Small Ubiquitin-related Modifier (SUMO)-specific Proteases

PROFILING THE SPECIFICITIES AND ACTIVITIES OF HUMAN SENPs

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Jowita Mikolajczyk1,†, Marcin Drag1,‡, Mikiós Békés1,¶, John T. Cao1,§, Ze’ev Ronai1, and Guy S. Salvesen2,†‡

From the Programs in 1Apoptosis and Cell Death Research and 3Signal Transduction, Burnham Institute for Medical Research, La Jolla, California 92037, 2Graduate Program in Molecular Pathology, University of California, San Diego, La Jolla, California 92093, and the 4Division of Medicinal Chemistry and Microbiology, Faculty of Chemistry, Wroclaw University of Technology, Wyzwescie Wyspianskiego 27, 50-370 Wroclaw, Poland

SENPs are proteases that participate in the regulation of SUMOylation by generating mature small ubiquitin-related modifiers (SUMO) for protein conjugation (endopeptidase activity) and removing conjugated SUMO from targets (isopeptidase activity). Using purified recombinant catalytic domains of 6 of the 7 human SENPs, we demonstrate the specificity of their respective activities on SUMO-1, -2, and -3. The primary mode of recognition of substrates is via the SUMO domain, and the C-terminal tails direct endopeptidase specificity. Broadly speaking, SENP1 is the most efficient endopeptidase, whereas SENP2 and -5–7 have substantially higher isopeptidase than endopeptidase activities. We developed fluorogenic tetrapeptide substrates that are cleaved by SENPs, enabling us to characterize the environmental profiles of each enzyme. Using these synthetic substrates we reveal that the SUMO domain enhances catalysis of SENP1, -2, -5, -6, and -7, demonstrating substrate-induced activation of SENPs by SUMOs.

Small ubiquitin-related modifier (SUMO)3 belongs to a family of ubiquitin-like proteins that, similar to ubiquitin, are conjugated to their substrates by a dedicated ligation system. Conjugation of SUMO in most cases results in altered subcellular localization of the modified protein, with consequent effects on its activity. The list of proteins subjected to SUMOylation is rapidly growing, and includes proteins localized in most subcellular compartments that are involved in the regulation of cell cycle, transcription, cell survival and death, DNA damage response, heat shock, and stress response, as well as endoplasmic reticulum and plasma membrane-associated proteins, receptors, and viral proteins (reviewed in Refs. 1–3).

Modification of proteins by SUMO is a dynamic and reversible process. The SUMO cycle begins when SUMO precursors are processed to remove short C-terminal extensions, thereby uncapping the C-terminal Gly-Gly motif that is essential for conjugation. SUMO ligases conjugate the protein, via its C-terminal carboxylate, to the side-chain lysine of target proteins to generate an isopeptide linkage. Eventually, SUMO is removed intact from its substrate SUMOylated proteins, and so the SUMOylation/deSUMOylation cycle regulates SUMO function. A group of proteases known as SENPs are involved in both the maturation of SUMO precursors (endopeptidase cleavage) and deconjugation of the targets (isopeptidase cleavage) (4, 5). Not all SENPs are SUMO-specific, indeed SENP8 is not a SUMO protease but functions on another small ubiquitin-related protein known as Nedd8 (6, 7). The defining characteristic of SENPs is their predicted conserved molecular scaffold, defined as members of peptidase clan CE (8), conserved catalytic mechanism, and their reported activity on SUMO or Nedd8 conjugated proteins (or the respective precursors).

