A new NEDD8-ligating system for cullin-4A

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NEDD8 is a ubiquitin (Ub)-like protein. Here we report a novel ubiquitinylation-related pathway for modification by NEDD8. NEDD8 was activated by an E1 (Ub-activating enzyme)-like complex, consisting of APP-BP1 and hUbα3 with high respective homologies to the amino-1997; Schwarz et al. 1998) as the E1- and E2-like enzymes, respectively, which resembles that of the recently described Smt3p/SUMO-1, as mentioned above (Johnson and Blobel 1997; Johnson et al. 1997; Schwarz et al. 1998), indicating that three distinct pathways for modification of Ub and Ub-like proteins exist in cells. Moreover, we report that NEDD8 is likely to be conjugated to Cul-4A via the carboxy-terminal Gly residue in a manner analogous to ubiquitinylation. Cul-4A is one member of a family of human cullin proteins (Kipreos et al. 1996). Yeast Cdc53p (a homolog of Hs–Cul-1) functions as a common subunit of the large Ub-protein ligase E3 complex responsible for a ubiquitinylation-dependent proteolytic pathway that regulates various biologically important processes, such as the cell cycle, metabolism (Mathias et al. 1996), and gene expression (for review, see Jackson 1996; Hershko 1997; Hoyt 1997). Therefore, the results obtained in this study suggest that modification of Cul-4A by NEDD8 has an important role for regulation of the cell cycle.

Results and Discussion

Identification of a NEDD8-activating enzyme

To explore the conjugating mechanism of NEDD8, we first attempted to identify the protein(s) that can interact with human 35S-labeled NEDD8 in rabbit reticulocyte lysates. Three major bands of 100, 66, and 30 kD were reproducibly detected in addition to unmodified35S-labeled NEDD8, and the 66- and 30-kD bands disappeared by treatment with the reducing reagent dithiothreitol (DTT) (Fig. 1A). To characterize these proteins, we isolated them from reticulocyte lysates by affinity chromatography with GST–NEDD8 fused protein as a ligand. Five proteins, 1–5 (see Fig. 1B), were eluted from the GST–NEDD8 column but not from the control GST resin. Proteins 1–3 were eluted by DTT (Fig. 1B, left); and proteins 4 and 5 by subsequent treatment with reduced glutathione, GSH (Fig. 1B, right).

Sequence analysis of the 62-kD protein 1 eluted from the GST–NEDD8 affinity column showed that it was almost identical to the known protein, APP–BP1 (Fig. 2A, which had been found to interact with the APP, or β-amyloid precursor protein (Chow et al. 1996). APP–BP1 showed strong similarity to the amino-terminal region of the Ub-activating enzyme E1; but this presumptive E1-like protein lacked the carboxy-terminal region containing the conserved Cys residue required for the formation of a thioester bond with Ub (Hatfield and Vierstra 1992; Dohmen et al. 1995), indicating that this protein perhaps differs from the DTT-sensitive 66-kD band shown in Figure 1A. In considering that the E1-like

[Key Words: Ubiquitin; ubiquitin-like protein; NEDD8; SUMO-1; cullin-4A; Cdc53]
enzyme for Smt3p is a heterodimer of Aos1p and Uba2p, which correspond to the amino- and carboxy-terminal regions, respectively, homologous to E1 (Johnson et al. 1997), we predicted that a hypothetical protein with a similarity to the carboxy-terminal region of E1 that is capable of interacting with APP-BP1 must be present. We believed that Uba3p deposited in the yeast genome database (PIR accession no. 554087), with unknown function (Hochstrasser 1997), might be a possible candidate. By computer analysis in public databases we found that it encodes a protein that has 42% identity to Ubc12p, a member of the Ub-conjugating enzyme E2 family in yeast (SWISS-PROT accession no. P52491) (Fig. 3A). Their sequence alignment showed that this newly identified protein is a human counterpart of yeast Ubc12p. Therefore, we named it hUbc12 and predicted that hUbc12 was incubated with 35S-labeled hUba3, the bound materials were eluted with 40 mM DTT (left) and subsequently with 30 mM GSH (right). Samples were subjected to SDS–PAGE and stained with silver. Proteins bound specifically to GST–NEDD8 are numbered at right (1–5).

