Differential Regulation of Sentrinized Proteins by a Novel Sentrin-specific Protease*

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Sentrin-1, also called SUMO-1, is a protein of 101 residues that is distantly related to ubiquitin and another ubiquitin-like protein, NEDD8. Here we report the cloning of a novel sentrin-specific protease, SENP1, which has no homology to the known de-ubiquitinating enzymes or ubiquitin C-terminal hydrolases. However, SENP1 is distantly related to the yeast Smt3-specific protease, Ulp1. A COS cell expression system was used to demonstrate the activity of SENP1 in vivo. When HA-tagged sentrin-1 was co-expressed with SENP1, the higher molecular weight sentrin-1 conjugates were completely removed. Surprisingly, the major sentrinized band at 90 kDa remained intact. The disappearance of the high molecular weight sentrin-1 conjugates also coincided with an increase in free sentrin-1 monomers. SENP1 is also active against proteins modified by sentrin-2, but not those modified by ubiquitin or NEDD8. In addition, sentrinized PML, a tumor suppressor protein that resides in the nucleus, was selectively affected by SENP1, whereas sentrinized RanGAP1, which is associated with the cytoplasmic fibrils of the nuclear pore complex, remained intact. The inability of SENP1 to process sentrinized RanGAP1 in vivo is most likely due to its nuclear localization because SENP1 is active against sentrinized RanGAP1 in vitro. The identification of a nuclear-localized, sentrin-specific protease will provide a unique tool to study the role of sentrinization in the biological function of PML and in the pathogenesis of acute promyelocytic leukemia.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF149770. To whom correspondence should be addressed: Division of Molecular Medicine, Dept. of Internal Medicine, The University of Texas-Houston Health Science Center, 6431 Fannin, Suite 4.200, Houston, TX 77030. Tel.: 713-500-6660; Fax: 713-500-6647; E-mail: eyeh@heart.med.uth.tmc.edu.

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The abbreviations used are: HA, hemagglutinin; EST, expressed sequence tag; bp, base pair(s); PCR, polymerase chain reaction; kb, kilobase(s); GST, glutathione S-transferase; RACE, rapid amplification of cDNA ends; RAα, retinoic acid receptor α; nt, nucleotide(s).
93000 on the 193-kb human genomic DNA led to the identification of another region (nt position 92516–92611) containing a conserved Cys site. The Cys surrounding sequence (PQQMNQSDCG) is 80% identical to the Cys site region of yeast Ulp1 (PQQPNGYDCG). Two specific oligonucleotides (one from nt position 98532–90041 and another from nt position 92516–92611) were used as primers for PCR amplification from a human placenta cDNA library. A 450-bp cDNA fragment was cloned, suggesting that the 193-kb DNA fragment contains a novel gene, which was later named SENP1. RACE of the 3′ end of SENP1 identified a stop codon at nt position 93643–93645 of the 193-kb DNA. RACE of 5′ end of the 450-bp cDNA led to the identification of the start codon for SENP1 at nt position 37672–37674. A 2511-bp cDNA fragment was cloned by PCR using primers from the RACE fragments. The full length of SENP1 (1.93 kb) was subcloned to pcDNA3 vector for further study. Direct sequencing of PCR fragments and comparison with genomic DNA confirmed the sequence of SENP1 cDNA.

**PCR, 5′-RACE, and Sequence Analysis—** Nested primers were synthesized on the basis of the information obtained from either the positive EST clones or from the genomic DNA. These primers were used to amplify the novel protease gene fragments by PCR from a human placenta cDNA library. Both PCR and RACE were performed as described previously (23). The nucleotide sequences were determined using dye terminator sequencing and an automated sequencer from Applied Biosystems Inc. (Foster City, CA).

**Plasmid Construction and Transfection—** The cDNA for ubiquitin, NEDD8, sentrin-1, sentrin-2, PML, and RanGAP1 were subcloned into pcDNA3-HA vector as described previously (2). The full-length cDNA fragment for SENP1 was cloned into pcDNA3-RGH vector using standard techniques. The plasmids described above were transfected into COS cells using LipofectAMINE (Life Technologies, Inc.) described previously (2).

**Western Blotting—** Protein samples were treated at 45 °C for 1 h in 300 μl of 2% SDS treating solution containing 5%-mercaptoethanol. Western blotting was performed using the protocol provided by ECL.

**Fig. 1.** Sequence alignment of SENP1 and Ulp1. Identical amino acids are shaded blocks. Accession numbers for SENP1 and Ulp1 are AF149770 and AAB68167, respectively. The underline represents the sequence of the “core domains” of yeast Ulp1 and its related enzymes. The asterisks mark likely residues of the catalytic triad (histidine, aspartate, and cysteine) and a glutamine residue predicted to form the oxyanion hole in the active site.

