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Enhanced SUMOylation of proteins containing a SUMO-interacting motif by SUMO-Ubc9 fusion

Eui Tae Kim [a], Kyeong Kyu Kim [a], Mike J. Matunis [b], Jin-Hyun Ahn [a,*]

[a] Department of Molecular Cell Biology, Samsung Biomedical Research Institute, Sungkyunkwan University School of Medicine, 300 Cheoncheondong Jangangu, Suwon, Gyeonggido 440-746, Republic of Korea
[b] Department of Biochemistry and Molecular Biology, Johns Hopkins University, Bloomberg School of Public Health, Baltimore, MD, USA

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ABSTRACT

Identifying new targets for SUMO and understanding the function of protein SUMOylation are largely limited by low level of SUMOylation. It was found recently that Ubc9, the SUMO E2 conjugating enzyme, is covalently modified by SUMO at a lysine 14 in the N-terminal alpha helix, and that SUMO-modified Ubc9 has enhanced conjugation activity for certain target proteins containing a SUMO-interacting motif (SIM). Here, we show that, compared to intact Ubc9, the SUMO-Ubc9 fusion protein has higher conjugating activity for SIM-containing targets such as Sp100 and human cytomegalovirus IE2. Assays using an IE2 SIM mutant revealed the requirement of SIM for the enhanced IE2 SUMOylation by SUMO-Ubc9. In pull-down assays with cell extracts, the SUMO-Ubc9 fusion protein bound to more diverse cellular proteins and interacted with some SIM-containing proteins with higher affinities than Ubc9. Therefore, the devised SUMO-Ubc9 fusion will be useful for identifying SIM-containing SUMO targets and producing SUMO-modified proteins.

Introduction

Covalent modification of proteins by small ubiquitin-like modifiers (SUMOs) causes changes in the intracellular localization and stability of proteins, and alters their ability to interact with other proteins and nucleic acids. In particular, these changes affect the functions of proteins involved in transcription, macromolecular transport, the maintenance of nuclear structure, nucleic acid DNA metabolism, cell signaling, and neurodegenerative disorders (for reviews, refer to [1–4]).

In the SUMOylation pathway, SUMO precursors are proteolytically processed at their C-terminal double-glycine motifs, activated by the formation of a thioester bond with the heteromeric E1 SUMO activation enzyme (SAE1/SAE2) in an ATP and Mg^{2+}-dependent reaction, and then transferred, again via a thioester bond, to E2 SUMO conjugation enzyme (Ubc9). SUMO is then transferred from Ubc9 to the lysine residues of substrates directly or with the help of E3 ligases [1]. Although several families of ubiquitin E3 ligases provide substrate specificity for ubiquitination, only a few mammalian SUMO E3 ligases, such as PIAS family members, RanBP2, and Pc2, have been reported [5–7]. Because Ubc9 is able to recognize and directly bind to substrates via a conserved motif, ψKXE/D (ψ, a bulky hydrophobic residue), which surrounds the modified lysine residues [8–11], both Ubc9 and E3 ligases appear to control substrate specificity for SUMOylation [1]. SUMOylation of proteins is reversibly regulated by a family of sentrin/SUMO-specific proteases that contain well-conserved residues of the catalytic triad (His, Asp, and Cys) [12,13]. In mammals, three SUMO proteins, SUMO-1, SUMO-2, and SUMO-3 have been identified, and SUMO-1 shows about 45% amino acid sequence identity to SUMO-2 and SUMO-3, which share approximately 95% sequence identity [14–18].

