THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 8, pp. 5256–5262, February 23, 2007

Two Novel Ubiquitin-fold Modifier 1 (Ufm1)-specific Proteases, UfSP1 and UfSP2*

Received for publication, November 15, 2006, and in revised form, December 20, 2006 Published, JBC Papers in Press, December 20, 2006, DOI 10.1074/jbc.M610590200

Sung Hwan Kang[‡], Gi Ryang Kim[‡1], Minu Seong[‡1], Sung Hee Baek[‡], Jae Hong Seol[‡], Ok Sun Bang[‡], Huib Ovaa[§], Kanako Tatsumi[¶], Masaaki Komatsu[¶], Keiji Tanaka[¶], and Chin Ha Chung[‡2]

From the [‡]School of Biological Sciences, Seoul National University, Seoul 151-742, Korea, the [§]Division of Cellular Biochemistry, Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, the Netherlands, and the [¶]Laboratory of Frontier Science, Tokyo Metropolitan Institute of Medical Science, Bunkyo-ku, Tokyo 113-8613, Japan

Ubiquitin-fold modifier 1 (Ufm1) is a recently identified new ubiquitin-like protein, whose tertiary structure displays a striking resemblance to ubiquitin. Similar to ubiquitin, it has a Gly residue conserved across species at the C-terminal region with extensions of various amino acid sequences that need to be processed *in vivo* prior to conjugation to target proteins. Here we report the isolation, cloning, and characterization of two novel mouse Ufm1-specific proteases, named UfSP1 and UfSP2. UfSP1 and UfSP2 are composed of 217 and 461 amino acids, respectively, and they have no sequence homology with previously known proteases. UfSP2 is present in most, if not all, of multicellular organisms including plant, nematode, fly, and mammal, whereas UfSP1 could not be found in plant and nematode upon data base search. UfSP1 and UfSP2 cleaved the C-terminal extension of Ufm1 but not that of ubiquitin or other ubiquitin-like proteins, such as SUMO-1 and ISG15. Both were also capable of releasing Ufm1 from Ufm1-conjugated cellular proteins. They were sensitive to inhibition by sulfhydryl-blocking agents, such as *N*-ethylmaleimide, and their active site Cys could be labeled with Ufm1-vinylmethylester. Moreover, replacement of the conserved Cys residue by Ser resulted in a complete loss of the UfSP1 and UfSP2 activities. These results indicate that UfSP1 and UfSP2 are novel thiol proteases that specifically process the C terminus of Ufm1.

Ubiquitin is a 76-amino acid polypeptide that is highly conserved from yeast to human. It is covalently ligated to a wide variety of target proteins through the action of ubiquitin-activating enzyme (E1),[3] ubiquitin-conjugating enzyme (E2), and ubiquitin-protein isopeptide ligase (E3). Proteins modified by multiple units of ubiquitin are degraded by the 26 S proteasome (1). Although the role as a tag for protein degradation by the proteasome has been known as a major function of ubiquitin, numerous other functions of ubiquitination have been identified (2–7). For example, monoubiquitination is not involved in the protein degradation pathway but plays a role in distinct cellular processes, such as histone regulation, endocytosis, and budding of retroviruses from the plasma membrane.

A number of other small proteins, so called ubiquitin-like molecules (Ubls), have been identified (8). These proteins are structurally related to ubiquitin and can be conjugated to various target proteins in a similar manner with ubiquitin (9–12). However, covalent attachment of Ubls does not result in degradation of the modified proteins but functions in a similar way to monoubiquitination. To date, nearly 10 Ubls including SUMO, NEDD8, and ISG15 have been identified. Of these, the best characterized Ubl is the mammalian SUMO-1 (13–17) that is conjugated to a variety of cellular proteins including transcription factors or their co-regulators.

Protein modification by Ub is a reversible process that is catalyzed by deubiquitinating enzymes (DUBs) (18–20). DUBs consist of five families that have distinct catalytic domain structures: the ubiquitin-specific protease family, the ubiquitin C-terminal hydrolase family, the ovarian tumor protease family, the Machado-Joseph disease protein family, and the Jab1/MPN/Mov34-domain protease family (21–25). Although the Jab1/MPN/Mov34-domain protease family members are metallo-proteases, the other family members are cysteine proteases. Protein modification by Ubls is also a reversible process that is catalyzed by Ubl-specific proteases (ULPs). For example, deconjugation of SUMO is conducted by SUMO-specific proteases, called SENP or SUSP (13, 16, 17). In cells, Ub and most Ubls are not synthesized as a free form but as precursors with C-terminal extensions. Thus, DUBs and ULPs play an important role in the generation of free Ub and Ubl monomers in addition to their role in the reversal of protein modification by matured Ub and Ubl molecules.

A new ubiquitin-like protein, Ufm1 (ubiquitin-fold modifier 1), has recently been identified (26). It shares only 16% sequence identity with Ub but displays a striking similarity in its tertiary structure to Ub (27). It has a single Gly at its C terminus, unlike Ub and most other Ubls that have a conserved C-terminal diglycine motif. Ufm1 in mouse and human is expressed as a precursor with a C-terminal Ser-Cys dipeptide extension that

* This work was supported by grants from the Korea Research Foundation (Grant KRF-2005-084-C00025) and the Korea Science and Engineering Foundation (Grant M10533010001-05N3301-00100) (to C. H. C.) and by a VIDI grant from the Netherlands Foundation for Scientific Research (to H. O.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Recipients of a fellowship from the BK21 Program.

[2] To whom correspondence should be addressed. Tel.: 82-2-880-6693; Fax: 82-2-871-9193; E-mail: chchung@snu.ac.kr.

