Monitoring Flux Through the SUMOylation Cascade Using the Transcreener® AMP/GMP Assay

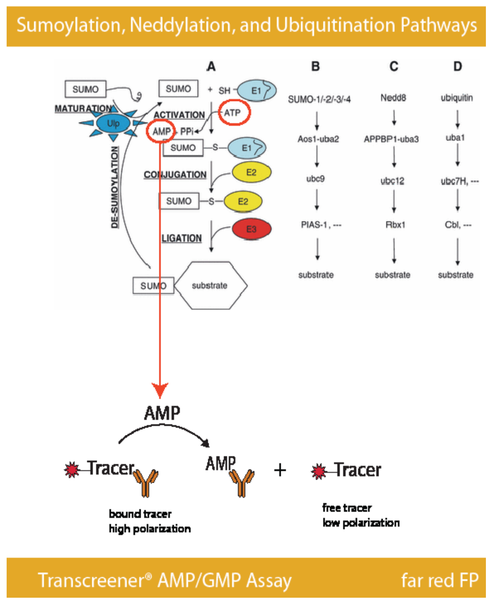
Meera Kumar, Matt Staeben, Robert G. Lowery and Karen M. Kleman-Leyer
BellBrook Labs, Madison, Wisconsin, USA 53711

Introduction

Conjugation of SUMO-1 to target protein substrates requires an E1-activating enzyme (SAE1/SAE2), an E2-conjugating enzyme (UbC9), and in certain cases a SUMO E3 ligase. E3 ligases have been associated with substrate recognition and localization *in vivo*, but their mechanism of action is still being elucidated. Because of the complexity of the system, there are few biochemical assays available for studying SUMOylation cascades. Those assays that have been developed are specific for a given E3/target protein pair. In the first step in the SUMO cascade, E1 utilizes the phosphodiester bond of ATP to drive the formation of a thioester bond between SUMO and a reactive cysteine, releasing AMP in the process. Here we used the Transcreener AMP/GMP Assay, which enables homogeneous immunodetection of AMP, for following protein SUMOylation events. This generic competitive fluorescence polarization assay, which utilizes a selective AMP/GMP antibody and a red-shifted nucleotide tracer, was used to detect AMP formation by SAE1/SAE2 alone or in combination with Ubc9. We next studied the effect of nucleoporin RanBP2 on this SUMOylation system. We demonstrated that in the presence of SAE1/SAE2 and Ubc9 this E3-like ligase undergoes poly/multisumoylation. We also showed that PYR41, a known inhibitor of ubiquitin activating enzyme UBE1, inhibited SAE1/SAE2 in a dose-dependency experiment. Overall, these studies demonstrate the concept of using AMP formation by E1 as a method to assess flux through the SUMOylation cascade and suggest that the Transcreener AMP/GMP Assay could be used to study similar protein modifications, including ubiquitination, isgylation, and neddylation.

Figure 1.

AMP Detection with the Transcreener® AMP/GMP FP Assay



Overview of sumoylation, neddylation and ubiquitination reactions. (A) The sumoylation pathway. Free SUMO is generated by either proteolytic maturation of a SUMO precursor or de-sumoylation of a sumoylated substrate by Ulp/SENPs. For conjugation, SUMO is firstly activated in an ATP-dependent reaction to form a thioester bond with the activating enzyme E1, then transferred to the conjugation enzyme E2, and finally conjugated to a substrate that is recruited by a ligase E3. (B–D) Comparison of the enzyme cascades involved in sumoylation, neddylation and ubiquitination reactions. (A) and (B) are modified from [1], and (C) and (D) from [29] and [7], respectively. (Figure and legend are from Tang Z. et al. 2008. FEBS Journal 275: 3003-3015).

The Transcreener assay platform relies on homogenous immunodetection of nucleotide products, enabling robust, generic detection of entire families of enzyme targets. This competitive , fluorescence polarization(FP), high throughput screening assay relys on highly selective antibodies that distinguish between nucleotides on the basis of a single phosphate group. With the Transcreener AMP/GMP assay, AMP generated during an ATP-utilizing enzyme reaction displaces a fluorescently-labeled nucleotide tracer from the bound antibody causing a decrease in the polarization signal. Here, we demonstrate the robust and simple detection of AMP during the activation of the Sumo E1-activating enzyme, SAE1/SAE2.

Figure 2.

