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Nedd8-modification of Cul1 is promoted by Roc1 as a Nedd8-E3 ligase and regulates its stability☆

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Abstract

SCF is a ubiquitin ligase and is composed of Skp1, Cul1, F-box protein, and Roc1. The catalytic site of the SCF is the Cul1/Roc1 complex and RING-finger protein Roc1. It was shown earlier that when Cul1 was co-expressed with Roc1 in Sf-9 cells in a baculovirus protein expression system, Cul1 was highly neddylated in the cell, suggesting that Roc1 may function as a Nedd8-E3 ligase. However, there is no direct evidence that Roc1 is a Nedd8-E3 in an in vitro enzyme system. Here we have shown that Roc1 binds to Ubc12, E2 for Nedd8, but not to Ubc9, E2 for SUMO-1 and Roc1 RING-finger mutant, H77A, did not bind to Ubc12. In in vitro neddylation system using purified Cul1/Roc1 complex expressed in bacteria, Roc1 promotes neddylation of Cul1. These results demonstrate that Roc1 functions as a Nedd8-E3 ligase toward Cul1. Furthermore, Roc1 and Cul1 were ubiquitinylated in a manner dependent on the neddylation of Cul1 in vitro. In addition, Cul1 was degraded through the ubiquitin–proteasome pathway, and a non-neddylated mutant Cul1, K720R, was more stable than wild-type in intact cells. Thus, neddylation of Cul1 might regulate SCF function negatively via degradation of Cul1/Roc1 complex.

Keywords: Cul1; Nedd8; Roc1; SCF; Ubiquitin; Ubiquitin-like protein; Ligase; Ubc12; Cdc34; RING-finger

Protein degradation by the ubiquitin–proteasome pathway plays an important role in many biological steps including cell-cycle progression and immune responses. Proteasomes recognize the poly-ubiquitinylated chain attached to the protein to be degraded. The ubiquitinylation of proteins requires three enzymes: E1, ubiquitin activating enzyme; E2, ubiquitin conjugating enzyme; and E3, ubiquitin ligase [1,2]. The ubiquitin ligase recognizes the protein targeted to be degraded. There are at least two types of ubiquitin ligases. One type is ubiquitin ligase having a HECT-domain [3,4] and the other is that having a RING-finger domain [5–8]. SCF is thought to be one of the RING-finger type ubiquitin ligases [9–11] and is composed of Skp1, Cul1 (or CDC53), F-box protein, and Roc1/Rbx1. The catalytic site of the SCF ubiquitin ligase is the Cul1/Roc1 complex and Roc1 is a RING-finger domain-containing protein [12–17]. This complex recruits the E2 and transfers a ubiquitin moiety from E2 to the target protein, which is bound by the F-box protein. This F-box protein recognizes the protein to be ubiquitinylated [18]. For example, Skp2 recognizes $p27^{kip1}$ [19,20] in the presence of cks1 [21,22] and β-TrCP recognizes IκBα [23,24] and β-catenin [25].

Cul1 in SCF complex was found to be modified by a ubiquitin-like protein Nedd8 [26,27]. Furthermore, Cul2 in VCB [28,29], another E3 complex, and all other cullin family members (Cul3, Cul4A, Cul4B, and Cul5) were also modified by Nedd8 [30]. Nedd8 modification (neddylation) of Cul1 was shown to be necessary for the activity of SCF toward $p27^{kip1}$ [31,32] or IκBα [33,34] in vitro; and Rub1, a Nedd8 homologue in fission yeast, was shown to be essential for cell viability [35]. Recently, neddylation of Cul1 was shown to enhance the binding of the ubiquitin-binding form of UbcH4 [36]. These

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findings suggest that the modulation of neddylation system is crucial for the regulation of SCF function.

Nedd8 modification is catalyzed by APP-BP1/Uba3 acting as E1 and Ubc12 as E2 [28,37,38] and may be regulated by Nedd8-E3 ligase. When CDC53, the budding-yeast homologue of Cul1, was co-expressed with Roc1 in insect cells in a baculovirus protein expression system, CDC53 was modified markedly by Nedd8 in a Roc1-dependent manner [39]. Therefore, Roc1 is thought to be a possible candidate for a Nedd8-E3 ligase, but the enhancement of neddylation by Roc1 in vitro has not been shown so far.

