A Dominant-negative UBC12 Mutant Sequesters NEDD8 and Inhibits NEDD8 Conjugation in Vivo*

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NEDD8, a novel ubiquitin-like protein, has been shown to conjugate to proteins in a manner analogous to ubiquitination and sentrinization. Recently, human UBC12 was identified as a putative NEDD8 conjugation enzyme (E2). While investigating the in vivo function of UBC12, we found that the point mutant, UBC12(C111S), showed a dominant-negative effect on NEDD8 conjugation. This mutant, with a single Cys-to-Ser substitution at the conserved Cys residue in the E2 family, could specifically inhibit NEDD8 conjugation. We observed the dominant-negative effect on NEDD8 conjugation to substrates, including the C-terminal fragment of cullin-2 (Cul-2-ΔN), full-length cullin-1, and also other uncharacterized target proteins. Interestingly, UBC12(C111S) formed a heterodimeric conjugate with NEDD8. This conjugate was stable under stringent conditions, including 6 M guanidine HCl, 8 M urea, 2% SDS, or 5% β-mercaptoethanol. Our results are consistent with the hypothesis that UBC12(C111S) sequesters the NEDD8 monomer by forming a UBC12(C111S)-NEDD8 conjugate and, in turn, inhibits the subsequent transfer of NEDD8 to its targets. To examine the biological role of NEDD8 conjugation, this dominant-negative form of UBC12 was applied to a cell growth assay. Overexpression of UBC12(C111S) led to inhibition of growth in U2OS and HEK293 cells. Thus, this dominant-negative form of UBC12 could be useful in defining the role of NEDD8 modification in other biological systems.

NEDD8 is a highly conserved 81-amino acid protein, which shares 60% identity and 80% homology with ubiquitin. The expression of 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 nuclear proteins, including the cullin family members (1, 12, 13). APP-BP1 and hUBA3 are homologous to the N- and C-terminal domains of the E1 ubiquitin-activating enzyme, respectively, and together fulfill E1-like functions for Rub1 activation. Rub1 conjugation also requires Ubc12p, a protein related to E2 ubiquitin-conjugating enzymes, which functions analogously to E2 enzymes in Rub1-protein conjugate formation (4).

The pathways of Rub1 and NEDD8 conjugation are also thought to be catalyzed by E1, E2, and E3 in a manner analogous to ubiquitination. In fact, yeast Rub1 conjugation has been shown to require at least three proteins, including Ula1p/Enr2p, Uba3p, and Ubc12p. In vivo, Ula1p/Enr2p and Uba3p are related to the N- and C-terminal domains of the E1 ubiquitin-activating enzyme, respectively, and together fulfill E1-like functions for Rub1 activation. Rub1 conjugation also requires Ubc12p, a protein related to E2 ubiquitin-conjugating enzymes, which functions analogously to E2 enzymes in Rub1-protein conjugate formation (4).

In the mammalian NEDD8 conjugation system, APP-BP1, hUBA3, and hUBC12 have been recently identified as human homologues of Ula1p/Enr2p, Uba3p, and Ubc12p, respectively (12, 15). APP-BP1 and hUBA3 are homologous to the N- and C-terminal domains of the E1 ubiquitin-activating enzyme, respectively. In vitro translated hUBA3 has been shown to conjugate to GST-NEDD8 by thioester linkage in the presence of APP-BP1 (12, 15). In vitro translated hUBC12 has also been shown to conjugate to GST-NEDD8 by thioester linkage in the presence of both APP-BP1 and hUBA3 (15). However, the in vivo functions of these human homologues are still unknown. It has not been shown in vivo that a heterodimeric complex of APP-BP1/hUBA3 has E1-like function in NEDD8 conjugation. Also, hUBC12 has not been shown to function as an E2-like enzyme in vivo. In order to test the function of hUBC12 in vivo, we generated and overexpressed mutants of hUBC12 in COS cells. One of the mutants, UBC12(C111S), clearly showed a dominant-negative effect on NEDD8 conjugation. This result...
strongly supports the hypothesis that hUBC12 plays a role of E2 in NEDD8 conjugation in vivo. In this paper, we characterized the overexpression of UBC12 (C111S) and investigated the mechanism of its dominant-negative effect.

**EXPERIMENTAL PROCEDURES**

**Cell Line and Culture Conditions**—Human embryonic kidney HEK293 cells and human osteosarcoma U2OS cells were purchased from American Type Culture Collection (Manassas, VA). Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics.