Identification of a NEDD8-conjugating enzyme

We next carried out sequence analysis of the peptide fragments derived from the 22-kD protein 3 that interacted with GST–NEDD8 (see Fig. 1B) and found that three of the fragments obtained had high similarity to a cDNA clone deposited in GenBank (accession no. T48884). We sequenced the cDNA clone entirely and found that it encodes a protein that has 42% identity to Ubc12p, a member of the Ub-conjugating enzyme E2 family in yeast (SWISS-PROT accession no. P52491) (Fig. 3A). Their sequence alignment showed that this newly identified protein is a human counterpart of yeast Ubc12p. Therefore, we named it hUbc12 and predicted that hUbc12 is a presumptive conjugating enzyme for NEDD8. Actually, the presumptive active Cys residue, required for the formation of a thioester bond between Ub and a family of E2 enzymes (Jentsch 1992; Hass and Siepmann 1997), is conserved in hUbc12 (see asterisk in Fig. 3A).

To validate this assumption, we examined whether 35S-labeled hUbc12 forms a thioester linkage with GST–NEDD8 in reticulocyte lysates. When 35S-labeled hUbc12 was incubated with GST–NEDD8, a new larger band was evident besides 35S-labeled hUbc12 but was not observed by treatment with DTT or when the mutated GST–NEDD8(Δ76G) mentioned above was used (Fig. 3B). Moreover, neither GST–Ub nor GST–SUMO-1 linked to hUbc12. These results indicate that hUbc12
presumably acts as an E2-like enzyme specific for the conjugation of NEDD8.

Finally, we attempted to clarify the nature of the 100-kD component linked to 35S-labeled NEDD8 that was resistant to treatment with DTT (see Fig. 1A, left lane). We detected two bands in the GSH eluate from the GST–NEDD8 column (Fig. 1B, right, bands 4 and 5). We found that protein 5, but not 4, a protein of ~120 kD, contained NEDD8 (perhaps GST–NEDD8) by immunoblotting with anti-NEDD8 antibody (data not shown) and therefore used it for chemical sequence analysis. Surprisingly it had a striking homology with Hs–cullin-4A (called simply Cul-4A), reported recently as a member of the cullin/Cdc53 family of proteins (Kipreos et al. 1996; Fig. 4A). We found the cDNA fragment of Cul-4A in our human cDNA bank (Kato et al. 1994) and called it Cul-4A(524C), because it covered the carboxy-terminal 524 amino acid residues, but lacked the short amino-terminal region. We next examined whether Cul-4A(524C) is modified by NEDD8. 35S-Labeled Cul-4A(524C) was modified by GST–NEDD8 in reticulocyte lysates, which was insensitive to treatment with DTT (Fig. 4B). We also found that the 20-kD fragment of Cul-4A covering the carboxy-terminal 171 amino acid residues, designated Cul-4A(171C), was sufficient for the formation of the linkage with GST–NEDD8 (Fig. 4C), indicating that NEDD8 is covalently linked to the carboxy-terminal region of Cul-4A. Moreover, no complex with 35S-labeled Cul-4A was formed when the mutated GST–NEDD8(D76G), GST–SUMO-1, or GST–Ub was used instead of GST–NEDD8, implying that NEDD8 is conjugated to Cul-4A via the carboxy-terminal Gly residue in a manner analogous to ubiquitinylation (Hershko and Ciechanover 1992) and that the presently described novel ligation pathway for NEDD8 did not catalyze formation of a linkage between Cul-4A and Ub or SUMO-1. These findings indicate strongly that Cul-4A is a major target protein for modification by NEDD8. Recently, Kamitani et al. (1997) also found that an ~90-kD NEDD8-modified protein, differing from RanGAP1, was detected in all mammalian cell lines tested. We presume that this protein is Cul-4A or is

