Published in final edited form as:

Nat Struct Mol Biol. 2006 December ; 13(12): 1069–1077. doi:10.1038/nsmb1172.

SUMO protease SENP1 induces isomerization of the scissile peptide bond

Linnan Shen^{1,3}, Michael H Tatham^{1,3}, Changjiang Dong^{2,3}, Anna Zagórska¹, James H Naismith², and Ronald T Hay¹

¹Centre for Interdisciplinary Research, School of Life Science, University of Dundee, DD1 5EH, UK

²Centre for Biomolecular Sciences, The University of St Andrews, KY16 9ST, UK

Abstract

Small ubiquitin-like modifier (SUMO)-specific protease SENP1 processes SUMO-1, SUMO-2 and SUMO-3 to mature forms and deconjugates them from modified proteins. To establish the proteolytic mechanism, we determined structures of catalytically inactive SENP1 bound to SUMO-1–modified RanGAP1 and to unprocessed SUMO-1. In each case, the scissile peptide bond is kinked at a right angle to the C-terminal tail of SUMO-1 and has the *cis* configuration of the amide nitrogens. SENP1 preferentially processes SUMO-1 over SUMO-2, but binding thermodynamics of full-length SUMO-1 and SUMO-2 to SENP1 and K_m values for processing are very similar. However, k_{cat} values differ by 50-fold. Thus, discrimination between unprocessed SUMO-1 and SUMO-2 by SENP1 is based on a catalytic step rather than substrate binding and is likely to reflect differences in the ability of SENP1 to correctly orientate the scissile bonds in SUMO-1 and SUMO-2.

SUMO has diverse roles in many biological processes and is required for normal growth and development in all eukaryotes studied to date. In the yeast *Saccharomyces cerevisiae*, there is a single version of SUMO known as Smt3 (ref. 1), whereas in vertebrates, three SUMO genes, SUMO-1, SUMO-2 and SUMO-3, are expressed. Whereas SUMO-2 and SUMO-3 are very similar (96% identity) they are less than 50% identical to SUMO-1. Proteomic analysis has indicated that there are distinct substrates for modification with SUMO-1 and SUMO-2/3 (refs. 2,3), and functional analysis has indicated that the different isoforms have distinct roles in transcriptional regulation⁴ and during the cell cycle⁵. Like most other ubiquitin-like modifiers (Ubls), the primary translation products of the *SUMO* paralogs need to be proteolytically processed to expose the C-terminal glycine residue that will be linked to target proteins.

SUMO modification is mediated by a SUMO-activating enzyme (E1; in human, SAE1 and SAE2), a SUMO-conjugating enzyme (E2; Ubc9) and, typically, a SUMO protein ligase (E3), and it results in formation of an isopeptide bond between the C-terminal carboxyl group of SUMO and the ϵ -amino group of lysine in the substrate protein. Lysine residues

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Correspondence should be addressed to J.H.N. (naismith@st-andrews.ac.uk) or R.T.H. (r.t.hay@dundee.ac.uk).

³These authors contributed equally to this work

Accession codes. Protein Data Bank: Coordinates have been deposited with accession codes 2IY0 (heterotrimer) and 2IY1 (heterodimer).

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

that act as acceptors for SUMO modification are usually located in a SUMO-modification consensus motif, ψ KxE (where ψ is a large hydrophobic residue and x is any residue). This motif is directly recognized by Ubc9 (refs. 6-8), but, except on RanGAP1, SUMO conjugation with only SAE1, SAE2 and Ubc9 is inefficient, and SUMO-specific E3 ligases are needed for efficient modification (reviewed in ref. 9). Both SUMO-2 and SUMO-3 have SUMO-modification consensus motifs that allow the formation of polymeric SUMO chains¹⁰.

Processing of SUMO precursors is mediated by SUMO-specific proteases that also remove SUMO from modified substrates and depolymerize poly(SUMO) chains. *S. cerevisiae* seems to express two SUMO-specific proteases, Ulp1 and Ulp2, which are cysteine proteases belonging to the family typified by the adenovirus protease^{11,12}. Yeast in which the Ulp1 gene is deleted are not viable, whereas yeast deleted for Ulp2 grow abnormally and are hypersensitive to DNA damage.

Eight genes for human proteins with substantial sequence homology to yeast Ulp1 (Senp proteins) have been identified¹³. However, they are not all specific for SUMO, as SENP8 (also called NEDP1 or DEN1) is specific for the ubiquitin-like protein NEDD8 (refs. 14-16). SENP1, SENP2 and SENP3 are SUMO-specific proteases that have distinct subcellular localizations, dictated by their nonconserved N-terminal regions (reviewed in ref. 9). SENP1 is nuclear and SENP3 is nucleolar, but differential splicing generates SENP2 proteins that can be cytoplasmic, nuclear pore localized or nuclear body localized. SENP5 is a nucleolar protein that preferentially deconjugates SUMO-2 and SUMO-3 and is required for cell division^{17,18}. Genetic analysis indicates SENP1 is required for normal mouse development¹⁹.

SENP1 processes SUMOs to mature forms and deconjugates them from modified proteins. To determine the mechanism of substrate recognition by SENP1 (ref. 20), we determined the structure of the catalytically inactive protease bound to SUMO-1–modified RanGAP1 and to unprocessed SUMO-1. In both structures, the scissile peptide bond is bent at a right angle to the C-terminal tail of SUMO-1 and adopts the *cis* configuration of the amide nitrogens. SENP1 preferentially processes SUMO-1 over SUMO-2 (refs. 21,22), but whereas substrate binding and K_m values for SUMO-1 and SUMO-2 cleavage are very similar, k_{cat} values differ by 50-fold. Therefore, the ability of SENP1 to differentially cleave SUMO-1 over SUMO-2 is not a consequence of preferential binding of substrate but is based on more efficient catalysis. This may result from differences in the ability of SENP1 to kink the scissile bonds in SUMO-1 and SUMO-2.