Thus, it is an important issue to clarify whether Roc1 functions as a Nedd8-E3 ligase and what is a function of the neddylation of Cul1. Here we show that Roc1 enhanced the neddylation of Cul1 and that this neddylation destabilized Cul1.

Materials and methods

Cell culture and DNA transfection. Human U2OS cells and COS7 cells were maintained in Dullbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were transfected with various kinds of plasmids by using FuGene 6 reagent (Roche) according to the manufacturer's instructions. When the stability of the Cul1 was monitored, the cells in several dishes were harvested 24 after transfection, mixed together, and then equal numbers of cells were inoculated again into each dish. Cycloheximide (10 μg/ml) was added to some dishes to inhibit protein synthesis.

Plasmid constructions and expression of recombinant proteins. To amplify the His-Cul1 sequence with bacterial ribosomal binding site (RBS-His-Cul1) by PCR, pTrcHis-Cul1 was used as a template. The PCR-generated fragment was cloned into the pGEX-6P-2. Next, the resultant plasmids were inserted by the Roc1 cDNA to fuse in-frame into a glutathione *S*-transferase (GST) sequence. The final constructed plasmid was designated as pGEX-6P-2-Roc1/RBS-His-Cul1. Recombinant GST- or His-Cul1, GST- or His-Roc1, GST-Skp1, GST-p53, His-Nedd8, His-APPBP1, and His-Uba3 proteins were expressed in a baculovirus expression system using pFastBac vector (Invitrogen) and Sf-9 cells as described previously [31]. GST-Roc1/His-Cul1, His-Cdc34, UbcH5c, Ubc12, and Nedd8 were expressed in *Escherichia coli* (BL21, *LysS*). The substitution mutants of Roc1 and Cul1 were prepared by the PCR amplification method using specific mutation primers.

Protein interaction assay. Sf-9 cells expressing GST-fusion proteins were suspended in lysis buffer containing 50 mM Tris–HCl (pH 7.4), 0.4 M NaCl, 3 mM $MgCl_2$, 0.1% NP-40, and 1 mM PMSF, and disrupted by sonication. GST-fusion proteins were captured with glutathione–Sepharose 4B (Amersham). GST-fusion proteins immobilized on beads and purified Ubc12 (5 μg) or Ubc9 (5 μg) were incubated in lysis buffer without NaCl at room temperature for 30 min. The beads were then washed four times with the lysis buffer without NaCl, and the bound proteins were eluted with SDS sample buffer, subjected to SDS–PAGE, and immunoblotted with anti-T7 tag antibody (Novagen) or anti-His6 tag (Qiagen) and peroxidase-conjugated anti-mouse secondary antibody. The immunoblotted proteins were detected by the ECL method (Amersham).

In vitro ubiquitinylation assay. Substrate immobilized on glutathione–Sepharose 4B (Amersham) was added to the reaction buffer (40 mM Tris–HCl [pH7.4], 5 mM $MgCl_2$, 2 mM ATP, and 1 mM DTT) containing ubiquitinylation enzymes (mouse Uba1 and His-Cdc34 or UbcH5c) and biotinylated ubiquitin. The in vitro ubiquitinylation assay was performed essentially as described previously using biotinylated ubiquitin [40]. The ubiquitinylated proteins were detected by Western blotting using peroxidase-conjugated avidin (Extravidin, Sigma) and the ECL method (Amersham).

In vitro neddylation system. Substrate immobilized on glutathione–Sepharose 4B was added to the ubiquitinylation reaction buffer containing neddylation enzymes (His-APP-BP1, His-Uba3, and Ubc12) and Nedd8. Modification of Cul1 by Nedd8 was done in vitro as described previously by using purified enzymes and Nedd8 [31]. The neddylated proteins were detected by Western blotting using avidin-peroxidase and the ECL method (Amersham Pharmacia). The Western blotting of Nedd8 or 6xHis-tagged protein was done by use of anti-Nedd8 antibody (Alexis) or anti-6xHis antibody (Qiagen), respectively. Anti-Cul1 antibody (Biosource) was used for detecting Cul1. The secondary antibody used was peroxidase-conjugated anti-mouse rabbit antibody (Dako).

