Characterization of NEDD8, a Developmentally Down-regulated Ubiquitin-like Protein*

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NEDD8 is a novel 81 amino acid polypeptide which is 60% identical and 80% homologous to ubiquitin. Northern blot analysis showed that the NEDD8 message was developmentally down-regulated. In adult tissues, NEDD8 expression was mostly restricted to the heart and skeletal muscle. Antiserum specific for NEDD8 detected a 6-kDa monomer in SK-N-SH, BJAB, and HL60 cell lysates. A 14-kDa band was also detected in BJAB, HL60, and SK-MEL28 but not in SK-N-SH and K562 cell lysates. An approximately 90-kDa band was detected in all cell lines tested. Thus, NEDD8 is likely to be conjugated to other proteins in a manner analogous to ubiquitination. However, the conjugation pattern of NEDD8 is entirely different from that of ubiquitin in all cell lines tested. To study NEDD8 conjugation in more detail, hemaggulutinin-epitope-tagged NEDD8 was expressed in COS cells. Western blot analysis revealed an NEDD8 monomer and a series of higher molecular weight NEDD8-conjugated proteins or NEDD8 multimers. Immunocytochemical analysis showed that NEDD8 expression was highly enriched in the nucleus and was much weaker in the cytosol. In contrast, ubiquitin expression was detectable equally well in the nucleus and cytosol. Mutational analysis showed that the C terminus of NEDD8 was efficiently cleaved and that Gly-76 was required for conjugation of NEDD8 to other proteins. Taken together, NEDD8 provides another substrate for covalent protein modification and may play a unique role during development.

Ubiquitin is one of the most conserved eukaryotic proteins which can be conjugated to other proteins through a well defined enzymatic pathway (1, 2). The importance of ubiquitination is underscored by its involvement in antigen processing, in cell cycle regulation, in degradation of tumor suppressors, in receptor endocytosis, and in signal transduction (3–10). Conjugation of ubiquitin to its target protein requires the initial activation of the conserved C-terminal Gly residue catalyzed by a specific ubiquitin-activating enzyme, E11 (1, 2, 11–13). Ubiquitin adenylation is formed by displacement of PPi from ATP and subsequently transferred to a thiol site in E1 with release of AMP. Next, ubiquitin is transferred to a ubiquitin-conjugating enzyme, E2, to form another thiol ester bond. Finally, ubiquitin is transferred from E2 to its target protein through an isopeptide linkage with the ε-amino group of the Lys residue of the target protein. The transfer of ubiquitin from E2 to the target protein requires the participation of a ligase, E3, in many instances. The biological specificity of the ubiquitination pathway appears to be regulated by a selective combination of E2 and E3 proteins (6). Currently, more than 30 E2 and 10 E3 proteins have been identified.

The complexity of the ubiquitination system is further compounded by the identification of other ubiquitin-like molecules, such as UCRP and sentrin. UCRP (ubiquitin cross-reactive protein) is a type 1 interferon-inducible protein which contains two ubiquitin domains (14). UCRP has been shown to be conjugated to a large number of intracellular proteins (15). The proteins which are modified by UCRP have not yet been identified. It is unknown whether UCRP and ubiquitin could share the same substrate. Recent results from Haas and co-workers (16) suggest that UCRP conjugation proceeds through an enzyme pathway distinct from that of ubiquitin with respect to the activation step. Our laboratory has recently reported the cloning of a novel ubiquitin-like protein, sentrin, that protects cells against both anti-Fas and tumor necrosis factor-induced cell death (17). We have further demonstrated that the C terminus of sentrin is efficiently processed, which allows sentrin to be transferred to a subset of nuclear proteins (18). It appears that sentrin targets a more limited substrate pool than ubiquitin (18). Mahajan et al. (19) and Matunis et al. (20) have independently identified a novel modification of RanGAP1 by a ubiquitin-like protein, respectively called SUMO-1 and GMP-1. Boddy et al. (21) and Shen et al. (22) have reported the interaction between PIC1 with PML or UBL1 with Rad51. Remarkably, PIC1, UBL1, SUMO-1, and GMP-1 are identical to sentrin.

