IN VITRO ANALYSIS OF SENP ENZYMES WITH SUMO C-TERMINAL DERIVATIVES, SUMO CHAINS and SUMOYLATED SUBSTRATES

Kal Lapan, David Proia, Shannon Fishman and Francesco Melandri
Boston Biochem Inc., Cambridge, MA 02139

INTRODUCTION

The small ubiquitin-related modifier (SUMO) is a highly conserved post-translational modifier, and four functionally distinct human SUMO isoforms exist. The physiological consequences of SUMOylation are distinct from that of ubiquitination; in general not targeting proteins for degradation but is implicated in cell cycle progression, nuclear import, target sub-nuclear localization, transcriptional regulation, and the modulation of protein-protein interactions and protein stability. SUMO chain formation and conjugation to target proteins is mediated by enzymes including an E1 (SAE1/SAE2), an E2 (UbcH9) and many different E3 ligases. UbcH9 is also able to mediate the conjugation of SUMO (in the absence of an E3) to lysine residues on a variety of cellular targets.

Modification by SUMO requires the presence of a SUMOylation motif which contains a yKxE consensus sequence. Within this sequence, y is a large hydrophobic residue (I, L, or V), K is the lysine that becomes modified, X is any residue and E is glutamic acid. The presence of this motif on a short peptide allows for successful SUMOylation with all SUMO isoforms. And SUMO itself contains this motif allowing for the formation of polymeric SUMO chains. We show that specific SUMO lysine mutations within this motif affects chain formation. Single lysine mutants of SUMO-2 or SUMO-3 have drastic effects on chain formation while SUMO-1 function is dictated by three different lysine residues. However, SUMOylation can occur on lysine residues located within non-consensus regions or may be dependent on secondary structure, such as the model substrate protein (the ubiquitin E2 conjugating enzyme UbcH1, E2-25K) which occurs at a lysine residue within an α-helix.

Similar to ubiquitination, SUMOylation is a reversible process performed by SUMO-specific proteases (SENPs) which function in both the maturation of SUMO precursor proteins and in the removal of SUMO from modified substrates. SENP1 and SENP2 enzymes were characterized with a panel of SUMO substrates including a SUMOylated protein or peptide and purified SUMO-2 and SUMO-3 chains. In addition, a kinetic analysis was performed using the fluorogenic SUMO-AMC substrate. SENPs are fully and specifically inhibited by SUMO derivatized at its C-terminus by aldehyde and vinyl-sulfone groups.

SENP SUBSTRATE: SUMO-AMC

SUMO-AMC (C-terminal derivative cleavage of fluorescent group)

Fluorogenic C-terminal derivatives of SUMO are useful for kinetic studies since the release of the fluorophore (AMC or AFC) results in signal which is directly proportional to activity. This assay format can be used to characterize and study the mechanism of action of SENP inhibitors such as SUMO-aldehyde or SUMO-vinylsulfone.

Enzyme	Substrate	nM AMC/ sec/µg	Inhibition: Ub Aldehyde	Inhibition: SUMO1 Aldehyde	Inhibition: NEDD8 Aldehyde
SENP1	SUMO1-AMC	630.7	None	Complete	None
SENP2	SUMO1-AMC	684.6	None	Complete	None

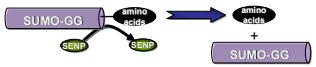
Table 1. Hydrolysis of SUMO-AMC by SENP enzymes and inhibition by SUMO-aldehyde proteins.

The specific activity of the deconjugating enzymes (5nM $SENP1_{CD}$,$SENP2_{CD}$) was determined with 400nM SUMO-AMC. Substrate hydrolysis was measured kinetically using a calibrated fluorimeter (λem:340, λex:465) at 37ºC. For inhibition studies, enzymes were pre-incubated with aldehyde derivatives (2µM) as indicated for 1hr. Residual SUMO-AMC hydrolysis was measured as described.

Conclusions: SENPs are specifically inhibited by the SUMO aldehyde derivative which is a reversible active site inhibitor. These enzymes show no cross-reactivity with closely related ubiquitin and NEDD8 proteins. Similar inhibition activities are observed with the corresponding vinylsulfone derivatives.

