Regulation of the NEDD8 Conjugation System by a Splicing Variant, NUB1L*

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NUB8 is a ubiquitin-like protein that controls vital biological events through its conjugation to target proteins. We previously identified a negative regulator of the NEDD8 conjugation system, NUB1, which works by recruiting NEDD8 and its conjugates to the proteasome for degradation. Recently, we found its splicing variant, NUB1L. It possesses an insertion of 14 amino acids that codes for a UBA domain. Structural study revealed that NUB1 has a NEDD8-binding site at the C terminus, whereas NUB1L has an additional site at the newly generated UBA domain. Interestingly, the sequence AL[3]X[4]LL[5]X[6]X[7]L was conserved in these NEDD8-binding sites among human and other mammals. Mutational studies revealed that at least three Leu residues in the conserved sequence are required for binding with NEDD8. Functional study suggested that the NEDD8-binding ability at the C terminus of NUB1 and NUB1L is mainly involved in the down-regulation of NEDD8, but the NEDD8-binding ability at the UBA2 domain of NUB1L is minimally or not involved at all. The NEDD8-binding ability at the UBA2 domain might be required for an unknown function of NUB1L.

NUB8 is a highly conserved 81-amino acid protein that shares 60% identity and 80% homology with ubiquitin. NEDD8 conjugates to a large number of target proteins (1), and this conjugation is thought to be catalyzed by four enzymes, NEDD8-carboxy-terminal hydrolase (2), NEDD8-activating enzyme, NEDD8-conjugating enzyme, and NEDD8-ligating enzyme, in a manner analogous to ubiquitination and sentrinization (also known as SUMO-conjugation) (3). So far, all of the known NEDD8 targets are cullin family members, and these include Cul-1, -2, -3, -4A, -4B, and -5 (4, 5). Each cullin family member appears to be a component of the SCF complex, a ubiquitin E3 ligase composed of Skp1, Cullin, F-box protein, and ROC1 (also called RBX1 or HRT1) (3, 6). For example, Cul-1 is a major component of an SCF complex that catalyzes the ubiquitination of IκBα, β-catenin, and p27 (Kip1) (7–9) and controls many biological events, such as cell-cycle transition, inflammation, and tumorigenesis. Recently, several groups reported that NEDD8 conjugation to Cul-1 is required for the ubiquitin-ligase activity of the Cul-1-containing SCF complex (10–13). These observations suggest that the NEDD8 conjugation system is involved in many important biological functions. Indeed, the NEDD8 conjugation system was shown to be essential for cell cycle progression and morphogenesis in mice (14) and for eye development in Drosophila (15).

Recently, we identified a novel down-regulator of the NEDD8 conjugation system, NUB1 (NEDD8 ultimate buster-1), using a yeast two-hybrid system with NEDD8 as bait (16). NUB1 is a NEDD8-interacting protein composed of 601 amino acid residues with a calculated molecular mass of 69.1 kDa. It is an interferon-inducible protein and predominantly localizes in the nucleus. In a biochemical analysis, we found that NUB1 overexpression led to a severe reduction in the NEDD8 monomer and its conjugates in cells (16). Surprisingly, this reduction was completely blocked by proteasome inhibitors (17). Furthermore, we found that NUB1 interacted with the 19 S proteasome activator (PA700) (17). These results strongly suggested that NUB1 recruits NEDD8 and its conjugates to the proteasome for degradation, making NUB1 a down-regulator in the NEDD8 conjugation system.

The ubiquitin-associated (UBA) domain is a small domain of about 40 residues that was initially identified in ubiquitination enzymes, including E2s, E3s, and other proteins linked to ubiquitination (18). Human NUB1 has two UBA domains, whereas NUB1 homologues of other species such as mouse (accession number: AF534114), Drosophila (accession number: AE003752), and Arabidopsis (accession number: AC007295) have three UBA domains. This suggests that there is an isoform of human NUB1 with three UBA domains. To isolate isoforms of NUB1, we recently screened the human cDNA library by plaque hybridization and identified a splicing variant of NUB1 that encodes a longer protein, termed NUB1L (accession number: AF459743). In the studies described here, we characterized NUB1L and compared NUB1 and NUB1L.