[3] The abbreviations used are: E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin-protein isopeptide ligase; Ub, ubiquitin; Ubl, ubiquitin-like molecules; ULP, Ubl-specific proteases; DUB, deubiquitinating enzymes; SUMO, small Ub-related modifier; UfSP, Ufm1-specific protease; MBP, maltose-binding protein; GST, glutathione *S*-transferase; CBD, chitin binding domain; Bz, *N*-benzoxycarbonyl; AMC, 7-amido-4-methylcoumarin; VME, vinylmethylester; MESNa, 2-mercaptoethanesulfonic acid; NEM, *N*-ethylmaleimide; HA, hemagglutinin.

needs to be processed prior to conjugation to target proteins. The matured Ufm1 is specifically activated by an E1-like enzyme, Uba5, and then transferred to its cognate E2-like enzyme, Ufc1. The Ufm1 system is conserved in metazoa and plants but not in yeast, implicating its important roles in various multicellular organisms. However, the enzymes responsible for the processing of Ufm1 precursor as well as for the reversal of protein conjugation by matured Ufm1 have not been identified so far. E3-like enzymes for the ligation of Ufm1 to target proteins have not been identified either.

In the present study, we report the isolation, cloning, and characterization of two novel mouse Ufm1-specific proteases, named UfSP1 and UfSP2. Like most DUBs and ULPs, UfSP1 and UfSP2 belong to the family of cysteine proteases. However, they show no sequence homology to previously known proteases. Both enzymes could process the C-terminal extension of Ufm1 precursor, thus generating matured Ufm1 for conjugation to target proteins. Moreover, they were capable of releasing free Ufm1 molecules from Ufm1-conjugated cellular proteins. Thus, UfSP1 and UfSP2 may play an important role in the reversal of protein modification by Ufm1 as well as in the processing of Ufm1 precursor.

EXPERIMENTAL PROCEDURES

Plasmids—pQE30-GST-Ufm1-Ecotin was constructed as described previously (28). UfSP1 and UfSP2 cDNAs encoding the 217-amino acid LOC70240 (GenBank™ accession number: NM_027356) and the 461-amino acid LOC192169 (accession number: NM_138668) were amplified from a mouse cDNA library, respectively. The PCR products were cloned into BamHI and SalI sites of pMAL-c2x (New England Biolabs). pGEX-Ufm1, pGEX-Ufm1-HA, pGEX-Ub-HA, pGEX-SUMO-1-HA, and pGEX-ISG15-HA were generated as described previously (26). To generate an in-frame fusion of Ufm1 with the intein-chitin binding domain (Ufm1-intein-CBD), the cDNA for matured Ufm1 that lacks the C-terminal Gly was amplified by PCR and cloned into the NdeI and SapI sites of pTYB1 vector (New England Biolabs). FLAG-tagged Ufm1-intein-CBD was then generated by inserting the coding sequence for FLAG into the NdeI site. Site-directed mutagenesis of UfSP1 and UfSP2 was performed using QuikChange site-directed mutagenesis kit (Stratagene) by following the manufacturer's instructions. All sequences of the above mentioned constructs were confirmed by DNA sequencing.

Protein Purification—MBP-, GST-, and intein-CBD-fused proteins were expressed in *Escherichia coli* Rosetta strain, and His_6-tagged proteins were in the M15 strain. They were then purified by using appropriate affinity resins.

Assay of Ufm1-processing Activity—Ufm1-processing activity was assayed by using His-GST-Ufm1-Ecotin as a substrate as described previously (28). Briefly, enzyme samples were incubated for 1 h at 37 °C with purified His-GST-Ufm1-Ecotin in 100 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA and 1 mM dithiothreitol. After incubation, the mixtures were heated for 10 min at 95 °C in a water bath. They were added with bovine serum albumin to a final concentration of 0.02% and centrifuged for 10 min at 20,000 × *g*. Aliquots of the supernatants were mixed with 2 μl of 1 μg/ml trypsin and incubated for 10 min at room temperature with 90 μl of 100 mM Tris-HCl buffer (pH 8.0) containing 200 mM $CaCl_2$. They were added with 10 μl of 1 mM *N*-benzoxycarbonyl-Arg-7-amido-4-methylcoumarin (Bz-R-AMC) on ice. The release of AMC from Bz-R-AMC was then monitored continuously by incubation of the samples at 37 °C in a fluorometer (FLUOSTAR optima). Ufm1-processing activity was also assayed by incubation of enzyme samples with GST-Ufm1-HA. After incubation, the mixtures were subjected to SDS-PAGE followed by staining with Coomassie Blue R-250 for separation of GST-Ufm1 from GST-Ufm1-HA.

Fractionation of Ufm1-processing Activity—To fractionate Ufm1-processing activity, extracts were prepared from mouse liver, brain, and kidney tissues and dialyzed against buffer A (25 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10% glycerol, 5 mM 2-mercaptoethanol). The dialyzed extracts were precipitated by 40–60% $(NH_4)_2SO_4$. Precipitated proteins were dialyzed against buffer A and loaded onto a Q-Sepharose column equilibrated with the same buffer. Fractions showing high activity toward His-GST-Ufm1-Ecotin were pooled, dialyzed against buffer B (20 mM KH_2PO_4/K_2HPO_4, pH 6.5, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol), and loaded onto a hydroxylapatite column. Unbound proteins were collected, dialyzed against buffer A containing 1.2 M with $(NH_4)_2SO_4$, and loaded onto a phenyl-Sepharose column. Active fractions, which were eluted with 0.2–0.4 M $(NH_4)_2SO_4$, were pooled, dialyzed against buffer A, and further fractionated by gel filtration chromatography on a Superose-12 column equilibrated with buffer A containing 0.1 M NaCl.

