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# UBE1DC1, an Ubiquitin-Activating Enzyme, Activates Two Different Ubiquitin-Like Proteins

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**Abstract** Ubiquitin and ubiquitin-like proteins are known to be covalently conjugated to a variety of cellular substrates via a three-step enzymatic pathway. These modifications lead to the degradation of substrates or change its functional status. The ubiquitin-activating enzyme (E1) plays a key role in the first step of ubiquitination pathway to activate ubiquitin or ubiquitin-like proteins. Ubiquitin-activating enzyme E1-domain containing 1 (UBE1DC1) had been proved to activate an ubiquitin-like protein, ubiquitin-fold modifier 1 (Ufm1), by forming a high-energy thioester bond. In this report, UBE1DC1 is proved to activate another ubiquitin-like protein, SUMO2, besides Ufm1, both in vitro and in vivo by immunological analysis. It indicated that UBE1DC1 could activate two different ubiquitin-like proteins, SUMO2 and Ufm1, which have no significant similarity with each other. Subcellular localization in AD293 cells revealed that UBE1DC1 was especially distributed in the cytoplasm; whereas UBE1DC1 was mainly distributed in the nucleus when was cotransfected with SUMO2. It presumed that UBE1DC1 greatly activated SUMO2 in the nucleus or transferred activated-SUMO2 to nucleus after it conjugated SUMO2 in the cytoplasm. J. Cell. Biochem. 104: 2324–2334, 2008. 

**Key words:** ubiquitin-activating enzyme E1; UBE1DC1; SUMO2; high-energy thioester bond; subcellular localization

Ubiquitin (UB) and ubiquitin-like proteins (UBLs) as a kind of protein modifications are activated in the first step of ubiquitin–proteasome pathway which is a highly complicated, temporally controlled and tightly regulated system of protein degradation [Weekes et al., 2003]. Ubiquitin–proteasome pathway plays major roles in a variety of basic physiological functions involved in cell cycle, apoptosis, antigen presentation, transcription, DNA damage and repair and so on. The system of ubiquitination conjugation is highly specific and selective, and it involves three steps [Willis and Patterson, 2006]. The ATP-dependent ubiquitin-activating enzyme (E1) forms a covalent bond with ubiquitin (UB) or ubiquitin-like proteins (UBLs) in the first step. E1 can be modified by covalent attachment of ubiquitin-like proteins (UBLs), such as Rub1/NEDD8, SUMO/Sentrin/Smt3/Ubl1, or APG8/APG12, which have structural similarity to ubiquitin, although interestingly UBLs are not known to form polymers on target proteins in sentrin, NEDD8 and APG12 modification pathway [Passmore and Barford, 2004]. The function of ubiquitination is known as targeting proteins to the proteasome for degradation, and promoting the down-regulation of membrane proteins through the lysosomal proteolytic system by serving as a signal for internalization into the endocytic pathway [Hicke, 1997; Hershko, 1999; Burger and Seth, 2004]. NEDD8 modification pathway plays an important role in ubiquitin-mediated proteolysis [Yeh et al., 2000]. The sentrinization pathway is essential for transcriptional inhibition and may even function as a ubiquitin antagonist, however it dose not play a role in ubiquitin-mediated proteolysis and degradation [Chupreta et al., 2005; Shi et al., 2006]. In the case of IkBa, sentrinization of IkBa may create a privileged pool of IkBa and is resistant to degradation [Yeh et al., 2000]. Similarly, sentrinization of p53 does not lead to degradation, but enhances its transactivation activity [Yeh et al., 2000].

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The ubiquitin-activating enzyme (E1) is a two-step intramolecular and ATP-dependent reaction to generate a high-energy E1-thiol-ester–ubiquitin intermediate [Passmore and Barford, 2004]. The activated ubiquitin or ubiquitin-like protein moieties are then transferred to E2. El proteins and their encoding genes have been isolated from rabbit, yeast, wheat, human, and mice. Initially, UBE1 localizes in both the nucleus and the cytoplasm and exists as two isoforms E1a (117 kDa) and E1b (110 kDa) in mammals [Handley et al., 1991; Schwartz et al., 1992; Trausch et al., 1993; Stephen et al., 1997]. In recent studies, at least four kinds of E1 (UBE1, AOS1/UBA2, APP-BP1/UBA3, and APG12) exist, each of which has different function in organism. The yeast genome encodes for a single ubiquitin-activating enzyme, UBA1, and inactivation of this gene is lethal [Glickman and Ciechanover, 2002]. In mice, an El gene essential for spermatogenesis has been isolated which is distinct from an El gene expressed in most other tissues, indicating a difference in El expression [Hatfield and Vierstra, 1992]. E1 has several conserved Cys residue, and one of them is the binding site where UB or UBLs conjugate to E1, and only can conjugate to highly specific UB or UBLs in present studies.

A new ubiquitin-activating enzyme, named ubiquitin-activating enzyme E1-domain containing 1 (UBE1DC1) was reported by our colleagues [Dou et al., 2005]. The cDNA of UBE1DC1 is 2654 base pairs in length and contains an open reading frame encoding 404 amino acids with conserved ThiF domain and ATP-binding domain (GXGXXG). The UBE1DC1 gene, consisting of 12 exons, is located at human chromosome 3q22. The result of RT-PCR shows that UBE1DC1 is expressed in most of human tissues [Dou et al., 2005]. The gene encoding UBE1DC1 is highly conserved with the homology in *Caenorhabditis elegans*, *Drosophila melanogaster* and *Arabidopsis thaliana*. UBE1DC1 activates the C-terminally processed ubiquitin-fold modifier 1 (Ufm1) by forming a high-energy thioester bond, and then activated Ufm1 is transferred to its cognate E2-like enzyme, Ufc1, in a similar thioester linkage [Komatsu et al., 2004]. The Cys250 of UBE1DC1 seems to be the most possible active site Cys residue. If an active site Cys residue within UBE1DC1 was changed to Ser, an O-ester bond instead of a thioester bond was formed with its respective modifier protein and the intermediates became stable even under reducing conditions [Komatsu et al., 2004].

