Mapping residues of SUMO precursors essential in differential maturation by SUMO-specific protease, SENP1

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SUMO (small ubiquitin-related modifier) is a member of the ubiquitin-like protein family that regulates cellular function of a variety of target proteins. SUMO proteins are expressed as their precursor forms. Cleavage of the residues after the 'GG' region of these precursors by SUMO-specific proteases in maturation is a prerequisite for subsequent sumoylation. To understand further this proteolytic processing, we expressed and purified SENP1 (sentrin-specific protease 1), one of the SUMO-specific proteases, using an *Escherichia coli* expression system. We show that SENP1 is capable of processing all SUMO-1, -2 and -3 *in vitro*; however, the proteolytic efficiency of SUMO-1 is the highest followed by SUMO-2 and -3. We demonstrate further that the catalytic domain of SENP1 (SENPI C) alone can determine the substrate specificity towards SUMO-1, -2 and -3. Replacement of the C-terminal

fragments after the 'GG' region of SUMO-1 and -2 precursors with that of the SUMO-3, indicates that the C-terminal fragment is essential for efficient maturation. In mutagenesis analysis, we further map two residues immediately after the 'GG' region, which determine the differential maturation. Distinct patterns of tissue distribution of SENP1, SUMO-1, -2 and -3 are characterized. Taken together, we suggest that the observed differential maturation process has its physiological significance in the regulation of the sumoylation pathway.

Key words: maturation, sentrin-specific protease 1 (SENPI), small ubiquitin-related modifier (SUMO), SUMO-specific proteases, sumoylation, ubiquitin-like protein.

INTRODUCTION

Ubiquitin and ubiquitin-like proteins are small polypeptides of approx. 8–11 kDa that covalently modify various intracellular proteins. In recent years, many researchers have focused on a new ubiquitin-like protein, SUMO (small ubiquitin-related modifier), and sumoylation has emerged as an important new protein post-translational modification that can mediate physiological and pathological responses in humans. In contrast with ubiquitination that targets conjugated proteins to 26 S proteasome-mediated degradation, the biological consequences of SUMO modification include nuclear targeting, formation of subnuclear structures, regulation of transcriptional activities and control of protein stability [1–4]. Much less is known about the regulation of sumoylation; however, recent findings reveal that sumoylation affects [5–7] or is affected by [8–10], other post-translational modifications such as phosphorylation and ubiquitination. Intriguingly, although SUMO molecules exist only in eukaryotes, some pathogenic prokaryotes and viruses are capable of utilizing [11–15] or abrogate [16–18] the sumoylation pathway in the host system.

SUMO is highly conserved in all species, from yeast to humans. In vertebrates, there are three SUMO family members, SUMO-1, -2 and -3, whereas in invertebrates there is only one SUMO gene [19]. All three SUMO proteins are expressed as their precursor forms and are widely distributed among different tissues [20,21]. In humans, SUMO-1 precursor exhibits 44 % sequence identity with SUMO-2 and -3, whereas the latter two are more similar to each other, they share 86 and 97 % sequence identity before and after proteolytic processing respectively [22,23] (Figure 1). Precursor processing involves cleavage of the residues after the conserved 'GG' region (in the present study, we named these residues as the tail of SUMO precursor) by the hydrolysis activity of

SUMO-specific protease. The exposed second glycine then forms a covalent bond with the ϵ -amino group of a substrate lysine residue at the ψ KxE motif by a cascade of SUMO E1, E2 and E3 ligases.

To regulate the effects of SUMO-conjugated proteins, sumoylation is reversed by SUMO-specific proteases, which release SUMO from their substrates [23]. SUMO-specific proteases are crucial as they function in both maturation and deconjugation. To date, several SUMO proteases, SENP1–7 (where SENP stands for sentrin-specific protease), have been identified in humans [2]. Structurally, the less conserved N-terminal domain of the protease is responsible for cellular localization and substrate specificity during deconjugation [24], whereas the conserved C-terminal domain catalyses the enzymic reaction. The crystal structure of yeast Ulp1-Smt3 has revealed essential elements for SUMO recognition, processing and deconjugation [25]. Until now, only SENP2 and SENP6 in human [26,27], ESD4 in plant [28] and Ulp1 in yeast [25] have been shown to be functional in SUMO maturation. Although some regulatory features of SUMO expression [21,29], conjugation [8–10,30] and deconjugation [28] in response to different cellular events have been reported, the specific functions of different SUMO proteases, are still poorly understood.