Identifying new SUMO targets and understanding the function of protein SUMOylation are largely limited by low level of SUMOylation. Recently, several studies have suggested that Ubc9 activity is regulated by direct interaction with SUMO or by its SUMOylation. SUMO was shown to bind directly to Ubc9 and this noncovalent interaction promoted the formation of SUMO chains [19,20]. In addition, many studies have suggested that SUMO-1 may be covalently conjugated to Ubc9 *in vitro* [21,22] and *in vivo* [23–26]. Knipscheer et al. recently reported that lysine 14 of Ubc9 is modified by SUMO-1, and that SUMO-modified Ubc9 has enhanced conjugating activity for certain targets that contain a SUMO-interacting motif (SIM) [27]. In this study, we further evaluated whether SUMO-Ubc9 fusion can be used to mimic SUMO-modified Ubc9. We found that, like the SUMO-modified form of Ubc9, the N-terminal SUMO fusion of Ubc9 enhances SUMOylation of proteins containing a SIM. Our data suggest that the SUMO-Ubc9 fusion construct may be useful for identifying or producing SUMO-modified proteins and for regulating the cellular SUMO pathway.

* Corresponding author. Fax: +82 31 299 6435.
E-mail address: jahn@med.skku.ac.kr (J.-H. Ahn).

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Materials and methods

Cell culture and transient DNA transfection. 293T and human foreskin fibroblast (HF) cells were grown and transfected as previously described [28].

Plasmids. Plasmids expressing GST-SAE2/SAE1 and His-Ubc9 have been described previously [29]. A pET-17b (Novagen)-based plasmid expressing T7-SUMO-1_{GG} was kindly provided by Ho Zoon Chae (Chonnam National University, Republic of Korea). Plasmids expressing flag-SUMO-1 and HA-IE2 were generated in a pSG5 background [30] and expression plasmid for myc-IE2 was generated on a background of pCS3-MT using Gateway technology (Invitrogen). Plasmids for GST-IE1, GST-PML VI, GST-IE2(135–289), and GST-Sp100A were generated in a pGEX-derived background also using Gateway technology [31]. Plasmid expressing GST-RanGAP1(NΔ419) has been previously described [11]. To generate plasmid expressing IE2 SIM mutant, the IVIS residues between 200 and 203 were replaced with A residues using the Stratagene QuickChange site-directed mutagenesis protocol. Ubc9 K14R mutant was generated using the same mutagenesis protocol. His-tagged SUMO-1-Ubc9(K14R) fusion was generated in a pDEST17 (Invitrogen) background by placing Ubc9 behind SUMO-1ΔGG, a truncated SUMO-1 without the six carboxyl-terminal amino acids including double-glycine residues. Plasmids for GFP-Ubc9 and GFP-SUMO-Ubc9 fusion constructs were also generated in a pDEST53 (Invitrogen) background by placing Ubc9 or SUMO-Ubc9 behind GFP using Gateway technology.

In vitro SUMOylation assays. Recombinant GST fusion proteins were expressed in *Escherichia coli*, and purified on glutathione–agarose 4B (Peptron) according to the manufacturer's instruments. His-tagged proteins were also produced in *E. coli,* and purified on Ni–NTA beads (Invitrogen) according to the manufacturer's procedure. T7-SUMO-1_{GG} was expressed in *E. coli* and purified on DEAE-Sephacel columns (Bio-Rad) and subsequent Sephacryl S-200 gel filtration chromatography (Amersham). Typical SUMOylation reactions were conducted in a 30 μl volume containing 70 nM GST-SAE2/SAE1, 0.1 ~ 3 μM His-Ubc9 or His-SUMO-Ubc9, and 9 μM SUMO-1_{GG} in buffer [50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, and 5 mM ATP]. Reaction mixes were incubated for 1 h at 37 °C. Reactions were terminated using SDS sample buffer containing β-mercaptoethanol, and reaction products were separated by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE).

Antibodies. Anti-His (H-3) and anti-GFP (B-2) mouse monoclonal antibody (MAb) conjugated with horseradish peroxidase (HRP), anti-Daxx rabbit PAb (M-112), and anti-GST MAb (B-14) were purchased from Santa Cruz. Anti-HA rat MAb (3F10) and anti-myc mouse MAb (9E10) conjugated with HRP were purchased from Roche. Anti-flag mouse MAb M2 was obtained from Sigma. Rabbit anti-PML PAb was described previously [29], anti-IE1 PAb was raised in rabbits using purified IE1 protein, and anti-Sp100 rabbit PAb was provided by Paul D. Ling (Baylor College of Medicine, Houston, TX, USA).

