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A proline-90 residue unique to SUMO-4 prevents maturation and sumoylation

David Owerbach [a,*], Eileen M. McKay [b], Edward T.H. Yeh [c], Kenneth H. Gabbay [a], Kurt M. Bohren [a]

[a] *Molecular Diabetes and Metabolism Section and the Harry B. and Aileen B. Gordon Diabetes Research Center, Department of Pediatrics, One Baylor Plaza, Baylor College of Medicine, Houston, TX 77030, USA*
[b] *Department of Pathology, Baylor College of Medicine, Houston, TX 77030, USA*
[c] *Department of Cardiology, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA*

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Abstract

Four small ubiquitin-related modifier (SUMO) genes have been identified in humans. However, little is known about the basic biology of SUMO-4. Here, we report that SUMO-4 differs from SUMO-1, -2, and -3 in that the maturation process of SUMO-4 to active form containing C-terminal di-glycine residues is inhibited by a unique proline residue located at position 90 (Pro-90). Although, both the hydrolase and isopeptidase activities of SUMO peptidases are significantly diminished by Pro-90 as compared to Gln-90 (glutamine) in mutated SUMO genes, only the defective hydrolase activity appears to be biologically relevant. Native SUMO-4, thus, appears to be unable to form covalent isopeptide bonds with substrates. A biological role of SUMO-4, through non-covalent interactions is proposed.

Keywords: SUMO-4; SUMO4; Isopeptidase; SENP1; SENP2; ULP1; Non-covalent interaction; Type I diabetes

SUMO proteins are involved in modification of cellular proteins and regulate various cellular processes, such as nucleo-cytoplasmic signal transduction, transcription, stress response, and mitosis/cell-cycle progression (reviewed in [1–3]). SUMOs belong to the superfamily of ubiquitin-like modifiers (UBLs) which share a common three-dimensional structure [4]. Four SUMO family members have been identified based on interaction with the SUMO-specific enzyme UBC9; SUMO-1 protein shares 48% identity with SUMO-2 and 46% with SUMO-3 [5,6]. The recently described SUMO-4 has a predicted 86% amino acid homology with SUMO-2, but its mRNA is expressed in a limited number of tissues, mainly kidney, lymph, and spleen [7,8].

SUMOs 1–3 are initially expressed as inactive precursors in which a carboxy-terminal proteolytic event exposes two C-terminal glycine residues (reviewed in [1–3]). The di-glycines are involved in the formation of an isopeptide bond with the ε-amino group of a lysine residue of a target protein. The activating enzyme E1 (heterodimer consisting of AOS1 and UBA2) and the conjugating enzyme E2 (UBC9) are required for sumoylation in vitro. In vivo, additional enzymes, called E3, appear to be required for efficient sumoylation. Very little is known about the functional differences of SUMO-1, -2, -3, and -4.

SUMO-4 is unique in that a polymorphism has been described in a conserved amino acid, M55V, which has been associated with susceptibility to type I diabetes in some genetic studies [7–10], although its possible role in diabetes is controversial and unclear at this time [11–13]. The Met residue is conserved across various SUMO family members and across species [14], suggesting that it may be biologically important. However, little is known about the basic biology of SUMO-4.

* Corresponding author. Fax: +1 832 825 3766.
E-mail address: davido@bcm.tmc.edu (D. Owerbach).

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Methods

Recombinant clones. SUMO-1, -2, -4M, and -4V which express active recombinant proteins ending in the C-terminal di-glycine residues in pCMV-Myc vector were previously described [7]. Mammalian expression vectors for SENP1 and SENP2 were previously described [15–17]. Plasmid constructs SUMO-1-94P, SUMO-2-90P, and SUMO-4-90Q were made using the Quickchange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA) utilizing PfuTurbo DNA polymerase and 25–35 bp oligonucleotides as described by Stratagene. Vectors pCMV-Myc-SUMO-4-55M90Pext, -55V90Pext, and -55M90Qext were made by site-directed mutagenesis of the original stop codon, so that the readthrough protein contained 17 additional amino acids found in the pCMV-Myc vector. The sequence of the expressed C-terminal of these vectors is di-glycines followed by VYSKGISRGTAAAGIQT.

SUMO-1 was previously cloned into pET-15b vector containing a His-Tag at the N-terminal and di-glycine residues at the C-terminal [7]. SUMO-1-94P (Pro-94) was made from this pET15-SUMO vector by site-directed mutagenesis as described above. SUMO-1ext and SUMO-1-94Pext were made by site-directed mutagenesis to mutate the stop codons in the SUMO-1 and 1p-pET-15b vectors, so that readthrough occurred, for an additional 23 amino acids found in pET-15b vector. The sequence of the expressed C-terminal of theses vectors is di-glycines followed by VSTDPAANKARKEAELAAATAEQ. The recombinant proteins were propagated in BL21 (DE3) transformed cells and recombinant proteins were purified utilizing His-bind Resin (Ni^{2+}) as described by the manufacturer (Novagen, Madison, WI). The nucleotide sequence of all recombinant clones was verified by nucleotide sequence analysis.

In vitro hydrolase assay. Ulp1 SUMO protease was purchased from Invitrogen, Carlsbad, CA. By definition, one unit of enzyme cleaves >85% of 2 μg control substrate in 1 hr at 30 °C. We digested 100 ng of purified SUMO-1ext or SUMO-1-94Pext, using either 1 unit of ULP1 enzyme, 8 μl of cell extract or control digestions lacking both. Cell extracts from K562, HepG2 or HEK293 cells were prepared fresh, as described for K562 cells above, except that mammalian protease inhibitor cocktail was omitted. A 10X digestion buffer was supplied by Invitrogen. Western blots were done on the reaction mixtures using polyclonal antibody for SUMO-1 (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:500 dilution.

