Characterization of a Family of Nucleolar SUMO-specific Proteases with Preference for SUMO-2 or SUMO-3*

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SUMOylation is a reversible process regulated by a family of sentrin/SUMO-specific proteases (SENPs). Of the six SENP family members, except for SENP1 and SENP2, the substrate specificities of the rest of SENPs are not well defined. Here, we have described SENP5, which has restricted substrate specificity. SENP5 showed SUMO-3 C-terminal hydrolase activity but could not process pro-SUMO-1 in vitro. Furthermore, SENP5 showed more limited isopeptidase activity in vitro. In vivo, SENP5 showed isopeptidase activity against SUMO-2 and SUMO-3 conjugates but not against SUMO-1 conjugates. Native SENP5 localized mainly to the nucleolus but was also found in the nucleus. The N terminus of SENP5 contains a stretch of amino acids responsible for the nucleolar localization of SENP5. N-terminal-truncated SENP5 co-localized with PML, a known SUMO substrate. Using PML SUMOylation mutants as model substrates, we showed that SENP5 can remove poly-SUMO-2 or poly-SUMO-3 from the Lys160 or Lys490 positions of PML. However, SENP5 could not remove SUMO-1 from the Lys160 or Lys490 positions of PML. Nonetheless, SENP5 could remove SUMO-1, -2, and -3 from the Lys65 position of PML. Thus, SENP5 also possesses limited SUMO-1 isopeptidase activity. We were also able to show that SENP3 has substrate specificity similar to that of SENP5. Thus, SENP3 and SENP5 constitute a subfamily of SENPs that regulate the formation of SUMO-2 or SUMO-3 conjugates and, to a less extent, SUMO-1 modification.

A broad array of cellular processes, such as cell division, differentiation, signal transduction, trafficking, and quality control, depend on the covalent modification of proteins by ubiquitin (Ub), a highly conserved 76-amino acid polypeptide (1). A variety of target proteins are modified by Ub through the action of a multi-enzyme system consisting of E1 (Ub-activating), E2 (Ub-conjugating), and E3 (Ub-ligating) enzymes. The discovery of Ub-like modifiers has further expanded our understanding of this particular form of post-translational protein modification (2, 3). In humans at least, 12 Ub-like molecules have been identified and defined by their sequence and structural homology to Ub. Of these, the best characterized is the SUMO family, which contains SUMO-1, SUMO-2, and SUMO-3, and the recently identified SUMO-4 (4–8). Native SUMO-4 appears to be unable to form covalent isopeptide bonds with substrates (9). Thus, it appears that SUMO-4 has a biological role that is accomplished through non-covalent interactions. SUMO-1 shows ~50% sequence identity to SUMO-2 and SUMO-3, which are almost identical. In contrast to SUMO-1, SUMO-2 and SUMO-3 harbor a consensus SUMOylation site at their N-terminal region and likely form polymers, as demonstrated for SUMO-2 (10). Studies on protein SUMOylation have mainly focused on SUMO-1, a protein of 101 amino acids that shares 18% sequence identity with Ub. More than 100 SUMOylated proteins have been identified to date, including transcription factors, signaling molecules, nuclear envelope proteins, and even cell membrane proteins (2, 11–14). SUMOylation is catalyzed by the E1-activating enzyme complex (Aos1/Uba2), the E2-conjugating enzyme (UBC9), and E3 ligases (Ran-BP2, Hec1, or PIAS family members) (15–19).

The covalent modification of proteins by SUMO is reversibly regulated by a family of sentrin/SUMO-specific proteases (SENPs) (2). The SENPs in yeast and mammals have well conserved residues of the catalytic triad (His, Asp, and Cys) and an invariant Gln residue predicted to help form the oxyanion hole in the active site (20, 21). Two SENPs in yeast, Ulp1 and Ulp2/Smt4, have been reported (20, 22). Ulp1 is an essential protein that co-localizes with nuclear pore proteins, whereas Ulp2 localizes predominantly to the nucleus. At least seven human proteases (SENPI–8) have a conserved catalytic domain homologous to that of yeast Ulp1 (2). However, SENP8 (also called DEN1 or NEDP1) has less similarity with other SENPs and has been shown to be a NEDD8-specific protease (23, 24).

