Evaluation of the activity and substrate specificity of the human SENP family of SUMO proteases

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ABSTRACT

Protein modification with the small ubiquitin-like modifier (SUMO) is a reversible process regulating many central biological pathways. The reversibility of SUMOylation is ensured by SUMO proteases many of which belong to the sentrin/SUMO-specific protease (SENP) family. In recent years, many advances have been made in allocating SENPs to specific biological pathways. However, due to difficulties in obtaining recombinant full-length active SENPs for thorough enzymatic characterization, our knowledge on these proteases is still limited. In this work, we used in vitro synthesized full-length human SENPs to perform a side-by-side comparison of their activities and substrate specificities. ProSUMO1/2/3, RanGAP1–SUMO1/2/3 and polySUMO2/3 chains were used as substrates in these analyses. We found that SENP1 is by far the most versatile and active SENP whereas SENP3 stands out as the least active of these enzymes. Finally, a comparison between the activities of full-length SENPs and their catalytic domains suggests that in some cases their non-catalytic regions influence their activity.

INTRODUCTION

The post-translational modification of proteins with the small ubiquitin-like modifier (SUMO) plays a central role in cell homeostasis by regulating a large number of key biological processes such as DNA repair, transcription and cell cycle progression [1], [2]. Mammals express three SUMO paralogues—SUMO1, and the nearly identical and functionally indistinguishable SUMO2 and SUMO3 [3], [4], [5], [6], [7]. The three SUMO paralogues are conjugated to target-proteins through an enzymatic cascade comprising one SUMO-activating enzyme [8], one SUMO-conjugating enzyme [9] and, in some cases, also a SUMO ligase [10]. This machinery activates the C-terminal glycine carboxyl group of SUMO in an ATP-dependent process and conjugates it to the ε-amino group of a lysine residue in target proteins [2], [11]. As with ubiquitination, proteins can be modified with a single SUMO molecule (monoSUMOylation) or with a chain of SUMO molecules (polySUMOylation) [2]. Polymeric chains are built by attaching a new SUMO moiety to lysine 11 of the previous SUMO molecule [6], [12]. Due to the fact that SUMO1 lacks this lysine residue, only SUMO2 and SUMO3 are involved in polySUMO chain elongation [12].

Protein modification by SUMO is a highly dynamic and transient process, readily reverted by the action of SUMO proteases, hydrolases that remove SUMO or SUMO chains from SUMOylated proteins [2], [13]. Most SUMO proteases belong to the so-called sentrin/SUMO-specific protease (SENP) family of cysteine proteases. In mammals there are six SENPs. They are often classified into three groups according to sequence relationships and predominant subcellular localization [2], [13], [14], [15]. The first group comprises SENP1 and SENP2, both localizing mainly to the nuclear periphery [16], [17], [18]. The second group consists of SENP3 and SENP5 [19], [20], which localize mostly to the nucleolus. The third group comprises SENP6 and SENP7 [21], [22], two enzymes mainly found in the nucleoplasm. All SENPs are structurally organized into a C-terminal catalytic domain and a non-conserved N-terminal region which comprises around two-thirds of their polypeptide chains [2], [13].

In addition to SENPs, three other SUMO proteases have been described recently in mammals: the deSUMOylating isopeptidases DeSI-1 and DeSI-2 [23], and the ubiquitin-specific protease-like 1 (USPL1) [24]. These enzymes are also cysteine proteases but they are very distantly related to the SENP family. Also, they probably act on a very restricted set of substrates because knockdown of any of these enzymes in cell lines does not lead to detectable alterations in the bulk of SUMO conjugated products [23], [24]. The minor roles of each of these enzymes in the global cell SUMOylation status [23], [24] contrast with the large accumulations of SUMOylated proteins detected in cells lacking any of the SENP family members [19], [22], [25], [26], [27], [28], [29].

Besides a major role in controlling the SUMOylation status of many proteins, SENPs are also involved in the de novo synthesis of SUMO [19], [20], [30], [31], [32]. Indeed, the three SUMO paralogues are expressed as precursor proteins (proSUMO1/2/3) that must be processed to yield the active/conjugatable proteins. Processing involves the removal of small peptide extensions that are present at the C termini of proSUMOs [2].

Although the involvement of SENPs in several biological processes is now well established, their substrates specificities and activities are still ill-characterized. A major problem in addressing this issue stems from the fact that, thus far, it has not been possible to obtain recombinant active full-length SENPs for thorough enzymatic characterization. Thus, most studies on SENP activity and

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specificity have used recombinant truncated proteins comprising only their catalytic domains in enzymatic assays with natural or artificial substrates [19], [22], [30], [33], [34], [35], [36], [37]. In the case of SENP3, even this type of data are lacking due to problems in obtaining a stable and active catalytic domain [30]. Other studies have looked at the reactivity of endogenous or epitope-tagged SENPs in cell extracts towards activity-based irreversible inhibitors such as SUMO1 and SUMO2 vinyl sulfones (VS) [21], [38], [39]. One of these studies revealed that (full-length) SENP1 reacts equally well with both SUMO1-VS and SUMO2-VS whereas all the other SENPs show a preference for SUMO2-VS [38]. Interestingly, it was also shown that the recombinant catalytic domains of SENPs no longer display a SUMO paralogue preference when tested with the activity-based inhibitors. Seemingly, the N-terminal non-catalytic domains of SENPs somehow modulate SENP activity/substrate specificity [2], [13], thus raising some doubts on whether or not the enzymatic data reported for SENP catalytic domains can be extrapolated to the full-length enzymes. Clearly, additional studies using intact enzymes and their natural substrates are needed to better understand the properties of these proteases.