**Antibodies**—Monoclonal Ab 12CA5 (Roche Molecular Biochemicals), 16B12 (Covance, Richmond, CA), and rabbit polyclonal HA.11 (Covance) are antibodies to the peptide sequence YPYDVPDYA of influenza hemagglutinin (HA). Mouse anti-RH (specific for the amino acid sequence RGSHHHHH) mAb was purchased from Qiagen (Santa Clara, CA). Mouse anti-c-myc (specific for the amino acid sequence EQKLISEEDL) mAb, 9E10, was purchased from Roche Molecular Biochemicals. Rabbit anti-polycystic-oocyte-NEDD8 antisemur was generated by immunization with a peptide corresponding to amino acids 20–32 (TD-KVERIKVERVE) (1). Rabbit polyclonal antibody to Cul-1 (catalog number 71-8700; recognizing human cullin-1) was purchased from Zymed Laboratories Inc. (San Francisco, CA).

**Plasmid and Transfection**—To express proteins tagged with epitope at the N terminus in COS cells, pcDNA3/HA-N (7), pcDNA3/3HA-N, pcDNA3/RH-N (1), pcDNA3/3RH-N, or pcDNA3/c-myc-N was used as described previously (1). Mammalian expression vector, pcDNA3/3HA-N or pcDNA3/3RH-N, was constructed by inserting triple-repeated epitope of HA or RH into pcDNA3 (Invitrogen, San Diego, CA). By using pcDNA3/3HA-N or pcDNA3/3RH-N, proteins can be tagged with triple-repeated epitope at their N terminus and expressed in mammalian cells. This system allows us to detect proteins expressed at low level, pcDNA3/c-myc-N was constructed for N-terminal tagging of c-myc epitope. c-myc adaptor duplexes were inserted into pcDNA3 for the construction of pcDNA3/c-myc-N. The human cDNA of UBC12, UBA3, UBC9, Cul-2-ΔN (13), or Cul-1 (GenBank™ accession number AF062536) was amplified by polymerase chain reaction (PCR) using appropriate primers from human testis cDNA library (CLONTECH, Palo Alto, CA). The cDNA of green fluorescent protein (GFP) was carried out by PCR from pEGFP-C1 (CLONTECH). These cDNAs were inserted into plasmid vectors described above. The sequence of each cDNA was confirmed by automated DNA sequencing. HA-tagged NEDD8 mutants such as HA-NEDD8(GGGLQRQ), HA-NEDD8(GGGG), HA-NEDD8(C111S), HA-NEDD8(CG111S), and HA-NEDD8(CG111A) were expressed by plasmids constructed previously (1). Plasmids were transfected into COS-M6 cells using LipofectAMINE (Life Technologies, Inc.). The transfected cells were harvested for Western blotting or coalt precipitation 20 h after transfection.

**Site-directed Mutagenesis**—A Cys-to-Ser or Cys-to-Ala substitution was generated in UBC12 at Cys-720, which is a NEDD8 conjugation site (13), by using the same method, a Cys-to-Ser substitution was generated in UBA3 at Cys-93 to make UBC9(C93S) and also in UBA3 at Cys-216 to make UBA3(C216S). In addition to E2 enzymes, Cul-1 was mutated by this method. The Lys-720, which is a NEDD8 conjugation site (13), was substituted to Arg to generate Cul-1(K720R). The mutated cDNAs were subcloned into pcDNA3/RH-N or pcDNA3/c-myc-N.

**Covalent Precipitation of RH- or 3RH-tagged Proteins**—To investigate the conjugation of NEDD8 to Cul-2-ΔN or UBC12(C111S), HA-tagged NEDD8 was co-expressed with RH-tagged Cul-2-ΔN or UBC12(C111S) in COS cells by the co-transfection method. Since the sequence of the RH tag is RGSHHHHHH, RH-tagged protein can be purified by covalently immobilized resin beads (TALON beads, CLONTECH) (13). The total cell lysate of the transfected expressing RH-tagged protein and HA-NEDD8 was prepared in lysis buffer (20 mM Tris-HCl, 6 mM guanidine 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 mM urea, 100 mM NaCl (pH 7.0)). Finally, the beads were washed twice with phosphate-buffered saline and treated for 1 h at 50 °C in sample treating solution containing 2% SDS and 5% β-mercaptoethanol, followed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

**Western Blot Analysis**—Western blotting was performed by the protocol of ECL detection system (Amersham Pharmacia Biotech) as decribed previously (7–9, 13). As a secondary antibody, horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG (Santa Cruz Biotechnology) was used.