Kumar et al. (23) have reported another ubiquitin-like protein, NEDD8 (Neural precursor cell-Expressed Developmentally down-regulated), using a subtraction cloning approach. NEDD8 encodes a small novel protein of 81 amino acids, which is 60% identical and 80% homologous to ubiquitin (24). Although the authors speculated that NEDD8 could also be con-

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* This work was supported by National Institutes of Health Grant HL-45851 (to E. T. H. Y.), an American Heart Association Established Investigator Award (to E. T. H. Y.), and an Arthritis Foundation Irene Degan Arthritis Investigator Award (to T. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2 The abbreviations used are: E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin-protein isopeptide ligase; HA, hemaggulutinin; UCRP, ubiquitin cross-reactive protein; NEDD8, neural precursor cell-expressed developmentally down-regulated; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; HRP, horseradish peroxidase; GST, glutathione S-transferase; mAb, monoclonal antibody.
jugated to other proteins, they did not provide any biochemical evidence for NEDD8 modification of other proteins. Here, we demonstrate that NEDD8 is indeed activated and transferred to other proteins in a process analogous to ubiquitination. Furthermore, NEDD8-conjugated proteins appear to reside predominantly in the nucleus. The characterization of NEDD8 adds another level of complexity to the process of protein ubiquitination. The specific antigeniser described in this manuscript will provide a powerful tool to study the biochemical and biological differences between NEDD8 modification and protein ubiquitination.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions—SK-MEL28, SK-N-SH, HL60, and K562 were purchased from American Type Culture Collection (Rockville, MD). BJAB and COS-M6 cells were generous gifts from Dr. Fred Wang (Harvard University) and Dr. Steve Goldring (Harvard Medical School), respectively. These cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and antibiotics.

Antibodies—12CA5 (Boehringer Mannheim) and 16B12 (AbCo, Richmond, CA) are mouse monoclonal antibodies to the peptide sequence (EDPVDYAA) influenza hemagglutinin (HA). Mouse anti-GST monoclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit polyclonal anti-human NEDD8 antisemur was generated by immunization with a peptide corresponding to amino acids 20–32 (TDKRIERKVEE). The rabbit polyclonal anti-ubiquitin antibody was purchased from Sigma.

Plasmid Construction and Transfection—To express HA-tagged proteins in COS-M6 cells, two vectors for N-terminal tagging (pDNA3/HA-N) and C-terminal tagging (pDNA3/HA-C) were constructed as described previously (18). HA adaptor duplexes were inserted into pDNA3 (Invitrogen, San Diego, CA), and then the DNAs of ubiquitin, senrin, Rad51, and NEDD8 mutants were carried out by polymerase chain reaction using appropriate primers followed by ligation into the vector pcDNA3/HA-N or pcDNA3/HA-C. Plasmid encoding RGS/H1K-tagged RanGAP1 was constructed by inserting the human RanGAP1 cDNA into pcDNA3/RH-N vector. The sequence of each insert was confirmed by automated DNA sequencing. Plasmids were transfected into COS-M6 cells using LipofectAMINE (Life Technologies, Inc). Transfected cells were harvested for Western blotting or immunostaining 16 h after transfection.

Northern Blot Analysis—A full-length cDNA fragment of human NEDD8 from the plasmid pcDNA3/NEDD8-HA was labeled with [α-32P]-dCTP by a megaprime labeling kit (Amersham). The bacteria were then lysed on ice by mild sonication and centrifuged at 45 °C for 1 h and then loaded onto SDS-polyacrylamide gels. The bacteria were then eluted on ice by mild sonication and centrifuged at 45 °C for 1 h and then loaded onto SDS-polyacrylamide gels.

RESULTS AND DISCUSSION

NEDD8 Is a Highly Conserved Ubiquitin-like Protein—NEDD8 was isolated from a subtraction library prepared by subtracting a cDNA library of mouse neural precursor cells with adult brain mRNA (23). It encodes an 81-amino acid polypeptide with 60% identity to human ubiquitin. Fig. 1 shows the amino acid alignment of human, mouse, and rat NEDD8 compared with ubiquitin. As shown, NEDD8 is highly conserved in mammalian species. There is only a single amino acid difference between the human and mouse NEDD8. Furthermore, its C terminus contains the Leu-Arg-Gly-Gly residues which are critical for conjugation of ubiquitin to other proteins. The Lys-48 residue, which plays an important role in the formation of multi-ubiquitin chains, is also conserved.

Immunostaining—Immunocytochemical staining was performed by using the avidin-biotin-HRP complex (ABC-HRP) method (25), using the VECTASTAIN ABC kit system (Vector, Burlingame, CA). Transfected COS-M6 cells on coverslip were fixed in 3.7% paraformaldehyde solution for 20 min and permeabilized in 0.1% Triton X-100 for 10 min at room temperature. After washing with PBS, the fixed cells were incubated with PBS containing 0.1% H2O2 for 10 min to quench endogenous peroxidase activity and then washed with PBS. The cells were incubated for 10 min with PBS containing 5% horse serum for blocking, followed by the further incubation with anti-HA antibody (16B12) for 30 min at 37 °C. After rinsing with PBS, the cells were incubated with biotinylated anti-mouse IgG (1:200) for 30 min at 37 °C, washed with PBS, and then treated with the ABC reagent (avidin-biotin-HRP complex) for 30 min at 37 °C. Finally, the enzymatic disclosing procedure was performed as reported previously (25).