Results

Roc1 is Nedd8-E3 ligase

Since Nedd8 is a ubiquitin-like proteins, and the mechanism of its conjugation to a target protein could be analogous to that operating in ubiquitin-conjugation, the Nedd8 conjugation system is thought to require three enzymatic steps. However, Nedd8-E3 ligase has not been identified so far; although it has been suggested to be Roc1, based on the data that Rbx1/Roc1 enhanced the neddylation of Cdc53, a yeast homologue of Cul1, when Sf-9 cells were co-infected with these proteins [39].

To determine whether neddylation of Cul1 depends on Roc1, we co-infected Sf-9 cells with baculoviruses containing GST-Cul1 and His-Roc1. In that case, the Cul1 was highly neddylated (Fig. 1A, lanes 3 and 4), whereas when the cells were infected with viruses containing only GST-Cul1, the modification was not detected (lane 1), as shown previously [39]. When the Cul1 expressed in Sf-9 cells was purified and then used in an in vitro neddylation assay, only the Cul1/Roc1 heterocomplex enhanced the neddylation of Cul1 (Fig. 1A, lane 4), but not neddylation-site mutant (K720R)/Roc1 heterocomplex or Cul1 alone (Fig. 1A, lanes 2 and 6). These data suggest that Roc1 may function as a Nedd8-E3 ligase. However, we cannot rule out the possibility that some Roc1 associated protein originated from insect cells promotes the neddylation of Cul1.

To demonstrate that Roc1 functions as Nedd8-E3 ligase, indeed, an in vitro neddylation assay system is necessary using recombinant proteins expressed in bacterial cells, which do not have a neddylation system. In order to construct an appropriate in vitro neddylation assay system, we co-expressed GST-Roc1 and His-Cul1 in bacterial cells. In this case, expressed proteins formed complex in a cell. Nedd8 and Ubc12 were expressed in *E. coli* and His-APP-BP1/His-Uba3 was expressed in Sf-9 cells; and then each protein was purified. When His-Cul1/GST-Roc1 was incubated in the presence or

