NUB1, a NEDD8-interacting Protein, Is Induced by Interferon and Down-regulates the NEDD8 Expression*

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NUB1, a ubiquitin-like protein, covalently conjugates to cullin family members. It appears to control vital biological events through its conjugation to cullins. To study how this conjugation pathway is regulated, we performed yeast two-hybrid screening by using NEDD8 as a bait and isolated a cDNA fragment encoding a potent down-regulator of the NEDD8 expression. Here, we report this novel regulator, NUB1 (NEDD8 Ultimate Buster-1). NUB1 is composed of 601 residues with a calculated 69.1-kDa molecular mass. It is an interferon-inducible protein and predominantly localized in the nucleus. The NUB1 message is specifically expressed in adult human testis, ovary, heart, and skeletal muscle tissues and is developmentally down-regulated in mouse embryos. In biochemical analysis, we found that NUB1 overexpression leads to severe reduction of NEDD8 monomer and NEDD8 conjugates in cells. This reduction is not due to down-regulation of NEDD8 transcription, but due to post-transcriptional mechanism. As expected from this activity, overexpression of NUB1 had a profound growth-inhibitory effect on U2OS cells. Thus, NUB1 is a strong down-regulator of the NEDD8 expression and appears to play critical roles in regulating biological events, including cell growth.

NUEDD8 is a highly conserved 81-amino acid protein that shares 60% identity and 80% homology with ubiquitin. Expression of the NEDD8 message is highly restricted to the heart and skeletal muscle in adult human tissues (1) and is developmentally down-regulated in mouse embryos (1, 2). NEDD8 and its yeast homologue, Rub1 (3, 4), belong to an expanding family of ubiquitin-like proteins that includes UCRP (5), sentrin-1/sentrin-2 (8), and sentrin-3 (9). These proteins share a common distinction; the mature form is always translated in precursor form, with one or more amino acids following a Gly-Gly dipeptide that forms the C terminus of the mature protein (10). In the NEDD8-conjugation process, the C-terminal tail of the precursor protein is cleaved off by a C-terminal hydrolase, such as UCH-L3 (11). The mature form has been shown to conjugate to a large number of nuclear proteins (1).

The pathway of 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 sentrinization (10, 12, 13).

All known NEDD8 targets in mammalian cells are cullin (Cul) family members, including Cul-1, -2, -3, -4A, -4B, and -5 (14, 15). Human Cul-1 is a major component of ubiquitin ligase, known as an SCF complex that catalyzes the ubiquitination of IκBα, β-catenin, and p27 (Kip1) (16–18). Interestingly, the NEDD8 conjugation to Cul-1 is required for the ubiquitin ligase activity of the SCF complex containing Cul-1 (19, 20). Thus, NEDD8 conjugation seems to be involved in many important biological functions, including NFκB signaling and cell-cycle regulation by p27, and must be strictly regulated. However, the regulation system of NEDD8-conjugation is still unclear, with the exception of the recent discovery of USP21, a novel isopeptidase for NEDD8-conjugated proteins (21).

To define the unknown regulators of NEDD8 conjugation, the yeast two-hybrid system was applied in this study. From library screening, we isolated a cDNA clone encoding a novel NEDD8-interacting protein, NUB1. Here, we report NUB1 as a strong down-regulator of the NEDD8 expression.

EXPERIMENTAL PROCEDURES

Cell Lines, Culture Conditions, and Interferon Treatment—We purchased the following human cell lines from American Type Culture Collection (Manassas, VA): rectal adenocarcinoma SW837, neuroblastoma SK-N-SH, malignant melanoma SK-MEL28, myeloid leukemia U937, Burkitt lymphoma Raji, T-cell leukemia Jurkat, chronic myelogenous leukemia K562, promyelocytic leukemia HL60, human embryonic kidney 293, osteosarcoma U2OS, renal cell carcinoma 786–0, and adrenal carcinocoma HeLa. COS-M6 cells were a generous gift from Dr. Steve Goldring of Harvard Medical School. SW837, SK-N-SH, SK-MEL28, human embryonic kidney 293, U2OS, 786–0, HeLa, and COS-M6 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and antibiotics. U937, Raji, Jurkat, K562, and HL60 cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics. Human interferon-β (IFN-β) was purchased from Calbiochem (La Jolla, CA). Antibodies—Mouse monoclonal antibody 16B12 (Covance; Richmond, CA) is an antibody to the peptide sequence YPYDVPDYA of inflammation hemagglutinin (HA). Rabbit anti-human NUB1 antisemper was generated by immunization with a GST fusion protein of NUB1 corresponding to amino acids 432–601 (UB1432-601). 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, plasmid vectors pcDNA3/HA-N (7) and pcDNA3/HR-N (8) were used as described pre-

