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Modification of Cullin-1 by Ubiquitin-like Protein Nedd8 Enhances the Activity of SCF^{skp2} toward p27^{kip1}

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The periodic expression of cell cycle proteins is important for the regulation of cell cycle progression. The amount of CDK inhibitor, p27^{kip1}, one such protein, seems to be regulated by the ubiquitin-proteasome system. The ubiquitin ligase (E3) toward p27^{kip1} is thought to be SCF^{skp2}. The activity of SCF^{skp2} was increased by the addition of Roc1 protein to the complex. Furthermore, the ubiquitination of p27^{kip1} seemed to be dependent on the phosphorylation of T187 of p27^{kip1} because the mutant T187A was not ubiquitinated at all in an *in vitro* ubiquitination system. Cullin-1, a component of SCF, is modified by ubiquitin-like protein Nedd8. The modification site of cullin-1 was shown to be K696 because the K696R mutant was not modified. When the effect of the Nedd8 modification on the SCF^{skp2} activity toward p27^{kip1} was investigated, the activity was markedly decreased by using the Nedd8-unmodified mutant cullin-1 (K696R), indicating that the modification may play an important role on the SCF^{skp2} activity toward p27^{kip1}.

Key Words: **p27^{kip1}; SCF^{skp2}; Nedd8; cullin-1; ubiquitin; ubiquitination; CDK inhibitor; *RUB1; CDC53.***

In mammals, cell-cycle progression is thought to be mainly regulated by the activity of CDK. When the cells enter G_0 phase, the amount of CDK inhibitor p27^{kip1} increases, which inhibits the activity of CDK4 or CDK6 (1). On the other hand, when the cells enter G_1 phase the amount of p27^{kip1} decreases to activate CDK4 or CDK6. Since the p27^{kip1} is degraded by the ubiquitin-proteasome system, the regulatory mechanism of its ubiquitination is important to clarify the regulation of cell-cycle progression (2–4). The ubiquitination of the protein is regulated by the phosphorylation of it. The phosphorylation of T187 of p27 is thought to be a prerequisite for the ubiquitination and is attributed to CyclinE/CDK2 complex (5–7). The SCF^{skp2}, consisting of cullin-1, skp1, and skp2, seems to function as a ubiquitin ligase (E3) toward p27 (3, 4). The p27 binding protein Jab1/Mov34 may function to accelerate the degradation of p27 by carrying p27 into cytoplasm (8).

Nedd8, a homologue of ubiquitin, is a modifier of cullin family proteins, and this modification is catalyzed by APPBP1/Uba3 as E1 and Ubc12 as E2 (9, 10). Recently, the cullin-1 in SCF^{skp2} was found to be modified by Nedd8 (11, 12). Furthermore, cullin-2 in VBC (11, 13), another E3 complex, is modified by it. More recently, cullin-1 to cullin-5 have also been found to be modified by it (10).

The modified cullin-1 is found in centrosomes, and the ratio of the modified cullin-1 to total cullin-1 increases when the cullin-1 is expressed together with Roc1 in Sf-9 cells in the baculovirus protein expression system (14). However, the role of this modification has not yet been clarified.

Here, we show that Nedd8 modification of cullin-1 enhances the ubiquitination of p27.

MATERIALS AND METHODS

Expression and mutation of the proteins. Cullin-1, skp1, skp2, Roc1, p27^{kip1}, cyclinE, CDK2, APPBP1, and hUba3 were expressed in a baculovirus expression system using Sf-9 cells according to the manufacturer's protocol (Clontech). In some experiments, the Sf-9 cells were co-infected with baculoviruses with inserts of various cDNAs. UbcH5, Ubc12, and Nedd8 were expressed in *E. coli* (*BL21, LysS*) by using the pET vector. Point mutation of p27 cDNA or Nedd8 cDNA was done by use of the PCR method as described previously (15).

In vitro ubiquitination assay. The Sf-9 cells expressing cullin-1, skp1, and skp2 were lysed in 50 mM Tris–HCl (pH 7.4), 3 mM $MgCl_2$, 1 mM phenylmethyl sulfonyl fluoride (PMSF), 0.1 M NaCl, and 0.1% NP-40, and the SCF complex (composed of these expressed proteins)

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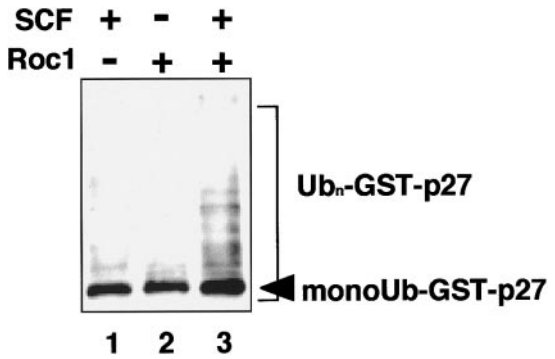