In the study, we further described the characteristic of ubiquitin-activating enzyme E1-domain containing 1 (UBE1DC1) in order to better understand its function and find out whether E1 can conjugate to multiple UB or UBLs. We identified that UBE1DC1 also could conjugate to small ubiquitin-like modifier (SUMO2) both in vitro and in vivo by immunological analysis. Furthermore, UBE1DC1 is distributed in the cytoplasm predominantly by subcellular localization, whereas UBE1DC1 is mainly distributed in the nucleus when cotransfected with SUMO2. It comes to the conclusion that UBE1DC1 activated two different ubiquitin-like proteins, and UBE1DC1 especially activated SUMO2 in the nucleus or transferred activated-SUMO2 to nucleus after it had activated SUMO2 in the cytoplasm.

## MATERIALS AND METHODS

### Reverse Transcription-PCR Analysis

Human multiple tissue cDNA templates reversed transcription products from multiple human cells by our lab, including the fetal brain, pancreas, liver, embryonic kidney, and embryonic kidney T, were used as templates in the RT-PCR analysis. The sequences of human *UBE1DC1A* (full length of UBE1DC1, NM_024818) specific primer pair were E1RT10P 5′-ATGGCGGAGTCTGTGGAGC-3′ (the primer binding sites are 1–19) and E1RT10M 5′-CATCCATGTTTGTCCAAGTTCA-3′ (the primer binding sites are 600–622), and *UBE1DC1B* (isoforms of UBE1DC1A, NM_198329) specific primer pair were E1RT11P 5′-ATGGCATTGAAACGAATGGGAA-3′ (the primer binding sites are 1–22), and E1RT11M 5′-CATCCATGTTTGTCCAAGTTCA-3′ (the primer binding sites are 433–455). PCR mixtures were heat-denatured at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing for 30 s at 55°C and extension at 72°C for 1 min. And G3PDH primers were used to amplify the same cDNAs as a control. Then, the PCR products were detected by electrophoresis with on 1.3% Metaphor agarose gel (FMC).

### Construction of cDNA Library and Recombination Plasmids

A cDNA library was set up in modified pBluescript II SK(+) vector with human fetal brain mRNA purchased from Clontech [Dou

et al., 2005]. The cDNA encoding human UBE1DC1A and UBE1DC1B were obtained by PCR from human fetal brain cDNA Library. They were then cloned into pET28b, pCMV-Myc, and pGEFP-C1 vector (Clontech). A point mutation of Cys to Ala at position 250 (or 194) was generated by PCR-based site-directed mutagenesis. Then UBE1DC1A$^{C250A}$ and UBE1DC1B$^{C194A}$ were cloned into pET28b. Similarly, the cDNA encoding human SUMO2 was cloned into pET28b and pCMV-HA vector. The cDNAs encoding human UB, SUMO1, ISG15, UBL5, URM1, ATG8, and Ufm1 were cloned into pET28b vector. All the primers used for these PCRs are listed in Table I. Positive recombinant plasmids were sequenced by ABI3730 sequencer.

### Expression and Purification of Recombinant Proteins

Recombination pET28b-UBE1DC1A, pET28b-UBE1DC1B, pET28b-SUMO2, pET28b-UB, pET28b-SUMO1, pET28b-ISG15, pET28b-UBL5, pET28b-URM1, pET28b-ATG8, pET28b-Ufm1, pET28b-UBE1DC1A$^{C250A}$, and pET28b-UBE1-DC1B$^{C194A}$ plasmids were respectively transformed into *Escherichia coli* Rosetta (Novagen). Then, they were added to LB medium supplemented containing 34 μg/ml chloromycetin and 20 μg/ml kanamycin, and incubated at 37°C until the culture reached $OD_{600}$ of 0.6–0.8. Induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) in final concentration for 8 h at 25°C, cells were harvested by centrifugation at 5,000 rpm for 5 min at 4°C. Subsequently cells were suspended in cool sodium phosphate buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, and 10 mM Imidazole, pH 7.4) at 10 ml/g wet weight. After treated by ultrasonic disintegrator, the cleaved product was harvested by centrifugation at 13,200 rpm for 20 min at 4°C. Then the supernatant was applied to Ni-NTA His-Bind Superflow (Qiagen). After washed with 50 mM sodium phosphate Buffer (10 mM $NaH_2PO_4$, 300 mM NaCl, and 50 mM Imidazole, pH 8.0), the target proteins were eluted by 250 mM Elution Buffer (5 mM Tris–HCl, 300 mM NaCl, and 250 mM imidazole, pH 8.0). Eluted fractions were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and bands were visualized by Coomassie blue staining by previously described standard procedures. The concentration of proteins in extracts was determined by the method of [Bradford, 1976], using bovine serum albumin (BSA) as the protein standard.