EXPERIMENTAL PROCEDURES

Cell Culture—HeLa cells (American Type Culture Collection, Manassas, VA) and COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and antibiotics. Antibodies—Mouse anti-RH antibody (specific for the amino acid sequences RGSHHHH and GGSIGSSH) was purchased from Qiagen (Santa Clara, CA). Mouse anti-FLAG antibody (M5) was purchased from Sigma Chemical Co. (St. Louis, MO). GST-32, mouse monoclonal antibody specific for glutathione S-transferase (GST), was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Rabbit anti-human NUB1 antisera was generated by immunization with a GST fusion protein of NUB1 corresponding to amino acids 432–601 (16). Rabbit
polyclonal anti-actin antibody (specific for the C-terminal actin fragment) was purchased from Sigma.

Plasmid Construction and Transfection—To express proteins tagged with epitope at the N terminus in mammalian cells, pcDNA3/RH-N and pcDNA3/FLAG-N were used as described previously (17, 19). To express NUB1L, the cDNA insert was subcloned into pcDNA3/RH-N from pPK-CMV/NUB1L, which was isolated by cDNA library screening. The other human cDNAs used in this report were described previously. These include ubiquitin (20), NEDD8 (1), sentrin-3 (21), USP21 (22), HHR23B (23), glucocorticoid receptor (GR) (24), and NUB1 (16). These cDNAs were inserted into the aforementioned plasmid vectors, and the plasmids were transfected into COS-M6 cells or HeLa cells using FuGENE6 (Roche Applied Science, Indianapolis, IN). The transfected cells were harvested for immunostaining or Western blotting 20 h after transfection.

Screening of cDNA Library by Plaque Hybridization—A human testis cDNA library constructed in the ZAP Express vector (Stratagene, La Jolla, CA) was screened with the probe of NUB1 cDNA fragment, which was labeled with [32P]dCTP by a Ready-To-Go DNA labeling kit (Amersham Biosciences, Piscataway, NJ). Approximately 1 × 109 plaques were transferred to Hybond-N+ replica filters (Amersham Biosciences). The filters were hybridized with the probe in ExpressHyb hybridization solution (Clontech, Palo Alto, CA) and washed in 0.1× SSC containing 0.1% SDS. Autoradiography was performed at ~80 °C. Determination of Ubiquitin-like Domain and Ubiquitin-associated Domain—The BL21 (DE3) strain, which was transformed with plasmid pGEX-2T (Genentech, San Francisco, CA) and pGEX-2TK plasmid (Amersham Biosciences), respectively. Cells were resuspended in lysis buffer (25 mm Tris-HCl (pH 7.5), 100 mm NaCl, 0.1% Nonidet P-40) containing protease inhibitor mixture (Roche Applied Science) and then lysed by brief sonication. The GST fusion proteins were purified as described previously (28). The crude bacterial lysate containing the tagged proteins was centrifuged at 14,000 × g for 5 min, and the supernatant was incubated for 3 h at 4 °C with glutathione-Sepharose beads (Amersham Biosciences). The beads were then washed four times with lysis buffer. The precipitated proteins on the beads were solubilized in sample buffer containing 2% SDS and 5% β-mercaptoethanol, followed by Western blot analysis using anti-RH antibody.