Humans contain seven SENPs (SENPs -1, -2, -3, -5, -6, -7, and -8), and several of these have been characterized as SUMO (or Nedd8) endopeptidases or isopeptidases. However, there exists almost no information on SENP6 and -7, and there is considerable diversity in the literature regarding the specificity of individual SENPs for distinct SUMOs. In the effort to define the relative selectivities within the family, we have expressed and purified the catalytic domains corresponding to human SENP1, -2, and -5, -6, -7, and -8 and systematically analyzed their endopeptidase and isopeptidase activity on SUMO-1, -2, and -3 and Nedd8. This study also presents for the first time a fluorogenic assay based on simple peptides developed for SENPs that allow us to determine the influence of the SUMO targets on SENP activity and activation.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—Catalytic domains of SENP2, SENP7, and SENP8 were amplified from a human fetal brain cDNA library, and the catalytic domains of SENP1, SENP3, SENP5, and SENP6 were amplified from a human keratinocyte library. The PCR products were cloned into the bacterial expression vector pET28a (Novagen) engineered to contain an N-terminal His tag. The plasmids expressing SUMO precursor proteins His-tagged at the C terminus were generated using Ncol-Xhol.
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restriction sites in pET28a. The NcoI restriction site incorporated a change from Ser at position 2 to Ala in SUMO-1 and SUMO-3 proteins, but SUMO-2 already contains Ala at this position. Because of some inconsistency in the literature regarding SUMO nomenclature (9–12), in this study we define human pro-SUMO-2 as ending with the C-terminal extension YY and human pro-SUMO-3 with VPESSLAGHSF according to the HUGO nomenclature recommendations. The coding sequence for pro-Nedd8 was amplified from a human fetal brain library and inserted into the pET28a plasmid yielding the construct His₆-Nedd8-His₆. C-terminally truncated SUMO constructs were generated from full-length constructs and inserted into the pET28a vector in-frame with an N-terminal His tag. The cDNA coding for human RanGAP1ΔN (amino acids 418–588) was amplified by PCR from human fetal brain library and cloned into the pET28a in-frame with an N-terminal FLAG sequence. A cDNA coding for Ub-9 SUMO-conjugating enzyme was obtained by PCR from a human fetal brain library and inserted into pGEX-2T vector (GE Healthcare). All constructs were verified by DNA sequencing.

Protein Expression in Escherichia coli—Recombinant SENP enzymes, SUMO proteins, and Nedd8 were produced in E. coli codon plus (Novagen). Production of SUMO proteins and Nedd8 was induced with 0.4 mM isopropyl-β-D-galactopyranoside at 37 °C for 3 h. Expression of SENPs was induced with 0.2 mM isopropyl-β-D-galactopyranoside at 30 °C for 3 h (for the expression of catalytic domains of SENP1, SENP2, SENP5, and SENP8) or at 25 °C for 5 h when expressing SENP6 and SENP7 catalytic domains. His-tagged proteins were purified using Ni-NTA-agarose and eluted with a 20–200 mM gradient of imidazole in 50 mM HEPES, pH 7.4, 100 mM NaCl. GST-Ubc9 was expressed in E. coli BL21 (DE3) under standard conditions provided by the manufacturer (Novagen), purified by affinity chromatography using glutathione-Sepharose, and eluted with 10 mM reduced glutathione in 50 mM Tris, pH 8.0. Protein purity was examined by SDS-PAGE, and concentrations of the purified proteins were determined from the absorbance at 280 nm based on the molar absorption coefficients determined from the Edelhoch relationship (13).

Tissue Culture, Transfection, and Protein Purification—HEK293A cells were grown in Dulbecco’s modified Eagle’s medium, containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. The cDNA constructs FLAG-RanGAP1ΔN was used to transfect HEK293A cells using Genejuice (Novagen) as recommended by the manufacturer. 36 h after transfection, cells were washed twice in PBS and lysed on ice in a buffer containing 50 mM HEPES, 200 mM NaCl, 0.1% Nonidet P-40, supplemented with the protease inhibitors 3,4-dichloroisocoumarin (100 μM) and E-64 (50 μM) (14). FLAG-RanGAP1ΔN was absorbed to anti-FLAG M2 affinity gel (Sigma) overnight at 4 °C, and beads were washed five times in Tris-buffered saline and eluted with two bead volumes of 150 μg/ml 3XFLAG peptide (Sigma).

