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SUMO-Mimicking Peptides Inhibiting Protein Sumoylation

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Abstract

The ubiquitin-like protein SUMO is transferred through a core E1-E2 cascade composed of the SUMO activating enzyme (SAE) and Ubc9 to modify cellular proteins and transmit important biological signals. SAE primarily recognizes the C-terminal tail of SUMO and catalyzes ATP condensation with the SUMO C-terminal carboxylate to activate its transfer through the cascade. Here, we used phage display to show that a broad profile of SUMO C-terminal sequences can be activated by SAE. Based on this, we developed 7-mer peptides that can (1) form thioester conjugates with SAE, (2) be transferred from SAE to Ubc9, and (3) be further transferred to the sumoylation target protein RanGAP1. Since these peptides recapitulate the action of SUMO in protein modification, we refer to them as “SUMO-mimicking peptides”. We found that once the peptides are conjugated to SAE and Ubc9, they block full-length SUMO from entering the cascade. These peptides can thus function as mechanism-based inhibitors of the protein sumoylation reaction.

Keywords

SUMO; SUMO activating enzyme; sumoylation; phage display; peptide inhibitor

Protein posttranslational modification by the small ubiquitin-related modifier (SUMO) mediates the transmission of diverse biological signals in the cell.^[1] It has been estimated that more than 10% of cellular proteins are modified by SUMO^[2] and the dynamic on and off switch of protein sumoylation regulates DNA repair,^[3] nuclear transport,^[4] gene expression,^[5] and the assembly of promyelocytic leukemia (PML) nuclear bodies in response to viral infection.^[6] Plant and mammalian species have multiple SUMO genes with four SUMO isoforms identified in the human genome.^[7] All SUMO proteins utilize the

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same enzymatic cascade consisting of the SUMO activating enzyme E1 (SAE) and the conjugating enzyme E2 (Ubc9) to catalyze protein sumoylation.^[8] Often a SUMO E3 ligase is involved to facilitate SUMO transfer to the substrate proteins.^[4b, 9] Malfunctions of the SUMO transfer cascade have been implicated in many diseases such as cancer, heart diseases, and neurodegenerative disorders including Huntington's, Parkinson's, and Alzheimer's disease.^[10] During cancer development, increased levels of SAE and Ubc9 promote tumor metastasis.^[11] In contrast, SAE and Ubc9 are deleted by ubiquitin (UB) mediated degradation during viral infection of the host cell.^[12] It has thus been suggested that components of the SUMO transfer cascade represent important drug targets similar to their counterparts in the enzymatic cascades for UB and the UB-like protein (UBL) Nedd8.^[13] Here we report that 7-mer peptides can function as SUMO mimics by forming thioester conjugates with SAE and Ubc9. By covalently loading onto the cascade enzymes, these peptides can block the transfer of the full-length SUMO protein to substrate proteins.

SAE catalyses the condensation between the SUMO C-terminal carboxylate and ATP to form a SUMO-AMP conjugate that will subsequently react with the catalytic Cys residue of SAE to form a SUMO~SAE thioester conjugate ("~" designates a thioester linkage) (Figure S1A).^[8a, 14] Crystal structures of SAE bound to SUMO captured the tandem actions of SAE when the SUMO C-terminal carboxylate is approaching ATP and when the SUMO-AMP and SUMO~SAE conjugates are formed at the enzyme active site (Figure 1 and S2).^[15] These structures showed that the C-terminal peptide of SUMO with the sequence ⁹²QEQTGG⁹⁷ (numbered referring to full-length SUMO1) extends into the ATP-binding pocket of SAE so that the carboxylate of the terminal Gly can react with ATP to afford the SUMO-AMP conjugate. Subsequently, a conformational change of SAE allows the catalytic Cys residue of the enzyme to attack the SUMO-AMP conjugate resulting in a thioester bond between SUMO and the Cys residue. Due to the important role of the SUMO C-terminal peptide in SAE recognition, we used phage display to profile the C-terminal sequence of SUMO that can be activated by SAE.

