Identification of the Activating and Conjugating Enzymes of the NEDD8 Conjugation Pathway

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NEDD8 is a ubiquitin-like molecule that can be covalently conjugated to a limited number of cellular proteins, such as Cdc53/cullin. We have previously reported that the C terminus of NEDD8 is efficiently processed to expose Gly-76, which is required for conjugation to target proteins. A combination of data base searches and polymerase chain reaction cloning was used to identify a cDNA encoding human UBA3, which is 38% identical to the yeast homologue, 22% identical to human UBA2, and 19% identical to the C-terminal region of human UBE1. The human UBA3 gene is located on chromosome 3p13 and gave rise to a 2.2-kilobase pair transcript that was detected in all tissues. Human UBA3 could be precipitated with glutathione S-transferase (GST)-NEDD8, but not with GST-ubiquitin or GST-sentrin-1. Moreover, human UBA3 could form a β-mercaptoethanol-sensitive conjugate with NEDD8 in the presence of APP-BP1, a protein with sequence homology to the N-terminal half of ubiquitin-activating enzyme. We have also cloned human UBC12 and demonstrated that it could form a thiol ester linkage with NEDD8 in the presence of the activating enzyme complex. Identification of the activating and conjugating enzymes of the NEDD8 conjugation pathway should allow for a more detailed study of the role of NEDD8 modification in health and disease.

NEDD8 is a novel 81-amino acid polypeptide that is 60% identical and 80% homologous to ubiquitin (1). Antiserum specific for NEDD8 detected a 6-kDa NEDD8 monomer and a series of NEDD8 multimers or NEDD8-conjugated proteins (2). However, in all cell lines tested, the conjugation pattern of NEDD8 is entirely different from that of ubiquitin (2). Immunocytochemical analysis showed that NEDD8-conjugated proteins were highly enriched in the nucleus. In contrast, ubiquitin-conjugated proteins were detected 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. The yeast homologue of NEDD8, Rub1, can also be conjugated to a limited number of cellular proteins, including Cdc53/cullin, a component of the SCF ubiquitin ligase (a complex composed of Cdc53, Skp1, and an F-box protein) that plays a critical role in the regulation of cell cycle progression (3, 4). A prominent 90-kDa NEDD8-modified protein was detected in all mammalian cells, which was consistent with the molecular mass of the NEDD8-cullin-1 conjugate (2). The substrate specificity of NEDD8 conjugation appears to be strictly regulated because NEDD8 cannot conjugate to PML and RanGAP1, two substrates of the sentrin family of ubiquitin-like proteins (2, 5). The function of NEDD8 modification is not known at present. Yeast mutants defective in Rub1 are viable, but sensitive to alterations in the levels of Cdc4, Cdc34, and Cdc53 (3). It is not known whether NEDD8 conjugation is also involved in targeting proteins to the proteasome.

Ubiquitin-mediated proteolysis is an important pathway of non-lysosomal protein degradation. This pathway involves a cascade of enzymatic reactions (6). The first step is the ATP-dependent activation of ubiquitin by a ubiquitin-activating enzyme (E1), leading to the formation of a thiol ester linkage between Gly-76 of ubiquitin and a cysteine residue of E1. In the second step, activated ubiquitin is transferred to a cysteine residue in one of several ubiquitin-conjugating enzymes (E2) to form another thiol ester conjugate. In the last step, an isopeptide bond is formed between Gly-76 of ubiquitin and a lysyl ε-amino group within a substrate protein, a reaction catalyzed either directly by the E2 enzyme or via a third enzyme designated ubiquitin-protein ligase. A single E1 enzyme has been characterized from human, mouse, wheat, and Saccharomyces cerevisiae (7). In yeast, Uba1 is essential for viability and encodes a 114-kDa activating enzyme required for ubiquitin conjugation (8). Another E1-like protein, Uba2, which encodes a 71-kDa protein that is similar to the C terminus of E1 proteins and bears a cysteine residue at a position similar to the active-site cysteine of Uba1, has been identified (9). Johnson et al. (10) demonstrated that Uba2 cooperates with another 40-kDa protein, termed Aos1 (activation of Smt3), the yeast homologue of the sentrin family of ubiquitin-like proteins (11). Interestingly, both human and yeast Aos1 and Uba2 share extensive homology with the N- and C-terminal halves of yeast ubiquitin-activating enzyme, Uba1 (2).