In Vitro SUMOylation—To generate mature SUMO proteins ending with a Gly-Gly motif, the corresponding precursor proteins were incubated in 50 mM Tris, pH 8.0, 20 mM NaCl, 2 mM DTT with SENP1 enzyme for 2 h at 37 °C. Uncleaved precursor, cleaved C-terminal extension, and enzyme all contain His tags and were thus removed from the digest mixture using Ni-NTA-agarose. The remaining supernatant contained mature SUMO proteins used for in vitro SUMOylation assays. In vitro SUMOylation reactions were carried out in a total volume of 100 μl of buffer containing 20 mM HEPES, pH 7.4, 5 mM MgCl₂, 20 μl of GST-Ubc9 at 50 μg/ml (SUMO-conjugating enzyme), 40 μl of RanGAP1ΔN, 10 μl of the respective mature SUMO at 50 μg/ml, 10 μl of 20 mM ATP, and 3 μl of Sae1/Sae2 (SUMO-activating enzyme; Boston Biochem) (15). After 2 h at 37 °C the SUMOylation mixture was depleted of Ubc-9 enzyme by using glutathione-agarose beads. The remaining supernatant (150 μl) was used as a source of SUMOylated RanGAP1ΔN.

SENPR Endopeptidase and Isopeptidase Assays—We routinely used an amiodiol/HCl, 8–18% SDS-PAGE linear gel system for protein analysis (16). But in the case of pro-SUMO and pro-Nedd8 processing, we found that Laemmli SDS-polyacrylamide 15% uniform gels gave better separation (17). To measure the ability of SENPs to cleave SUMO-1, SUMO-2, SUMO-3, or Nedd8 precursors, substrate (5 μM) was incubated with purified recombinant enzymes at the indicated concentrations at 37 °C for 1 h in buffer containing 50 mM Tris, pH 8.0, 20 mM NaCl, 5 mM DTT, and the reaction was stopped with Laemmli sample buffer. Samples were subjected to Laemmli SDS-PAGE (15% gel) followed by Coomassie Blue staining. The fraction of remaining precursor protein was calculated from the densitometric intensity of bands, and this was plotted against enzyme concentration to calculate the concentration of enzyme required to decrease 50% of precursor (EC₅₀). To monitor isopeptidase activity of SENPs, 5 μM of SUMOylated RanGAP1ΔN was incubated with various concentrations of enzyme in total 10 μl reactions for 1 h at 37 °C. Cleavage products were resolved on SDS-PAGE and transferred onto polyvinylidene difluoride membrane by Western blotting. The removal of SUMO from RanGAP1ΔN was determined by M2 primary antibodies, followed by peroxidase-conjugated mouse secondary antibody and Super Signal detection (Pierce). For quantification, nonsaturated chemiluminescence signal was captured directly from the membrane by a CCD camera using ChemImage 4000 software (Alpha Innotech), or by quantitative densitometry through scanning bands on films, and EC₅₀ calculated. The values are either the average of three experiments ± S.D.

SENPR Activity on Peptide Substrates—Synthetic tetrapeptide substrates corresponding to the cleavage site in the natural SUMO and Nedd8 protein substrates were synthesized as follows. Based on the results from scanning a combinatorial substrate library, we used Ac-QTGG-AFC for SENP1, -2, and -5 and Ac-LRGG-AFC for SENP -6, -7, and -8. The synthesis of these substrates will be described elsewhere. Activity of recombinant SENPs was assayed in low salt buffer (Tris buffer): 50 mM Tris, pH 8.0, 20 mM NaCl, 5 mM DTT and in sodium citrate buffer (25 mM Tris, pH 8.0, 0.8 M sodium citrate, 5 mM DTT). Standard enzyme assay studies (100-μl reaction) were

length of SENP3, SENP6, and SENP7 catalytic domains (Fig. 1A) was selected based on a secondary structure prediction using Jpred (19), following alignment to the SENP2 catalytic domain, which represents one of the best characterized members of the family from a structural perspective (10, 20). SENP8 does not have an N-terminal extension, and thus we encoded the full-length protein. The domains were expressed in E. coli as N-terminal His-tagged proteins and purified using Ni-NTA-agarose. Recombinant proteins migrate in SDS-PAGE with sizes corresponding to the predicted molecular weight (Fig. 1B). SENP1, -2, and -8 yielded about 10–20 mg of protein per liter of E. coli culture, and purified material was substantially homogeneous. SENP -5, -6, and -7 were expressed at lower yield, about 0.5–1 mg per liter of E. coli culture, and the lower yield resulted in some E. coli proteins contaminating the final preparations of SENP6 and -7 (Fig. 1B). The initial SENP3 construct (S353TPSR) and SENP5 construct (S536DFSN) yielded insoluble proteins, but additional truncation of the N termini produced a soluble recombinant SENP5 (Fig. 1). However, similar truncation for SENP3 resulted in accumulation of an insoluble protein, and we were thus unable to further characterize SENP3. All soluble recombinant SENPs used in this study elute as uniform peaks with the expected monomeric size in gel filtration, indicating that the chosen constructs are correctly folded (data not shown).

