Biochemical and Biophysical Research Communications **257,** 100–105 (1999)
Article ID bbrc.1999.0339, available online at http://www.idealibrary.com on IDEAL®

Identification of NEDD8-Conjugation Site in Human Cullin-2

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Received February 9, 1999

NEDD8 is a novel ubiquitin-like protein that has been shown to conjugate to nuclear proteins in a manner analogous to ubiquitination and sentrinization. Recently, human cullin-4A was reported to be conjugated by a single molecule of NEDD8. Here, we show that human cullin-2 is also conjugated by a single molecule of the NEDD8. The C-terminal 171-amino-acid residues in human cullin-2 are sufficient for NEDD8-conjugation. In addition, the equivalent C-terminal fragments of other cullins have been shown to be conjugated by NEDD8. Mapping of the NEDD8-conjugation site revealed that Lys-689 in human cullin-2 is conjugated by NEDD8. Interestingly, the Lys residue at position 689 in cullin-2 is conserved in all cullin family members, including human cullin-1, -2, -3, -4A, -4B, and -5 and yeast cullin (Cdc53), suggesting the possibility that other cullin family members are conjugated by NEDD8/Rub1 at a Lys residue of equivalent position.

NEDD8 is a highly conserved 81-amino acid protein, which is 60% identical and 80% homologous to ubiquitin. The message expression of NEDD8 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, such as UCRP (5), sentrin-1/SUMO1 (6–9), sentrin-2 (10), and sentrin-3 (11). These proteins share a common distinction in which the mature form of proteins 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 (12). In the NEDD8-conjugation process, the C-terminal tail of the precursor protein is cleaved off by C-terminal hydrolase such as UCH-L3 (13). The mature form has been shown to conjugate to a large number of nuclear proteins (1). The conjugation is thought to be catalyzed by NEDD8-activation enzyme (E1) and NEDD8-conjugation enzyme (E2) in the presence of ATP. Recently, Osaka *et al.* have identified human Uba3/APP-BP1 and Ubc12 as heterodimeric E1 and E2 in NEDD8-conjugation pathway, respectively (14). They have also reported that human cullin-4A (Cul-4A) is conjugated by a single molecule of NEDD8. In yeast, Cdc53 (also known as yeast cullin) has been shown to be conjugated by a single molecule of Rub1 (an yeast homologue of mammalian NEDD8) (3,4). Cdc53 is a common subunit of the SCF complex, which consists of Skp1, Cdc53, and F-box protein and acts as an E3 ubiquitin ligase (15,16). The Cdc53-containing SCF catalyzes ubiquitination of cell-cycle related proteins such as Sic1 and the ubiquitinated proteins are degraded by proteasome (16–18). Although the precise role of NEDD8/Rub1-conjugation has not been elucidated, NEDD8/Rub1 might modify the function of Cul-4A/Cdc53 and participate in the regulation of cell-cycle progression (3,12). In contrast with ubiquitination, NEDD8/Rub1-conjugation does not target Cul-4A/Cdc53 for proteolytic degradation (3,14).

Human Cul-4A and yeast Cdc53 belong to the cullin family, which includes Cul-1, Cul-2, Cul-3, Cul-4B, and Cul-5 (19). The sequence similarity of the cullin family extends across all protein domains, but is greatest at the C-terminus. Strikingly, these proteins terminate with the Tyr-rich motif Y-X_2-R-X_{6-7}-Y/F-X-A, termed the cullin motif. In addition, the sequences of cullins contain a potential nuclear localization signal (NLS). The biological functions of human cullins are not as clear as that of yeast Cdc53. Human Cul-1 has been shown to associate with Skp1/Skp2 complex to form SCF complex (20–22). This complex appears to function as a ubiquitin E3 ligase. Human Cul-2 binds to von

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0006-291X/99 $30.00

Hippel-Lindau gene product (pVHL) through elongin B and elongin C to form Cul-2-VBC complex and negatively regulate hypoxia-inducible mRNAs such as vascular endothelial growth factor (VEGF) and glucose transporter Glut1 mRNAs (23,24). Human Cul-3 message has been shown to be suppressed by salicylate treatment (25). Human Cul-4A gene has been demonstrated to be amplified and overexpressed in primary breast cancers (26). Thus, cullin family members appear to be involved in a variety of biological functions.

