Construction of a mouse Aos1-Uba2 chimeric SUMO-E1 enzyme, mAU, and its expression in baculovirus-insect cells

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SUMO (small ubiquitin-related modifier) is a highly conserved protein that is covalently attached to target proteins. This posttranslational modification, designated SUMOylation, is a major protein-conjugation-driven strategy designed to regulate structure and function of cellular proteins. SUMOylation consists of an enzymatic cascade involving the E1-activating enzyme and the E2-conjugating enzyme. The SUMO-E1 enzyme consists of two subunits, a heterodimer of activation of Smt3p 1 (Aos1) and ubiquitin activating enzyme 2 (Uba2), which resembles the N- and C-terminal halves of ubiquitin E1 (Uba1). Herein, we describe the rational design of a single polypeptide version of SUMO-E1, a chimera of mouse Aos1 and Uba2 subunits, termed mAU, in which the functional domains appear to be arranged in a fashion similar to Uba1. We also describe the construction of a mAU plasmid for expression in a baculovirus-insect cell system and present an in situ SUMOylation assay using the recombinant mAU. Our results showed that mAU has SUMO-E1 activity, thereby indicating that mAU can be expressed in baculovirus-insect cells and represents a suitable source of SUMO-E1.

Introduction

Ubiquitin is a 76 amino acid residue protein, which possesses the typical “β-grasp” or “ubiquitin-fold” structure, wherein four β-sheets pack around a central α-helix. Ubiquitin was first described as a “heat-stable polypeptide of the ATP dependent proteolytic system”. It was subsequently found to function as a posttranslational protein modifier, which covalently attached to target proteins via an isopeptide linkage between the carboxyl terminus of ubiquitin and the ε-amino group of lysines on target proteins, thereby regulating protein stability and function. Posttranslational modification by ubiquitin, called ubiquitinylation, plays important roles in regulating a wide variety of cellular signals involved in cell growth, differentiation, and death. When the nuclear pore targeting of Ran GTPase activating enzyme 1 (RanGAP1) via specific interaction with the nuclear pore component, Ran-binding protein 2/358 kDa nuclear pore protein (RanBP2/Nup358), was first investigated nearly 15 years ago, it became clear that protein modification included a ubiquitin-like modifier, a ~100 residue polypeptide, termed SUMO (small ubiquitin-related modifier). SUMO possesses the typical “β-grasp” or “ubiquitin-fold” structure and covalently attaches to target proteins in a manner similar to ubiquitin, a process referred to as SUMOylation. Like ubiquitinylation, SUMOylation plays a role in regulating many cellular processes, including in genetic and/or epigenetic regulation, cell structural maintenance, cell signaling, and cell cycle progression.

In this paper, we summarize the enzymatic cascade of the SUMOylation reaction and structure of the enzymes involved in SUMOylation. In particular,
we focus on the SUMO-activating enzyme E1, the first enzyme required for initiating the SUMOylation cascade. To reduce the number of recombinant proteins required for the in vitro SUMOylation reaction and to eliminate formation of the heterodimer of the two subunits of the SUMO-E1 enzyme, we generated a linear fusion protein consisting of the two subunits and expressed this construct in a baculovirus-insect cell system. The genetic manipulation and heterologous expression of a functional linear-type of SUMO-E1 should aid researchers interested in characterizing the SUMO pathway, as well as help to bioengineer E1 enzymes involved in other SUMO/ubiquitin-like conjugation pathways.