**Immunoprecipitation**—The immunoprecipitation method described by Wada et al. (18) was modified as follows. 5 × 10⁶ COS cells were transfected with empty vector or the plasmid for expression of 3HA-Cul-1, followed by trypsination 20 h after transfection. Cell pellets were transferred to liquid nitrogen and lysed in 1.25 ml of RIPA buffer (50 mM Tris-HCl (pH 7.4), 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl) containing 1 mM phenylmethylsulfonyl fluoride (μg/ml), 10 μg/ml aprotinin, 1.5 μg/ml pepstatin, 1 mM Na₃VO₄, and 5 μM N-ethylmaleimide. The cell lysate was performed for 30 min on ice, and DNA in the sample was sheared with a 22-gauge needle. After centrifugation at 100,000 × g for 30 min at 4 °C, the supernatants were added to 10 μg of mouse anti-HA mAb (12CA5) coupled to 25 μl of protein G-Sepharose beads (Amersham Pharmacia Biotech). The bead suspensions were rocked for 3 h at 4 °C. Beads were washed five times with RIPA buffer. The immunoprecipitates were treated with 30 μl of 2% SDS treatment solution containing 5% β-mercaptoethanol, and 3 μl of solubilized samples were loaded onto 8% SDS-PAGE gels. Then, Western blotting was performed using three different antibodies. Rabbit anti-HA antibody (HA.11) and anti-Cul-1 antibody were used to detect 3HA-tagged Cul-1 derivatives. Rabbit anti-NEDD8 antibody was used to detect 3HA-tagged Cul-1 conjugated with endogenous NEDD8.

**Cell Growth Assay**—Naviglio’s method (19) was modified and performed for this assay. U2OS cells (2.3 × 10⁶) or HEK293 cells (1.0 × 10⁶) were plated in a 6-cm dish and transfected by FuGENE 6 (Roche Molecular Biochemicals) either with 5 μg of control empty pcDNA3 vector, pcDNA3/RH-UBC12(C111S), or pcDNA3/RH-UBC12(wild type). After 24 h, the cells were washed twice with phosphate-buffered saline and incubated with fresh medium containing 10% fetal calf serum and 1 mg/ml G418. The medium was changed every 2 days. Ten days after transfection, drug-resistant cells were harvested and counted. The data was statistically analyzed by Fisher's protected least significant difference method.

**RESULTS AND DISCUSSION**

**Dominant-negative Effect of UBC12(C111S) on NEDD8 Modification**—To examine the in vivo function of NEDD8-related enzymes, we first generated two mutants, hUBA3(C216S) and hUBC12(C111S). hUBA3(C216S) has a single Cys-to-Ser substitution at the conserved Cys residue (Cys-216) in the E1 family. Since hUBA3 was a component of the putative E1 enzyme for NEDD8 activation, and its Cys-216 was recently shown to link to NEDD8 by a thioester bond (15), hUBA3(C216S) was expected to be a dominant-negative mutant for the inhibition of the NEDD8 modification pathway. In addition, we generated a mutant, hUBC12(C111S), of putative E2 enzyme for NEDD8 conjugation, hUBC12(C111S) has a single Cys-to-Ser substitution at the conserved Cys residue (Cys-111) in the E2 family. Since hUBC12 appeared to link to NEDD8 via a thioester linkage, hUBC12(C111S) was also expected to show a dominant-negative effect on NEDD8 conjugation. By using hUBA3(C216S) and hUBC12(C111S), we examined whether they have a dominant-negative effect on NEDD8 modification. As shown in Fig. 1A, there was no difference in the NEDD8 conjugation pattern between control (lane 2) and UBA3(C216S)-expressing sample (lane 3), indicating that the expression of UBA3(C216S) did not affect NEDD8 conjugation. The expression level of UBA3(C216S), however, was poor in COS cells. This weak expression might be the reason for its ineffectiveness as a dominant-negative mutant. In contrast, the overexpression of UBC12(C111S) inhibited NEDD8 conjugation (lane 4), because bands of NEDD8-conjugated proteins could not be detected except for the reduced band of p90. Interestingly, a prominent ~33-kDa doublet (indicated by a square bracket) could be detected by both anti-HA mAb (lane 4) and anti-RH mAb (lane 9) in the lysate co-expressing HA-NEDD8 and RH-UBC12(C111S). The doublet was predicted to be a heterodimeric complex, where NEDD8 was bound to...
Inhibition of NEDD8 Conjugation by UBC12 Mutant

FIG. 1. Dominant-negative effect of UBC12(C111S) on NEDD8 conjugation. A, UBC12(C111S) inhibits NEDD8 conjugation. HA-tagged NEDD8 was transiently co-expressed in COS cells with empty vector (lanes 2 and 7), RH-tagged UBA3(C216S) (lanes 3 and 8), RH-tagged UBC12(C111S) (lanes 4 and 9), and both RH-UBA3(C216S) and RH-UBC12(C111S) (lanes 5 and 10). The co-transfection was performed by LipofectAMINE. Twenty hours after the co-transfection, the total cell lysate was prepared from COS cells and analyzed by Western blotting (WB) using anti-HA mAb (16B12) to detect NEDD8 conjugation (lanes 1–5) and anti-RH mAb to show the expression level of UBA3(C216S) and UBC12(C111S) (lanes 6–10). B, UBC12 a(C111A) does not show dominant-negative effect on NEDD8 conjugation. HA-tagged NEDD8 was transiently co-expressed in COS cells with empty vector (lanes 2 and 6), RH-tagged UBC12(C111S) (lanes 3 and 7), and RH-tagged UBC12- C111A) (lanes 4 and 8). The co-transfection was performed by LipofectAMINE. Twenty hours after the co-transfection, the total cell lysate was prepared from COS cells and analyzed by Western blotting using anti-HA mAb (16B12) to detect NEDD8 conjugation (lanes 1–4) and anti-RH mAb to show the expression level of UBC12 mutant (lanes 5–8). A nonspecific band is indicated by an asterisk.