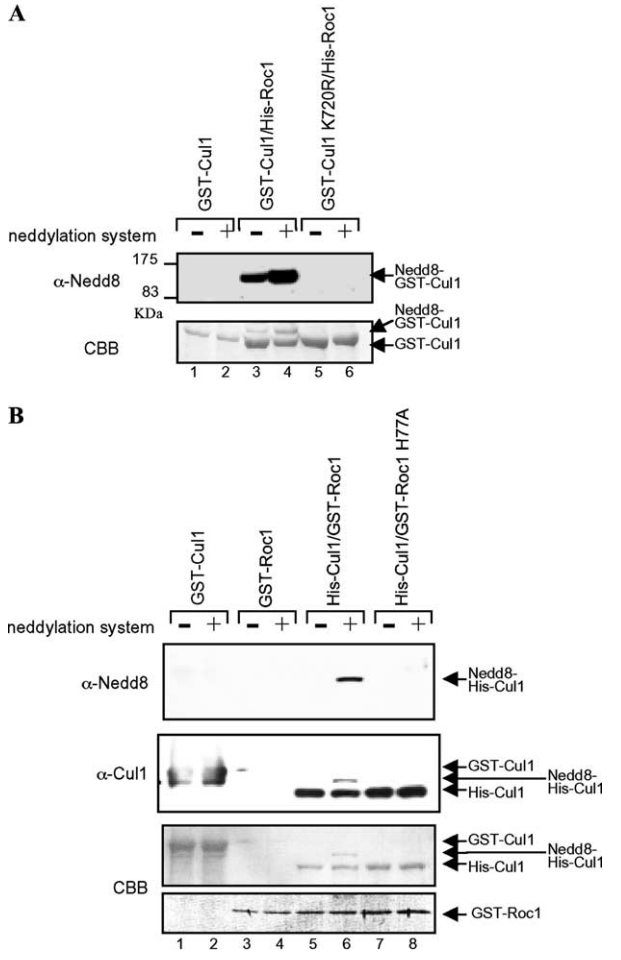

Fig. 1. Roc1 enhances neddylation of Cul1 in a RING-finger-dependent manner in vitro. (A) GST-Cul1 (lanes 1 and 2), GST-Cul1/His-Roc1 (lanes 3 and 4), and GST-Cul1 (K720R)/His-Roc1 (lanes 5 and 6) were expressed in Sf-9 cells in the baculovirus protein expression system. Total cell extracts were prepared and GST-Cul1 and its binding protein (Roc1) were immobilized on glutathione–Sepharose 4B beads. The proteins loaded into lanes 2, 4, and 6 had been subjected to in vitro neddylation. Neddylated Cul1 was detected by Western blotting with anti-Nedd8 antibody. (B) GST-Cul1, GST-Roc1, GST-Roc1/His-Cul1, and GST-Roc1 (H77A)/His-Cul1 were co-expressed in *E. coli*. Total cell extracts were prepared, and GST-Cul1, GST-Roc1, and GST-Roc1 bound His-Cul1 were pulled down using glutathione–Sepharose 4B beads. Then beads were subjected to neddylation in vitro, and neddylated Cul1 was detected by Western blotting with anti-Nedd8 antibody (upper panel) or anti-Cul1 antibody (middle panel). Coomassie staining of proteins is shown (lower two panels).

absence of His-APP-BP/His-Uba3 (Nedd8-E1), Ubc12 (Nedd8-E2), or Nedd8, neddylated Cul1 was detected by the anti-Nedd8 antibody or anti-Cul1 antibody (Fig. 1B). Cul1 was neddylated only in the presence of both neddylation enzymes and Roc1 (Fig. 1B, lane 6). The modification of Cul1 was dependent on the RING-finger domain of Roc1, because the H77A mutant, a RING-finger domain mutant, did not enhance the modification (Fig. 1B, lane 8). These data strongly suggest that Roc1 is a Nedd8-E3 ligase functioning in a RING-finger domain-dependent manner.

Binding of Ubc12 to Roc1

The RING finger-type ubiquitin ligase (E3) binds E2 in a RING-finger domain-dependent manner as clearly described in the case of c-Cbl and Ubc7 [41]. SUMO-E3 ligases, PIAS family proteins, also can bind Ubc9, an E2 for SUMO, in a RING-finger-like domain-dependent manner [42].

Since Roc1 has a RING-finger domain in its carboxyl terminus, Ubc12 (Nedd8-E2) would be expected to bind to the RING-finger domain of Roc1. In an in vitro binding assay using glutathione–Sepharose 4B, Ubc12 could bind to GST-Roc1 in either GST-Roc1 expressed alone or in GST-Roc1 expressed as a Cul1/Roc1 heterocomplex, but not to GST-Skp1, a component of SCF (Fig. 2A). However, Ubc9, an E2 for SUMO, could not bind to GST-Roc1 (Fig. 2B). p53 could bind to Ubc9 as a control. The same binding assay was then performed with RING-finger mutant H77A. Ubc12 could bind to neither GST-Roc1(H77A) nor GST-Roc1(H77A)/His-Cul1 (Fig. 2C). These data indicated that Roc1, not Cul1, binds Ubc12 in a RING-finger domain-dependent manner.

Self-ubiquitinylation of the Cul1/Roc1 complex

During the construction of in vitro neddylation assay system, we noticed that Cul1 and Roc1 were self-ubiquitinylated. Since the neddylation of Cul1 was shown to be necessary for ubiquitin ligase activity toward $p27^{kip1}$ or IκBα, we examined whether this modification was necessary for the self-ubiquitinylation of Roc1/Cul1 complex. Using Cdc34 as E2 enzyme, both Roc1 and Cul1 in complex were self-ubiquitinylated in a neddylation system domain-dependent manner (Fig. 3A, lane 6). However, in the presence of the Roc1 mutant, H77A, Cul1/Roc1(H77A) did not show self-ubiquitinylation activity (Fig. 3A, lane 8). In the case of using neddylation-site mutant Cul1 (K720R), neither Roc1 nor mutant Cul1 was self-ubiquitinylated (data not shown). These findings indicated that neddylation of Cul1 was necessary for the self-ubiquitinylation activity of Roc1/Cul1 complex. Roc1 expressed solely did not show the self-ubiquitinylation activity (Fig. 3A, lanes 3 and 4). When the same experiments were performed using UbcH5c as E2 enzyme, both Roc1 expressed solely and Cul1/Roc1 complex could catalyze the self-ubiquitinylation independent of the neddylation of Cul1 (Fig. 3B, lanes 3 and 4). These results suggested that the neddylation of Cul1 enhances the ubiquitinylation activity of Cul1/Roc1 complex through Cdc34 but not UbcH5c in vitro.