Materials and Methods

Plasmids and proteins

The cDNAs encoding human full-length SENP1/2/5/6 were optimized for Escherichia coli expression (GenScript) and cloned into the NdeI/XhoI sites of pET28a. The plasmids encoding human full-length SENP3 and SENP7 in pET28a were described before [40]. Flag-tagged pcDNA5/FRT/TO-derived constructs of wild type (WT) SENP7, of a mutant lacking seven putative SUMO-interacting motifs (SIMs) in the N-terminal region (SM) and of the catalytic inactive C992A mutant (CA) were kindly provided by Dr. Joanna R. Morris (University of Birmingham, UK) [41]. These plasmids were digested with KpnI/XhoI and the DNA inserts were cloned into pcDNA3.1, which contains a T7 promoter, for in vitro synthesis. The pET28a-derived plasmids coding for the catalytic domains of human SENP1/2/5–7 were a kind gift from Dr. Guy Salvesen [30]. The cDNA encoding SENP3 catalytic domain, SENP3(307–574), was amplified by PCR using as template SENP3-pET28a and cloned into the NdeI/XhoI sites of pET28a. A plasmid encoding untagged human full-length RanGAP1 (RanGAP1-pET23a) was obtained from Addgene (plasmid #13387) [42]. The plasmid ΔC1-PEX5-pET28a was described elsewhere [43].

Recombinant full-length His6-SENP1 was expressed in Rosetta 2(DE3) E. coli cells with 0.5 mM IPTG for 3 h at 30 °C. After cell lysis in buffer A (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 10% (w/v) glycerol) supplemented with 1:500 (v/v) protease inhibitors cocktail (Sigma), 0.25 mg/ml PMSF and 1 mM DTT, inclusion bodies were washed twice with the same buffer in the presence of 1% (w/v) Triton X-100. The plasmid pTYB-SUMO2 [40] was used to produce HA-tagged SUMO2 vinyl methyl ester (HA-SUMO2-VME) as previously described [44], excluding the ion-exchange purification step. Plasmids encoding SUMO2, SUMO3, Ubc9 and SAE1/SAE2 (SUMO E1) were kindly provided by Dr. Frauke Melchior [45], [46] and used to prepare the respective recombinant proteins as described [47]. Recombinant untagged SUMO1, proSUMO1-3, polySUMO2 and polySUMO3 were purchased from Boston Biochem or Enzo Life Sciences. Radiolabeled proteins were synthesized in vitro using pET- or pcDNA3.1-based plasmids with the TNT® T7 Quick Coupled Transcription/Translation System (Promega), in the presence of EasyTag™ L-[^{35}S]-Methionine (Perkin Elmer, specific activity > 1000 Ci (37.0 TBq)/mmol), according to the manufacturer's instructions. Translation reactions were stopped by incubation with 1 mM cycloheximide (Sigma) for 10 min at 30 °C.

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Covalent labeling of SENPs with HA-SUMO-VME

In vitro synthesized ^{35}S-labeled full-length SENPs (0.5–1 µl), proteins comprising their catalytic domains alone (0.02–0.1 µl) or HeLa cell total homogenates (200 µg of total proteins, prepared as described [40]) were incubated with 0.2–0.4 µM HA-SUMO2-VME for 15 min at 25 °C in 20 µl (final volume) of buffer A supplemented with 2 mM EDTA-NaOH, pH 8.0, 0.15 µg/µl BSA and 1 mM DTT. Reactions were stopped with Laemmli sample buffer and analyzed by SDS-PAGE/autoradiography.

RanGAP1 monoSUMOylation assay

Full-length RanGAP1 was synthesized in vitro for 45 min and treated with 1 mM cycloheximide for 10 min at 30 °C to prevent further protein synthesis. Samples were then diluted twofold with buffer A supplemented with 1 mM DTT, 2 mM $MgCl_2$ and 2 mM ATP (final concentrations) and incubated for 40 min at 37 °C in the presence of 50 nM E1, 100 nM Ubc9 and 1 µM of either SUMO1 or SUMO2 or SUMO3. SUMOylation reactions were terminated by depleting ATP with 5 U/ml (final concentration) of apyrase (grade VII, Sigma) for 10 min at 30 °C.

Densitometric analyses

In vitro synthesized full-length SENP1, HeLa cell total proteins and different amounts of recombinant full-length SENP1 were analyzed by SDS-PAGE/western-blot using an anti-SENP1 antibody. The approximate levels of ^{35}S-labeled SENP1 produced in the rabbit reticulocyte lysate (RRL) and of the endogenous enzyme in HeLa cells were determined by direct comparison with the recombinant protein, as assessed by densitometric analyses of western-blots using ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997–2014). To estimate the relative stoichiometries of the in vitro synthesized SENPs to be used in the activity assays (Section 2.5.), aliquots of the RRLs containing the ^{35}S-labeled proteins were subjected to SDS-PAGE/autoradiography followed by densitometry analyses, taking into account the methionine content of each enzyme. Full-length SENP1/2/3/5/6/7 contain 14, 15, 15, 17, 14 and 14 methionines, respectively, whereas the corresponding catalytic domains contain 11, 9, 10, 7, 10 and 6 methionine residues, respectively.

SENP activity assays

The activity of in vitro synthesized SENPs was assessed by HA-SUMO2-VME labeling. Approximate equimolar amounts of each SENP (see Section 2.4.) were then used in the activity assays unless otherwise indicated. The total volume of RRL used in each reaction was made equal by addition of a mock-translated and cycloheximide-treated RRL. A control RRL containing an in vitro synthesized unrelated protein, ^{35}S-labeled ΔC1-PEX5 [43], was also included in many assays as specified. The activity assays were performed at 25 °C in the presence of 0.6–2 µl of monoSUMOylated RanGAP1 or 250 ng of polySUMO chains in 10 µl of buffer A supplemented with 2 mM EDTA-NaOH, pH 8.0, 0.15 µg/µl BSA and 2 mM DTT. For the activity assays with SUMO precursors, 200–500 ng of proSUMOs were directly added to 6 µl of normalized RRLs (i.e., mock plus SENP-containing RRL) and incubated at 25 °C. The approximate concentrations of SENP1 in these assays were 6–7 nM, 3–5 nM and 1–2 nM for proSUMOs, RanGAP1–SUMOs and polySUMO chains, respectively, as judged from the determinations with the anti-SENP1 antibody (Section 2.4.). In the activity assays containing the catalytic domains and SENP7 mutants, the incubation times and/or amounts of