In order to examine the dominant-negative effect of UBC12 mutant on NEDD8 conjugation in more detail, we generated another point mutant, UBC12(C111A), which has a Cys-to-Ala substitution at residue 111. By using the same method as described above, we estimated the dominant-negative effect. As shown in lane 4 of Fig. 1B, NEDD8-conjugated proteins could be detected above the p90 band, indicating that the overexpression of UBC12(C111S) did not synergize with UBC12(C111S).

UBC12(C111S) by a covalent linkage (see below). This 33-kDa protein was not a complex linked through a thioester bond because it was stable in SDS-treating solution containing 5% β-mercaptoethanol. Although the 33-kDa doublet was detected strongly in lane 4, the HA-NEDD8 monomer was not detected, most likely due to the consumption of NEDD8 monomer in the formation of the 33-kDa complex. Thus, the UBC12(C111S) mutant is extremely useful because it sequesters NEDD8 monomers and prevents the formation of NEDD8 conjugates. We also examined whether UBA3(C216S) could synergize with UBC12(C111S) (lanes 5 and 10). No difference could be detected between both samples shown in lanes 4 and 5, suggesting that the overexpression of UBA3(C216S) did not synergize with UBC12(C111S).

In order to examine the dominant-negative effect of UBC12 mutant on NEDD8 conjugation in more detail, we generated another point mutant, UBC12(C111A), which has a Cys-to-Ala substitution at residue 111. By using the same method as described above, we estimated the dominant-negative effect. As shown in lane 4 of Fig. 1B, NEDD8-conjugated proteins could be detected above the p90 band, indicating that the overexpression of UBC12(C111A) (lane 4) did not inhibit NEDD8 conjugation as much as UBC12(C111S) (lane 3). The UBC12(C111S) formed a ∼33-kDa doublet (lane 3) but UBC12(C111A) did not, suggesting that the Ser-111 residue in UBC12(C111S) was involved in the generation of the heterodimeric complex NEDD8-UBC12(C111S). Furthermore, this stable conjugation of UBC12(C111S) with NEDD8 might be involved in its dominant-negative effect on NEDD8 conjugation. UBC12(C111S) might capture unconjugated NEDD8 molecules and prevent the transfer of NEDD8 molecules to their target proteins.

In lanes 4 and 5 of Fig. 1A and lane 3 of Fig. 1B, we detected a 33-kDa doublet. When we made the expression plasmid RH-UBC12(C111S), we did not remove the original start codon from UBC12(C111S) cDNA. Since the cDNA of RH-UBC12(C111S) contains two start codons, the expression plasmid generates RH-tagged UBC12(C111S) and untagged UBC12(C111S) in COS cells. In Fig. 1, A and B, we used total cell lysates expressing both RH-tagged UBC12(C111S) and untagged UBC12(C111S). The upper band of the doublet (lanes 4 and 5 of Fig. 1A, lane 3 of Fig. 1B) is most likely HA-NEDD8 conjugated RH-UBC12(C111S), whereas the lower band is most likely HA-NEDD8-conjugated UBC12(C111S).

Conjugate Formation between NEDD8 and UBC12(C111S)—When HA-NEDD8 was co-expressed with RH-UBC12(C111S) in COS cells, a 33-kDa protein complex was generated (lanes 4 and 9 of Fig. 1A and lanes 3 and 7 of Fig. 1B). The complex was detected by both antibodies against HA tag and RH tag, implying that the complex was formed from HA-NEDD8 and RH-UBC12(C111S). To prove this hypothesis, the complex was purified by TALON precipitation method and characterized by Western blotting. As shown in Fig. 2A, RH-UBC12(C111S) was precipitated as 26-, 31-, and 33-kDa proteins (lane 7). The 26-kDa protein represented RH-UBC12(C111S) unconjugated with HA-NEDD8 because the molecular size was equivalent to RH-UBC12(wild type) (lane 6) and RH-UBC12(C111A) (lane 8). The 33-kDa protein represented RH-UBC12(C111S) conjugated with HA-NEDD8 because it could be detected by anti-HA.
Inhibition of NEDD8 Conjugation by UBC12 Mutant