**Enzymatic Mechanism and Structure of SUMO-E1**

SUMOylation is an important mechanism involved in regulating protein structure and function, and therefore should be under stringent enzymatic control. SUMOylation involves an enzymatic cascade composed of at least two components. Initially, SUMOylation requires activation by the E1-activating enzyme, a heterodimer of activation of Smt3p 1 (Aos1) and the ubiquitin activating enzyme 2 (Uba2) (Fig. 1A). The E1 enzyme catalyzes the formation of a thioester-linked complex between SUMO and the E2 enzyme. This process is initiated by activation of the carboxyl terminus of SUMO by adenylation, followed by a thioesterification reaction in which SUMO is conjugated to a cysteine residue at the active site of Uba2 in the E1 enzyme. SUMO is then transferred to the active site cysteine of the E2 enzyme, Ubc9, via a trans-thioesterification reaction. A SUMO-charged E2 enzyme and substrate are finally bound with or without the assistance of a distinct class of SUMO E3-ligases, resulting in the activated SUMO bound to the substrate through an isopeptide linkage. In many cases, SUMO-E1 and -E2 enzymes are sufficient to conjugate SUMO to substrate proteins, which is in contrast to the essential requirement of three components, E1, E2, and E3, in the ubiquitin conjugation reaction.

The SUMO-E1 enzyme of the Aos1-Uba2 heterodimer was originally discovered in the yeast *Saccharomyces cerevisiae.* Successive studies revealed the existence of a yeast homolog of Aos1 and Uba2 in humans, called SUMO activating enzyme 1 (Sae1) and Sae2. By comparing the amino acid sequences of the SUMO-E1 enzyme (Aos1-Uba2 heterodimer) with the ubiquitin-E1 enzyme (a single linear polypeptide Uba1), the similarities of Aos1 to the N-terminal and Uba2 to the C-terminal halves of Uba1 were observed (Fig. 1B). The crystal structures of human SUMO-E1 and that of the partly resolved mouse Uba1 showed that both E1 enzymes possess three modular domains. The first ThiF-like domain of SUMO-E1 contributes
to adenylation and is composed of Aos1 and the N-terminal portion of Uba2. ThiF is an enzyme required for the thiamine biosynthetic pathway in *Escherichia coli*. The second cysteine-containing domain (CCD) contains an enzymatic active cysteine residue that is responsible for thioesterification. The carboxyl terminal ubiquitin-fold domain (Ufd) binds to SUMO-E2, Ubc9, and recruits SUMO-E2 to facilitate the transfer of the thioesterified SUMO to the E2 enzyme. It is predicted that the spatial order of these domains must be restricted and further implies that, during adenylation, thioesterification, and trans-thioesterification processes, these three domains in the E1 enzyme undergo dynamic remodeling and conformational changes in a regulated manner.

Currently, six kinds of proteins that show similarity to SUMO-E1 and ubiquitin-E1 enzymes have been identified and most have been recognized as ubiquitin-like proteins, such as NEDD8, ISG15, FAT10, URM-1, UFM-1, and ATG8/12. Among them, the two E1 enzymes for SUMO and NEDD8 consist of two subunits, whereby each subunit resembles the N- and C-terminal halves of ubiquitin E1. Since neither of the subunits alone show E1 activity, the heterodimeric structure of the SUMO- and NEDD8-E1 enzymes lends itself to several modes of regulation that would not be applicable for a linear-type E1, such as Uba1. However, the reason for this structural complexity and the physiological significance of the heterodimer formation of SUMO- and NEDD8-E1 enzymes remains unresolved.

**Construction of mAU and its Expression in a Baculovirus and/or Insect Cell System**

Although the biochemical and physiological function of heterodimer formation of the SUMO-E1 enzyme remains unresolved, we inferred that by preserving the three-domain structure commonly observed in E1 enzymes, SUMO-E1 may function without the requirement to dimerize. In other words, as long as the three domains were arranged properly in E1, two subunits of Aos1 and Uba2 could be fused into a single-linear polypeptide, as observed for ubiquitin-E1 Uba1: the domain order of chimeric Aos1 and Uba2, namely mAU, was seemingly similar to Uba1 (Fig. 1B). In addition, if mAU possessed SUMO-E1 activity, a reduction in the number of recombinant proteins required to perform the in vitro SUMOylation reaction would be observed, thereby making the experimental procedure simpler and more efficient. We also anticipated improvement in the folding efficiency of the linear fusion of mAU compared with the protein folding of the two subunits of Aos1 and Uba2, which are independently expressed and then assembled in the test tube.

