A New 30-kDa Ubiquitin-related SUMO-1 Hydrolase from Bovine Brain*

(Received for publication, July 17, 1999, and in revised form, August 27, 1999)

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SUMO-1 is a ubiquitin-like protein functioning as an important reversible protein modifier. To date there is no report on a SUMO-1 hydrolase/isopeptidase catalyzing the release of SUMO-1 from its precursor or SUMO-1-ligated proteins in mammalian tissues. Here we found multiple activities that cleave the SUMO-1 moiety from two model substrates, 125I-SUMO-1 generating protease termed Ulp1 (Ubl-specific protease 1) which possesses both C-terminal processing and isopeptidase activities. The enzyme had the ability to cleave SUMO-1 not only from its precursor but also from a SUMO-1-ligated RanGAP1 conjugate, in bovine brain extracts. Of them, a major SUMO-1 C-terminal hydrolase had been partially purified by successive chromatographic operations. The enzyme had the ability to cleave SUMO-1 not only from its precursor but also from a SUMO-1-ligated RanGAP1 but did not exhibit any significant cleavage of the ubiquitin- and NEDD8-precursor. The activity of SUMO-1 hydrolase was almost completely inhibited by N-ethylmaleimide, but not by phenylmethanesulfonyl fluoride, EDTA, and ubiquitin-aldehyde known as a potent inhibitor of deubiquitinating enzymes. Intriguingly, the apparent molecular mass of the isolated SUMO-1 hydrolase was approximately 30 kDa, which is significantly smaller than the recently identified yeast Smt3/SUMO-1 specific protease Up1. These results indicate that there are multiple SUMO-1 hydrolase/isopeptidases in mammalian cells and that the 30-kDa small SUMO-1 hydrolase plays a central role in processing of the SUMO-1 precursor.

* This work was supported in part by CREST, Japan Science and Technology Corporation (JST) (to M. K., N. S., and K. T.). 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.

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Recent progress in the study of the ubiquitin system has revealed the existence of multiple molecules that are structurally related to ubiquitin (reviewed in Refs. 1 and 2). These proteins are called ubiquitin-like proteins (Ubl), but their biological roles have been largely unknown. Of these, SUMO-1 (small ubiquitin-related modifier), human homologue of yeast Smt3 and also called UBL1, sentrin, PIC1, GMP1, or SMT3C, is capable of modifying a wide variety of cellular proteins, such as RanGAP1, a Ran-GTPase-activating protein (3), IxBa, an inhibitor for NF-κB transcriptional factor (4), and PML, a promyelocytic leukemia protein (5). It was suggested that SUMO-1 ligation influences subcellular localization of RanGAP1 and PML (6–8). Moreover, SUMO-1 conjugation is suggested to stabilize IxBa because SUMO-1 occupies the Lys residue undergoing ubiquitylation prior to proteasomal degradation (4).

Recently, the ubiquitin-related ligating system for yeast Smt3 (9, 10) and human SUMO-1 (11, 12) were found to be activated by a heterodimeric E1 and conjugated by Ubc9 as the E2-like enzyme in the ubiquitin pathway. Moreover, it has been reported that both SUMO-1 and Smt3 synthetases as proproducts are processed to generate the C-terminal glycine residue prior to its conjugation to appropriate target proteins (13). The cleavage of the extra peptides to expose the C-terminal glycine residue should be essential for their ligation to target proteins through an isopeptide linkage, which is analogous to ubiquitylation (14, 15). The enzyme(s) catalyzing the maturation of the SUMO-1 precursor, however, has not yet been reported in any mammalian cells and tissues. In this paper, we report that the soluble extract of bovine brain contains one major SUMO-1 C-terminal hydrolase that cleaves SUMO-1 from the SUMO-1 precursor (abbreviated pSUMO-1) fused with αNH-GSMHISPEPESESSEEE-HYC (designated as pSUMO-1-gsPESTc). Intriguingly, the partially purified enzyme also shows isopeptidase activity capable of releasing SUMO-1 from a SUMO-1-RanGAP1 conjugate. Recently, it was reported that Saccharomyces cerevisiae has a unique Smt3/SUMO-1 generating protease termed Ulp1 (Ub)-specific protease 1 which possesses both C-terminal processing and isopeptidase activities (16). However, the presently described 30-kDa SUMO-1 C-terminal hydrolase appears to differ from yeast Ulp1 or its structurally related putative yeast protease Ulp2/Smt4 with a predicted molecular mass of 72 or 117 kDa, respectively. The physiological significance of the newly identified SUMO-1 C-terminal hydrolase is discussed.