In order to address the question, RH-UBC12(C111S) was co-expressed in COS cells with wild type or C-terminal deletion mutants of HA-tagged NEDD8. After we confirmed the protein expression level of HA-tagged NEDD8 wild type and mutants in the total cell lysates (upper panel of Fig. 2B), the co-expressed RH-UBC12(C111S) was precipitated from the lysates with TALON beads. Then, the precipitates were analyzed by Western blotting to detect RH-UBC12(C111S) conjugated with endogenous or HA-tagged NEDD8. The NEDD8-UBC12(C111S) complex was resistant to denaturing conditions, including 6 M guanidine HCl, 8 M urea, and 2% SDS (see “Experimental Procedures”) and also stable under reducing conditions such as 5% β-mercaptoethanol. This stability indicated that the complex was formed by a strong covalent bond but not a thioester linkage. Although UBC12(wild type) and UBC12(C111A) shared a similar amino acid residue with UBC12(C111S) at position 111, they did not form the stable complex with NEDD8. These results suggest that the hydroxyl group of Ser-111 in UBC12(C111S) plays an important role in forming the linkage between UBC12(C111S) and NEDD8.

NEDD8 has been shown to utilize its Gly-76 for the thioester linkage with UBC12(wild type) (12). It is not known whether NEDD8 also utilizes the same Gly-76 for the conjugation with UBC12(C111S). In order to address this question, RH-UBC12(C111S) was co-expressed in COS cells with wild type or C-terminal deletion mutants of HA-tagged NEDD8. We also lysed the samples in the denaturing buffer containing 6 M guanidine HCl and used to examine the protein expression level of HA-NEDD8. After SDS-PAGE, Western blotting was performed using anti-HA mAb (upper panel). The transfected cells were also lysed in the denaturing buffer containing 6 M guanidine HCl and used for the purification of RH-UBC12(C111S) by covalent bond. The 31-kDa protein was most likely RH-UBC12(C111S) conjugated by endogenous NEDD8 from COS cells. This was further supported by the observation that anti-HA could not detect the 31-kDa band (lane 3). In contrast with RH-UBC12(C111S) conjugated by endogenous NEDD8 from COS cells, UBC12(C111A) does not. HA-NEDD8 was co-expressed in COS cells with empty vector (lane 1) and UBC12(C111A) (lane 2). In order to examine the dominant-negative effect of UBC12(C111S) on NEDD8 Conjugation to Cul-2 Fragment—In order to examine the dominant-negative effect of UBC12(C111S) on NEDD8 conjugation to a specific substrate, we used the C-terminal 171 amino acids of human cul2 (Cul2-ΔN) that has been shown to be a sufficient substrate for NEDD8 conjugation (13). The Cul2-ΔN can be conjugated by NEDD8 much more intensively than full-length Cul2, even when expressed without promoting proteins for NEDD8 conjugation such as vHL, elongin B, and elongin C (18, 20). As shown in Fig. 3A, we first characterized the NEDD8 conjugation to Cul2-ΔN. When 3RH-tagged Cul2-ΔN was co-expressed with an empty vector, two protein bands (28-kDa and 34-kDa) could be detected in TALON precipitates by Western blotting using anti-RH mAb (lane 2). The upper 34-kDa band was weakly detected by anti-NEDD8 anti-
serum, whereas the lower 28-kDa band was not (lane 5). This result indicated that the 28-kDa protein was an un conjugated form of 3RH-Cul-2-ΔN, and the 34-kDa protein was 3RH-Cul-2-ΔN conjugated with endogenous NEDD8 of COS cells. When 3RH-tagged Cul-2-ΔN was co-expressed with 3HA-tagged NEDD8, three protein bands (28, 34, and 39 kDa) could be detected in TALON precipitates by Western blotting using anti-RH mAb (lane 3). The 39-kDa band was detected by both anti-NEDD8 antisera (lane 6) and anti-HA mAb (lane 9), indicating that it was 3RH-Cul-2-ΔN conjugated with 3HA-NEDD8.

By using this NEDD8 conjugation system of Cul-2-ΔN, the dominant-negative effect of UBC12(C111S) was further tested. As shown in upper panel of Fig. 3B, overexpression of UBC12(C111S) suppressed the generation of 34- and 39-kDa proteins (lane 3). Notably, the 34-kDa protein generation was completely inhibited. In contrast, the generation of these proteins could not be inhibited by control (GFP) (lane 1) or UBC12(wild type) (lane 2). Thus, NEDD8 conjugation to Cul-2-ΔN was inhibited by UBC12(C111S) but not by UBC12(wild type) or GFP. In addition to the dominant-negative effect, we detected conjugates consisting of NEDD8 and UBC12 (lower panel of Fig. 3B). UBC12(C111S) formed conjugates with endogenous NEDD8 of COS cells and co-expressed 3HA-NEDD8 (lane 3), whereas UBC12(wild type) could not (lane 2). Taken together, UBC12(C111S) inhibited NEDD8 conjugation to Cul-2-ΔN by sequestering NEDD8.