In this paper, we report the molecular cloning of a human cDNA encoding a protein homologous to yeast Uba3, which has recently been shown to be involved in the activation of Rub1 (3, 4).

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† The abbreviations used are: E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; PCR, polymerase chain reaction; kb, kilobase pair(s); EST, expressed sequence tag; THC, tentative human consensus.

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We show that UBA3 is homologous to the C-terminal half of E1 and can form a thiol ester conjugate with NEDD8 in the presence of APP-BP1, which is homologous to the N-terminal half of E1. Furthermore, we have cloned human UBC12 and demonstrate that it could form a thiol ester linkage with NEDD8 in the presence of APP-BP1 and UBA3. The identification of the specific E1 complex and E2 for NEDD8 should provide further stimuli to study the role of NEDD8 modifications in health and disease.

**EXPERIMENTAL PROCEDURES**

**Materials—** All chemical reagents were purchased from Sigma unless otherwise noted. DNA restriction endonucleases and T4 ligase were from New England Biolabs Inc. (Beverly, MA) or Roche Molecular Biochemicals. Human and mouse cDNA libraries, multiple-tissue Northern blots, and the PCR cloning kit were obtained from CLONTECH (Palo Alto, CA). The TNT® T7-coupled reticulocyte lysate system was purchased from Promega (Madison, WI). ATP, [α-32P]dCTP, T[α-33]Smethionine, Hyperfilm MP, and the GST protein purification kit were from Amersham Pharmacia Biotech. Prestained molecular mass markers and SDS-polyacrylamide gel electrophoresis reagents were obtained from Bio-Rad. Oligonucleotides were synthesized with a Beckman DNA synthesizer. Model olig1000. Sequences of the primers used in this study are as follows: olig1, CTGGATCCAAATGGCTGTTGA; olig2, GAAATACCAATTTATATTGGAATCG; olig3, CTGGAGAGATTCAGTGCTCGGTTCGA; olig4, TATCTATTCGATGGAGTTT; olig5, GAAGACTTCTACCATCTGGCTAG; olig6, GCAGTAACATTCCCCTTTAGTTAG; olig7, ACGGATGGTCTGATTTG; olig8, GCAGTAACATTCCCCTTTAGTTAG; olig9, CTGGATCCTAC; olig10, GACGTTCTCGCTGAAG; and olig11, GCAGTAACATTCCCCTTTAGTTAG.