Transfections and Western blotting. K562 cells were grown at 37 °C and 5% CO_2 in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum (FCS), 1% penicillin–streptomycin, and 2 mM glutamine in T175 flasks to confluency. Cells (1/15 of a T175 at confluency) were seeded into 6-well plates and transfected with DNA using lipofectamine 2000 as described by the manufacturer (Invitrogen, Carlsbad, CA). The cells were transfected with 5 μg DNA/well of pCMV-Myc-SUMO plus 5 μg of either pCMV-Myc control or SENP1 or SENP2 expression constructs. After 24-h incubation, wells were washed with ice-cold PBS and whole cell extracts were prepared in 100 μl of 50 mM Hepes [pH 8.0], 0.1 mM EDTA, 1 mM DTT, 12.5 mM $MgCl_2$, 20% glycerol, 0.1 M KCl, and 1% Triton X-100 containing 1 μl of mammalian protease inhibitor cocktail from Sigma (St. Louis, MO). After 5 min of incubation on ice, the lysed cells were sonicated for 5 s, centrifuged for 5 min at 10,000 rpm, and the supernatant was collected. Ten microliters of extract was combined with Nupage LDS sample buffer containing reducing agent (Invitrogen, Carlsbad, CA), heated for 5 min at 95 °C, and then resolved on 10% SDS–PAGE (NuPAGE, Invitrogen, Carlsbad, CA). Gels were then transferred onto nitrocellulose using an electroblotting apparatus from Novex (San Diego, CA). Blots were blocked in 5% non-fat dried milk and probed with monoclonal antibody for cMyc (Clontech, Palo Alto, CA) at a 1:2000 dilution as directed by Clontech. Anti-mouse-HRP secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was then applied to the blots and bands were visualized using an ECL chemiluminescence kit (Amersham–Pharmacia, Piscataway, NJ) and autoradiography (Kodak X-OMAT film, Rochester, NY). Autoradiographs were scanned by Vista Scan software using the transmission mode on a UMAX, Astra 2400SCT scanner (Dallas, TX). The selected bands were quantified using Scion Image for Windows software (Release Beta 4.0.2). The regions containing SUMO-HMW or 70 kDa were compared relative to non-conjugated SUMO.

Results

UBL isopeptidases have two activities, one that removes the isopeptide bond from the ε-amino group of a lysine residue of a target protein and the second that cleaves the unprocessed native SUMO through a hydrolase activity, to make the SUMO protein end in C-terminal di-glycine residues. The latter activity is the maturation process that is required for sumoylation to occur. We initially tested if Gln/Pro-94 in SUMO-1 (Pro-90 in SUMO-4), which is located to the N-terminal side of the di-glycines (Gly-96-Gly-97 in SUMO-1 and Gly-92-Gly-93 in SUMO-4), affects the maturation activity, since this Gln has been shown to be important in the interaction with SENP2 and ULP1, human and yeast SUMO isopeptidases [18]. Fig. 1 demonstrates isopeptidase digestion in vitro, of recombinant purified SUMO-1ext or SUMO-1-94Pext (SUMO-1Pext), both containing a C-terminal extension of 23 amino acids after the di-glycines (sequence shown in Methods). Purified yeast SUMO isopeptidase ULP1, a homolog of human SENP2, or extracts from human cell cultures (K562, HepG2 or HEK293 [human embryonic kidney] cells) were incubated with the recombinant SUMOs (Fig. 1). Using ULP1, more than 90% of the SUMO-1ext is cleaved (Fig. 1, lane 1), while SUMO-1Pext is completely resistant to digestion (Fig. 1, lane 3). Extracts from all three of the mammalian cells tested give similar results to those of ULP1 (Fig. 1, lanes 6 and 5 shown for K562 cell extracts only). The cell extracts presumably contain human SENP1 and SENP2 and additional SENPs [19], and therefore, the lack of digestion of SUMO-1-94Pext must be due to resistance to the whole class of isopeptidases. Since SUMO-1ext (94Q) is cleaved by both ULP1 and human cell extracts, the partially artificial nature of C-terminal 23 base pair extension cannot be the basis of our results. Indeed, previous studies

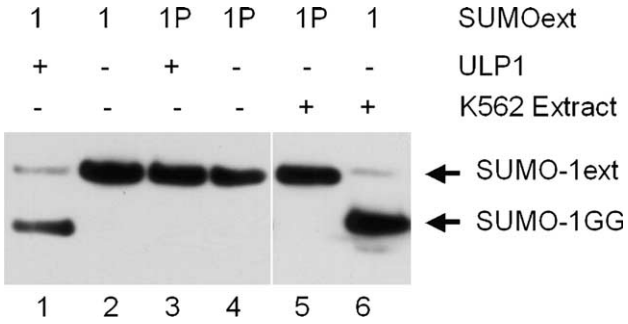

Fig. 1. Effect of Pro-94 on in vitro digestion of purified SUMO-1ext by ULP1 and K562 extract. Arrows indicate the location of SUMO-1 extended by 23 amino acids after the di-glycines and fully processed SUMO ending in C-terminal di-glycines. Control digestions lacking ULP1 or K562 extract are shown in lanes 2 and 4. ULP1 was present in lanes 1 and 3. K562 extract was present in lanes 5 and 6. SUMO-1ext was present in lanes 1, 2, and 6. SUMO-1Pext was present in lanes 3–5. Proteins are detected with SUMO-1 antibody.