Specificity of Dominant-negative Effect of UBC12(C111S) To examine the enzymatic specificity of the dominant-negative effect by UBC12(C111S), we tested whether NEDD8 conjugation to Cul-2-ΔN can be inhibited by a mutant of an E2 enzyme for other ubiquitin-like protein. For this purpose, UBC9, a sentrin-conjugating enzyme (21), was applied to the experimental system described above. We generated UBC9(C93S), which has a Cys-to-Ser substitution at the enzymatic active site (Cys-93), and then tested its dominant-negative effect on NEDD8 conjugation to Cul-2-ΔN. As shown in the upper panel of Fig. 4A, UBC9(C93S) did not inhibit NEDD8 conjugation to Cul-2-ΔN (lane 2) as well as UBC9(wild type) (lane 1) or empty vector (lane 3). Also, UBC9(C93S) did not form any conjugates with endogenous NEDD8 or 3HA-tagged NEDD8 in COS cells (lane 2 of lower panel). Thus, the dominant-negative effect on NEDD8 conjugation to Cul-2-ΔN was not led by an E2 mutant that catalyzes other ubiquitin-like proteins.

In order to show the specificity of dominant-negative effect by UBC12(C111S) in more detail, we examined whether UBC12(C111S) inhibited conjugation between other ubiquitin-like proteins and their substrates. For this purpose, we used sentrin-1 (6–9) and its specific substrate RanGAP1. As shown in the upper panel of Fig. 4B, UBC12(C111S) (lane 3), as well as GFP (lane 1) and UBC12(wild type) (lane 2), did not inhibit sentrinization of RanGAP1. Also, UBC12(C111S) did not form any conjugates with endogenous sentrin-1 or HA-tagged sentrin-1 in COS cells (lane 3 of lower panel).

Finally, we examined whether UBC12(C111S) stably conjugated with ubiquitin or sentrin-1. For this purpose, RH-UBC12(C111S) was co-expressed with 3HA-tagged NEDD8, ubiquitin, or sentrin-1 (Fig. 4C). After we confirmed the expression level of 3HA-tagged proteins in the total cell lysates (upper panel of Fig. 4C), the co-expressed RH-UBC12(C111S) was precipitated from the lysates with TALON beads. Then, the precipitates were analyzed by Western blotting to detect RH-UBC12(C111S) conjugated with 3HA-proteins. As shown in the lower panel of Fig. 4C, UBC12(C111S) formed a stable conjugate only with NEDD8 (lanes 4 and 10) but not with ubiquitin (lanes 5 and 11) or sentrin-1 (lanes 6 and 12). Thus, UBC12(C111S) forms a conjugate with NEDD8 specifically, and its dominant-negative effect is specific to NEDD8 conjugation system.

Dominant-negative Effect of UBC12(C111S) on NEDD8 Conjugation to Full-length Cullin-1— By using Cul-2-ΔN as a substrate, we showed a dominant-negative effect of UBC12(C111S) on NEDD8 conjugation in Fig. 3B. Although the dominant-negative inhibition was clear, the substrate was an “artificial” protein. In order to examine the effect on a “natural” substrate, we chose full-length Cul-1 which was recently shown to be conjugated by NEDD8 (13). Before testing the dominant-negative effect, we characterized NEDD8 conjugation to full-length Cul-1 by using the same assay system as used in Fig. 3B. We expressed 3HA-tagged Cul-1 in COS cells, and we purified 3HA-Cul-1 derivatives from the total cell lysate by immunopre-
Inhibition of NEDD8 Conjugation by UBC12 Mutant