Labeling of UfSPs with FLAG-Ufm1-VME—FLAG-tagged Ufm1-vinylmethylester (FLAG-Ufm1-VME) was synthesized as described previously (29). FLAG-Ufm1-intein-CBD that had been bound to chitin affinity resin was treated with 50 mM 2-mercaptoethanesulfonic acid (MESNa) to generate FLAG-Ufm1-MESNa (29). Gly-VME was added to 0.5 ml of FLAG-Ufm1-MESNa (1 mg/ml) to a final concentration of 0.25 M followed by the addition of 75 μl of 2 M *N*-hydroxysuccinimide and 30 μl of 2 M NaOH. After incubation at 37 °C for 6 h, the reaction was terminated by treatment with 30 μl of 2 M HCl. The samples were dialyzed against 50 mM sodium acetate (pH 4.5) and loaded onto an S-Sepharose cation exchange column equilibrated with 50 mM sodium acetate (pH 4.5). After washing with 5 column volumes of the same buffer, bound proteins (*i.e.* FLAG-Ufm1-VME) were eluted by stepwise increase in NaCl concentration. For labeling UfSPs with FLAG-Ufm1-VME, enzyme samples were incubated with FLAG-Ufm1-VME for 2 h at 37 °C in 100 mM Tris-HCl buffer (pH 8.0) containing 5 mM EDTA and 10% glycerol. They were then subjected to SDS-PAGE followed by immunoblot with anti-FLAG M2 antibody (Sigma).

Identification of UfSP1 Labeled by FLAG-Ufm1-VME—The active fractions from Superose-12 column were incubated for 12 h at 37 °C with 0.35 mg of FLAG-Ufm1-VME. The samples were added with anti-FLAG antibody that had been conjugated to Sepharose beads. After incubation of the mixtures overnight at 4 °C, beads were collected by centrifugation and washed five times with 0.5 ml of 50 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl. Bound proteins were eluted with 100 mM glycine

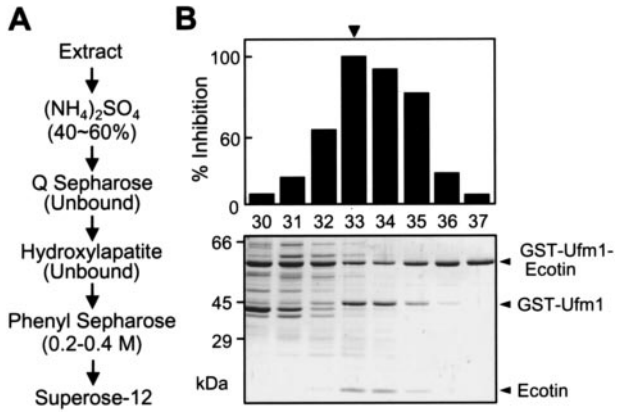

FIGURE 1. **Fractionation of Ufm1-processing activity.** *A*, protocol for fractionation of Ufm1-processing activity is summarized. *B*, active fractions eluted from a phenyl-Sepharose column were subjected to gel filtration on a Superose-12 column. Fractions of 0.5 ml were collected, and aliquots of them (50 μl) were incubated for 1 h at 37 °C with 2 μg of His-GST-Ufm1-Ecotin. The mixtures were then subjected to assay for their ability to inhibit trypsin activity. The *arrowhead* indicates the fraction where the peak of a marker protein chymotrypsinogen A (25 kDa) was eluted (*upper panel*). The same mixtures were also subjected to SDS-PAGE in 12% gels, and proteins in the gels were stained with Coomassie Blue R-250 (*lower panel*).

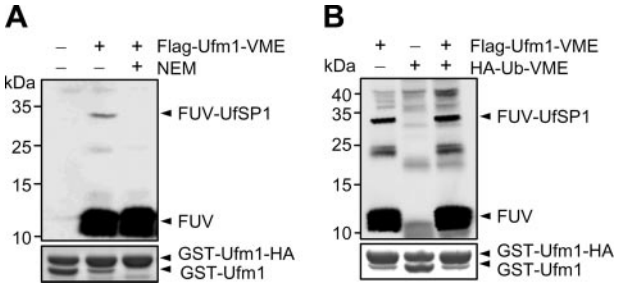

FIGURE 2. **Labeling of UfSP1 by FLAG-Ufm1-VME.** *A*, the S12 fraction was incubated in the absence or presence of 5 mM NEM for 10 min at 37 °C. The mixtures were further incubated with FLAG-Ufm1-VME for the next 1 h. They were then subjected to SDS-PAGE in 12% gels followed by immunoblot with anti-FLAG antibody (*upper panel*). The same samples were also assayed for Ufm1-processing activity by incubation with 5 μg of GST-Ufm1-HA (*lower panel*). *FUV* denotes FLAG-Ufm1-VME. *B*, the S12 fraction was incubated with HA-Ub-VME for 10 min at 37 °C. The mixtures were further incubated for the next 30 min in the absence or presence of FLAG-Ufm1-VME. They were then subjected to SDS-PAGE followed by immunoblot with the mixture of anti-FLAG and anti-HA antibodies (*upper panel*). The same samples were also assayed for Ufm1-processing activity as above (*lower panel*).

(pH 3.0) at 4 °C and dialyzed against distilled water. Dialyzed samples were concentrated to 30 μl, subjected to SDS-PAGE, and silver-stained. Protein bands with the sizes of 30–35 kDa were cut out from the gels and subjected to mass spectrometric analysis using a Q-TOF micro-tandem mass spectrometer (IN2GEN Co.).

Northern Analysis—Northern blot was performed using mouse adult total RNA blot (Seegene) and ExpressHyb (BD Biosciences) solution. All procedures followed the manufacturer's instructions. As probes, the entire coding sequence for UfSP1 and the nucleotide sequence of 1–717 for UfSP2 were labeled using a Random Primer DNA labeling kit (Takara).