Expression and Purification of GST Fusion Proteins—The expression and purification were performed essentially as described previously (26). Logarithmically growing culture (500 ml, A600 0.8) of Escherichia coli BL 21 (Stratagene, La Jolla, CA) transformed with the pGEX-2TK (Pharmacia Biotech Inc.) recombinants were incubated with 0.1 mM isopropyl-b-D-thiogalactopyranoside (Stratagen) at room temperature for 2.5 h. The cells were then pelleted, resuspended in 50 ml of ice-cold NETN buffer (100 mM NaCl, 1 mM EDTA, 20 mM Tris (pH 8.0), 0.5% Nonidet P-40) containing 100 μg/ml egg white lysozyme (Sigma). The bacteria were then lysed on ice by mild sonication and centrifuged at 40,000 × g for 30 min at 4 °C. Bacterial supernatants were rocked for 1 h at 4 °C with 750 μl of glutathione-Sepharose beads (Pharmacia), which had been previously washed three times and resuspended (final concentration 1.1, μ/l) in NETN. The glutathione-Sepharose beads were then washed three times with NETN. For analysis of bound bacterial proteins, the beads were incubated in the sample buffer containing 2% SDS and at 45 °C for 1 h and then loaded onto SDS-polyacrylamide gels.

Imunoabsorption—1 ml of diluted anti-NEDD8 antiserum (1:1000) was incubated overnight with GST or GST-NEDD8 fusion protein-coated beads. The beads were removed by centrifugation, and the supernatant was used for Western blotting analysis.

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Tissue-specific Expression and Developmental Down-regulation of NEDD8 Message—To determine the expression of NEDD8 in human tissues, Northern blot analyses were performed using 32P-labeled human NEDD8 cDNA as probe. As shown in Fig. 2, NEDD8 message is highly enriched in the heart and skeletal muscle but much lower in all other tissues.
Since NEDD8 was originally isolated as a developmentally down-regulated message in the mouse brain, we also tested its expression in the developing mouse embryo. As shown in Fig. 3, NEDD8 message was highest in the day 11 mouse embryo and was markedly decreased in day 15 and day 17 embryos.

Detection of NEDD8 Monomer and Conjugates with a Polyclonal NEDD8-specific Antiserum—To further characterize NEDD8 protein expression in tissues or cell lines, polyclonal antibody specific for NEDD8 was generated. As shown in Fig. 4, anti-NEDD8 antiserum reacted specifically with GST-NEDD8 fusion protein (lane 4) but not with GST-ubiquitin (lane 3). In contrast, anti-GST antibody was able to detect both GST-NEDD8 and GST-ubiquitin (lanes 5 and 6). The expression of NEDD8 was surveyed in five different cell lines using NEDD8-specific antiserum as described above. As shown in Fig. 5, antiserum specific for NEDD8 identified a 6-kDa monomer in SK-N-SH, BJAB, and HL60 (arrowhead). A 14-kDa band was also observed in BJAB, HL60, and SK-MEL28. Furthermore, a 90-kDa band was observed in all cell lines tested. The identity of the 14- and 90-kDa bands is unknown. In a similar analysis with anti-ubiquitin antiserum (Fig. 6), a 6-kDa band was observed in SK-N-SH, BJAB, HL60, and K562 (arrowhead). Furthermore, a ladder of high molecular weight ubiquitinated proteins was observed in Western blot analysis. Comparing Figs. 5 and 6, it is quite evident that the expression pattern of NEDD8 is distinct from that of ubiquitin.

NEDD8 Expression in COS Cells—To study the biochemistry of NEDD8 modification in more detail, HA-epitope-tagged NEDD8 was transfected into COS cells as described previously (18). Transfected cell lysates were analyzed by Western blot analysis with anti-HA antibody. HA-epitope-tagged ubiquitin served as a control. As shown in Fig. 7, NEDD8 expression and conjugation are clearly distinct from that of ubiquitin modification. Two anti-HA monoclonal antibodies were used in Western blot analyses. 12CA5 is more sensitive than 16B12. However, three nonspecific bands (~25, 45, and 75 kDa) were observed in the 12CA5 blot as previously reported (18). Fig. 7 clearly demonstrates that NEDD8 modification is much less extensive than ubiquitination. The prominent 90-kDa band in the HA-NEDD8-transfected COS cell lysate is also of interest (arrowhead). However, it is unlikely that this band is NEDD8-modified RanGAP1 (see below). The immunoblot, using a more...
sensitive anti-HA antibody (12CA5), revealed a series of faint bands in a ladder-like pattern similar to those in the HA-ubiquitin lane. These bands could represent conjugation of proteins by NEDD8 multimers because the Lys-48 residue is conserved in NEDD8. However, this hypothesis remains to be confirmed.