To date, two cullin family members, Cul-4A and Cdc53, have been shown to be conjugated by NEDD8/Rub1 (3,4,14). Amino acid sequences of Cul-4A and Cdc53 also share high similarity with those of other cullins (19), suggesting that other cullins might be conjugated by NEDD8 and their biological functions might be altered by this post-translational modification. In this paper, we chose a well-characterized cullin family member, human Cul-2, as a target and examined whether it could be conjugated by NEDD8. Furthermore, the NEDD8-conjugation site of human Cul-2 was determined.

MATERIALS AND METHODS

Cell lines and culture conditions. COS-M6 cells were maintained in Dullbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum and antibiotics.

Antibodies. 16B12, a mouse monoclonal antibody (mAb) to the peptide sequence YPYDVPDYA of influenza hemagglutinin (HA), was purchased from BAbCo (Richmond, CA) and used for the detection of HA-tagged proteins. Rabbit polyclonal anti-c-myc (specific for the amino acid sequence EQKLISEEDL) antibody was purchased from Santa Cruz Biotechnology.

Plasmid construction and transfection. To express HA-tagged NEDD8 in COS cells, pcDNA3/HA-NEDD8 was transfected as described previously (1). The Cul2-ΔN cDNA was amplified from human brain cDNA library (CLONTECH) by polymerase chain reaction (PCR) using primers Cul-2CF (5′-CTCAGGATCCGCCATGGTTACAACATACCA-3′) and Cul-2R (5′-TCAGGAATTCACGCGACGTAGCTGTATTC-3′). The full-length open reading frame of human Cul-2 cDNA was amplified from the same cDNA library by PCR using primers Cul-2F (5′-CGAAGGATCCATGTCTTTGAAACCAAGAGTAGTAG-3′) and Cul-2R. These PCR products were subcloned into pcDNA3/MH-N. The DNA sequences of inserts were confirmed by an automated sequencer. pcDNA3/MH-N is a mammalian expression vector for N-terminal tagging of both c-myc and polyhistidine epitopes. Using pcDNA3 (Invitrogen) as an original plasmid, pcDNA3/MH-N was made by the insertion of adaptor duplexes encoding MEQKLISEEDLSGSHHHHHH. The MH-tagged proteins can be purified by nickel- or cobalt-charged beads due to polyhistidine sequence in MH-tag. In addition, the tagged proteins can be detected by anti-c-myc-antibody due to c-myc-epitope in MH-tag. To express HA-tagged NEDD8, MH-tagged full-length Cul-2 and its mutants, the plasmids were transfected into COS cells using LipofectAMINE (Life Technologies, Inc.) as described previously (1,27). The transfected cells were harvested for TALON-beads purification 16 h after the incubation.

Site-directed mutagenesis. A Lys-to-Arg substitution was generated in the C-terminal Cul-2 fragment (Cul-2-ΔN) at Lys-621, Lys-689, or Lys-719 by using PCR-based site-directed mutagenesis as described previously (28). Full-length Cul-2 with a mutation at Lys-689 was generated by non-PCR-based site directed mutagenesis in order to avoid PCR-error. Transformer Site-Directed Mutagenesis Kit was purchased from CLONTECH for this purpose. The mutated cDNAs were subcloned into pcDNA3/MH-N.