The aim of the present study was to characterize the maturation process of the three SUMO precursors by SENP1. Here, we demonstrate that the maturation efficiencies of SUMO-1, -2 and -3 are different under the catalysis of SENP1. We designed the C-terminal catalytic domain of SUMO protease SENP1 (SENPI C) from the secondary structure prediction and the three-dimensional structure of yeast Ulp1 [25]. Previous studies have shown a similarly designed SENPI C function in desumoylation [31]. Our results show that SENPI C carries proteolytic activity for SUMO maturation and displays comparable substrate specificity with

Abbreviations used: GST, glutathione S-transferase; SENP1, sentrin-specific protease 1; SUMO, small ubiquitin-related modifier; for brevity, the single-letter system for amino acids has been used, V94, for example means Val⁹⁴.

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SUMO-1	MSDQEAKEPSTEDLGDKKEG-EYIKLVIGQDSSEIHFKVMITHLKLKESYCORQGVPM	59
SUMO-2	MADEKPKK-----GVKTENNHNILKVGQDGSVVQFKIKRHTPLSKLMKAYCERQGLSM	55
SUMO-3	MSEKPKK-----GVKTEN-DHINKLVAGQDGSVVQFKIKRHTPLSKLMKAYCERQGLSM	54

SUMO-1	NSLRFLFEGQRIADNHTPKELGMEEDVISVYQQTGGHSTV-----	101
SUMO-2	RQIRFPFDGQPINSTDTPAQLEMEDEDTIDVFQQTGGVY-----	95
SUMO-3	RQIRFPFDGQPINSTDTPAQLEMEDEDTIDVFQQTGGVPESSLAGHSF	103

Figure 1 Multiple protein sequence alignment of human SUMO-1, -2 and -3

The sequences of SUMO-1, -2 and -3 are shown. Residues that are completely conserved are in boldface and the 'GG' cleavage site is boxed.

full-length SENP1. This indicates that the catalytic domain alone distinguishes the three SUMO precursors. Through mutagenesis we further identify that the two amino acids immediately after the 'GG' cleavage site are essential for conferring the substrate specificity. This provides an insight into the mechanism and regulation of SUMO maturation. Notably, SUMO-2 and -3 precursors have 96 % sequence identity, and recent studies have shown protein substrates conjugated with SUMO-2 or -3 have similar, if not identical, biological consequences [32–36]. Together with the distinct expression pattern of the SUMO precursors and the protease, the biological significances of the observed differential maturation efficiencies are discussed.

EXPERIMENTAL

Plasmid construction

Human SENP1, SENP1C, wild-type and various SUMO mutants were amplified from a human control cDNA library of Human MTC™ panel I (BD Biosciences, Franklin Lakes, NJ, U.S.A.) by PCR. SENP1 and SENP1C were cloned into an expression vector pTWO-E encoding an N-terminal His₆ tag (gift from Dr A. Oliver, The Institute of Cancer Research, London, U.K.) between *NheI* and *EcoRI*, whereas SENP1C was cloned into expression vector pGEX-6P-1 (Amersham Biosciences, Uppsala, Sweden) between *EcoRI* and *NotI* for the expression of GST (glutathione S-transferase) fusion proteins. To assay for the SUMO C-terminal hydrolysis activity of a protease, SUMO precursors were engineered to have C-terminal extensions of a GST (amino acids 1–220, GenBank® accession no. AAB37346). Therefore, the amplified SUMO DNA was cloned into pTWO-E between *NdeI* and *BamHI*, followed by insertion of the fragment of GST between *BamHI* and *XhoI*. All clones were sequenced and shown to be identical with those previously reported for SENP1, SUMO-1, -2 and -3 (GenBank® accession nos. Q9P0U3, AAH53528, AAH68465 and NP_008867 respectively).

