Targeting of NEDD8 and Its Conjugates for Proteasomal Degradation by NUB1*

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NEDD8 is a ubiquitin-like protein that controls vital biological events through its conjugation to cullin family members. Recently, we identified a negative regulator of the NEDD8 conjugation system, NUB1, which interacts with NEDD8 and down-regulates NEDD8 expression post-translationally (Kito, K., Yeh, E. T. H., and Kamitani, T. (2001) J. Biol. Chem. 276, 20603–20609). Here, we show that NUB1 possesses a ubiquitin-like domain at the N-terminal region and binds to S5a of the 19 S proteasome activator (PA700). A GST pull-down assay revealed that the overexpression of NUB1 leads to a greater precipitation of NEDD8 conjugates with GST-S5a, suggesting that NUB1 might have an adaptor function between S5a and NEDD8. Furthermore, proteasome inhibitors completely block NUB1-mediated down-regulation of NEDD8 expression. These results suggest that NUB1 recruits NEDD8 and its conjugates to the proteasome for degradation, providing a direct functional link between the NEDD8 conjugation system and the proteasomal degradation pathway.

NEDD8 is a highly conserved 81-amino acid protein that shares 60% identity and 80% homology with ubiquitin. NEDD8 has been shown to conjugate to a large number of nuclear proteins (1). NEDD8 conjugation is thought to be catalyzed by three enzymes, termed E1 (NEDD8-activating), E2 (NEDD8-conjugating), and E3 (NEDD8-ligating), in a manner analogous to ubiquitination and senitization (also known as SUMO conjugation) (2–4). All of the known NEDD8 targets in mammalian cells are cullin family members, including Cul-1, -2, -3, -4A, -4B, and -5 (5, 6). Human Cul-1 is a major component of ubiquitin ligase known as a SCF complex that catalyzes the ubiquitination of IκBα, β-catenin, and p27 (Kip1) (7–9). Interestingly, NEDD8 conjugation to Cul-1 is required for the ubiquitin ligase activity of the SCF complex (10, 11). This finding implies that NEDD8 modifies the molecular function of other cullin family members as well as Cul-1. Thus, NEDD8 conjugation could be involved in many important biological functions, and its conjugation and deconjugation systems have to be strictly regulated. Recently, we tried to define the unknown regulators of NEDD8 conjugation using a yeast two-hybrid system with NEDD8 as a bait. From the library screening, we isolated a cDNA clone encoding a novel NEDD8-interacting protein, NUB1. We demonstrated that NUB1 overexpression leads to severe inhibition of NEDD8 expression by a post-transcriptional mechanism (12). Thus, NUB1 acts as a strong down-regulator of NEDD8 conjugation. In this report, we describe the mechanism of action by which NUB1 down-regulates NEDD8 expression.

EXPERIMENTAL PROCEDURES

Cell Culture—Human osteosarcoma U2OS cells and cervical adenocarcinoma HeLa cells were purchased from American Type Culture Collection (Manassas, VA). COS-M6 cells were generous gifts from Dr. Steve Goldring (Harvard Medical School). These cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and antibiotics.

Antibodies—Mouse anti-HA antibody (16B12) was purchased from Covance (Richmond, CA). Mouse anti-RH antibody (specific for the amino acid sequence RGSHHHH) was purchased from Qiagen (Santa Clara, CA). Rabbit anti-human NUB1 antiserum was generated by immunization with a GST fusion protein of NUB1-(432–601), corresponding to amino acids 432–601 (12). Rabbit anti-MBP antibody was purchased from New England Biolabs (Beverly, MA). Mouse monoclonal antibodies against Rpt5 and α-subunits were purchased from Affinity Research Products Ltd. (Mamhead, Exeter, UK). Mouse anti-FLAG antibody (M5) was purchased from Sigma. Rabbit anti-NEDD8 antibody was generated by immunization with a GST fusion protein of NUB1-(432–601), corresponding to amino acids 432–601 (12). Rabbit anti-MBP antibody was purchased from New England Biolabs (Beverly, MA). Mouse monoclonal antibodies against Rpt5 and α-subunits were purchased from Affinity Research Products Ltd. (Mamhead, Exeter, UK). Mouse anti-FLAG antibody (M5) was purchased from Sigma. Rabbit anti-NEDD8 antibody was generated by immunization with a GST fusion protein of human NEDD8, followed by purification with protein G column. To remove the reactivity to GST and the cross-reactivity to ubiquitin, the purified antibody was absorbed with GST-ubiquitin immobilized on glutathione-Sepharose beads. After the immunoaabsorption, the anti-NEDD8 antibody was used for Western blotting.