**TABLE I. The Oligonucleotides Primers of Recombination Plasmid**

| Primer | Orientation | Nucleotide sequence | RE site |
|---|---|---|---|
| pET28b-UBE1DC1A | Sense | 5′-CTAGCTAGCATGGCGGAGTCTGTGGAGC-3′ | *Nhe*I |
| | Antisense | 5′-CCGCTCGAGAGAAATACAATATATCCCAGTCC-3′ | *Xho*I |
| pET28b-UBE1DC1B | Sense | 5′-CTAGCTAGCATGGCATTGAAACGAATGGGAA-3′ | *Nhe*I |
| | Antisense | 5′-CCGCTCGAGAGAAATACAATATATCCCAGTCC-3′ | *Xho*I |
| pEGFP-C1-UBE1DC1A | Sense | 5′-CCGCTCGAGCGATGGCGGAGTCTGTGGAGC-3′ | *Xho*I |
| | Antisense | 5′-CGGGATCCCTACATATTCTTCATTTTGGCCA-3′ | *Bam*HI |
| pEGFP-C1-UBE1DC1B | Sense | 5′-CCGCTCGAGCGGCATTGAAACGAATGGGAATTG-3′ | *Xho*I |
| | Antisense | 5′-CGGGATCCCTACATATTCTTCATTTTGGCCA-3′ | *Bam*HI |
| pCMV-Myc-UBE1DC1A | Sense | 5′-CGGAATTCGGGCGGAGTCTGTGGAGCGC-3′ | *Eco*RI |
| | Antisense | 5′-CCGCTCGAGAGAAATACAATATATCCCAGTCC-3′ | *Xho*I |
| pCMV-Myc-UBE1DC1B | Sense | 5′-CGGAATTCGGGCATTGAAACGAATGGGAATTG-3′ | *Eco*RI |
| | Antisense | 5′-CCGCTCGAGAGAAATACAATATATCCCAGTCC-3′ | *Xho*I |
| pET28b-SUMO2 | Sense | 5′-CTAGCTAGCATGGCCGACGAAAAGCCCAA-3′ | *Nhe*I |
| | Antisense | 5′-CCGCTCGAGCTAACCTCCCGTCTGCTGTTGG-3′ | *Xho*I |
| pCMV-HA-SUMO2 | Sense | 5′-CGGAATTCGGGCCGACGAAAAGCCCAAGG-3′ | *Eco*RI |
| | Antisense | 5′-CCGCTCGAGCTAACCTCCCGTCTGCTGTTGG-3′ | *Xho*I |
| pET28b-UB | Sense | 5′-CTAGCTAGCATGCAGATCTTCGTGAAAACCC-3′ | *Nhe*I |
| | Antisense | 5′-CCGCTCGAGCTAACCACCTCTCAGACGCAGG-3′ | *Xho*I |
| pET28b-SUMO1 | Sense | 5′-CTAGCTAGCATGTCTGACCAGGAGGCAAAA-3′ | *Nhe*I |
| | Antisense | 5′-CCGCTCGAGCTAACCCCCCGTTTGTTCCTGAT-3′ | *Xho*I |
| pET28b-ISG15 | Sense | 5′-CGGCTAGCGGCTGGGACCTGACGGTG-3′ | *Nhe*I |
| | Antisense | 5′-CCCTCGAGCTAGCCTCCCCGCAGGCGCA-3′ | *Xho*I |
| pET28b-UBL5 | Sense | 5′-CTAGCTAGCATGATCGAGGTTGTTTGCAACG-3′ | *Nhe*I |
| | Antisense | 5′-CCGCTCGAGGGCAGGAAGATGAGGATTCTC-3′ | *Xho*I |
| pET28b-URM1 | Sense | 5′-CTAGCTAGCATGGCTGCGCCCTTGTCAG-3′ | *Nhe*I |
| | Antisense | 5′-CCGCTCGAGGCCCAGGCCCAGAGAAGG-3′ | *Xho*I |
| pET28b-ATG8 | Sense | 5′-CTAGCTAGCATGAAGTTCGTGTACAAAGAAGA-3′ | *Nhe*I |
| | Antisense | 5′-CCGCTCGAGCCAGCTCAGGGGCAGCAG-3′ | *Xho*I |
| pET28b-Ufm1 | Sense | 5′-CTAGCTAGCATGTCGAAGGTTTCCTTTAAGAT-3′ | *Nhe*I |
| | Antisense | 5′-CCGCTCGAGTCGTATGTTCCAAGTAGCAGAT-3′ | *Xho*I |

### In Vitro Activity Assay

The thioester formation reaction system contained 5 μg UB or UBLs (SUMO1, SUMO2, ISG15, UBL5, Ufm1, URM1 and ATG8) purified proteins, 10 μg UBE1DC1A or UBE1DC1B purified protein and reaction buffer with 47.5 mM Tris–HCl (pH 7.5), 2 mM ATP, 5 mM $MgCl_2$ and 0.2 mM DTT. The mixture was incubated for 60 min at 37°C and analyzed followed by SDS–PAGE (12% polyacrylamide gradient), and then transferred onto nitrocellulose membrane (Schleicher and Schuell). The membranes were incubated in primary anti-His mouse antibody (Clontech), followed by secondary HRP-conjugated anti-mouse IgG antibody (Calbiochem). Each experiment was repeated at least three times.

### Immunological Analysis In Vivo

AD293 cell line which is derived directly from the HEK293 cell line but has been transfected with a gene that can improve cell adherence [Zhang et al., 2006], grew in Dulbecco's modified Eagle's medium (DMEM) containing 10% newborn calf serum (NCS), and then were seeded and transfected with recombinant pCMV-Myc-UBE1DC1A, pCMV-Myc-UBE1DC1B, and pCMV-HA-SUMO2 plasmids (4 μg/DNA) using Lipofectamine reagent (Invitrogen) in the tissue culture plates for 30 h. Subsequently, the cells were solubilized in lysis buffer with protease inhibitors set (Roche) and immunoprecipitated with protein A/G Plus agarose (Santa Cruz) and anti-Myc mouse antibody. Then, the mixture was washed three times, and solubilized in lysis buffer followed by electrophoresis in SDS-polyacrylamide gel. After the target protein were transferred onto nitrocellulose membrane (Schleicher and Schuell), the membranes were incubated in primary anti-Myc or anti-HA mouse antibody (Clontech), followed by secondary HRP-conjugated anti-mouse IgG antibody (Calbiochem). Subsequently, the expressed proteins were visualized by the stabilized ECL reagent (Promega).

### Analysis of UBE1DC1A and UBE1DC1B Subcellular Localization

$2 \times 10^5$ AD293 Cells were transfected with recombination pEGFP-C1-UBE1DC1A and pEGFP-C1-UBE1DC1B plasmids (1 μg/DNA) using Lipofectamine reagent (Invitrogen) on coverslips in six-well tissue culture plates for 24 h. Then, cells were washed by PBS three times and fixed in PBS containing 4% paraformaldehyde and 0.2% Triton X-100 for 15 min. Subsequently, cells washed by PBS three times were counterstained with 2.5 mg/ml DAPI (4′, 6′-diamidino-2-phenylindole) for 10 min, and then washed with PBS three times. Similarly, $2 \times 10^5$ AD293 Cells were cotransfected with recombination pEGFP-C1-UBE1DC1A and pCMV-HA-SUMO2 (or pEGFP-C1-UBE1DC1B and pCMV-HA-SUMO2) plasmids (1 μg/DNA) on coverslips in six-well tissue culture plates for 24 h. Then, cells were fixed in PBS containing 4% paraformaldehyde and 0.2% Triton X-100 for 15 min, and were blocked with TBST containing 5% BSA for 1 h. Subsequently, cells were incubated in primary anti-HA mouse antibody (Tiangen) for 1 h, followed by secondary Cy3-Conjugated Affinipure Goat Anti-Mouse IgG (H + L) antibody (Product Tech Group) for 1 h. After washed by PBS three times, cells were counterstained with 2.5 mg/ml DAPI for 10 min, and then washed with PBS three times. The cells were viewed using R2100 Confocal Microscope (BioRad) with appropriate filters and the photos were captured with software Leica DC viewer. In addition, the blank pEGFP-C1 was used as control.