SENP SUBSTRATE: SUMO precursor proteins

Inactive Proforms of SUMO (C-terminal cleavage of additional residues)



SUMO precursors are inactive proforms that contain additional amino acids beyond the conserved C-terminal glycine residues. These residues are removed by SENP enzymes *in vivo* to generate [illegible] mature SUMO proteins. SENP family enzymes have distinct preferences for the processing [illegible] SUMO-1, SUMO-2 or SUMO-3 paralogs. ¹Residues upstream (C-terminal) to the conserved GG-m[illegible] appear to influence the rate at which the different SUMO precursors are processed by various SENP e[illegible]

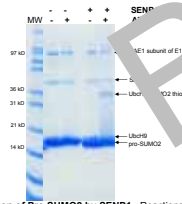


Figure 1. Processing and activation of Pro-SUMO2 by SENP1. Reactions contained E1 (100nM), UbcH9 (5µM), pro-SUMO2 (50µM) in the presence or absence of Mg-ATP (10mM) and/or with SENP1 (100nM) as indicated. Samples were incubated at 37°C for 60 minutes, and proteins were separated by non-reducing SDS-PAGE.

Conclusions: Pro-SUMO proteins have an inactive C-terminus and cannot form a thioester complex with UbcH9 or SUMO chains. SENPs can process the C-terminus to generate mature or active SUMO protein that is able to be activated by the E1 enzyme and tranferred to the UbcH9 active site.

¹Mikolajczyk J.,*et al.* (2007) JBC 282:26217-26224.

SENP SUBSTRATE: SUMO CHAINS

SUMO Chains (Isopeptide linkage cleavage)

Unanchored SUMO polymeric chains that are linked via isopeptide bonds are substrates and are disassembled *in vivo* to recycle the protein monomers.

ΨKxE

SUMO-1: ¹MSDQEAKPSTEDLGDKKE—GEYIKLKVIGQDS SE IHFKVKMTH LK KLKES[50]
SUMO-2: ¹MAD----EKP------KEGVKTENNDHINLKVAGQDGSVVQFKIKRHTPLSKLMKA[46]
SUMO-3: ¹MSE----EKP------KEGVKTE--NDHINLKVAGQDGSVVQFKIKRHTPLSKLMKA[45]

SUMO-1: [51]YCQRQGVPMNSLRFLFEGQRIADNHTPKELGMEEEDVIEVYQEQTGGHSTV[101]
SUMO-2: [47]YCERQGLSMRQI RFRFDGQPINETDTPAQLEMEDEDTIDVFQQQTGGVY[95]
SUMO-3: [46]YCERQGLSMRQI RFRFDGQPINETDTPAQLEMEDEDTIDVFQQQTGGVPESSLAGHSF[103]

Figure 2. Primary sequence alignment of SUMO-1, SUMO-2 and SUMO-3 proteins. Residues in blue are identical, the consensus SUMOylation motif (ψKxE) is underlined. The precursor forms for each SUMO contains the residues beyond the conserved C-terminal GG. Conserved lysines that play a critical role in SUMO polymerization are indicated as (K). SUMO-1 contains a total of 11 lysine residues, with some (K) not found in SUMO-2 and SUMO-3, which both have a total of 7 lysines.

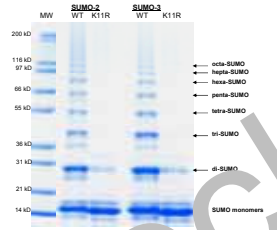


Figure 3. *In vitro* mediated SUMO-2/3 chain formation is abrogated by mutation of the conserved lysine 11 to arginine. SUMO chains were synthesized in the presence of SUMO E1 and Ubc[illegible] enzymes. [illegible] products were visualized by 16% SDS-PAGE.