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enzyme used were adjusted to obtain partial substrate hydrolysis. SENP-containing RRLs used in the assays with monoSUMOylated RanGAP1 were pretreated with apyrase (see Section 2.3.) to prevent reactivation of the exogenously added SUMO-conjugation machinery. Reactions from the activity assays with RanGAP1–SUMO were stopped with Laemmli sample buffer, separated in 8 or 12% SDS-PAGE and blotted onto a nitrocellulose membrane, followed by autoradiography to detect the radiolabeled proteins (SENPs, RanGAP1 and ΔC1-PEX5). In the activity assays with SUMO precursors and polySUMO chains, two aliquots of each sample were withdrawn after stopping the reactions. One aliquot was resolved in 16% SDS-PAGE and analyzed by western-blot with anti-SUMO1 or anti-SUMO2/3 antibodies to detect proSUMOs, polySUMO chains and their hydrolysis products. The second aliquot was analyzed by 8% SDS-PAGE/autoradiography to detect the radiolabeled SENPs. The stoichiometric ratios of SENPs were then reassessed by densitometry analyses (see Section 2.4.) and are presented in the figures. The contribution to SENP2 and SENP3 intensity signals from the band of the RanGAP1 doublet that co-migrates with those enzymes in some gels was estimated and subtracted by analyzing the intensity of the other RanGAP1 band and the stoichiometry of the RanGAP1 doublet in other lanes. All assays were performed at least three times; representative experiments are shown.

Antibodies

Mouse monoclonal anti-SUMO1 (21C7) [5] and anti-SUMO2/3 (8A2) [48] antibodies were developed by Michael J. Matunis and obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at the University of Iowa, Department of Biology, Iowa City, IA 52242, USA. Rabbit polyclonal anti-SENP1 antibody (A302-927A-1) was purchased from Bethyl Laboratories, Inc. These antibodies were detected on western-blots using alkaline phosphatase-conjugated anti-mouse or anti-rabbit IgGs (Sigma).

Results

As shown previously [40], full-length human SENPs synthesized in a RRL-based in vitro transcription/translation system are quite active as assessed by their reactivity toward the activity-based irreversible inhibitor SUMO2 vinyl methyl ester (SUMO2-VME; see Fig. 1A). Furthermore, the amounts of protein produced by the RRL, although orders of magnitude lower than those typically obtained using in vivo expression systems, are considerable. Indeed, as shown in Fig. 1B, 1 µl of RRL produces approximately 3 ng of SENP1, an amount similar to that present in 100–200 µg of total proteins from HeLa cells. These two findings led us to conclude that in vitro synthesized SENPs might be used in enzymatic assays aiming at better understanding their substrate specificities and relative activities. The strategy developed for this purpose turned out to be very simple because the endogenous SUMO protease activity of the RRL is relatively low. Indeed, with very few exceptions (e.g., see Fig. 2C, lower right panel), the activities detected in RRLs containing a negative control protein were always much lower than those detected in RRLs containing SENPs. This obviated the need to purify SENPs from the RRL before proceeding with the activity assays. Thus, aliquots of the in vitro translation reactions were simply mixed with a test substrate in an appropriate buffer and incubated (see Section 2.5. for details). Because one of our aims was also to gather qualitative information on the relative activities of the different SENPs, their relative stoichiometries in each of the experiments presented below were estimated by densitometric analyses of autoradiographs (see legends to figures and Section 2.4. for details). Fig. 2A shows the results obtained with SUMO1/2/3 precursors. From all human SENPs, only SENP1 and SENP2 show detectable processing

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activity, with the former being more active on proSUMO1 and the latter preferring proSUMO2 and proSUMO3. We next assessed the isopeptidase activity of the different SENPs using as a substrate monoSUMOylated RanGAP1, a nuclear pore protein and a canonical target of SUMOylation [5,49]. Radiolabeled RanGAP1 modified with SUMO1 or SUMO2 was produced in vitro (Fig. 2B; see Section 2.3.) and incubated with radiolabeled SENPs. Note that RanGAP1 migrates in SDS-gels as a double band, a result of phosphorylation events occurring in the RRL [50]. As shown in Fig. 2C (upper left panel), SENP1 is by far the most active isopeptidase achieving complete deconjugation of RanGAP1–SUMO1 in 5 min or less. SENP2 also hydrolyzes the RanGAP1–SUMO1 conjugate but this activity was detectable only after a 1-h incubation (upper right panel). Different results were obtained with RanGAP1–SUMO2 (Fig. 2C, lower panels). In this case, SENP1 is again the most active isopeptidase, but SENP2 now shares with SENP5 the second largest activity (left panel). No hydrolysis of RanGAP1– SUMO2 was detected in the 5 min incubation assay when testing SENP3, SENP6 and SENP7 (Fig. 2C, left panel). A longer incubation did reveal some hydrolysis of this substrate in the SENP3/6/7-containing RRLs. However, the amounts of RanGAP1–SUMO2 hydrolyzed in these samples are similar to or, at best, only slightly larger than the one obtained with a negative control RRL (right panel, compare lane C with lanes 3, 6 and 7). Substituting RanGAP1–SUMO3 for RanGAP1–SUMO2 in these assays yielded the same results (data not shown).

