Read et al. (26) have recently reported that Nedd8 modification activates the SCFβ-TRCP-dependent ubiquitination of IκBα. They further demonstrate that Nedd8 does not affect the K14 for SCFβ-TRCP binding to IκBα, nor does it significantly alter the ability of CUL1 to form complexes with ROC1, Skp1 and β-TRCP. In this study we have provided evidence that Nedd8 modification does not affect the ROC1-CUL1 interaction (Figs. 2 and 4). In addition, Podust et al. (36) and Morimoto et al. (37) have shown that the Nedd8 conjugation pathway is essential for proteolytic targeting of p27 by ubiquitination. Although our study is in accord with these papers, it provides unequivocal evidence that the conjugation of Nedd8 to CUL1 at Lys-720 by the combined action of Nedd8, APP-BP1/Uba3, and Ubc12 is sufficient to activate the Ub polymerization activity of the ROC1-CUL1 complex. These findings have led us to conclude that Nedd8 is a novel regulator of the efficiency of polyubiquitin chain synthesis and, hence, promotes rapid turnover of protein substrates.

Our studies have shown that the bacterially expressed, unmodified ROC1-CUL1 complex, when used in high levels, supported Ub ligation (Fig. 1B). Furthermore, elimination of Nedd8 modification by replacing Lys-720 with arginine reduced, but did not abolish, the ability of SCFHHOS/β-TRCP-ROC1 to support the ubiquitination of IκBα (data not shown; see Ref. 26). These results demonstrate that the biochemical role of Nedd8 conjugation is to activate, but is not essential for, the Ub polymerization activity of the ROC1-CUL1 complex, which...
promotes the synthesis of polyubiquitin chains covalently attached onto substrates such as phosphorylated IκBα. These studies further imply that Nedd8 pathway depends on the intracellular levels of CUL1 (and/or other members of the cullin family proteins). It is thus possible that the Nedd8 modification is indispensable for cell growth where cullin concentrations are limiting.

Several questions arise from the results of this study. First, how is the Nedd8 conjugation to CUL1 regulated? Does the conjugation solely depend on the levels of Nedd8, whose expression is developmentally down-regulated and is high in selective tissues such as heart and skeletal muscles (32)? Second, is the SCF-Roc1 function down-regulated by removal of the Nedd8 conjugate from CUL1? Preliminary analysis suggests that there is an activity in HeLa extracts that cleaves Nedd8 from conjugate from CUL1. If such an enzyme(s) does exist, what physiological role does it play in cell proliferation?

Third, how does NEDD8 activate the ROC1-CUL1 Ub ligase? It is possible that the NEDD8 molecule conjugated to the Lys-720 residue of CUL1 alters the ROC1-CUL1 structure in favor of promoting ubiquitination. Preliminary experiments using 720 residue of CUL1 alters the ROC1-CUL1 structure in favor of promoting ubiquitination. Preliminary experiments using GST-based pull-down assays indicated that the HeLa extract-activated ROC1-CUL1 did not promote the formation of a stable complex between ROC1-CUL1 and Cdc34 more efficiently than the untreated complex (data not shown). However, this does not rule out the possibility that the Nedd8-conjugated ROC1-CUL1 may recognize Cdc34 or Cdc34-S-Ub more rapidly than the unmodified complex and that such an encounter instantly induces a conformational change that dissociates Cdc34 (or Cdc34-S-Ub), leading to the transfer of a thiol ester-bound Ub to a substrate or another Ub receptor molecule.

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