AMP Formation by SAE1/SAE2 (E1-activating enzyme) is SUMO-dependent

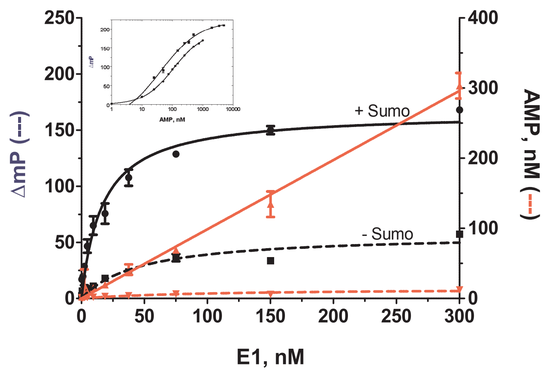


Table 1. Stoichiometric production of AMP by E1

E1, nM	Experimental AMP, nM	Theoretical AMP, nM
150	134 (±8.6)	150
75	69 (±4.2)	75
37.5	41 (±7.4)	37.5
18.7	19 (±4.1)	18.7
9.3	8 (±3.1)	9.3

Eq.1 $E1 + ATP + Sumo \longrightarrow E1_{\text{-AMP-Sumo}} + PPi$

Eq.2 $E1_{\text{-AMP-Sumo}} \longrightarrow E1_{\text{-S-CO-Sumo}} + AMP$

Eq.3 $E1_{\text{-S-CO-Sumo}} + ATP + Sumo \longrightarrow E1^{\text{-AMP-Sumo}}_{\text{-S-CO-Sumo}} + PPi$

An increase in the millipolarization shift (ΔmP) is observed with increasing E1 in the presence of SUMO. Converting the mP values to µM AMP using a standard curve prepared with ATP/AMP samples (inset) revealed a production of 1 mole of AMP per mole E1 enzyme when the reaction was allowed to proceed to completion (Table 1). This is in agreement with the proposed mechanism of E1 activation by SUMO and ATP (Eq. 1 and 2). The SUMO E1-activating reactions (10 µL) contained 5 µM SUMO and 5 µM ATP and were performed at 25°C for 24 hours and stopped by adding an equal volume of 20 mM EDTA, 4 nM tracer and 26 µg/mL antibody. The control reactions were performed under similar conditions without SUMO. Error bars represent the standard error of the mean mP shift (n=3).

Figure 3.

AMP production by E1 is enhanced by E2 and E2 + E3

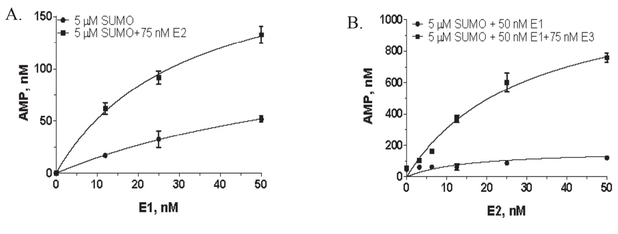


Table 2. AMP production by E1+E2 and E1 + E2 + E3

E2, nM	Experimental + 50 nM E1 AMP, nM	Theoretical* + 50 nM E1 AMP, nM	Experimental + 50 nM E1 + 75 nM E3 AMP, nM	Theoretical* +50 nM E1+ 75 nM E3 AMP, nM
50	119 (±7.2)	100	758 (±4.8)	175
25	86 (±4.3)	75	600 (±6.2)	150
12.5	61 (±5.4)	62.5	368 (±7.1)	137.5
0	47 (±3.4)	50	53 (±1.4)	50

* Theoretical values based on one SUMO site per enzyme

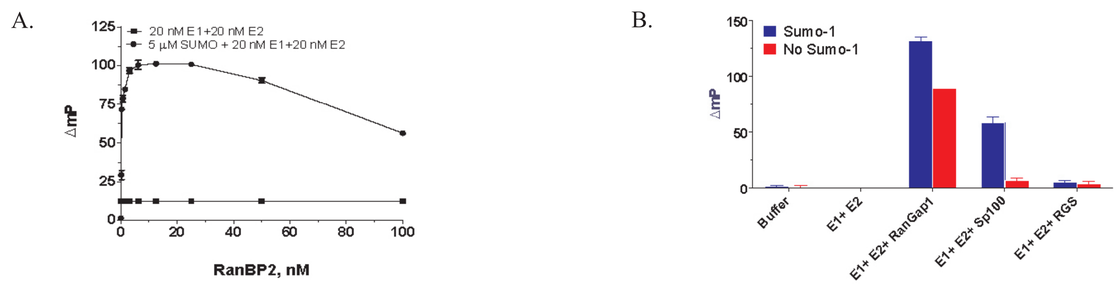
Eq.4 $E1^{\text{-AMP-Sumo}}_{\text{-S-CO-Sumo}} + E2_{\text{-SH}} \text{ ----> } E2_{\text{-S-CO-Sumo}} + E1^{\text{-AMP-Sumo}}$