RESULTS

Structure RanGAP1–SUMO-1 bound to SENP1 C603A

SENP1 cleaves both isopeptide bonds, during deconjugation of SUMO from substrates, and peptide bonds, during activation of SUMO precursors. To examine the recognition of these dissimilar substrates, we purified a complex of a catalytically inactive SENP1 C603A mutant (415–644) bound to a SUMO-1–modified fragment of RanGAP1 (residues 418–587). This complex, SENP1(C603A)–RanGAP1–SUMO-1, was crystallized and X-ray diffraction data were collected. The structure was solved by molecular replacement using the individual components of the complex as search models.

The overall structure of SUMO-1–modified RanGAP1 bound to SENP1 C603A has a right-angled 'L'-shaped arrangement. The vertex is formed by SENP1, which is joined on one arm to SUMO-1 and on the other arm to RanGAP1 (Fig. 1). The structures of the three component proteins in the complex are largely identical to previous descriptions^{21,23}.

The arrangement of SUMO-1 and SENP1 is very similar to that observed in the complexes of SENP1 with SUMO-1 and SUMO-2, SENP2 with SUMO-1 and NEDD8 with the related protease NEDP1 (refs. 21,23-26). The C-terminal five residues of SUMO-1 adopt an elongated strand structure that fits into a central cleft in SENP1. The strand is stabilized by a number of hydrogen bonds between SUMO-1 and SENP1. The Gly-Gly motif at the C terminus of SUMO-1 is capped by Trp465 of SENP1, effectively enclosing the C terminus inside a tunnel. This cap enforces the requirement for Gly-Gly at the C terminus of all SUMO paralogs. Three residues before the Gly-Gly motif in SUMO-1 make a series of hydrogen bonds and van der Waals contacts with SENP1. The C-terminal strand of SUMO-1 is conserved or conservatively changed in SUMO-2 and SUMO-3 and accounts for about half of the SUMO-1 contacts with SENP1. Contacts are made mainly with main chain atoms of SENP1; these atoms are in the same positions in SENP2, which has an almost identical fold. In SUMO-1, Asn60, Arg63, Leu65, Phe66, Glu67, Gly68 and Arg70 form the other main region of contact with SENP1. Notably, only half of these residues are conserved: Arg70 is found as proline and Leu65 as arginine in SUMO-2. The contact surface buries a total of 2,100 Å² for both proteins. However, analysis of the interface using parameters derived from a systematic study of protein-protein complexes, by the CCP4 PISA server at the European Bioinformatics Institute (http://www.ebi.ac.uk/msd-srv/prot_int/pistart.html), suggests that the interface is less complementary than is normally seen in protein-protein complexes. This is consistent with an earlier hypothesis that it is the interaction of the conserved C-terminal strand of the SUMO proteins with the main chain of the Senp family that provides the principal means of recognition^{21,23}. The remainder of the interactions are relatively weak and nonconserved. This provides a structural explanation for the observation that proteins modified by any one of the three SUMO proteins are deconjugated with similar efficiencies by SENP1 and SENP2, despite substantial divergence in their sequences.

Applying the same analysis to the SENP1-RanGAP1 interface reveals that it lacks any hydrogen bonds and buries only approximately 700 Å² of surface in total for both proteins. In addition to the relatively small buried surface area, the PISA server analysis indicates that this interface is markedly outside the range seen in known protein-protein complexes. We therefore conclude there is unlikely to be any specific, biologically relevant recognition of unmodified RanGAP1 by SENP1. The interactions that exist are clustered around Lys524 of RanGAP1, which is linked by the isopeptide bond to the C terminus (Gly97) of SUMO-1, positioned at the active site of SENP1. This lack of interaction is key to the function of SENP1, as it must remove SUMO paralogs from a variety of conjugated proteins.

The third interface in the complex is between SUMO-1 and RanGAP1. This buries a mere 320 Å² of surface area of both proteins, with the only point of contact, aside from the isopeptide link, being a van der Waals interaction between Pro566 of RanGAP1 with Gly93 and Thr95 of SUMO-1. Again, this lack of interaction is consistent with the biological requirement for SUMO to be attached to and removed from many different substrates. The presence of RanGAP1 does alter the position of the conserved Gln92 of SUMO-1, compared with SENP1-SUMO-2 and SENP2-SUMO-1 complexes. Without this relatively minor change, Gln92 of SUMO-1 would clash with Glu571 of RanGAP1.

The side chain of Lys524 adopts a conformer that forms a right angle between RanGAP1 and SUMO-1 (Fig. 1 and Supplementary Fig. 1 online). The isopeptide bond is located adjacent to residue 603 of SENP1, which in wild-type protein is the catalytic cysteine. With the exception that the side chain of His533 (part of the catalytic triad) has an altered conformation, there is little appreciable difference between the SENP1-SUMO-1 structure observed in the SENP1(C603A)-RanGAP1-SUMO-1 complex or in the complex of the SUMO-1 precursor bound to SENP1 C603A described in more detail below (Fig. 2).

Comparing the relative orientations of SUMO-1 and RanGAP1 when bound to SENP1 C603A and RanGAP1–SUMO-1 bound to RanBP2 and Ubc9 (ref. 23) reveals a striking difference. In SENP1(C603A)–RanGAP1–SUMO-1, the two proteins are set at 90° to each other, forming an L shape; but in the Ubc9–RanBP2–RanGAP1–SUMO-1 tetramer, they are colinear (Fig. 1b). To achieve the L shape, the isopeptide bond adopts a *cis* configuration of the amide nitrogens ($\psi = -30^\circ$; Fig. 1d). In other structures of SUMO linked to substrate^{23,27,28}, the isopeptide bond adopts the *trans* conformation of the amide nitrogens ($\psi = 160^\circ$; Fig. 1d), commonly seen in β -sheets and energetically more favorable. The colinear arrangement of SUMO-1 and RanGAP1 found in the Ubc9–RanBP2 complex cannot be accommodated by SENP1, as Lys524 of RanGAP1 would clash with Ser601 of SENP1 (Fig. 1d). It is the requirement to avoid this interaction that presumably forces the higher-energy orientation of the isopeptide bond with $\psi = 30^\circ$.

The right-angled isopeptide bond constrains the surface topology of the protease, with the surface of SENP1–SUMO-1 upon which RanGAP1 sits being quite smooth, with a concave curve at the junction of SUMO-1 and SENP1. The channel in which the C terminus of SUMO-1 sits is almost level with the surface, and its sides present a low profile that slopes down across the SENP1 surface.

