Generation of SUMO-1 modified proteins in *E. coli*: towards understanding the biochemistry/structural biology of the SUMO-1 pathway

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Abstract Here, we developed a binary vector system that introduces a synthetic SUMO-1 conjugation pathway into *Escherichia coli* and demonstrated that large amounts of sumoylated Ran GTPase activating protein 1 C-terminal region (RanGAP1-C2), Ran binding protein 2 internal repeat domain, p53 and promyelocytic leukemia were efficiently produced. The sumoylated recombinant RanGAP1-C2 appeared to retain the in vivo properties, since it was specifically sumoylated at lysine 517 as expected from in vivo studies. Our findings indicate the establishment of a biosynthetic route for producing large amounts of sumoylated recombinant proteins that will open up new avenues for studying the biochemical and structural aspects of the SUMO-1 modification pathway.

Key words: Posttranslational modification; SUMO-1; Ran GTPase activating protein 1; Ran binding protein 2; Promyelocytic leukemia; p53

1. Introduction

With the help of the E1-activating enzyme composed of a Aos1/Uba2 heterodimer and the E2-conjugating enzyme Ubc9 (ubiquitin conjugating enzyme 9), SUMO-1 (small ubiquitin-related modifier-1) becomes covalently attached to acceptor proteins [1–5]. This posttranslational modification is referred to as 'sumoylation'. Sumoylation is likely to constitute a new and important mechanism through which the structure and/or function of target proteins are regulated. However, little is known about the biochemical and structural differences between non-modified and sumoylated target proteins. This is due, in part, to the difficulties of producing large amounts of recombinant sumoylated proteins of interest in bacteria such as *Escherichia coli*.

Here, we describe the establishment of a binary vector system that allows simultaneous expression of SUMO-E1, SUMO-E2, SUMO-1 and an acceptor protein, leading to efficient sumoylation of the protein of interest in *E. coli*. Our data indicate that the bacterial expression/modification system is a powerful tool for the rational design and large-scale synthesis of a wide variety of sumoylated proteins, which might be invaluable for biochemical and structural studies of the SUMO-1 conjugation and de-conjugation pathways.

2. Materials and methods

2.1. DNA constructs

Mouse Aos1 and Uba2 were amplified by polymerase chain reaction (PCR) [5] using the following oligonucleotides: 5′-AGGGATCCCCATGGTAGAGAAGGAGGAGGCTGGC-3′ (coding strand of Aos1), 5′-AGGAATTCTCACTGGGGACCAAGGCACTC-3′ (anticoding strand of Aos1), 5′-AGGAATTCCCATGGCACTGTCGCGGGGGTTG-3′ (coding strand of Uba2), and 5′-AGTCTAGATCAGTCTAACGCTATGACGTCA-3′ (anticoding strand of Uba2). The underlined sequences represent *Bam*HI, *Eco*RI, and *Xba*I sites, respectively. The amplified Aos1 and Uba2 fragments were introduced into pGEX (Amersham-Pharmacia) and pET (Novagen) vectors, resulting in the formation of pGEX-Aos1, pGEX-Uba2, pET-(His)6-Aos1, and pET-(His)6-Uba2, respectively. To prepare an Aos1-Uba2 chimeric construct, the amplified fragments of Aos1 and Uba2 were digested with *Bam*HI+*Eco*RI and *Eco*RI+*Xba*I, respectively, and inserted into a *Bam*HI+*Xba*I-digested pGEX-KG vector. The resultant plasmid, pGEX-AU, encoded a glutathione *S*-transferase (GST)-Aos1-Uba2 fusion protein. The expression plasmids of *Xenopus* Ubc9, Ran GTPase activating protein 1 C-terminal region (RanGAP1-C2), human SUMO-1, Ran binding protein 2 internal repeat domain (RanBP2-IR), p53 and promyelocytic leukemia (PML) were described previously [5–8].