RESULTS

Fractionation of Ufm1-processing Activity—To identify the proteases capable of processing the C-terminal extension of Ufm1, we adapted the recently developed method for assaying DUBs by using His-GST-Ufm1-Ecotin as the substrate (28). This method utilizes an unusual property of the *E. coli* trypsin inhibitor protein Ecotin, which is stable even after heating at 100 °C (30). After incubation of His-GST-Ufm1-Ecotin with enzyme samples, one of the reaction products, His-GST-Ufm1, was precipitated by boiling in a water bath. The supernatant containing the other reaction product (*i.e.* heat-stable Ecotin) was then assayed for its ability to inhibit trypsin. Using this assay method, we fractionated Ufm1-processing activity from mouse tissue extracts as described under "Experimental Procedures." The purification procedure was summarized in Fig. 1*A*. In the final Superose-12 column chromatography step, a peak of trypsin inhibitory activity was eluted in the fraction that corresponds to a size of about 25 kDa (Fig. 1*B*, *upper panel*). To verify that the trypsin inhibitory activity is indeed mediated by Ufm1-processing activity, His-GST-Ufm1-Ecotin was incubated with the same column fractions for 1 h at 37 °C, and the mixtures were subjected to SDS-PAGE followed by staining with Coomassie Blue R-250. The extents of substrate cleavage into His-GST-Ufm1 and Ecotin correlated well with those of trypsin inhibitory activity (Fig. 1*B*, *lower panel*), indicating that Ufm1-processing activity is responsible for the trypsin inhibition. The active fractions (number 33–35) were pooled, concentrated to 0.5 ml, and referred to as the S12 fraction.

Labeling of UfSP1 by FLAG-Ufm1-VME—Aliquots of the active fractions from Superose-12 column (*i.e.* before pooling and concentration) were subjected to SDS-PAGE followed by silver staining. However, we could not find any relevant protein band that matched Ufm1-processing activity (data not shown). To identify the protein(s) responsible for Ufm1-processing activity, FLAG-Ufm1-VME was synthesized and incubated for 1 h at 37 °C with the S12 fraction in the presence or absence of 5 mM *N*-ethylmaleimide (NEM), a sulfhydryl blocking agent. The incubation mixtures were then subjected to SDS-PAGE followed by immunoblot with anti-FLAG antibody. A new 34-kDa band appeared in the mixture incubated with FLAG-Ufm1-VME in the absence of NEM but not in its presence (Fig. 2*A*, *upper panel*), suggesting that a Cys residue of putative Ufm1-specific protease is labeled by FLAG-Ufm1-VME. To determine the effect of FLAG-Ufm1-VME on Ufm1-processing activity, the S12 fraction was also incubated with His-GST-Ufm1-HA for 1 h at 37 °C. Both FLAG-Ufm1-VME and NEM strongly inhibited the Ufm1-processing activity of the enzyme sample (Fig. 2*A*, *lower panel*). Collectively, these results suggest that the protein labeled with FLAG-Ufm1-VME represents an Ufm1-specific protease (UfSP). Henceforth, the labeled protein in the S12 fraction is referred to as UfSP1.

To determine whether the labeling by FLAG-Ufm1-VME is specific to UfSP1, HA-Ub-VME was synthesized and incubated with the enzyme for 10 min at 37 °C. The mixture was further incubated in the absence or presence of FLAG-Ufm1-VME for the next for 30 min. Fig. 2*B* (*upper panel*) shows that UfSP1 can be labeled by FLAG-Ufm1-VME whether or not HA-Ub-VME is present. Moreover, HA-Ub-VME showed little or no effect on Ufm1-processing activity of UfSP1 or on the ability of FLAG-Ufm1-VME to inhibit the UfSP1 activity (Fig. 2*B*, *lower panel*). These results suggest that UfSP1 specifically reacts with FLAG-Ufm1-VME.

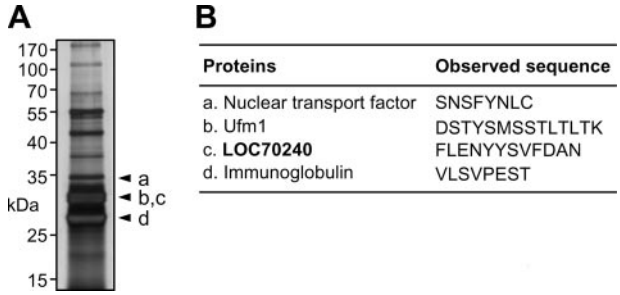

FIGURE 3. **Identification of UfSP1 by mass spectrometry.** *A*, the S12 fraction was incubated with FLAG-Ufm1-VME followed by immunoprecipitation with anti-FLAG antibody that had been conjugated to Sepharose beads. Proteins bound to the beads were eluted with glycine-HCl, pH 3.0, subjected to SDS-PAGE in 12% gels, and visualized by silver staining. The *arrowheads* (*a*–*d*) correspond to the proteins in *B*, which were identified by mass spectrometric analysis. *B*, the gels corresponding to the size of 30–35 kDa were sliced, digested by trypsin, and subjected to mass spectrometric analysis. Proteins that were identified from the peptide sequences are listed.

Identification of UfSP1 by Mass Spectrometry—To identify UfSP1, the S12 fraction was incubated with FLAG-Ufm1-VME followed by immunoprecipitation with anti-FLAG antibody. Precipitates were subjected to SDS-PAGE followed by silver staining (Fig. 3*A*). Protein bands in the gels with the sizes ranging from 30 to 35 kDa were then sliced, treated with trypsin, and subjected to tandem mass spectrometric analysis. Proteins that matched with the identified sequences included Ufm1 and a previously uncharacterized hypothetical mouse protein LOC70240 (accession number: gi 13899211) in addition to immunoglobulin and nuclear transport factor, which were not expected to interact with FLAG-Ufm1-VME (Fig. 3*B*). The estimated size of LOC70240, consisting of 217 amino acids, is 23 kDa, and that of FLAG-Ufm1-VME is about 10 kDa. Therefore, the sum of their sizes is well correlated with the 34-kDa band that appeared upon incubation of FLAG-Ufm1-VME with the S12 fraction. These results strongly suggest that LOC70240 corresponds to UfSP1.