FIG. 1. Roc 1 enhances ubiquitination of p27^{kip1} *in vitro*. SCF^{skp2}, consisting of cullin-1, skp1, and skp2, was mixed with Roc1, and the ubiquitination reaction toward p27 was carried out as described under Materials and Methods except that the reaction mixture also contained HeLa cell lysate (4×10^5 cells/ml). Lane 1, the reaction mixture without Roc1 and with SCF^{skp2}; lane 2, with Roc1 and without SCF^{skp2}; lane 3, with Roc1 and SCF^{skp2}.

was bound to a TALON metal affinity resin (Clontech), which binds 6xHis-tagged cullin-1, and eluted from the resin by use of 2 mM imidazole. The Sf-9 cells expressing GST-p27 and CyclinE/CDK2 were lysed in 50 mM Tris–HCl (pH 7.4), 3 mM $MgCl_2$, 1 mM PMSF, 0.1 M NaCl, and 0.1% NP-40, and GST-p27 was purified by glutathione-Sepharose 4B affinity resin. When 6xHis-tagged Roc1 was used, the protein was purified by use of the TALON resin as described above. The *in vitro* ubiquitination was carried out essentially as described previously, using biotin-labeled ubiquitin (15, 16). Briefly, the reaction mixture containing 40 mM Tris–HCl, 2 mM ATP, 1 mM dithiothreitol, mouse E1 (1 μg), E2 (UbcH5, 3 μg), SCF^{skp2} (2 μg), GST-tagged p27^{kip1} (150 ng), and biotinylated ubiquitin was incubated for 30 min at 25°C. After incubation, the GST-tagged p27^{kip1} was pelleted after having been bound by glutathione-Sepharose 4B, and the resin was applied to SDS-polyacrylamide gel electrophoresis (SDS–PAGE). The ubiquitinated p27^{kip1} was detected by Western blotting using avidin-peroxidase and the ECL method (Amersham Pharmacia).

Nedd8 modification system. The Sf-9 cells expressing APPBP1 and hUba3 were lysed; and the mixture containing APPBP1 (500 ng), hUba3 (50 ng), hUbc12 (3 μg), SCF^{skp2} (2 μg), and Nedd8 or GST-Nedd8 (1 μg) was incubated at 25°C for 30 min. After incubation, the SCF^{skp2} containing 6xHis-tagged cullin-1 were purified by use of TALON beads. When the Nedd8-modified SCF^{skp2} was used for the *in vitro* ubiquitination assay, HeLa cell lysate (1×10^6 cells) was added to the Nedd8 modification system. The *in vitro* ubiquitination assay was performed as described above. The Western blotting of Nedd8 or 6xHis-tagged protein was done by use of anti-Nedd8 antibody (Alexis) or anti-6xHis antibody (Qiagen), respectively, and peroxidase- conjugated anti-mouse rabbit antibody (Dako).

RESULTS AND DISCUSSION

Ubiquitination of p27 by SCF^{skp2} in the presence of Roc1 and Nedd8 modification. The Roc1 protein was found to be a binding protein of cullin family proteins and to activate the ubiquitination of IκBα by SCF$^{\beta\text{-TrCP}}$ (17, 18). To know whether the Roc1 protein is also important in the activity of SCF^{skp2} toward p27, we carried out the ubiquitination assay in the presence or absence of Roc1 protein. As shown in Fig. 1, the ubiqutination of p27 increased markedly in the presence of Roc1. Cullin-1, a component of SCF^{skp2}, has been shown to be modified by Nedd8, a ubiquitin-like protein. So to determine whether the Nedd8 modification is important for the activity of SCF, we carried out the ubiquitination of p27 with or without the Nedd8 modification system, i.e., APPBP1/hUba3, hUba12, and Nedd8. The ubiquitination of p27 was markedly increased in the presence of this modification system. In contrast, the p27 (T187A) was not ubiquitinated at all with or without the Nedd8 modification system (Fig. 2). However, even without the Nedd8 modification system, some ubiquitination of p27 was apparent, perhaps due to modification of cullin-1 by Nedd8 in Sf-9 cells during its expression. To confirm the effect of Nedd8 modification of cullin-1 on ubiquitination of p27, we examined the effect of Nedd8 un-modified form of cullin-1 on the ubiquitination.