2.2. Construction of the binary vector system

The Ubc9-coding region fused to the T7 promoter was prepared by PCR using pT7-Ubc9 as a template. The fragment was digested with *Nhe*I and inserted into the *Xba*I site of pGEX-AU, resulting in the formation of the p-I plasmid that produces E1 and E2 enzymes under the control of Tac and T7 promoters, respectively. To create the p-II plasmid, a SUMO-1 fragment was amplified by PCR and inserted into the *Nde*I-*Hin*dIII site of pACYC-T7 vector [9], resulting in the formation of pACYC-SUMO-1. The RanGAP1-C2-coding region fused to (His)6 and T7 tag and the T7 promoter was amplified by PCR and inserted into the *Bgl*II site of pACYC-SUMO-1. The resulting plasmid, designated p-II(SUMO-1+RanGAP1-C2), generates (His)6-T7-tagged RanGAP1-C2 and SUMO-1 under the control of T7 promoters. The RanGAP1-C2 gene in the p-II plasmid can be replaced by any protein-coding sequence of interest for sumoylation in *E. coli*. Bacteria harboring both the p-I and p-II plasmids were selected by Luria–Bertani medium containing 100 mg/l ampicillin and 50 mg/l chloramphenicol.

2.3. Expression and purification of recombinant proteins

The pGEX, pET, and pACYC expression plasmids were introduced

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Abbreviations: SUMO-1, small ubiquitin-related modifier-1; Ubc9, ubiquitin conjugating enzyme 9; RanGAP1, Ran GTPase activating protein 1; RanBP2, Ran binding protein 2; PML, promyelocytic leukemia; SDS, sodium dodecyl sulfate; IPTG, isopropyl-β-D-thiogalactopyranoside; DTT, dithiothreitol

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into *E. coli* BL21(DE3) and expression of the recombinant proteins was induced with 0.2 mM isopropyl-β-D-thiogalactose (IPTG) at 25°C. Purification of GST fusion proteins and (His)6 fusion proteins was carried out as described previously [5,6,8]. The purified GST fusion proteins were incubated with thrombin to remove the GST moiety.

2.4. In vitro sumoylation assay

To prepare biotinylated SUMO-1, approximately 2.5 mg of GST-SUMO-1 was incubated with 1.2 mg of EZ-Link™PEO-maleimide-activated biotin (Pierce) followed by thrombin cleavage of the GST moiety. A typical in vitro sumoylation reaction was carried out in the reaction buffer containing 50 mM Tris–HCl, pH 7.6, 50 mM NaCl, 2 mM $MgCl_2$, 0.1 mM dithiothreitol (DTT), 5 mM ATP.

3. Results

3.1. AU activates SUMO-1 in vitro

Ubiquitin E1 (Uba1) enzyme is a single subunit protein. Several E1 enzymes for ubiquitin-like proteins, including SUMO-E1 (Aos1/Uba2 heterodimer), consist of two subunits that resemble the N- and C-terminal halves of ubiquitin E1 [3–5]. But the reason for this remains unclear. To mimic ubiquitin E1 and investigate whether a single peptide version of SUMO-E1 mediates the sumoylation reaction, we first designed a fused Aos1 and Uba2 subunit, designated AU, and prepared a bacterial expression plasmid followed by production of the recombinant GST-AU protein (Fig. 1A,B). We then tested whether GST-AU conferred the expected enzymatic activity in vitro. As shown in Fig. 1C, GST-AU formed a DTT-sensitive adduct with SUMO-1 and transferred SUMO-1 to Ubc9 in the presence of ATP. It was also demonstrated that GST-AU and Ubc9 together were able to sumoylate the C-terminal region of RanGAP1 (RanGAP1-C2 wt) in vitro (Fig. 1D). We further showed that SUMO-1 wild type (wt), but not SUMO-1G96 mutant, was conjugated to RanGAP1-C2 wt, and found that neither SUMO-1 nor SUMO-1G96 was conjugated to RanGAP1-C2 K517R mutant, in which the SUMO-1 acceptor lysine residue is mutated to arginine (Fig. 1D). These data indicate that the chimeric AU has the ability to activate the SUMO-1 conjugation pathway and catalyzes sumoylation with a similar specificity to the authentic Aos1/Uba2 heterodimer.