Immunoblot analysis. For SUMOylation assays in transfected cells, cells were washed with PBS containing 5 mM NEM, and samples were prepared by boiling cells in SDS loading buffer. The clarified cell extracts were then separated by SDS–PAGE followed by the standard procedure of immunoblotting [32].

Pull-down assays. HF cells (2×10^7) were lysed with cold PBS, 1% NP-40, 5% glycerol, and 1% complete protease inhibitor cocktail (Sigma). The cell extracts were pre-cleared with Ni–NTA beads, and incubated with 100 μg of His-Ubc9 or His-SUMO-Ubc9 fusion proteins immobilized to Ni–NTA agarose beads for 6 h at 4 °C. Beads were then washed with 20 mM Tris–HCl (pH8.0) containing 100 mM NaH_2PO_4 and 25 mM imidazole. The bound proteins were eluted with SDS loading buffer at 100 °C and analyzed by Coomassie Blue staining or immunoblotting analysis.

Results and discussion

The SUMO-Ubc9 fusion protein has a higher conjugating activity for substrates containing a SIM than wild-type Ubc9

It was recently suggested that the SUMO-modified form of Ubc9 has enhanced conjugating activity for certain SIM-containing targets [27]. Since the identified SUMOylation site lies close to the N-terminus, we asked whether the N-terminal SUMO fusion of Ubc9 also enhances the conjugating activity compared to intact Ubc9. We generated the SUMO-fused Ubc9(K14R) fusion construct (Fig. 1A). In this fusion protein, the original SUMO conjugation site (K14) of Ubc9 is abolished. Since the SUMO fused to the N-terminus of Ubc9 lacks double-glycine residues, this SUMO moiety is not processed by SUMO proteases. Both Ubc9 and SUMO-Ubc9 proteins were expressed in *E. coli* with a His tag at their N-termini, and partially purified for use in *in vitro* assays (Fig. 1B).

We first compared the conjugating activities of Ubc9 and SUMO-Ubc9 using SUMO substrates without a SIM, such as RanGAP1(NΔ419), IE1, and PML VI [33,34]. In *in vitro* SUMOylation assays using these substrates, the conjugating activities of wild-type Ubc9 and the SUMO-Ubc9 fusion were comparable (Fig. 2A). We next tested two other SUMO substrates such as Sp100A [27,35] and IE2 of HCMV [29], which contain a SIM. *In vitro* SUMOylation assays showed that the SUMO-Ubc9 fusion protein had greater conjugating activity for both Sp100A (by 2.5-fold) and IE2 (by 9-fold) than wild-type Ubc9 (Fig. 2B). This result suggests that the SUMO-Ubc9 fusion may functionally mimic the reported activity of the SUMO-modified form of Ubc9.