Endopeptidase Versus Isopeptidase Activity—SENP5s have two types of activity. They can process the precursors of SUMOs as endopeptidase substrates and the isopeptidase activity of SUMO attached to target proteins, described here as isopeptidase activity. SENP1, -2, -3, -5, and -6 have each been previously demonstrated to possess endopeptidase activity on at least one pro-SUMO as endopeptidase substrate. By comparing the efficiency of SENPs on the distinct substrates, we were able to demonstrate substantial differences in specificity within the family (Fig. 2 and supplemental Table 1). SENP1 stood out as by far the most efficient endopeptidase of the family, and SENP2 and -5 had very similar but far less endopepti-
dase activity. In contrast, SENP2 and -5 were almost as efficient isopeptidases as SENP1. SENP6 and -7 were almost without endopeptidase activity. They also had low isopeptidase activity, although this was higher than their endopeptidase activity (compare supplemental Tables 1 and 2). Interestingly, pro-SUMO-3 was an extremely bad endopeptidase substrate but was deconjugated as efficiently as SUMO-2 and was better than SUMO-1 for all SENPs (Fig. 2). As expected, SENP8 showed no activity on any pro-SUMOs, but excellent cleavage of pro-Nedd8 (supplemental Table 1).

FIGURE 2. Activity of SENPs on protein substrates. A, endopeptidase activity. 5 μM pro-SUMOs were incubated with the indicated range of enzyme in 30 μl reaction at 37 °C for 1 h. Reactions were stopped by boiling in Laemmli sample buffer, and cleavage products were separated in 15% SDS-polyacrylamide gel, and stained with Coomassie Blue. E indicates the enzyme. B, isopeptidase activity. In vitro SUMOylated FLAG-RanGAPΔN was incubated for 1 h with the indicated range of SENP concentrations. Reaction products were detected by immunoblotting with FLAG antibodies followed by horseradish peroxidase-conjugated secondary antibodies and chemiluminescence. EC50 values were calculated for each SENP as described under “Experimental Procedures.” For a convenient comparison of relative activities, the y axis is represented by the reciprocal of EC50 (histograms, lower panels).

The sequences of mature SUMO-2 and -3 are 96% identical but with totally different extensions (Fig. 3A), leading to the possibility that the marked difference in the activity of SENP2, -5, -6, and -7 for pro-SUMO-2 over pro-SUMO-3 may be directed mainly by the sequence variation in the C-terminal tails. Indeed, it had been illustrated for SENP1 and SENP2 that the efficiency of pro-SUMO cleavage may depend on the sequences corresponding to these regions (9, 10). We tested the importance of the tails by swapping regions between pro-SUMO-2 and pro-SUMO-3 to create SUMO-3 with the SUMO-2 tail and designated this hybrid pro-SUMO-3/2. In each SENP studied, the pro-SUMO-3/2 hybrid was a better substrate than pro-SUMO-3 (see Fig. 3B for examples), importantly almost recapitulating the activity on the preferred substrate pro-SUMO-2 (supplemental Table 1). This result strongly indicates that, although SUMO-2 and SUMO-3 share a high degree of identity, endopeptidase cleavage studies using either one should be extended with caution to the other. We conclude that the C-terminal tails play a pivotal role in defining endopeptidase specificity of SENPs in the discrimination of pro-SUMO-2 and pro-SUMO-3.