Plasmid Construction and Transfection—To express proteins tagged with epitope at the N terminus in mammalian cells, pcDNA3/HA-N (13) or pcDNA3/RH-N (14) was used as previously described (1). The human cDNAs used in this work were described previously: ubiquitin (13), NEDD8 (1), NEDD8-G (1), sentrin-2 (also known as SUMO-3/SMT3B) (14), sentrin-2-G (14), and USP21 (15). The cDNA of EGFP (enhanced green fluorescent protein) was amplified by polymerase chain reaction from pEGFP-1 (CLONTECH) using appropriate primers. These cDNAs were inserted into the aforementioned plasmid vectors. The sequence of each cDNA was confirmed by automated DNA sequencing. Plasmids were transfected into COS-M6 cells using FuGENE 6 (Roche Molecular Biochemicals). The transfected cells were harvested for Western blotting 20 h after transfection.

Treatment with Proteasome Inhibitors—N-Acetyl-l-leucinal-l-leucinal-l-norleucinal was purchased from Sigma. MG132 and lactacystin were purchased from Calbiochem. 1 × 10⁶ COS-M6 cells were transfected with FuGENE 6. After overnight culture, the culture medium was replaced with fresh medium containing proteasome inhibitor such as N-acetyl-l-leucinal-l-leucinal-l-norleucinal (LLnL) (50 μM), MG132 (40 μM), or lactacystin (20 μM). The transfectants were cultured at 37°C with these proteasome inhibitors for 6 h. Then the cells were harvested, and the total cell lysates were prepared for Western blot analysis.

The abbreviations used are: GST, glutathione S-transferase; HA, hemagglutinin epitope; RH, RGS-poly(His); MBP, maltose-binding protein; TBS, Tris-buffered saline; EGFP, enhanced green fluorescent protein; PAGE, polyacrylamide gel electrophoresis.

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Western Blotting—Protein samples were treated at 45 °C for 1 hour in 150 µl of 2% SDS treating solution containing 5% β-mercaptoethanol. After SDS-PAGE, Western blotting was performed using the protocol provided with the ECL detection system (Amersham Pharmacia Biotech). As secondary antibodies, horseradish peroxidase-conjugated antibodies against mouse IgG or rabbit IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used.

Determination of Ubiquitin-like Domain—The ubiquitin-like domain was determined by the ProfileScan program via the World Wide Web.

**FIG. 1. Inhibition of NEDD8 expression by overexpression of NUB1.** A, reduction of NEDD8-conjugated proteins by NUB1 overexpression. In COS cells, HA-tagged NEDD8 was coexpressed with empty vector (lane 2), RH-tagged NUB1 (lane 3), or EGFP (lane 4). Total cell lysates were prepared from transfectants and analyzed by Western blotting using anti-HA antibody to detect HA-NEDD8 and proteins conjugated with HA-NEDD8 (upper panel) and anti-RH antibody to detect the coexpressed proteins (lower panel). As a control, HA-ubiquitin (lanes 5–7) or HA-sentrin-2 (lanes 8–10) was also coexpressed with empty vector, RH-NUB1, or RH-EGFP, followed by Western blot analysis. Non-specific bands are indicated by an asterisk. Note that in lane 7, a band of ubiquitin monomer is reduced (upper panel), and ladder-like bands are detected above EGFP (lower panel). The ladder-like bands may be ubiquitinated EGFP formed from the consumption of ubiquitin monomer. B, reduction of NEDD8 monomer by NUB1 overexpression. In COS cells, HA-tagged NEDD8-G was coexpressed with empty vector (lane 2) or RH-NUB1 (lane 3). As a control, HA-tagged sentrin-2-G was also coexpressed with empty vector (lane 4) or RH-NUB1 (lane 5). Total cell lysates were prepared from transfectants and analyzed by Western blotting using anti-HA antibody to detect the unconjugated form of HA-tagged proteins (upper panel) and anti-RH antibody to detect RH-NUB1 (lower panel). NEDD8-G indicates a deletion mutant of NEDD8 lacking a C-terminal fragment from Gly-76 to Gln-81. Sen2-G indicates a deletion mutant of sentrin-2 lacking a C-terminal fragment from Gly-93 to Tyr-95. These mutants are unable to form conjugates with target proteins.