Designation for SENP1C and mutations of SUMO proteins

SENP1C was cloned from Met-427 to the stop codon of SENP1 according to the secondary structure prediction (PredictProtein Server), multiple sequence alignment and the crystal structure of Ulp1 [25]. To construct mutants SUMO-1M and -2M, the residues after the C-terminal 'GG' region of SUMO-3 are used to substitute for that of SUMO-1 and -2. This was done by PCR with specially designed primers in which the 5'-end is complementary to the residues before the 'GG' region of SUMO-1 or -2 and the 3'-end encodes the residues after the C-terminal 'GG' region of SUMO-3. A similar strategy was used to construct mutant SUMO-3M so that the residues after the C-terminal 'GG' region of SUMO-3 are substituted for that of SUMO-1. For mutants SUMO-2/V94H (Val⁹⁴ → His), SUMO-2/Y95P and SUMO-2/V94H/Y95S, SUMO-2 cDNA was amplified with primers carry-

ing the corresponding mutation at residues 94, 95 and both 94 and 95 respectively.

Protein expression and purification

Recombinant SENP1C was expressed in *Escherichia coli* BL21 by induction with 0.1 mM isopropyl β -D-thiogalactoside at 25 °C for 12 h. Cell pellets were resuspended in buffer I (500 mM NaCl, 10 mM Tris/HCl, pH 8.0, 0.2 mM benzamidine, 0.2 mM PMSF, 0.5 mM EDTA and 2 mM dithiothreitol) and lysed by sonication on ice. Lysate was clarified by centrifugation at 40 000 g for 1 h and the protein was purified by affinity chromatography on glutathione–Sephadex beads (Amersham Biosciences). The GST tag was cleaved from the fusion protein by PreScission protease and SENP1C was further purified by Superdex 75 chromatography column (Amersham Biosciences). Expression of His-SUMO-1–GST fusion protein (SUMO-1 fusion protein) in *E. coli* BL21 was induced by 0.1 mM isopropyl β -D-thiogalactoside at 37 °C for 4.5 h. Cell pellets were resuspended in buffer II (500 mM NaCl, 10 mM Tris/HCl, pH 8.0, 0.2 mM benzamidine and 0.2 mM PMSF). Lysate was centrifuged for 40 000 g for 1 h. The soluble fraction was loaded on to a nickel agarose column (Qiagen) under standard conditions followed by size exclusion chromatography (Superdex 75; Amersham Biosciences). Expression and purification of SENP1 and other SUMO fusion proteins are the same as described above, with the exception that SENP1 was only partially purified by nickel affinity chromatography as the yield was too low for further purification. SENP1C and the SUMO fusion proteins were purified to beyond 95 % homogeneity.

In vitro assays and Western blotting

To assay the hydrolysis activity of SENP1 and SENP1C *in vitro*, 4 μ g (0.1 nM) of purified SUMO precursors was incubated for 20 min at 37 °C in the absence (control) or presence of various concentrations of SENP1 or SENP1C. Reaction mixtures in a total volume of 50 μ l contained 150 mM NaCl, 10 mM Tris/HCl and 2 mM dithiothreitol. After incubation, the reactions were terminated by adding 5 $\times$ protein loading dye [10 % (w/v) SDS] and subjected to SDS/PAGE analysis. For immunoblotting analysis, briefly, the nitrocellulose membrane was blocked in 10 % non-fat milk in PBST (PBS and 0.2 % Tween 20) before the addition of primary monoclonal anti-His antibody (Amersham Biosciences) and incubated for 30 min. Signals were detected by ECL® chemiluminescence kit (Amersham Biosciences) after incubating with anti-mouse-horseradish peroxidase antibody (Amersham Biosciences).

Tissue distribution analysis of SENP1, SUMO-1, -2 and -3

Endogenous transcription levels of human SENP1, SUMO-1, -2 and -3 precursors in various tissues were determined by PCR using Human MTC™ panel I and II (BD Biosciences) and the following specific primers. SUMO-1: Fwd, 5'-GCTTATCATATGTC-TGACCAGGAGGCAAAACCT-3'; Rev, 5'-CATACTGGATCC-AACTGTTGAATGACCCCCCGTT-3'. SUMO-2: Fwd, 5'-TTC-TGTCATATGATGGCCGACGAAAAGCCCAAGG-3'; Rev, 5'-ATAACAGGATCCGTAGACACCTCCCGTCTGTGT-3'. SUMO-3: Fwd, 5'-TCTATGATGATGTCCGAGGAGAAG-CCCAAGG-3'; Rev, 5'-ATTATAGGATCCGAAACTGTGCCC-TGCCAGGCTG-3'. SENP1: Fwd, 5'-TCCACAAGAAGTGCA-GCTTATA-3'; Rev, 5'-CATCTGTAGCAGCTGTCTGTAA-3'.