Cobalt precipitation of MH-tagged Cul-2. To investigate the conjugation of NEDD8 to Cul-2, MH-Cul-2 was coexpressed with HA-NEDD8 in COS cells by the cotransfection method. Since the sequence of the MH tag is EQKLISEEDLSGSHHHHHH, MH-Cul-2 can be purified by cobalt-immobilized resin beads (TALON-beads, CLONTECH). The total cell lysate of the transfectants expressing MH-Cul-2 and HA-NEDD8 was prepared in lysis buffer (20 mM Tris-HCl, 6 M Guanidium-HCl, 100 mM NaCl (pH 8.0)). DNA in the sample was sheared with a 22-gauge needle, and then the lysate was centrifuged at 100,000 × g for 30 min at 15°C. The supernatant was incubated with TALON-beads for 1 h at room temperature. The beads were washed once with lysis buffer, followed by washing twice with washing buffer (20 mM Tris-HCl, 15 mM imidazole, 8 M Urea, 100 mM NaCl (pH 7.0)). Finally, the beads were washed twice with PBS and treated in SDS treating solution for SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Western blot analysis. Protein samples were treated at 45°C for 1 h in 30 μl of 2% SDS treating solution containing 5% β-mercaptoethanol. For SDS-PAGE, 3 μl of the solubilized sample was loaded on 8 or 10% polyacrylamide gel, and proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore) with an electroblotting system (Hoefer Scientific Instruments, Piscataway, NJ) for 2 h at a constant voltage of 100 V. The membrane was blocked at room temperature for 1 h with TBS-T (20 mM Tris (pH 7.5), 137 mM NaCl, 0.1% Tween 20) containing 5% dry milk (Carnation, Glendale, CA), incubated with a primary antibody in TBS-T containing 1% dry milk for 1 h, and washed for 5 min with TBS-T three times. The membrane was then incubated for 1 h with horseradish peroxidase (HRP)-conjugated anti-mouse IgG or anti-rabbit IgG (Santa Cruz). Specific bands were detected using an enhanced chemiluminescence system (Amersham).

RESULTS AND DISCUSSION

NEDD8-conjugation to C-terminal Cul-2 fragment (Cul-2-ΔN). Osaka *et al.* reported that Cul-4A, one of the human Cdc53 homologues, is conjugated with NEDD8 (14). They also showed that the C-terminal 171-amino acid residues of Cul-4A (Cul-4A-ΔN) are sufficient for NEDD8-conjugation. Since human Cul-2 is highly homologous to Cul-4A and is the best characterized human cullin, we examined NEDD8-conjugation to Cul-2 using equivalent C-terminal fragment (Cul-2-ΔN) as a substrate. As shown in Fig. 1A, Cul-2-ΔN also contains the same number of amino acids (171 a.a.) as Cul-4A-ΔN. The cDNA encoding Cul-2-ΔN was isolated by PCR from human brain cDNA library. Then, the C-terminal fragment was tagged with MH-epitope (c-myc + polyhistidine) at the N-terminus and co-expressed with HA-tagged NEDD8 in COS cells. The transfected cells were lysed in denaturing buffer and MH-tagged Cul-2-ΔN was purified with cobalt-coated beads. As shown in Fig. 2, the precipitates of MH-Cul-2-ΔN were analyzed by Western blotting using anti-c-myc antibody to detect Cul-2-ΔN derivatives (lane 7) and anti-HA antibody to detect NEDD8-conjugated Cul-2-ΔN (lane 2). In lane 7, both NEDD8-conjugated and unconjugated forms of Cul-

