Identification of a Novel Isopeptidase with Dual Specificity for Ubiquitin- and NEDD8-conjugated Proteins*

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Covalent conjugation of proteins by ubiquitin or ubiquitin-like molecules is an important form of post-translational modification and plays a critical role in many cellular processes. Similar to the concept of phosphorylation and dephosphorylation, these conjugates are regulated by a large number of deconjugating enzymes. Here, we report the cloning of a 2,141-base pair DNA fragment from human placenta cDNA library by a strategy that involves expressed sequence tag data base searching, polymerase chain reaction, and rapid amplification of cDNA ends. Nucleotide sequence analysis revealed that the cloned cDNA contains an open reading frame of 1,143 base pairs encoding a novel protease, USP21, which is composed of 381 residues with a calculated molecular mass of 43 kDa. The human USP21 gene is located on chromosome 1q21 and encodes a member of the ubiquitin-specific protease family with highly conserved Cys and His domains. The activity and specificity of USP21 were determined by using a COS cell expression system in vivo. We showed that USP21 is capable of removing ubiquitin from ubiquitinated proteins as expected. Furthermore, USP21 is capable of removing NEDD8 from NEDD8 conjugates but has no effect on Sentrin-1 conjugates. As expected from its biochemical activity, overexpression of USP21 has a profound growth inhibitory effect on U2OS cells. Thus, USP21 is the first ubiquitin-specific protease shown to have dual specificity for both ubiquitin and NEDD8 and may play an important role in the regulation of cell growth.

Ubiquitin (Ub) is a highly conserved 76-amino acid protein involved in a variety of cellular functions, including regulation of intracellular protein degradation, cell cycle progression, and signal transduction (1, 2). This small polypeptide is covalently attached to cellular proteins by an enzymatic cascade that begins with ATP-dependent activation of Ub-activating enzyme (E1) to form a high energy E1-Ub thiol ester. The activated Ub is then transferred to the SH group of a Cys residue in the active site of a Ub-conjugating enzyme (E2 or Ubc). Finally, in the presence of a Ub-protein ligase (E3), the C-terminal Gly residue of Ub is conjugated via an isopeptide bond to the ε-amino group of a Lys residue on the target protein. Sequential conjugation of Ub to a previously conjugated Ub by linking the ε-amino group of one Ub lysine residue to the C terminus of another Ub results in the formation of polyubiquitin chain, which targets proteins for degradation by the 26 S proteasome (3, 4). Regulation of these processes requires precise timing and specific recognition of different substrates at appropriate cellular milieu, thought to be mediated by a combinatorial usage of different E2 and E3 enzymes and by a large family of deubiquitinating enzymes (5, 6).

Deubiquitinating enzymes are ubiquitin-specific thiol-proteases that cleave either linear ubiquitin precursor proteins or ubiquitin conjugates. Some deubiquitinating enzymes appear to perform an editing function, which controls the fidelity of the conjugation process, thus preventing inappropriate deconjugation of cellular proteins. Two families of deubiquitinating enzymes have been identified on the basis of in vitro activities and/or sequence identity. The ubiquitin C-terminal hydrolases (UCHs) are small (approximately 25 kDa) thiol proteases that share amino acid sequence identity and cleave esters and amides from the C terminus of ubiquitin (5). Unrelated in sequence to the UCHs are the ubiquitin-specific proteases (UBPs), a large family of proteases differing greatly in length but characterized by sequence similarity in several regions: the Cys box, the His box, and six other blocks of amino acid sequence identity (5).

In recent years, ubiquitin-like proteins, such as UCRP/ISG15, Sentrin-1, and NEDD8, have greatly expanded the scope of post-translational protein modification (7–13). These ubiquitin-like proteins are translated in precursor form, with one or more amino acids following a Gly-Gly dipeptide, which forms the C terminus of the mature protein. The proteases specific for Sentrin-1 and Smt-3 are structurally different from the Ubiquitin-specific UBPs and the UCHs (14, 15). UCH-L3, originally identified as a ubiquitin-specific hydrolase, has been shown by our laboratory to possess a C-terminal hydrolase activity for NEDD8 (8, 16, 17). However, the isopeptidase responsible for NEDD8 deconjugation has not been identified. Here, we report the identification of a novel protease, USP21 (ubiquitin-specific protease 21), which is not only specific for ubiquitin conjugates but also functions as a NEDD8-specific isopeptidase.