All plasmid constructs were made using standard techniques. Each construct was sequenced to verify the correct frame as well as the proper sequence of any linker introduced during the cloning procedure. The plasmids used as templates for in vitro transcription and translation were constructed based on 1.3-kb cDNA containing the entire coding sequence of UBA3 was amplified from a human placenta cDNA library by PCR using an oligonucleotide forward primer (5'-GGCGGATCTTAAGAAGTTAGTTGATGGTGGG-3') containing a HindIII site (underlined) and a reverse primer (5'-GGCGGATCTTCAGAAGTTAGTTGATGGGTGGG-3') containing a HindIII site (underlined). The PCR product was digested with HindIII and KpnI and ligated with the 1.6-kb HindIII-KpnI fragment of pcDNA3 to construct pcDNA3-UBA3. pcDNA3-APP-BP1 and pcDNA3-UBC12 were constructed in a similar way, except that the HindIII-KpnI fragment of APP-BP1 and the 0.55-kb HindIII-KpnI fragment of UBC12 were used. For the generation of the GST fusion plasmid, a DNA segment encoding the first 76 amino acids of human UBA3 was amplified by PCR and gel-purified and concentrated using a GeneClean kit (Bio 101, Inc., La Jolla, CA) according to the manufacturer’s instructions. A human α-actin probe was used as a control to determine equal RNA loading. The cDNA probe was labeled with [α-32P]dCTP (3000 Ci/mmol) using the Random Primers DNA labeling system (Life Technologies, Inc.), and unincorporated nucleotide was removed by chromatography on a NucTrap column (Stratagene, La Jolla, CA). The probe was filter-sterilized and used at 1 at 42 °C in 0.5× SSPE (1× SSPE = 0.16 × NaCl, 20 mM NaPO4, and 1× Na2EDTA, pH 7.2), 0.5% SDS, and 100 μM herring sperm DNA and prehybridized for 16 h at 42 °C in 30% formamide. Hybridization was performed with the 32P-labeled probe overnight. The nylon sheet was washed twice with 2× SSC and 0.1% SDS at room temperature for 10 min and with 0.2× SSC and 0.1% SDS at 55 °C for 10 min. Autoradiograms were obtained by exposing the blot to x-ray film with an intensifying screen at −80 °C for 2 days.

**Expression and Purification of GST Fusion Proteins—** The plasmids pGEX-UB, pGEX-NEDD8, and pGEX-sentrin-1 were used for expression of GST fusion proteins. The pGEX expression vectors were introduced into competent BL21 bacteria, and expression of GST fusion proteins was induced by isopropyl-β-D-thiogalactopyranoside and purified as described previously (12).

In Vitro Translation and Binding Assays—For in vitro translation and binding assays, pUC8-BP1, and UBC12 proteins, pcDNA3-APP-BP1, pcDNA3-UBA3, and pcDNA3-UBC12 were used as templates for in vitro transcription and translation using TNT T7 polymerase (Promega) under conditions recommended by the supplier. Translation reactions were performed in TNT T7-coupled rabbit reticulocyte lysates in a final volume of 50 μl. Reaction mixtures contained 25 μl of lysate, 40 μCi of [35]S-methionine (1000 Ci/mmol), 15 units of RNAsin (Promega), 50 μM amino acid mixture (minus methionine), and 1 μg of DNA. The reactions were incubated for 90 min at 30 °C and stopped by the addition of 3 volumes of Laemmli buffer. Translation products were analyzed by 12% SDS-polyacrylamide gel electrophoresis. Gels were fixed,
treated in Amplify solution (Amersham Pharmacia Biotech), dried, and processed for autoradiography. For in vitro binding between NEDD8 and UBA3, 10 μl of in vitro translated UBA3 with or without 20 μl of translated APP-BP1 were incubated with 50 μl of glutathione-Sepharose beads containing ~1 μg of GST-ubiquitin, GST-NEDD8, or GST-sentrin-1 for 30 min at 25 °C. For thiol ester bond formation between NEDD8 and UBC12, 5 μl of translated unlabeled UBA3 and APP-BP1 and 10 μl of translated 35S-labeled UBC12 were incubated with 50 μl of glutathione-Sepharose beads containing GST fusion protein for 12 h at 4 °C.