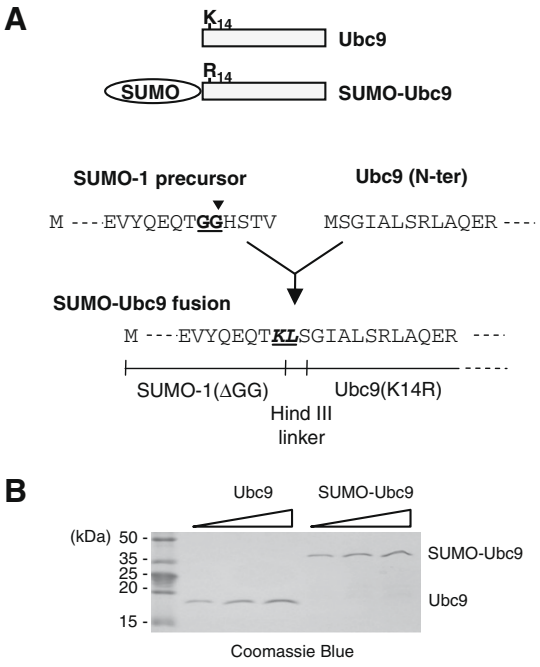

Fig. 1. The structure of the SUMO-Ubc9 fusion protein. (A) The structures of Ubc9 and SUMO-Ubc9 fusion proteins are shown. In the SUMO-Ubc9 fusion protein, the SUMO lacking the C-terminal six amino acids was fused to the N-terminus of Ubc9(K14R) through a linker of two amino acids (Lys and Leu). The normal processing site (after underlined double-Gly residues) on SUMO precursor is indicated as an arrowhead. (B) The amounts (0.5, 1, and 1.5 μg) of His-Ubc9 and the His-SUMO-Ubc9 construct used in the *in vitro* assays were compared by Coomassie Blue staining.