For SUMO-1, -2 and -3, the cDNAs were amplified for 30 cycles in a programme of 30 s at 94 °C, 36 s at 58 °C and 90 s at 72 °C. For SENP1, the cDNAs were amplified by the same programme with the exception that the annealing temperature was 55 °C. The

Figure 2 Hydrolysis of SUMO-1, -2 and -3 fusion proteins by SENP1

(A) Analysis of the partially purified SENP1 by SDS/PAGE. (B) SDS/PAGE analysis of SUMO-1, -2 and -3 maturation reactions. Purified His-SUMO-1, -2 and -3-GST fusions (0.1 nM) were incubated with 2 μ g of partially purified SENP1 for 20 min at 37 °C in 50 μ l of reaction mixture. The control (–) reactions do not contain SENP1. After incubation, 12 μ l of each reaction mixture was subjected to SDS/PAGE analysis. (C) Proteolytic activity of SENP1 shown in (B) is subjected to immunoblotting analysis using an anti-His antibody.

DNA products were detected by 1 % agarose gel staining with ethidium bromide.

RESULTS AND DISCUSSION

SENP1 purification and its activity in SUMO maturation

Full-length SENP1 was poorly expressed in *E. coli* BL21 and only several 100 μ g of SENP1 could be partially purified (Figure 2A). The *in vitro* hydrolysis activity of SENP1 in SUMO maturation was studied and detected by SDS/PAGE and immunoblotting (Figures 2B and 2C). To distinguish the SUMO precursors and their mature forms on gels, a GST module was inserted at the C-terminus of the precursors. Proteolytic cleavage at the 'GG' region by the protease will release a 16 kDa mature form and a 27 kDa GST module. When 2 μ g of partially purified SENP1 was added to the assay, over 90 % of SUMO-1 and -2 were hydrolysed; however, surprisingly, only 50 % of SUMO-3 was hydrolysed. To examine the substrate specificity of SENP1 in SUMO maturation, different concentrations of SENP1 were tested (Figure 3). When the SENP1 dosage reduced from 2 to 0.4 μ g, substrate preferences were clearly illustrated; the maturation efficiency is in the order of SUMO-1 (90 %), SUMO-2 (50 %) and SUMO-3 (10 %). Furthermore, cleavage of SUMO-3 could not be detected when 0.08 μ g of SENP1 was added. These results imply that SENP1 is capable of processing all SUMO-1, -2 and -3 *in vitro* but with different efficiencies. Since the maturation reaction is the first committed step for subsequent sumoylation, the different maturation efficiencies catalysed by SENP1 may regulate the availability of different SUMO proteins for conjugation.

The catalytic domain of SENP1 determines its substrate specificity in the SUMO maturation process

The N-terminal domains of SUMO proteases have been suggested to control the substrate specificity during desumoylation [2,24].

Figure 3 Substrate specificity of SENP1 in SUMO maturation

Different amounts of SENP1 (2, 0.4 and 0.08 μ g) were used to hydrolyse 0.1 nM of His-SUMO-1, -2 and -3-GST fusions for 20 min at 37 °C in 50 μ l of reaction mixture. After incubation, 12 μ l of the reaction mixture was subjected to SDS/PAGE analysis.

Figure 4 SENP1C carries the same substrate specificity with SENP1

(A) Analysis of the purified SENP1C by SDS/PAGE. (B) Substrate specificity of SENP1C in SUMO maturation. Different amounts of SENP1C (0.2, 0.05, 0.01 and 0.002 μ g) were used to hydrolyse 0.1 nM each of His-SUMO-1, -2 and -3-GST fusions for 20 min at 37 °C in 50 μ l of reaction mixture. After incubation, 12 μ l of reaction mixture was subjected to SDS/PAGE analysis.