We previously used phage display to profile the recognition of the C-terminal sequence of UB and Nedd8 by their respective E1 enzymes, namely the UB activating enzyme UAE and Nedd8 activating enzyme NAE.^[16] Here we used the same approach to display a library of SUMO1 isoform on the M13 phage surface with randomized sequences replacing ⁹²QEQTG⁹⁶ at the C-terminus of SUMO1. We kept Gly⁹⁷ unchanged in the library since it was reported that this residue at the very C-terminus of SUMO is absolutely required for its activation by SAE.^[17] We reacted the phage library with immobilized SAE in the presence of ATP (Figure S1B). SUMO variants recognized by SAE for the activation reaction were covalently bound to the plate by forming thioester conjugates with SAE. The corresponding phage particles were retained by the plate and after washing the plate, they were released by hydrolysing the SUMO~SAE conjugate with dithiothreitol (DTT).

We carried out five rounds of selections of the SUMO library. Stepwise increase in phage recovery from the selection reaction comparing to the controls in which either SAE or ATP was left out indicated convergence of the library to SAE active clones (Table S1). Sequencing the selected phage clones after round 6 revealed a clear profile of SUMO C-terminal sequences that are reactive with SAE (Figure 1C and S3). The first prominent

feature of the selected SUMO clones is that they all have a ⁹⁶GG⁹⁷ terminus matching the native SUMO despite the fact that Gly96 was randomized in the library. This suggests that the double Gly motif of the SUMO C-terminus is indispensable for SAE activation. Second, Glu93 tends to be replaced by bulky aromatic residues, Trp, Tyr and Phe, indicating that the negative charge of the Glu side chain may not be required for SAE recognition. Instead increasing the steric bulk of residues at this position improves the binding of SUMO to SAE. Third, residues at positions 92, 94 and 95 are more flexible among the selected SUMO clones. Gln92 in the wild type (wt) SUMO is still present among the selected clones but it can be substituted with the homologous Glu residue, or residues of smaller sizes such as Ala, Ser, or Thr. At position 94, the wt Gln residue is selected along with Ser, Thr, Val, or His. Thr95 in the wt SUMO is most often replaced with Ser, Gly, Leu, Ile, and Val, which are all residues of comparatively small size. These results suggest that except residue 93, where bulky aromatic side chains are favoured, and residue 96, which must be a Gly, residues 92, 94 and 95 are not specifically recognized by SAE.

The crystal structure of SAE in complex with SUMO and ATP reflects the broad specificity of SAE with SUMO residues 92–95 (Figure 1 and S2). In the complex structure, Glu93 of SUMO is bound in a pocket defined by Arg119, Glu138, Glu147, Tyr 159 and Lys164 of the Uba2 subunit of SAE, however, considerable extra space remains in the binding pocket. SUMO residues Gln92, Gln94 and Thr95 are exposed to the large opening between SUMO and SAE so these residues do not appear to be critical for the interaction of SUMO with SAE. The crystal structures of the SUMO-SAE complexes have also been solved showing SUMO in conjugation with the AMP analogues AMSN and AVSN. [15b] SUMO-AMSN mimics the binding mode of SUMO after SAE catalyses the condensation of ATP and the SUMO C-terminal carboxylate to form the SUMO-AMP conjugate. The structure of SUMO-AVSN bound to SAE mimics the binding mode of SUMO during the formation of a thioester conjugate with SAE. In either structure, Glu93 of SUMO interacts non-specifically with SAE and the flanking residues at positions 92, 94 and 95 of SUMO are still in the opening space. Thus at the various stages of SUMO binding to and activating by SAE, there will be considerable room for sequence variations of the SUMO C-terminal residues.

To probe if the sequence variations in the SUMO mutants from phage selection can be accommodated by SAE at different catalytic states, we modelled the S50 (⁹²SFVSGG⁹⁷) and S90 (⁹²QYVSGG⁹⁷) C-terminal sequences into the SAE-SUMO complexes in the presence of ATP, AMSN or AVSN (Figure S2). We found that Glu93 in wt SUMO can be replaced by a Phe (S50) or a Tyr (S90) residue without interfering with the binding pocket in SAE. Tyr93 in S90 may even introduce an extra hydrogen bond between the side chain hydroxyl groups of Tyr93 and Thr415 in the Uba2 subunit of SAE. Other side chain variations at positions 92, 94 and 95 can be readily accommodated by SAE as well as shown by the modelled structures. These results suggest that structurally SAE is capable of activating SUMO C-terminal sequences with substantial promiscuity. It is quite likely that the phage-selected SUMO C-terminal sequences are better recognized by SAE than the wt SUMO sequence. We thus assayed the activity of SUMO variants with SAE.