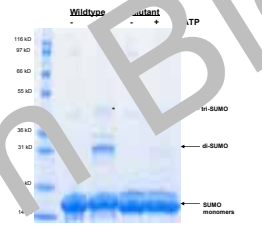


Figure 4. *In vitro* SUMO-1 chain formation is abrogated by the mutation of conserved lysines 6, 16 and 17 to arginine. Asterisk denotes tri-SUMO.

SUMO-1 Protein	Chain Formation
SUMO-1 wildtype	• • • • • •
SUMO-1 K7R	• • •
SUMO-1 K16R	• • • • •
SUMO-1 K17R	• • • •
SUMO-1 K7R, K16R	• •
SUMO-1 K7R, K17R	•
SUMO-1 K16R, K17R	•
SUMO-1 K7R, K16R, K17R	none
SUMO-1 methylated	none

Table 2. Effect of SUMO-1 lysine mutants on *In vitro* mediated SUMO-1chain formation. SUMO chains were synthesized in the presence of SUMO E1 and UbcH9 enzymes and various SUMO-1 proteins as in Figure 3. The effect of each protein on di-SUMO formation is indicated, wildtype with 100% di-SUMO formation.

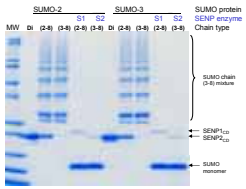


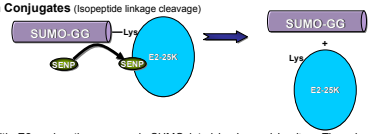
Figure 5. Deconjugation of SUMO-2 and SUMO-3 chains by sumo-specific isopeptidases SENP1 and SENP2. Reactions contained SUMO chains (2µg) in 50 mM Hepes, 50 mM NaCl, 5 mM DTT incubated in the presence of 500 nM SENP1 (S1) or SENP2 (S2) for 60 minutes at 37ºC. Products were visualized by 16% SDS-PAGE.

Conclusion: SUMO chains are formed through isopeptide linkages to lysine residues within the conserved consensus motif. Mutation of these lysines reduce SUMO chain formation. The deSUMOylating enzymes SENP1 and SENP2 hydrolyze free purified SUMO-2 and SUMO-3 chains of all lengths *in vitro*.

References: Okuma T.,*et al.* (1999) BBRC 254:693-698. Tatham M.H.,*et al.* (2001) JBC 276:35368-35374. Pedrioli P.G.,*et al.* (2006) Nat. Meth. 3:533-539.
Yang S-H.,*et al.* (2006) EMBO J. 25:5083-5093

SENP SUBSTRATE: MONO-SUMOylated E2-25K

SUMO Protein Conjugates (Isopeptide linkage cleavage)



E2-25K, a ubiquitin E2 conjugating enzyme is SUMOylated *in vivo* and *in vitro*. The primary acceptor site (Lys14) is unique since it is not located within the known consensus motif (ΨKxE) and functions only in the context of an α-helix. This indicates that secondary structure elements may also be important for SUMO conjugation and/or deconjugation of targets. The E2 enzyme is linked to SUMO via an isopeptide linkage and is a substrate for SENPs.

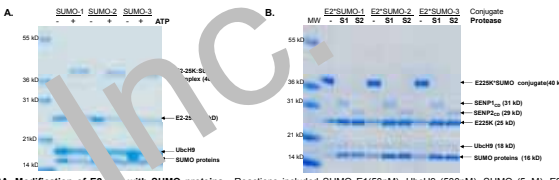


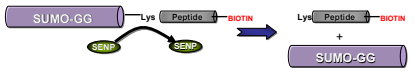
Figure 6A. Modification of E2-[illegible] with SUMO proteins. Reactions included SUMO E1(50nM), UbcH9 (500nM), SUMO (5µM), E2-25K (5µM) in buffer (50mM Hepes pH8.0, 100mM NaCl) and were incubated at 37°C for 60 minutes. Products were visualized by 16% SDS-PAGE.
Figure 6B. Deconjugation of E225K*SUMO protein conjugates by SENP1 and SENP2. E2-25K was modified with SUMO proteins using SUMO-1, SUMO-2 and SUMO-3 conjugation kits (K-710, K-715, K-720). SENP (500nM) was incubated with proteins for 60 minutes at 37°C. Products were visualized by 16% SDS-PAGE.