To investigate if the N-terminal domain of SENP1 is required for controlling the substrate specificity in maturation, we constructed SENP1C encompassing only the catalytic domain (residues 427–643) of SENP1. This construct was created according to the secondary structure prediction, multiple sequence alignment and the crystal structure of yeast Ulp1 [25]. The hydrolysis activity of purified SENP1C was studied by *in vitro* assay as described for the full-length SENP1. The recombinant SENP1C is enzymically active and exhibits a similar pattern of substrate specificity as SENP1 in SUMO maturation (Figures 4A and 4B). This result reveals that it is the catalytic domain that differentiates the maturation efficiencies.

Residues after the 'GG' region determine the maturation efficiencies of SUMO precursors

As SUMO-2 and -3 share higher sequence similarity, we anticipated their rates of maturation would be very similar. Surprisingly, the cleavage efficiency of SUMO-2 is more akin to that of SUMO-1. From the sequence alignment in Figure 1, we examined if the residues in the tail of SUMO-3 precursor hindered the catalysis. To verify our hypothesis, three chimaeras were constructed. SUMO-1M and -2M in which the tail of SUMO-1 and -2 precursors were replaced by that of SUMO-3, and -3M where the tail

Figure 5 Substrate specificity of SENP1 is conferred by the tails of SUMO proteins

Analysis of the effect of the tail in maturation efficiency. **(A)** Schematic diagram showing the construction of chimaeras SUMO-1M, -2M and -3M by swapping the tails after the 'GG' cleavage site. Only the protein sequence at the tail is shown. **(B)** SENP1 (2 and 0.4 μ g) and **(C)** SENP1C (0.2 and 0.01 μ g) were added to the assay mixture containing 0.1 nM of SUMO-1M, -2M or -3M. Reactions were incubated for 20 min at 37°C in 50 ml mixture. After incubation, 12 μ l of the reaction mixture was analysed by SDS/PAGE. The control (-) reactions do not contain SENP1 or SENP1C.

of SUMO-3 precursors was replaced by that of SUMO-1 (Figure 5A). In reactions catalysed either by SENP1 or SENP1C (Figure 5B), the maturation efficiencies of SUMO-1M and -2M are comparable with that of SUMO-3, whereas that of SUMO-3M is similar to that of SUMO-1. These results show that the tail can modulate the maturation efficiencies of SUMO precursors. Although the overall sequence of SUMO-1, -2 and -3, especially of SUMO-2 and -3, are similar to each other, the length and amino acid sequence of their tails are diverse.

To identify the residues involved in maturation efficiency, three further mutants were constructed on the basis of SUMO-2 as it has the shortest tail of the three precursors. We noticed that the two residues after the 'GG' site are HS, VY and VP in SUMO-1, -2 and

Figure 6 The first and second amino acids of the tails of SUMO proteins are important in SUMO maturation reactions

(A) A schematic diagram showing the mutations made on the basis of SUMO-2. The 'GG' cleavage site is in boldface. *, locations of the mutations. **(B)** SDS/PAGE analysis of SUMO maturation with different SUMO mutants. SENP1C (0.01 μ g) was added in the reaction mixture and experiments were performed as described in Figure 5(B) and in the Experimental section. The control (-) reactions do not contain SENP1C.

-3 respectively. Therefore the rationale of this analysis is to create three mutants that allow us to examine the effect of the amino acid sequence after the 'GG' region in the maturation process. In comparing the maturation efficiencies of mutants SUMO-2/V94H, SUMO-2/Y95P and SUMO-2/V94H/Y95S with those of the wild-type precursors, we were able to distinguish the role of the amino acid sequence C-terminal to the cleavage site. The three mutants were assayed using the same condition as described above. The results show that the SENP1C can hydrolyse SUMO-2/V94H and SUMO-2/V94H/Y95S as efficiently as hydrolysing SUMO-1. Substitution of Tyr-95 to proline in SUMO-2 reduced the maturation rate to that of SUMO-3 (Figure 6). These results demonstrated that although the sequence of SUMO-1, -2 and -3 are not identical, the differences in the N-terminal of the cleavage site do not contribute towards proteolytic efficiency (Figure 5). Instead, His-98 in SUMO-1 confers the highest maturation efficiency observed as described in Figures 3 and 4, whereas Pro-94 in SUMO-3 is responsible for the lowest maturation efficiency. In summary, this experiment implies that the first two residues of the tail play a crucial role in controlling the maturation efficiency.