This low profile is essential to permit the approach of RanGAP1, as any large loop in this region of protein would clash with RanGAP1 (and presumably many other SUMO-modified substrates) and prevent binding. The concave surface impression at the junction between SENP1 and SUMO would accommodate diverse target proteins, allowing them to undergo the right-angled bending of the isopeptide bond required for deconjugation.

Structure of precursor SUMO-1 bound to SENP1 C603A

To establish the mechanism of SUMO processing by SENP1, we crystallized a complex of SENP1 C603A bound to full-length SUMO-1 (SUMO-1-FL) and determined the structure by X-ray crystallography. The SENP1(C603A)–SUMO-1-FL complex crystallizes as the heterodimer with two complete copies in the asymmetric unit. As expected, the complex, except residues 98–101 of SUMO-1, is almost identical to previously determined structures^{21,26,29} and to the SENP1–SUMO-1 component of SENP1(C603A)–RanGAP1–SUMO-1. This is the first complex of a SUMO protease bound to its full-length substrate, and the scissile bond is clearly visible (Fig. 2 and Supplementary Fig. 1). Again, the peptide bond has the *cis* arrangements of the amide nitrogen atoms that was also seen for the isopeptide bond (Fig. 2b-d). This suggests that the *cis* arrangement of nitrogens is an important requirement for cleavage by the Senp proteases. Biochemical studies^{21,22} show that SENP1 prefers SUMO-1 as a substrate and that this preference is due to the presence of the histidine residue immediately after the Gly-Gly motif of SUMO-1. In the *cis* amide conformation, the side chain of His98 of SUMO-1 (the first residue on the C-terminal side of the scissile bond, P1') points up and away from the surface of SENP1. We had hypothesized it might bind in a negatively charged cleft in SENP1, but this is not the case. Instead, Ser99 of SUMO-1 (P2') occupies this cleft. The side chain of Ser99 makes a hydrogen bond to a water molecule that is in turn hydrogen bonded to Asp550 of SENP1 (part of the catalytic triad). Simply by choosing another rotamer, Ser99 can be substituted by any other amino acid residue without causing a steric clash, explaining the lack of discrimination for this position. The residues on the C-terminal side of Ser99 make no contacts with the protein and have no role in recognition. The side chain of His98 makes a hydrogen bond to Gly600 of SENP1 (Fig. 2c).

SEN1 binding changes SUMO substrate conformations

The two structures of SEN1 C603A in complex with the SUMO-1 precursor and RanGAP1–SUMO-1 suggest that formation of the *cis* arrangement of the amide nitrogens is an important feature of the proteolytic mechanism of this enzyme. To confirm that SEN1 substrates undergo a major structural change upon SEN1 binding in solution, a fluorescence resonance energy transfer (FRET)-based assay was developed that detects conformational changes. Venus yellow fluorescent protein (YFP) and enhanced cyan fluorescent protein (ECFP) either were fused in a single polypeptide at the N and C termini of a SUMO precursor or were linked to RanGAP1 and SUMO, respectively, which were then linked together (Fig. 3a,b). As the FRET intensity is proportional to the proximity of the fluorophores³⁰, changes in FRET are consistent with changes in the distance between fluorescent groups. The intensity of the FRET signal for each of the four SEN1 substrates was monitored in solution in the presence of increasing concentrations of SEN1 C603A. Binding of SEN1 C603A results in dose-dependent changes in FRET signals for all four SEN1 substrates (Fig. 3c). The precursor forms of SUMO-1 and SUMO-2 show a clear increase in the FRET signal with increasing concentrations of SEN1 C603A (Fig. 3c). Binding of SUMO-1–modified RanGAP1 to SEN1 C603A results in a positive FRET change (Fig. 3c), but the SEN1 C603A–induced FRET change in YFP–RanGAP1–SUMO-2–ECFP is negative (Fig. 3c). The latter result indicates a net increase in the distance between the fluorophores upon binding, probably as a result of the increased flexibility of the attached fluorophores in this substrate. Control reactions replacing SEN1 C603A with glutathione *S*-transferase (GST) showed no appreciable changes in FRET signals (data not shown). These data are consistent with the findings of the crystallographic analysis and suggest that SEN1 binding is accompanied by a conformational change in the substrate, although it is also possible that changes in FRET intensity could be caused by clashes or interactions of the CFP or YFP with the bound SEN1.

Thermodynamics of SUMO binding by SEN1 C603A

Residues on the C-terminal side of the diglycine motifs in the three SUMO paralogs largely confer SUMO-type processing specificity in SEN1 (ref. 22) and SEN2 (ref. 29) and may contribute to the binding affinity of the SUMO precursors for the proteases. To test this hypothesis, isothermal titration calorimetry (ITC) was used to study the binding thermodynamics of SEN1 C603A with SUMO-1-FL, SUMO-2-FL, RanGAP–SUMO-1, mature SUMO-1 (bearing the Gly-Gly motif; SUMO-1-GG), mature SUMO-2 (SUMO-2-GG) and RanGAP1 alone (Fig. 4). Comparison of the calculated dissociation constants and thermodynamic parameters (Table 1) shows that SUMO-1-FL and SUMO-2-FL bind SEN1 C603A similarly ($K_d = 787$ and 492 nM, respectively, and free energy change $\Delta G = -8.5$ and -8.8 kcal mol⁻¹, respectively). By contrast, much higher-affinity binding is observed for RanGAP1–SUMO-1 and the two ‘products’ of protease action, SUMO-1-GG and SUMO-2-GG: all three of these bind with K_d between 6 and 11 nM and ΔG between -11.1 and -11.4 kcal mol⁻¹. Thus, although SEN1 preferentially processes SUMO-1-FL over SUMO-2-FL, their binding affinities for SEN1 C603A are very similar. The products of the processing reaction, mature SUMO-1 and SUMO-2, are bound substantially more tightly, with 100-fold lower K_d values and 3 kcal mol⁻¹ lower ΔG values than the two full-length substrates. RanGAP1 shows no evidence of binding directly to SEN1, consistent with structural analysis. Although SUMO-1-FL and SUMO-2-FL have similar K_d and ΔG values for binding to SEN1 C603A, the thermodynamic parameters reveal that enthalpic and entropic changes make very different contributions to the overall free energy change for these two substrates. A similar situation was observed for RanGAP1–SUMO-1 and SUMO-1-GG (Table 1). Thus, rates of cleavage by the protease²¹ do not correlate with the affinity of the enzyme for its substrate, and the products of the reaction have a high affinity for the protease.