## RESULTS

### Identification of the Human UBE1DC1A and UBE1DC1B Gene

By large-scale cDNA sequencing, we isolated a novel isoforms (named UBE1DC1B) of full length gene *UBE1DC1A* from the human fetal brain. The cDNAs of *UBE1DC1A* and *UBE1DC1B* contain an open reading frame encoding 404 amino acids and 348 amino acids respectively with analogical conserved ThiF domain and ATP-binding domain (GXGXXG) by blast analysis. The *UBE1DC1B* gene consisting of 11 exons without the first one (Fig. 1A), is located at human chromosome 3q22 by the BLAST-N (http://www.ncbi.nlm. nih.gov/blast) searching against the nr database of GenBank. The result of RT-PCR shows that *UBE1DC1A* is expressed in most human tissues [Dou et al., 2005]. The result of the BLAST-N searching against the EST (Expressed sequence tags) database of GenBank shows that *UBE1DC1B* is also expressed in most of the human tissues, including fetal liver, testis, fetal eyes, lens, eye

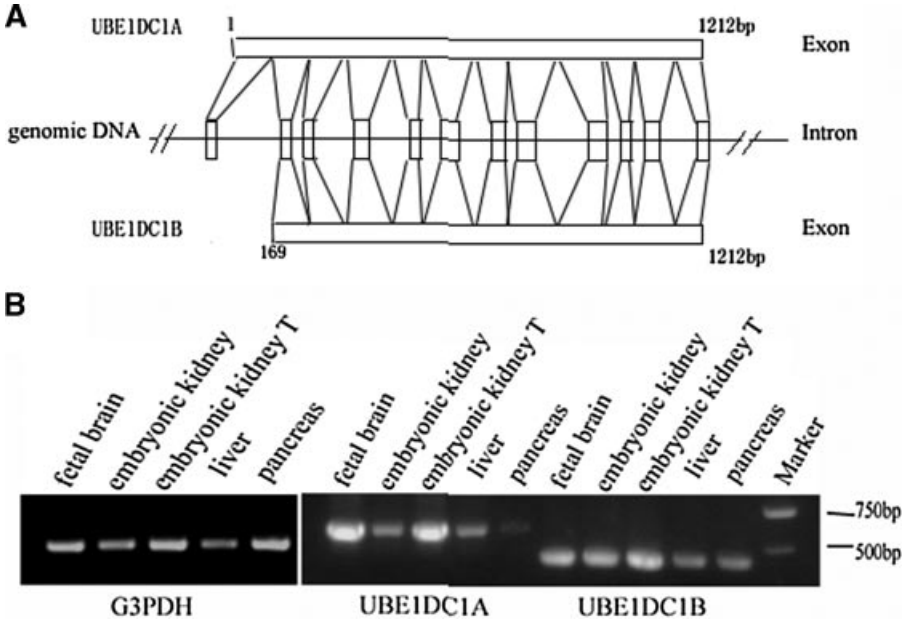


**Fig. 1.** Identification of *UBE1DC1A* and *UBE1DC1B* gene. **A**: Genomic structures of *UBE1DC1A and UBE1DC1B* gene. The UBE1DC1A gene consisting of 12 exons is located at human chromosome 3q22. The UBE1DC1B gene, which lacks the N-terminal of UBE1DC1 and consists 11 exons, is less than UBE1DC1A in length and located at human chromosome 3q22. **B**: Tissue distribution of *UBE1DC1A* and *UBE1DC1B* mRNA. Reverse transcription-PCR analysis of human cDNA for UBE1DC1A, UBE1DC1B and G3PDH. Prenormalized cDNAs from five human tissues were reversed from human cells and employed as a template in PCR reactions containing UBE1DC1A, UBE1DC1B or G3PDH specific primers.

anterior segment, optic nerve, retina, retina foveal and macular, RPE and choroid. To observe the tissue expression and distribution of the *UBE1DC1A* and *UBE1DC1B* cDNA, reverse transcription-PCR analysis was used by amplification of human cDNA. The products of *UBE1DC1A* and *UBE1DC1B* were expressed in fetal brain, pancreas, liver, embryonic kidney, and embryonic kidney T. The length of the transcription produce *UBE1DC1A* and *UBE1DC1B* in these tissues were the anticipative 622 and 454 bp, respectively (Fig. 1B). The results implicated that UBE1DC1B was a novel splice variant of full length gene of *UBE1DC1A*.

## Expression and Purification of Recombinant Proteins

The UBE1DC1A, UBE1DC1B, UBE1DC1A$^{C250A}$, UBE1DC1B$^{C194A}$, SUMO2, UB, SUMO1, ISG15, UBL5, URM1, ATG8, and Ufm1 genes were cloned into pET28b vector and then were expressed in *E. coli* Rosetta. The soluble target proteins were purified by chromatography of Ni-NTA His-Bind Superflow (Qiagen), and detected by 12% or 15% SDS–PAGE, respectively. All of the purified recombinant proteins were eluted in the 250 mM Elution buffer (Fig. 2).

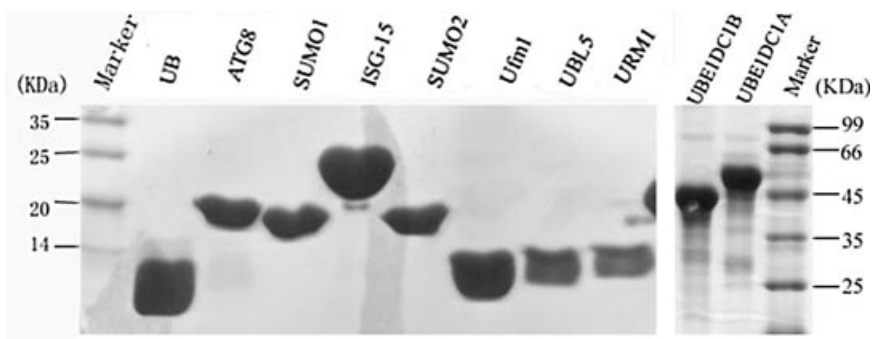


**Fig. 2.** SDS–PAGE of purified recombinant proteins. Lanes 2–11 respectively were UB, SUMO2, Ufm1, ATG8, SUMO1, ISG15, UBL5, URM1, UBE1DC1B, and UBE1DC1A purified proteins. All of the purified recombinant proteins were eluted in 250 mM Elution buffer.