EXPERIMENTAL PROCEDURES

Preparation of Recombinant ubiquitin or Ubl Fused with the PESTc or gsPESTc Sequence and Radioiodination—Recombinant ubiquitin-PESTc was prepared as previously reported (17). For the preparation of Ubl substrates, we used Ubl precursor forms with extension peptides (pUbl); i.e. SUMO-1-HSTV and NEDD8-LRQ fused with gpPESTc were constructed and abbreviated as pSUMO-1-gsPESTc and pNEDD8-gsPESTc, respectively. The DNA fragments encoding ubiquitin or Ubl-gsPESTc or SUMO-1 (G97A)-gsPESTc, in which Gly at position 97 of the C-terminal end of the mature form of SUMO-1 was substituted with Ala and fused with gpPESTc, were synthesized using polymerase chain reaction and ligated into the expression vector pGEX-2T (Amersham Pharmacia Biotech). The purified recombinant proteins were radiola-

1 The abbreviations used are: Ubl, ubiquitin-like; pUbl, Ubl precursor; GST, glutathione S-transferase; DTt DH, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; MES, 4-morpholinoeethanesulfonic acid; DUB, deubiquitinating enzyme; CHO, aldehyde.
beled with Na$_{125}$I using IODO-BEADS (Pierce) according to the recommendation of the manufacturer. The specific activity of iodinated ubiquitin-PESTc and pUbi-gsPESTc was about 5 x 10$^5$ cpm/µg protein.

**Assay of SUMO-1 C-terminal Hydrolase—**Ubiquitin or ubiquitin-related proteins fused with PESTc or gsPESTc (0.1–0.5 µg of 125I-labeled substrate or 4 µg of non-labeled substrate) were incubated at 37 °C for 2 h for the 125I-labeled substrate or 3 h for the non-labeled substrate in a total volume of 100 µl of reaction mixtures containing 100 mM Tris-HCl (pH 7.8), 1 mM dithiothreitol (DTT), 1 mM EDTA, 5% (v/v) glycerol, and a proper amount of SUMO-1 hydrolase. To validate the generation of SUMO-1 from the fusion protein, the reaction products were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) followed by autoradiography or staining with Coomassie Blue R-250. The intensity of the resulting bands was determined using a Fuji BAS-2500 Bio-imaging analyzer.

**Assay of Release of GST-SUMO-1 from GST-SUMO-1-35S-RanGAP1 Conjugate—**GST-SUMO-1-35S-RanGAP1 was synthesized using 75 µl of rabbit reticulocyte lysate as reported previously (18). After the reaction, 100 µl of glutathione beads was added and incubated for 45 min at 4 °C. The glutathione beads were collected by centrifugation and washed three times with ice-cold phosphate-buffered saline. The beads were used for the assay immediately. For the detection of isopeptidase activity, 10 µl of the beads was incubated with 10 µl of each column fraction for varying times at 37 °C. The reaction was stopped by the addition of 10 µl of SDS-sample buffer. After 4–10% SDS-PAGE, the gel was dried and the signals were detected by autoradiography. The conversion of SUMO-1–35S-RanGAP1 conjugate to 35S-RanGAP1 was estimated by an imaging analyzer.