3.2. The recombinant AU is less active than Aos1/Una2 heterodimer

It should be noted that the assays shown in Fig. 1C,D are all endpoint, not rate, determinations and might not represent a meaningful test of enzymatic efficiency. Additionally, the E1 proteins used in these experiments were GST and (His)6 fusion proteins that may not be optimally active. Therefore, we removed the GST and (His)6 moieties from the fusion proteins (Fig. 2A) and compared AU with the Aos1/Uba2 heterodimer for its sumoylation efficiency of RanGAP1-C2, both by a time course and by titrating the amount needed. As

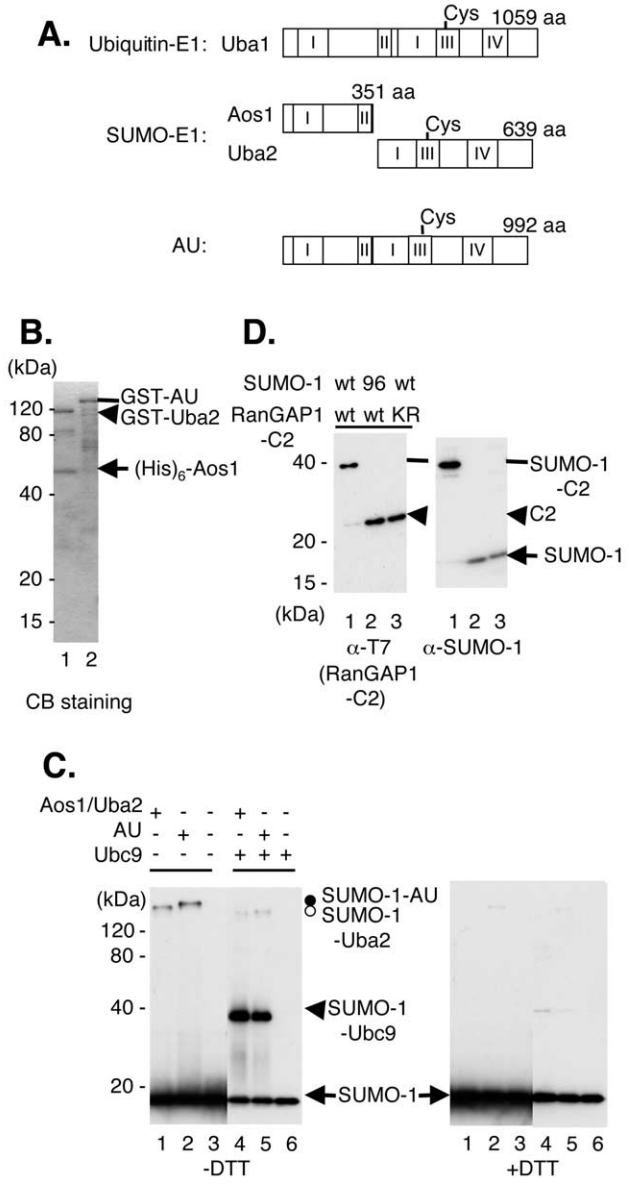

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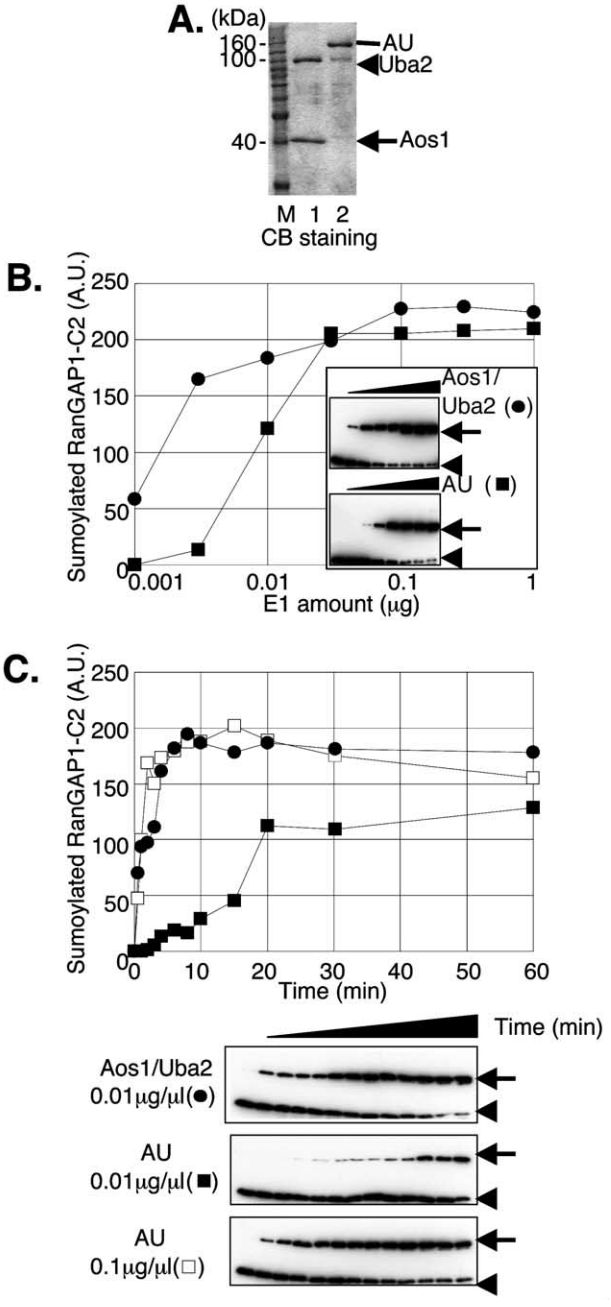
Fig. 1. The recombinant GST-AU protein functions as a SUMO-E1 enzyme. A: Schematic representation of mouse Uba1, Aos1, Uba2 and AU proteins [3,4]. B: Purification of recombinant GST-AU protein. Recombinant (His)6-Aos1 (arrow), GST-Uba2 (arrowhead) and GST-AU (line) proteins were expressed. The purified proteins (0.2 μg/lane) were separated in a 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel and stained with Coomassie blue. Protein size markers are indicated on the left. C: GST-AU activates SUMO-1. 0.1 μg of biotinylated SUMO-1 was incubated with 0.5 μg of purified (His)6-Aos1/GST-Uba2 or GST-AU, as indicated, in the presence of 5 mM ATP at 37°C for 20 min. The reaction mixtures were subjected to 12% SDS–PAGE in the absence (left panel) or presence (right panel) of DTT followed by immunoblotting analysis using avidin-conjugated horseradish peroxidase. Arrows indicate the position of free biotinylated SUMO-1. The arrowhead represents the position of SUMO-1-Ubc9. The dot shows the position of SUMO-1-GST-AU. The circle indicates the position of SUMO-1-GST-Uba2. D: SUMO-1 