Much of our knowledge of the behavior of SENPs has come from studies of SENP1 and SENP2. These have shown that SENP1 localizes to the nucleus, excluding the nucleolus (21), and that SENP2, also called SUSP1, localizes to the nucleoplasmic face of the nuclear pore complex (25, 26). Both SENP1 and SENP2 are able to remove SUMO from all SUMO-modified proteins. SENP1 can also regulate a large family of transcription factors and their co-regulators (13). Although both SENP1 and SENP2 can regulate c-JUN-dependent transcription, SENP1 mediates its effect through the desumoylation of p300, whereas SENP2 exerts its effect via PML (27, 28). Two additional SENPs, SENP3 (SMT3P1) and SENP6 (SUSP1), have also been reported (29, 30). However, their mechanisms of actions are largely unknown.

Here we have reported our findings regarding SENP5, a novel SUMO-specific protease that has specificity for SUMO-2 and SUMO-3 but less so for SUMO-1. Because SENP3 is closely related to SENP5, we also investigated its substrate specificity. Interestingly, we found SENP3 is also active against SUMO-2 and SUMO-3 conjugates but, like SENP5, less so against SUMO-1 conjugates. Thus, SENP3 and SENP5 constitute a new subfamily of SENPs that share considerable sequence homology and exhibit similar substrate specificities.
A Second Family of SUMO-specific Proteases

MATERIALS AND METHODS

Cell Lines and Culture Conditions—COS-7 and HeLa cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and antibiotics. Transfections were performed in genetin-free medium.

Antibodies—Mouse anti-RGS (specific for the amino acid sequence RGS1) monoclonal antibody was purchased from Qiagen (Santa Clara, CA). 16B12 (Babco, Richmond, CA) is a mouse monoclonal antibody to the peptide sequence YPYDVPDYA of influenza hemagglutinin (HA). The coding region of SENP5 was subcloned into pGEX-5X1, a prokaryotic expression vector for the expression of recombinant enzyme in Escherichia coli. The fusion protein was used as an immunogen for generating polyclonal anti-SENP5 anti-serum. After purification, this anti-serum was used for Western blot analysis and immunohistochemical staining.

cDNA Cloning of the Human SENPs—To clone human SENPs, we initially performed a tBLASTn search of the data bases for a human-expressed sequence tag (EST) using the coding sequence of SENP1. 102 positive EST sequences were identified in the initial screen. After further analysis, one EST clone (AA134543) was found to have a 595-bp sequence similar to the “core domain” of SENP1. The nucleotide sequence of this EST clone was used to perform another BLAST search of the EST data base. One of the positive EST clones (A1651270) was shown to partially overlap with AA134543 and contain most of the 3’-non-coding region. Another positive EST clone (AA342658) extended the cDNA sequence toward the 5’-end for an additional 500 bp. The combined nucleotide sequence from these three EST clones was extended to a 1.5-kb cDNA fragment, which was confirmed by PCR amplification. The cDNA was extended toward the 5’-end by performing rapid amplification of the 3’-cDNA ends (RACE) using the oligonucleotides generated from a cDNA library vector and AA342658. A 2801-bp cDNA fragment was cloned by PCR using primers from the RACE fragment and A1651270. The full length of SENP5 (2.2 kb) was subcloned to a pcDNA3 vector for further study. Direct sequencing of the PCR fragments and comparison with genomic DNA confirmed the sequence of SENP5 cDNA.

PCR, RACE, and Sequence Analysis—Nested primers were synthesized on the basis of the information obtained from the positive EST clones. 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 (16). 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 cDNAs for Ub, NEDD8, SUMO-1, SUMO-2, and SUMO-3 were subcloned into a pcDNA3-HA vector as described previously (31). The full-length cDNA fragments for SENP3 and SENP5 were cloned into a pcDNA3-RGS vector using standard techniques. The plasmids described above were transfected into COS-7 cells using Lipofectamine (Invitrogen) as described previously (4).