Lastly, we tested human SENPs on polySUMO2 chain disassembly. As shown in Fig. 3, all SENPs display at least some activity with this substrate. Interestingly, SENP1 and SENP2 seem to be at least as active (if not more) as SENP6 and SENP7, the two enzymes typically associated with polySUMO disassembly. Also, the activity of SENP6 on polySUMO2 chains seems larger than that of SENP7. However, due to the fact that in vitro synthesis of both SENP6 and SENP7 also results in the synthesis of significant amounts of truncated fragments (some of which may be catalytically active) this observation should be taken with caution. SENP3 and SENP5 are the least efficient SENPs in polySUMO2 chain disassembly (lower panel, lanes 3 and 5, respectively). The same results were obtained with polySUMO3 chains (data not shown). As stated above (Section 1.) there are some data suggesting that the non-catalytic N-terminal domains of SENPs may somehow modulate the activities of these enzymes. The availability of active full-length human SENPs prompted us to compare their activities with those of the corresponding catalytic domains. The catalytic domains of SENP1/ 2/5/6/7 used in these experiments were those defined before by other authors [30] (see Section 2.1.). For SENP3, the previously defined catalytic domain (residues ^{35}3–574; [30]) turned out to be inactive, as assessed by reaction with SUMO2-VME (data not shown), and thus we used instead a protein comprising residues 307–574 of SENP3, the shortest SENP3 protein tested that reacted quantitatively with SUMO2-VME (see Fig. 4A). Note that the experimental conditions used in these assays for each SENP/catalytic domain pair were not the same and differ from those used in Fig. 3. Specifically, the incubation times and/or the amounts of enzyme in the assays were changed in order to avoid less informative situations of complete substrate hydrolysis. The results of this comparison using polySUMO2 chains as substrate are shown in Fig. 4B. No obvious differences between the activities of full-length SENP2 and SENP3 and their catalytic domains were observed in these experiments. For SENP1 we did observe a larger activity with its catalytic domain than with the full-length enzyme. This difference, however, is modest, as increasing the amount of full-length SENP1 in the assay by a factor of ~2 yields approximately the activity observed for its catalytic domain. Furthermore, no difference in the activities of full-length SENP1 and its catalytic domain was detected when using RanGAP1–SUMO2 as substrate (Fig. 4C). In contrast, marked differences between the activities of full-length enzymes and their catalytic domains were observed for SENP5, SENP6 and SENP7 with polySUMO2 chains,

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but in these cases in the opposite direction (Fig. 4B). That is, their catalytic domains are much less active than the corresponding full-length enzymes. Again, for the reasons stated above these differences should be taken with caution in the cases of SENP6 and SENP7 (see also below). The lower activity of SENP5 catalytic domain compared to the full-length enzyme is also easily detectable when using RanGAP1–SUMO2 as a substrate (Fig. 4C). The non-catalytic N-terminal domains of several SENPs contain one or more SIMs [51]. These SIMs might act as substrate recruiting platforms and thus they could explain why full-length SENP5/6/7 are more active than the corresponding catalytic domains in the polySUMO2 hydrolysis assays presented above. To test this possibility, we used a SENP7 mutant protein that lacks seven of these SIMs and which was recently shown to be rather inefficient in homologous recombination DNA repair, a SUMO-dependent process that requires catalytically active SENP7 [41]. Unexpectedly, as shown in Fig. 5B, the mutant SENP7 protein is as efficient as SENP7 in disassembling polySUMO2 chains. Apparently, these SENP7 SIMs are not relevant for the enzyme activity, at least in these in vitro assays.

Discussion

From the qualitative assessment of SENP activities presented in this work (see Table 1) it is clear that SENP1 is one of the most active SENPs and the only one for which we could detect activity with all the substrates tested. Two observations are worth noting. The first is the almost exclusive capacity of SENP1 to cleave the two SUMO1-containing substrates used here. Indeed, under our experimental conditions, no other SENP cleaved proSUMO1 and only SENP2 displayed some activity on RanGAP1–SUMO1. This finding should not be taken as evidence to conclude that only SENP1 is capable of cleaving proSUMO1—as shown before, recombinant full-length SENP2 as well as the catalytic domains of SENP2 and SENP5 is also able to cleave proSUMO1 in vitro, when used at concentrations several fold larger than the ones used here [19,30,32,33,35]. What is apparent from our results is that SENP1 is by far the most efficient SENP acting on SUMO1-containing substrates, a finding re-enforcing the idea that this protease plays an essential role in all processes involving SUMO1 [29,52]. The second interesting result regards the activity of full-length SENP1 on polySUMO2/3 chain disassembly. Previous studies have shown that the catalytic domain of SENP1 is more active on polySUMO2 chain hydrolysis than those of SENP6 and SENP7 [22,53], but whether or not these findings were valid for the full-length enzymes remained unknown. Here, we show that full-length SENP1 is one of the most active enzymes, if not the most efficient, towards polySUMO2/3 chains. Furthermore, our data suggest that this is probably an intrinsic property of its catalytic domain (see Fig. 4B). Thus, the idea that disassembly of polySUMO2/3 chains is a function performed mainly by SENP6 and SENP7 [2,15,54], seems unlikely. SENP2, the enzyme more closely related to SENP1, also presents a large catalytic activity and broad substrate specificity but, in contrast to SENP1, displays a strong preference for SUMO2/3-containing substrates. These findings are consistent with data reported previously for the recombinant catalytic domain of this protease [30,35], as well as with the fact that Senp2 knockout mouse embryos present a strong accumulation of SUMO2/3 conjugates and depletion of free SUMO2/3, while the levels of free and conjugated SUMO1 remain essentially normal [27]. Our data also suggest that full-length SENP2, and not SENP1, is the most active SENP acting on proSUMO2 and proSUMO3, a result that differs from previous data showing that the catalytic domain of SENP1 is slightly more active than that of SENP2 in cleaving proSUMO2/3 [30]. Regardless of this difference, which given the non-quantitative nature of the assays used here and in that study may in fact be not significant, it is clear that SENP1 and SENP2 are by far the most active enzymes acting on proSUMO2/3. Due to