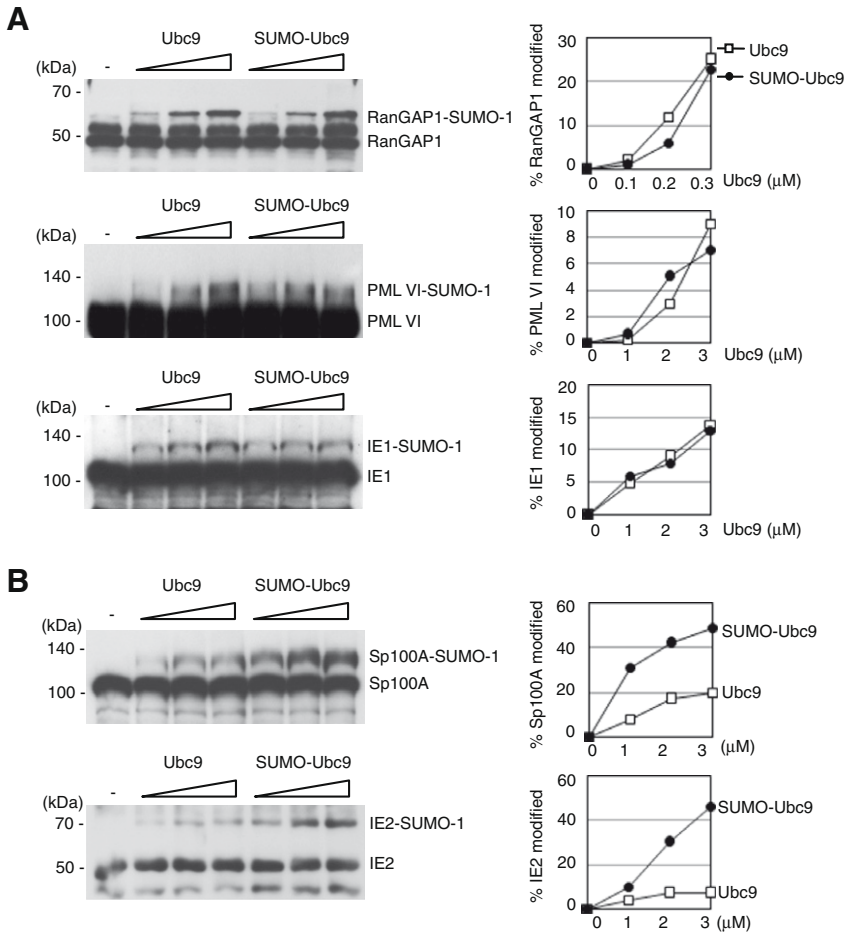

Fig. 2. Comparison of *in vitro* SUMO conjugating activities for diverse substrates between Ubc9 and SUMO-Ubc9 fusion. (A) *In vitro* SUMOylation reactions were conducted using GST-RanGAP1(NΔ419), GST-PML VI, or GST-IE1 with increasing amounts of His-Ubc9 or His-SUMO-Ubc9 (0.1, 0.2, and 0.3 μM for RanGAP1(NΔ419), or 1, 2, or 3 μM for PML VI or IE1). The reaction products were analyzed by SDS–PAGE (12% for RanGAP1(NΔ419) and 8% for PML VI or IE1) and immunoblotted with anti-GST (for RanGAP1), anti-PML, or anti-IE1 Ab. The amounts of unmodified and SUMO-modified proteins were quantified by counting using Scion Image software (Scion Corporation) and the % of protein modified over total amount of protein was indicated. (B) Similar *in vitro* SUMOylation assays were performed with GST-Sp100A or GST-IE2(135–289). The reaction products were analyzed by SDS–PAGE [8% for Sp100A and 12% for IE2(135–289)] and immunoblotted with anti-GST [for IE2(135–289)] or anti-Sp100 Abs. The amounts of SUMOylated proteins were quantitated as above.

Enhanced IE2 SUMOylation by the SUMO-Ubc9 fusion protein occurs in a SIM-dependent manner

To investigate whether the enhanced conjugating activity of the SUMO-Ubc9 fusion protein requires a SIM in target proteins, an IE2 SIM mutant was used. IE2 contains a SIM (IVISDSEEE from 200 to 208) that resembles one of the several proposed consensus sequences of a SIM, i.e., *h*-*h*-*X*-S-*X*-S/T-*a*-*a*-*a* (*h*, hydrophobic; *a*, acidic; *X*, any amino acid) [36]. We produced IE2(135–289), which contained the SUMOylation sites (K175 and K180) and a SIM [29], and its SIM mutant version, IE2(mSIM), which contained substitutions of four consecutive IVIS residues between 200 and 203 to A residues (Fig. 3A). In *in vitro* SUMOylation assays, the SUMO-Ubc9 construct efficiently modified wild-type IE2 (up to 40% of total IE2) but not SIM mutant (Fig. 3B, top). The SUMOylation level of the IE2 SIM mutant was not significantly increased by Ubc9 or the SUMO-Ubc9 construct (Fig. 3B, bottom). These results demonstrate that the enhanced conjugating activity of SUMO-Ubc9 for IE2 compared to wild-type Ubc9 depends on the presence of a SIM within IE2.

We also compared the conjugating activities of wild-type Ubc9 and SUMO-Ubc9 fusion protein in co-transfected cells. The results showed that the SUMO-Ubc9 fusion protein had higher conjugating activity for IE2 in transfected cells than wild-type Ubc9 (Fig. 3C, left), and this enhanced activity of the SUMO-Ubc9 construct was not detected for the IE2 SIM mutant (Fig. 3C, right). These results indicate that the enhanced SUMOylation of IE2 by the SUMO-Ubc9 fusion protein occurs in a SIM-dependent manner.

The SUMO-Ubc9 fusion protein more efficiently interacts with SIM-containing substrates than Ubc9

In an effort to evaluate whether the SUMO-Ubc9 fusion protein can be useful in identifying new SUMO substrates, we compared the binding patterns of wild-type Ubc9 and SUMO-Ubc9 fusion proteins to cellular proteins using pull-down assays. The results showed that SUMO-Ubc9 bound to more diverse cellular proteins than Ubc9 (Fig. 4A, left panel). Furthermore, the SUMO-Ubc9 fusion protein showed higher binding affinities for Sp100 proteins and Daxx, two known SIM-containing substrates [27,35] (Fig. 4A, right two panels). These data suggest that SUMO-Ubc9 may be useful in identifying new SUMO substrates, as well as in producing the SUMO-modified form of SIM-containing proteins.