**RESULTS**

**Identification of SUMO-1 C-terminal Hydrolase from Bovine Brain—**Whole bovine brains were homogenized in three volumes of 20 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA and 1 mM DTT in a Potter-Elvehjem homogenizer and centrifuged at 100,000 × g for 1 h at 100,000 × g. This supernatant, but scarcely the particulate fraction, hydrolyzed 125I-ubiquitin-PESTc and 125I-pNEDD8-gsPESTc. The extracts also showed strong activity capable of cleaving 125I-ubiquitin-PESTc and 125I-pNEDD8-gsPESTc. To analyze them, the cytosolic proteins (20 g of protein) were mixed with 850 ml of Q-Sepharose resin for 2 h at 4 °C. After the unabsorbed fraction was pooled, the resin was packed in a column, and proteins adsorbed on the resin were eluted with a linear gradient of 0–0.8 M NaCl. The 125I-ubiquitin-PESTc- and 125I-pNEDD8-gsPESTc-degrading activities were eluted at several peaks (19), but little or no 125I-pSUMO-1-gsPESTc-degrading activity was detected in all of the column fractions eluted with the salt gradient. However, a strong activity against 125I-pSUMO-1-gsPESTc degradation together with considerable activities of 125I-ubiquitin-PESTc and 125I-pNEDD8-gsPESTc degradations were observed in the flow-through fraction (data not shown).

To separate the activities against the 125I-pSUMO-1-gsPESTc degrading enzyme in the flow-through fraction, (NH$_4$)$_2$SO$_4$ fractionation was carried out (Fig. 1A). Although a small amount of the degrading activity against 125I-pSUMO-1-gsPESTc was detected in the 25–50% fraction, most of the activity was detected in the 50–75% fraction, (left panel). In contrast, the major degrading activities against 125I-ubiquitin-PESTc (middle panel) and 125I-pNEDD8-gsPESTc (right panel) were recovered in the 0–50% (NH$_4$)$_2$SO$_4$ fraction. Thus, it is likely that the enzyme(s) responsible for the generation of SUMO-1 differs from those for releasing ubiquitin or NEDD8.

**Chromatographic Separation of SUMO-1 Hydrolase—**The resulting 50–75% (NH$_4$)$_2$SO$_4$ fraction was dialyzed against 50 mM MES buffer (pH 6.0) containing 1 mM DTT at ambient temperature, it (1.3 g) was divided into three pools, and each pool was applied onto an S-Sepharose column (70 ml) that had been equilibrated with the same buffer. Proteins adsorbed on the resin were then eluted with 400 ml of a linear gradient of 0–0.8 M NaCl. The 125I-pSUMO-gsPESTc degrading activities were eluted as a single peak around 0.25 M NaCl concentration (Fig. 1B). In contrast, most of the degrading activities of 125I-ubiquitin-PESTc and 125I-pNEDD8-gsPESTc were not bound to the S-Sepharose column.

It is notable that SUMO-1 is conjugated to RanGAP1 for...
regulation of nuclear protein transportation (reviewed in Refs. 1 and 3). Therefore, it is of interest to examine whether the SUMO-1 C-terminal hydrolase has activity capable of releasing SUMO-1 from the SUMO-1-RanGAP1 conjugate. For this, we prepared GST-SUMO-1-35S-RanGAP1 as a substrate and asayed SUMO-1 isopeptidase activity by measuring the release of 35S-RanGAP1 from the GST-SUMO-1-35S-RanGAP1 conjugate. The obvious isopeptidase activity was found in the bovine brain extracts, and subsequent investigation revealed that most of the activities were not adsorbed on a Q-Sepharose column, although a weak activity was found to be bound loosely to the resin (data not shown). The flow-through fraction containing most of the activity was loaded on an S-Sepharose column, and proteins adsorbed on the resin were eluted as described in Fig. 1B. As shown in Fig. 1C, two isopeptidase activities were eluted around 0.2 and 0.4 M NaCl concentrations. The former weak activity overlapped the 125I-pSUMO-1-gsPESTc degrading activity, and the latter activity was estimated to be an apparent molecular size of over 80 kDa by subsequent molecular sieve chromatography (data not shown). In this work, we focused on the SUMO-1 C-terminal hydrolase to explore further biochemical properties.