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1 The abbreviations used are: Cul, human cullin; IFN, interferon; HA, hemagglutinin epitope; RH, RGS-poly(His); HRP, horseradish peroxidase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; UCH, ubiquitin C-terminal hydrolases; UBA, ubiquitin-associated domain; NLS, nuclear localization signal; SCF, Skp1-Cullin-F-box; kb, kilobases(s); bp, base pair(s); PBS, phosphate-buffered saline; GST, glutathione S-transferase; E1, activating enzyme; E2, conjugating enzyme; E3, ligating enzyme.
vously (1). The human cDNAs used in this study have been described previously: ubiquitin (7), NEDD8 (1), sentrin-1 (7), and Ubc12/C111S (22). These cDNAs were inserted into the aforementioned plasmid vectors. The sequence of each cDNA was confirmed by automated DNA sequencing. Plasmids were transfected into mammalian cells using FuGENE 6 (Roche Molecular Biochemicals). The transfected cells were processed for immunostaining, Western blotting, or Northern blotting 20 h after transfection.

**Yeast Two-hybrid Screening**—Yeast strain L40 was purchased from Invitrogen (Carlsbad, CA). Prey vector pGAD10 was purchased from CLONTECH (Palo Alto, CA). The bait plasmid pHybLex/HA- NEDD8-GG (11) was transformed into L40 using the lithium acetate method (6). The transformants were plated on YPD medium containing adenosine and Zeocin (YPAD/Zeoc) and selected for 2 days at 30 °C. The L40 clone carrying pHybLex/HA-NEDD8-GG was cultured in YPAD/Zeo medium and sequentially transformed with 500 μg of human heart cDNA (CLONTECH) fused to GAL4 DNA-activating domain vector, pGAD10. The transformed cells were incubated for 6 days at 30 °C on selection plates (Ura-, Lys-, His-, Leu-, and Zeocin-) and assayed for β-galactosidase activity on filter papers as described in the protocol of CLONTECH.

**Domain Search by Research Tools in Web Sites**—Domain search of NUB1, rabbit antiserum against GST-NUB1150–20604 (22, 24), was preabsorbed with either GST or GST-NUB1432–601 and used for Western blot analysis as a primary antibody. For this preabsorption, 1 ml of diluted anti-NUB1 antiserum (1:1000) was incubated overnight with GST or GST-NUB1432–601 fusion protein-coated beads. After the incubation, the beads were removed by centrifugation. The supernatant was filtered, diluted to 1:10,000 with 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 0.1% beads were removed by centrifugation. The supernatant was filtered, by polymerase chain reaction and purified by a Qiagen II gel extraction kit (Qiagen Inc.). These fragments were labeled with alkaline phosphatase by cross-linker using the AlkPhos Dystem kit (Amersham Pharmacia Biotech). After prehybridization for 1 h and hybridization with alkaline phosphatase-labeled probe overnight at 55 °C, the blots were washed five times. The signal was detected by the chemiluminescent method using CDP-Star (Amersham Pharmacia Biotech).

The radioactive probe was hybridized to the membrane in ExpressHyb hybridization solution (CLONTECH) and washed in 0.1× SSC and 0.1% SDS. Autoradiography was performed at 80 °C. The membrane was repeatedly hybridized with other probes after removing the previous probe.

**RESULTS**

**Yeast Two-hybrid Screening**—To identify NEDD8-interacting proteins, we screened ~1 × 10^9 primary library transformants as reported previously (11). A total of 450 colonies grew on the selection plates, 18 of which stained positive when tested for β-galactosidase expression. Subsequent analysis of DNA sequencing indicated that 7 of the 18 clones encoded human UCH-L3 (11). In addition, 1 of the 18 clones turned out to be a human homologue of mouse BS4 (GenBank accession no. U27462.1).