GST Fusion Protein Expression and Purification in E. coli—The full-length cDNA of SENP1 or SENP5 was amplified by PCR from a human placenta cDNA library. The PCR product was then subcloned into pGEX-5X1 (Amersham Biosciences Inc.) using EcoRI and Sall restriction sites to generate pGEX-SENP1 or pGEX-SENP5. E. coli BL21 cells carrying pGEX-SENP1 or pGEX-SENP5 or pGEX-5X1 were then grown to saturation in 10 ml of Luria Bertani broth containing 50 μg/ml of ampicillin and then transferred to 500 ml of Luria Bertani broth for expansion to an absorbance (at 600 nm) of 0.8. After the addition of isopropyl-β-D-thiogalacto-pyranoside (final concentration 0.1 mM), the culture was incubated at room temperature for 3 h. The cells were then sonicated in lysis buffer (20 mM Tris, pH 8.0, 1 mM EDTA, 100 mM NaCl, 1% Igepal CA-630, 100 μg/ml of egg white lysozyme), and bacterial debris was removed by centrifugation. The sonicated lysate was incubated at 4°C for 1 h with 500 μl of 50% slurry of glutathione-Sepharose 4B beads. After centrifugation, glutathione S-transferase fusion protein-bound beads were washed three times, and the protein was eluted from the beads.

In Vitro Translation and SENP Protease Activity—For in vitro expression of RGS-RanGAP1, SUMO-1-HA, and SUMO-3-HA proteins, the pcDNA3-RGS-RanGAP1, pcDNA3-SUMO-1-HA, and pcDNA3-SUMO-3-HEA plasmids were used as templates. Proteins were translated by using an in vitro transcription and translation kit (TNT T7-coupled rabbit reticulocyte lysates; Promega) according to the instruction of the supplier. All translated proteins were confirmed by using Western blot analysis. For the isopeptidase activity, 5 μl of in vitro translated and SUMO-conjugated RanGAP1 was incubated at 30°C for 60 min with ~0.05 μg of GST-SENP1 or 5 μg of GST-SENP5 in a reaction mixture containing 150 mM NaCl, 1 mM dithiothreitol, and 10 mM Tris-HCl, pH 8.0. For the C-terminal hydrolase activity, 5 μl of in vitro translated SUMO-1-HEA or SUMO-3-HEA was incubated at 30°C for 60 min with ~0.05 μg of GST-SENP1 or 5 μg of GST-SENP5 in a reaction mixture containing 150 mM NaCl, 1 mM dithiothreitol, and 10 mM Tris-HCl, pH 8.0. The samples were loaded onto a 10% SDS-Polyacrylamide gel and detected by Western analysis with anti-RGS or anti-HA antibodies.

TALON Bead Precipitation of RH-tagged Proteins—To investigate the effect of SENP3 or SENP5 on SUMO-2 conjugation of PML-K160, we purified RH-tagged PML-K160 by TALON bead precipitation and analyzed their SUMOylation by Western blotting. Because the sequence of the RH tag is RGS1HHHHHH, RH-tagged PML-K160 can be purified by cobalt-immobilized resin beads (TALON beads; Clontech).

The total cell lysate of the transfectants expressing RH-tagged PML-K160 and HA-SUMO-2 with different SENPs and SENPm was prepared in lysis buffer (20 mM Tris-HCl (pH 8.0), 6 mM guanidine-HCl, and 100 mM NaCl). DNA in the sample was sheared with a 22-gauge needle, and then the lysate was centrifuged at 100,000 g for 30 min at 15°C. The supernatant was incubated with TALON beads for 1 h at room temperature. The beads were washed once with lysis buffer, followed by two washings with washing buffer (20 mM Tris-HCl (pH 7.0), 15 mM imidazole, 8 mM urea, and 100 mM NaCl). The beads were then washed twice with phosphate-buffered saline and treated for 1 h at 50°C in sample treating solution containing 2% SDS and 5% β-mercaptoethanol. Finally, the solubilized RH-PML-K160 was analyzed by Western blotting.

Western Blotting Analysis—HA-tagged SUMO-1, SUMO-2, SUMO-3, NEDD8, or Ub were co-expressed with RGS-tagged SENP5, RGS-tagged SENP3, or control vector in COS-7 cells. Protein samples were treated at 42°C for 1 h in 300 μl of 2% SDS treating solution containing 5% mercaptoethanol. Western blotting was performed using the protocol provided with the ECL detection system (Amersham Biosciences Inc.). Horseradish peroxidase-conjugated antibodies against mouse IgG or rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) were used as secondary antibodies.

Immunohistochemical Localization of Proteins—First, we transfected plasmids encoding RGS-tagged SENP5 into HeLa cells. HeLa cells were grown at 37°C on round coverslips in 6-well plates (Corning Glass, Inc., Corning, NY) until they reached ~80% confluency. Sixteen hours after the cells had been plated on the round glass coverslips, the cells were fixed at room temperature for 15 min with freshly prepared 4% paraformaldehyde in phosphate-buffered saline, after which 10% goat
serum (Invitrogen) was used to block the background activity. For the immunohistochemical staining of pcDNA3-RGS-SENP5-transfected cells, monoclonal mouse anti-RGS or polyclonal rabbit anti-SENP5 was used to visualize the RGS-SENP5. Cellular nuclei (chromatin) were stained with 1:1000 4,6-diamidino-2-phenylindole (Sigma-Aldrich).