To test whether a single-polypeptide version of SUMO-E1 has enzymatic activity, we designed a chimera consisting of the Aos1 and Uba2 subunits, and expressed the Aos1-Uba2 chimeric protein, mAU, in *Escherichia coli*. Briefly, cDNA fragments containing full-length mouse Aos1 and Uba2 were amplified by polymerase chain reaction (PCR), followed by ligation into the pGEX-KG vector (GE Healthcare). The resulting plasmid, pGEX-mAU, carried a Aos1-Uba2 fused fragment, in which the *EcoRI* site was present between the Aos1 and Uba2 fragments for purpose of ligating these two fragments. GST-mAU expressed in bacteria possessed SUMO-E1 activity and purification of the recombinant protein could be performed. However, the yield and purity of the GST-mAU expressed in bacteria was not satisfactory: less than 1 μg of GST-mAU was obtained with multiple degradation products from 100 ml of bacterial culture (Fig. 2C). Therefore, we started to generate a bacmid DNA vector for expression of mAU in a baculovirus and/or insect cell system with the intention to dramatically improve the yields of recombinant mAU. To this end, using pGEX-mAU as a template for PCR, the mAU fragment was amplified, followed by cloning into the NotI and Xbal sites of the pFastBac-HT-C vector (Fig. 2A; Invitrogen). The resulting plasmid was termed pFastBac-His mAU, and was used to express a His-mAU recombinant protein from pFastBac-His mAU (Fig. 2B). It should be noted that the *EcoRI* site was present as a linker sequence between the Aos1 and Uba2 fragments because of the template used in the PCR, as described above. We speculated that the linker ensured the sufficient flexibility of the ThiF-like domain to carry out the intra-molecular interactions required during activation of the SUMO molecule. However, it remains unclear as to whether there is a linker polypeptide more effective than the linker currently present in the His-mAU construct described herein.

Kanemaru and Saitoh reported that reasonable levels of the His-mAU recombinant protein were expressed in baculovirus-insect cells. The His-mAU recombinant protein expressed in baculovirus-insect cells was reasonably soluble and stable in an aqueous environment, and therefore could be purified by conventional affinity column chromatography: 1–2 mg of the 130 kDa protein was obtained from 100 ml SF9 cell culture and the 130-kDa His-mAU recombinant protein can be purified efficiently by conventional nickel affinity chromatography. When compared with GST-mAU expression in *E. coli*, the full-length His-mAU recombinant proteins were induced efficiently with less degraded products in baculovirus-insect cells (Fig. 2C). As shown in Figure 2D, the baculovirus-insect expressed mAU showed enzymatic activity of SUMO-E1, at least, in the in situ SUMOylation assay. The assay revealed enhanced nuclear rim staining, which represented an accumulation of GFP-SUMO-1 conjugated to the nuclear pore complex, when the digitonine-permeabilized and paraformaldehyde-fixed HeLa cells were treated with the SUMOylation buffer containing His-mAU. In contrast, GFP signals in the reaction containing the SUMOylation buffer without His-mAU exhibited negligible GFP signals. These data indicate that the His-mAU expressed in baculovirus-insect cells possesses SUMO-E1 enzymatic activity. According to our preliminary experiment, the enzyme activity of mAU produced from baculovirus was approximately the same as that produced from *E. coli*. However, more detailed in vitro SUMOylation experiments remain to be performed, including comparison of the kinetic properties.
parameters, such as $K_m$ and/or $K_d$ values, of recombinant mAU protein expressed in baculovirus-insect cells with the bacterially expressed mAU. Given that the mAU expressed in bacteria was 10-fold less active than the Aos1-Uba2 heterodimer expressed in bacteria,27 it should also be investigated whether the enzymatic activity of the recombinant His$_6$-mAU is equal to that of the baculovirus-insect cell-expressed Aos1-Uba2 heterodimer. For this purpose, we are preparing the recombinant Aos1-Uba2 heterodimer complex in a baculovirus-insect cell system.