RESULTS AND DISCUSSION

Molecular Cloning of the Human UBA3 cDNA—A full-length cDNA sequence encoding a protein with significant homology to the human E1 protein was identified by a combination of data base searches and PCR amplification described under “Experimental Procedures.” The nucleotide and deduced amino acid sequences of one of the cloned human cDNAs are shown in Fig. 1. The predicted amino acid sequence of the cloned human cDNA is most similar to yeast Uba3 (38.3% identity) and less similar to yeast Uba2 (20.8% identity) and the C-terminal half of the E1 enzyme (19.2% identity). We named this protein UBA3 because of its close similarity to yeast Uba3 (Fig. 2) and its ability to form a thiol ester bond with NEDD8 (see below). The UBA3 cDNA contains an open reading frame initiating with an ATG codon (nucleotides 85–87) and terminating with a TAA codon (nucleotides 1411–1413). The ATG triplet is preceded by an in-frame stop codon at nucleotides 1331, and therefore, it is the most likely candidate for the initiation codon. Another ATG codon is located at nucleotides 322–325, but the functional UBA3 was found to initiate from the first ATG codon (see below). The 84-bp non-coding region has a high AG content (72%). At the 3′-end of the cDNA, two potential polyadenylation signals are observed: AAAATAAA is present at nucleotide 1779, and AATAAAA is located at nucleotide 2101. This open reading frame encodes a protein (UBA3) of 442 amino acids residues. The calculated molecular mass and pI, based on the cDNA-derived amino acid sequence of human UBA3, are 49.3 kDa and 5.13, respectively. Compared with the amino acid sequence for yeast Uba3, human UBA3 has an extra 46 amino acids at its N terminus and an additional 90 amino acids at its C terminus (Fig. 2).

Two important motifs are present in the known E1 enzymes: one is the consensus sequence for a nucleotide-binding site, GXXGXXGCE (amino acids 475–482 in human UBE1); and the other is the consensus sequence PZCTXXXXP surrounding the essential cysteine in E1 enzymes (amino acids 630–639 in human UBE1), which becomes linked to ubiquitin in an E1-ubiquitin thiol ester linkage. As shown in Fig. 3, both UBA2 and UBA3 have an almost identical ATP-binding region and a significantly conserved active-site motif. The last amino acid residue in the active-site motif of yeast and human E1 is

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**Fig. 1.** Nucleotide sequence of the coding and flanking regions of human UBA3. The deduced amino acid sequence is given below the nucleotide sequence in single-letter code. The asterisk indicates the stop codon, and the potential polyadenylation signal is underlined. The nucleotide sequence was determined on both strands by automated sequencing.

**Fig. 2.** Sequence alignment of human, yeast, and mouse UBA3. Amino acids that are identical to the corresponding position in yeast (Y), human (H), and mouse (M) are shaded. The numbers correspond to the sequence of UBA3.
asparagine. In contrast, the last amino acid residue in the active-site motif of yeast and human UBA2 is serine, whereas it is arginine for yeast and human UBA3. Site-directed mutagenesis experiments will be required to determine whether this substitution contributes to the substrate preference for different activating enzymes.

**Southern and Northern Blot Analyses**—Southern blot analysis of human genomic DNA completely digested with either BamHI or BglII using a probe for UBA3 revealed the presence of a single hybridization signal (data not shown). This observation indicates the existence of one copy of the UBA3 gene per chromosome. Northern blot analysis revealed a single 2.2-kb band in all tissue samples, but the message level of UBA3 was highest in the heart and skeletal muscle and lowest in the liver (Fig. 4).

**Chromosomal Localization of the Human UBA3 Gene by EST Mapping**—When the human UBA3 cDNA sequence was used to search the GenBank™ EST data base using the BLASTN program, 63 positive EST clones were identified. All of these EST clones were used to check the Human Gene Map data base. Three of them, W73812 (nucleotides 1601–2120), N71576 (nucleotides 1810–2120), and T17227 (nucleotides 1815–2120), have been mapped to chromosome 3 between D3S125 and D3S131 microsatellite markers at 91–97 centimorgans (National Center for Biotechnology Information). Thus, the UBA3 gene is located on chromosome 3p13. The E1-like gene, UBE1L, is also located on chromosome 3 (3p21), whereas UBE1 is located on chromosome X, and UBA2 is located on chromosome 19.2

**Isolation and Characterization of Mouse UBA3**—We also searched the mouse EST data base to identify mouse UBA3. A
mouse and human UBA3, using BestFit with default parameters, showed 94% identity between the two sequences. The level of conservation between the two proteins is even greater (98.7% identical) (Fig. 2). Interestingly, both human and mouse UBA3 contain almost identical N- and C-terminal amino acids, which are absent from yeast Uba3. The structure/function relationship of these N- and C-terminal extensions awaits further investigation.