Cloning, Expression, and Characterization of UfSP1 and UfSP2—BLAST search (31) for the identified sequence led to the finding of an additional hypothetical mouse protein LOC192169 (accession number: AAH05503) that is homologous to UfSP1 (Fig. 4*A*). LOC192169, named as UfSP2, is comprised of 461 amino acids with an estimated size of about 46 kDa. Although the C-terminal region (238–461) of UfSP2 showed 33.3% identity and 45.6% similarity in amino acid sequence to UfSP1, the extended N-terminal sequence is unique to UfSP2. Similar to DUBs and ULPs, both UfSP1 and UfSP2 have highly conserved Cys and His residues that form a catalytic triad for cysteine proteases (Fig. 4*B*). However, neither UfSP1 nor UfSP2 showed any sequence similarity to known DUBs and ULPs, indicating that UfSPs form a new subfamily of cysteine proteases.

To investigate the expression of UfSP1 and UfSP2 mRNAs, Northern analysis was performed using ^{32}P-labeled full-length cDNA of UfSP1 (651 bp) and 5′-region of UfSP2 (717 bp) as probes. Although UfSP1 mRNA was expressed in all tissues tested, its level was significantly higher in brain, heart, kidney, and skeletal muscle than in other tissues (Fig. 4*C*). Unlike UfSP2 mRNA, two transcripts for UfSP1 were detected in most of the tissues examined, which might have been derived from alternative splicing or the use of alternative promoters. The levels of UfSP2 mRNA in brain, kidney, stomach, skeletal muscle, and testis were higher than those in other tissues.

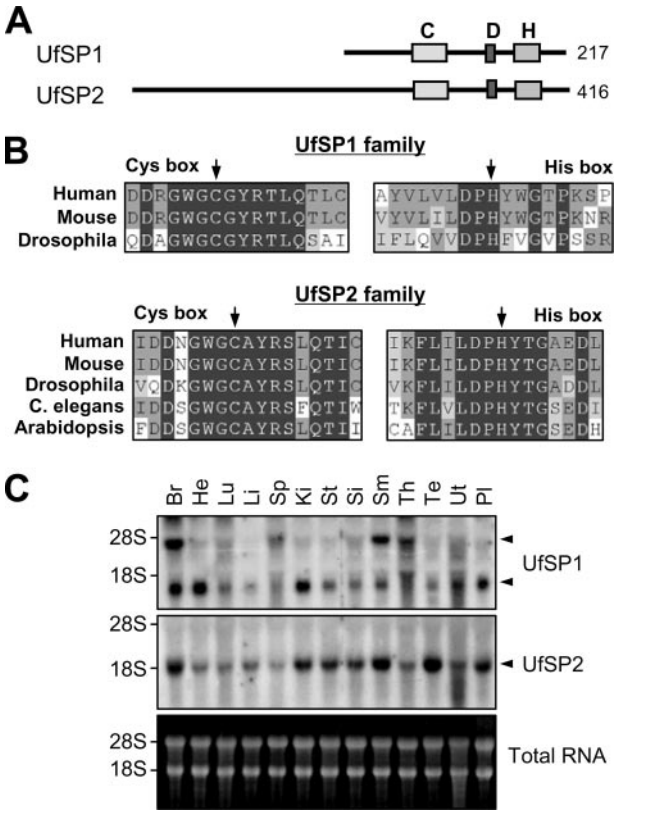

FIGURE 4. **Alignment of amino acid sequences of UfSP1 and UfSP2 and expression of their mRNAs.** *A*, the primary structures of UfSP1 and UfSP2 were schematically shown. The *black lines* indicate the non-conserved amino acid sequences, whereas the *boxes* represent the conserved Cys, Asp, and His boxes. *B*, the conserved Cys and His box domain sequences of UfSP1 and UfSP2 in various species were aligned. The identical and similar amino acids were shaded in *black* and *gray*, respectively. The *arrows* indicate the active site Cys and His residues. *C*, total RNAs were isolated from the indicated mouse tissues. They were then subjected to Northern blot analysis using the cDNAs for UfSP1 and UfSP2 as their probes.

The cDNAs for UfSP1 (accession number: NM_027356) and UfSP2 (accession number: NM_138668) were cloned into pMAL-c2x. MBP-fused UfSP1 and UfSP2 proteins were expressed in *E. coli* and purified by using amylose affinity resin (Fig. 5*A*). Mutant forms of UfSP1 and UfSP2, in which Cys-53 and Cys-294, respectively, in the Cys boxes were replaced by Ser, were also purified by using the same affinity resin. We then examined whether the Cys residues serve as the reactive sites for FLAG-Ufm1-VME. Purified UfSPs and their mutant forms were incubated in the absence or presence of FLAG-Ufm1-VME for 10 min at 37 °C. After incubation, the samples were subjected to SDS-PAGE followed by immunoblot with anti-FLAG antibody. Fig. 5*B* shows that both UfSP1 and UfSP2, but not their mutants (UfSP1/C53S and UfSP2/C294S), can be labeled by FLAG-Ufm1-VME, indicating that the Cys residues are the Ufm1-VME reactive sites.