is conjugated to RanGAP1-C2 in vitro by GST-AU. The conjugation reactions were performed with 5 mM ATP, 0.1 μg of biotinylated wild-type SUMO-1, 0.1 μg of (His)6-Ubc9 and 0.5 μg of GST-AU (lane 1). To demonstrate the specificity of the reaction, inactive forms of biotinylated SUMO-1 (SUMO-1G96: 0.1 μg) and a RanGAP1-C2K517R mutant (RanGAP1-C2KR: 0.1 μg) were included in similar reactions (lanes 2 and 3). Reaction mixtures were fractionated by 12% SDS–PAGE followed by immunoblotting analyses using an anti-T7 antibody to detect RanGAP1-C2 (lanes 1–3, left panel) or an anti-SUMO-1 antibody (lanes 1–3, right panel). The arrow indicates the position of free biotinylated SUMO-1. The arrowhead shows the position of RanGAP1-C2. The thin line represents the position of sumoylated RanGAP1-C2.

shown in Fig. 2B,C, the Aos1/Uba2 heterodimer approximately 10-fold more efficiently sumoylated RanGAP1-C2 than AU, suggesting that AU may in fact be less active than the Aos1/Uba2 heterodimer. However, it should be noted that AU overcame the sumoylation rate when a 10-fold excess amount of AU was added to the reaction, indicating that AU efficiently mediates sumoylation if it is over-produced (Fig. 2C).

Collectively, the results indicate that AU functions as a SUMO-E1 enzyme and imply that the heterodimeric structure of the SUMO-E1 enzyme is not totally essential for the enzymatic activity per se. Since the linear fusion of Aos1 and Uba2 would not only negate the heterodimerization process of the two proteins but also eliminate the possibility of unbalanced production of the two proteins, we predicted that AU greatly facilitates the synthetic circuit of sumoylation and that the idea of simply fusing the Aos1 and Uba2 subunits would be neat for the *E. coli* expression/modification system described below.