RESULTS

Characterization of SENP5 Gene and cDNA—Using the full-length SENP1 protein sequence as a query in a tBLASTn sequence search, we detected three positive EST clones (T70794, N42548, and AW167097) that were homologous to the C-terminal of SENP1 (21). Extension of the cloned cDNA by RACE resulted in the identification of a 2802-bp cDNA clone from a human placenta cDNA library that contained an open reading frame of 2265 bp encoding a protein of 755 amino acids. The protein encoded by this open reading frame was named SENP5 (2). Data base searches also identified SENP5 orthologs in monkey, dog, rat, and mouse, but no detectable homologs were present in \textit{Saccharomyces cerevisiae}, \textit{Drosophila melanogaster}, \textit{Caenorhabditis elegans}, \textit{Fugu rubripes}, or \textit{Danio rerio}. Although all SENPs share a conserved catalytic domain in the C-terminal region, the domain of SENP1 was more homologous to that of SENP2 (59%) than to that of either SENP3 (42%) or SENP5 (44%) (Fig. 1A). In contrast, the catalytic domain of SENP5 was 62% identical to that of SENP3 but only 44% and 42% identical to that of SENP1 and SENP2, respectively. Thus, SENP5 is more closely related to SENP3 than to either SENP1 or SENP2. Furthermore, SENP5 and SENP3 shared a 40% identity at another region directly preceding the catalytic domain, 507–573 amino acids of SENP5 and 321–392 amino acids of SENP3 (Fig. 1A). A similar conserved region is shared by SENP1

![FIGURE 1. A, comparison of the SUMO-specific proteases SENP1, SENP2, SENP3, and SENP5. The C-terminal catalytic domains are shown in block. The conserved region immediately adjacent to the catalytic domain is shown in gray. SENP1 and SENP2 share an additional conserved domain in the N-terminal. B, Western blot of endogenous SENP5 detected in three different cell lines. Preimmune serum (left panel) and anti-SENP5 serum (right panel) were used to detect endogenous SENP5 in three different human cell lines, PC3, HeLa, and MCF7. Endogenous SENP5 bands are marked with an asterisk. C, SENP5 is localized in the nucleolus and nuclei of HeLa cells transfected with RGS-tagged SENP5 as shown by fluorescence microscopy. a, the anti-SENP5 serum was used to identify exogenous RGS-SENP5 (four cells at lower right) and endogenous SENP5 (four cells at upper left indicated by arrowheads). b, anti-RGS antibody was used to identify exogenous RGS-SENP5 only. c, 4,6-diamidino-2-phenylindole staining. d, staining with anti-SENP5 antibody showing localization of endogenous SENP5. e, staining with anti-B23 antibody showing localization of endogenous B23, a nucleolar protein.](image-url)
and SENP2, in that the 173–201-amino acid region of SENP1 was 55% identical to the 196–223-amino acid region of SENP2 (Fig. 1A). Thus, our analysis of sequence homology showed SENP1 and SENP2 to be more closely related and SENP3 and SENP5 to be more closely related.

We found the SENP5 gene is located on human chromosome 3 and spans ∼39 kb of contiguous DNA. Further, we found that the SENP5 gene is composed of nine distinct exons ranging between 48 and 1544 bp. Both the 5′- and 3′-acceptor splice sites in each of the introns followed the GT-AG consensus sequence for eukaryotic genes. Exon 1 encodes the 5′-untranslated region, exon-2 encodes the remaining 5′-untranslated region and first 196 amino acids, exons 3 through 8 encode most of the amino acids, and exon 9 (∼399 bp) contains the final 37 codons and an extensive 3′-untranslated region of ∼288 bp.