Catalytic discrimination of SUMO paralogs by SENP1

Binding affinity alone cannot explain why SENP1 cleaves SUMO-1-FL, RanGAP1-SUMO-2 and RanGAP1-SUMO-1 much more efficiently than SUMO-2-FL²¹, but conversion of the scissile bond to the *cis* configuration may be rate limiting and could therefore allow discrimination between SUMO-1-FL and SUMO-2-FL. To analyze the mechanism of discrimination between SUMO substrates by SENP1, a FRET-based assay that faithfully mimics cleavage of unmodified forms of SUMO was used to determine k_{cat} and apparent K_m values for isopeptidase and C-terminal hydrolase activities of SENP1. Steady-state kinetic analysis comparing the initial rates of cleavage of SUMO-1-FL, SUMO-2-FL, RanGAP1-SUMO-1 and RanGAP1-SUMO-2 at different substrate concentrations (Fig. 5a,b) are consistent with previous data. Thus, SUMO-2-FL is a poor substrate for SENP1, whereas SUMO-1-FL and the two RanGAP1 conjugates are cleaved more efficiently. Apparent K_m values for all substrates, including SUMO-2-FL, are between 98 nM and 242 nM (Table 2). Whereas the turnover number (k_{cat}) varies moderately, from 224 min⁻¹ to 1,090 min⁻¹, among RanGAP1-SUMO-1, RanGAP1-SUMO-2 and SUMO-1-FL, the value for SUMO-2-FL is at least 50-fold lower (4.51 min⁻¹). These data suggest that residues on the C-terminal side of the diglycine motif of SUMO-2 affect catalysis, rather than affinity for SENP1, when compared with SUMO-1-FL and the conjugated SUMOs.

Product inhibition of SENP1 by SUMO-1/2-GG

ITC analysis indicated that SENP1 has a high affinity for the SUMO products of its reaction. Thus, SUMO-GG could act as an inhibitor of catalysis. To address this possibility, reaction rates were determined in the presence of increasing concentrations of SUMO. SUMO-1-GG and SUMO-2-GG inhibit cleavage of RanGAP1-SUMO-1, RanGAP1-SUMO-2, SUMO-1-FL and SUMO-2-FL (Fig. 5c,d). As inhibition can be assumed to be competitive²², IC₅₀ values can be determined and are between 1,400 and 2,300 nM for all substrates (Table 2). Thus, the apparent K_m values calculated by the Michaelis-Menten analysis (Fig. 5a,b and Table 2) will be higher than the actual K_m . K_m values for each substrate can be calculated from the equation

$$K_i = \text{IC}_{50} / (1 + [\text{substrate}] / K_m) - 1$$

using the IC₅₀ and the K_d measured by ITC, because $K_i = K_d \text{ product}^{31}$. All derived and calculated kinetic parameters are summarized in Table 2. Notably, for all substrates studied, the actual K_m is between 1.8 nM and 2.8 nM, which is between 60 and 90 times smaller than the apparent values.

DISCUSSION

Previous structural analysis of the Senp family of Ubl proteases has established how these proteases recognize the Ubl domains of SUMO or NEDD8 (refs. 12,21,24-26,29). However, these complexes were necessarily of truncated substrates, as peptide bond cleavage had already taken place and one of the products had dissociated. We have obtained complexes of SENP1 C603A bound to full-length SUMO precursors and to SUMO-1 linked by an isopeptide bond to RanGAP1. Structural analysis has provided details of substrate recognition and revealed that in both cases the scissile peptide bond is kinked at a right angle to the C-terminal tail of SUMO-1 owing to *cis* arrangement of amide nitrogens (Figs. 1 and 2). A *trans* arrangement of the nitrogens in either the isopeptide or α -peptide would cause the P1' substituent (on the C-terminal side of the scissile peptide bond) to clash with the loop containing Ser601 of SENP1. This loop is also present in SENP2 (ref. 29) and the NEDD8-specific protease (NEDP1)^{24,25}. Notably, NEDP1 has a bulkier tyrosine rather than a serine that may further constrict this channel and explain why NEDD8 (unlike SUMO) has

Gly-Gly immediately after the scissile bond. Although the change in substrate conformation from *trans* to *cis* seems to be a characteristic of the Senp family of proteases, further structural analysis will be required to establish whether this is also the case for the ubiquitin-specific proteases^{32,33}.

When RanGAP1-SUMO-1 is bound to Ubc9 and RanBP2, the isopeptide bond is found in the extended (nitrogens *trans*) orientation²³. NMR analysis of SUMO-1-modified RanGAP1 suggests that the isopeptide linkage is flexible and in an extended orientation³⁴. The C terminus of SUMO-1 is kinked when bound to the ubiquitin-conjugating enzyme E2-25K²⁸ and thymine DNA glycosylase²⁷, but this kink is in the Gly-Gly motif before isopeptide bond and the isopeptide bonds have an extended conformation. The *cis* arrangement of the amide nitrogens in the scissile peptide bond positions the carbonyl carbon so that the predicted trajectory of the nucleophilic attack by the sulfur of the active site cysteine is close to the optimal 90° (ref. 35). As the peptide bond adopts the *cis* conformation, it also has the effect of altering the position of the carbonyl oxygen such that the C=O bond is now pointing directly toward a number of backbone amide nitrogens that constitute the oxyanion hole of the protease. This would help stabilize the developing negative charge on the oxygen as the tetrahedral intermediate forms. During serine-protease catalysis, which is mechanistically similar to cysteine-protease catalysis, a scissile peptide bond bound at the active site is in the *cis*-amide configuration³⁶, and studies of the self-cleaving GyrA intein have revealed that the scissile peptide bond is in the *cis* configuration³⁷. Although the presence of a *cis* peptide at the scissile bond does not prove a requirement for *trans*-*cis* isomerization, it does suggest that the conformation of the scissile peptide bond is an important factor in catalysis.