**FIG. 2. In vitro interactions of NUB1.** A, interaction of NUB1 with NEDD8. Poly-His-tagged ubiquitin, NEDD8, sentrin-1, sentrin-2, and sentrin-3 (lanes 1–5) were expressed in bacteria and purified with TALON beads. These proteins were eluted and separated by SDS-PAGE. The gel was stained with Coomassie Blue (upper panel). In the lower panel, a pull-down assay of NUB1 was performed by using the TALON beads coated with poly-His-tagged ubiquitin and ubiquitin-like proteins. The solution of recombinant NUB1 protein (50 ng/ml) was incubated with ubiquitin and ubiquitin-like proteins immobilized on TALON beads (lanes 7–11). After incubation, the beads were washed and treated with 2% SDS solution to elute proteins. The eluted proteins were analyzed by Western blotting using rabbit anti-NUB1 antibody (12). As a control, 3 ng of NUB1 protein was loaded on lane 6. B, interaction of NUB1 with S5a. GST (lane 1) and GST-fused NUB1 (lane 2) were expressed in bacteria and purified with glutathione-Sepharose beads. MBP and MBP-fused S5a were expressed in bacteria and purified with amylose resin beads. These proteins were eluted and precipitated by GST (lane 3) or GST-NUB1 beads (lane 4). Precipitated proteins were detected by Western blotting using rabbit anti-MBP antibody.
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Co-fractionation of proteasome with NUB1- and NEDD8-conjugated proteins. A, co-fractionation of proteasome with

prokaryotic expression plasmid pTrcHisA (Invitrogen). Escherichia coli BL21 cells were transformed with these plasmids. Transformed cells were grown logarithmically (A_{600} = 0.8) in 2 ml of LB medium containing 50 μg/ml ampicillin. Then the expression of His₆-tagged ubiquitin-like proteins was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside at room temperature for 2.5 h. Cells were pelleted and resuspended in 150 μl of TBS buffer (50 mM Tris-HCl [pH 7.5], 100 mM NaCl), followed by sonication for 15 s. After centrifugation, the supernatant was washed with TBS buffer five times and treated with sample treating buffer containing 2% SDS and 5% β-mercaptoethanol. The protein samples were analyzed by Western blotting using rabbit anti-NUB1 antibody.

In Vitro Interaction between NUB1 and S5a—The cDNA of S5a was amplified by polymerase chain reaction using appropriate primers from human testis cDNA library (Life Technologies, Inc.) and inserted into pMAL-c2 (New England Biolabs) to make pMAL/S5a. E. coli BL21 cells were transformed with plasmids pMAL-c2 and pMAL/S5a. Recombinant proteins MBP and MBP-S5a were purified from the lysate of the BL21 cells by amylose resin beads and eluted using the protocol provided by New England Biolabs. MBP and MBP-S5a were used for the GST pull-down assay as described previously (17).

Western Blotting of Co-fractionation Samples—Fractions of 26 and 20 S proteasomes were purchased from Bostonbiochem (Cambridge, MA) and Calbiochem, respectively. These proteasome fractions were derived from rabbit reticulocyte lysate. Approximately 0.7 μg of protein of each fraction was solubilized in 2% SDS treating solution and loaded on an SDS-PAGE gel, followed by Western blotting analysis. To show the co-fractionation of NUB1 or NEDD8 conjugates in proteasome fraction, rabbit anti-NUB1 antibody (12) or rabbit anti-NEDD8 antibody (see above) was used, respectively. In addition to proteasome fractions, total cell lysates of HeLa cells (NUB1-expressing cell line) and U2OS cells (cell line with undetectable NUB1 expression) (12) were examined as controls.