3.3. *Biosynthesis of sumoylated RanGAP1-C2 in* E. coli

To produce sumoylated recombinant proteins through a biosynthetic pathway in *E. coli*, we next constructed a binary vector system by modifying pGEX and pACYC plasmids. The generated plasmids were designated p-I and p-II (Fig. 3A). Due to the plasmid compatibility, *E. coli* can be co-transformed with p-I and p-II to produce four foreign proteins, E1 (AU), E2 (Ubc9), SUMO-1 and RanGAP1-C2. In the p-I plasmid, AU is designed to be expressed as a GST fusion protein. Although the fusion of the GST moiety to the N-terminus at the AU protein appears to reduce its E1 activity (data not shown), we thought it is convenient to monitor and purify the expressed E1 protein in *E. coli*.

After *E. coli* BL21(DE3) was transformed with p-I and p-II(SUMO-1 wt+RanGAP1-C2 wt) plasmids, the bacteria were cultivated in the presence of IPTG and the total lysate was analyzed by immunoblotting (Fig. 3B). As expected, we found the expression of four foreign proteins as well as sumoylated RanGAP1-C2 around 40 kDa, suggesting that the SUMO conjugation pathway was reconstituted in *E. coli*. Comparing the band intensities between free SUMO-1 and the 40 kDa conjugated bands, ~90% of the total expressed SUMO-1 was subjected to sumoylation of RanGAP1-C2 (lane 1). On the other hand, ~90% of the total expressed RanGAP1-C2 appeared to be sumoylated (lane 9). Importantly, we hardly detected other proteins modified by SUMO-1 in the total bacterial lysate. These results indicate that the exogenously expressed SUMO-1 is selectively conjugated to the exogenously expressed RanGAP1-C2, but not to the bacterial host proteins (lane 1).

The sumoylated RanGAP1-C2 generated in the *E. coli* expression/modification system could be purified using Ni^{2+} beads (Fig. 3C). Using purified bovine serum albumin as a