Effect of Nedd8 unmodified mutant cullin-1 on SCF^{skp2} activity. Recently, the mammalian cullin-2 was found to be modified at K689 by Nedd8 (13). When human cullin-1 was aligned with cullin-2, the Nedd8 modification site of cullin-2 corresponded to K696 in cullin-1, as shown in Fig. 3. Therefore, the K696 residue was modified to R696, and the Nedd8 modification of this mutant cullin-1 was tested. After the wild-type or mutant cullin-1 had been subjected to the Nedd8 modification system, the modification of it was

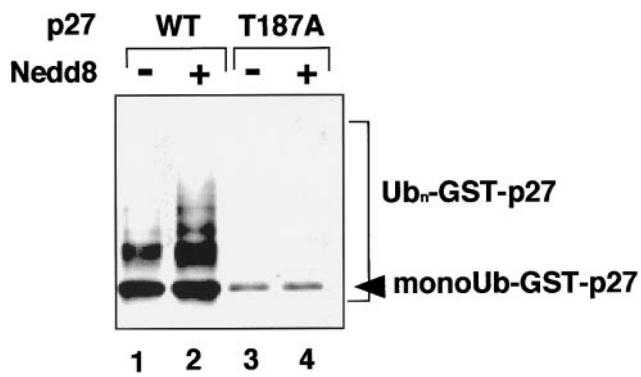

FIG. 2. Nedd8 modification of cullin-1 and the phosphorylation of T187 of p27 enhance the ubiquitination of p27. The Sf-9 cells were co-infected with GST-tagged p27 or GST-tagged p27 (T187A) and Cyclin E/CDK2. Nedd8 modification and the *in vitro* ubiquitination assay were carried out by use of GST-tagged p27 or GST-tagged p27 (T187A) as described under Materials and Methods. Lane 1, ubiquitination of GST-tagged p27 without Nedd8 modification; lane 2, ubiquitination of GST-tagged p27 with Nedd8 modification; lane 3, ubiquitination of GST-tagged p27 (187A) without Nedd8 modification; lane 4, ubiquitination of GST-tagged p27 (187A) with Nedd8 modification.

FIG. 3. Alignment of Nedd8 modification site of cullin-2 with cullin-1 sequence. The K696 of cullin-1 seems to be the putative Nedd8 modification site.

checked. By Western blotting using anti-Nedd8 antibody, the modification of wild-type cullin-1 was detected, but that of the mutant cullin-1 was not (Fig. 4A). Also, when the GST-tagged Nedd8 was used in the modification system, the Nedd8 modified wild-type cullin-1 was detected, but the modified mutant cullin was not (Fig. 4B). The Nedd8-modified form of cullin-1 had a much larger molecular weight than the unmodified cullin-1 (lanes 2, 4, and 6 in Fig. 4B). After the Nedd8 modification reaction had been carried out in the presence of wild-type or mutant cullin-1, the ubiquitination of p27 under the two conditions was compared. Although the SCF^{skp2} containing Nedd8-modified cullin-1 catalyzed the multi-ubiquitinated chain formation of p27 (lane 2 in Fig. 5), the SCF^{skp2} containing mutant cullin-1 did not catalyze the multi-ubiquitinated chain formation of p27 (Fig. 5).

Here we showed that Nedd8 modification of cullin-1 enhanced the activity of SCF^{skp2} toward $p27^{kip1}$. The *in vitro* ubiquitination assay in mammalian system showed that the modification is essential for the SCF^{skp2} activity, although the disruption of *RUB1*, the yeast *Saccharomyces cerevisiae* homologue of Nedd8 gene, showed that the gene was not essential (19). In the yeast system, the over-expression of Cdc53 or Hrt1, the yeast homologue of Roc1, may compensate for the defect of Rub1 modification.

Further, we showed that Roc1 also enhanced the activity, indicating that SCF^{skp2} may also use the Roc1/cullin-1 heterodimer as the catalytic site, as shown for $SCF^{\beta\text{-TrCP}}$. The modification may be necessary for the efficient assembly of SCF components, and/or be necessary for the catalytic activity of the Roc1/cullin-1