We predicted that SENP1 promotes the isomerization of the amide nitrogens from the *trans* to the *cis* orientation. This was confirmed by FRET studies, which indicated that SUMO-1-FL and SUMO-2-FL undergo a conformational change upon binding to SENP1 that is consistent with the kinking of the C terminus.

Comparison of the structure of SENP1 alone with the SENP1 thiohemiacetal-linked SUMO-2 complex²¹ indicates that Trp465, which is required for catalysis, adjusts its conformation to close down over the Gly-Gly motif, forming a tunnel. We hypothesized that accompanying structural changes as the tryptophan alters its conformation are required to bring the catalytic triad into functional alignment, thus enforcing an inactive state in the absence of the correct substrate²⁵. On the basis of our experimental results, we propose the model outlined in Figure 6 for SENP1-mediated proteolysis. As the Gly-Gly motif enters the channel, the Ser601 loop selects a *cis* arrangement of the amide nitrogens. In this conformation, the peptide (or isopeptide) bond is aligned for nucleophilic attack and formation of the first acyl intermediate. Dissociation of the cleaved product is likely to be rapid, as neither the lysine residue (isopeptide) nor C-terminal residues (full-length SUMO) are recognized by SENP1. This would lead to rapid hydrolysis of the acyl-enzyme but relatively slow release of the tightly bound cleaved SUMO product.

SENP1 processes SUMO-1-FL and deconjugates RanGAP1-SUMO-2 much more efficiently than it processes SUMO-2-FL. For all these substrates, K_m is very similar (1.8 to 2.8 nM); but there are great differences in k_{cat} , with processing of SUMO-1-FL and deconjugation of SUMO-2 RanGAP1 having $k_{cat} = 224 \text{ min}^{-1}$ and $1,090 \text{ min}^{-1}$, respectively, compared with 4.5 min^{-1} for SUMO-2-FL processing. ITC analysis shows that SUMO-1-FL and SUMO-2-FL bind SENP1 C603A with essentially identical free energy changes. Thus, substrate binding is not the basis of selectivity. The large difference in k_{cat} implies that substrates are discriminated after initial binding. There is little difference in the rate at which SUMO-1 and SUMO-2 are deconjugated from RanGAP1, and this limits the

discriminatory step to a point before or at the formation of the first acyl intermediate. Subsequent steps are identical for both the deconjugation and processing reactions. We propose that it is the ability of the enzyme to position the scissile peptide bond for cleavage that differs between SUMO-1-FL and SUMO-2-FL substrates. Thus, in terms of the scheme proposed (Fig. 6), k_2 for SUMO-1 would be relatively large compared to k_2 for SUMO-2. The only residues on the C-terminal side of the cleavage site that make contact with SENP1 are those in the P1' and P2' positions. In SUMO-1-FL, this sequence is His-Ser, whereas in SUMO-2-FL it is Val-Pro. Mutational analysis has indicated that histidine in the P1' position has a positive impact on processing, whereas proline in the P2' position has a negative impact on processing²². Structural analysis can explain the preference for histidine at P1', as reorientation of the scissile peptide bond allows the side chain of His98 to make a hydrogen bond with the main chain of Gly600 of SENP1. Any amino acid residue can be accommodated without steric clashes at the P2' position; the negative effect of proline in this position in SUMO-2-FL is likely to be due to its rigid structure having an inhibitory effect on orientation of the scissile peptide bond. This could also explain the large difference in k_{cat} between deconjugation of SUMO-2 and processing of SUMO-2-FL, as the lysine side chain would impose no constraints on isomerization. Precedents for such an effect of proline come from studies of Bowman-Birk serine-protease inhibitors, which, when bound at the protease active site, have a well-defined rigid structure in which the scissile peptide bond is held in the nonproductive *trans* configuration. These inhibitors contain a *cis*-proline at the P3' position that is necessary for biological activity³⁸ and is predicted to prevent *trans-cis* isomerization.

It should be noted that there is a divergence of opinion in the literature as how SUMO-2 and SUMO-3 are designated. The sequence definitions we use to describe SUMO-2 and SUMO-3 have been described previously²¹. SENP2 cleaves SUMO-3 (which has Val-Tyr at P1' and P2') more quickly than it cleaves SUMO-1. Unlike SENP1, SENP2 lacks the cleft in which Ser99 sits. The side chain of Gln499 of SENP2 would seem likely to clash with Ser99 in the side chain conformation we observed. We suggest that SENP2 would alter the conformation of the main chain at the P2' site of SUMO-1 to bind and cleave it. In doing so, we predict, it perturbs the position of His98, disrupting its hydrogen bond to Gly600. The preference for a valine at the P1' site in SUMO-2 and SUMO-3 may be because the side chain of this smaller residue is easier to reposition than the histidine. SENP2 lacks a cleft but has a hydrophobic surface that may favor a bulky hydrophobic residue at the P2' site, explaining why the preferred substrate is SUMO-3, with a tyrosine at P2'.

An unusual characteristic of SENP1 is that the enzyme has a high affinity for the SUMO products of the reaction. During deconjugation, SENP1 does not interact with the protein to which SUMO is attached, and thus all of the specificity of the reaction must come from the interaction with SUMO. The high affinity for product is therefore likely to be a side effect of this requirement to recognize SUMO.

Although SUMO processing selectivity seems to reside within the protease domain examined here, it seems unlikely that this domain would be capable of discriminating between different substrates. Given the apparent selectivity of these proteases *in vivo*¹⁹, it is probable that the unique N-terminal regions of the protease provide substrate specificity by targeting the protease to unique subcellular locations or by directly recognizing substrate.

METHODS

Complementary DNA cloning and protein expression and purification

RanGAP1 residues 418–587 (RanGAP1₄₁₈₋₅₈₇), mature SUMO-1₁₋₉₇, precursor SUMO-1₁₋₁₀₁ (plus an additional cysteine residue at the C terminus) and SENP1₄₁₅₋₆₄₄

were subcloned into pHIS-TEV-30a (see Acknowledgments). Proteins were expressed in *Escherichia coli* BL21(DE3) and purified by nickel affinity chromatography²¹.