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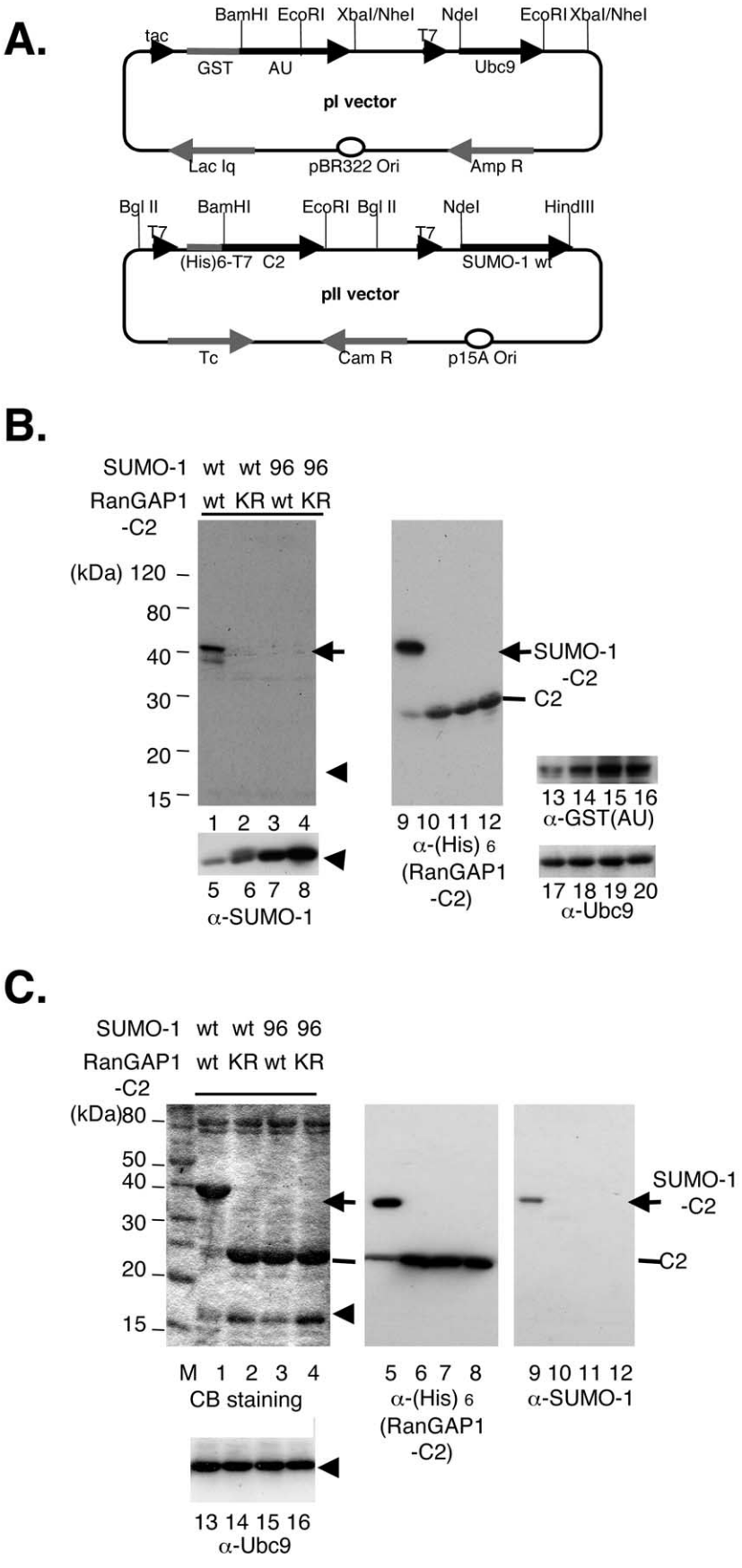
Fig. 2. AU is less active than the authentic Aos1/Uba2 heterodimer. A: Purification of recombinant AU protein. Recombinant GST-Aos1, (His)6-Uba2 and (His)6-AU proteins were incubated with thrombin to remove GST and (His)6 moieties from the fusion proteins. The purified proteins (0.2 μg/lane) were analyzed on a 12% SDS–PAGE gel followed by Coomassie blue staining. The positions of recombinant Aos1 (lane 1), Uba2 (lane 1) and AU (lane 2) proteins are indicated by arrow, arrowhead and line, respectively. Protein size markers are indicated on the left. B: AU sumoylates RanGAP1-C2 less efficiently than Aos1/Uba2 heterodimer does. Different amounts of either AU or Aos1/Uba2 complex as indicated were incubated with 0.1 μg of Ubc9, 0.1 μg of SUMO-1 and 0.1 μg of RanGAP1-C2 at 30°C for 10 min in the sumoylation buffer. The reaction mixture was then analyzed by immunoblotting using anti-T7 tag antibody to detect non-modified (arrowhead) and sumoylated (arrow) RanGAP1-C2 (shown in the inset). The signals of modified proteins in the each lane were quantified using the image analyzing system. A. U. represents arbitrary unit. C: Comparison of time-dependent sumoylation mediated by AU versus Aos1/Uba2 heterodimer. 0.01 μg of either AU or Aos1/Uba2 complex was incubated with 0.1 μg of Ubc9, 0.1 μg of SUMO-1 and 0.1 μg of RanGAP1-C2 at 30°C for 0, 0.5, 1, 3, 4, 6, 8, 10, 12, 15, 20, 30 or 60 min in the sumoylation buffer. The reaction mixture was then analyzed by immunoblotting using anti-T7 tag antibody to detect non-modified (arrowhead) and sumoylated (arrow) RanGAP1-C2. The signals of modified proteins in the each lane were quantified using the image analyzing system. A. U. represents arbitrary unit.


A.
BamHI EcoRI XbaI/NheI NdeI EcoRI XbaI/NheI
tac
T7
GST AU
Ubc9
pI vector
Lac Iq
pBR322 Ori
Amp R
Bgl II BamHI EcoRI Bgl II NdeI HindIII
T7
(His)6-T7 C2
SUMO-1 wt
pII vector
Tc
Cam R
p15A Ori
B.
SUMO-1 wt wt 96 96
RanGAP1 wt KR wt KR
-C2
(kDa) 120
80
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SUMO-1
-C2
C2
1 2 3 4
5 6 7 8
α-SUMO-1
9 10 11 12
α-(His) 6
(RanGAP1
-C2)
13 14 15 16
α-GST(AU)
17 18 19 20
α-Ubc9
C.
SUMO-1 wt wt 96 96
RanGAP1 wt KR wt KR
-C2
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-C2
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-C2)
9 10 11 12
α-SUMO-1
13 14 15 16
α-Ubc9