NUB1. Purified fractions of 26 and 20 S proteasomes were purchased and used to examine co-fractionated proteins. Approximately 0.7 μg of protein of each fraction was analyzed by Western blotting using rabbit anti-NUB1 (upper panel), mouse anti-Rpt5 (middle panel), and mouse anti-α-subunits (lower panel). Rpt5, a component of the 19 S proteasome activator, and α-subunits, components of the 20 S proteasome, were shown to demonstrate the protein amount and purity of each proteasome fraction. As controls, we used total cell lysates of HeLa cells (an NUB1-expressing cell line) and U2OS cells (a cell line with undetectable NUB1 expression) (12). B, demonstration of the specificity of anti-NEDD8 antibody. GST (lane 1), GST-ubiquitin (lane 2), GST-NEDD8 (lane 3), GST-sentrin-1 (lane 4), GST-sentrin-2 (lane 5), and GST-sentrin-3 (lane 6) were expressed in bacteria and purified with glutathione-Sepharose beads. The purified fusion proteins (0.3 μg/lane) were electrophoresed in 12% SDS-PAGE and stained with Coomassie Blue (upper panel). The fusion proteins (3 ng/lane) were also used for Western blotting with rabbit anti-NEDD8 antibody (lower panel). C, co-fractionation of proteasome with NEDD8-conjugated proteins. To examine whether NEDD8-conjugated proteins were co-fractionated with proteasome, purified proteasomes of 26 and 20 S (see A) were used. Approximately 0.7 μg of protein of each fraction was analyzed by Western blotting using rabbit anti-NEDD8 (see B). As controls, we demonstrated NEDD8 monomer and its conjugated proteins in total cell lysates of HeLa cells and U2OS cells.
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RESULTS AND DISCUSSION

Reduction of NEDD8 Expression by NUB1 Overexpression—To investigate how NUB1 affects NEDD8 expression, we first used the COS cell coexpression assay. HA-tagged NEDD8 was coexpressed in COS cells with empty vector (control), RH-tagged NUB1, or EGF-P (control). Further controls were created by coexpression of HA-tagged ubiquitin or sentrin-2 (SUMO-3/SMT3B) with empty vector, RH-tagged NUB1, or EGF-P. As shown in the upper panel of Fig. 1A, when HA-NEDD8 was coexpressed with empty vector (lane 2) or RH-EGFP (lane 4), a 7-kDa band of unconjugated HA-NEDD8 and high molecular mass bands of HA-NEDD8-conjugated proteins were strongly detected. This NEDD8 expression pattern was identical to the pattern we found in our previous study (1). When HA-NEDD8 was coexpressed with RH-NUB1 (lane 3), the same bands were detected, but they were much weaker than those of lanes 2 and 4. This result indicated that NUB1 inhibits the expression of NEDD8 monomer and its conjugates. In contrast, when HA-ubiquitin (lane 6) or HA-sentrin-2 (lane 9) was coexpressed with RH-NUB1, overexpression of NUB1 did not lead to reduction of ubiquitination or sentrinization. Thus, the down-regulation by NUB1 was specific to the NEDD8 conjugation system. Next, we confirmed that NUB1 affects the expression of NEDD8 monomer by using a NEDD8 mutant, NEDD8-G, which has a C-terminal deletion from Gly-76 to Gln-81 and is unable to form conjugates with target proteins (1). As a control, we used a sentrin-2 mutant, sentrin-2-G, which has a similar C-terminal deletion from Gly-93 to Tyr-95 and is unable to form conjugates with target proteins (14). As shown in the upper panel of Fig. 1B, overexpression of RH-NUB1 strongly reduced the protein expression of HA-NEDD8-G (lane 2 versus lane 3) but not HA-sentrin-2-G (lane 4 versus lane 5). This finding confirmed that NUB1 also specifically reduced the protein expression of the unconjugated form of NEDD8.

In Vitro Interaction between NUB1 and NEDD8—NUB1 was originally isolated by yeast two-hybrid system using NEDD8 as a bait, indicating that NUB1 interacts with NEDD8 directly or indirectly in vivo. In Fig. 1, we showed that the effect of NUB1 overexpression was specific to NEDD8, suggesting a specific interaction of NUB1 with NEDD8. To examine whether NUB1 interacts with NEDD8 directly or indirectly and whether the interaction is specific to NEDD8 or not, an in vitro interaction assay was performed. We expressed poly-His-tagged ubiquitin and ubiquitin-like proteins, including NEDD8, sentrin-1 (SUMO-1), sentrin-2 (SUMO-3/SMT3B), and sentrin-3 (SUMO-2/SMT3A), in bacteria and purified the proteins using TALON beads (Fig. 2A, upper panel). Then the bead-immobilized proteins were used for a pull-down assay of NUB1. As shown in the lower panel of Fig. 2A, NUB1 was precipitated with poly-His-tagged NEDD8 but not with ubiquitin, sentrin-1, sentrin-2, or sentrin-3. Thus, NUB1 showed the direct interaction specific to NEDD8 in vitro.