**Human UBA3 and APP-BP1 Are Required for NEDD8 Activation**—Since UBA3 has a conserved ATP-binding site and an active site of E1, we tested whether UBA3 would be able to form a thiol ester linkage with either ubiquitin or ubiquitin-like proteins. As shown in Fig. 5A (lane 1), in vitro translated UBA3 resulted in two major bands (40 and 49 kDa). This is most likely due to differential usage of the two ATG codons (nucleotides 13–14 and 322–325) in the UBA3 sequence. The in vitro translated UBA3 was incubated with GST-ubiquitin (lane 2), GST-NEDD8 (lane 4), or GST-sentrin-1 (lane 6) at room temperature for 30 min in the presence of ATP. The GST beads were extensively washed, and the precipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis. As shown, two bands (40 and 49 kDa) could be precipitated by GST-NEDD8, but not by GST-ubiquitin or GST-sentrin-1. 

Thus, UBA3 appears to bind preferentially to NEDD8. However, a higher molecular mass UBA3-NEDD8 conjugate could not be detected (lane 4). Since UBA3 is homologous to the C-terminal half of E1, it is likely that another protein homologous to the N-terminal half of E1 is required for the formation of a UBA3-NEDD8 conjugate. Initially, we tested whether human AOS1 would be able to complement UBA3 in the formation of the UBA3-NEDD8 conjugate. However, human AOS1 could not complement UBA3 and appeared to be specific for the sentrinization pathway. Thus, we tested an AOS1 homologue, APP-BP1, for its ability to complement UBA3 in the formation of the UBA3-NEDD8 conjugate. In vitro translated APP-BP1 was precipitated by GST-NEDD8 in the presence of both UBA3 and APP-BP1 (lane 5). The position of the GST-NEDD8-UBC12 conjugate (55 kDa) is indicated. UBC12 could not be precipitated by GST-ubiquitin (UB) and GST-NEDD8, respectively, in the presence of UBA3 and APP-BP1 UBC12 (lanes 2 and 4; lane 5, precipitated by GST-NEDD8 in the presence of both UBA3 and APP-BP1; lane 6, the sample from lane 5 treated with 5% β-mercaptoethanol (2-ME).
jugate because APP-BP1 does not contain the active-site cysteine. Furthermore, the UBA3 C216S mutant, which contains a substitution in the active site, could not form an 82-kDa conjugate (Fig. 5B). The thiol ester bond formation between UBA3 and NEDD8 was dependent on the presence of ATP (Fig. 5C).

Next, we showed that the 82-kDa band was sensitive to 5% β-mercaptoethanol treatment, suggesting that it behaved like a thiol ester conjugate (Fig. 6). These results support the suggestion that formation of a UBA3-NEDD8 conjugate is dependent on the presence of APP-BP1. Our results are also consistent with recent reports that Rub1, the yeast homologue of NEDD8, can form a thiol ester bond with yeast Uba3 (3, 4). Moreover, Ula1/Enr2, the yeast homologue of APP-BP1, is also required for the activation of Rub1(3, 4). Taken together, there is a remarkable conservation in the enzymology in the activation of NEDD8 and Rub1.

Identification and Cloning of Human UBC12—We have previously identified UBC9 as a specific conjugating enzyme in the sentrinization pathway (12). While testing the specificity of the conjugating enzymes in the sentrinization pathway, we used NEDD8 as a control and failed to observe a thiol ester bond formation between NEDD8 and eight known human E2 proteins, including UCH15B, UCH6, H1P2, HHR6A, HHR6B, E2-EPF, UBCH-ben, and UBC9 (data not shown). Thus, we sought to identify additional human E2 proteins that could conjugate to NEDD8. We used the amino acid sequence from the conjugation site of human UBC9 (YPSPPCKDFFHIHPNVPSGTVCL) as a query to search the TIGR Tentative Human Consensus Sequence data base. 18 positive THC fragments were identified. After further analysis, one THC fragment (THC210627) was found to have a conserved sequence different from known E2 proteins. We designed two primers, oligo7 and oligo8, according to the information from THC210627. PCR amplification from a human placenta cDNA library resulted in the isolation of a 750-base pair cDNA. The nucleotide sequences and predicted amino acids are shown in Fig. 7.