Activity of SENPs on Short Synthetic Substrates—Until this study, all published measurements and characterizations of SENP activity had been defined using full-length SUMO proteins. However, there is no a priori reason to expect that short synthetic reporter substrates, commonly used to define other protease families, could not be used with SENPs. Despite extensive interactions between SUMO and SENPs, the catalytic apparatus is arranged in a similar manner to proteases from other families that are very active on short substrates, and the structures of SENP1, -2, and -8, as well as the structure of the yeast homolog Ulp1, have defined active site clefts (10, 11,
Therefore, we designed peptidyl substrates to define the relative contribution of the active site interactions and the extended (often called exosite) interactions, between substrate and enzyme. Importantly, the use of tetra-peptide fluorogenic substrates allows us to define critical catalytic properties of the enzymes. We designed the substrates based on the last four residues of SUMOs and Nedd8, with an AFC fluorogenic reporter replacing the C-terminal tails (see “Experimental Procedures”). In this way we were able to assay enzyme activity of SENP1 and -2 on Ac-QTGG-AFC, and SENP8 on Ac-LRGG-AFC. Using the same buffer conditions, no measurable activity was detected for SENP5, -6, and -7 on either of the substrates.

To determine the optimal conditions in which SENPs exhibit the highest level of amidolytic activity, a series of assays was conducted over a range of pH values and salt concentrations (Fig. 4). The activity of SENP1, -2, and -8 rose steadily from pH 6.0 reaching a maximum at about pH 8.5, after which there was a gradual decrease (Fig. 4A). The midpoint of the ascending limb was around pH 8, which is consistent with ionization of the catalytic His-Cys dyad in the active site, typical of other cysteine proteases (29). Elevated concentrations of the Hofmeister-effect salt Na$_2$citrate had a marked effect on activity of SENP1 and SENP2 (Fig. 4C), much more than could be accounted for by simple ionic issues, as evidenced by the minimal effect of NaCl (Fig. 4B).

**Substrate-induced Activation of SUMO-cleaving SENPs**—The observation that a Hofmeister-effect salt can enhance the activity of SENPs has several possible explanations, one of which is that the active site of the enzyme is not in an optimal orientation for catalysis. For example, the Hofmeister-effect salts can order the active site, as demonstrated for the protease prostate-specific antigen (30), possibly because of general desolvation effect on the protein. Thus, Na$_2$citrate may optimize loops surrounding the active site and promote catalysis in a manner similar to the binding of the natural SUMO substrates. Crystal structures of SENP1 (11) and SENP2 (10) with or without SUMO substrates bound indicate that the enzymes...
undergo small structural rearrangements upon substrate binding. The availability of synthetic substrates enabled us to explore whether these conformational changes had any measurable impact on enzyme activity. Mature SUMO did not enhance enzyme activity, which is probably because of spatial clashes between the C terminus of SUMO and the synthetic substrate that would overlap in the enzyme active site. To avoid this steric clash, yet retain the basic fold of SUMO, we deleted six amino acids from the C terminus of mature SUMO-1, 2, and 3 (we call the respective derivatives SUMO-ΔC to signify this deletion) to accommodate the tetrapeptide substrate at the active site (Fig. 5, A and B). Activity of all SENPs was greatly enhanced by at least one of the truncated SUMOs (Fig. 5C and supplemental Table 3). Activity of SENPs was assayed at various molar ratios of truncated SUMO, and the lowest amount that gave maximal enzyme activity was considered optimal. We used the ratio (SENP:SUMO-ΔC) 1:5 for SENP1 and -2 and 1:10 for SENP5, -6, and -7. SENP1 and SENP5 did not distinguish between SUMOs, and enzyme activity was induced almost equally by truncated SUMO-1–3 (Fig. 5C). SENP2, -6, and -7, on the other hand, showed strong preferences toward SUMO-2/ΔC and SUMO-3/ΔC (Fig. 5C).

Experiments investigating the effect of SUMO-ΔC on enzyme activity were carried out at low ionic strength (20 mM NaCl). Increasing the salt concentration decreased the effect of truncated SUMO (data not shown), indicating that polar interactions revealed in crystal structures are important in SUMO binding and SUMO-induced activation. In contrast to the SUMO-specific SENPs, the Nedd8-specific SENP8 was minimally activated by Na2citrate, and truncated Nedd8 had no effect (Fig. 5C).