In Vitro Interaction between NUB1 and S5α—NUB1 is composed of 601 residues and has a calculated molecular mass of 68.1 kDa. It possesses several domains, including two coiled coils, two ubiquitin-associated domains, a bipartite nuclear localization signal, and a PEST sequence (12). Recently, we found an additional domain in NUB1, called the ubiquitin-like domain, that represents a new class of proteasome-interacting motif (18). The domain was located at the N-terminal region of NUB1 from Ile-85 to Val-147 between two coiled coil regions. So far, the ubiquitin-like domain has been characterized well in Rad23 and BAG-1. Rad23 interacts with S5α, a subunit of the

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**Fig. 4. Pull-down assay of NEDD8 monomer and its conjugates with GST-S5a.** A, pull-down assay of ubiquitin and ubiquitin-like proteins. Empty vector or plasmid encoding FLAG-tagged ubiquitin, NEDD8, or sentrin-2 was transfected into COS cells. After overnight culture, the transfectants were treated with 20 μM MG132 for 4 h. Total cell lysates were also prepared from the transfectants, and FLAG-tagged proteins were detected by Western blotting using anti-FLAG antibody (lanes 1–4). The total cell lysates were also used for pull-down assay with GST (lanes 5–8) or GST-S5a (lanes 9–12). After pull-down, the precipitates were analyzed by Western blotting using anti-FLAG/M5 antibody. B, effect of NUB1 overexpression on pull-down of NEDD8-conjugated proteins. In COS cells, empty vector or plasmid encoding FLAG-tagged NEDD8, sentrin-2, or ubiquitin was cotransfected with empty vector (lanes 1, 3, 5, and 7) or plasmid encoding RH-tagged NUB1 (lanes 2, 4, 6, and 8). After overnight culture, the transfectants were treated with 20 μM MG132 for 4 h. Total cell lysates were prepared from the transfectants and used for a pull-down assay by glutathione beads coated with GST-S5a. The precipitates were analyzed by Western blotting using anti-FLAG/M5 antibody.

**GST Pull-down Assay to Precipitate Ubiquitin and Ubiquitin-like Proteins—**COS-M6 cells were transfected with empty vector or plasmid DNA encoding FLAG-tagged ubiquitin, NEDD8, or sentrin-2. After overnight culture, the transfected cells were treated with 20 μM MG132 for 4 h, followed by the preparation of total cell lysate in TBS buffer (25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 20 mM MgCl2, 0.2% Nonidet P-40). The lysate was centrifuged at 100,000 × g at 4 °C for 30 min, and the supernatant was incubated with GST or GST-S5a immobilized on glutathione-Sepharose beads (Amersham Pharmacia Biotech) at 4 °C for 1 h. After incubation, the beads were washed five times with TBS buffer. Precipitated proteins were solubilized in 2% SDS treating solution at 45 °C for 1 h and analyzed by Western blotting using anti-FLAG antibody. The GST-S5a fusion protein was generated by pGEX-2TK/Nedd8. The S5a cDNA was inserted into pGEX-2TK (Amersham Pharmacia Biotech). GST-S5a was expressed in BL21 cells and purified by glutathione-Sepharose beads as described previously (16).
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The COS cells were cultured for 6 h in the absence (lanes 1–3) or presence of proteasome inhibitors (lanes 4–6). Total cell lysates were prepared from the COS cells and analyzed by Western blotting using anti-HA antibody to detect unconjugated and conjugated forms of HA-NEDD8 (upper panel) and anti-RH antibody to detect RH-NUB1 (lower panel). Note that an asterisk indicates a very prominent NEDD8-protein conjugate of 80–90 kDa, which is an uncharacterized protein and appears slightly resistant to NUB1 overexpression. B, effect of proteasome inhibitors on USP21 function in cells expressing HA-NEDD8. HA-NEDD8 was coexpressed in COS cells with empty vector (lane 2) or RH-NUB1 (lanes 3–6). The COS cells were cultured for 6 h in the absence (lanes 1–3) or presence of proteasome inhibitors (lanes 4–6). Total cell lysates were analyzed by Western blotting using anti-HA antibody (upper panel) and anti-RH antibody (lower panel). Note that an asterisk indicates a very prominent NEDD8-protein conjugate of 80–90 kDa, which is an uncharacterized protein and appears slightly resistant to USP21 overexpression. C, effect of proteasome inhibitors on NUB1 function in cells expressing HA-NEDD8-G. HA-NEDD8-G was coexpressed in COS cells with empty vector (lane 2) or RH-NUB1 (lanes 3–6). The COS cells were cultured for 6 h in the absence (lanes 1–3) or presence of proteasome inhibitors (lanes 4–6). Total cell lysates were analyzed by Western blotting using anti-HA antibody to detect HA-NEDD8-G (upper panel) and anti-RH antibody to detect RH-NUB1 (lower panel).
fraction. Neither the monomer nor the conjugated form of NEDD8 could be detected in 20 S proteasome. These results suggested that NEDD8 conjugates are associated with the 19 S proteasome activator. In this experiment, the NEDD8 monomer could not be detected in the fraction of the 26 S proteasome. The NEDD8 monomer might be rapidly degraded by proteasome (see below).