RESULTS

Domain Structure of Human NUB1 and Its Homologues in Other Species—To examine NUB1 homologues in other species, we searched the protein data base of GenBank™ and found the homologues of Drosophila and Arabidopsis. These homologues had three UBA domains. In Arabidopsis, we isolated a putative full-length cDNA from the testis cDNA library of BALB/c mouse. It revealed that the cDNA sequence, BS4, had 77% amino acids. Unlike NUB1 and the other homologues we examined, BS4 had a longer C-terminal region next to the UBA repeats. In the C-terminal region, BS4 shared homology with NUB1 in other species have three UBA domains, as described above (Fig. 1). The C-terminal region, BS4 shared no homology with NUB1 possessing three UBA domains. To isolate this possible isoform of BS4, we recently screened the human testis cDNA library by plaque hybridization and obtained seven positive clones. Subsequent analysis by DNA sequencing showed that three of the seven clones had a 3113-bp insert
NUBI (Human)

NUBIL (Human)

(Drosophila)

(Arabidopsis)

Fig. 1. Protein structure of human NUB1, NUBIL, and their homologues. Domains of human NUB1, NUBIL, and their homologues in other species have been examined. The amino acid sequences were deduced from the cDNA sequences, which were obtained from the GenBank™ data base using the following accession numbers: NUB1, AF300717; NUBIL, AF459743; mouse homologue, AF534114; Drosophila homologue, AE003752; and Arabidopsis homologue, AC007295. The amino acid sequences are represented as diagrams showing the deduced domains such as the ubiquitin-like domain (UBL) and the ubiquitin-associated domain (UBA). Amino acid residues are numbered.

containing a single open reading frame from nucleotides 78 to 1883 (Fig. 2, Clone 1). The cDNA sequence of Clone 1 was identical to that of NUB1, which was previously reported by our group (accession number: AF300717) (16). Another two of the seven clones had a 2687-bp insert containing a single open reading frame from nucleotides 37 to 1842 (Fig. 2, Clone 2 (accession number: AY129295)). Although the non-coding regions at both the 5'- and 3'-ends were shorter than those of Clone 1, its coding region (nucleotides 37–1842) was identical to that of Clone 1 (nucleotides 78–1883). The remaining two of the seven clones had a 3114-bp insert containing a single open reading frame from nucleotides 37 to 1884 (Fig. 2, Clone 3 (accession number: AF459743)). The non-coding region at the 5'-end (nucleotides 1–36) was identical to that of Clone 2 (nucleotides 1–36), whereas the non-coding region at the 3'-end (nucleotides 1885–3114) was identical to that of Clone 1 (nucleotides 1884–3113). The coding region of Clone 3 was 42 bp longer than that of Clone 1 and Clone 2 because of a single insertion from nucleotides 1390 to 1431. As a result, Clone 3 coded for a longer form of NUB1 that had an insertion of 14 amino acids. We termed this isoform NUBIL.

Alternative Splicing of NUB1 Gene and the Domain Structure of Its Product.—To determine how the cDNA of NUBIL was generated, we searched the genomic data base of GenBank™ and found the genomic sequence of human NUB1 in chromosome 7q36 (accession number: AC005486). Sequence analysis revealed that the NUB1 gene was composed of 15 exons and that the 42-bp insertion in the NUBIL cDNA was generated by alternative splicing of an extended exon 12 of the NUB1 gene. As described above, the 42-bp insertion of the cDNA resulted in a 14-amino acid insertion between residues 451 and 452 in the protein sequence of NUB1 (Fig. 1, see also Fig. 5C). This short insert in NUBIL led to the generation of an additional UBA domain (termed UBA2) between the two original UBA domains (termed UBA1 and UBA3). Thus, we found a novel isoform, NUBIL, containing three UBA domains as initially expected (see Figs. 1 and 5C).

Subcellular Localization of NUBIL.—The subcellular localization of NUBIL was determined in HeLa cells transfected with a plasmid containing an insert of RH-NUBIL cDNA. As controls, we transfected HeLa cells with either an expression plasmid alone or a plasmid containing an insert of RH-USP21 or RH-NUB1 cDNA. The cells were then fixed, permeabilized, and stained with anti-RH antibody. As shown in Fig. 3, RH-USP21 was detected equally in both the cytosol and the nucleus, whereas RH-NUB1 and RH-NUBIL were mainly found in the nucleus. The nuclear localization of NUB1 and NUBIL is consonant with the fact that both molecules have an NLS located from Arg-414 to Arg-431 (16). However, NUB1 and NUBIL were also detected weakly in the cytosol.