Distinct expression patterns of SENP1, SUMO-1, -2 and -3

To elucidate further the biological significance of the differential maturation efficiencies of SUMO-1, -2 and -3, the expression patterns of SENP1, SUMO-1, -2 and -3 are characterized by PCR using a human cDNA library (Human MTC™ panel I and II;

Figure 7 Tissue distribution of SENP1, SUMO-1, -2 and -3

Tissue distributions of SENP1, SUMO-1, -2 and -3 were analysed by PCR using normalized Human MTCTM panel I and II (BD Biosciences) as templates. PCRs were performed as described in the Experimental section. The PCR products of SENP1, SUMO-1, -2 and -3 were separated on 1% agarose gel by the same sequence: lane 1, thymus; lane 2, kidney; lane 3, lung; lane 4, prostate; lane 5, pancreas; lane 6, spleen; lane 7, liver; lane 8, leukocyte; lane 9, brain; lane 10, placenta; lane 11, testis; lane 12, colon; lane 13, ovary; lane 14, small intestine; lane 15, heart; lane 16, muscle. The results were detected by staining the agarose gel with ethidium bromide.

BD Biosciences). Previous reports have shown a tissue-specific distribution of SUMO-2 in humans by Northern-blot analysis [20], but in the present study, extensive tissue samples were analysed. The expression pattern of SUMO-2 is similar to that previously reported in common tissues, notably in heart, liver, brain, placenta, lung and kidney. When comparing the expression patterns of the three SUMO precursors, SUMO-1 and -2 are widely distributed, but the latter is less expressed in kidney, lung and leukocyte. Interestingly, SUMO-3 is expressed predominantly in liver only. SENP1 is highly expressed in testis, and detectable in thymus, pancreas, spleen, liver, ovary and small intestine (Figure 7). For those tissues with undetectable SENP1 levels, other SUMO-specific proteases may contribute to SUMO maturation, as SENP2 and SENP6 are also functional in SUMO maturation [26,27].

During the preparation of this paper, the structure of the catalytic domain of human SENP2 and its complex with SUMO-1 was published [37]. It is shown that the catalytic domain of SENP2 displays differential abilities to process the three SUMO precursors in maturation. However, the processing efficiency of SENP2 towards the SUMOs is in the order of SUMO-2, -1 and -3. This contradicts our observed substrate specificity of SENP1, although the two SUMO proteases share the highest sequence identity among the seven known SENPs. Moreover, we demonstrate that the substrate specificity is determined by the catalytic domain based on the very similar hydrolysis activities of SENP1 and SENP1C. In Reverter and Lima's study, the Gly–Gly insertion mutants suggested that the amino acid side-chains after the cleavage site contribute to the SENP2 substrate specificity. The present study based on the proteolytic cleavage of various SUMO mutants allows us to locate specifically the two residues, His-98 in SUMO-1 and Pro-94 in SUMO-3 corresponding to the efficient and inefficient cleavage respectively.

Our results in concert with the recent findings for SENP2 [37] strongly suggest that the seven known human SUMO-specific proteases carry distinct substrate specificities in maturation and deconjugation. Other SUMO proteases may exist that process SUMO-3 more efficiently than SUMO-1 and -2, otherwise maturation of SUMO-3 would always be suppressed. To this end, we attempted to purify other proteases (SENP6 and SENP7) to identify a protease, if any, which can specifically process SUMO-3. However, the resultant recombinant protein was expressed in an insoluble form and no activity was observed (results not shown).

The existence of several SUMO precursors in vertebrates, coupled with their differential processing by different SENPs, suggest that the control of the proteolytic processing is of physiological importance. Such regulation controls the availability of the different SUMO proteins for their cognate substrates. Investigation of the changes of SUMO precursors under *in vivo* overexpression of proteases will provide insights to understand the biological significance of our findings.

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