**Effect of NUB1 Overexpression on the Association of NEDD8 Conjugates with S5a**—In Fig. 3, we demonstrated that NEDD8 conjugates are co-fractionated with the 19 S proteasome activator. To explain this co-fractionation, there are three possibilities. The first possibility is that NEDD8 directly binds to S5a of the 19 S proteasome activator. The second possibility is that NUB1 is an adaptor between S5a of the 19 S proteasome activator and NEDD8 conjugates. The third possibility is that S5a interacts with the NUB1-NEDD8 complex by mutual binding. To examine these possibilities, we overexpressed FLAG-tagged NEDD8 in COS cells (Fig. 4A). As shown in lane 3, FLAG-NEDD8-expressing cell lysate revealed 6.5-kDa NEDD8 monomer and higher molecular mass NEDD8 conjugates. These proteins were examined by GST pull-down assay using GST or GST-S5a immobilized on glutathione beads. GST did not precipitate any proteins (lane 7), whereas GST-S5a precipitated a trace amount of NEDD8 conjugates but not NEDD8 monomer (lane 11). As positive and negative controls, FLAG-ubiquitin and FLAG-sentrin-2 were expressed in COS cells (lanes 2 and 4) and pulled down with GST-S5a (lanes 10 and 12), respectively. GST-S5a precipitated ubiquitinated proteins (lane 10) but not sentrinnized proteins (lane 12). These results support the second or third possibility described above.

Next, we examined the effect of NUB1 overexpression on the association of NEDD8 conjugates with S5a (Fig. 4B). FLAG-NEDD8 was coexpressed in COS cells with empty vector (lane 3) or RH-tagged NUB1 (lane 4), followed by precipitation with GST-S5a-coated beads. As controls, empty vector (lanes 1 and 2), FLAG-tagged sentrin-2 (lanes 5 and 6), and ubiquitin (lanes 7 and 8) were expressed and analyzed by the same method. As shown in lanes 3 and 4, NEDD8 conjugates could be more effectively precipitated by GST-S5a when NUB1 was coexpressed. In comparison, sentrin and sentrinnized proteins could not be precipitated even when NUB1 was coexpressed (lane 6). Although ubiquitinated proteins could be precipitated, no effect of NUB1 overexpression was observed (lane 7 versus lane 8). These results support the first possibility that NUB1 is an adaptor between S5a and NEDD8 conjugates. In Fig. 3C, NEDD8 conjugates, which were less than 220 kDa in size, could be detected in the fraction of the 26 S proteasome. In Fig. 4, A and B, however, GST-S5a only precipitated NEDD8 conjugates with higher molecular weight (>220,000 kDa). NEDD8 conjugates, which were less than 220 kDa in size, might bind to endogenous S5a and could not be precipitated by GST-S5a.