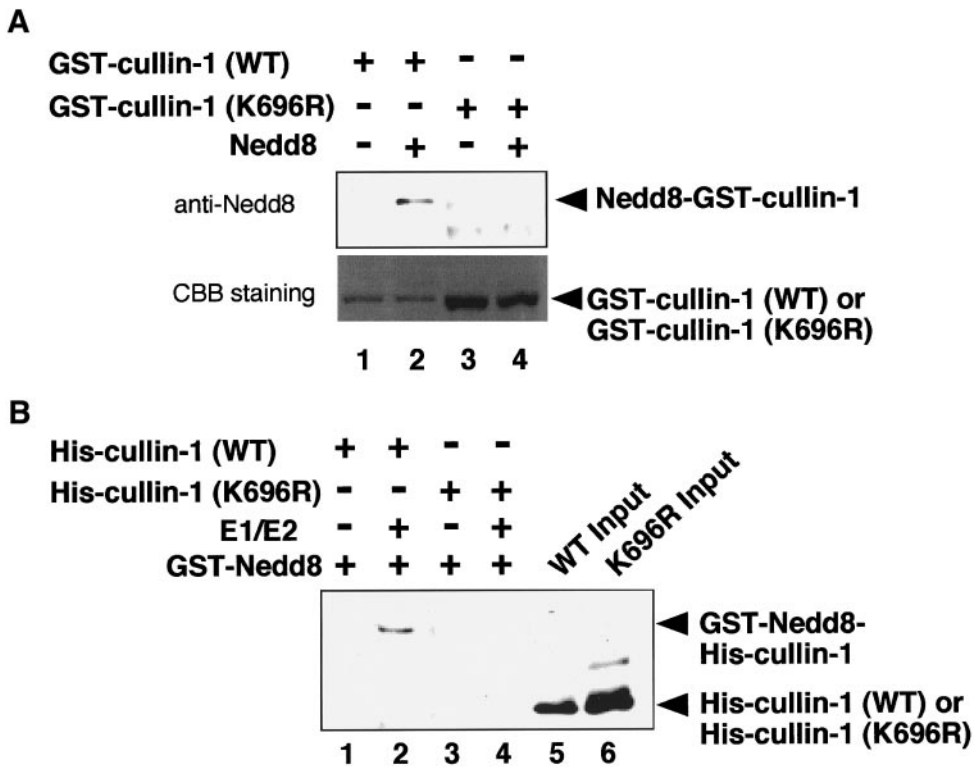

FIG. 4. K696 of cullin-1 is the Nedd8 modification site. (A) After the reactions indicated at the top of the figure had been carried out, the GST-tagged cullin-1 was pelleted by glutathione-Sepharose 4B beads, and the Nedd8-modified cullin-1 was detected with anti-Nedd8 antibody (Alexis). The cullin-1 proteins were detected by staining with coomassie brilliant blue R-250 (CBB). Nedd8-modified cullin-1 was only detected in lane 2. (B) After reactions indicated, GST-tagged Nedd8 was pelleted by glutathione-Sepharose 4B beads, and GST-Nedd8-modified 6xHis-tagged cullin-1 was detected with anti-6xHis antibody (Qiagen). The wild-type cullin-1 and mutant-type cullin-1 used in the reaction are shown in lanes 5 and 6 as a control of Western blotting. Nedd8- modified His-cullin-1 was only detected in lane 2.

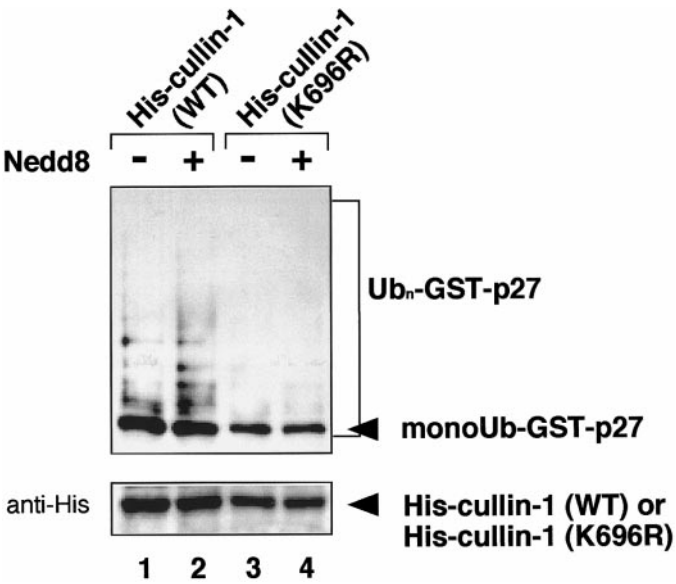

FIG. 5. SCF^{skp2} containing the Nedd8 un-modified form of cullin-1 did not have ubiquitin ligase activity toward p27. SCF^{skp2} containing wild-type cullin-1 (lanes 1 and 2), or K696R mutant cullin-1 (lanes 3 and 4) was used in a ubiquitination assay toward $p27^{kip1}$, with (lanes 2 and 4) or without (lanes 1 and 3) the Nedd8 modification reaction. The cullin-1 proteins were detected with anti-6xHis antibody (Qiagen). Multi-ubiquitinated forms of p27 were apparent in lane 2, and some in lane 1, but none were found for the mutant cullin-1.

heterodimer. Thus, the Nedd8 modification of SCF^{skp2} is probably essential for its activity.

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