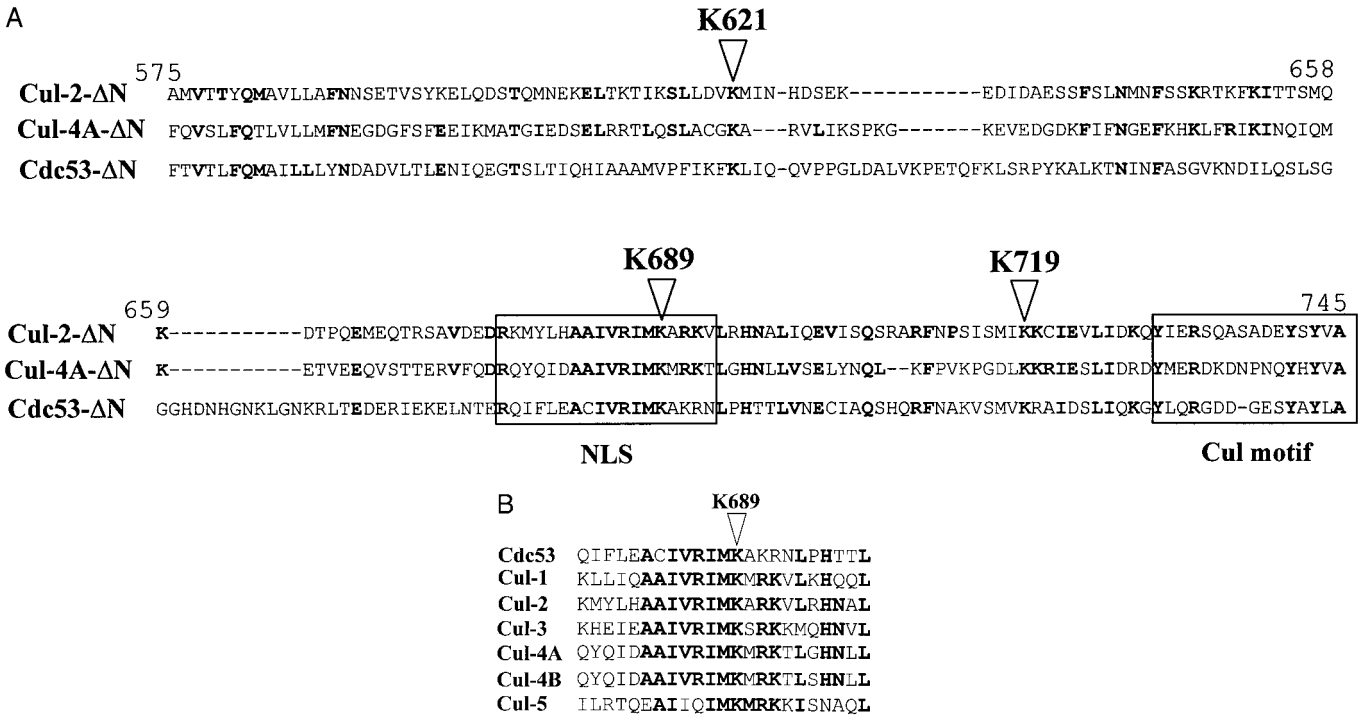

FIG. 1. Amino acid sequences of cullin family members. **(A)** C-terminal alignment of human cullins and yeast Cdc53. Identical amino acids are printed in *bold type*. Lys-621, Lys-689, and Lys-719 of human Cul-2 were indicated by *open triangles*. The nuclear localizing signal (NLS) and cullin motif are *boxed*. **(B)** Alignment at NEDD8-conjugation region of Cul-2. The GenBank accession numbers for yeast Cdc53, human Cul-1, Cul-2, Cul-3, Cul-4A, Cul-4B, and Cul-5 (also known as VACM-1) are U43564, U58087/AF062536, AF126404, AF062537/AF064087, U58090/AB012193, U58091, X81882, respectively.

2-ΔN were detected. More than 90% of the Cul-2-ΔN was detected as conjugated forms showing a 33-kDa doublet band in Western blotting (lane 7). The upper band of the doublet is most likely Cul-2-ΔN modified by HA-NEDD8, and the lower band is most likely Cul-2-ΔN modified by native NEDD8 of COS cells (lane 7). This is further supported by the observation that only the upper band of the doublet was detected by anti-HA antibody in lane 2. The unconjugated form of Cul-2-ΔN was weakly detected as a 26-kDa band (lane 7). Thus, the C-terminal 171-amino acid peptide of Cul-2 is covalently conjugated with NEDD8.

Determination of NEDD8-conjugation site on C-terminal Cul-2. In yeast, Cdc53 (a homologue of mammalian cullins) has been shown to be conjugated by Rub1 (a homologue of mammalian NEDD8) (3,4). In human, the C-terminal 171-amino acid fragment of Cul-4A has been shown to be sufficient for NEDD8-conjugation (14), suggesting that the target Lys residue of NEDD8-conjugation is in the C-terminal 171-amino acids. Since we have found that NEDD8 conjugates to the equivalent C-terminal fragment of Cul-2 (Fig. 2, lane 2 and 7), the target Lys residue should also reside in the C-terminal 171-amino acids of Cul-2. As shown in Fig. 1A, there are 16 Lys residues in the C-terminal sequence of Cul-2. When the C-terminal sequence of Cul-2 is aligned with those of Cdc53 and Cul-4A, only three Lys residues are found to be conserved among these three C-terminal sequences as indicated by open triangles in Fig. 1A. The target Lys residue of NEDD8/Rub1-conjugation most likely belongs to one of these three Lys residues.