DISCUSSION
In this study we report the comparison of members of the human SENP family in terms of their endopeptidase and
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isopeptidase specificity. We present for the first time their activity on small synthetic substrates, which allowed us to discover substrate-induced activation for some members of the family, as well as a definition of their optimal biochemical parameters for activity. There are two main caveats to our study. First, we lack data on SENP3 because of difficulties in obtaining sufficient soluble material from expression in E. coli. SENP4 was not included in our study because, although it is annotated in some data bases, we could find no ESTs in Gen/EMBL encoding the protein, suggesting it is likely a pseudogene. Second, we expressed only the C-terminal catalytic domains and not full-length proteins, with the exception of the deNEDDylating enzyme SENP8, which does not contain an N-terminal extension. The N-terminal regions of SENPs presumably influence the biological properties of the enzymes in vivo and may even influence their inherent biochemical properties as enzymes. However, there is currently no way to predict how they influence the function of the enzymes because they contain no recognizable features of any known protein domain. Nevertheless, our in vitro characterization allows us to compare the inherent properties of the conserved catalytic domains without interference from the other regions.

In terms of endopeptidase activity on SUMO precursors, our studies are consistent with published data for SENP1 (9) and SENP2 (10) showing that SENP1 prefers pro-SUMO-1 > pro-SUMO-2 > pro-SUMO-3, whereas SENP2 pro-SUMO-2 > pro-SUMO-1 > pro-SUMO-3. SENP5 demonstrated substrate preferences almost identical to SENP2. Previous literature is conflicted regarding the relative specificities of SENP5, with opposing conclusions regarding the preference of the enzyme form pro-SUMOs (18, 22). This is because of different construction of the C-terminal tails, discussed below. Our data demonstrate that SENP5 is closest to SENP2 in its endopeptidase and isopeptidase activities. SENP6 and -7 were substantially less active as endopeptidases, and in our hands cleaved only pro-SUMO-2 at a detectable rate. This is consistent with the demonstration that SENP6 may not function in vivo as a pro-SUMO-activating enzyme (31), and we suggest that the same is probably true for SENP7. The isopeptidase activity, using SUMO-conjugated RanGAP1 as substrate, showed substantial alterations in SENP specificity. SUMO-3 became a much better substrate when conjugated to RanGAP1 than when it is a precursor. This was seen using recombinant enzyme and substrate, and also in whole cell extracts containing endogenous SENP activity. Moreover, in relation to SENP1, all SENPs are better isopeptidases than they are endopeptidases. This suggests that SENP1 may be the most common SUMO endopeptidase in vivo, which is consistent with its wide tissue distribution (9). Perhaps the most obvious structural feature that separates the poor endopeptidases, SENP6 and -7, from other members of the family is the substitution of the critical Trp-410 residue (SENP2 numbering) for Phe. This residue forms a lid to the flap that gates entry of the Gly-Gly region of SUMO to the active site of SENPs, and its replacement by mutation to Ala in SENP2 negates activity (20). Possibly Phe at this position prohibits endopeptidase activity but allows isopeptidase activity, and future mutagenesis studies should resolve this.

Clearly the C-terminal tails of pro-SUMOs are pivotal determinants of specificity as demonstrated by the following: (i) swapping the tails of pro-SUMO-2 and -3, and (ii) isopeptidase versus endopeptidase activity. Indeed, this conclusion was reached for SENP1 and -2 following mutagenesis of the tails of pro-SUMOs (9, 10), and we now show this is a property of all the SUMO-specific SENPs tested here. Thus the C-terminal tail is the primary structure that allows SENPs to discriminate between SUMO-2 and -3. The significance of the sequence of the C-terminal tail can now explain the conflicting results of previous studies on SENP5 (18, 22). These studies used different constructs to replace the natural tails of pro-SUMOs to visualize endopeptidase activity, and thus we obtained quite opposite results. There are currently no data regarding the importance of the pro-Nedd8 tail and SENP8 specificity.