Neddylated Cul1 is unstable

If Cul1/Roc1 complex is self-ubiquitinylated in a cell, the ubiquitinylated complex will be degraded by

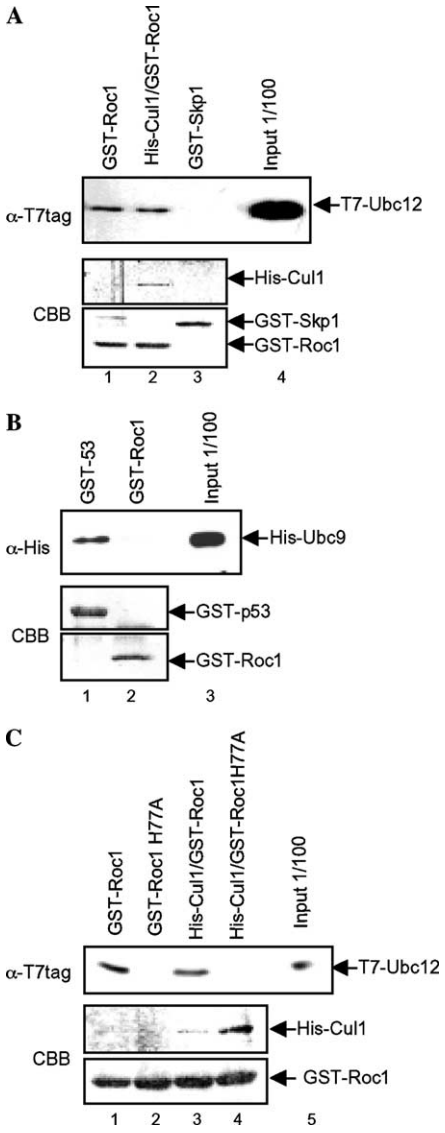

Fig. 2. Roc1 interacts with Ubc12, an E2 for neddylation in vitro. (A) GST-Roc1 (lane 1), GST-Roc1/His-Cul1 (lane 2), or GST-Skp1 (lane 3) was expressed in Sf-9 cells in the baculovirus protein expression system. Total cell extracts were prepared and GST-fusion proteins were immobilized on glutathione–Sepharose 4B beads and then incubated with purified Ubc12. After extensive washing, Ubc12 bound to each protein was detected by Western blotting with anti-T7tag antibody (upper panel). Coomassie staining of proteins is shown (lower two panels). (B) Binding assay was done as shown in (A) by using His-Ubc9, an E2 for sumoylation. (C) Binding assay was done as shown in (A) using GST-Roc1(H77A) mutant in place of GST-Roc1 wild-type.