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To characterize SENP5 in more detail, we generated a polyclonal anti-serum against SENP5 using the GST-SENP5 protein as an immunogen. Anti-SENP5 serum was also used to detect the expression of native SENP5 in three different cell lines, PC3, HeLa, and MCF7 cells, derived from prostate, blood, and breast tissue, respectively. As shown in Fig. 1B, an ∼87-kDa band, consistent with the predicted molecular mass of SENP5, was identified in all cell lysates of the three different cell lines when blotted with anti-SENP5 serum, whereas the preimmune serum did not reveal this band. This anti-serum was also used to identify the location of endogenous SENP5 in HeLa cells. First, we transfected plasmids encoding RGS-tagged SENP5 into HeLa cells. As shown in Fig. 1C–a, anti-SENP5 antiserum detected both the endogenous SENP5 and the exogenously expressed RGS-tagged SENP5. Further, both anti-SENP5 antisemur (Fig. 1C–a) and anti-RGS antibody (Fig. 1C–b) staining indicated that SENP5 localizes to both the nucleolus and the nucleus. 4,6-Diamidino-2-phenylindole staining for the nucleus is shown in Fig. 1C–c. In a separate staining experiment in which HeLa cells were stained with anti-SENP5 anti-serum to detect the endogenous SENP5 and with anti-B23 antibody to detect the nucleolar protein nucleoplasmin/NPM/ 

**Cellular Localization and Expression of Endogenous SENP5**

To determine whether SENP5 also has this ability, we expressed and purified GST-SENP5 fusion proteins in bacteria. The HA tag was fused to the C-terminal end of SUMO-1 to generate a SUMO-1-HA fusion protein that could be used as a substrate to demonstrate SUMO-specific C-terminal hydrolase activity. The HA-tagged SUMO-1 could be detected by anti-HA antibody. As expected, however, SUMO-1-HA was absent in the sample treated with GST-SENP1, suggesting that the HA tag was removed from the SUMO-1-HA fusion protein by GST-SENP1 (Fig. 2A). In contrast, GST-SENP5 could not remove the HA tag from the SUMO-1-HA fusion protein, suggesting that GST-SENP5 does not have C-terminal hydrolase activity against SUMO-1. In terms of the protein sequence, SUMO-2 and SUMO-3 belong to the same subgroup of the SUMO family because they are almost identical. SUMO-1, however, is only ∼50% identical to SUMO-2 and SUMO-3. To determine whether GST-SENP5 functions as a C-terminal hydrolase against SUMO-2/3 subfamily, we generated SUMO-3-HA fusion protein. As shown in Fig. 2A, both GST-SENP1 and GST-SENP5 could remove the HA tag from the SUMO-3-HA fusion protein, indicating that GST-SENP5 has C-terminal hydrolase activity against SUMO-3 but not against SUMO-1.

**Preference of SENP5 for SUMO-2 and SUMO-3 Conjugates in Vivo**

When we tested for SENP5 isopeptidase activity against SUMO conjugates in vitro and in vivo, we found one clear SUMO-conjugated band when RanGAP1 was translated in vitro (Fig. 2B). Further, when GST-SENP1 or GST-SENP5 was used to digest SUMO-RanGAP1 in vitro, both GST-SENP1 and GST-SENP5 proved to have SUMO-specific isopeptidase activity (Fig. 2B). However, the protease activity of GST-SENP1 is 100-fold higher than GST-SENP5 (data not shown). Alternatively, SENP5 may not be able to remove all SUMO family members from SUMO-RanGAP1 because in vitro translated RanGAP1 could be modified by SUMO-1, -2, or -3 (also see description below).

To demonstrate the protease activity of SENP5 in vivo, HA-tagged SUMO-1, SUMO-2, SUMO-3, NEDD8, or ubiquitin was co-expressed with RGS-tagged SENP5 or control vector in COS-7 cells. Protein modification in whole-cell lysates was detected using an antibody against the HA tag. A greater portion of higher molecular weight SUMO conjugates was removed when HA-tagged SUMO-2 or SUMO-3 was co-expressed with RGS-tagged SENP5 than when HA-tagged SUMO-3, NEDD8, or Ub was co-expressed with RGS-tagged SENP5 (Fig. 3). However, the cleavage of the SUMO-2 conjugates appeared to be less complete than that of the SUMO-3 conjugates. The SENP5(C713A) catalytic inactive mutant, which was transfected in place of SENP5 and served as a control, exhibited no activity against either SUMO-2 or SUMO-3. Nonetheless, the expression levels of both SENP5 and SENP5(C713A) were identical in all lanes, thus ruling out the possibility that the differences were due to differences in the expression of the wild-type and mutant protease (Fig. 3). In addition, SENP5 demonstrated a preference for SUMO-2 and SUMO-3 because it could not process Ub, NEDD8, or SUMO-1 conjugates in vivo.