Tissue Distribution of NUB1 and NUBIL.—To identify the expression of NUB1 and NUBIL, we employed RT-PCR. As shown in Fig. 4B, the NUB1 message was detected almost equally in all examined tissues other than the pancreas (lane 13). In contrast, the NUBIL message could not be detected equally. The NUB1 message in the prostate (lane 3), leukocyte (lane 7), liver (lane 10), and skeletal muscle (lane 11) was detected much less than that in the other tissues.

Activity of NUBIL in the Down-regulation of NEDD8 Expression.—The COS cell coexpression assay was used to examine the functions of NUB1 and NUBIL. In brief, FLAG-tagged NEDD8 was coexpressed in COS cells with empty vector, RH-tagged glucocorticoid receptor (GR), NUB1, or NUBIL. As shown in Fig. 5, when FLAG-NEDD8 was coexpressed with empty vector (lane 2) or RH-tagged GR (lane 3), we clearly detected a 7.0-kDa band of unconjugated FLAG-NEDD8 and high molecular mass bands of NEDD8-conjugated proteins. This NEDD8 expression pattern was identical to the one we observed previously (1). When FLAG-NEDD8 was coexpressed with RH-NUB1 (lane 4) or RH-NUBIL (lane 5), both the unconjugated and conjugated forms of NEDD8 were dramatically reduced. This result suggested that NUB1 and NUBIL possess the ability to down-regulate the expression of NEDD8 monomer and its conjugates. As controls, we also coexpressed FLAG-tagged ubiquitin or sentrin-3 with empty vector, RH-GR, RH-NUB1, or RH-NUBIL. As shown in Fig. 5 (B and C), the overexpression of NUB1 and NUBIL did not reduce the expression of ubiquitin or sentrin-3 (lanes 4 and 5). Thus, the down-regulation induced by NUB1 and NUBIL was specific to NEDD8.

We previously reported that the reduction of NEDD8 expres-
NUB1L led to a dramatic reduction of NEDD8 expression (lane 2 versus lane 3). As expected, all of the proteasome inhibitors prevented the reduction in NEDD8 conjugates caused by NUB1L overexpression (lanes 4–6). However, they did not restore the levels of unconjugated NEDD8. This is probably because the unconjugated NEDD8 was quickly consumed for the conjugation of its targets during the treatment with proteasome inhibitors.