On each fractionation in S-Sepharose chromatography (Fig. 1B), the fractions with the highest activity against 125I-pSUMO-1-gsPESTc degradation (shown by top bar) were pooled and dialyzed against 10 mM potassium phosphate (pH 6.8) containing 1 mM DTT. They were divided into seven pools and applied to a pre-packed hydroxylapatite column (bed volume, 10 ml) that had been equilibrated with the same buffer. After washing the column with 5 bed volumes of the buffer, the adsorbed materials were eluted with 120 ml of linear gradient of 0–0.3 M potassium phosphate buffer (pH 6.8). The 125I-pSUMO-1-gsPESTc-degrading activity was recovered as a single peak at 0.15 M potassium phosphate concentration (Fig. 2A).

Subsequently, the peak fractions of the activity of each fractionation were pooled, dialyzed against 20 mM Tris-HCl (pH 7.5) and 1 mM DTT, and applied on a Hi-Trap heparin-Sepharose CL-6B column (bed volume, 5 ml) in the same buffer. Proteins adsorbed on the resin were then eluted with 25 ml of a linear gradient of 0–1.2 M NaCl. The 125I-pSUMO-1-gsPESTc-degrading activity was eluted as a single peak at 0.4 M NaCl concentration (Fig. 2B). The fractions with the highest activity against 125I-pSUMO-1-gsPESTc degradation (shown by top bar) were pooled and used for further analysis.

Some Biochemical Properties of the Isolated SUMO-1 Hydrolase—For the first time, we examined the molecular mass of the enzyme by molecular sieve chromatography with a Superdex 200-pg column (Amersham Pharmacia Biotech). The apparent molecular mass of the SUMO-1 hydrolase was estimated to be approximately 30 kDa (Fig. 3). As shown in Fig. 4A (left panel), the enzyme activity was almost completely inhibited by N-ethylmaleimide, but not by phenylmethanesulfonyl fluoride nor EDTA (the entire reaction mixture contains 1 mM EDTA), indicating that it is probably a thiol protease, like many deubiquitinylating enzymes reported so far (16). How-
ever, SUMO-1 hydrolase was not inhibited by ubiquitin-aldehyde (ubiquitin-CHO) known as a potent inhibitor of deubiquitinating enzymes (20). When the digestion products of pSUMO-1-gsPESTc were separated by reverse phase-high performance liquid chromatography and analyzed by automated Edman degradation and mass spectrometry, the SUMO-1 hydrolase produced the HSTVGSMHISPPEPESEEEEEHYC peptide (data not shown). We also measured the cleavage of SUMO-1(G97A)-gsPESTc, in which Gly at position 97 of the C-terminal end of the mature form of SUMO-1 was replaced by Ala and fused with gsPESTc, by the partially purified enzyme. As shown in Fig. 4A (middle panel), SUMO-1(G97A)-gsPESTc was scarcely cleaved. These results indicate that the isolated SUMO-1 hydrolase is capable of hydrolyzing the precise site to produce mature SUMO-1, and thus it can act as a SUMO-1 processing enzyme. Moreover, SUMO-1 hydrolase rapidly degraded pSUMO-1-gsPESTc, but not ubiquitin-gsPESTc and pNEDD8-gsPESTc (Fig. 4A, right panel), suggesting that the isolated enzyme is specific for the generation of SUMO-1. Taken together, this indicated that the isolated enzyme is the SUMO-1-specific protease.

We next examined whether the presently described SUMO-1 hydrolase has de-conjugation activity. To test this activity, the column fractions eluted from hydroxylapatite chromatography (see Fig. 2A, note that a different preparation was used for this experiment) were incubated with GST-SUMO-1-35S-RanGAP1, and then the products were analyzed by SDS-PAGE and autoradiography. As shown in Fig. 4B, cleaved products (70-kDa non-modified form) were detected in fractions 37–44 (lower panel). The fractions with high GST-SUMO-1-35S-RanGAP1-cleaving activity were in fairly good agreement with those with 125I-pSUMO-1-gsPESTc-degrading activity (upper panel). These results strongly indicate that SUMO-1 hydrolase has the SUMO-1-isopeptidase activity capable of releasing the SUMO-1 moiety from the SUMO-1-RanGAP1 conjugate.