**FIGURE 2**. A, SENP5 has C-terminal hydrolase activity against SUMO-3-HA, but not SUMO-1-HA, whereas SENP1 is active against both SUMO-1-HA and SUMO-3-HA. B, lane 1 shows the input sample of translated RGS-RanGAP1. Both SENP1 (lane 3) and SENP5 (lane 2) can remove SUMO from SUMO-RanGAP1 in vitro. The concentration of GST-SENP5 is 100-fold higher than GST-SENP1.
Respective Effects of SENP5 and Truncated Forms of SENP5—As described above, SENP5 is a nucleolar, SUMO-specific protease with preference for SUMO-2 and SUMO-3. To define the specific regions of SENP5 responsible for the intracellular localization and protease activity of the enzyme, we created a series of deletion mutants of SENP5, as shown in Fig. 4A. Most of the full-length SENP5 was present in the nucleolus (Fig. 4C). However, when the first 168 amino acids of SENP5 were removed, the resultant truncated SENP5, SP5d168, was localized to both the nucleus and cytosol, suggesting that the N-terminal region of SENP5 is responsible for its nucleolar localization. Interestingly, the truncated SENP5, SP5d310, co-localized with PML (Fig. 4D). To define the minimal sequence required for SENP5 to exert its catalytic activity, a series of deletion mutants were tested using SUMO-2-modified PML-K65(K160R,K490R) as a substrate. This showed that the deletion of amino acids 1–571 did not eliminate the protease activity, whereas the deletion of an additional 29 amino acids (SP5d590) did (Fig. 4B). Thus, the catalytic activity of SENP5 is independent of its N-terminal sequences and is conferred by the catalytic domain alone.

Fine Mapping of Substrate Specificity of SENP5 Using PML Mutants—Because the nuclear form of SENP5 co-localizes with PML, we chose PML as a substrate to further define substrate specificity of SENP5. We have previously shown that wild-type PML could be modified by SUMO-1, -2, and -3 and that Lys65 in the RING finger domain, Lys160 in the B1 Box, and Lys490 in the nuclear localization signal constitute three major SUMOylation sites (32). To further test the specific protease activity of SENP5, we first used a COS-7 cell expression system and wild-type PML as a substrate. As shown in Fig. 5, when the wild-type PML was co-expressed with different SUMOs in COS-7 cells, multiple SUMOylated bands could be observed (Fig. 5). As expected, the conjugated bands were completely removed when SENP1 was expressed (Fig. 5). In contrast, the expression of SENP5 only partially affected the pattern of PML SUMOylation (Fig. 5), suggesting that the protease activity of SENP5 is more restricted than that of SENP1.

To further define the specificity of SENP5, we used three different PML mutants (PML-K160(K65R,K490R), PML-K490(K65R,K160R), and PML-K65(K160R,K490R)) as substrates. These mutants had only a single Lys residue left for SUMO modification, thus allowing for a detailed mapping of substrate specificity. Both SENP1 and SENP5 could remove SUMO-1, -2, or -3 from the Lys65 site of PML (Fig. 5). Thus, protease activity of SENP5 is not restricted to SUMO-2 and SUMO-3. However, when SUMO-1 was co-expressed with PML-K160(K65R,K490R) or PML-K490(K65R,K160R), SENP5 could not remove SUMO-1 from the Lys65 or Lys490 conjugation sites (Fig. 5).
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FIGURE 4. A, a series of SENP5 deletion mutants were used to define the region responsible for nucleolar localization and protease activity. B, SP5d571 is active against SUMO-2-modified PML-K65(K160, K490), whereas SP5d590 is inactive. C, wild-type SENP5 is localized in the nucleolus, whereas the N-terminal truncated mutant (SP5d168) is present in the nucleus and cytoplasm. D, wild-type SENP5 could not co-localize with PML, but the N-terminal-truncated SENP5 mutant could co-localize with PML.

| Isopeptidase activity | Cellular localization |
|-----------------------|----------------------|
| SP5WT                 | Nucleolus            |
| SP5d168               | Nucleus and cytoplasm|
| SP5d310               | Nucleus and cytoplasm|
| SP5d511               | Nucleus and cytoplasm|
| SP5d671               | Nucleus and cytoplasm|
| SP5d900               | Nucleus and cytoplasm|