control, we determined the amount of purified sumoylated RanGAP1-C2 on a Coomassie blue-stained gel and estimated that the yield was approximately 2.5 mg from a 1 l culture (data not shown). It should be noted that Ubc9 was co-purified with sumoylated RanGAP1-C2 (Fig. 3C, lanes 1 and 13), suggesting that the functional interaction between these proteins was stably maintained in *E. coli* [10,11]. Importantly, we failed to detect the 40 kDa band in lysate from the bacteria expressing mutant SUMO-1 and/or mutant RanGAP1-C2 (Fig. 3B,C, lanes 2–4, 10–12), indicating that sumoylation in *E. coli* occurred specifically via an isopeptide bond between Lys517 of RanGAP1 and glycine at the C-terminus of SUMO-1 as predicted from in vivo studies [6,10,11].

3.4. Biosynthesis of sumoylated p53, PML and RanBP2-IR in E. coli

To demonstrate that the binary vector system is broadly applicable, we examined several proteins that have previously been shown to be sumoylated in vivo and/or in vitro, including the suppressor oncogene product, p53 [12,13], the nuclear domain protein, PML [14], and the cytoplasmic filament component of the nuclear pore complex, RanBP2 [6,15]. As shown in Fig. 4, (His)6-T7-p53, (His)6-T7-PML and (His)6-T7-RanBP2-IR were efficiently sumoylated in the *E. coli* expression/modification system, indicating that the system is applicable to a wide variety of SUMO-1 acceptor proteins. The yields of sumoylated p53, PML and RanBP2-IR were approximately 40 μg, 5.0 μg and 500 μg from 1 l of culture, respectively, suggesting that the efficiency of this system varies with substrate proteins. It should be noted that the SUMO-1 moiety of all the sumoylated recombinant proteins described above could be released by a SUMO-specific isopeptidase (Y. Uchimura, T. Nishida, and H. Saitoh, unpublished results), indicating that the conjugations were each mediated by an isopeptide-bond.

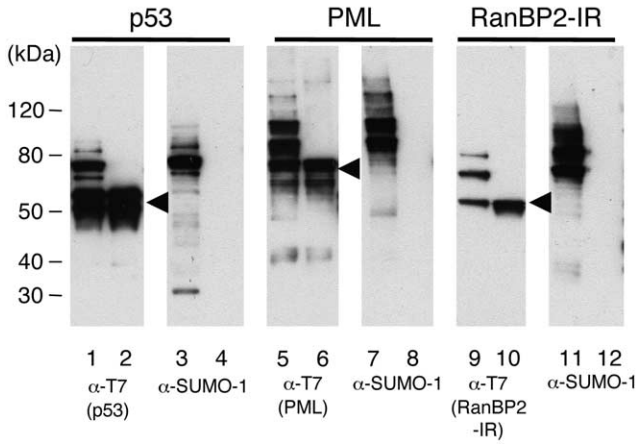

Fig. 4. RanBP2-IR, PML and p53 can be modified by SUMO-1 in *E. coli*. Total lysates from each bacterial culture were incubated with Ni^{2+} beads and the proteins bound to the beads were analyzed by SDS–PAGE followed by immunoblotting analyses using anti-T7 (lanes 1, 2, 5, 6, 9 and 10) or anti-SUMO-1 (lanes 3, 4, 7, 8, 11 and 12) antibodies. Lanes 1 and 3: p-I plus p-II(SUMO-1 wt+(His)6-T7-tagged p53), lanes 2 and 4: p-I plus p-II(SUMO-1G96+(His)6-T7-tagged p53), lanes 5 and 7: p-I plus p-II(SUMO-1 wt+(His)6-T7-tagged PML), lanes 6 and 8: p-I plus p-II(SUMO-1G96+(His)6-T7-tagged PML), lanes 9 and 11: p-I plus p-II(SUMO-1 wt+(His)6-T7-tagged RanBP2-IR), and lanes 10 and 11: p-I plus p-II(SUMO-1G96+(His)6-T7-tagged RanBP2-IR). Arrowheads indicate the positions of non-modified (His)6-T7-p53, (His)6-T7-PML and (His)6-T7-RanBP2-IR, respectively.