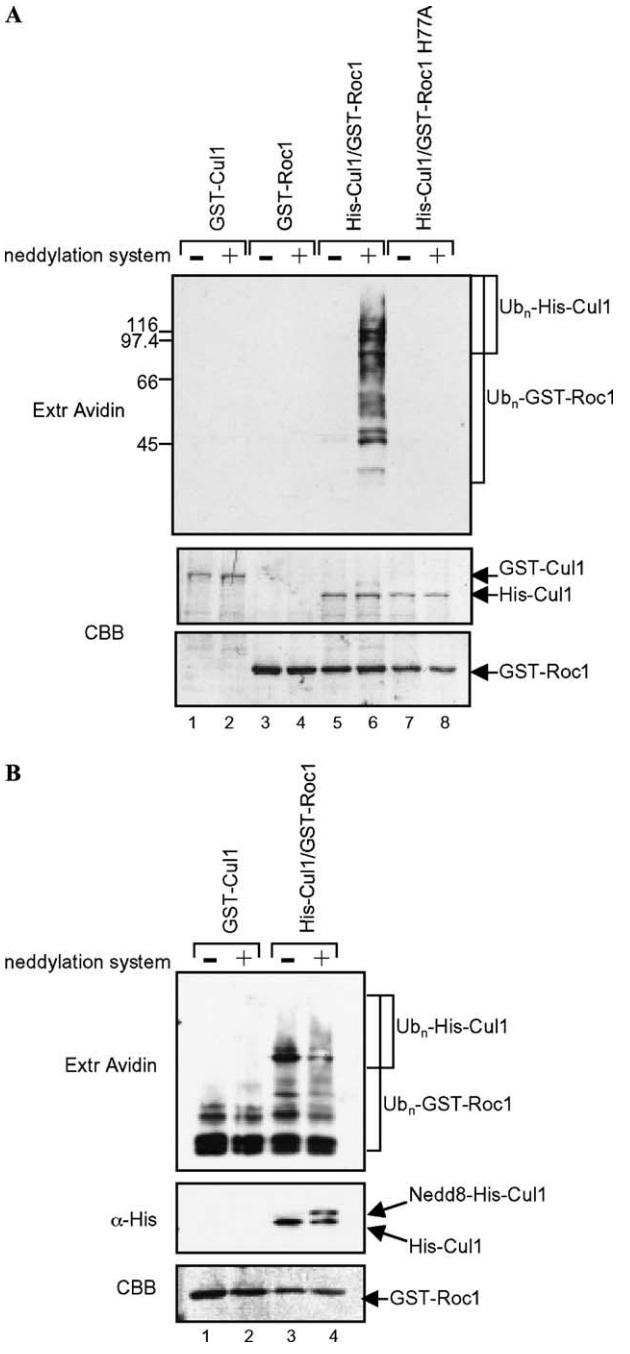

Fig. 3. Self-ubiquitinylation of Cul1/Roc complex is dependent on the neddylation of Cul1 in vitro. (A) GST-Cul1, GST-Roc1, GST-Roc1/His-Cul1, or GST-Roc1 (H77A)/His-Cul1 was expressed in *E. coli*. Total cell extracts were prepared and GST-fusion proteins were immobilized on glutathione–Sepharose 4B beads. After incubation with (lanes 2, 4, 6, and 8) or without the neddylation system (lanes 1, 3, 5, and 7), beads were subjected to the in vitro ubiquitinylation reaction using Cdc34 as E2 and biotinylated ubiquitin. Biotinylated proteins were detected by peroxidase conjugate avidin (ExtrAvidin, upper panel). Coomassie staining of proteins is shown (lower two panels). (B) Experiments were done as shown in (A) except for using UbcH5c as E2 in the in vitro ubiquitinylation reaction.

proteasomes. To examine the involvement of proteasomes in Cul1 degradation, we estimated the effects of a proteasome inhibitor, MG132, on the protein levels of Flag-Cul1 expressed in U2OS cells. As shown in Fig. 4A, Cul1 was accumulated in the presence of MG132. If neddylation of Cul1 was necessary for the self-ubiquitinylation, K720R mutant might be stable than wild-type in a cell. In order to assess the effect of neddylation on the stability of Cul1, we evaluated the half life of