p90 Is Not NEDD8-conjugated RanGAP1—The prominent 90-kDa band shown in Fig. 7 is reminiscent of RanGAP1 modified by sentrin. To test this hypothesis, a COS cell co-transfection experiment was performed. RanGAP1 was tagged in the N terminus with the amino acid sequence of RGSHHHHHH, termed RH tag, which allowed for facile purification of the RanGAP1 fusion protein with nickel resin beads. Plasmids encoding for HA-tagged sentrin, HA-tagged ubiquitin, HA-tagged NEDD8, and HA-tagged Rad51 were co-transfected with plasmid encoding RH-RanGAP1 into COS cells as described above. The lysates prepared from the transfectants were precipitated first with nickel resin beads following extensive washing. The precipitates were analyzing with Western blot analysis using anti-HA antibody. As shown in Fig. 8, sentrinized RanGAP1 is readily observed in lane 4. However, neither HA-tagged ubiquitin (lane 2) nor HA-tagged NEDD8 (lane 3) were able to form a conjugate with RanGAP1. The negative control, HA-tagged Rad51, also could not conjugate to RanGAP1. The effectiveness of the transfection protocol was confirmed in a separate Western blot analysis utilizing anti-RH monoclonal antibody. As shown in lanes 5–8, both unmodified RanGAP1 and sentrinized RanGAP1 could be observed equally well in all samples. It should be noted that p90 in lanes 5–7 was derived from RanGAP1 modified by native sentrin in COS cells. This is further supported by the observation of a doublet observed near p90 in the HA-sentrin-transfected sample (lane 8). The upper band is most likely HA-sentrin-modified RanGAP1, and the lower band is most likely native sentrin-modified RanGAP1 (lane 8). Taken together, NEDD8 could not form a conjugate with RanGAP1. The identity of p90 in Fig. 7 remains to be elucidated.

Predominant Nuclear Localization of NEDD8-modified Proteins—Transfected COS cells were also stained with anti-HA antibody (16B12) as described previously (18). As shown in Fig. 9, HA-epitope-tagged NEDD8 is expressed predominately in the nucleus with minor cytosolic expression. In contrast, HA-epitope-tagged ubiquitin is distributed evenly in both nucleus and cytosol. The cellular localization pattern of NEDD8 is reminiscent of sentrin, which is even more restricted to the nuclear compartment (18).

C-terminal Processing Requirement of NEDD8—The C terminus of human NEDD8 contains Gly-Gly-Leu-Arg-Glu distal to the conserved Gly-75 and Gly-76 residues. In order for NEDD8 conjugation to occur, this C-terminal tail must be processed. As shown in Fig. 10, when the HA-tag was placed at the C terminus of NEDD8, it is efficiently cleaved in transfected COS cells. Thus, anti-HA antibody could not detect any NEDD8 monomer or NEDD8-modified proteins. This is further confirmed by the anti-NEDD8 immunoblot. As shown, anti-NEDD8 antisera could detect a 6-kDa band (native NEDD8) in COS cells transfected with vector alone and a 6-kDa (native or processed NEDD8) and 7-kDa (HA-tagged NEDD8) doublet. The upper band (HA-tagged NEDD8) is absent in cell lysates prepared from COS cells transfected with NEDD8 tagged with...
HA at the C terminus. Limited substitution of Ala for Gly at 64, 76, or 76 positions did not affect processing of the C terminus of NEDD8.

Since the C terminus of NEDD8 contains four consecutive Gly residues, it is important to determine which Gly residue is involved in the formation of NEDD8 conjugates. As shown in Fig. 9, Gly-76 is required for the formation of NEDD8 conjugates. Thus, in the HA-NEDD8-Gly-75 transfectant, only the NEDD8 monomer is detected. Taken together, the C-terminal processing and conjugation requirement is identical to that of ubiquitin and sentrin.

The identity of proteins modified by NEDD8 is currently unknown. Candidate proteins for NEDD8 modification are nuclear proteins expressed in the heart and skeletal muscles or in early development (see Figs. 2 and 3). Furthermore, it is unknown whether a protein can be modified by either ubiquitin or NEDD8 or both. Because both ubiquitin and NEDD8 contain the conserved Lys-48 residue, it is also of interest to determine whether multimers of NEDD8 and ubiquitin exist. The NEDD8-specific antiserum described in this report will provide an excellent tool to examine these issues in the future.

Acknowledgments—We thank L. Gong and L. Caskey for critical reading of this manuscript.

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