**Structure of NUB1, a Human Homologue of Mouse BS4**—Using yeast two-hybrid screening, we isolated 3,113 bp of cDNA from a human heart cDNA library (Fig. 1A). This cDNA encodes a predicted 69.1-kDa protein of 601 amino acids. Multiple termination codons were found in the other two reading frames. The ATG initiation codon was contained within a nearly perfect Kozak consensus sequence, which is necessary for efficient translation (25). A presumptive polyadenylation signal was found 28 bp upstream from the 3′ end of the cDNA. A BLAST search of the entire data base through the National Center for Biotechnology Information (Bethesda, MD) showed that this predicted protein was 76.9% identical to mouse BS4 in the amino acid sequence, indicating that this protein is a human homologue of mouse BS4. Since this protein negatively regulates the NEDD8 expression as described below, we designated this protein as NUB1 (NEDD8 Ultimate Buster-1), and its cDNA sequence was submitted to GenBank (accession no. AF300717). Although the cDNA sequence of mouse BS4 was registered in GenBank, we could not find any publication on its biological function in the Medline data base. Thus, the function of proteins NUB1 and BS4 was totally unknown.

To predict the protein function, we searched consensus sequences or domains in NUB1 using several research tools in Web sites. As shown in Fig. 1B, there were two coiled coil regions at the N-terminal side of NUB1. The first coiled coil region was located from Leu-36 to Ala-67. The second coiled coil region was located from Val-155 to Thr-203. At the C-terminal side of NUB1, there were two UBDs, a bipartite NLS and a...
PEST sequence. The first UBA domain was located from Asp-376 to Asn-413. The second UBA domain was located from Ser-477 to His-514. The UBA domain has been reported to occur in subsets of the ubiquitin-conjugation enzymes (E2), ubiquitin ligases (E3), and deubiquitinating enzymes (26). Between these two UBA domains, a bipartite NLS was located from Arg-414 to Arg-431. Finally, a PEST sequence (27) was located from His-514 to His-568. Based on the domain search data, we predicted that NUB1 was a nuclear protein and its turnover was rapid because of the PEST motif.

Detection of Endogenous NUB1 Expression in Various Cell Lines—To characterize protein expression of NUB1 in human cell lines, rabbit polyclonal antiserum specific for NUB1 was generated. The expression of NUB1 was surveyed in 12 different cell lines. The expression data are shown in Fig. 1. The results indicate that NUB1 is expressed at high levels in all cell lines tested.

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Fig. 1. Structure of NUB1 molecule. A, nucleotide and predicted protein sequence of NUB1. The presumptive polyadenylation signal AATAAA is underlined. The initiation codon fits within the Kozak consensus sequence GCC(A/G)CCATG, in 7 of 10 bases (indicated by dots). The termination codon TAA is indicated by an asterisk. The nucleotide sequence was determined on both strands by automated sequencing. The nucleotide sequence has been submitted to the GenBank data base and assigned accession no. AF300717. The predicted amino acid sequence is given below the nucleotide sequence in single-letter code.

B, schematic representation of deduced domains. CC, the coiled coil domain; UBA, the ubiquitin-associated domain; NLS, the nuclear localization signal; PEST, the PEST sequence.
ent human cell lines and COS cells by Western blotting. The antiserum was preabsorbed with either GST (Fig. 2, upper panel) or GST-NUB1 (data not shown) to demonstrate specificity of the immunoreactivity to NUB1. As shown in the upper panel of Fig. 2, a 69-kDa band specific for NUB1 was strongly observed in SK-N-SH, Raji, K562, and 786–0 cells (arrowhead). A moderate signal was detected in SW837, 293, HeLa, and COS cells. In SK-MEL28, Jurkat, and HL60 cells, the band was very weak. Although the 69-kDa band was undetectable in U937 and U2OS cells, a longer exposure allowed us to detect the faint band. In contrast to this blotting, the antiserum preabsorbed with GST-NUB1 could not detect anything (data not shown). Thus, our antiserum specifically detected endogenous NUB1, and its expression level varied widely among cell lines.

**IFN-induced Expression of NUB1 Protein**—The sequence information on BS4, a mouse homologue of human NUB1, was deposited to GenBank on June 21, 1995, and assigned to accession number U274621. Although the title of the data base entry was “BS4: an interferon-inducible gene with novel regulatory properties,” BS4 has not been published in the literature so far. To confirm whether NUB1 is an INF-inducible protein, HeLa, 293, and U2OS cells were cultured with human IFN-β and the induction of NUB1 was examined by Western blotting. As shown in the upper panel of Fig. 3A, treatment with 250 units/ml IFN-β for 16 h induced expression of the NUB1 protein in HeLa cells (~10-fold) (lane 1 versus lane 2) and 293 cells (~5-fold) (lane 3 versus lane 4) but not in U2OS cells (lane 5 versus lane 6). Thus, we found that NUB1 is an IFN-inducible protein and that HeLa cells are very sensitive to this induction.