Figure 2. Identification of an E1-like APP–BP1/hUba3 heterodimer for activation of NEDD8. (A) Sequence alignment of two peptides of the rabbit 62-kD protein associated with GST–NEDD8 (band 1 in Fig. 1B) with human APP–BP1 (Chow et al. 1996). Partial amino acid sequences of the fragments of the 62-kD band digested with lysylendopeptidase were determined with a protein sequencer and are shown above the APP–BP1 sequences in italics. (X) An unidentified residue. The identical amino acids are boxed in black. (B) Primary structure of human Uba3 (Hs) deduced from the nucleotide sequence of a human cDNA and its sequence alignment with yeast Uba3p (Sc) (PIR accession no. S54087). Amino acid residues are numbered from the amino terminus. Identical amino acids are boxed in black. The motif shown (●) is the consensus sequence for a nucleotide binding site. The asterisk indicates the essential Cys residue conserved in E1 enzymes that becomes linked to Ub in an E1-Ub thioester linkage. (C) Thioester linkage between 35S-labeled hUba3 and GST–NEDD8. 35S-Labeled hUba3 and/or 35S-labeled APP–BP1 were cosynthesized for 60 min at 30°C in vitro in a 5 µl reticulocyte lysate transcription/translation system in the presence of 0.35 µg of unlabeled GST–NEDD8, GST–NEDD8(D76G), GST–SUMO-1, or GST–Ub, and a part of each (2.5 µl) was analyzed as in Fig. 1A. Arrowheads indicate the 35S-labeled hUba3–GST–NEDD8 complex. (D) Complex formation between 35S-labeled (His)6–APP–BP1 and 35S-labeled APP–BP1. 35S-labeled (His)6–APP–BP1 and 35S-labeled APP–BP1 were synthesized individually for 60 min at 30°C in vitro, and 1 µl of each was analyzed (lanes 1–3), as described in Fig. 1A. After samples (5 µl) of 35S-labeled (His)6–APP–BP1 or 35S-labeled APP–BP1 had been incubated with 35S-labeled hUba3 (5 µl) for 30 min at 30°C, half of each sample was applied onto a Ni-chelate column. After the column had been washed with 50 mM Na-phosphate buffer (pH 8.0) containing 0.5 M NaCl, the materials eluted with 100 mM EDTA (lanes 4,5) were analyzed as in Fig. 1A.
in its family of proteins, although the nature of this 90-kD protein has not yet been characterized.

In the present study, we reported a novel modification system of NEDD8, consisting of the APP–BP1/hUba3 complex and hUbc12, which are related to E1 and E2 enzyme, respectively, in the ubiquitinylation pathway. This NEDD8-ligation pathway resembles that of Smt3p/SUMO-1, as mentioned in the introductory section (Johnson and Blobel 1997; Johnson et al. 1997; Schwarz et al. 1998). Intriguingly, quite recently Rub1p, a presumptive yeast homolog of mammalian NEDD8 displaying 59% amino acid identity to human NEDD8, was found to be ligated to target protein through Ula1p/Uba3p and Ubc12p as the E1- and E2-like enzymes, respectively (Liakopoulos et al. 1998). In addition to the similarities in Uba3 and Ubc12 proteins between humans and yeast (Figs. 2B and 3A), APP–BP1 and Ula1p show high sequence similarity, that is, 26% amino acid identity, indicating evolutionary conservation of the post-translational protein-modifying system for Rub1p/NEDD8 and a common role in eukaryotes.

So far, it can be concluded that three different systems operate for activation of Ub and Ub-like proteins: A Ub-activating enzyme, E1, consisting of a single polypeptide, and two heterodimeric E1-like complexes, Aos1p/Uba2p and APP–BP1/hUba3, for activation of Smt3p/SUMO-1 and Rub1p/NEDD8, respectively (this study; Johnson et al. 1997; Liakopoulos et al. 1998; for review, see Hochstrasser et al. 1998), although there is no direct evidence that APP–BP1 and hUba3 form a heterodimer. It is notable that Ub is conjugated by multiple species of Ubc, whereas Smt3p/SUMO-1 and NEDD8 each use a specific conjugating enzyme, Ubc9 and hUbc12, respectively (this study; Johnson and Blobel 1997; Schwarz et al. 1998). Taken together, it is conceivable that three distinct pathways for modification of Ub and Ub-like proteins exist in both yeast and mammalian cells.

Here, we reported that NEDD8 was likely to be conjugated to Cul-4A via the carboxy-terminal Gly residue in a manner analogous to ubiquitinylation (Fig. 4B). Moreover, we observed that the carboxy-terminal domain of Cul-4A, which has been conserved in various species (Kipreos et al. 1996), was sufficient for the conjugation of NEDD8 (Fig. 4C), indicating that the sites accepting NEDD8 exist in this region. One interesting aspect is that Cul-4A is one member of a family of human cullin proteins that have high sequence homologies (Kipreos et al. 1996). So far, 6 species of human cullin family proteins including Cul-1, Cul-2, Cul-3, Cul-4B, and Cul-5 in addition to Cul-4A have been reported...
(Kipreos et al. 1996). Whether NEDD8 is also ligated to other cullin family proteins awaits further study.