**Conclusion**

We have described the rational design and construction of a recombinant chimera consisting of a linear fusion of Aos1 and Uba2. The resulting chimeric polypeptide, mAU, encompasses three domains functionally indispensable for E1 enzymatic activity. Since the yield of the recombinant mAU expressed in baculovirus-insect cells is reasonable, we highly recommend the baculovirus-insect cell system for expression of mAU. In addition, our strategy for bioengineering SUMO-E1 enzymes should be equally applicable to the E1 enzymes of other SUMO/ubiquitin family members.

**Disclosure of Potential Conflicts of Interest**

No potential conflict of interest was disclosed.
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References

1. Hershko A, Ciechanover A. The ubiquitin system. Annu Rev Biochem 1998; 67:425-79; PMID:9759494; http://dx.doi.org/10.1146/annurev.biochem.67.1.425

2. Ciechanover A, Hid Y, Hershko A. A heat-stable polypeptide component of an ATP-dependent proteolytic system from reticulocytes. 1978. Biochem Biophys Res Commun 2012; 425:565-70; PMID:22925675; http://dx.doi.org/10.1016/j.bbrc.2012.08.025

3. Mahajan R, Delphin C, Guan T, Gerace L. The ubiquitin-like protein Smr3p is activated for conjugation to other proteins by an Aos1p/Ubap2p heterodimer. EMBO J 1997; 16:5509-19; PMID:9312010; http://dx.doi.org/10.1093/emboj/16.18.5509

4. Saitoh H, Pu R, Cavenagh M, Dasso M. RanBP2 provides mechanistic insights into SUMO activation and E2 recruitment to E1. EMBO J 2005; 24:439-51; PMID:15660128; http://dx.doi.org/10.1038/sj.emboj.7600552

5. Saitoh H, Pu R, Cavenagh M, Dasso M. RanBP2 associates with Ubc9p and a modified form of RanGAP1. Proc Natl Acad Sci U S A 1997; 94:3736-41; PMID:9108084; http://dx.doi.org/10.1073/pnas.94.8.3736

6. Bayer P, Arnhold A, Metzger S, Mahajan R, Melchior F, Jaenicke R, Becker J. Structure determination of the small ubiquitin-related modifier SUMO-1. J Mol Biol 1998; 280:275-86; PMID:9654451; http://dx.doi.org/10.1006/jmbi.1998.1839

7. Tang Z, Hecker CM, Scheschonka A, Betz H. Protein interactions in the sumoylation cascade: lessons from X-ray structures. FEBS J 2008; 275:3003-15; PMID:18492068; http://dx.doi.org/10.1111/j.1742-4658.2008.06495.x

8. Geiss-Friedlander R, Melchior F. Concepts in sumoylation: a decade on. Nat Rev Mol Cell Biol 2007; 8:497-56; PMID:18005952; http://dx.doi.org/10.1038/nrm2295

9. Keister O. SUMO junction-what’s your function? New insights through SUMO-interacting motifs. EMBO Rep 2007; 8:550-5; PMID:17545995; http://dx.doi.org/10.1038/sj.emboj.7400980

10. Cuberas-Ports C, Matunis MJ. SUMO: a multifaceted modifier of chromatin structure and function. Dev Cell 2013; 24:1-12; PMID:23328396; http://dx.doi.org/10.1016/j.devcel.2012.11.020

11. Sarge KD, Park-Sarge OK. SUMO and its role in human diseases. Int Rev Cell Mol Biol 2011; 288:167-83; PMID:21482412; http://dx.doi.org/10.1016/B978-0-12-386041-5.00004-2

12. Johnson ES, Schwenkhorst I, Dohmen RJ, Blobel G. The ubiquitin-like protein Smr3p is activated for conjugation to other proteins by an Aos1p/Ubap2p heterodimer. EMBO J 1997; 16:5509-19; PMID:9312010; http://dx.doi.org/10.1093/emboj/16.18.5509

13. Okuma T, Honda R, Ichikawa G, Tsumagari N, Yasuda H. In vitro SUMO-1 modification requires two enzymatic steps, E1 and E2. Biochem Biophys Res Commun 1999; 254:693-8; PMID:9920803; http://dx.doi.org/10.1006.bbrc.1998.9995