In order to determine the position of the target Lys residue, we generated three mutants of Cul-2-ΔN which have a single Lys-to-Arg (K-to-R) substitution at the position of the conserved Lys residue (Lys-621, Lys-689, or Lys-719). The three mutants were tagged with MH-epitope at the N-termini and co-expressed with HA-tagged NEDD8 in COS cells. The transfected COS cells were lysed in the denaturing buffer and MH-tagged Cul-2-ΔN mutants were purified with cobalt-coated beads. As shown in Fig. 2, precipitated mutant proteins were analyzed by Western blotting using anti-c-myc antibody (lane 8-10) and anti-HA antibody (lane 3-5). The NEDD8-conjugation to each mutant was compared with that of wild type (lane 2) or negative control (lane 1). As shown in lane 3-5, two mutants, Cul-2-ΔN(K621R) and Cul-2-ΔN(K719), were conjugated by NEDD8, but not Cul-2-ΔN(K689R). This result indicates that Lys-689 is the actual site of NEDD8-conjugation. Interestingly, Cul-2-ΔN(K621R) (lane 3) was conjugated by NEDD8 as intensely as the wild type of Cul-2-ΔN (lane 2), whereas Cul-2-ΔN(K719R) (lane 5) was much less conjugated. The

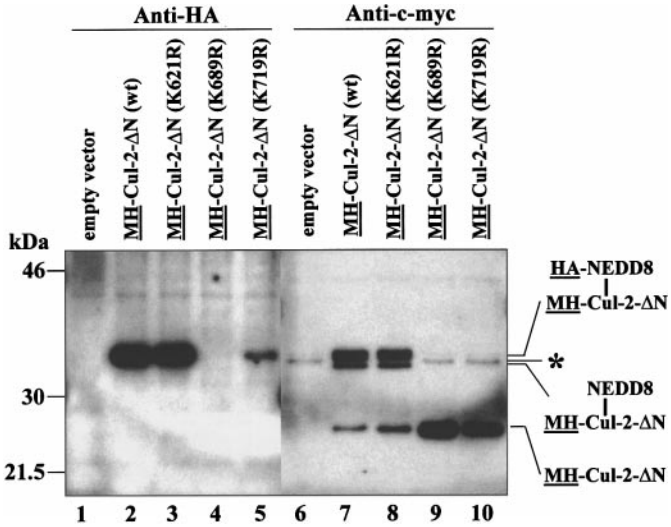

FIG. 2. NEDD8-conjugation to C-terminal fragment of human Cul-2. HA-NEDD8 was co-expressed with wild type (lane 2, 7) or Lys-to-Arg substitution mutant (lane 3-5, 8-10) of Cul-2 C-terminal fragment tagged with c-myc and poly-His (MH-Cul-2-ΔN). MH-Cul-2-ΔN was precipitated by cobalt-charged resin beads. Both modified and unmodified Cul-2-ΔN could be detected by Western blotting using anti-c-myc antibody (lanes 7, 8, 10). Anti-HA antibody could only detect HA-NEDD8-modified Cul-2-ΔN in lane 2, 3, and 5. The identity of each band is indicated on the *right-hand margin*. Non-specific bands are indicated by an *asterisk*.

C-terminal 21-amino acid residues of Cdc53 have been shown to be required for Rub1-conjugation (3). Since Lys-719 is located closely to this sequence in Cul-2 (Fig. 1A), the substitution at Lys-719 might partially affect the NEDD8-conjugation.