EXPERIMENTAL PROCEDURES

Identification of USP21—To clone USP21, we used a similar strategy as previous described (14, 18, 19). We first performed a TBLASTN search of the human EST data base using the conserved sequences previously identified as the Cys and His boxes (5). This search identified...
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transfection of COS-M6 Cells—COS-M6 cells were grown to approxi-
ately 80% confluency in Dulbecco's modified Eagle's medium contain-
ing 10% fetal bovine serum, 1% antibiotics, and 10% fetal bovine
serum. Cells were transfected using LipofectAMINE (Life
Technologies, Inc.), and unincorporated nucleotide was removed by
hybridization and hybridization were performed under conditions rec-
ommended by the supplier. The blot was dried and autoradiographed
with an x-ray film and intensifying screen at −70 °C for 3 days.

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serum at 37 °C. Cells were transfected using LipofectAMINE (Life
Technologies, Inc.). 5 μg of plasmid and 25 μg of lipid were added to 500
μl of Dulbecco's modified Eagle's medium (serum- and antibiotic-free)
and then incubated for 30 min at room temperature. After this time, the
lipid/DNA mix was diluted 5-fold with Dulbecco's modified Eagle's
medium (containing 10% fetal bovine serum, 1% antibiotics, and
10% fetal bovine serum). After incubation for another 24 h, cells were washed
phosphate-buffered saline and incubated with fresh 10% serum-containing
medium. After incubation for another 24 h, the cells were washed twice with
phosphate-buffered saline and collected in 10% serum-containing
medium. After incubation for another 24 h, cells were washed twice with
phosphate-buffered saline, rinsed off the plates with 0.05% trypsin,
0.2% EDTA in phosphate-buffered saline, and then split at different dilutions
and subcultured into medium containing 500 μg/ml G418. After 14 days of
incubation drug-resistant colonies were counted after trypan blue
staining.

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Cell Growth Assay—U2OS cells (8 × 103) were plated in a 10-cm dish
and transfected by LipofectAMINE either with 5 μg of control empty
pcDNA3 vector or of the same vector containing the USP21 sequence in
the same orientation. After 24 h, the cells were washed twice with
phosphate-buffered saline and resuspended in Dulbecco's modified Eagle's
containing medium. After incubation for another 24 h, cells were washed twice with
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Northern Blot Analysis of Human USP21—To study the expression
pattern of the USP21 gene, a commercially available Northern blot
(CLONTECH, Palo Alto, CA) containing 8 μg of poly(A)+ RNA from
eight different tissues was used. The full-length coding region for
USP21 was used as template. The cDNA probe was labeled with
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RESULTS AND DISCUSSION

Molecular Cloning of the Human USP21 cDNA—Using a combined PCR cloning and EST data base search technique described previously (14, 18, 19), a 2141-base pair cDNA fragment was cloned from a human placenta cDNA library. The nucleotide and deduced amino acid sequence of the cloned cDNA are shown in Fig. 1. The cloned cDNA has the following features: 1) a translation initiation codon (ATG) is located at nucleotide positions 727–729, preceded by a 726-base pair 5′-untranslated sequence. The ATP triplet is preceded by an in-frame stop codon at nucleotide positions 514–516. (2) A translation termination codon TGA is located at nucleotide positions 1869–1872, followed by a 826-base pair 3′-untranslated region of 269 nucleotides. At the 3′ terminus a potential polyadenylation signal, AATAAA, is present at nucleotide 2128. (3) The cloned fragment encodes a 381-amino acid polypeptide, called USP21 (approved by the Human Genome Nomenclature Committee). The calculated molecular mass for USP21 is 43,031 Da. The amino acid sequence of USP21 was compared with sequences in several data bases using the BLAST network service at the National Center for Biotechnology Information. The results indicated that USP21 is a novel member in the ubiquitin-specific protease family as shown in Fig. 2. Similar to DUB-1, a deubiquitinating enzyme with growth-suppressing activity, USP21 contains all eight conserved sequence motifs originally identified in the yeast ubiquitin-specific proteases (5). These domains include a conserved cysteine residue and two histidine residues, respectively, that presumably form part of the active site of these thiol proteases (Fig. 2).