Next, we examined the dose dependence of NUB1 protein induction by using HeLa cells treated for 16 h with various concentrations of IFN-β. As shown in the upper panel of Fig. 3B, NUB1 protein was moderately expressed in the untreated cells (lane 1), and it could be induced with IFN-β in a dose-dependent manner (lanes 2–6). Furthermore, the time course of NUB1 induction was examined by using HeLa cells treated with 250 units/ml IFN-β for various times. As shown in the upper panel of Fig. 3C, the expression level of NUB1 protein was not changed by 2 h after treatment (lanes 1–3), whereas it increased with time from 4 h to 16 h after treatment (lanes 4–6). Taken together, these data indicated that the NUB1 protein can be induced by IFN-β in a dose- and time-dependent manner. In addition to IFN-β, IFN-γ was also examined for its effects on induction of NUB1. In HeLa cells, IFN-γ could induce NUB1 protein as well as IFN-β (data not shown).

**Tissue-specific Expression and Developmental Down-regulation of NUB1 Message**—To determine the expression of NUB1 message in human tissues, Northern blot analyses were performed using 32P-labeled human NUB1 cDNA as probe. As shown in Fig. 4A (upper panel), 3.5 kb of NUB1 message was weakly detected in testis, ovary, heart, and skeletal muscle. In all other tissues, the message signal was much weaker or undetectable. In addition, a 2.3-kb smaller band was strongly detected in the testis but not in the other tissues. As shown in the middle panel, NEDD8 message was detected on the iden-
tical blot. Interestingly, NEDD8 message was enriched in the ovary, heart, and skeletal muscle. This tissue specificity is similar to that of NUB1. Since NEDD8, which interacts with NUB1, was originally isolated as a developmentally down-regulated message in the mouse brain, we also examined the expression of NUB1 message in developing mouse embryos. As shown in the upper panel of Fig. 4B, 3.0-, 2.5-, and 2.0-kb NUB1 messages were detected. The 3.0-kb message was strongest, the 2.5-kb message was moderate, and the 2.0-kb message was weakest. These NUB1 messages were strongest in the day 7 mouse embryo and were markedly decreased in the day 11, day 15, and day 17 embryos (upper panel). In contrast, the strength of the NEDD8 message peaked in the day 11 mouse embryo and was markedly decreased in the day 15 and day 17 embryos (middle panel). Thus, messages of NUB1 and NEDD8 were developmentally down-regulated differently.

IFN-induced Expression of NUB1 Message—We defined whether IFN regulates the transcription of NUB1. HeLa cells were treated for 16 h with various concentrations of IFN-β, and the level of NUB1 message was analyzed by Northern blotting (Fig. 4C, upper panel). The NUB1 message could not be detected in IFN-β-untreated cells (lane 1), whereas it was induced by 0.4 units/ml IFN-β (lane 2) and increased in a dose-dependent manner (lanes 2–6). Thus, IFN-β up-regulates the transcription of NUB1.

Subcellular Localization of NUB1—The subcellular localization of NUB1 was determined. HeLa cells were treated with 16 h with various concentrations of IFN-β, and the level of NUB1 message was analyzed by Northern blotting using a fragment of NUB1 cDNA as a probe (upper panel). The blot was subsequently reprobed by GAPDH as a control (lower panel). The positions of 28S and 18S RNAs are indicated on the left-hand margin.

**FIG. 4. Northern blot analysis of NUB1.** A, expression of NUB1 mRNA in a variety of human tissues. B, expression of NUB1 mRNA in mouse embryos of different developmental stages. Samples of poly(A)^+ RNA (2 μg) from indicated sources were run on a denaturing gel, transferred to a nylon membrane, and hybridized with a 32P-labeled cDNA fragment of human NUB1 (upper panel), NEDD8 (middle panel), or β-actin (lower panel). Open arrows indicate isoforms of NUB1 message. RNA size markers are shown in kilobases (kb). C, IFN-mediated induction of NUB1 mRNA. HeLa cells were treated for 16 h with human IFN-β at concentrations of 0 (lane 1), 0.4 (lane 2), 2 (lane 3), 10 (lane 4), 50 (lane 5), or 250 units/ml (lane 6). Total RNA samples were extracted and analyzed by Northern blotting using a fragment of NUB1 cDNA as a probe (upper panel). The blot was subsequently reprobed by GAPDH as a control (lower panel). The NUB1 or GAPDH message is indicated by an arrowhead. The positions of 28S and 18S RNAs are indicated on the left-hand margin.