We used an ATP-PP_i exchange assay^[18] to measure the rate of formation of the SUMO-AMP conjugate as the consequence of ATP condensation catalysed by SAE. We assayed the

five most abundant SUMO variants after six rounds of selection. These clones included S38, S46, S90 and S50 with aromatic Trp, Tyr and Phe residues replacing the wt Glu93, and S45 that still has a wt Glu93. Kinetic measurements showed that all mutants were reactive with SAE. S38 underwent the exchange reaction at a rate slightly higher than wt SUMO while mutants S90 and S50 were two and three times higher in $k_{\text{cat}}/K_{1/2}$ than wt SUMO (Figure S4A and Table 1). SUMO mutants S45 and S46 were also reactive with SAE except that their activities ($k_{\text{cat}}/K_{1/2}$) were more than 35-fold lower than that of wt SUMO, S50 or S90. The C-terminal sequences of S46 ($^{92}\text{QYTGGG}^{97}$) and S90 ($^{92}\text{QYVSGG}^{97}$) just differ by the two residues immediately before the $^{96}\text{GG}^{97}$ motif. The large difference in their activities suggests that subtle change to the SUMO C-terminal sequence may have a significant effect on SAE recognition. Despite their low activities, S45 and S46 were selected in parallel to S90 and they were among the most abundant clones enriched by phage selection. The decoupling of clone abundance and their activities with SAE suggests that other factors such as phage display efficiency, growth rate of the *E. coli* cells secreting the phage, and the folding stability of the SUMO variants anchored on phage surface may have all played a role in the enrichment of specific phage clones besides the reactivity of SAE with the SUMO variants.

We also found that S38, S50 and S90, which displayed an ATP-PP_i exchange activity higher than wt SUMO, can form thioester conjugates with SAE as shown by Western blot analysis of the SUMO loading reactions. Furthermore, the SUMO variants can be transferred from SAE to the E2 enzyme Ubc9 and to the SUMO substrate protein RanGAP1^[4a] as demonstrated by the detection of SUMO~Ubc9 and SUMO-RanGAP1 conjugates (Figure 2A). S45 and S46, which exhibited significantly lower $k_{\text{cat}}/K_{1/2}$ rates than wt SUMO, were not found to form SUMO conjugates with SAE, Ubc9 and RanGAP1. These results demonstrate that the SAE-Ubc9 cascade can accommodate the C-terminal variations in the SUMO clones and that the SUMO variants can be transferred by the enzyme cascade to the substrate proteins with a similar efficiency as wt SUMO. Interestingly we observed that RanGAP1 conjugates with wt SUMO, S50 or S90 can be cleaved at similar efficiencies by the common deSUMOylases SENP1 and SENP2 (Figure S5).^[19] Thus S50 and S90 have no apparent differences from wt SUMO in substrate modification and proteolytic removal despite the differences in their C-terminal sequences

Encouraged by the high activity of the SUMO variants S50 and S90 with SAE, we decided to test if the 7-mer peptides pS50 and pS90 corresponding to the C-terminal sequences of S50 and S90 (residues 91–97) could be activated by SAE. We previously found that short peptides corresponding to the C-terminal sequences of UB and Nedd8 variants from phage selection can be activated by UAE and NAE.^[16b–d] ATP-PP_i exchange assay showed that the pS50 and pS90 peptides could be activated by SAE with a $k_{\text{cat}}/K_{1/2}$ 75-fold higher than the peptide with the wt SUMO sequence (Figure S4B and Table 2). However, the activities of the pS50 and pS90 peptides in the ATP-PP_i reaction are much smaller than those of full-length SUMO or the corresponding SUMO variants. This is mainly due to the significantly increased $K_{1/2}$ values of the peptides (233 μM for S50 and 262 μM for S90) compared to full-length SUMO and its variants with $K_{1/2}$ -values in the range of 0.48–0.64 μM . This

result suggests that, besides the C-terminal peptide, other parts of the SUMO globular domain also make significant contributions to the SUMO-SAE interaction.

We then assayed if the pS50 and pS90 peptides can form thioester conjugates with SAE and Ubc9, and can be further transferred to the sumoylation substrate RanGAP1. We conjugated biotin to the N-terminal amino group of either pS50 or pS90 and incubated the peptides with SAE and Ubc9 as well as the substrate RanGAP1 in the presence of ATP. The peptide transfer reaction was subsequently analysed by electrophoresis and Western blot probed with a streptavidin – horseradish peroxidase (HRP) conjugate. The Western blot revealed that biotin-labelled pS50 and pS90 could be transferred to SAE, Ubc9 and eventually to RanGAP1 (Figure 2B). In contrast, the biotin-labelled pSUMO peptide was incapable of conjugating to SAE and Ubc9 and to RanGAP1. This proves that pS50 and pS90 peptides identified by phage selection are recognized by SAE more efficiently during the activation reaction and have a higher activity in modifying RanGAP1 than the pSUMO peptide. Since the pS50 and pS90 peptides recapitulate the action of full-length SUMO during transfer by the SAE-Ubc9 cascade to modify SUMO substrates, we refer to these peptides as “SUMO-mimicking peptides”.