### Activity of UBE1DC1A and UBE1DC1B In Vitro

UBE1DC1 that conjugated to ubiquitin-fold modifier 1 (Ufm1) was reported. Therefore, whether UBE1DC1 forms an intermediate complex with other ubiquitin-like proteins was investigated. The thioester formation assay was performed using recombinant proteins expressed in *E. coli*. First, we identified that UBE1DC1A could not activate UB, SUMO-1, ISG-15, UBL5, URM1 and ATG8, but could activate SUMO2 and Ufm1 in vitro by SDS–PAGE and immunoblotting with anti-His antibody in preliminary experiment (Fig. 3). Then, we subsequently determined whether UBE1DC1can activate SUMO2 in vitro. Recombinant 6His-tagged UBE1DC1A, 6His-tagged UBE1DC1A$^{C250A}$, 6His-tagged UBE1DC1-B$^{C194A}$, 6His-tagged UBE1DC1B and 6His-tagged SUMO2 were purified, respectively, mixed and incubated in reaction buffer and then analyzed by SDS–PAGE and immunoblotting with anti-His antibody at either reducing or nonreducing conditions. The intermediate complex of 6His-UBE1DC1A-6His-SUMO2 was clearly observed when the mixture was applied at nonreducing conditions (Fig. 4A, lanes 4 and 9), but it was not observed when ATP or 6His-SUMO2 was excluded from the mixture (Fig. 4A, lanes 1–3 and 5), or when the mixture was loaded in the presence of a reducing agent dithiothreitol (DTT) (Fig. 4A, lane 7). The intermediate was not detected, when 6His-UBE1DC1A$^{C250A}$ was incubated with SUMO2 (Fig. 4A, lane 10). Similarly, the intermediate complex of 6His-UBE1DC1B-6His-SUMO2 was observed when the mixture was applied at nonreducing conditions (Fig. 4B, lanes 4 and 9), whereas it was not observed in other conditions (Fig. 4B, lanes 1–3 and 5–8). The intermediate was not observed, when 6His-UBE1DC1B$^{C194A}$ was substituted for 6His-UBE1DC1B in the mixture (Fig. 4B, lane 10). The results presumably indicate that UBE1-DC1 conjugates to SUMO2 and forms an intermediate with SUMO2 in Cys 250 (or Cys194) by high-energy thioester bond in vitro.

### Conjugation of SUMO2 to UBE1DC1A and UBE1DC1B In Vivo

It indicated that UBE1DC1 conjugated to SUMO2 and formed an intermediate with SUMO2 by high-energy thioester bond in vitro, so we further detect whether UBE1DC1 could conjugate SUMO2 in vivo. Myc-UBE1DC1A and Myc-UBE1DC1B with HA-SUMO2 were expressed in AD293 cells. Each cell lysate was prepared by immunoprecipitate with anti-Myc antibody. When Myc-UBE1DC1A was coexpressed with HA-SUMO2, the intermediate of Myc-UBE1DC1A–HA-SUMO2 complex shifted to higher molecular weight than expressed Myc-UBE1DC1A and could be detected by immunoblotting analysis with anti-HA or anti-Myc antibody (Fig. 5A and B). Similarly, the intermediate of Myc-UBE1D-C1B–HA-SUMO2 complex was observed by immunoblotting with anti-HA (Fig. 5A) and anti-Myc antibody (Fig. 5B), when Myc-UBE1DC1B was coexpressed with HA-SUMO2. The high-energy thioester bond of the

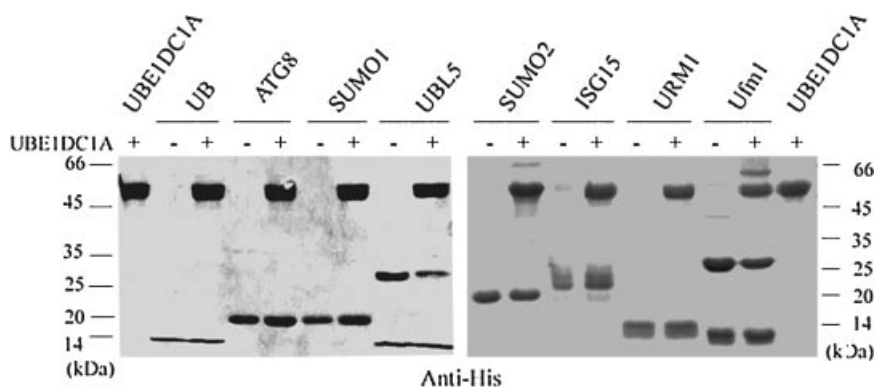


**Fig. 3.** Activating assay of UBE1DC1A with UBLs in vitro. Purified recombinant 6His-UBE1DC1A (10 μg) (lanes 1, 3, 5, 7, 9, 11, 13, 15, and 17) was incubated for 60 min at 37°C with some of the following: 5 μg of purified several members of UBLs (UB, ATG8, SUMO1, UBL5, SUMO2, ISG15, URM1, and Ufm1) (5 μg) recombinant proteins (lanes 2–15) and 2 mM ATP (lanes 3, 5, 7, 9, 11, 13, 15, and 17). Those lanes were all analyzed by SDS–PAGE and immunoblotting with anti-His antibody.