Our results demonstrate that the SUMO-Ubc9 fusion protein has greater conjugating activity than wild-type Ubc9 for SIM-containing SUMO targets (Sp100 and IE2), whereas their activities were comparable for SUMO targets without a SIM (RanGAP1(NΔ419), PML VI, and IE1). Importantly, as in the case of

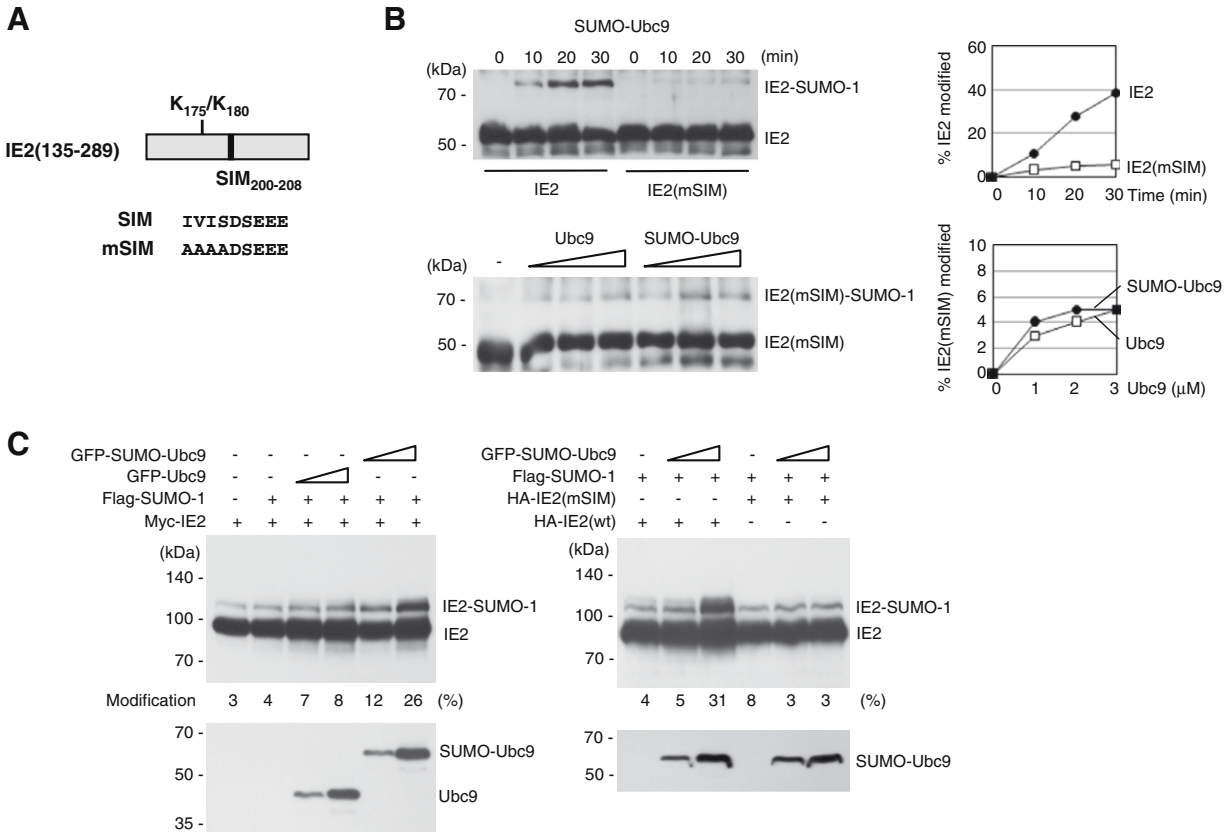

Fig. 3. SIM-dependent enhancement of IE2 SUMOylation by the SUMO-Ubc9 fusion construct. (A) The structures of IE2 containing the SUMOylation sites (K174 and K180) and an intact or mutant SIM. (B) *In vitro* SUMOylation reactions were conducted using GST-IE2(135–289) or GST-IE2(135–289/mSIM) with increasing amounts (1, 2, or 3 μM) of His-Ubc9 or His-SUMO-Ubc9. The reaction products were analyzed by SDS–PAGE (12%) and immunoblotted with anti-GST Ab. The amounts of SUMOylated proteins were quantitated as in Fig. 2A. (C) HF cells were co-transfected with the indicated plasmids. At 48 h, the total cell extracts were prepared and separated by SDS–PAGE (8%). Immunoblotting was performed with anti-myc Ab and anti-HA Ab to detect unmodified or SUMO-modified IE2 (full-length) (Top panels). The expression levels of wild-type Ubc9 and of the SUMO-Ubc9 fusion construct are determined by immunoblotting with anti-GFP Ab (bottom panels). The% numbers of protein modified over total amount of protein were indicated.