YFP³⁹ or ECFP fusion proteins, or those containing both fluorophores, were expressed from the pHIS-TEV-30a plasmid. cDNA encoding RanGAP1_{418–587} (ref. 40), SUMO-1_{1–101} (NCBI Entrez Protein CAA67898), SUMO-1_{1–97}, SUMO-2_{1–103} (NCBI Entrez Protein CAG46970) or SUMO-2_{1–93} (ref. 10) was cloned into the ECFP- or YFP-modified pHIS-TEV30a. Fluorescent proteins were expressed and purified by nickel affinity chromatography followed by anion-exchange chromatography. Protein concentrations were determined from the known extinction coefficients of YFP (Venus) and ECFP and the measured optical density of protein samples at 515 nm and 435 nm, respectively.

Preparation of YFP-RanGAP1-SUMO-1-ECFP and YFP-RanGAP1-SUMO-2 -ECFP

His₆-YFP-RanGAP1 was conjugated to His₆-ECFP-SUMO-1-GG and His₆-ECFP-SUMO-2-GG in 7-ml reactions as described¹⁰. His₆-proteins were repurified by nickel affinity chromatography, with a 1 M salt wash to remove any traces of SAE2, SAE1 or Ubc9 that might be noncovalently associated, and anion-exchange chromatography.

Preparation and crystallization of SENP1(C603A)–RanGAP1–SUMO-1 and SENP1(C603A)–SUMO-1-FL complexes

His₆-RanGAP1 was conjugated to SUMO-1 as above and purified using nickel affinity chromatography. SENP1 C603A was added to His₆-RanGAP1-SUMO-1 conjugates in a 1.2:1 molar ratio and the complex was purified by nickel affinity chromatography. The His₆ tag was removed by incubation with the tobacco etch virus (TEV) protease²¹. The TEV protease and the His₆ tag were subsequently removed by nickel affinity chromatography. The complex between His₆-SUMO-1-FL and SENP1 C603A was formed in a similar way using a 1:1.2 molar ratio. Both complexes were dialyzed against 20 mM Tris-HCl (pH 7.5), 50 mM NaCl and 1 mM DTT and concentrated to 19 mg ml^{−1}. Crystals were grown at 20 °C using sitting drop vapor diffusion. Crystals grew within 2 d from equal volumes of protein solution described above and a reservoir solution containing 0.2 M ammonium citrate and either 20% (w/v) PEG 3,350 (pH 5.0), for SENP1(C603A)–RanGAP1–SUMO-1 complex, or 4% (w/v) PEG 8,000, 0.1 M Tris-HCl (pH 8.0), for SENP1(C603A)–SUMO-1-FL complex.

Structure determination

Both the SENP1(C603A)–RanGAP1–SUMO-1 complex and SENP1(C603A)–SUMO-1-FL complex crystals were soaked in their mother liquor with water replaced by 17% (v/v) glycerol before being cryo-cooled to 100 K. Data were collected on a Rigaku R-axis IV image plate with X-rays from a micromax 007 copper anode generator. Data were indexed with MOSFLM⁴¹ and merged in SCALA⁴² as implemented in CCP4. Both structures were solved using molecular replacement with PHASER⁴³ in CCP4. The individual components (PDB 2BZP, 1Y8R and 1Z5S) were used as molecular replacement models. The resulting structures were refined using REFMAC5 (ref. 44) with TLS parameters. In the SENP1(C603A)–RanGAP1–SUMO-1 complex, the density for side chains of much of the SUMO molecule is weak, apart from areas where SUMO-1 is in contact with the SENP1, in which the SUMO-1 side chains are well ordered. The main chain of SUMO-1 is ordered throughout. The isopeptide bond in the SENP1(C603A)–RanGAP1–SUMO-1 complex was included in the model only when there was clear density (Supplementary Fig. 1) and was restrained in the same way as a normal peptide bond. The SENP1 C603A and RanGAP1 molecules are largely well ordered. In the SENP1(C603A)-SUMO-1-FL complex, the two copies of the assembly were tightly restrained to be very similar. The r.m.s. deviation for all 2,483 atoms in each assembly from each other is 0.07 Å. The additional residues on the C-

terminal side of the Gly-Gly motif were added only once the electron density was clear (Supplementary Fig. 1). The high B -factor of the structure is reflected in the slightly higher R -factors of the structure than one might expect at this resolution. In the heterotrimer, 88% of residues are in the core region, whereas in the heterodimer, 90% are in the core region. The full details of the models are given in Table 3.

Isothermal titration calorimetry

ITC experiments were performed at 30 °C in PBS with 0.5 mM β -mercaptoethanol using a VP-ITC microcalorimeter (MicroCal) and data were analyzed using Origin software (MicroCal v5.0). SENP1 C603A was titrated at 100 μ M into the reaction cell for the RanGAP1-SUMO-1 experiment and used in the reaction cell at a concentration of 10 μ M for SUMO-2-FL or 5 μ M for the remaining samples. Binding between SENP1 C603A and RanGAP1, both at 100 μ M, was not detected. Data were processed so that errors represent one s.e.m. of the best fit values calculated by Origin of the fit of the data from a single experiment to the single-binding site model, using nonlinear regression.

Detection of SENP1-induced conformational changes in substrates by fluorescence resonance energy transfer

Samples (triplicate) were analyzed in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM β -mercaptoethanol and 0.1 mg ml⁻¹ BSA at room temperature in 40- μ l volumes in 384-well plates, using a BMG Labtech NOVOstar fluorimeter. Twenty milliliters of 1,000 nM fluorescent SUMO protein was mixed with 20 μ l of either buffer alone or a solution of SENP1 C603A, to a final concentration of protease between 20 and 15,000 nM, and equilibrated for 30 min before reading. Incident light was 405 nm and signals were measured at 480 nm (direct ECFP excitation, E_{480}) and 530 nm (FRET excitation, E_{530}). The E_{480}/E_{530} ratio was then compared between samples. All readings were standardized against the 'buffer alone' control and data were represented as 'relative FRET change'.