Expression of USP21 Transcripts in Human Tissues—To study the expression of USP21, we designed a probe that has little homology to the other UBP genes. On a Northern blot analysis, a single transcript of approximately 2.2 kilobases was detected in all the tissues, indicating that the isolated cDNA was likely to be full length (Fig. 3). In addition, USP21 was expressed at different levels in the tissues examined. The highest level of USP21 expression was found in the heart, pancreas, and skeletal muscle. USP21 messages could also be detected in the brain, placenta, liver, and kidney but are very low in the lung.

Chromosomal Localization of the Human USP21 Gene by EST Mapping—The human USP21 cDNA sequence was used to search the human EST data base using the BLASTN program, and 42 positive EST clones were identified. All of these EST clones were used to check the Human Gene Map data base. 11 of them (DDBJ/EMBL/GenBank™ accession numbers T33337, T33539, T16677, R54635, H42874, W74194, R45549, R56564, H17877, N64752, and R76797) have been mapped to chromosome 1 between D1S484 and D1S426 microsatellite 1869–1872, followed by a 3′-untranslated region of 269 nucleotides. At the 3′ terminus a potential polyadenylation signal, AATAAA, is present at nucleotide 2128. (3) The cloned fragment encodes a 381-amino acid polypeptide, called USP21 (approved by the Human Genome Nomenclature Committee). The calculated molecular mass for USP21 is 43,031 Da. The amino acid sequence of USP21 was compared with sequences in several data bases using the BLAST network service at the National Center for Biotechnology Information. The results indicated that USP21 is a novel member in the ubiquitin-specific protease family as shown in Fig. 2. Similar to DUB-1, a deubiquitinating enzyme with growth-suppressing activity, USP21 contains all eight conserved sequence motifs originally identified in the yeast ubiquitin-specific proteases (5). These domains include a conserved cysteine residue and two histidine residues, respectively, that presumably form part of the active site of these thiol proteases (Fig. 2).

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USP21 Is Specific for NEDD8, but Not for Sentrin Conjugates—We next tested the activity of USP21 against other ubiquitin-like proteins using the COS cell expression system described above. As shown in Fig. 5, when RH-tagged NEDD8 was expressed in COS cells, a series of bands in a ladder-like pattern representing NEDD8-conjugated proteins were observed (lanes 1 and 4). However, when RH-tagged NEDD8 was co-expressed with HA-tagged USP21 in the COS cells, the higher molecular weight NEDD8 conjugates were completely removed (lane 5). The disappearance of the high molecular weight NEDD8 conjugates also coincided with the accumulation of free NEDD8 monomers (lane 5). As expected, the (C37A)USP21 point mutant also lost its activity against NEDD8 conjugates (lane 6). Furthermore, USP21 has no effect on Sentrin conjugates, demonstrating that USP21 is not a generalized isopeptidase for all ubiquitin-like proteins (lanes 10–12). These results are consistent with the previous observation that the Sentrin-specific protease, SENP1, is structurally different from UBPs (14). NEDD8 is 60% identical and 80% homologous to ubiquitin, whereas sentrin is only 18% identical and 48% similar to ubiquitin. Thus, it is not entirely surprising that NEDD8 and ubiquitin could share similar UBPs. To test whether other UBPs may also be active against NEDD8 conjugates, we tested the effect of DUB-1 on NEDD8 (lane 2) or Sentrin conjugates (lane 8). As shown in Fig. 5, DUB-1 has no activity against either NEDD8 or Sentrin conjugates. This observation suggests that USP21 is a unique UBP with dual specificity for NEDD8 and ubiquitin.

USP21 Inhibits U2OS Cell Growth—Naviglio et al. (21) have previously shown that UBPY(USP8), another UBP family member, inhibits U2OS cell growth. We asked whether USP21 also has similar biological properties. As shown in Fig. 6, USP21 inhibited cell growth by up to 80% as compared with control in two separate experiments. In contrast, SENP1, a Sentrin-specific protease, only showed 15% inhibition of cell growth. These data suggest that USP21 has a profound effect on cell growth regulation similar to UBPY. In conclusion, USP21 is a novel member of the UBP family that appears to possess dual specificity for both NEDD8 and ubiquitin conjugates and may play important role in the regulation of cell growth.

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