Specificity of dominant-negative effect of UBC12(C111S) does not show dominant-negative effect on NEDD8 conjugation to Cul-2-ΔN. 3RH-Cul-2-ΔN and 3HA-NEDD8 were co-expressed with c-myc-tagged UBC9(wild type (wt)) (lane 1), UBC9(C93S) (lane 2), or empty vector (negative control) (lane 3) in COS cells. The total cell lysates of transfectants were analyzed by Western blotting using anti-RH mAb to detect 3RH-Cul-2-ΔN derivatives (upper panel) and anti-c-myc mAb to detect UBC9 wild type or the mutant (lower panel). B, UBC12(C111S) shows no effect on sentrinization to RanGAP1. RH-tagged RanGAP1 and HA-tagged sentrin-1 were co-expressed with c-myc-tagged GFP (negative control) (lane 1), UBC12(wild type) (lane 2), or UBC12(C111S) (lane 3) in COS cells. The total cell lysates of transfectants were analyzed by Western blotting using anti-RH mAb to detect RH-RanGAP1 derivatives (upper panel) and anti-c-myc mAb to detect GFP or UBC12 (lower panel). C, UBC12(C111S) forms a stable conjugate with NEDD8 but not with ubiquitin and sentrin-1. RH-tagged UBC12(C111S) was co-expressed with 3HA-tagged NEDD8 (lanes 4 and 10), ubiquitin (lanes 5 and 11), or sentrin-1 (lanes 6 and 12) in COS cells. The transfected cells were lysed in the sample treating solution containing 2% SDS and 5% β-mercaptoethanol and used to examine the protein expression level of 3HA-tagged proteins. After SDS-PAGE, Western blotting was performed using anti-HA mAb (16B12) (upper panel). The transfected cells were also lysed in the denaturing buffer containing 6 M guanidine HCl and were used for the purification of RH-tagged UBC12(wild type) or UBC12(C111S) by cobalt-coated beads (TALON) (lower panel). Western blotting analysis was performed by using anti-RH mAb to detect RH-UBC12 derivatives (lanes 1–6) and anti-HA mAb (16B12) to detect RH-UBC12 conjugated by 3HA-tagged ubiquitin or ubiquitin-like proteins (lanes 7–12). As a negative control, empty vector alone (lanes 1 and 7) or RH-UBC12(wild type) (lanes 2 and 8) was also expressed in COS cells.

In this paper, we showed that UBC12(C111S) specifically forms a stable complex with NEDD8 and inhibits NEDD8 conjugation to target proteins such as Cul-2-ΔN, full-length Cul-1, and other uncharacterized proteins. To explain the molecular mechanism of this dominant-negative inhibition, here we propose a potential model.

In NEDD8 conjugation, the following pathway was proposed using mouse anti-HA mAb. The immunoprecipitates were analyzed by Western blotting using rabbit anti-HA antibody and anti-Cul-1 antibody to detect 3HA-Cul-1 derivatives and anti-NEDD8 antibody to detect 3HA-Cul-1 conjugated with endogenous NEDD8. As shown in Fig. 5A, 80- and 85-kDa proteins were immunoprecipitated clearly (lane 2). By using anti-Cul-1 antibody, we confirmed that these two proteins were Cul-1 derivatives (lane 4). The precipitated 85-kDa protein could be detected by anti-NEDD8 antibody, but the 80-kDa protein could not (lane 6). This result indicated that the 85-kDa protein was 3HA-Cul-1 conjugated with endogenous NEDD8, whereas the 80-kDa protein was an unconjugated form of 3HA-Cul-1. Interestingly, more than 90% of 3HA-Cul-1 was tran-siently expressed as an unconjugated form (lanes 2, 4 and 6).

Next, we tested the dominant-negative effect of UBC12(C111S) on NEDD8 conjugation to full-length Cul-1. As shown in upper panel of Fig. 5B, overexpression of UBC12(C111S) suppressed the generation of the 85-kDa protein (lane 4). The expression pattern of full-length Cul-1 (lane 4) was almost identical to that of Cul-1(K720R) (lane 5), which has a Lys-to-Arg substitution at the NEDD8 conjugation site (Lys-720) and cannot be conjugated with NEDD8 (13). In contrast, the generation of the 85-kDa protein could not be inhibited by control (lane 2) or UBC12(wild type) (lane 3). Thus, NEDD8 conjugation to full-length Cul-1 was inhibited by UBC12(C111S) but not by UBC12(wild type) or empty vector expression. In addition, we detected a conjugate consisting of NEDD8 and UBC12 (lower panel of Fig. 5B). UBC12(C111S) formed a conjugate with endogenous NEDD8 of COS cells (lane 4), whereas UBC12(wild type) could not form any conjugates (lane 3). Taken together, UBC12(C111S) inhibited NEDD8 conjugation to Cul-1 by sequestering of NEDD8.

A Model to Explain Dominant-negative Effect of UBC12(C111S)—In this paper, we showed that UBC12(C111S) specifically forms a stable complex with NEDD8 and inhibits NEDD8 conjugation to target proteins such as Cul-2-ΔN, full-length Cul-1, and other uncharacterized proteins. To explain the molecular mechanism of this dominant-negative inhibition, here we propose a potential model.
Inhibition of NEDD8 Conjugation by UBC12 Mutant