Steady-state kinetic analysis of SENP1

The FRET-based assay⁴⁵ was adapted to monitor the progress of both isopeptidase and C-terminal hydrolase activities of SENP1. YFP-RanGAP1-SUMO-1-ECFP, YFP-RanGAP1-SUMO-2-ECFP, YFP-SUMO-1-FL-ECFP and YFP-SUMO-2-FL-ECFP (Fig. 3a,b) were used to test isopeptidase and hydrolase activities of SENP1 in FRET assays. Protease assays were performed at 30 °C in 25- μ l volumes in 384-well plates, using a BMG Labtech NOVOstar fluorimeter, with either 0.625 nM or 10 nM SENP1. Assays were in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM β -mercaptoethanol and 0.1 mg ml⁻¹ BSA. For derivation of apparent Michaelis-Menten constants, we used a range of SUMO substrate concentrations from 1,600 nM to 16.12 nM, in 0.6-fold dilutions, and determined initial rates. Incident light was 405 nm and signals were measured at 480 nm (direct ECFP excitation) and 530 nm (FRET excitation). The E_{480}/E_{530} ratio is directly proportional to SUMO substrate concentration. Experiments were carried out in triplicate, and apparent K_m and V_{max} values were calculated by nonlinear regression analysis using Prism (Graphpad Software). Note that rates were standardized for SENP1 concentration, effectively making $V_{max} = k_{cat}$.

IC₅₀ values for inhibition of SENP1 by SUMO-1-GG and SUMO-2-GG were determined with substrates at 500 nM plus SUMO-1 from 20,480 nM to 20 nM. IC₅₀ and actual K_m values were derived by fitting to equation (1)³¹. Experiments were carried out in triplicate, and nonlinear regression was used to fit the measured cleavage rates at each SUMO-GG concentration to the 'one-site competition' model (Prism, Graphpad Software) to give IC₅₀ values.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

pHis-TEV-30a was a kind gift from H. Liu, University of St. Andrews. This work was supported by CRUK, AICR and the Wellcome Trust. Structural analysis was carried out in the Scottish Structural Proteomics Facility, which is funded by the Biotechnology Biological Science Research Council and The Scottish Funding Council. J.H.N. is a Biotechnology Biological Science Research Council career development fellow.

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Figure 1.

Structure of the complex between SENP1 C603A and SUMO-1-modified RanGAP1. **(a)** Overall structure of the complex. RanGAP1 is shown in red ribbon, SENP1 C603A in blue and SUMO-1 in turquoise. Isopeptide bond between Lys524 of RanGAP1 and Gly97 of SUMO-1 is shown as sticks with carbon yellow, nitrogen blue and oxygen red. **(b)** A superposition of the SENP1(C603A)–RanGAP1–SUMO-1 complex with the Ubc9–RanBP2–RanGAP1–SUMO-1 complex²³. Only RanGAP1 (magenta) and SUMO-1 (teal) are shown from the Ubc9–RanBP2–RanGAP1–SUMO-1 complex; Ubc9 and RanBP2 are omitted. The isopeptide bond between Lys524 of RanGAP1 and Gly97 of SUMO-1 is shown as sticks with carbon green, nitrogen blue and oxygen red. The superposition is based on the 97 C α atoms of SUMO-1. **(c)** Details of the complex in **a**. Residues mentioned in the text are indicated. **(d)** Details of the superposition in **b**. The more common *trans* orientation of the amide nitrogens in the isopeptide seen in the Ubc9–RanBP2–RanGAP1–SUMO-1 complex would result in severe steric clashes with Ser601 of SENP1. This forces the isopeptide to adopt a *cis* arrangement of the amide nitrogens.

Figure 2.

Structure of full-length SUMO-1 bound to SENP1 C603A. **(a)** SENP1(C603A)-SUMO-1-FL complex. Cyan, SENP1; purple, SUMO-1-FL. SENP1 is effectively identical to earlier descriptions. **(b)** Superposition of SENP1(C603A)-RanGAP1-SUMO-1 complex with SENP1(C603A)-SUMO-1-FL complex. In the superposed RanGAP1-SUMO-1 complex, RanGAP1 is in red, SENP1 is in dark blue and SUMO-1 is in turquoise. Isopeptide bond is depicted as in Figure 1a. **(c)** Detail of the complex in **a**, with SENP1 in dark blue and carbons of SUMO-1-FL in pink. Residues mentioned in the text are indicated. Dotted line denotes hydrogen bond. **(d)** The same *cis* arrangement of nitrogens is seen in the SENP1(C603A)-SUMO-1-FL processing complex and in the SENP1(C603A)-RanGAP1-SUMO-1 deconjugating complex (colored as in **b** and **c**).

Figure 3.

SENP1 C603A induces a conformational change in substrates in solution. **(a)** Diagram of FRET substrates for SENP1 processing (C-terminal hydrolase) activity against full-length SUMO-1 or SUMO-2. Linear fusions YFP-SUMO-FL-ECFP (listed N terminus to C terminus) were purified from bacteria. The sequence on the C-terminal side of the scissile bond (arrowhead) is shown. **(b)** Diagram of FRET substrates for SENP1 deconjugation (isopeptidase) activity. Bacterially produced YFP-RanGAP1 was conjugated to either ECFP-SUMO-1-GG or ECFP-SUMO-2-GG *in vitro* and the conjugates purified. **(c)** Conformational change in RanGAP1-SUMO and SUMO-FL upon SENP1 C603A binding in solution. YFP-RanGAP1-SUMO-1-ECFP, YFP-RanGAP1-SUMO-2-ECFP, YFP-SUMO-1-FL-ECFP or YFP-SUMO-2-FL-ECFP (1,000 nM each) was mixed with indicated concentrations of the inactive protease mutant SENP1 C603A. FRET signals (Methods) were standardized against 'buffer alone' control. Binding of SENP1 C603A to the FRET SUMO constructs results in a change in the intensity of the FRET signal that represents a change in the distance between the two fluorophores. Measurements were in triplicate; error bars (± 1 s.e.m.) are obscured by chart symbols.