Increased Interaction of NUB1L with NEDD8—In our initial study, we isolated the cDNA of the C-terminal fragment (Glu-372 to Asn-601) of NUB1 by a yeast two-hybrid screening using NEDD8 as bait (16). This indicated that the NEDD8-binding site is located on this C-terminal region of NUB1. We therefore hypothesized that the inserted sequence in the C-terminal region of NUB1L may exert an influence on its interaction with NEDD8. To test this hypothesis, we examined the interaction of NEDD8 with the C-terminal region of NUB1 (termed NUB1c) or NUB1L (termed NUB1Lc) by a liquid culture assay of the yeast two-hybrid system in which β-galactosidase activity was measured to quantify the interaction. Before the assay, we prepared cDNAs of NUB1c and NUB1Lc. NUB1c is the C-terminal fragment (Glu-372 to Asn-601) of NUB1, and NUB1Lc is the C-terminal fragment (Glu-372 to Asn-615) of NUB1L. These cDNAs were inserted into a plasmid, pGBK7 (Gal4 DNA-binding domain vector for Gal4-BD fusion). We also used a plasmid, pGADT7 (Gal4 DNA-activating domain vector for Gal4-AD fusion), in which a cDNA of NEDD8-G had been inserted (pGADT7/NEDD8-G). Because NEDD8-G has a C-terminal deletion from Gly-76 to Gln-81 and is unable to form conjugates with target proteins, its use allowed us to examine the interaction of NUB1c and NUB1Lc with NEDD8 itself, not with the target proteins of NEDD8 (1, 17). For the yeast two-hybrid assay, yeast cells were cotransformed with pGADT7/-NEDD8-G and pGBK7 plasmid described above. As shown in the lower panel of Fig. 7A, yeast cells transformed with pGADT7/NEDD8-G alone (negative control) produced low levels of β-galactosidase. Yeast cells transformed with pGADT7/-NEDD8-G and pGBK7/HHR23B (negative control) also produced low levels of β-galactosidase due to the lack of interaction between NEDD8 and HHR23B. In contrast, yeast cells transformed with pGADT7/NEDD8-G and pGBK7/-NUB1c displayed high levels of β-galactosidase because of the interaction between NEDD8 and NUB1c. Yeast cells transformed with pGADT7/-NEDD8-G and pGBK7/-NUB1Lc displayed even higher levels of β-galactosidase indicating a stronger interaction between NEDD8 and NUB1Lc. Importantly, NUB1Lc produced levels of β-galactosidase almost double those produced by NUB1c. Although the result of the β-galactosidase assay was clear, it was possible that the observed difference was due to the differing expression levels between NUB1c and NUB1Lc. To rule out this possibility, we examined the expression levels of NUB1c and NUB1Lc in yeast cells by Western blotting using rabbit anti-NUB1 antibody. As expected, the expression levels of these two proteins were the same (Fig. 7A, upper panel), suggesting that the binding ability of the C-terminal NUB1L (Glu-372 to Asn-615) was almost twice that of the C-terminal NUB1 (Glu-372 to Asn-601) in yeast cells. To confirm this finding, a GST pull-down assay was also performed. Poly-His-tagged full-length NUB1 and NUB1L were expressed in E. coli and precipitated with GST-NEDD8 immobilized on beads. As shown in Fig. 7B, GST-NEDD8 could precipitate NUB1L more effectively than NUB1 (lane 3 versus lane 6), supporting the result of liquid β-galactosidase assay.

Identification of NEDD8-binding Sites on NUB1 and NUB1L—We formulated two hypotheses to explain the increased ability of NUB1L to bind to NEDD8. The first was that...
Neddylation Regulated by NUB1L

**Specific reduction of NEDD8 by NUB1L overexpression.**

A, effect of NUB1L on NEDD8 expression. In COS cells, FLAG-tagged NEDD8 was coexpressed with empty vector (lane 2), RH-tagged GR (glucocorticoid receptor) (lane 3), NUB1 (lane 4), or NUB1L (lane 5). Total cell lysates were prepared from transfectants and analyzed by Western blotting using anti-FLAG antibody to detect the coexpressed proteins (middle panel). To demonstrate an equal loading amount of total cell lysates, Western blotting using anti-actin antibody was also performed (lower panel). B, effect of NUB1L on ubiquitin expression. In COS cells, FLAG-ubiquitin was coexpressed with empty vector (lane 2), RH-tagged GR (lane 3), NUB1 (lane 4), or NUB1L (lane 5). Total cell lysates were prepared from transfectants and analyzed by Western blotting using anti-RH antibody to detect the coexpressed proteins (middle panel). An open arrowhead indicates an unconjugated form of FLAG-ubiquitin, ubiquitin, or sentrin-3. Molecular size markers are shown in kilodaltons.

B, effect of NUB1L on sentrin-3 expression. In COS cells, FLAG-sentrin-3 was coexpressed with empty vector (lane 2), RH-tagged GR (lane 3), NUB1 (lane 4), or NUB1L (lane 5). Total cell lysates were prepared from transfectants and analyzed by Western blotting using anti-FLAG antibody (upper panel) or RH epitope (lower panel). An open arrowhead indicates an unconjugated form of FLAG-NEDD8, ubiquitin, or sentrin-3. Molecular size markers are shown in kilodaltons.