We rationalized that if the pS50 and pS90 peptides can be loaded on SAE and Ubc9, they should also be able to block the SUMO protein from being transferred through the SAE-Ubc9 cascade to modify RanGAP1. To test this hypothesis we incubated SAE, Ubc9 and RanGAP1 with increasing concentrations of pSUMO, pS50 and pS90 for one hour to allow peptide loading onto SAE and Ubc9. We then added 5 μ M HA-tagged SUMO to the reaction to allow SUMO transfer through the cascade. The formation of SUMO conjugates with SAE, Ubc9 and RanGAP1 was subsequently analysed by Western blot probed with an anti-HA antibody (Figure 2C). We found that pSUMO could not block SUMO transfer to RanGAP1 even at a peptide concentration as high as 500 μ M. In contrast RanGAP1 modification by SUMO was almost completely blocked in the presence of 200 μ M of pS50, or 100 μ M pS90. Correspondingly the formation of SUMO~SAE and SUMO~Ubc9 conjugates were diminished with increasing concentrations of pS50 and pS90, suggesting that the SUMO-mimicking peptides inhibit RanGAP1 sumoylation by blocking SUMO transfer through the SAE-Ubc9 cascade.

To assay if pS50 and pS90 can directly compete with full-length SUMO in reacting with SAE, we incubated SAE with 1 μ M SUMO and increasing concentrations of the peptides for 10 minutes. The reactions were then quenched and analysed by Western blot to probe for the formation of SUMO~SAE conjugates with an anti-HA antibody (Figure S6). The amount of SUMO~SAE conjugate formation was quantified by densitometry analysis of the corresponding bands on the Western blot. The IC₅₀-values of the peptides inhibiting SUMO loading onto SAE were calculated by plotting the amount of SUMO~SAE formed with the concentration of the peptide inhibitor. We found that pS50 and pS90 have IC₅₀-values of 125 μ M and 131 μ M, respectively, in inhibiting 1 μ M of SUMO to form thioester conjugates with SAE. This proves that the SUMO-mimicking peptides pS50 and pS90 can function as competitive inhibitors of SAE to block SUMO activation.

In this study we used phage display to establish that a broad profile of SUMO C-terminal sequences can be recognized by SAE to activate SUMO transfer to the substrate proteins. The profile of SAE reactive SUMO variants shows that there needs to be a Gly-Gly motif at the end of SUMO and a common presence of bulky aromatic residues Phe, Tyr or Trp at position 94, the -5 position from the C-terminal end of SUMO. The -5 residue of UB and the UBL protein Nedd8 have been found to be a key recognition site for their respective E1s, UAE and NAE. By phage selection, we found the -5 Arg in UB, which ends with ⁷¹LRLRGG⁷⁶ is absolutely required for UAE recognition while the -5 Ala in Nedd8, which terminates in ⁷¹LALRGG⁷⁶, is preferred for NAE recognition but may be replaced with residues of similar sizes such as Gly, Ser and Leu.^[16a, 16d] In contrast SAE prefers the replacement of -5 Glu in SUMO with Trp, Tyr and Phe that are much larger in size and of very different charge characteristics than the native Glu residue. This indicates that SAE is more promiscuous than UAE and NAE in binding to the C-terminal sequence of SUMO.