FIGURE 5. SUMO-specific protease activity of SENP1 and SENP5 shown using wild-type PML, PML-K65(K160R,K490R), PML-K160(K65R,K490R), and PML-K490(K65R,K160) as substrates. Vector (lanes 2, 8, and 14), SENP1 (lanes 3, 9, and 15), SENP1 catalytic mutant (lanes 4, 10, and 16), SENP5 (lanes 5, 11, and 17), or SENP5 catalytic mutant (lanes 6, 12, and 18) were co-transfected with PML or PML mutants with SUMO-1 (lanes 2–6), SUMO-2 (lanes 8–12), or SUMO-3 (lanes 14–18). The control samples contain PML (panel A, lanes 1, 7, and 13) or PML-K65 (panel B, lanes 1, 7, and 13) or PML-K160 (panel C, lanes 1, 7, and 13) or PML-K490 (panel D, lanes 1, 7, and 13). PML and SUMO-modified PML were identified by immunoblotting with anti-RGS antibody. PML monomer, PML-K65 monomer, PML-K160 monomer, or PML-K490 monomer is marked with an asterisk.
whereas SENP1 could readily remove SUMO-1 from these two sites. Thus, the ability of SENP5 to deconjugate SUMO-1 is limited to the Lys65 site of PML. Interestingly, SENP5 could remove SUMO-2 or SUMO-3 from the Lys160 and Lys490 conjugation sites (Fig. 5). Thus, SENP5 can remove SUMO-2 or SUMO-3 from Lys160 or Lys490.

Preference of SENP3 for SUMO-2 and SUMO-3—Because SENP3 is closely related to SENP5, we next tested whether SENP3 has a substrate specificity like that of SENP5, using a protocol similar to that used to detect the desumoylation activity of SENP5. As shown in Fig. 6, when HA-tagged SUMO-2 or SUMO-3 was co-expressed with RGS-SENP3, a major portion of higher molecular weight SUMO conjugates was removed. Replacement of the wild-type SENP3 with a SENP3(C532A) mutant significantly increased the expression of the SUMO-2 or SUMO-3 conjugates, suggesting that this SENP3 mutant functions as a dominant-negative protein. The expression levels of both SENP3 and the SENP3(C532A) catalytic mutant were then determined and shown to be identical in all lanes, thus ruling out the possibility that the differences were due to differences in the expression of the wild-type and mutant protease (Fig. 6). The activity of SENP3 was further determined to be specific for SUMO-2 and SUMO-3 because it could not process Ub, NEDD8, or SUMO-1 conjugates. Thus, SENP3 appears to also be specific for SUMO-2 and SUMO-3 conjugates, but not for SUMO-1.

To further define the specificity of SENP3, we used PML-K65, PML-K160, or PML-K490 as a substrate. Both SENP3 and SENP5 could remove SUMO-1 from the Lys65 site of PML (Fig. 7). In addition, when SUMO-2 or SUMO-3 was co-expressed with SENP3, SENP3 demonstrated substrate specificity similar to that of SENP5 (Fig. 7).

Finally, to determine whether SENP3 or SENP5 has the ability to remove mono-SUMO-2 from PML, HA-tagged SUMO-2 was co-expressed with RGS-tagged PML-K160. Unmodified PML-K160 and HA-SUMO-2-modified PML-K160 were precipitated with TALON beads and blotted with either anti-RGS antibody (left panel) or anti-HA antibodies (right panel). As shown, either SENP3 or SENP5 can remove SUMO-2 from SUMO-2-PML-K160, whereas SENP3 or SENP5 catalytic mutants could not.
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FIGURE 8. SUMO-2-specific protease activity of SENP3 and SENPS shown using PML-K160 as substrate. COS-1 cells were transfected with expression vectors for SENP1 (lanes 5 and 10), SENP3 (lanes 1 and 6), SENP3m (lanes 2 and 7), SENP5 (lanes 3 and 8), SENP5m (lanes 4 and 9), RGS-PML-K160 (lanes 1–10), and HA-SUMO-2 (lanes 1–10) as indicated, and cell extracts were lysed in 6 M guanidine HCl. RH-PML-K160 in the lysate was then precipitated by cobalt-coated TALON beads and analyzed by Western blotting using anti-HA antibody to detect HA-SUMO-2-conjugated PML-K160 and anti-RH antibody to detect the SUMOylated and non-conjugated PML-K160.