We next examined whether UfSP1 and UfSP2 indeed have Ufm1-processing activity. Purified UfSPs and their mutant forms were incubated with His-GST-Ufm1-HA. Both UfSP1 and UfSP2, but not their mutant forms, were capable of releas-

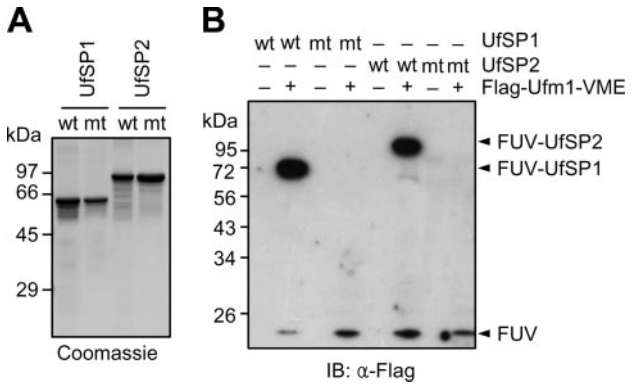

FIGURE 5. **Labeling of the catalytic Cys residues in UfSP1 and UfSP2 by FLAG-Ufm1-VME.** *A*, MBP-fused UfSP1 and UfSP2 (*wt*) were purified by using amylose affinity resin. MBP-fused UfSP1/C53S and UfSP2/C294S (*mt*) were also purified as above. Aliquots (3 μg each) of them were subjected to SDS-PAGE in 10% gels followed by staining with Coomassie Blue R-250. *B*, MBP-UfSPs (2 μg each) were incubated with or without 1 μg of FLAG-Ufm1-VME for 1 h at 37 °C. The samples were then subjected to SDS-PAGE followed by immunoblot (*IB*) with anti-FLAG antibody. *FUV* denotes FLAG-Ufm1-VME.

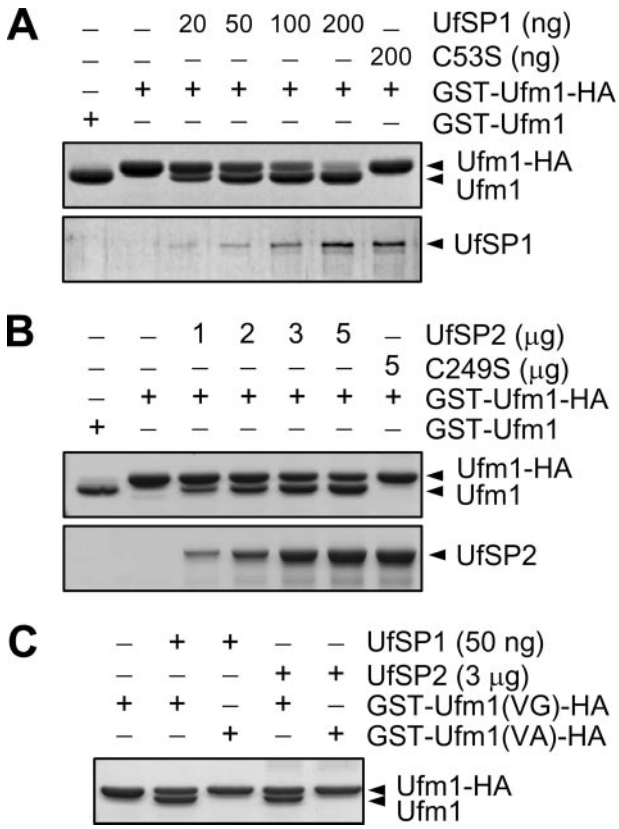

FIGURE 6. **Ufm1-processing activities of UfSP1 and UfSP2.** *A*, increasing amounts of MBP-UfSP1 or 200 ng of its mutant form (C53S) were incubated with GST-Ufm1-HA (5 μg) for 1 h at 37 °C. After incubation, the mixtures were subjected to SDS-PAGE followed by staining with Coomassie Blue R-250 (*upper panel*) or silver staining (*lower panel*). *B*, experiments were performed as in *A* but using the indicated amounts of MBP-UfSP2 or its mutant form (C249S). *C*, UfSP1 (50 ng) and UfSP2 (3 μg) were incubated with 5 μg of wild-type (GST-Ufm1(VG)-HA) or its mutant form (GST-Ufm1(VA)-HA). After SDS-PAGE, proteins in *B* and *C* were stained by Coomassie Blue R-250.

ing HA from GST-Ufm1-HA (Fig. 6), indicating that Cys-53 and Cys-294 serve as the catalytic residues in UfSP1 and UfSP2, respectively. Notably, UfSP2 was much less efficient in the hydrolysis of GST-Ufm1-HA than UfSP1. Although 50 ng of UfSP1 cleaved about 50% of GST-Ufm1-HA (Fig. 6*A*), nearly 3 μg of UfSP2 was required to hydrolyze it to a similar extent (Fig. 6*B*). Collectively, these results suggest that the maturation of Ufm1 precursor is catalyzed mainly by UfSP1 in cells. To determine whether UfSP1 and UfSP2 indeed cleave the peptide bond linked to the C-terminal Gly residue of Ufm1, the enzymes were incubated with GST-Ufm1(VA)-HA, in which the C-terminal Gly of Ufm1 was replaced by Ala. Unlike GST-Ufm1(VG)-HA, GST-Ufm1(GA)-HA was not cleaved by either of the enzymes (Fig. 6*C*). These results indicate that both UfSP1 and UfSP2 are authentic Ufm1-processing proteases.