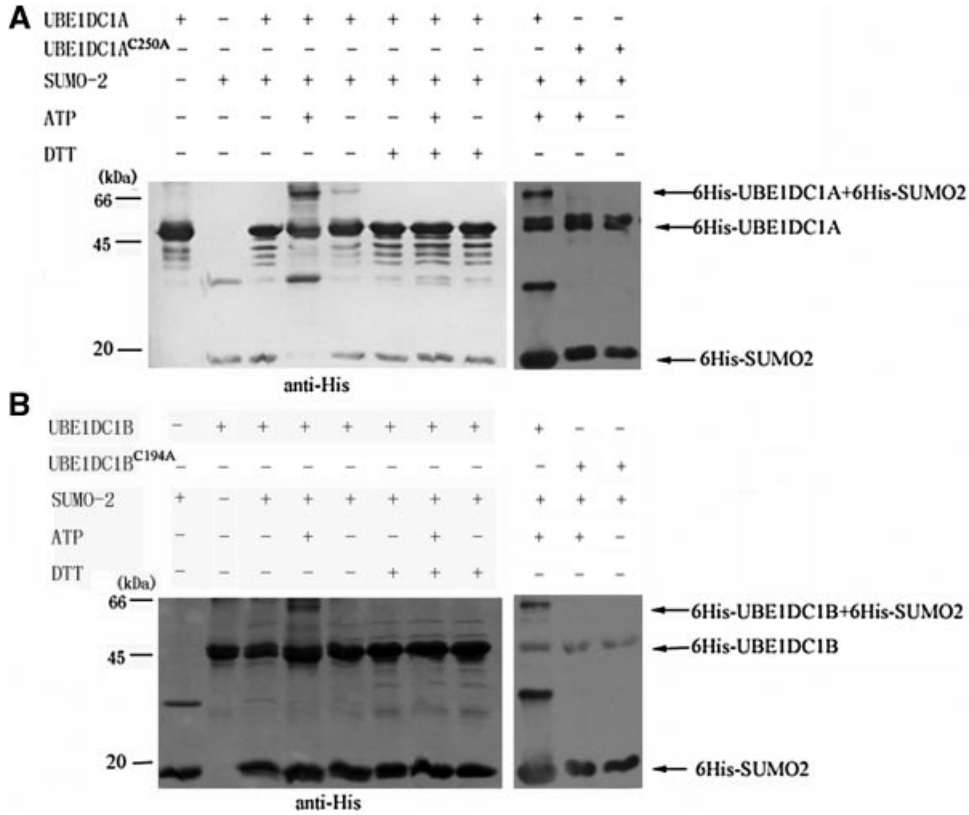


**Fig. 4.** Activating assay of UBE1DC1A and UBE1DC1B with SUMO2 in vitro. **A**: Purified recombinant 6His-UBE1DC1A (10 μg) (lanes 1, 3–9) was incubated for 60 min at 37°C with some of the following: 5 μg of purified recombinant 6His-SUMO2 (5 μg) (lanes 3–8), 2 mM ATP (lanes 4, 7 and 9), and 100 mM DTT (lanes 6–8). Lane 2 was purified recombinant 6His-SUMO2 (5 μg) incubated for 60 min at 37°C. Purified recombinant 6His-UBE1DC1A$^{C250A}$ (10 μg) (lanes 10 and 11) was incubated for 60 min at 37°C with some of the following: 5 μg of purified recombinant 6His-SUMO2 (5 μg) (lanes 10 and 11), 2 mM ATP (lanes 10). **B**: Purified recombinant 6His-UBE1DC1B (10 μg) (lanes 2–9) was incubated for 60 min at 37°C with some of the following: 5 μg of purified recombinant 6His-SUMO2 (5 μg) (lanes 3–8), 2 mM ATP (lanes 4, 7, and 9), and 100 mM DTT (lanes 6–8). Lane 1 was Purified recombinant 6His-SUMO2 (5 μg) incubated for 60 min at 37°C. Purified recombinant 6His-UBE1DC1B$^{C194A}$ (10 μg) (lanes 10 and 11) was incubated for 60 min at 37°C with some of the following: 5 μg of purified recombinant 6His-SUMO2 (5 μg) (lanes 10 and 11), 2 mM ATP (lanes 10). Those lanes were all analyzed by SDS–PAGE and immunoblotting with anti-6His antibody.

intermediate Myc-UBE1DC1A–HA-SUMO2 and Myc-UBE1DC1B–HA-SUMO2 complex would be interrupted, when cell lysate was prepared with DTT (Fig. 5). These results indicate that the intermediate is presumably a complex with exogenetic UBE1DC1 and SUMO2, and UBE1DC1 forms an intermediate with SUMO2 by high-energy thioester bond in vivo.

### Subcellular Localization of UBE1DC1A and UBE1DC1B

It is reported that the ubiquitin-activating enzyme (E1) is distributed both in the nucleus and the cytoplasm. To determine whether the ubiquitin-activating enzyme E1-domain containing 1 (UBE1DC1) is the same as E1s, the pEGFP-C1-UBE1DC1A (or pEGFP-C1-UBE1DC1B) and pCMV-HA-SUMO2 fusion plasmid was cotransfected into AD293 cells. After 24 h of expression, the GFP-tagged UBE1DC1A and UBE1DC1B were detected mainly in the cytoplasm (Fig. 6), whereas UBE1DC1A (or UBE1DC1B) was distributed predominantly in the nucleus and diffusely in the cytoplasm when they cotransfected with SUMO2 (Fig. 6). The control protein is distributed throughout the whole cell, as expected.

## DISCUSSION

Under many circumstances, the changing of cellular proteins' physical and physiological properties is due to chemical modifications, and an important sort of protein modifications is adding appendages [Dou et al., 2005]. Ubiquitin and ubiquitin-like proteins are a kind of such