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difficulties in obtaining stable and active recombinant versions of SENP3, the catalytic properties of this SENP have remained poorly characterized. Indeed, besides some data showing that SENP3 reacts with SUMO2/3-VS but not with SUMO1-VS [38–40] and prefers SUMO2/3-modified substrates [20,39,55], very little was known. Here we show that although the in vitro synthesized full-length SENP3 is fully active, as assessed by its quantitative reaction with HA-SUMO2- VME, the enzyme displays only a minor isopeptidase activity. This finding is intriguing when we consider that knockdown of SENP3 in human/mouse cells leads to a strong accumulation of SUMO2/3 conjugates [25,28], a phenomenon consistent with SENP3 controlling the global levels of SUMO2/3-ylated conjugates. One possibility to explain this paradox is to assume that our assay lacks some SENP3-activating factor. If so, our data might suggest that this activating factor does not act by a de-repressing mechanism on its non-catalytic N-terminal domain because the catalytic domain of SENP3 alone also displays a minor isopeptidase activity. Another possibility is to assume that SENP3 acts on a large number of substrates but that all these substrates have some SENP3-interacting domain which, clearly, does not exist in the substrates used here. Clearly, further data are necessary to understand the properties of SENP3. SENP5 is as efficient as SENP2 in hydrolyzing RanGAP1–SUMO2/3 but shows no detectable activity with RanGAP1–SUMO1 even after prolonged incubations, suggesting a very strong paralogue preference for SUMO2/3. By contrast, it is one of the least active SENPs on polySUMO2/3 chains. This suggests that SENP5 is probably more important in removing monoSUMO2/3 from SUMOylated substrates than in disassembling polySUMO2/3 chains. Interestingly, in contrast to the results on SENP1/2/3, the catalytic domain of SENP5 is considerably less active than the full-length enzyme. This suggests that the N-terminal non-catalytic domain of SENP5 modulates its activity. Whether the N-terminal domain of SENP5 increases its substrate affinity or the catalytic efficiency of the enzyme remains to be determined. As expected from previous findings [21,22,30,33], SENP6 and SENP7 display a clear preference for polySUMO2/3 chains with very little or no activity towards the other tested substrates. However, as stated above, our results also show that SENP1 and SENP2 are at least as active as SENP6/7, suggesting that SENP6/7 are not alone in the task of disassembling polySUMO2/3 chains. As with SENP5, we found that the catalytic domains of SENP6 and SENP7 seem to be less active than the corresponding full-length enzymes. Since all these SENPs possess putative SIMs at their non-catalytic N-terminal domains, we considered the possibility that their N-terminal domains might somehow contribute for substrate recruitment. Unexpectedly, we found that a biologically compromised SENP7 mutant lacking seven of these SIMs [41] is as active as the normal protein in polySUMO2 disassembly. Apparently, these SIMs are not important for SENP7 polySUMO disassembly activity, at least in our assays. Naturally, given the difficulties in defining SIMs based solely on primary structure analyses (the SIM is quite a degenerate motif [51,56–58]), there may be additional SIMs in the N-terminal domain of SENP7 playing some role in substrate recruitment. However, it is also possible that the larger activity of full-length SENP7 in comparison with its catalytic domain is due to a stabilizing effect of its N-terminal domain over its catalytic domain, leading to an increase in the catalytic efficiency of the enzyme. Clearly, additional data are necessary to clarify these issues. In summary, here we describe a simple in vitro approach to study the properties of SENPs. The data collected, although of qualitative nature, provide a global perspective on the relative activities and substrates specificities of these biochemically intractable enzymes. Such an approach will be surely useful in any future work aiming at unraveling the structural/functional relationships of these proteases.

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Table 1. Summary of the relative activities and substrate specificities of SENPs.

The relative activities of the six SENPs for a given substrate are qualitatively classified from the maximum activity detected (+++) to non-detectable (–). Note that this classification cannot be used to compare the activity of a given SENP on different substrates because different experimental conditions were used for each substrate.

	SENP1	SENP2	SENP3	SENP5	SENP6	SENP7
ProS1	+++	–	–	–	–	–
ProS2	++	+++	–	–	–	–
ProS3	++	+++	–	–	–	–
RanGAP1–S1	+++	+	–	–	–	–
RanGAP1–S2/3	+++	++	–/+	++	–/+	–/+
PolyS2/3	+++	+++	+	+	+++	++

S1/2/3, SUMO1/2/3.

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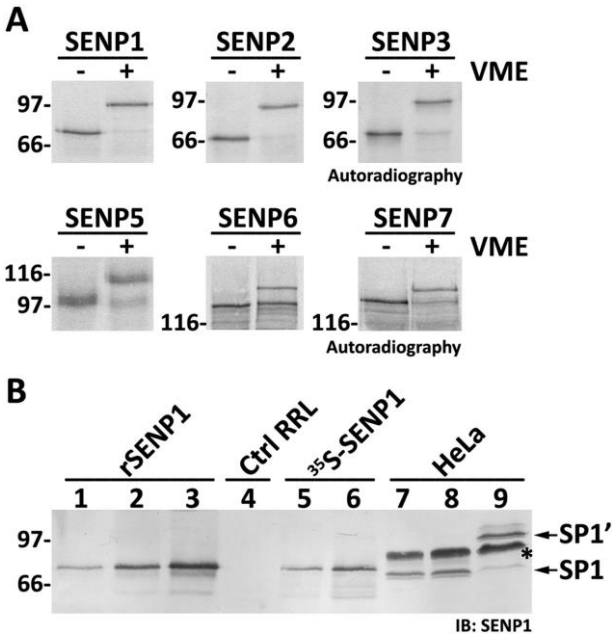