**Effect of Proteasome Inhibitors on NUB1-mediated Down-regulation of NEDD8 Expression**—To examine the involvement of proteasome in NUB1 function, RH-NUB1 was coexpressed with HA-NEDD8 in COS cells in the presence of various proteasome inhibitors, including N-acetyl-L-leucinyl-L-leucinyl-L-norleucinyl (LLnL), MG132, and lactacycin (22). As a control, RH-USP21, an isopeptidase that cleaves NEDD8 conjugation, was coexpressed with HA-NEDD8. As shown in the upper panel of Fig. 5A, the overexpression of NUB1 led to a dramatic reduction of NEDD8 monomer and NEDD8-conjugated proteins (lane 2 versus lane 3). Surprisingly, all of the proteasome inhibitors prevented the reduction caused by NUB1 overexpression (lanes 4–6). In contrast, the proteasome inhibitors did not block the reduction caused by USP21 expression (Fig. 5B, upper panel). Finally, the effect of proteasome inhibitors was tested using NEDD8-G, a C-terminal deletion mutant of NEDD8 (see Fig. 1B). As shown in Fig. 5C, overexpression of NUB1 led to severe reduction of the expression of NEDD8-G monomer (lane 2 versus lane 3). This reduction was prevented by all of the proteasome inhibitors (lanes 4–6). Thus, we confirmed that NUB1 reduces the protein expression of unconjugated NEDD8 through a proteasome pathway. Taken together, our findings indicate that NUB1 interacts with S5a in 19 S proteasome activator to recruit both unconjugated and conjugated proteasome for proteasomal degradation.

Through conjugation to cullins, NEDD8 appears to regulate many biological events. For example, NEDD8 conjugation to Cul-1 plays critical roles in cell cycle transition by promoting ubiquitination of p21 (CIP1/WAF1), cyclin D, and p27 (Kipl) (11, 23). Furthermore, it controls the NF-κB signaling pathway by promoting ubiquitination of IkBa (10). Thus, various biological events seem to be regulated by NEDD8 conjugation to cullins and also other target proteins. Recently, we reported two down-regulators of the NEDD8 conjugation pathway. One is USP21, a novel isopeptidase with dual specificity for ubiquitin- and NEDD8-conjugated proteins (15). The other is an artificial mutant of UBC12 that sequesters NEDD8 and inhibits NEDD8 conjugation (24). Most recently, COP9/signalosomes has been shown to promote the cleavage of NEDD8 conjugation (25). In the studies described here, we demonstrated the down-regulation of NEDD8 expression by NUB1. Interestingly, the mechanism of action was totally different from that of other down-regulators, such as USP21, UBC12 mutant, and COP9/signalosome. Our results, thus, provide a direct link between the NEDD8 conjugation system and the proteasomal degradation pathway.

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4. Yeh, E. T. H., Gong, L., and Kamitani, T. (2000) Gene 251, 220,000 kDa). NEDD8 conjugates, which were less than 220 kDa in size, might bind to endogenous S5a and could not be precipitated by GST-S5a.

**Effect of Proteasome Inhibitors on NUB1-mediated Down-regulation of NEDD8 Expression**—To examine the involvement of proteasome in NUB1 function, RH-NUB1 was coexpressed with HA-NEDD8 in COS cells in the presence of various proteasome inhibitors, including N-acetyl-L-leucinyl-L-leucinyl-L-norleucinyl (LLnL), MG132, and lactacycin (22). As a control, RH-USP21, an isopeptidase that cleaves NEDD8 conjugation, was coexpressed with HA-NEDD8. As shown in the upper panel of Fig. 5A, the overexpression of NUB1 led to a dramatic reduction of NEDD8 monomer and NEDD8-conjugated proteins (lane 2 versus lane 3). Surprisingly, all of the proteasome inhibitors prevented the reduction caused by NUB1 overexpression (lanes 4–6). In contrast, the proteasome inhibitors did not block the reduction caused by USP21 expression (Fig. 5B, upper panel). Finally, the effect of proteasome inhibitors was tested using NEDD8-G, a C-terminal deletion mutant of NEDD8 (see Fig. 1B). As shown in Fig. 5C, overexpression of NUB1 led to severe reduction of the expression of NEDD8-G monomer (lane 2 versus lane 3). This reduction was prevented by all of the proteasome inhibitors (lanes 4–6). Thus, we confirmed that NUB1 reduces the protein expression of unconjugated NEDD8 through a proteasome pathway. Taken together, our findings indicate that NUB1 interacts with S5a in 19 S proteasome activator to recruit both unconjugated and conjugated proteasome for proteasomal degradation.
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