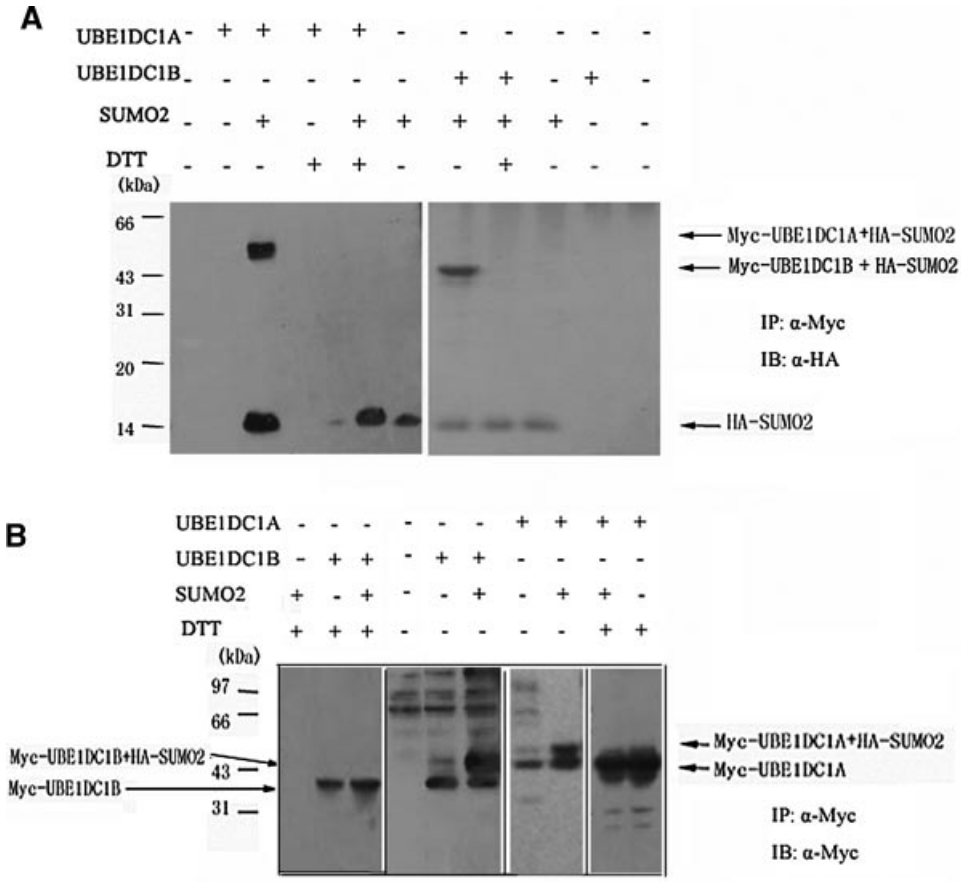


**Fig. 5.** Immunoblotting analysis of UBE1DC1 in vivo. **A**: Myc-UBE1DC1A (or Myc-UBE1DC1B) was coexpressed with HA-SUMO2 and analyzed by immunoprecipitate with anti-Myc mouse antibody and immunoblotting with anti-HA mouse antibody. Myc-UBE1DC1A was coexpressed with HA-SUMO2 at nonreducing or reducing conditions with anti-HA antibody in lanes 3 and 4, respectively. Lane 6 and 9 were expressed-SUMO2 protein without Myc-UBE1DC1A, and lane 2 was expressed-UBE1DC1A without SUMO2. Myc-UBE1DC1B was coexpressed with HA-SUMO2 at nonreducing or reducing conditions with anti-HA antibody in lanes 7 and 8, respectively. Lane 6 was expressed-SUMO2 protein without Myc-UNE1DC1B, and lane 10 was expressed-UBE1DC1B without SUMO2. Lanes 1 and 11 was control with cell lysate. **B**: Myc-UBE1DC1A (or Myc-UBE1DC1B) was coexpressed with HA-SUMO2 and analyzed by immunoprecipitate and immunoblotting both with anti-Myc antibody. Myc-UBE1DC1A was coexpressed with HA-SUMO2 at nonreducing or reducing conditions with anti-Myc antibody in lane 8 or 9, respectively. Lanes 7 and 10 was expressed-UBE1DC1A protein without HA-SUMO2 at nonreducing or reducing conditions respectively. Myc-UBE1DC1B was coexpressed with HA-SUMO2 at nonreducing or reducing conditions by with anti-Myc antibody in lane 6 or 3, respectively. Lanes 5 and 2 was expressed-UBE1DC1B protein without HA-SUMO2 at nonreducing or reducing conditions, and lane 1 was expressed-SUMO2 protein at reducing conditions. Lane 4 was control with cell lysate.

molecular modifiers, sharing similar steps. The ubiquitination system requires ubiquitin-activating enzyme E1s to activate ubiquitin or ubiquitin-like proteins. For example, the homologs of ubiquitin activating enzyme E1 (UBE1, also named UBA1) in sentrin and NEDD8 modification pathway are AOS1/UBA2 and APPBP1/UBA3 complexes, respectively [Yeh et al., 2000]. AOS1 and APPBP1 are similar to the N-terminal of UBA1, while UBA2 and UBA3 are similar to the C-terminal of UBA1. It is clear that the sentrinization, APG12 and NEDD8 modification define other enzymatic pathways distinct from ubiquitination [Yeh et al., 2000].

In this study, UBE1DC1 has conserved ThiF domain and ATP-binding domain (GXGXXG) which is similar to UBA1, and the UBE1DC1 sequence displays more than 35% identity to the C-terminal of UBE1. We identified that UBE1DC1 conjugated to SUMO2 both in vivo and in vitro. UBE1DC1 did not activate UB, SUMO1, ISG15, UBL5, URM1 and ATG8, but activated SUMO2 and Ufm1 in vitro (Figs. 3 and 4). When Myc-UBE1DC1A and