**FIG. 5. Immunocytochemical localization of NUB1.** HeLa cells were transfected with empty vector (Control), plasmid with a HA-USP21 cDNA insert (USP21), plasmid with a HA-NUB1 cDNA insert (NUB1), or plasmid with a HA-NEDD8 cDNA insert (NEDD8). The transfected HeLa cells were fixed, permeabilized, and immunostained with anti-HA antibody. The bar indicates 20 μm.

Fig. 5. Immunocytochemical localization of NUB1. HeLa cells were transfected with empty vector (Control), plasmid with a HA-USP21 cDNA insert (USP21), plasmid with a HA-NUB1 cDNA insert (NUB1), or plasmid with a HA-NEDD8 cDNA insert (NEDD8). The transfected HeLa cells were fixed, permeabilized, and immunostained with anti-HA antibody. The bar indicates 20 μm.
Down-regulation of NEDD8 by NUB1

In COS cells, HA-tagged NEDD8 was co-expressed with empty vector (lane 1) or RH-tagged NUB1 (lane 2). 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. As controls, HA-ubiquitin (lanes 3 and 4) or HA-sentrin-1 (lanes 5 and 6) was also co-expressed with empty vector or RH-NUB1 and subjected to Western blot analysis. Nonspecific bands are indicated by an asterisk. Molecular size markers are shown in kilodaltons.

**DISCUSSION**

IFNs are a group of cytokines with pleiotropic cellular effects. One prominent effect of IFNs is their potent antimitogenic action, which can be observed on both malignant and nonmalignant cells of many different origins. To explain this antimitogenic effect, multiple mechanisms have been proposed (28). In this study, we found three important properties of NUB1. First, IFN induces NUB1. Second, NUB1 negatively regulates the NEDD8 expression. Third, NUB1 inhibits cell growth. These findings suggest that treatment with IFN causes NUB1 induction, resulting in inhibition of the NEDD8 expression and affecting cell growth. Thus, NUB1-mediated inhibition of cell growth may be one of the mechanisms of the antimitogenic effect of IFNs.

The first down-regulator is USP21, which has isopeptidase activity with dual specificity for ubiquitin- and NEDD8-conjugated proteins. The second down-regulator is a dominant-negative UBC12 mutant that sequesters NEDD8 and inhibits NEDD8 conjugation. These proteins inhibit U2OS cell growth (21, 22). We asked whether NUB1 has a similar biological property. As shown in Fig. 6, when HA-NEDD8 was co-expressed with RH-NUB1 (lane 2), we could not detect both the unconjugated and conjugated forms of NEDD8 except for a 80-kDa band. This result suggested that NUB1 negatively regulates the protein expression of NEDD8 monomer and its conjugates. In contrast, when RH-NUB1 was co-expressed with HA-ubiquitin (lane 4) or HA-sentrin-1 (lane 6), the overexpression of NUB1 did not lead to a reduction of the expression of ubiquitin or sentrin-1. Thus, the down-regulation by NUB1 was specific to NEDD8.

**Effect of NUB1 on Transcription of NEDD8 Message**—Although we showed the inhibitory effect of NUB1 on expression of the NEDD8 protein, the molecular mechanism was unclear. At which step did NUB1 act as an inhibitor? To address this question, we examined the effect of NUB1 on expression of the NEDD8 message. As shown in Fig. 7, overexpression of RH-NUB1 did not reduce the expression level of HA-NEDD8 message (lane 2 versus lane 3, upper panel). This result suggested that NUB1 inhibits NEDD8 expression post-transcriptionally.

**Inhibitory Effect of NUB1 on Cell Growth**—We previously reported two down-regulators of the NEDD8 conjugation pathway. The first down-regulator is USP21, which has isopeptidase activity with dual specificity for ubiquitin- and NEDD8-conjugated proteins. The second down-regulator is a dominant-negative UBC12 mutant that sequesters NEDD8 and inhibits NEDD8 conjugation. These proteins inhibit U2OS cell growth (21, 22).

As shown in Fig. 8, overexpression of NUB1 inhibited cell growth up to 83% as compared with control. UBC12(C111S), a dominant-negative UBC12 mutant, also inhibited cell growth, up to 89%.
controlled by conjugation of NEDD8 to cullins. Since the NEDD8 expression is down-regulated by NUB1, the expression level of NUB1 may control many biological events, including NFκB signaling, and biological responses to hypoxia.

With this study, we showed that overexpression of NUB1 leads to reduction of NEDD8 monomer and its conjugates. This reduction probably results from the down-regulation of NEDD8 expression by NUB1. Since NUB1 does not affect NEDD8 expression at message level, the reduction appears to be caused by a post-transcriptional mechanism. Further studies are required to define the mechanism.

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