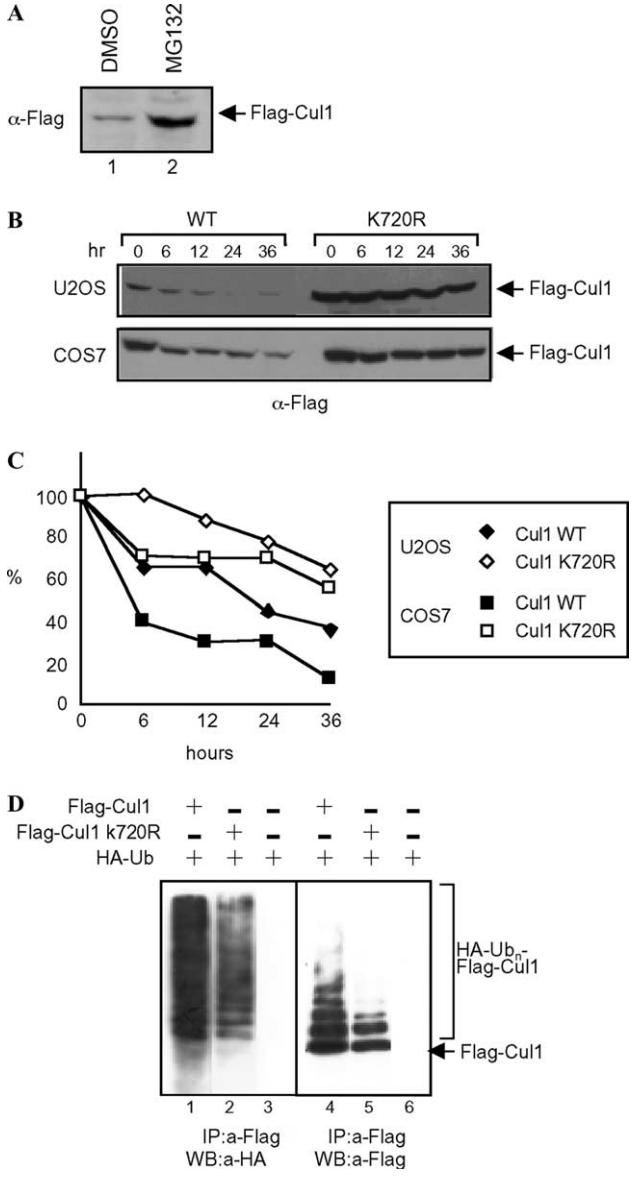

Fig. 4. K720R mutant Cul1 is more stable than wild-type Cul1. (A) U2OS cells were transfected with pFlag-Cul1 and treated with 20 μM MG132 (lane 2) or DMSO (lane 1) as a vehicle control for 10 h. The levels of Flag-Cul1 expression were determined by Western blotting with anti-Flag antibody. (B) U2OS cells or COS7 cells were transfected with pFlag-Cul1 (wild-type or K720R). Twenty-four hours after transfection, the cells were harvested and then re-inoculated into dishes. After an additional 24-h incubation, the cells were harvested at the times indicated. The levels of Flag-Cul1 expression were determined by Western blotting with anti-Flag antibody. (C) The relative amounts of Cul1 were estimated by quantification of band intensity from data in (B) using an imaging densitometer. (D) Cul1 is self-ubiquitinylated in intact cells. U2OS cells were co-transfected with expression plasmids encoding Flag-Cul1 (wild-type or K720R) and HA-Ubiquitin. The cells were treated with 20 μM MG132 for 12 h. Flag-Cul1 (wild-type or K720R) was immunoprecipitated (IP) from total cell extracts with anti-Flag antibody. Bound proteins were subjected to Western-blotting assay (WB) using anti-HA antibody (left panel) or anti-Flag antibody (right panel).

wild-type or K720R mutant in a cell. As expected, when wild-type Flag-Cul1 was expressed in U2OS cells, its half life was about 18 h, whereas that of the mutant one was about 48 h (Figs. 4B and C). Similar data were obtained by using COS7 cells. These findings suggest that neddylation accelerates Cul1 degradation through the ubiquitin–proteasome pathway.

To detect the ubiquitinylated Cul1 in intact cells, Flag-Cul1 (wild-type or K720R) was co-expressed with HA-ubiquitin in U2OS cells. Wild-type Cul1 was markedly ubiquitinylated (Fig. 4D, lanes 1 and 4), but K720R mutant Cul1 was less ubiquitinylated than the wild-type one (Fig. 4D, lanes 2 and 5). The difference between wild-type and K720R mutant in the ubiquitinylation in intact cells may reflect differences in their stabilities.