Substrate Specificity of UfSPs—To determine the substrate specificity of UfSPs, purified enzymes were incubated with GST-Ufm1-HA, GST-Ub-HA, GST-SUMO1-HA, and GST-ISG15-HA. Fig. 7*A* shows that both UfSPs can cleave the C terminus of Ufm1 but not that of Ub, SUMO1, or ISG15. These results demonstrate that UfSPs act specifically on the C terminus of Ufm1. To determine whether UfSPs can release Ufm1 molecules that are conjugated to cellular proteins via isopeptide bonds, His-FLAG-Ufm1-HA was stably expressed in NIH3T3 cells. Ufm1-conjugated proteins were then pulled down from the cell lysates by treatment with Ni^{2+}-nitrilotriacetic acid-agarose resin. The resins were then incubated with purified UfSPs and subjected to SDS-PAGE followed by immunoblot with anti-FLAG antibody. At least five Ufm1-conjugated proteins with approximate sizes of 80, 70, 65, 45, and 32 kDa were generated (Fig. 7*B*). Incubation of the proteins with UfSP1 led to a decrease in their intensity, although to different extents. These results suggest that UfSP1 cleaves off Ufm1 from the Ufm1-conjugated cellular proteins. UfSP2 also reproducibly reduced the intensity of 45- and 32-kDa bands but only to a small extent. Thus, it remains unclear whether the 45- and 32-kDa Ufm1 conjugates represent true substrates for UfSP2. On the other hand, the active site mutants, UfSP1/C53S or UfSP2/C294S, could not hydrolyze any of them. These results demonstrate that UfSP1 can act on Ufm1-conjugated proteins as well as on Ufm1 precursor protein. These results also suggest that UfSP2 may have tighter substrate specificity on Ufm1-conjugated proteins than UfSP1, likely due to the presence of a unique N-terminal extension that may confer substrate specificity.

DISCUSSION

In the present study, we report the isolation, molecular cloning, and characterization of two novel Ufm1-specific proteases, named UfSP1 and UfSP2. Both of the recombinant enzymes were capable of processing Ufm1 precursor and deconjugating Ufm1-modified cellular proteins. However, tissue extracts showed low Ufm1-processing activity toward Ufm1-peptide fusion, such as Ufm1-HA (data not shown). Therefore, for detection and isolation of Ufm1-processing activity from tissue extracts, we adapted a recently developed method for assaying DUBs using His-GST-Ufm1-Econtin as a substrate instead of His-GST-Ub-Ecotin (28). Using this method, we could partially purify UfSP1 from tissue extracts. However, we were unable to identify a protein that is responsible for Ufm1-processing activity, most likely due to the very low level of endogenous UfSP1 protein. To this end, we covalently labeled UfSP1 with FLAG-Ufm1-VME and identified its partial sequence by mass spec-

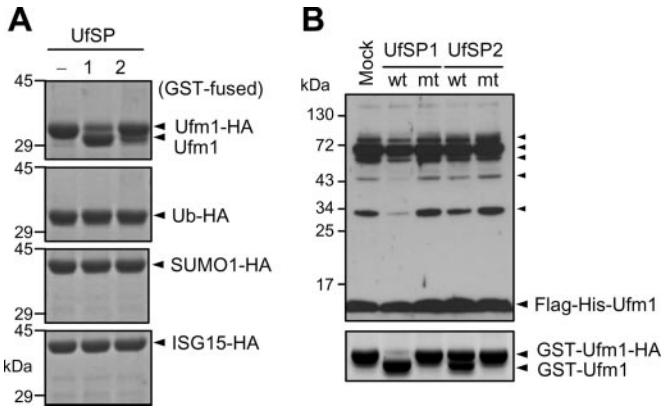

FIGURE 7. **Substrate specificity of UfSP1 and UfSP2.** *A*, UfSP1 (50 ng) and UfSP2 (2 μg) were incubated with 5 μg of GST-Ufm1-HA, GST-Ub-HA, GST-SUMO1-HA, or GST-ISG15-HA for 1 h at 37 °C. After incubation, the mixtures were subjected to SDS-PAGE in 12% gels followed by staining with Coomassie Blue R-250. *B*, cellular proteins that had been conjugated by FLAG-His-Ufm1 were pulled down by nickel-nitrilotriacetic acid resins from NIH 3T3 cells, which are stably expressing FLAG-His-Ufm1. Precipitates were incubated with 0.1 μg of UfSP1 (*wt*), 3 μg of UfSP2 (*wt*), or the same amounts of their mutant forms (*mt*) for 2 h at 37 °C. Precipitates were also incubated as above but without the proteases as a control (*Mock*). After incubation, the mixtures were subjected to SDS-PAGE followed by immunoblot with anti-FLAG antibody (*upper panel*). UfSPs and their mutant forms were also incubated with GST-Ufm1-HA for the same period for determining their Ufm1-processing activities (*lower panel*).

trometry. Moreover, data base searching with the identified sequence of UfSP1 led to the finding of an additional Ufm1-processing enzyme, named UfSP2. UfSP1 and UfSP2 show no sequence homology to previously known proteases, indicating that they represent novel Ufm1-specific proteases.

Like DUBs and ULPs, UfSP1 and UfSP2 show typical features of cysteine proteases. Their activities are inhibited by NEM, although we could not exclude a possibility that the inhibitory effect of NEM might be due to its non-selective modification of cysteine residue(s) located outside the active site. Moreover, replacement of the active site Cys by Ser resulted in complete inactivation of UfSP1 and UfSP2. In addition, both of the purified enzymes, but not their mutant forms, could be covalently labeled by FLAG-Ufm1-VME, indicating that the active site Cys sulfhydryl is the reactive nucleophile covalently modifying the VME moiety. The activity of cysteine proteases typically depends on the catalytic action of Cys and His residues that are usually assisted by an Asp or Asn residue (19). Sequence alignment revealed the presence of highly conserved His and Asp residues, suggesting that these residues together with the active site Cys form the catalytic triad of UfSPs.

The conserved sequences around catalytic motifs and the sizes of UfSPs offer a basis to group UfSPs into two families: the UfSP1 family and the UfSP2 family. UfSP1 family members that have a size around 25 kDa are present in fly, mouse, and human, but not in plant or nematode. On the other hand, UfSP2 family members have a size larger than 40 kDa and can be found in most multicellular organisms, including *Caenorhabditis elegans* and *Arabidopsis*. In addition, UfSP2 family members have an N-terminal extension, which is not found in the UfSP1 family. Moreover, the amino acid sequences that form the Cys and His boxes in UfSP1 family are distinct from those of UfSP2 family. Nevertheless, the Gly-Trp-Gly-Cys motif of the Cys box and the Asp-Pro-His motif of the His box are well conserved in both UfSP families. The distance between the two motifs is also nearly the same in all UfSP1 and UfSP2 family members (data not shown). These features in the amino acid sequences of UfSP families suggest that significant selective pressure has existed for the maintenance of their active site structures in multicellular organism during evolution.