Recently a number of natural products including ginkgolic acid, anacardic acid, and kerriamycin B have been reported to bind and inhibit SAE.^[20] Synthetic compounds of phenyl urea derivatives and the AMP analogue MLN4924 which was designed to inhibit NAE have also been found to inhibit SAE.^[13d, 21] Furthermore, full-length SUMO with its C-terminal carboxylate conjugated to a non-hydrolyzable adenosine sulfamide has been shown to bind to SAE and inhibit free SUMO from forming thioester conjugates with SAE.^[22] Our study shows that the identified peptides represent an interesting starting point for the development of a completely different class of inhibitors. As shown by ATP-PP_i exchange assay, the SUMO-mimicking peptides can be activated by SAE at a rate that is 75-fold higher than the pSUMO peptide with the C-terminal sequence of wt SUMO. We also found that the peptides can form thioester conjugates with the catalytic Cys residue of SAE and can be transferred to Ubc9 and then to SUMO modification target RanGAP1. The SUMO-mimicking peptides thus compete with SUMO for loading on SAE, and, once they are loaded on either SAE or Ubc9 or are covalently conjugated to SUMO substrate RanGAP1, they block the enzyme active sites or the substrate modification sites for regular SUMO conjugation. Hence these peptides provide a unique catalysis-based mechanism to inhibit SUMO transfer through each and every step of the enzymatic cascade. The cellular activity of these SUMO mimicking peptides in inhibiting protein sumoylation is worth to be further characterized.

Supplementary Material

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Acknowledgments

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Figure 1.

Binding of the SUMO C-terminal peptide to SAE and profile of the SUMO C-terminal sequences recognized by SAE. A) Crystal structure of SAE in complex with SUMO and ATP (PDB ID 1Y8R). The Sae1 subunit of the heterodimeric SAE is shown in pink, its Uba2 subunit in grey, SUMO in green and the ATP molecule as a ball-and-stick model. B) Enlarged view of the SUMO-SAE interaction at the C-terminus of SUMO. Residues involved in key interactions are labelled in green (SUMO residues) and grey (SAE residues). C) Representative SUMO clones selected by phage display based on SAE catalyzed SUMO activation. Red stars on top of the wild-type (wt) SUMO sequence highlight residues being randomized in the library. The number of times each clone appears among the clones sequenced after round 5 selection is also shown. See Figure S3 for a comprehensive alignment of the phage selected SUMO clones.

Figure 2.

Transfer of SUMO variants and the SUMO-mimicking peptides through the SAE-Ubc9 cascade to sumoylation target RanGAP1. A) Transfer of HA tagged SUMO variants to RanGAP1 through SAE and Ubc9. The Western blot was probed with a mouse anti-HA antibody and an anti-mouse IgG antibody conjugated to horseradish peroxidase (HRP). B) Transfer of biotin-labelled peptides pS50 and pS90 to RanGAP1. The western blot was probed with a streptavidin-HRP conjugate. C) Inhibition of SUMO loading on SAE and Ubc9 and inhibition of RanGAP1 sumoylation by the SUMO-mimicking peptides. pS50 and pS90 were incubated with SAE, Ubc9 and RanGAP1 for one hour followed by the addition

of 5 μ M HA-SUMO to the reaction mixture. The Western blot was probed with a mouse anti-HA antibody and an anti-mouse IgG conjugated to HRP. Bands marked with a star match the size of a SUMO~PCP-Uba2 thioester conjugate.

Table 1

Kinetic characterization of the ATP-PP_i exchange reactions of SUMO variants and the SUMO-mimicking peptides catalyzed by SAE. The C-terminal sequences of the SUMO variants and the sequences of the SUMO-mimicking peptides are shown.

SUMO variants	K _{1/2} (μM)	k _{cat} (min ⁻¹)	k _{cat} /K _{1/2} (μM ⁻¹ min ⁻¹)
SUMO (⁹¹ YQEQTG ⁹⁷)	0.64 ± 0.17	36 ± 4.1	56
S38 (⁹¹ YQWSSGG ⁹⁷)	0.68 ± 0.12	44 ± 11	65
S45 (⁹¹ YEEHIGG ⁹⁷)	4.5 ± 1.3	2.5 ± 0.7	0.56
S46 (⁹¹ YQYTGGG ⁹⁷)	2.4 ± 0.64	3.8 ± 0.5	1.6
S50 (⁹¹ YSFVSGG ⁹⁷)	0.48 ± 0.05	75 ± 5.7	160
S90 (⁹¹ YQYVSGG ⁹⁷)	0.59 ± 0.07	54 ± 8.2	92
SUMO mimicking peptides			
pSUMO (⁹¹ YQEQTG ⁹⁷)	ND	ND	1.3 × 10 ⁻³
pS50 (⁹¹ YSFVSGG ⁹⁷)	233 ± 34	22 ± 2.3	9.4 × 10 ⁻²
pS90 (⁹¹ YQYVSGG ⁹⁷)	262 ± 17	25 ± 4.9	9.5 × 10 ⁻²