DISCUSSION

We previously reported that ubiquitin-PESTc acts as a substrate for the sensitive and quantitative assays of various deubiquitinating enzymes (DUBs) and found a set of novel DUBs in the chicken skeletal muscle (17) and bovine brain (19). In this paper, we designed pSUMO-1-gsPESTc to look for a SUMO-1 hydrolase that cleaves the SUMO-1 moiety from the model substrate. The purified SUMO-1 hydrolase activity was inhibited by the sulfhydryl-blocking agent N-ethylmaleimide, suggesting that its intrinsic activity is catalyzed by a mechanism similar to other sulfhydryl proteases such as DUBs. However, the SUMO-1 hydrolase was not inhibited by ubiquitin-CHO and did not cleave ubiquitin-PESTc, indicating that this enzyme’s role is specific to the SUMO-1-modifying pathway.

During the preparation of this manuscript, Li and Hochstrasser (16) reported a new yeast protease, termed Ulp1, which is structurally unrelated to known DUBs. Ulp1 releases SUMO-1/Smt3 from SUMO-1-RanGAP1 and is a precursor of Smt3, indicating that Ulp1 has both Smt3 precursor processing and isopeptidase activities. Ulp1 is a thiol enzyme, but it is not sensitive to ubiquitin-CHO nor has ubiquitin C-terminal hydrolase activity. Because the catalytic properties of Ulp1 appear similar to those of the 30-kDa SUMO-1 hydrolase from bovine brain, one might predict that these two enzymes could be related to each other. Although we cannot rule out this possibility, our enzyme seems to differ from Ulp1, because the 30-kDa SUMO-1 hydrolase that we purified is significantly smaller than the 72-kDa of Ulp1. Moreover, we also detected chromatographically other distinct activities capable of cleaving SUMO-1 from SUMO-1-35S-RanGAP1 that was eluted at a different peak from the 30-kDa SUMO-1 C-terminal hydrolase on the Q-Sepharose and S-Sepharose chromatography. All our data indicate that the 30-kDa SUMO-1 C-terminal hydrolase is not a mammalian orthologue of yeast Ulp1 and that mammalian cells contain multiple SUMO-1 isopeptidases. Li and Hochstrasser (16) also found a putative 117-kDa protease named Ulp2 (equivalent to Smt4) in yeast genome, which is structurally related to Ulp1, although it is unknown whether Ulp2 has the SUMO-1 hydrolase/isopeptidase activities. Our computer-assisted homology analysis in a human cDNA data base reveals the existence of at least five different putative proteins having a conserved presumptive catalytic domain homologous to yeast Ulp1 and Ulp2 (not shown). This knowledge together with our present findings suggests that a family of SUMO-1 specific proteases that are structurally related are universally present in a wide variety of eukaryotic cells.

In budding yeast, increasing evidence has indicated that Smt3 modification is involved in cell cycle control, especially in G1/M progression of the cell cycle (1, 3). The balance of Smt3 conjugation and deconjugation to the multiple proteins, such as Cdc3 (21), appears to be dynamically regulated in a cell cycle-dependent manner, and both the processing and deconjugation activity of Ulp1 are involved to be involved in the normal growth of budding yeast (16). Therefore, it is of interest to investigate whether the SUMO-1 modification fluctuates in the mammalian cell cycle and whether the 30-kDa SUMO-1 hydrolase is involved in this regulation.

Recently, a growing number of proteins is found to be covalently modified and regulated by SUMO-1, but all of the proteins identified so far seem to use a single ligating pathway (9–13). If the modification of each protein is regulated, the deconjugation process catalyzed by multiple SUMO-1 isopeptidase seems to be important, even though the presently described 30-kDa SUMO-1 hydrolase contributes to this process awaits further study. The cDNA cloning of this enzyme, which is in progress, would provide a useful tool for clarifying the physiological roles.

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