In this study for the first time we report the use of synthetic substrates to assay activity of SENPs. The QTGG sequence used as a basis for the fluorogenic tetrapeptides encompasses the common recognition motif found in all three SUMOs, and LRGG encompasses the recognition motif found in Nedd8. Calculated turnover rates of the synthetic substrates were 2–3 orders of magnitude lower than the corresponding natural proteins, emphasizing the importance of the SUMO domain in substrate recognition. A similar disproportional reactivity had been observed previously when the de-ubiquitinating enzymes isopeptidase T and USP2 were compared on tetrapeptide fluorogenic substrates versus full-length ubiquitin conjugated to a C-terminal fluorogenic leaving group (32, 33). Notwithstanding the low rates, we were able to employ the small substrates to demonstrate the influence of pH and salts on SENP activity. The enzymes showed a pH profile typical of other mammalian cysteine proteases and an unexpected enhancement of activity in high concentrations of the Hofmeister-effect salt Na2citrate. This effect is typical of protein loop or domain ordering (30), although one can observe only a very small degree of reordering of loops encompassing the active site in SENP2 when substrate-bound structures are compared with unbound ones (10). These observations led us to suspect that complex formation of SENPs with SUMOs may induce the enzyme to undergo local rearrangements to enhance catalysis. The enhancement of enzyme efficiency in the presence of truncated SUMOs equaled the effect of Na2citrate, but it was still significantly lower than the efficiency of natural substrate cleavage. One reason could be that reconstituting the substrate from two parts (truncated SUMO plus the tetrapeptide sequence from the synthetic substrate) does not allow for optimal interactions with the enzyme. In our reconstituted substrate two residues (QE in SUMO-1 and QQ in SUMO-2 and -3) are missing (Fig. 5). Although the missing residues do not seem to make crucial contacts with the enzyme in the available crystal structures, the juxtaposition of a free carboxylate from truncated SUMO may interfere with the acetyl group at the N terminus of the synthetic substrate. Alternatively, the lack of the C-terminal tail, which is clearly important for substrate specificity, may decrease binding energy and catalysis of the synthetic substrate.

The ability of truncated SUMOs to substantially enhance activity is evidence for substrate-induced activation, a property not reported previously for members of this family but found in...
some members of the distantly related families of DUBs (33–38). The key mechanistic distinction between ubiquitin-mediated substrate activation of DUBs and SUMO-mediated activation of SENPs is that, with respect to synthetic substrates that probe the active site of the enzymes, full-length ubiquitin activates DUBs (32), but full-length SUMO does not activate SENPs. In the DUBs the catalytic residues are radically misaligned when bound structures are compared with unbound, explaining why the enzyme is incompetent in the absence of ubiquitin. A minor distortion in the residue that forms the oxyanion pocket in SENP2 is seen in one structure (20), but overall the bound and bound states of SENP1 and -2 both seem to have catalytic residues in the same orientation. Substrate-induced activation of SENP1, -2, and -5, -6, and -7 is specific (because truncated Nedd8 does not activate), reproducible, and substantial. Yet the mechanism is not apparent from the available crystal structures. Consequently, the substrate-induced activation must have another explanation in SENPs, or possibly the unbound crystal structures do not represent the protein conformations highly populated in solution. Interestingly, SENP8 exhibited only a minor component of substrate-induced activation by Nedd8ΔC. This surprised us because crystal structures of bound and unbound SENP8 reveal a drastic changes in the loop that locks the substrate to the enzyme, currently the major conformational change observed within the available structures (SENP1, -2, and -8).

SEPs can cleave tetrapeptide substrates with low efficiency, and because the human proteome contains numerous QTGG or LRGG sequences, one could imagine that such proteins may be adventitiously cleaved. To overcome this, SENPs have adopted a mechanism-based strategy where the SUMO domain enhances catalysis, thereby intensifying selectivity for SUMO precursors. A secondary specificity region shared by all SENPs is at the C-terminal side of the scissile bond, where distinctions are made between the otherwise almost identical SUMO-2 and -3. Our results reveal essentially no discrimination between SUMO-2 and -3 when attached to the model conjugation target RanGAP1, but strong endopeptidase discrimination based on the distinct C-terminal pro-SUMO tails, and from this perspective it is fascinating that the sequence of the SUMO-2 tail (-VY) is conserved in mammals, but there is absolutely no conservation in sequence or length of the SUMO-3 tail. Given the evident importance of the C-terminal tail sequences, we predict that not all SUMOylation sites in target proteins will be treated equally, even by the same SENP, pointing the way to future understanding of the regulation of deSUMOylation specificity.

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