Discussion

Roc1 functions as a Nedd8-E3 ligase toward Cul1

The Cul1/Roc1 complex has been shown to be a catalytic site of SCF, a ubiquitin ligase. Furthermore, Cul1 is not only self-ubiquitinylated but also neddylated. Nedd8 is one of the ubiquitin-like proteins and the mechanism of its conjugation to a target protein could be analogous to that of ubiquitin-conjugation. The ubiquitin conjugation system requires three enzymes, E1, E2, and E3 or ubiquitin ligase. The Nedd8 conjugation system also seems to require three enzymes, but Nedd8-E3 ligase has not been clearly identified so far; although it might be Roc1, based on the data that Roc1 enhanced the neddylation of Cul1 when Sf-9 cells were co-infected with these proteins [39]. The ubiquitin ligases bind E2 and the RING-finger-type ubiquitin ligase (E3) probably binds E2 mainly at that RING-finger domain [41]. If Roc1 is indeed a Nedd8 ligase toward Cul1, since Roc1 has RING-H2 finger domain, it should bind Ubc12 at the RING-finger domain. Also, a Roc1 mutant, in which this domain is disrupted, should no longer bind Ubc12. The data described herein demonstrated these characteristics of Roc1. To confirm that Roc1 was indeed acting as a Nedd8-E3 ligase, we needed to prepare an appropriate in vitro neddylation assay system. When Cul1/Roc1 complex was expressed in Sf-9 cells, Cul1 was highly neddylated. The non-neddylated Cul1 could be a better substrate than the neddylated one for an in vitro neddylation assay system. In addition, we were afraid that recombinant Roc1 purified from insect cells might bind some neddylation-stimulation factors originated from the cells, because the insect cells have a neddylation system. Therefore, we purified recombinant Cul1/Roc1 complex expressed in bacterial cells, which does not have a neddylation

system, and used it in vitro neddylation assay system. In this assay system, Roc1 enhanced the neddylation of Cul1in a RING-finger domain-dependent manner. Taken together, our data strongly suggest that Roc1 is a Nedd8-E3 ligase toward Cul1. The RING-finger domain of Roc1 is thought to be the binding site of ubiquitin-E2 as proposed in the case of RING-finger-type ubiquitin ligase. We can easily speculate that Roc2 could also be a Nedd8-E3 ligase. Further Roc1 in the VBC complex could function as a Nedd8-E3 ligase toward Cul2.

Neddylated Cul1 has self-ubiquitinylation activity and is degraded through the ubiquitin–proteasome pathway

We found in this study that Cul1/Roc1 complex has self-ubiquitinylation activity through the Cdc34 or UbcH5c. Cdc34-catalyzed self-ubiquitinylation activity of Cul1/Roc1 complex depends on the neddylation of Cul1. In contrast, UbcH5c-catalyzed self-ubiquitinylation activity of Cul1/Roc1 complex is independent of the neddylation. Recently, other researchers also reported similar data [43].

We also could find that Cul1 is ubiquitinylated and degraded through the ubiquitin–proteasome pathway in a cell. In addition, a non-neddylated mutant Cul1(K720R) was less ubiquitinylated and more stable than wild-type intact cells. These results suggest that the neddylation of Cul1 stimulates the degradation of Cul1/Roc1 complex, leading to decreased SCF activity. Recent study shows that overexpression of Rbx1 stabilizes the SCF substrate in *Arabidopsis* [44]. Although the mechanism is unclear, hyper-neddylation of Cul1 seems to regulate SCF function negatively. On the other hand the neddylation of Cul1 was shown to be necessary to enhance the ubiquitinylation of $p27^{kip1}$ [31,32] and IκBα [33,34] or for the polymerization of the ubiquitin moiety [43] by the SCF complex. We propose, therefore, that the neddylation of Cul1 regulates the SCF complex both positively and negatively. The data described here further suggest that Cul1/Roc1 complex acts as a RING-H2 finger-type ubiquitin ligase, only when neddylation of Cul1 occurs. Possibly, F-box protein at first binds to the target protein and then the SCF complex forms without neddylation of Cul1. The neddylation of Cul1, through APP-BP1/Uba3 as E1 and Ubc12 as E2, would activate the ubiquitin ligase activity of SCF. In this model, inactive SCF, being bound to the target protein, would be activated by neddylation of Cul1 to ubiquitinylate the target protein. In that time, Cul1/Roc1 complex in SCF would also be ubiquitinylated and could be degraded by proteasomes after ubiquitinylation of the target protein. This model provides the auto-regulation of the activity of SCF. The ubiquitinylation of Skp2 could be included in this model.

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