enhanced SUMOylation of Sp100 by the SUMO-modified Ubc9 [27], the enhanced SUMOylation of IE2 by the SUMO-Ubc9 fusion protein requires the intact SIM within IE2. Moreover, our results using pull-down assays demonstrate that, like the SUMO-modified form of Ubc9, the SUMO-Ubc9 fusion protein has enhanced binding activities for SIM-containing substrates. Therefore, the SUMO-Ubc9 fusion protein and the SUMO-modified form of Ubc9 appear to share common property in substrate recognition. It might be possible that the SUMO fused to the N-terminus of Ubc9 folds back to the SUMO position of SUMO-modified Ubc9 in the crystal structure [27], with providing a similar binding site for target proteins.

Our abilities to identify new SUMO targets and to understand the function of protein SUMOylation are largely limited by low levels of SUMOylation. In this regard, the devised SUMO-Ubc9 fusion

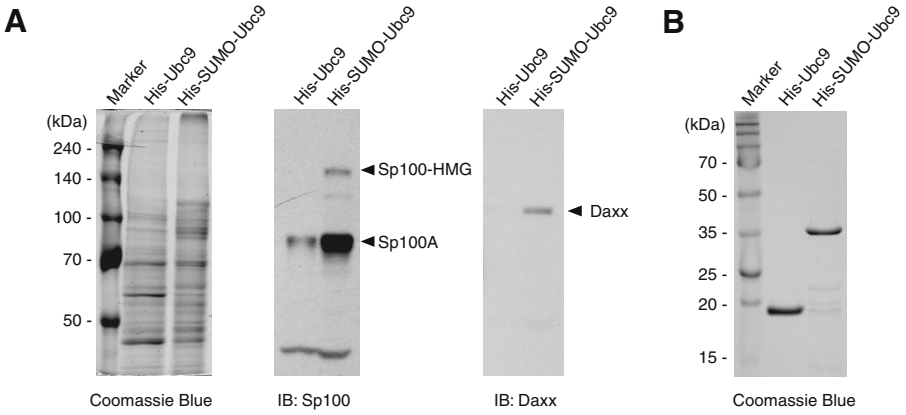

Fig. 4. Comparison of the abilities of Ubc9 and SUMO-Ubc9 to bind to cellular proteins by pull-down assays. (A) The pre-cleared cell lysates prepared from HF cells were incubated with His-Ubc9 or His-SUMO-Ubc9 fusion proteins. The proteins bound to His-Ubc9 or His-SUMO-Ubc9 were partially purified over Ni–NTA resion, separated by SDS–PAGE, and visualized by Coomassie Blue staining (left panel) and immunoblotting with anti-Sp100 or anti-Daxx Abs (right two panels). (B) Five % of the immobilized His-Ubc9 and His-SUMO-Ubc9 fusion proteins used in the binding reactions were shown by Coomassie Blue staining as input controls.

protein should be useful for indentifying, producing and characterizing SUMO-modified proteins *in vitro* and *in vivo*. Our findings also support the notion that SUMOylation of Ubc9 has a regulatory role in the SUMO pathway. In addition to SUMO targets, SIMs are also found in the SAE2 of E1 [36] and in E3 ligases such as PIAS proteins and RanBP2 [37]. Therefore, in addition to its proposed role in target discrimination [27], it is also possible that SUMOylation of Ubc9 may regulate its recognition of E1 and E3 enzymes. In this regard, the SUMO-Ubc9 fusion protein may also be useful for regulating the cellular SUMO pathway.

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