To detect 3HA-Cul-1 derivatives and rabbit anti-NEDD8 antibody (lanes 1, 3, 4) or 3HA-Cul-1 (lanes 2, 4, 6) was expressed in COS cells. Total cell lysates were prepared and 3HA-Cul-1 was immunoprecipitated with mouse anti-HA mAb (12CA5). The immunoprecipitates were analyzed by Western blotting using rabbit anti-HA antibody (HA.11) (lanes 1 and 2) and anti-Cul-1 (lanes 3 and 4) to detect 3HA-Cul-1 derivatives and rabbit anti-NEDD8 antibody (lanes 5 and 6) to detect NEDD8-conjugated 3HA-Cul-1. B, UBC12(C111S) inhibits NEDD8 conjugation to full-length Cul-1. 3RH-Cul-1(wild type) was co-expressed with empty vector (lane 2), RH-UBC12(wild type) (lane 3), or RH-UBC12(C111S) (lane 4) in COS cells. As a control, 3RH-Cul-1(K720R), which cannot be conjugated by NEDD8, was co-expressed with empty vector (lane 5). The total cell lysates of transfectants were analyzed by Western blotting using anti-HA mAb (16B12) to detect 3HA-Cul-1 derivatives (upper panel) and anti-RH mAb to detect UBC12 (lower panel). The identity of each band is indicated on the right-hand margin. Previously (12, 15). At the initial step, NEDD8 binds to a thiol site in E1 complex (APP-BP1/UBA3). Then, NEDD8 is transferred to a NEDD8 conjugation enzyme, UBC12. The wild type of UBC12 contains a Cys residue at the position 111. The thiol group of Cys-111 is thought to be an acceptor for the transfer of NEDD8, because this Cys residue is conserved in all UBC family members, and this site is the actual acceptor for the ubiquitin transfer in ubiquitination (22). In the final step, a NEDD8 E3 ligase, such as VBC complex (von Hippel-Lindau protein-elongin B-elongin C) (18, 20), appears to transfer NEDD8 from the NEDD8-UBC12 conjugate to target proteins. Since all human cullins can be conjugated with NEDD8 (13), the NEDD8 conjugation may regulate cullin function and affect cell cycle transition. To examine this hypothesis, we applied UBC12(C111S) to a cell growth assay. As shown in Fig. 6, the overexpression of UBC12(C111S) caused the inhibition of cell growth in U2OS and HEK293 cells. This result implied that UBC12(C111S) inhibited NEDD8 conjugation to cullins and affected cell cycle transition. NEDD8 conjugation may be required for the biological function of cullins. To date, the biological function of NEDD8 is unknown. However, cullin family members have been identified as target proteins of NEDD8 conjugation. Since cullins form E3 ubiquitin ligase such as SCF (26–30) or SCF-like complex (31), NEDD8 might modify the ligase activity. Interestingly, the NEDD8 conjugation site has been recently mapped to a Lys residue in its putative nuclear localizing signal sequence of cullins (19). The NEDD8 conjugation to cullins might control their nuclear translocation. As shown in Fig. 6, UBC12(C111S), which sequesters NEDD8 in vivo, will provide a useful tool to investigate these potential biological functions of NEDD8 conjugation.

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Fig. 6. Growth inhibitory effect of UBC12(C111S) on U2OS and HEK293 cells. U2OS or HEK293 cells were plated in a 6-cm dish and transfected either with control empty pcDNA3 vector, pcDNA3/RH-UBC12(C111S), or pcDNA3/RH-UBC12(wild type). After 24 h, the cells were washed and incubated with fresh medium containing 1 mg/ml G418. The medium was changed every 2 days. Ten days after transfection, drug-resistant cells were harvested and counted. The data were statistically analyzed, and p values were calculated by Fisher’s protected least significant difference method.

Inhibitory Effect of UBC12(C111S) on Cell Growth—Yu et al. (23) showed that human Cul-1 associates with Skp1-Skp2 complex and regulates p21 (CIP1/WAF1) and cyclin D proteins. Feng et al. (24) showed that Cul-2 is required for the G1-to-S phase transition and mitotic chromosome condensation in Caenorhabditis elegans. Cul-3 has also been shown to target cyclin E for ubiquitination and control S phase in mammalian cells (25). Thus, cullin family members regulate cell cycle transition. Since all human cullins can be conjugated with NEDD8 (13), the NEDD8 conjugation may regulate cullin function and control cell cycle transition. To examine this hypothesis, we applied UBC12(C111S) to a cell growth assay. As shown in Fig. 6, the overexpression of UBC12(C111S) caused the inhibition of cell growth in U2OS and HEK293 cells. This result implied that UBC12(C111S) inhibited NEDD8 conjugation to cullins and affected cell cycle transition. NEDD8 conjugation may be required for the biological function of cullins.

To date, the biological function of NEDD8 is unknown. However, cullin family members have been identified as target proteins of NEDD8 conjugation. Since cullins form E3 ubiquitin ligase such as SCF (26–30) or SCF-like complex (31), NEDD8 might modify the ligase activity. Interestingly, the NEDD8 conjugation site has been recently mapped to a Lys residue in its putative nuclear localizing signal sequence of cullins (19). The NEDD8 conjugation to cullins might control their nuclear translocation. As shown in Fig. 6, UBC12(C111S), which sequesters NEDD8 in vivo, will provide a useful tool to investigate these potential biological functions of NEDD8 conjugation.
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