Conjugation of NEDD8 to UBC12 Requires the Involvement of APP-BP1 and UBA3—Next, the ability of human UBC12 to form thiol ester conjugates with ubiquitin, NEDD8, or sentrin-1 was determined. As shown in Fig. 8 (lanes 2–4), only GST-NEDD8, but not GST-ubiquitin or GST-sentrin-1, could specifically precipitate in vitro translated UBC12. However, we could not observe an NEDD8 and UBC12 conjugate in the absence of E1 (lane 3). When APP-BP1 and UBA3 were added to the reaction mixture, a 55-kDa conjugate could be detected (lane 5). This band most likely represents GST-NEDD8 conjugated to UBC12 via a thiol ester linkage because it disappeared when the sample was reduced with 5% β-mercaptoethanol (lane 6). Thus, UBC12 is the only E2 tested that was able to form a conjugate with NEDD8. This observation is also consistent with the recent report of the critical role of yeast Ubc12 in the conjugation of Rub1 (4).

Distinct Pathways for Activation and Conjugation of Ubiquitin-like Proteins—Ubiquitination has proven to be one of the major protein modification pathways and plays a critical role in many cellular processes (14–16). The complexity of the ubiquitination system is further compounded by the identification of other ubiquitin-like molecules, such as UCRP (ubiquitin cross-reactive protein), sentrin, and NEDD8. UCRP is a type 1 interferon-inducible protein that contains two ubiquitin domains (17). UCRP has been shown to conjugate to a large number of intracellular proteins (18). Proteins modified by UCRP have not yet been identified. Recent results from the Haas laboratory (19) suggest that UCRP conjugation proceeds through an enzyme pathway distinct from that of ubiquitin, at least with respect to the activation step. However, the true identity of the activating enzyme for UCRP is not known. The sentrin family of ubiquitin-like proteins consists of three family members that have similar substrate specificity. We have shown that sentrin-1, sentrin-2, and sentrin-3 are able to conjugate to RanGAP1 and PML (5). The yeast homologue of sentrin is Smt-3, which utilizes an activating enzyme complex consisting of Uba2 and Aos1 (10). A similar complex is also required for the activation of the sentrin family members. Interestingly, both sentrin and Smt-3 utilize UBC9 as the conjugating enzyme (10, 12). We have shown previously that NEDD8 can be covalently conjugated to a limited number of cellular proteins (2). In this report, we cloned and characterized both the activating and conjugating enzyme complexes for NEDD8. Our results are consistent with reports from the yeast system (3, 4). Taken together, the enzymatic machinery responsible for the activation and conjugation of ubiquitin-like proteins diverged from the ubiquitination system early in evolution (Fig. 9).

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FIG. 9. Summary of activating enzymes for the ubiquitin and ubiquitin-like proteins, sentrin, and NEDD8. The sizes for UBE1, AOS1, APP-BP1, UBA2, and UBA3 are 1011, 346, 532, 670, and 442 base pairs, respectively. APP-BP1 is 22% identical and 46% similar to the N-terminal region of UBE1 (37–426 amino acids) and 21% identical and 45% similar to AOS1 (L. Gong and E. T. H. Yeh, manuscript in preparation). UBA3 is 19% identical and 42% similar to the C-terminal region of UBE1 (427–1011 amino acids) and 22% identical and 44% similar to UBA2 and AOS1 (L. Gong and E. T. H. Yeh, manuscript in preparation). The positions for the ATP-binding region and the active-site motif are shown in Fig. 3.
NEDD8-specific Activating and Conjugating Enzymes

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