The ligation of NEDD8 to Cul-4A was essentially the same as the recently observed conjugation of Rub1p to yeast Cdc53p (homolog of Hs–Cul-1) (Lammer et al. 1998; Liakopoulos et al. 1998). Yeast Cdc53p functions as a common component of a large Ub–protein ligation complex (called SCF Ub ligase) that regulates multiple cellular functions, such as G1/S progression of the cell cycle (Mathias et al. 1996; for review, see Jackson 1996; Hershko 1997; Hoyt 1997), gene expression (Li and Johnston 1997), and methionine biosynthesis (Patton et al. 1998). Of them, it is of interest to consider a relationship between the NEDD8 ligation system and the function of the SCF Ub ligase responsible for the ubiquitinylation of cell-cycle factors involved in the G1/S transition of the cell cycle (for review, see Jackson 1996; Hershko 1997; Hoyt 1997). Recently, Liakopoulos et al. (1998) reported that modification of yeast Cdc53p by Rub1p may affect optimal assembly or function of the SCF complex, although RUB1, UBA1, UBA3, and UBC12, all components of the NEDD8 ligation system, are not essential for viability (Lammer et al. 1998; Liakopoulos et al. 1998). Moreover, a deletion of ENR2 (equivalent to ULA1) is synthetic lethal with temperature-sensitive alleles of cdc34 (i.e., UBC3) and enhances the phenotypes of cdc4, cdc53, and skp1, all of which are components of the SCF Ub–ligase complex, implying that the Rub1p ligation pathway is linked closely to cell-cycle regulation (Lammer et al. 1998). Consistent with this notion, the mutation of hamster SMC, encoding a protein nearly identical to APP–BP1, is responsible for cell-cycle defects in the ts41 cell line (Handel and Weintraub 1992; Hochstrasser 1998). In considering these observations, the novel pathway for the ligation of NEDD8 to Cul-4A described here may provide new insight into understanding of the regulatory mechanism for ubiquitinylation mediated by the SCF Ub ligase.

Materials and methods
Biochemical analysis
Chemical analysis of proteins that interacted with the GST–NEDD8 fused protein was performed as follows: Fifty milliliters of rabbit reticulocyte lysate including 1 mM ATP, 1 mM MgCl2, 0.5 mM DTT, 2 mM PMSF, 20 µg/ml aprotinin, and 20 µM leupeptin was incubated for 15 min at 30°C with 5 mg of GST–NEDD8 and then mixed at 4°C for 1 hr with 2 ml of GSH–Sepharose 4B (Pharmacia). After SDS-PAGE, the protein fragments obtained by digestion with lysylendopeptidase were resolved by reverse-phase HPLC and sequenced by automated Edman degradation, as reported previously (Kawasaki et al. 1990).

Membrane–biological analysis
The cDNAs encoding human NEDD8 (accession no. D23662), SUMO-1, and Cul-4A were found in our human full-length cDNA library prepared with a multifunctional shuttle vector, pKa1 (Kato et al. 1994). Note that the Cul-4A cDNA lacked the short amino-terminal region and so was designated Cul-4A(524C), because it covered the carboxy-terminal 524 amino acid residues (see Fig. 4A). To make deletion mutants of the Cul-4A cDNA, we digested the cDNA with EcoRI to remove the amino-terminal region, which left the carboxy-terminal 171 amino acid residues; thus, we termed the deleted cDNA Cul-4A (171C). The cDNA cloned into pUbba3 and pUbcl2 and T7- and T3-primed transcripts were translated in vitro in a rabbit reticulocyte lysate including 1 mM ATP, 1 mM MgCl2, 0.5 mM DTT, 2 mM PMSF, 20 µg/ml aprotinin, and 20 µM leupeptin was incubated for 15 min at 30°C with 5 mg of GST–NEDD8 and then mixed at 4°C for 1 hr with 2 ml of GSH–Sepharose 4B (Pharmacia). The materials were then loaded onto a column and washed with 20 ml of 50 mM Tris-HCl (pH 8.0) containing 0.5 M NaCl. After washing, the absorbed materials were eluted with 20 mM Tris-HCl (pH 8.0), 40 mM DTT, and subsequently with 20 mM Tris-HCl (pH 8.0), 30 mM GSH. The eluate was concentrated by ultrafiltration and then dialyzed against PBS before use. The translation products were resolved by SDS-PAGE and immunostained with specific antibodies. The antibodies were raised in rabbits against the synthetic peptides from proteins stained with Coomassie Brilliant Blue R-250 after separation by sodium dodecyl sulphate-polyacrylamide gel

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Acknowledgments
We thank Kazuo Kamemura, Akihiko Komuro, Toshiaki Suzuki, Nobuyuki Tanahashi, and our colleagues for advice throughout this study.

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*Genes Dev.* 1998, 12:
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