**Fig. 2.** Genomic organization of SENP1. Panel A shows that SENP1 is located in 12q13.1 and spans about 61 kb. SENP1 gene is composed of 18 distinct exons. Panel B shows the exon and intron organization of the human SENP1 gene. The 5′ acceptors GT and the 3′ donor AG are underlined. The exonic sequences are printed in bold.
Fig. 3. SENP1 is a sentrin-specific protease. A, HA-sentrin-1 was co-expressed in COS cells with vector control (lane 1), His-SENP1 (lane 2), and Myc-SENP1 (lane 3). HA-ubiquitin was co-expressed in COS cells with vector control (lane 4), His-SENP1 (lane 5), and Myc-SENP1 (lane 6). Total cell lysates were analyzed by immunoblotting with an anti-HA monoclonal antibody. Sentrinized proteins, p90, sentrin-1 monomer, ubiquitin ladder, and ubiquitin monomer are indicated. B, HA-sentrin-2 was co-expressed in COS cells with vector control (lane 1) or His-SENP1 (lanes 2). Total cell lysates were analyzed by immunoblotting with an anti-HA monoclonal antibody. Sentrinized proteins, p90, sentrin-2 monomer are indicated. C, HA-NEDD8 was co-expressed in COS cells with vector control (lane 1) or His-SENP1 (lanes 2). Total cell lysates were analyzed by immunoblotting with an anti-HA monoclonal antibody. NEDD8-modified proteins and NEDD8 monomer are indicated.

RESULTS AND DISCUSSIONS

cDNA Cloning and Genomic Organization of SENP1—Li et al. (22) recently reported a novel protease, Ulp1, specific for Smt3, the yeast homologue of sentrin-1. In the same report, a human EST sequence homologous to Ulp1 was tentatively termed HsUlp1. Using the partial-length HsUlp1 cDNA as a query in a BLASTn sequence search, we detected a human clone containing one conserved histidine residue present in all Ulp1-related proteins from different species (22). Further analysis of the translated sequence from the 193-kb genomic DNA fragment (accession number AC004801). This 209-bp region contains one conserved cysteine residue.
determine whether this genomic DNA encodes a functional protein, we amplified a 450-bp cDNA fragment from a human placenta cDNA library with PCR using primers based on the conserved histidine and cysteine site sequences. Extension of the cloned cDNA by RACE resulted in the identification of a 2511-bp cDNA clone from a human placenta cDNA library. The 2511-bp cDNA clone contains an open reading frame of 1929 bp, encoding a protein of 643 amino acids (Fig. 1). The predicted coding region of this open reading frame is preceded by an in-frame stop codon and has a consensus sequence commonly associated with initiation methionines (27). The protein encoded by this open reading frame was named SENP1 (sentrin-specific protease-1) because it possesses a protease activity against sentrin-modified proteins but not ubiquitin or NEDD8-modified proteins in vivo (see below).

As shown in Fig. 1, SENP1 is 21% identical and 50% similar to yeast Ulp1. The similarity between SENP1 and yeast Ulp1 is confined primarily to the C-terminal region of ~200 amino acids, within which an ~90-residue segment has been proposed to form a core structure common to a diverse and widespread group of cysteine proteases (22). Similar to yeast Ulp1, SENP1 contains the four conserved catalytic residues of an adenoviral protease (22).

The SENP1 gene is located in 12q13.1 because it is carried by the Homo sapiens 12q13.1 PAC RPCI–228P16 fragment (Roswell Park Cancer Institute Human PAC Library; accession number AC004801). It spanned about 61 kb of contiguous DNA (Fig. 2). The SENP1 gene is composed of 18 distinct exons ranging between 39 and 487 bp. Both the 5’ and 3’ acceptor splice sites in each of the introns followed the GT-AG consensus sequence for eukaryotic genes (Fig. 2). Both exon 1 (112 bp) and exon 2 (47 bp) encoded most of the 5’-untranslated region, whereas exon 3 contained the remaining 14 bp of the 5’-untranslated region plus the first 36 amino acids. Exons 5 through 17 encoded most of the amino acids, with exon 18 (~487 bp) containing the final 20 codons and an extensive 3’-untranslated region of ~423 bp.