4. Discussion

Although an in vitro conjugation reaction is convenient for producing sumoylated recombinant proteins, it may prove difficult to sumoylate proteins on a large scale without a large amount of effort. In this report, we have demonstrated a novel and versatile system that can produce large amounts of functional sumoylated proteins of interest. To our knowledge, this study is the first demonstration that the introduction of the SUMO-1 conjugation system has little conflict with endogenous metabolic pathways and allows the production of large amounts of sumoylated proteins in *E. coli*.

In the process of developing this system, we revealed AU, a linear fusion product of Aos1 and Uba2, functions as the SUMO-E1 in vitro. This observation indicates that the heterodimeric structure of the SUMO-E1 enzyme is not essential for the E1 enzymatic activity per se. However, it implies that the Aos1/Uba2 heterodimer lends itself to several modes of regulation that would not be applicable for linear-type E1. It will be interesting to test whether overexpression of AU in mammalian cells causes uncontrolled sumoylation of cellular proteins, then influences the cell cycle progression and/or cellular differentiation.

Currently, we have succeeded in producing SUMO-2-conjugated proteins in *E. coli* using a similarly designed system (manuscript in preparation). We therefore believe that other isopeptide-bond-mediated protein–protein conjugation systems such as ubiquitinylation [16], neddylation [17], and Apg12 conjugation [18] could be introduced into *E. coli* by the binary vector system and that proteins modified by these respective modifiers could be produced in large amounts. However, it remains to be elucidated whether the protease(s) that cleaves the isopeptide bonds of ubiquitin, Nedd8, or Apg12 with the acceptor proteins is present in *E. coli*. If such an isopeptidase(s) is present in *E. coli*, then possible

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Fig. 3. Production of sumoylated RanGAP1-C2 in *E. coli*. A: Structures of the p-I(AU+Ubc9) and p-II(SUMO-1 wt+RanGAP1-C2 wt) plasmids. B: Sumoylation of RanGAP1-C2 at lysine 517 in *E. coli*. *E. coli* harboring the p-I and p-II plasmids carrying the wild-type and/or mutant SUMO-1/RanGAP1-C2 as indicated was cultured in the presence of IPTG. The total lysate was separated by 5–20% SDS–PAGE followed by immunoblotting analyses with anti-SUMO-1 (lanes 1–8), anti-(His)6 (lanes 9–12), anti-GST (lanes 13–16), or anti-Ubc9 (lanes 17–20) antibodies. Anti-(His)6 and anti-GST antibodies detected the expressed recombinant RanGAP1-C2 and GST-AU, respectively. Lanes 5–8 are longer-exposed images of lanes 1–4. Arrows indicate the position of sumoylated RanGAP1-C2. The line shows the position of non-modified RanGAP1-C2. Arrowheads represent the position of free SUMO-1. C: Expression and purification of sumoylated RanGAP1-C2. The total lysates from bacteria harboring the plasmids as in B were incubated with Ni^{2+} beads and the proteins associated with the beads were analyzed by 5–20% SDS–PAGE followed by Coomassie blue staining (lanes 1–4). The bound proteins were also analyzed by immunoblotting using anti-(His)6 (lanes 5–8), anti-SUMO-1 (lanes 9–12), or anti-Ubc9 (lanes 13–16) antibodies. Arrows indicate the position of sumoylated RanGAP1-C2. Thin lines show the position of non-modified RanGAP1-C2. Arrowheads represent the position of Ubc9.

reduction of the yield of the modified proteins would have to be considered.

It is obvious that sumoylation is important in controlling protein's interaction, stability and activity in eukaryotic cells and we are just starting to appreciate the SUMO-1 'code'. We believe that the *E. coli* expression/modification system described here will contribute to determining the complexity of the code, that is, the number of distinct sumoylated states that can be specified.

It would be interesting to determine the structures of sumoylated proteins using either X-ray crystallography or nuclear magnetic resonance spectroscopy. We are also interested in generating antibodies that could specifically discriminate modified from non-modified proteins in vivo using sumoylated recombinant proteins either as immunogens themselves or as proteins for screening.

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