DISCUSSION

Sumoylation is a reversible process that is regulated by a family of SUMO-specific proteases (SENP) (2). From the standpoint of sequence homology, the SENP family can be divided into four subfamilies. All SENPs share a conserved catalytic domain, starting from the sequence DXXTL at the C-terminal end. The catalytic domain of SENP1 is 59, 42, and 44% identical to that of SENP2, SENP3, and SENP5, respectively. As shown in Fig. 2, SENP1 and SENP2 share a conserved sequence (MEKE1LXXQXXGQDIEK-SXAFXXITTR) preceding their catalytic domain and thus constitute the first SENP subfamily. The third subfamily of the SENPs consists of SENP6 and SENP7, which have a long insertion in their conserved catalytic domain. The fourth subfamily has one member, SENP8; although SENP8 shares the SENP catalytic domain, it is actually active against NEDD8 conjugates. One of the reasons why SENP8 may not be able to process SUMO conjugates is that SENP8 lacks some of the SUMO-binding sites (33). In particular, the side chains of Arg156 and Trp257 of SENP2 interact with the conserved SUMO-1 β3-β4 loop. However, Met and Phe are substituted in these sites in SENP8. SENP2 Phe441, which is identical within the rest of the SENP family members but is substituted to Glu in SENP8, appears important for the interaction between SENP2 and SUMO-1 (34).

In keeping with other findings, our current study suggests that the SENP subfamilies are functionally distinct as well. In particular, we found that SENP3 and SENP5 prefer SUMO-2 and SUMO-3 conjugates, but not SUMO-1 conjugates. This is unlike SENP1 and SENP2, which can process all of the SUMO substrates in vivo (21, 25, 26). Of the four members of the SUMO family that have been identified, SUMO-2, -3, and -4 are closely related, whereas SUMO-1 is distantly related to the other three SUMOs. In addition, using an antibody that recognizes SUMO-2 and -3, but not SUMO-1, it was shown that SUMO-2 and SUMO-3 account for a greater percentage of total cellular protein modification than does SUMO-1 (35). It was further shown that there is a large pool of free, non-conjugated SUMO-2 and SUMO-3 and that the conjugation of SUMO-2 or SUMO-3 to high molecular mass proteins was induced when cells were subjected to protein-damaging stimuli such as acute temperature fluctuations (35). It also appears that different SUMOs have different substrates. For example, SUMO-2 and SUMO-3 are conjugated poorly, if at all, to a major SUMO-1 substrate, the Ran GTPase-activating protein RanGAP1 (35). Further, in experiments using a proteomics approach, SUMO-1 was shown to have different substrates from those of SUMO-2 and SUMO-3 (36, 37). Thus, the existence of a particular SENP subfamily with a preference for SUMO-2 and SUMO-3, but not for SUMO-1, would allow for more specific regulation of these substrates. In this study, we did not use SUMO-4 as a modifier because full-length SUMO-4 cannot be processed to reveal the di-Gly-Gly residues and cannot be conjugated to any substrates (9).

As noted earlier, SENPs can also be distinguished on the basis of their subcellular localizations. For example, SENP1 localizes to the nucleus, excluding the nucleolus, whereas SENP2 localizes to the nuclear envelope (21, 25, 26). SENP3 localizes to the nucleolus, whereas SENP6 localizes to the cytosol (29, 30). Interestingly, we found SENP5 to also be highly enriched in the nucleolus. The nucleolus is a dynamic structure that disassembles and reforms during each cell cycle around the rRNA gene clusters (38). More than 200 different proteins have been identified in nucleolus preparations (39). Several of these participate in the processing of preribosomal RNA and in the modification and assembly of mature ribosome subunits, but the functions of many are uncharacterized or are associated with extranucleolar activity. In the nucleolus, more than 20 subsets of heterogeneous nuclear ribonucleoproteins form large complexes with primary RNA polymerase II transcripts, which play important roles in regulating multiple steps in mRNA biogenesis. Five subsets of heterogeneous nuclear ribonucleoproteins have been demonstrated to be modified by SUMO (40). Therefore, the presence of both SENP3 and SENP5 in the nucleolus may eliminate the SUMO-2 and SUMO-3 modification of nucleolar proteins, while allowing SUMO-1 conjugates to persist. In conclusion, we have defined two subfamilies of the SENPs. SENP1 and SENP2 are active against SUMO-1, SUMO-2, and SUMO-3, whereas SENP3 and SENP5 prefer SUMO-2 and SUMO-3 conjugates.

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