NEDD8-conjugation to full-length human Cul-2 at Lys-689. In addition to Cul-2-ΔN, we tested NEDD8-conjugation for both wild type and mutant of full-length human Cul-2. We isolated a full-length cDNA of Cul-2 by PCR from human brain cDNA library. Then, the cDNA was inserted into pcDNA3/MH-N for tagging MH-epitope at the N-terminus. A mutated Cul-2 construct with a single Lys-to-Arg substitution at Lys-689 was also generated by using site-directed mutagenesis. As shown in Fig. 3, the MH-tagged full-length Cul-2 was co-expressed with empty vector as a negative control (lane 1) or HA-tagged NEDD8 (lane 2, 3) in COS cells. The transfected cells were lysed in denaturing buffer and MH-tagged Cul-2 proteins were purified with cobalt-coated beads, followed by Western blotting using anti-c-myc antibody to detect Cul-2 derivatives (upper panel) and anti-HA antibody to detect HA-NEDD8-conjugated Cul-2 (lower panel). When MH-Cul-2 was co-expressed with HA-NEDD8, an unconjugated form of MH-Cul-2 was detected as a major band at the position of ~75-kDa (lane 2 of upper panel). In addition, a NEDD8-conjugated form was weakly detected at the position of ~80-kDa (lane 2 of upper panel). The 80-kDa band was also detected by anti-HA antibody (lane 2 of lower panel), indicating that MH-Cul-2 is conjugated by HA-NEDD8. In contrast with the intensive NEDD8-conjugation to C-terminal Cul-2 fragment, less than 5% of full-length Cul-2 was conjugated by NEDD8 (lane 2 of upper panel). The N-terminal 574-amino acids of full-length Cul-2 might negatively regulate the NEDD8-conjugation to the C-terminal 171-amino acids. When MH-Cul-2 with a single Lys-to-Arg substitution at Lys-689 (MH-Cul-2(K689R)) was co-expressed with HA-NEDD8, an unconjugated form was detected (lane 3 of upper panel). Conjugated form of Cul-2(K689R) was not detected (lane 3 of upper and lower panels), indicating that Lys-689 is the NEDD8-conjugation site in full-length Cul-2. Taken together, the full-length Cul-2 is covalently conjugated with NEDD8 at Lys-689 and the N-terminal domain of Cul-2 might negatively regulate the conjugation.

Pause *et al.* have investigated the subcellular localization of Cul-2 and von Hippel-Lindau gene product (pVHL) by using COS cell expression system (23). Overexpression of Cul-2 alone results in its exclusive localization to the cytosol, even though Cul-2 has a nuclear localizing signal (NLS). In contrast, pVHL localized predominantly to the nucleus. Interestingly, Cul-2 can be relocalized to the nucleus when it is co-expressed with pVHL. The nuclear translocation of Cul-2 might be regulated by the NEDD8-conjugation, because the conjugation site, Lys-689, is located in the

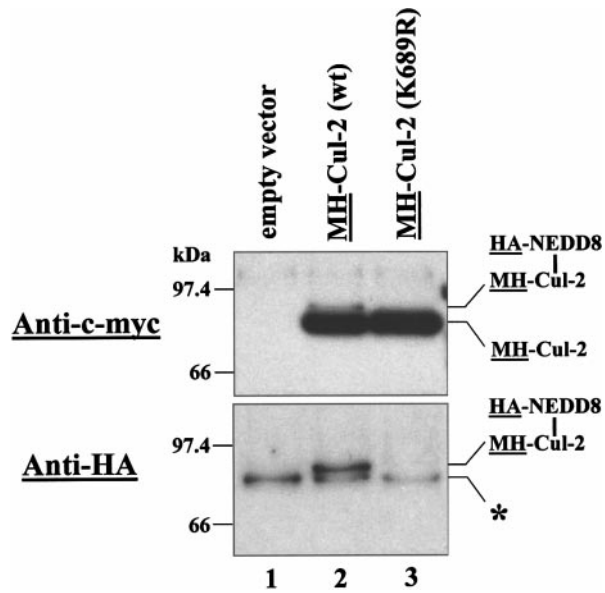

FIG. 3. NEDD8-conjugation to full-length human Cul-2 at Lys-689. HA-NEDD8 was co-expressed with empty vector (lane 1), wild type (lane 2) or K689R-substitution mutant (lane 3) of full-length Cul-2 tagged with c-myc and poly-His (MH-Cul-2). MH-Cul-2 was precipitated by cobalt-charged resin beads. Both modified and unmodified Cul-2 could be detected by Western blotting using anti-c-myc antibody (lane 2, upper panel). Anti-HA antibody could only detect HA-NEDD8-modified Cul-2 in lane 2 of lower panel. The identity of each band is indicated on the *right-hand margin*. Non-specific bands are indicated by an *asterisk*.