Conclusion: E2-25K is modified with a single SUMO (all isoforms) in the presence of SUMO-specific conjugating E1 and E2 enzymes. Approximately 80% of the E2-25K is modified under the reaction conditions described. Both SENP1 and SENP2 enzymes completely hydrolyze the E2-25K-SUMO conjugates to regenerate the E2 enzyme and SUMO protein.

Pichler A., *et al.* (2005) Nat. Struct. Mol. Biol. 12:264-269. Hoege C., *et al.* (2001) Nature 419:135-141

SENP SUBSTRATE: MONO-SUMOylated PEPTIDE

SUMO Peptide Conjugates (Isopeptide linkage cleavage)



Small peptides that contain the SUMOylation consensus sequence (yKxE) can be modified by all SUMO isoforms in the presence of SUMO conjugating enzymes. The resulting SUMO:peptide complex contains a bona fide isopeptide linkage with the C-terminus of SUMO covalently conjugated to a lysine residue on the peptide. In this case the peptide is biotinylated which facilitates the sensitive detection of complex cleaved by SENP enzymes.

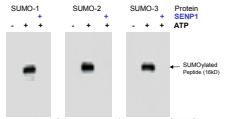


Figure 7. Western blot detection of consensus peptide (SP-300) modification by SUMO proteins and deconjugation by SENP1. The peptide containing the SUMOylation consensus motif was modified using SUMO Conjugation Kits (K-710, K-715, K-720). After conjugate formation, SENP (200nM) and DTT (5mM) were added and reaction incubated for 1hr at 37°C. Biotinylated peptide was detected with avidin-HRP.

Conclusion: A peptide containing the minimal SUMOylation consensus sequence is efficiently modified with a single SUMO-1, SUMO-2 or SUMO-3 protein in the presence of SUMO E1 and E2 conjugating enzymes in the presence of ATP. This covalent peptide-SUMO conjugate is a SENP substrate and allows for convenient detection via the biotin group on the peptide.

SUMMARY

- There are seven human SENP enzymes (SENP-1, -2, -3, -5, -6, -7, -8) that have been identified but their exact biochemical specificities have not yet been fully elucidated. There are several SENP substrates available to study endopeptidase or isopeptidase activities of known or newly identified enzymes.
- SENP endopeptidase activity can be studied kinetically with fluorescent substrates such as SUMO-AMC or SUMO-AFC. These substrates can be used for inhibitor identification of SENP enzymes in high-throughput assay formats. SENP activity can be effectively inhibited by both SUMO-aldehyde and SUMO-vinylsulfone derivatives. SENP enzymes show high specificity for SUMO since they do not hydrolyze other UBL-AMC derivatives, and are similarly not inhibited by aldehyde derivatives of ubiquitin and NEDD8.
- SUMO precursor proteins processing by SENP enzyme to produce mature forms also depends on endopeptidase activity. It has been shown that SENPs discriminate between the SUMO isoforms based on the primary structure of the substrate C-terminus[1]. It is also proposed that SENP1 is the most common SUMO endopeptidase *in vivo* since it has high activity and widespread tissue distribution.
- SUMO chains are obviously *in vivo* substrates for SENPs, and highly purified versions are also efficient *in vitro* substrates to monitor isopeptidase activity.
- Additionally, SENPs can also process a small peptide that is mono-SUMOylated indicating that substrate recognition and activity isn't dependent on the presence of full-length SUMO. Mono-SUMOylated E2-25K is also a SENP substrate even though in this case, the SUMO is attached to a lysine residue not located within the canonical consensus motif. It is not known if the recognition and cleavage mechanism of this isopeptide bond is similar to those of substrates with SUMO attached via the traditional ΨKxE motif. There are potentially other/novel classes of SUMOylation sites which might influence SENP activity and substrate preference.
- Using a complete panel of different SENP substrates is important to fully elucidate the deSUMOylation specificities of the SENP enzyme family.

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