Fig. 1. In vitro synthesized full-length SENPs are active. A. ^{35}S-labeled full-length SENPs were synthesized in vitro and incubated at 25 °C in the absence (–) or presence (+) of HA-SUMO2-VME (VME). Reactions were analyzed by SDS-PAGE/autoradiography. B. SENP1 levels in HeLa cells and in the rabbit reticulocyte lysate. Increasing amounts of bacterially expressed recombinant full-length SENP1 (rSENP1, lanes 1–3; 1.5, 3 and 6 ng, respectively), in vitro synthesized SENP1 (^{35}S-SENP1, lanes 5–6; 0.5 and 1 µl, respectively) and HeLa cell total proteins (HeLa, lanes 7–8; 100 and 200 µg, respectively) were subjected to SDS-PAGE/western-blot and probed with an anti-SENP1 antibody. The RRL synthesizes 3.2 ± 0.3 ng/µl of ^{35}S-labeled SENP1 and HeLa cells contain 2.1 ± 0.6 ng of endogenous SENP1 per 100 µg of total proteins, as determined by densitometric analysis (see Section 2.4.). An RRL containing an in vitro synthesized control protein (see Section 2.5.) was also analyzed (Ctrl RRL, lane 4; 1 µl), as well as an aliquot of HeLa cell total proteins (200 μg) previously subjected to HA-SUMO2-VME labeling (lane 9). Unmodified (SP1) and modified (SP1′) SENP1 species are indicated. Asterisk, non-specific band detected by the anti-SENP1 antibody in HeLa cells. Molecular masses of protein standards in kDa are shown to the left of the panels.

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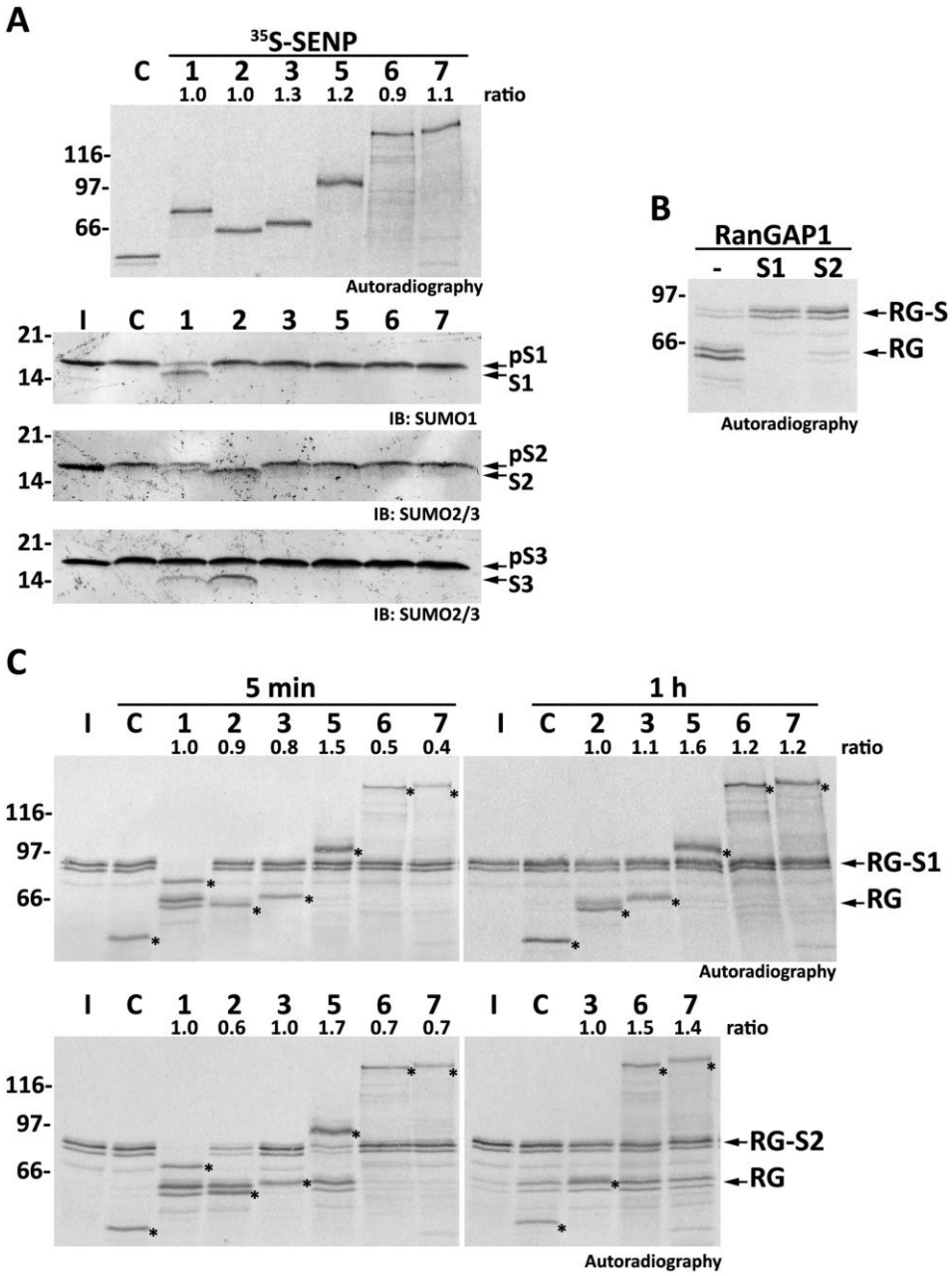

Fig. 2. Activity profiling of SENPs with SUMO precursors and monoSUMOylated substrates. A. Similar amounts of ^{35}S-labeled full-length SENP1-3/5–7 were incubated for 2 h at 25 °C with proSUMO1 (pS1), proSUMO2 (pS2) or proSUMO3 (pS3). At the end of the incubation, two aliquots of each sample were withdrawn. One aliquot was run on a 16% SDS-PAGE followed by western-blot with anti-SUMO1 or anti-SUMO2/3 antibodies to detect both the SUMO precursors and their hydrolysis products SUMO1 (S1), SUMO2 (S2) and SUMO3 (S3). The second aliquot was subjected to 8% SDS-PAGE/autoradiography to detect the radiolabeled SENPs (1–7, upper panel). Their stoichiometric ratios as determined by densitometry analysis are also shown (see Section 2.4. for details). B. In vitro synthesized full-length RanGAP1 (–) was subjected to SUMOylation with either