C, effect of NUB1L on sentrin-3 expression. In COS cells, FLAG-sentrin-3 (Sen3) was coexpressed with empty vector (lane 2), RH-tagged GR (lane 3), NUB1 (lane 4), or NUB1L (lane 5). Total cell lysates were prepared from transfectants and analyzed by Western blotting using anti-FLAG epitope (upper panel) or RH epitope (lower panel). An open arrowhead indicates an unconjugated form of FLAG-NEDD8, ubiquitin, or sentrin-3. Molecular size markers are shown in kilodaltons.

In summary, NUB1 possesses a NEDD8-binding site at the C terminus, but NUB1L has an additional site for binding with
NUB1L (FLAG-NEDD8) was coexpressed in COS cells with empty vector (lane 2) or RH-NUB1L (lanes 3–6). The COS cells were then cultured for 6 h in the absence of proteasome inhibitors (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-FLAG antibody to detect unconjugated and conjugated forms of FLAG-NEDD8 (upper panel) and anti-RH antibody to detect RH-NUB1L (lower panel). Molecular size markers are shown in kilodaltons.

NEDD8. As expected, the additional site in NUB1L was located at the UBA2 domain that includes the inserted sequence (Fig. 8C). Thus, we concluded that the greater ability of NUB1L to bind to NEDD8 originates with the additional binding site.

**NEDD8-binding Motif in NUB1 and NUB1L**—To predict amino acid residues that are responsible for NEDD8 binding, we aligned amino acid sequences of NEDD8-binding sites at the UBA2 domain and the C-terminal region obtained from human NUB1, human NUB1L, mouse NUB1 homologue, and cow NUB1 homologue (29). As shown in Fig. 9A, this alignment revealed that four amino acid residues, one Ala and three Leu, were conserved and found in the sequence A(Leu)(Leu)(Leu)(X) (Fig. 9B). Thus, we concluded that the greater ability of NUB1L to bind to NEDD8-G was strongly, but not completely, inhibited by the additional site in NUB1L that had either an Ala-to-Val substitution at Ala-557 or a Leu-to-Ala substitution at Leu-453, Leu-468, or Leu-469 (see Figs. 8A, 8C, and 9A). We also generated mutants of the C-terminal NUB1 (that had either an Ala-to-Val substitution at Ala-557 or a Leu-to-Ala substitution at Leu-453, Leu-468, or Leu-469) and the C-terminal NUB1L that had an Ala-to-Val substitution at Ala-557 or a Leu-to-Ala substitution at Leu-453, Leu-468, or Leu-469 (see Figs. 8A, 8C, and 9A). The yeast two-hybrid assay revealed that the interaction of M13 and LM2 mutants of the C-terminal NUB1L that had an Ala-to-Val substitution at Ala-557 or a Leu-to-Ala substitution at Leu-453, Leu-468, or Leu-469 (see Figs. 8A, 8C, and 9A). The yeast two-hybrid assay revealed that the interaction of M13 and LM2 with NEDD8-G was strongly, but not completely, inhibited by all of these Leu-to-Ala substitutions (data not shown). In contrast, Ala-to-Val substitutions did not affect the interaction at all (data not shown). These results suggest that at least three Leu residues in the sequence A[X] Leu[X] Leu[X] Leu are important for the interaction with NEDD8.