Figure 4.

Thermodynamics of substrate and product binding by SENP1 C603A. ITC was used to study the thermodynamic changes effected by binding of SENP1 to SUMO-1-FL, SUMO-2-FL, RanGAP1-SUMO-1, SUMO-1-GG, SUMO-2-GG or RanGAP1 (as indicated). Experiments were repeated on three separate occasions with very similar results. Thermodynamic parameters are indicated in Table 1.

Figure 5.

Steady-state kinetic analysis of isopeptidase and C-terminal hydrolase activities of SENP1 for SUMO-1 and SUMO-2 substrates. **(a,b)** Relationship between initial rate of cleavage by SENP1 and concentration of substrate, for RanGAP1-SUMO-1, RanGAP-SUMO-2, SUMO-1-FL and SUMO-2-FL. FRET-based assays (Methods) used the substrates diagrammed in Figure 3a,b. Initial rates at different substrate concentrations were fit using nonlinear regression to the Michaelis-Menten equation. We used 0.625 nM SENP1 in all assays except those containing SUMO-2-FL, which contained 10 nM SENP1. **(c,d)** Effect of product inhibition on SENP1 isopeptidase and C-terminal hydrolase activities. FRET-based SENP1 protease assays had a fixed concentration of the SUMO substrate (500 nM) with indicated range of SUMO-1-GG and SUMO-2-GG concentrations. Charts contain error bars (± 1 s.e.m.), which in most cases are obscured by chart symbols. Kinetic parameters are indicated in Table 2.

Figure 6.

Proposed mechanism for cleavage of substrates by SENP1. It is likely that two reversible steps occur before catalysis. First, there is an association between substrate (SUMO-target) and SENP1 that stimulates the opening of the tryptophan tunnel. Second, closing of the tryptophan tunnel causes *trans-cis* isomerization of the amide nitrogens of the scissile bond of the substrate. Chemical catalysis can then proceed, with hydrolysis and dissociation of the target being essentially irreversible. Finally, the product, SUMO-GG, dissociates from the SUMO-binding site in SENP1 before another round of catalysis can occur.

Table 1

Thermodynamic parameters

Protein sample	Binding sites, n	Dissociation constant, K_d (nM)	Enthalpy change, ΔH (kcal mol ⁻¹)	Entropy change, ΔS (kcal mol ⁻¹ K ⁻¹)	Free energy change, $\Delta G = \Delta H - T\Delta S$ (kcal mol ⁻¹)
SUMO-1-FL	1.001	787 ± 90.4	-18.5 ± 0.605	-33.2	-8.48 ± 0.605
SUMO-2-FL	1.001	492 ± 55.2	-4.38 ± 0.244	14.4	-8.76 ± 0.244
RanGAP1-SUMO-1	1.004	10.7 ± 3.12	-7.95 ± 0.091	10.4	-11.1 ± 0.091
SUMO-1-GG	1.001	6.22 ± 0.95	-15.8 ± 0.083	-14.6	-11.4 ± 0.083
SUMO-2-GG	1.000	8.56 ± 1.13	-14.5 ± 0.074	-10.8	-11.2 ± 0.074

Thermodynamic parameters for SENP1 C603A binding to substrates and products of the isopeptidase and C-terminal hydrolase reactions, calculated from the ITC experimental data shown in Figure 4. Errors represent one s.d. from the mean of the best-fit values, calculated by Origin (MicroCal), fitting the data from a single experiment to the single-binding site model using nonlinear regression. Experiments were repeated on three separate occasions with very similar results

Table 2

Kinetic parameters

SENPI substrate	$K_{m(app)}$ (nM) ^a	K_m (nM)	k_{cat} (min ⁻¹)	k_{cat}/K_m (nM ⁻¹ min ⁻¹)	IC ₅₀ (nM)
RanGAP1-SUMO-1	149 ± 15.2	2.24 ± 0.735	496 ± 15.3	251 ± 89.0	1,480 ± 275
RanGAP1-SUMO-2	242 ± 24.1	2.75 ± 0.635	1,090 ± 37.3	423 ± 111	1,600 ± 167
SUMO-1-FL	98.4 ± 5.05	1.80 ± 0.441	224 ± 3.16	133 ± 34.3	1,770 ± 172
SUMO-2-FL	126 ± 12.1	1.976 ± 0.477	4.51 ± 0.13	2.42 ± 0.592	2,230 ± 255

Summary of kinetic parameters of SENPI isopeptidase activity against RanGAP1 modified with SUMO-1 and SUMO-2 and C-terminal hydrolase activity against immature forms of SUMO-1 and SUMO-2. Errors represent one s.d. from the mean of the best-fit values calculated by Prism (Graphpad Software), fitting the triplicate data to the Michaelis-Menten equation using nonlinear regression.

^a $K_{m(app)}$, apparent K_m .

Table 3
Data collection and refinement statistics

	SENPI-SUMO-1-RanGAP	SENPI-SUMO-1
Data collection		
Space group	$P4_32_12$	$P4_32_12$
Cell dimensions <i>a</i> , <i>b</i> , <i>c</i> (Å)	83.4, 83.4, 158.0	141.2, 141.2, 98.9
Resolution (Å)	38–2.77 (2.87–2.77) ^a	100–2.46 (2.56–2.46)
<i>R</i> _{merge}	0.128 (0.514)	0.091 (0.405)
<i>I</i> / σI	14.1 (2.1)	14.7 (2.1)
Completeness (%)	100 (100)	98 (95)
Redundancy	5.0 (5.1)	4.1 (3.0)
Refinement		
Resolution (Å)	38–2.77 (2.87–2.77)	100–2.46 (2.56–2.46)
No. reflections	13161	34278
<i>R</i> _{work} / <i>R</i> _{free}	22.8 (34.1) / 27.9 (36.9)	24.9 (30.0) / 28.1 (37.6)
No. atoms		
Protein	3,709	5,126
Water	6	113
<i>B</i> -factors		
Protein	39	59
Water	37	58
R.m.s. deviations		
Bond lengths (Å)	0.010	0.014
Bond angles (°)	1.2	1.5

^aValues in parentheses are for highest-resolution shell.