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SUMO1 (S1) or SUMO2 (S2). Unconjugated (RG) and SUMOylated RanGAP1 (RG–S) are indicated. C. ^{35}S-labeled full-length SENPs (1–7) were incubated at 25 °C for 5 min (left panels) or 1 h (right panels) in the presence of RanGAP1–SUMO1 (RG–S1, upper panels) or RanGAP1–SUMO2 (RG–S2, lower panels). Samples were analyzed by SDS-PAGE/autoradiography to detect both RanGAP1 (RG) and SENPs (asterisks). Molar ratios of SENPs were determined as in B. Lanes C contain an irrelevant in vitro synthesized protein (asterisk) to control the activity of endogenous SENPs from the RRL. Lane I, SENP substrate used in each reaction.

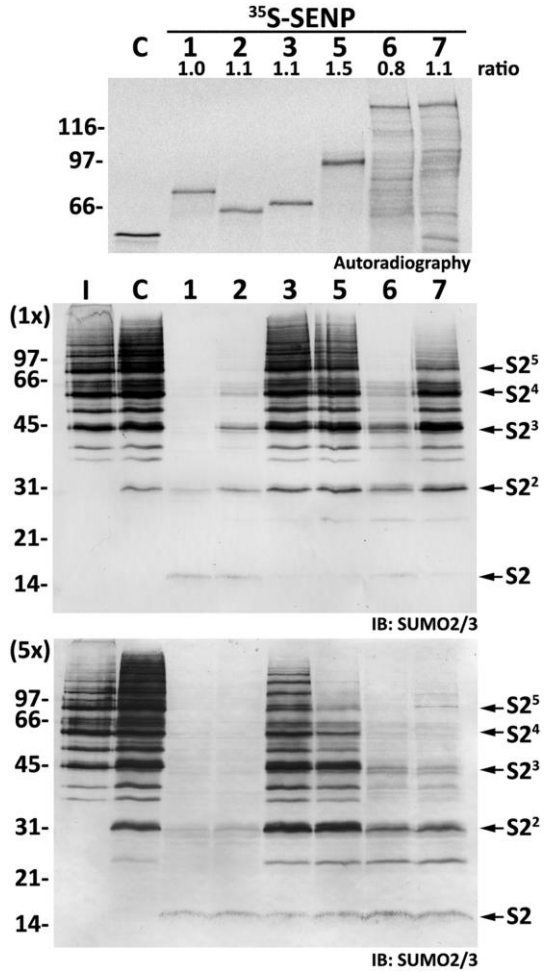

Fig. 3. PolySUMO2 chain disassembly activity of SENPs. In vitro synthesized full-length SENPs (1–7, upper panel) were incubated for 2 h at 25 °C with polySUMO2 chains (1 ×, middle panel). Similar reactions containing fivefold more SENPs (5 ×) were also prepared (lower panel). After stopping the reactions, two aliquots were withdrawn. One aliquot was analyzed in 16% SDS-PAGE followed by western-blot with an anti-SUMO2/3 antibody. SUMO2 (S2) and chains containing two to five SUMO moieties (S22–S25) are indicated. Note that monomeric SUMO2 runs in these gels together with the very abundant hemoglobin protein from the reticulocyte lysate, partially hampering its detection in western-blots. The other aliquot was analyzed by 8% SDS-PAGE/autoradiography to detect radiolabeled SENPs (upper panel). Their stoichiometric ratios are also indicated (see legend to Fig. 2). An unrelated in vitro synthesized protein (C) was included in these assays to control for background activity. Lane I, polySUMO2 chains used in each reaction.