**NEDD8-binding Ability of Full-length NUB1 and NUB1L with Leu-to-Ala Substitutions**—To confirm the effect of these Leu-to-Ala substitutions on the interaction of NUB1 with NEDD8, we next performed a GST pull-down assay. In this assay, RH-tagged full-length NUB1 was expressed in bacteria and precipitated with GST or GST-NEDD8 immobilized on beads. The precipitates were then analyzed by Western blotting using anti-RH antibody to detect RH-NUB1. As shown in the yeast two-hybrid assay, we detected a weak interaction between NEDD8 and full-length NUB1 molecules with a single substitution of Leu-to-Ala (data not shown). To completely abolish the ability of NUB1 to bind to NEDD8, we substituted both Leu-573 and Leu-577 with Ala at the C-terminal region in full-length NUB1 (see Figs. 8C and 9A). As shown in the upper panel of Fig. 9B, the wild-type NUB1 could be precipitated with GST-NEDD8 (lane 3), but the mutant NUB1 with both L573A and L577A could not (lane 6), indicating that the mutant could not interact with NEDD8.
Next, NUB1L with Leu-to-Ala substitutions were examined. In this assay, RH-tagged full-length NUB1L was expressed in bacteria and precipitated with GST or GST-NEDD8 immobilized on beads. The precipitates were analyzed by Western blotting using anti-RH antibody to detect RH-NUB1L. Because NUB1L possesses two NEDD8-binding sites, we generated three mutants of full-length NUB1L. The first mutant had two substitutions at the UBA2 domain (L464A and L468A). The second mutant had two substitutions at the C-terminal region (L587A and L591A). The third mutant had four substitutions, two at the C-terminal region (L587A and L591A) and two at the UBA2 domain (L464A and L468A) (see Figs. 8C and 9A). Using the wild type and these mutants of the full-length NUB1L, a GST pull-down assay was performed. As shown in the upper panel of Fig. 9C, GST-NEDD8 precipitated the wild type (lane 3), the first mutant with two substitutions at the UBA2 domain (lane 6), and the second mutant with C-terminal substitutions (lane 9), but it did not precipitate the third mutant with four substitutions at both the UBA2 domain and the C-terminal region (lane 12). This result indicated that the first and second mutants could still interact with NEDD8 through the intact C-terminal region or the intact UBA2, but this ability was totally abolished in the third mutant as a result of the four substitutions.

**NUD8-buster Function of NUB1 and NUB1L Lacking NEDD8-binding Ability**—In the previous section, we demonstrated that the interaction of NUB1 and NUB1L with NEDD8 was abolished by Leu-to-Ala substitutions in their C-terminal region and UBA2 domain in vitro. In this section, we examined whether these substitutions affected the function of NUB1 and NUB1L in vivo. First, we focused on the C-terminal substitutions of L573A and L577A in NUB1. An RH-epitope was tagged to wild-type NUB1 and its mutant with substitutions of L573A and L577A. These RH-tagged proteins and empty vector (control) were coexpressed with FLAG-tagged NEDD8 in COS cells. Total cell lysate was then prepared from the COS transfectants and analyzed by Western blotting using anti-RH antibody to detect the expression of FLAG-NEDD8. As shown in Fig. 10A, the overexpression of RH-tagged wild-type NUB1 caused a dramatic reduction in the FLAG-NEDD8 monomer and its conjugates (lane 2 versus lane 3). In contrast, the overexpression of the RH-tagged NUB1 mutant did not cause any reduction in the FLAG-NEDD8 monomer and its conjugates (lane 2 versus lane 4), suggesting that the C-terminal interaction of NUB1 with NEDD8 was required for its NEDD8-buster function to occur.