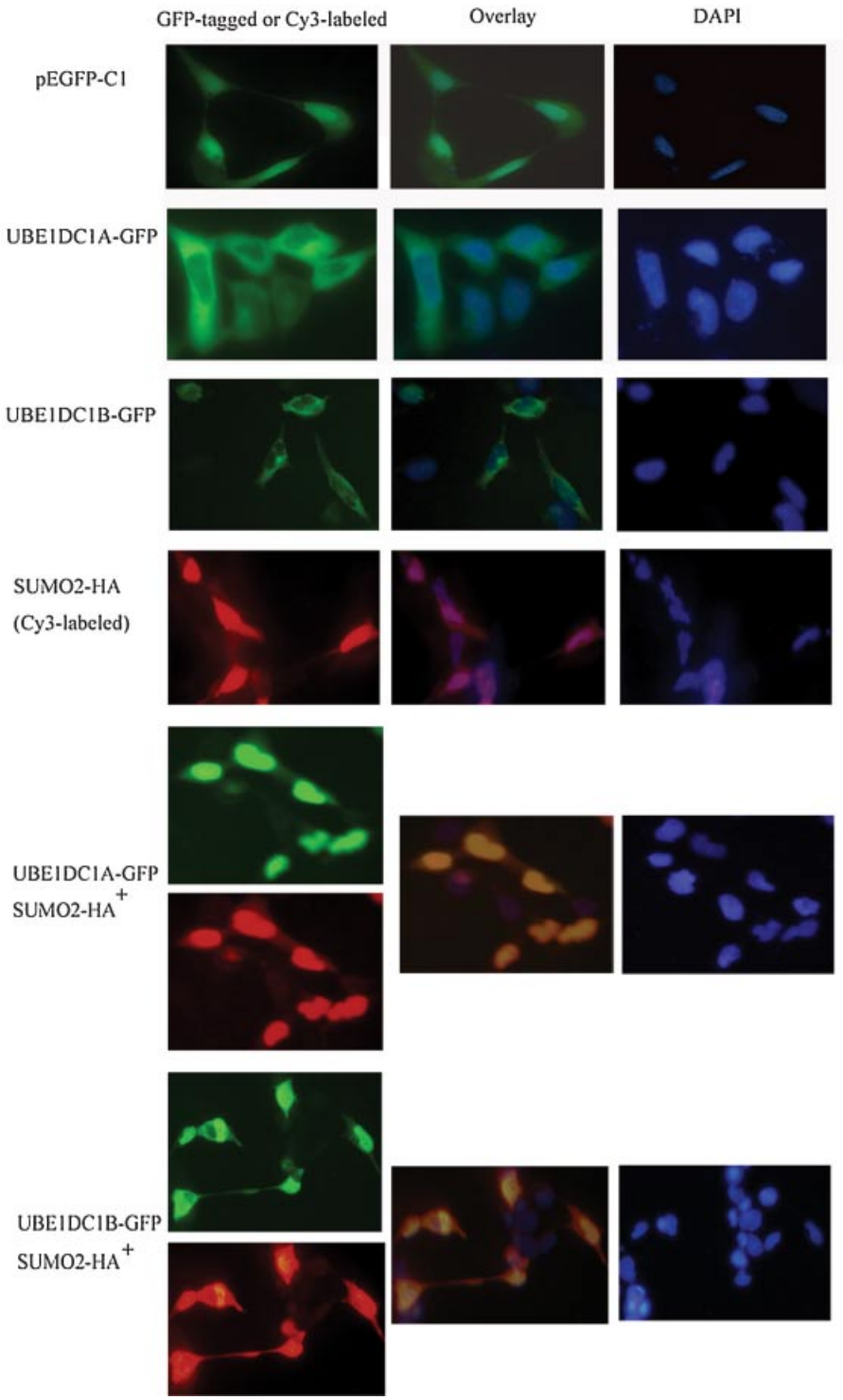


**Fig. 6.** Subcellular location of hUBE1DC1A-GFP and hUBE1DC1B-GFP fusion protein with or without SUMO2 in transfected AD293 cells. The left panels showed fusion protein visualized under blue or green fluorescence. The middle panels were produced by merging several kinds of signals from fusion proteins and nucleuses. The right panels showed the corresponding nuclear staining with DAPI. The hUBE1DC1A-GFP and hUBE1DC1B-GFP fusion proteins were distributed predominantly in the cytoplasm, whereas hUBE1DC1A-GFP (or hUBE1DC1B-GFP) was distributed in the nucleus predominantly when they were cotransfected with SUMO2. The pEGFP control is distributed throughout the whole cell as expected. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Myc-UBE1DC1B were coexpressed with HA-SUMO2 respectively, the intermediate shifted to greater molecular weight as showed by immunoblotting with anti-HA and anti-Myc antibody. Similarly, the intermediate of 6His-UBE1DC1A-6His-SUMO2 and 6His-UBE1D-C1B-6His-SUMO2 complexes were clearly observed when the mixture was applied at nonreducing conditions. But the intermediate complexes were not observed when the mixtures were loaded in the presence of a reducing agent DTT, or when 6His-UBE1DC1A$^{C250A}$ (6His-UBE1DC1B$^{C194A}$) was incubated with SUMO2. So, UBE1DC1 could activate SUMO2 by forming a high-energy thioester bond at the active site Cys250, and the N-terminal of UBE1DC1 was not essential for activating SUMO2, because UBE1DC1B which lacks the N-terminal of UBE1DC1 also could activate SUMO2. We further determined that the GFP-tagged UBE1DC1A and UBE1DC1B were detected mainly in the cytoplasm, whereas UBE1DC1A (or UBE1DC1B) was distributed in the nucleus predominantly and diffusely in the cytoplasm when they were cotransfected with SUMO2 (Fig. 6). The results presumably indicated that the N-terminal of UBE1DC1 was unrelated to localization in vivo, and UBE1DC1 especially activated SUMO2 in the nucleus or transferred activated-SUMO2 to nucleus after it conjugated SUMO2 in the cytoplasm. Moreover, it was reported that UBE1DC1 activated the C-terminally processed ubiquitin-fold modifier 1 (Ufm1) by forming a high-energy thioester bond [Komatsu et al., 2004]. So UBE1DC1 activated SUMO2 and Ufm1 by the same way as UBE1, not as a heterodimeric E1 complex as formed by AOS1/Uba2 and APP-BP1/Uba3 [Pelzer et al., 2007].

Initially, it was reported that ubiquitin-activating enzyme E1 activated single species of UB or UBL proteins. For example, UBE1 and UBE1L2 only activate ubiquitin, and then activated-ubiquitin is transferred to ubiquitin-conjugating enzyme (E2) in a similar thioester linkage. APPBP1–UBA3 (APPBP1 is homologous to the N-terminal half of UBA1, whereas UBA3 is homologous to the C-terminal half) is the heterodimeric E1 enzyme for the ubiquitin-like protein NEDD8 [Passmore and Barford, 2004], and can activate NEDD8/Rub1 to be transferred to UBC12. Moreover, NEDD8 exhibits more than 45% sequence identity with Rub1. Apg7, an E1 enzyme in the Apg12 conjugation system activates two different ubiquitin-like proteins, Apg12 and Apg8, and assigns them to specific E2 enzymes, Apg10 and Apg3, respectively [Ichimura et al., 2000]. AOS1/UBA2 (also named SAE1/SAE2) in sentrinization pathway cannot activate ubiquitin or NEDD8, but can activate sentrin (like SUMO1, SUMO2 and SUMO3) and deliver it to UBC9. The SUMO proteins for modification pathway are activated by specific E1-activating (SAE1/SAE2) [Chupreta et al., 2005]. Moreover, it was reported that UBE1DC1 activated Ufm1 to Ufc1 (E2-like enzyme) [Komatsu et al., 2004]. The SUMO2 protein exhibits no significant similarity with Ufm1 protein by aligning two sequences using Blast, while SUMO1 protein exhibits 44% sequence identity with SUMO2 and SUMO3 proteins. Therefore, UBE1DC1 can activate two different UBLs, both Ufm1 and SUMO2, through the studies of our laboratory and Komatsu's laboratory. It presumably indicated that UBE1DC1 mainly conjugated SUMO2 in nucleus or transferred activated-SUMO2 to nucleus after it activated SUMO2 in the cytoplasm, and activated-SUMO2 and activated-Ufm1 conjugated to distinct cohorts of E2s in ubiquitination pathway. Unexpected complexity was revealed that dual E1 (UBE1L2) activation systems for ubiquitin differentially regulate E2 (Use1, Cdc34A, and Cdc34B) enzyme charging. Uba6 is required for charging a previously uncharacterized Uba6-specific E2 (Use1), whereas Ube1 is required for charging the cell-cycle E2s Cdc34A, and Cdc34B [Jin et al., 2007]. It might be another complexity that UBE1DC1 presumably is a new kind of ubiquitin-activating enzyme E1 which can activate two different ubiquitin-like proteins and conjugates SUMO2 and Ufm1 to distinct cohorts of E2s. Further research is required to determine other interacting partners with UBE1DC1 to understand the mechanism and function of UBE1DC1.

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