Eq.5 $E2_{\text{-S-CO-Sumo}} + RanBP2_{\text{-NH2}} \longrightarrow E2_{\text{-SH}} + RanBP2_{\text{-NH-CO-Sumo}}$

AMP production is enhanced when E2 (A) and E2 + E3 (B) is added to E1 in the presence of SUMO. Based on the proposed model of E1 activation (Eq. 4, 5), the predicted amount of AMP is produced by E1 in the presence of 50 nM E2, but not with 50 nM E2 + 75 nM E3 (> 4-fold AMP is generated vs. the theoretical values (Table 2)). This suggests that E3 has multiple SUMOylation sites or is polySUMOylated. A) E1 was serially titrated in a 10 µL reaction containing 5 µM SUMO and 5 µM ATP, with or without 75 nM E2(Ubc9). B) E2 was serially titrated in a sumoylation reaction containing 5 µM SUMO, 5 µM ATP, and 50 nM E1 in the presence or absence of 75 nM RanBP2, an E3 ligase. All reactions (n=3) were run at 25°C for 24 hours and stopped by adding an equal volume of 20 mM EDTA, 4 nM tracer and 26 µg/mL antibody.

Figure 4.

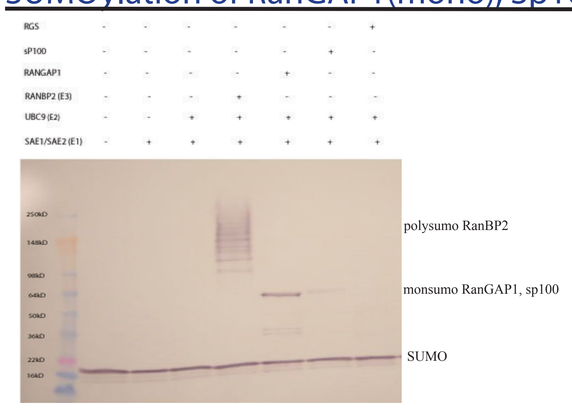
AMP Generation During the SUMOylation of E3 ligase (RanBP2) and other proteins



A) AMP is produced by E1 when E3 ligase, RanBP2, isSUMOylated with E2. RanBP2 was titrated in the presence of 5 µM SUMO, 5 µM ATP, 20 nM E1 and 20 nM E2 and the reactions were incubated at 25°C for 5 hours before adding an equal volume of EDTA (20 mM), tracer (4 nM) and AMP antibody (26 µg/mL). B) AMP is produced by E1 when E2 conjugating enzyme alone SUMOylates RanGap1 and Sp100, but not RGS. The SUMOylation reactions were performed under the same SUMOylation conditions in A) but in the presence of 2 µM RanGap-1, 2 µM sP100, or 1 µM RGS instead of RanBP2 (n=3).

Figure 5.

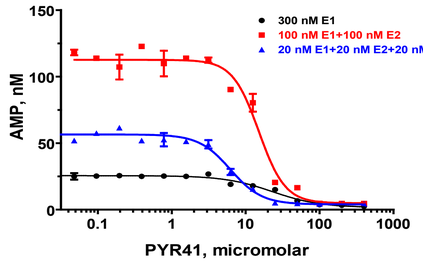
SUMOylation of RanGAP1(mono), Sp100(mono), and RanBP2(poly) with E1 and E2



Western blot analysis of E3 ligase, RanBP2 (100 nM), RanGap1 (1 µM), sP100 (1 µM) and RGS (1 µM) probed with an anti-SUMO antibody. Reactions were prepared with 50 nM E1, 50 nM E2, 5 mM ATP and 5µM SUMO in a 20 µL reaction volume at 37°C for 30 minutes. Proteins were resolved by SDS-PAGE in a 4-12% polyacrylamide gel followed by western blotting with an anti-SUMO antibody. The positions of SUMOylated products are indicated on the right edge of the panel. Molecular weight protein standards are labeled on the left side of the immunoblot.