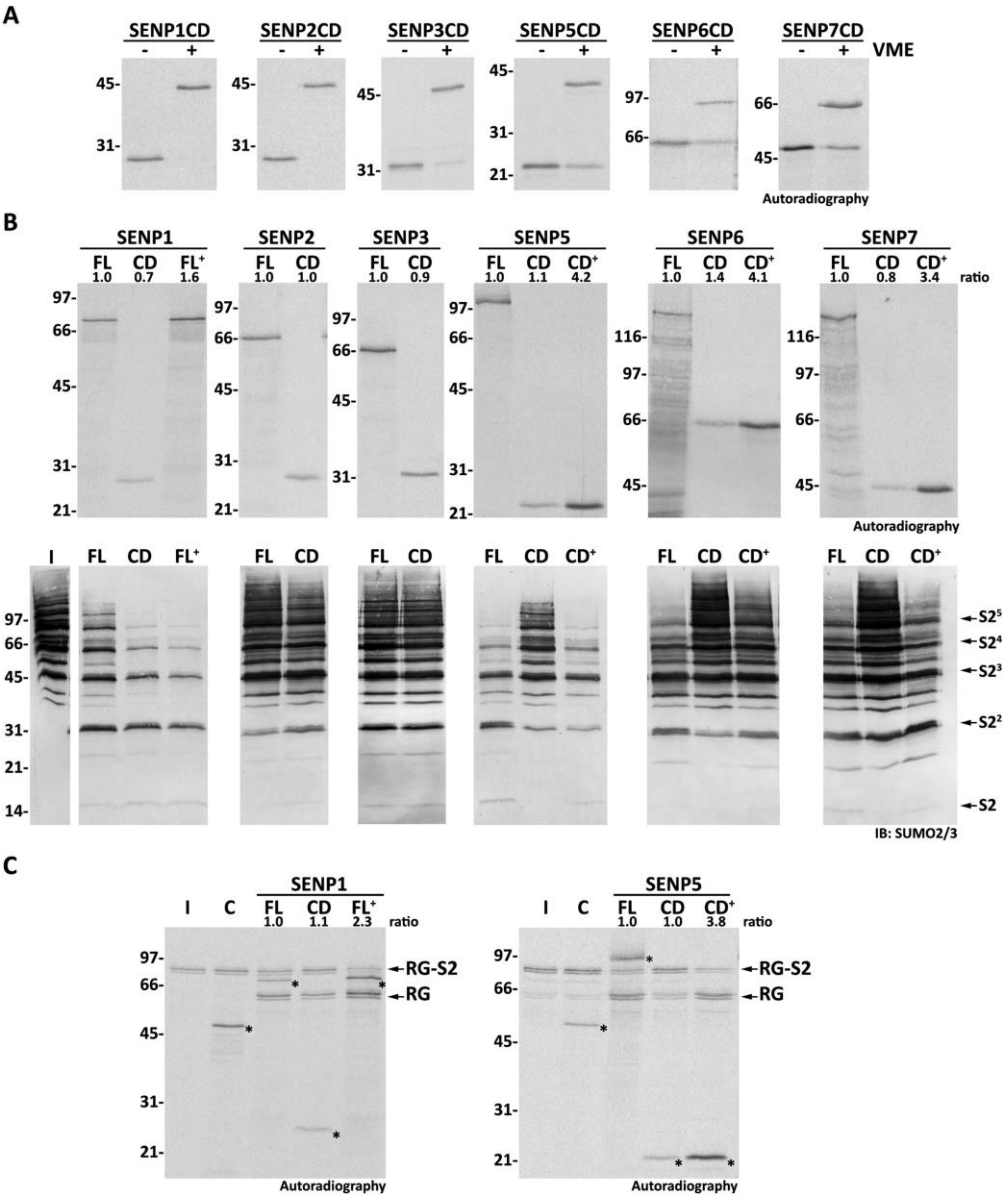

Fig. 4. Influence of the N-terminal domains of SENPs on their catalytic activity. A. ^{35}S-labeled versions of the catalytic domains (CD) of SENPs were synthesized in vitro and incubated in the absence (–) or presence (+) of HA-SUMO2-VME (VME) followed by SDS-PAGE/autoradiography analysis. B. Full-length SENPs (FL) and similar stoichiometric amounts of their catalytic domains alone (CD) were incubated with polySUMO2 chains for 5 min (SENP1/2) or 1 h (SENP3/5–7) at 25 °C. Some reactions contained a molar excess of full-length enzyme (FL+) or catalytic domain (CD+). Two aliquots were withdrawn from each sample. One aliquot was analyzed by 16% SDS-PAGE/western-blot with an anti-SUMO2/3 antibody to detect polySUMO2 chains and their hydrolysis products (lower panels). SUMO2 (S2) as well as chains containing two to five SUMO2 units (S22–S25) are indicated. The other aliquot was subjected to SDS-PAGE (8 or 12%)/autoradiography to detect the radiolabeled SENPs (upper panels). For each SENP, the stoichiometric ratios between the full-length enzyme and the catalytic domain are shown (see also

legend to Fig. 2). C. In vitro synthesized full-length SENP1/5 and their respective catalytic domains were incubated for 5 min at 25 °C using RanGAP1–SUMO2 (RG–S2) as substrate and analyzed by SDS-PAGE/autoradiography. SENP molar ratios are also shown. The radiolabeled enzymes as wells as a control protein (C) are indicated by asterisks. Lane I, SENP substrate used in each lane.

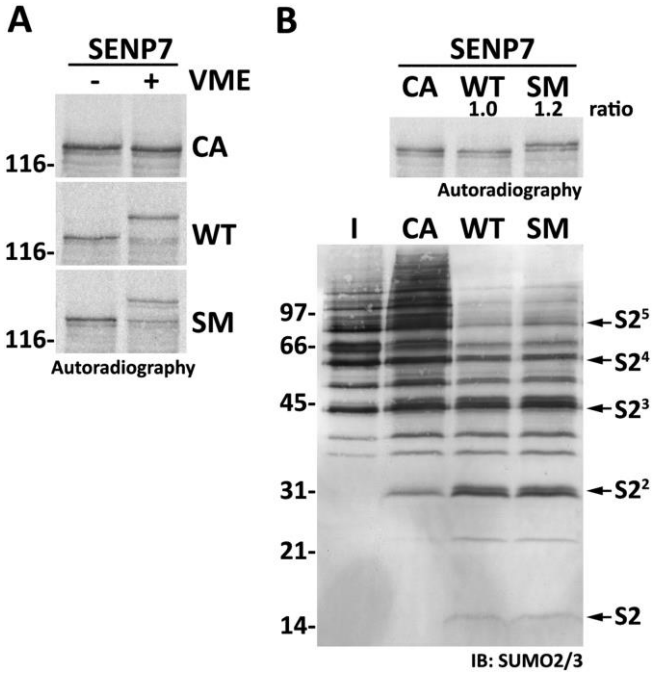

Fig. 5. A SENP7 mutant lacking seven putative SIMs is as active as the wild type enzyme on polySUMO2 chain disassembly. A. Radiolabeled wild type SENP7 (WT), a SIM mutant (SM) and a catalytic inactive C992A mutant (CA) were synthesized in vitro, incubated at 25 °C in the absence (–) or presence (+) of HA-SUMO2-VME (VME) and analyzed by SDS-PAGE/autoradiography. B. The wild type (WT), the SIM mutant (SM) and the catalytic inactive (CA) full-length versions of SENP7 were used in activity assays with polySUMO2 chains for 1 h at 25 °C. An aliquot of each reaction was analyzed by 16% SDS-PAGE/western-blot with an anti-SUMO2/3 antibody (lower panel). SUMO2 (S2) and chains containing two to five SUMO moieties (S22–S25) are indicated. A second aliquot was subjected to 8% SDS-PAGE/autoradiography to detect the radiolabeled SENP7 versions used in the assays (upper panel). The stoichiometric ratio between wild type SENP7 and the SIM mutant is shown (see also legend to Fig. 2). Lane I, polySUMO2 chains used in each reaction.