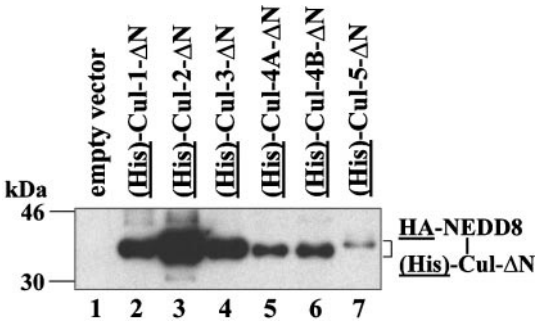

FIG. 4. NEDD8-conjugation to C-terminal fragments of human cullins. HA-NEDD8 was co-expressed with empty vector (lane 1) or poly(His)-tagged cullin C-terminal fragment ((His)-Cul-ΔN) (lane 2-7). (His)-Cul-ΔN was precipitated by cobalt-charged resin beads. HA-NEDD8-conjugated (His)-Cul-ΔN could be detected by Western blotting using anti-HA antibody.

NLS sequence. Interestingly, one of the conjugation sites of sentrin-1 in PML has been also mapped in the NLS sequence (29), suggesting the possibility that sentrinization might regulate the nuclear localization of PML. Thus, Lys residue in NLS might be a common target of ubiquitin-like proteins.

Lys-689 of Cul-2 is conserved in other cullins and NEDD8 conjugates to all human cullins. Human Cul-2 belongs to the cullin family (19). The sequence similarity of the cullin family has been shown to extend across all protein domains, but is greatest at the C-terminus. Interestingly, Lys-689 is a NEDD8-conjugation site in Cul-2 and the Lys residue is also conserved in Cdc53 and Cul-4A (Fig. 1A). As shown in Fig. 1B, this Lys residue is conserved in all cullins including human Cul-1, Cul-2, Cul-3, Cul-4A, Cul-4B, Cul-5, and yeast Cdc53. This suggests the possibility that NEDD8 might modify all cullins at the same position equivalent to Lys-689 of Cul-2. In order to confirm NEDD8-conjugation to other human cullins, we co-expressed HA-tagged NEDD8 in COS cells with poly(His)-tagged human cullin C-terminal fragments. Cul-1-ΔN, Cul-2-ΔN, Cul-3-ΔN, Cul-4A-ΔN, Cul-4B-ΔN, or Cul-5-ΔN consists of 176, 171, 177, 171, 171, or 188-amino acids of C-terminal cullin fragment, respectively. poly(His)-tagged cullin fragments were precipitated by TALON beads as described previously (1,10,11,29). After the precipitation, NEDD8-conjugation of cullin fragments were examined by Western blotting using anti-HA antibody. As shown in Fig. 4, all human cullins could be conjugated by NEDD8. Cul-2-ΔN was strongly conjugated by NEDD8 (lane 3), while Cul-5-ΔN was weakly conjugated (lane 7).

Some of the cullin family members have been shown to assemble functional protein complexes. For example, Cdc53 assembles a ubiquitin E3 ligase, which is called SCF. Human Cul-2 assembles a complex with VBC (pVHL-elongin B-elongin C). Thus, both cullins of Cdc53 and Cul-2 form functional protein-complexes. Since these cullins are modified by Rub1/NEDD8, this post-translational modification might change the function of complexes. Alternatively, it might affect or promote the complex formation. Cul-2(K689R), which cannot be modified by NEDD8, will provide a useful tool to study the biological function of NEDD8-conjugation.

ACKNOWLEDGMENTS

We thank Dr. T. Fukuda-Kamitani for editorial assistance. This work was supported in part by National Institutes of Health Grant HL-45851 (to E.T.H.Y.), the DREAM project (to E.T.H.Y.), an American Heart Association Established Investigator Award (to E.T.H.Y.), and an Arthritis Foundation Irene Dugan Arthritis Investigator Award (to T.K.).

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