**SENP1 Is a Sentrin-specific Protease**—A COS cell expression system was used to demonstrate the activity of SENP1 in vivo. Briefly, HA-tagged sentrin-1 was introduced into COS cells by liposome-mediated transfection as described previously (2). Total cell lysates were prepared 16 h after transfection for Western blot analysis using anti-HA antibody. As shown in Fig. 3A, lane 1, a 90-kDa band and higher molecular weight sentrin-1 conjugates were detected. When HA-tagged sentrin-1 was co-expressed with His-tagged SENP1, the higher molecular weight sentrin-1 conjugates were completely removed (lane 2). However, the 90 kDa band, which most likely represents sentrinized RanGAP1, remained intact. The disappearance of the high molecular weight sentrin-1 conjugates also coincided with the accumulation of free sentrin-1 monomers. A similar pattern was observed when Myc-tagged SENP1 was co-expressed with His-tagged SENP1, the higher molecular weight sentrin-1 conjugates were completely removed (lane 2). However, the 90 kDa band, which most likely represents sentrinized RanGAP1, remained intact. The disappearance of the high molecular weight sentrin-1 conjugates also coincided with the accumulation of free sentrin-1 monomers. A similar pattern was observed when Myc-tagged SENP1 was co-expressed with His-tagged SENP1, the higher molecular weight sentrin-1 conjugates were completely removed (lane 2).

The inability of SENP1 to reduce the 90-kDa band suggests that SENP1 cannot remove sentrin from all sen-
trinized proteins. Thus, we tested the effect of SENP1 on two specific sentrin conjugates, RanGAP1 and PML. RanGAP1 is a 70-kDa cytosolic protein that can be modified by a single molecule of sentrin-1 (28, 29). Sentrinized RanGAP1 (90 kDa) is a component of the nuclear pore complex and plays a role in regulating nuclear transport. When HA-tagged RanGAP1 was expressed in COS cells, a 70-kDa unmodified form of RanGAP1 and a 90-kDa band corresponding to sentrinized RanGAP1 were observed (Fig. 4A, lane 1). Co-expression of SENP1 was unable to remove native sentrin from HA-tagged RanGAP1 (lane 2). This is consistent with the results shown in Fig. 3, A and B, which show that the 90-kDa band is resistant to SENP1. Li et al. (22) have shown that SUMO-1 (sentrin-1)-modified RanGAP1 could be cleaved by Ulp1 using an in vitro assay. To further explore the difference between our in vivo results and Li’s in vitro observations, we tested GST-tagged SENP1 fusion protein in an in vitro assay (Fig. 4B). As shown, sentrinized RanGAP1 could be effectively processed by GST-SENP1 but not by GST in vitro. The discrepancy between the in vitro and in vivo activity of SENP1 against sentrinized RanGAP1 could be explained by the observation that SENP1 is localized in the nucleus (see below). Thus, the nuclear localized SENP1 does not have access to sentrinized RanGAP1, which is attached to the cytoplasmic fibrils of the nuclear pore complex. This interpretation is also supported by our previous finding that the majority of high molecular weight sentrinized proteins, which are sensitive to SENP1 (Fig. 3, A and B), are localized in the nucleus (2). To test this hypothesis further, we studied the in vivo activity of SENP1 against a well studied nuclear protein, PML. As expected, native sentrin was completely removed from sentrinized PML in COS cells expressing His-tagged SENP1 (Fig. 4A, lane 4).

The importance of cellular localization in the activity of SENP1 is also supported by the following co-localization experiment in which Myc-tagged SENP1 was transfected into HEp-2 cells. Fig. 5, D-F, in a field with seven cells, two HEp2 cells have been transfected with SENP1-containing plasmid and express SENP1 in the nucleus (red). In these two transfected cells, sentrin-1 (green) is localized in a nuclear rim pattern consistent with the nuclear-pore localization of RanGAP1. In contrast, in the five cells that were not transfected, sentrin-1 is localized not only in the nuclear rim, but also in nuclear dots consistent with the localization of PML-containing nuclear body (also see Fig. 5, A-C). Thus, SENP1 appears to selectively remove sentrin-1 from the PML-containing nuclear body but not from the nuclear rim where RanGAP1 is localized. Thus, the cellular localization results in HEp2 cells (Fig. 5) are consistent with the Western blot results of the COS cells (Fig. 4). Our results also suggest that removal of sentrin-1 from the PML-containing nuclear body does not destabilize it. PML, a RING finger protein with tumor suppressor activity, has been implicated in the pathogenesis of acute promyelocytic leukemia that arises following a reciprocal chromosomal translocation that fuses the PML gene with the retinoic acid receptor α (RARα) gene (30). In acute promyelocytic leukemia, two forms of PML-RARα fusion proteins have been reported. Remarkably, both forms of PML-RARα fusion proteins could not be sentrinized in vivo (10). Further experiments are needed to study the role of SENP1 in regulating the biological function of PML and in the pathogenesis of acute promyelocytic leukemia.

In summary, SENP1 is a sentrin-specific protease that preferentially removes sentrin from sentrinized proteins in the nucleus. Survey of the data base reveals at least four other human EST sequences that have significant homology with SENP1. Thus, it is likely that additional sentrin-specific protease will be identified in the near future that is localized in different cellular compartments and is involved in the regulation of different sentrinized proteins in vivo.

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