In a second experiment, we examined how the function of NUB1L was affected by substitutions in the UBA2 domain (L464A and L468A) and/or substitutions in the C terminus (L587A and L591A). We first generated three mutants of NUB1L. To abolish the interaction of its UBA2 domain with NEDD8, we generated a mutant with substitutions of L464A and L468A. To abolish the interaction of its C-terminal region with NEDD8, we generated a mutant with substitutions of L587A and L591A. To abolish the interaction of both sites with NEDD8, we generated a mutant with substitutions of L587A and L591A (see Figs. 8C and 9A). Using the wild-type NUB1L and these NUB1L mutants, their function was then examined. The proteins were tagged with RH-epitope to allow detection. These RH-tagged proteins and empty vector (control) were then coexpressed with FLAG-tagged NEDD8 in COS cells. Total cell lysate was prepared from the COS trans-
fectants and analyzed by Western blotting using anti-FLAG antibody to detect the expression of FLAG-NEDD8. As shown in the upper panel of Fig. 10B, the mutant NUB1L with two substitutions (L464A and L468A) in the UBA2 domain caused a severe reduction in the FLAG-NEDD8 monomer and its conjugates (lane 2 versus lane 4) as well as the wild-type NUB1L.
Leu residues in A(4) and assay using substitution mutants of NUB1 and NUB1L, three on the results of yeast two-hybrid assay and GST pull-down action with NEDD8. The NEDD8-binding ability at the UBA2 domain and the C-terminal region (lane 2 versus lane 6). These results suggested that the C terminus is mainly involved in NEDD8-buster function through its interaction with NEDD8.

**DISCUSSION**

We previously reported our findings of a novel NEDD8-binding protein, NUB1, that recruits NEDD8 monomer and its conjugates to the proteasome for degradation (16, 17). Recently, we identified a splicing variant of human NUB1, termed NUB1L. This isoform had a short insertion of 14 amino acids coded by an alternatively spliced mRNA of exon 12. Interestingly, this insertion generated an additional UBA domain between the two original UBA domains in NUB1L. Thus, NUB1 has two UBA domains, whereas NUB1L has three UBA domains, which are conserved in other species. So far, no NUB1 homologue possessing two UBA domains has been reported in other species.

Structural study revealed that NUB1 has a NEDD8-binding site at the C terminus, whereas NUB1L has an additional NEDD8-binding site at the newly generated UBA domain, UBA2. Comparing the amino acid sequences of the C terminus and the UBA domain in human, mouse, and cow, a NEDD8-binding motif could be formulated. It appears that the NEDD8-binding site contains the sequence, A(L4)L(X15)LL(L)X3L. Based on the results of yeast two-hybrid assay and GST pull-down assay using substitution mutants of NUB1 and NUB1L, three Leu residues in A(L4)L(X15)LL(L)X3L are important for the NEDD8 binding. The requirement of an Ala residue in this motif is unclear. Although an Ala to Val substitution had no effect on the NEDD8 binding, it might result from the high similarity between Ala and Val residues. Taken together, at least three Leu residues are required for the NEDD8 binding in NUB1/NUB1L.

Functional study established that NUB1L has the activity to down-regulate NEDD8 expression as well as NUB1. Repeated experiments showed that the down-regulation of NEDD8 by NUB1L is slightly greater than that by NUB1. The mutational study using a wild-type NUB1L and its three mutants revealed that the wild-type NUB1L and the first mutant, which lacked the ability to interact with NEDD8 at the UBA2 domain, showed a high NEDD8-buster activity. The second mutant, which lacked the ability to interact with NEDD8 at the C terminus, and the third mutant, which lacked the ability to interact with NEDD8 at both sites, showed almost no activity. These results suggested that the down-regulation of NEDD8 is mainly contributed by the NEDD8-binding site at the C terminus in NUB1L. Although the UBA2 domain also has an affinity to NEDD8, it minimally or does not contribute to the down-regulation of NEDD8. The NEDD8-binding ability at the UBA2 domain might be required for an unknown function of NUB1L.

We previously detected two major messages of 3.1–3.5 kb and 2.3–2.7 kb in the Northern blot analysis for NUB1 isoforms (16). Although we did not identify these mRNAs, the longer message might be the mRNAs of Clone 1 and Clone 3 that encode NUB1 and NUB1L, respectively, and the shorter message might be the mRNA of Clone 2 that encodes NUB1 (Fig. 2). In this study, we demonstrated that the tissue distribution of NUB1/NUB1L message is different from that of NUB1 message. The alternative splicing might control the balance between NUB1 and NUB1L and hence regulate the NEDD8 conjugation system in each tissue.