Covalent modification of proteins by Ub and Ubls is a key mechanism for the control of cellular processes as diverse as cell proliferation, differentiation, and apoptosis. Reversal of this modification, catalyzed by DUBs and ULPs, also functions in the control of diverse cellular processes by regulating the fate and function of the proteins modified by Ub and Ubls. For example, the cellular functions of DUBs include the regulation of proteasome activity, protein stability, signal transduction, DNA repair, chromatin dynamics and transcription, and endocytosis. However, the cellular function of protein modification by Ufm1 or its reversal by UfSPs remains unknown because no target protein for Ufm1 modification has yet been identified. Identification of Ufm1 target proteins is currently under investigation.

REFERENCES

1. Glickman, M. H., and Ciechanover, A. (2002) *Physiol. Rev.* **82,** 373–428
2. Deng, L., Wang, C., Spencer, E., Yang, L., Braun, A., You, J., Slaughter, C., Pickart, C., and Chen, Z. J. (2000) *Cell* **103,** 351–361
3. Terrell, J., Shih, S., Dunn, R., and Hicke, L. (1998) *Mol. Cell* **1,** 193–202
4. Hicke, L. (2001) *Nat. Rev. Mol. Cell Biol.* **2,** 195–201
5. Hofmann, R. M., and Pickart, C. M. (1999) *Cell* **96,** 645–653
6. Galan, J. M., and Haguenauer-Tsapis, R. (1997) *EMBO J.* **16,** 5847–5854
7. Spence, J., Gali, R. R., Dittmar, G., Sherman, F., Karin, M., and Finley, D. (2000) *Cell* **102,** 67–76
8. Kerscher, O., Felberbaum, R., and Hochstrasser, M. (2006) *Annu. Rev. Cell Dev. Biol.* **22,** 159–180
9. Tanaka, K., Suzuki, T., and Chiba, T. (1998) *Mol. Cells* **8,** 503–512
10. Hochstrasser, M. (2000) *Nat. Cell Biol.* **2,** E153–E157
11. Melchior, F., Schergaut, M., and Pichler, A. (2003) *Trends Biochem. Sci.* **28,** 612–618
12. Yeh, E. T., Gong, L., and Kamitani, T. (2000) *Gene* (*Amst.*) **248,** 1–14
13. Johnson, E. S. (2004) *Annu. Rev. Biochem.* **73,** 355–382
14. Gill, G. (2005) *Curr. Opin. Genet. Dev.* **15,** 536–541
15. Dohmen, R. J. (2004) *Biochim. Biophys. Acta* **1695,** 113–131
16. Kim, K. I., Baek, S. H., and Chung, C. H. (2002) *J. Cell. Physiol.* **191,** 257–268
17. Hay, R. T. (2005) *Mol. Cell* **18,** 1–12
18. D'Andrea, A., and Pellman, D. (1998) *CRC Crit. Rev. Biochem. Mol. Biol.* **33,** 337–352
19. Amerik, A. Y., and Hochstrasser, M. (2004) *Biochim. Biophys. Acta* **1695,** 189–207
20. Nijman, S. M., Luna-Vargas, M. P., Velds, A., Brummelkamp, T. R., Dirac, A. M., Sixma, T. K., and Bernards, R. (2005) *Cell* **123,** 773–786
21. Kim, J. H., Park, K. C., Chung, S. S., Bang, O., and Chung, C. H. (2003) *J. Biochem.* (*Tokyo*) **134,** 9–18
22. Evans, P. C., Smith, T. S., Lai, M. J., Williams, M. G., Burke, D. F., Heyninck, K., Kreike, M. M., Beyaert, R., Blundell, T. L., and Kilshaw, P. J. (2003) *J. Biol. Chem.* **278,** 23180–23186
23. Balakirev, M. Y., Tcherniuk, S. O., Jaquinod, M., and Chroboczek, J. (2003) *EMBO Rep* **4,** 517–522
24. Burnett, B., Li, F., and Pittman, R. N. (2003) *Hum. Mol. Genet.* **12,** 3195–3205
25. Verma, R., Aravind, L., Oania, R., McDonald, W. H., Yates, J. R., III, Koonin, E. V., and Deshaies, R. J. (2002) *Science* **298,** 611–615
26. Komatsu, M., Chiba, T., Tatsumi, K., Iemura, S., Tanida, I., Okazaki, N., Ueno, T., Kominami, E., Natsume, T., and Tanaka, K. (2004) *EMBO J.* **23,** 1977–1986

27. Sasakawa, H., Sakata, E., Yamaguchi, Y., Komatsu, M., Tatsumi, K., Kominami, E., Tanaka, K., and Kato, K. (2006) *Biochem. Biophys. Res. Commun.* **343,** 21–26
28. Kang, S. H., Park, J. J., Chung, S. S., Bang, O. S., and Chung, C. H. (2005) *Methods Enzymol.* **398,** 500–508
29. Hemelaar, J., Borodovsky, A., Kessler, B. M., Reverter, D., Cook, J., Kolli, N., Gan-Erdene, T., Wilkinson, K. D., Gill, G., Lima, C. D., Ploegh, H. L., and Ovaa, H. (2004) *Mol. Cell. Biol.* **24,** 84–95
30. Chung, C. H., Ives, H. E., Almeda, S., and Goldberg, A. L. (1983) *J. Biol. Chem.* **258,** 11032–11038
31. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) *J. Mol. Biol.* **215,** 403–410