14. Gong, E. Li, B. Milias S, Yeh E.T. Molecular cloning and characterization of human AOS1 and UBA2, components of the sentrin-activating enzyme complex. FEMS Lett 1999; 448:185-9; PMID:10217437; http://dx.doi.org/10.1111/j.1574-695X.2001.tb07167.x

15. Lois LM, Lima CD. Structures of the SUMO E1 provide mechanistic insights into SUMO activation and E2 recruitment to E1. EMBO J 2005; 24:439-51; PMID:15660128; http://dx.doi.org/10.1038/sj.emboj.7600552

16. Szczepanski RH, Filipek R, Bochtler M. Crystal structure of a fragment of mouse ubiquitin-activating enzyme. J Biol Chem 2005; 280:22206-11; PMID:15774460; http://dx.doi.org/10.1074/jbc.M502583200

17. Groettrup M, Pelzer C, Schmidke G, Hofmann K. Complementarity of the sulfur transfer system in thiamin biosynthesis. J Biol Chem 2005; 280:275-86; PMID:15653560; http://dx.doi.org/10.1074/jbc.2008.01.005

18. Lorencz S, Cantor AJ, Rape M, Kuriyan J. Macromolecular juggling by ubiquitylation enzymes. BMC Biol 2013; 11:65; PMID:23832333; http://dx.doi.org/10.1271/bbb.130070

19. O'Kuma T, Honda R, Ichikawa G, Tsumagari N, Yasuda H. In vitro SUMO-1 modification requires two enzymatic steps, E1 and E2. Biochem Biophys Res Commun 1999; 254:693-8; PMID:9920803; http://dx.doi.org/10.1006(bbrc.2012.08.025)

20. Furukawa K, Mizushima N, Noda T, Ohsumi Y. A protein conjugation system in yeast with homology to biosynthetic enzyme reaction of prokaryotes. J Biol Chem 2000; 275:7462-5; PMID:10713047; http://dx.doi.org/10.1074/jbc.275.11.7462

21. Komatsu M, Chiha T, Tatsuki S, Iemura S, Tanida I, Okazaki N, Ueno T, Komaminato E, Natsume T, Tanaka K. A novel protein-conjugating system for Ufm1, a ubiquitin-fold modifier. EMBO J 2004; 23:1977-86; PMID:15075706; http://dx.doi.org/10.1038/sj.emboj.7600205

22. Mizushima N, Noda T, Yoshimori T, Tanaka Y, Ishii T, George MD, Klononsky DJ, Osumi M, Ohsumi Y. A protein conjugation system essential for autophagy. Nature 1998; 395:395-9; PMID:9759731; http://dx.doi.org/10.1038/26060

23. Uchimura Y, Nakao M, Saitoh H. Generation of SUMO-1 modified proteins in E. coli: towards understanding the biochemistry/structural biology of the SUMO-1 pathway. FEBS Lett 2004; 564:85-90; PMID:15094046; http://dx.doi.org/10.1016/j.febslet.2003.11.011

24. Kanemaru A, Saitoh H. High-yield expression of mouse Aos1-Ub2a-fusion SUMO-activating enzyme, mAU, in a baculovirus-insect cell system. Biosk Biotechnol Biochem 2013; 77:1575-8; PMID:23832333; http://dx.doi.org/10.1271/bbb.130070

25. Saitoh N, Uchimura Y, Tachibana T, Sugahara S, Saitoh H, Nakao M. In situ SUMOylation analysis reveals a modulatory role of RanBP2 in the nuclear rim and PML bodies. Exp Cell Res 2006; 321:1418-30; PMID:16688858; http://dx.doi.org/10.1016/j.yexcr.2006.01.013

26. Muramatsu M, Uwada J, Matsumoto N, Saitoh H. A simple in situ cell-based sumoylation assay with potential application to drug screening. Biosci Biotechnol Biochem 2010; 74:1473-5; PMID:20622442; http://dx.doi.org/10.1271/bbb.100801