Figure 6.

Dose-dependency Inhibition of E1 with compound PYR41



Identification of an SUMO E1 inhibitor, PYR41, using the Transcreener AMP/GMP assay. Deconvolution of SUMO cascade in identifying the E1 inhibitor was carried out by serially titrating PYR 41 in the presence of 1) 300 nM E1, 5 µM SUMO and 5 µM ATP (black); IC_{50} = 22 µM, 2) 100 nM E1, 100 nM E2, 5 µM SUMO and 5 µM ATP (red); IC_{50} = 15 µM and 3) 20 nM E1, 20 nM E2, 20 nM E3, 5 µM SUMO and 5 µM ATP (blue); IC_{50} = 6 µM. Reactions were performed at 25°C for five hours and stopped by adding 20 mM EDTA, 4 nM tracer and 26 µg/mL of AMP antibody.

Summary

- SUMO-dependent AMP formation by E1 activating enzyme, SAE1/SAE2, can be detected using the Transcreener®AMP/GMP Assay.
- An enhancement of AMP production by E1 was seen in the presence of E2 (Ubc9) and E2 + E3 (RanBP2).
- E2 can SUMOylate target proteins such as RanGAP1 and Sp100 *in vitro* without E3 ligase.
- E3 ligase is polySUMOylated in the presence of E1 and E2.
- PYR41, a known ubiquitin E1 inhibitor, was also shown to inhibit SUMO-activating enzyme E1(SAE1/SAE2).

Materials and Methods

SUMO Activating Enzyme E1 (SAE1/SAE2), SUMO Conjugating Enzyme E2 (Ubc9), E3 ligase (RanBP2), SUMO-1, sp100, and RanGAP1 were purchased from Boston Biochem (Cambridge, MA) or Biomol (Plymouth Meeting, PA). Anti SUMO-1 antibody (AM1200a) was purchased from Abgent (San Diego, CA). PYR41 (UBE1-41) was purchased from Biogenova (Boston, MA). SUMOylation reactions were performed in 10 µL: 5 µL of 2X enzyme mix consisting of 50 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 2 mM EGTA, 1% DMSO, and 0.02% Brij-35 in the presence or absence of 10 µM SUMO and 5 µl of 10 µM ATP. Reactions were initiated with the addition of ATP and were incubated at 25°C for 4-24 hrs ensuring that the reactions were in the linear phase (< 10% ATP conversion). AMP was then detected by adding 10 µL of the Transcreener®AMP/GMP assay reagents consisting of 4 nM AMP/GMP Alexa®Fluor 633 tracer, 9 µg/mL of AMP/GMP Antibody in 50 mM HEPES and 0.02% Brij-35. The final 20 µl assays were performed in black Corning 384-well, round bottom plates (catalog # 3676) and were allowed to equilibrate for 1 hour before reading the plates on a Tecan Safire^{2TM} multiplate reader (Ex_{635} and Em_{670}). The data were fit to a variable slope, sigmoidal dose response curve or fit using Michaelis Menton equation using GraphPad Prism software.

References:

Pichler, A., Gast, A., Seeler J., Dejean, A. and Melchior, F. The Nucleoporin RanBP2 has Sumo1 E3 Ligase Activity. Cell, 2002, 108:109-120.
Bernier-Villamor V, S.D., Matunis MJ, Lima CD., Structural basis for E2-mediated SUMO conjugation revealed by a complex between ubiquitin-conjugating enzyme Ubc9 and RanGAP1. Cell, 2002. 108(3): p. 345-56.
Desterro JM, Rodriguez, M. S., Kemp GD, Hay RT., Identification of the enzyme required for activation of the small ubiquitin-like protein SUMO-1. J Biol Chem, 1999. 274(15): p. 10618-24.
Gocke CB, Y.H., Kang J., Systematic identification and analysis of mammalian small ubiquitin-like modifier substrates. J Biol Chem, 2005. 280(6): 5004-12.
Guo B, Y.S., Witty J, Sharrocks AD., Signalling pathways and the regulation of SUMO modification.. Biochem Soc Trans, 2007. 35(Pt 6):1414-8.
Tang, Z., Hecker, C.M., Scheschonka, A. and Heinrich, B. Protein interactions in the sumoylation cascade-lessons from X-ray structures. FEBS Journal, 275 (2008) 3003-3015

www.BellBrookLabs.com