Small Ubiquitin-like Modifier (SUMO) Recognition of a SUMO Binding Motif

A REVERSAL OF THE BOUND ORIENTATION*

Received for publication, June 28, 2005, and in revised form, September 6, 2005. Published, JBC Papers in Press, October 3, 2005, DOI 10.1074/jbc.M507059200

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Sumoylation has recently been identified as an important mechanism that regulates protein interactions and localization in essential cellular functions, such as gene transcription, subnuclear structure formation, viral infection, and cell cycle progression. A SUMO binding amino acid sequence motif (SBM), which recognizes the SUMO moiety of modified proteins in sumoylation-dependent cellular functions, has been consistently identified by several recent studies. To understand the mechanism of SUMO recognition by the SBM, we have solved the solution structure of SUMO-1 in complex with a peptide containing the SBM derived from the protein PIASx (KV/DVDDLTIEKS/S/DDEEDPPAKR). Surprisingly, the structure reveals that the bound orientation of the SBM can reverse depending on the sequence context. The structure also reveals a novel mechanism of recognizing target sequences by a ubiquitin-like module. Unlike ubiquitin binding motifs, which all form helices and bind to the main β-sheet of ubiquitin, the SBM forms an extended structure that binds between the α-helix and a β-strand of SUMO-1. This study provides a clear mechanism of the SBM sequence variations and its recognition of the SUMO moiety in sumoylated proteins.

Post-translational modification by the small ubiquitin-like modifiers (SUMO)‡ is an important mechanism that regulates a wide variety of cellular functions such as gene transcription, subnuclear structure formation, viral infection, and cell cycle progression (1–5). In mammalian cells, four SUMO paralogues have been identified (6–8). SUMO-2, -3, and -4 are closely related and share more than 80% amino acid sequence identity. However, these proteins are less than 50% identical to SUMO-1. The in vivo functions of SUMO-2, -3, and -4 modifications are still not well understood, but it is known that some of their differences are in localization- and tissue-specific expression (6, 9). SUMO modifies a large number of proteins, and recent proteomic studies indicate that as much as 5% of the yeast proteome are SUMO substrates (10–13).

‡ This work is supported by National Institutes of Health Grant CA94595 (to Y. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The atomic coordinates and structure factors (code 2ASQ) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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2 The abbreviations used are: SUMO, small ubiquitin-like modifier; SH, Src homology; SBM, SUMO binding motif; HPLC, high performance liquid chromatography; ITC, isothermal titration calorimetry; NOESY, nuclear Overhauser effect (NOE) spectroscopy; r.m.s.d., root mean square deviation.

The mechanisms by which sumoylation regulates cellular functions are poorly understood. Current data suggest that the SUMO moiety of SUMO-modified proteins provides a platform for binding other proteins, and thus, SUMO serves as a module in protein interaction networks. Protein-protein interactions, which govern the intricate and dynamic networks of cellular functions and regulation, often involve only a limited number of common modules, such as SH2, SH3, and PDZ domains and short amino acid sequence motifs that bind to these modules (14). The ubiquitin-like structures are special types of modules that can be either part of a protein or covalently attached to other proteins enzymatically (15, 16). An obvious advantage of attaching ubiquitin-like modules enzymatically is gaining the ability to turn on and off protein-protein interactions quickly by conjugation and deconjugation of these modules. There are two lines of evidence which suggest that SUMO is likely to function as a module in mediating protein interactions. First, sumoylation has been shown to regulate protein interactions. For example, SUMO-1 modification of RanGAP-1 results in association of RanGAP1 with the nuclear pore protein RanBP2/Nup358 (17, 18), and the SUMO binding motif in PIASXα is responsible for its interaction with the SUMO-modified transcription factor Elk-1 to confer transcriptional activation (19). Second, the available structures of SUMO-modified proteins suggest that covalently linked SUMO and substrate proteins are structurally independent from each other, like “two beads on a string” (20, 21).

Because the SUMO proteins appear to provide platforms for binding other proteins, identification of SUMO binding motifs (SBM) on interacting proteins is critical for uncovering the sumoylation-mediated protein interaction networks. Several recent studies have attempted to identify the consensus sequences of SUMO using NMR spectroscopy (22) or yeast two-hybrid screening (13, 23). These studies have identified similar consensus sequences, which include clusters of Val, Ile, Leu, and acidic residues. The non-covalent SUMO binding sites of several proteins also contain similar sequences, such as in the Epstein-Barr virus nuclear antigen 3C (EBNA3C) with the sequence DDDVIEVIDVETTE (24) and in the homeodomain-interacting kinase PKM (Mx-interacting protein kinase) containing a sequence VSVTISSDTDEEE (25). These sequences are similar to that of the SUMO-1 binding site of PIASX (VDVIDL), determined by our previous study (22). There is also the possibility that other SUMO binding sequences may exist (26, 27).

The physiological relevance has been demonstrated for the SBM that we have previously identified (28). An investigation combining site-directed mutagenesis of SUMO-1 and -2 dependent transcriptional inhibition. This surface is identical to the region that we have shown to bind to the SBM (22). Because transcriptional repression is a common function of sumoylation, this result suggests that the SUMO-SBM interaction is...
responsible for recruiting cellular factors that mediate the transcriptional inhibitory functions of SUMO.

The SBM is different from the SUMO-1 modification consensus sequence (ΦKXE) found in SUMO-1 substrate proteins. SBM binds SUMO non-covalently, but the ΦKXE motif does not bind to SUMO-1 noncovalently. Instead, it binds Ubc9 noncovalently for covalent SUMO attachment (29).

Although the consensus sequences of the SBM that are identified from most studies are similar, the consensus sequence is still not clear. In this report we present an NMR structural study of SUMO-1 in complex with a peptide containing such a SBM (KDVVDLTISSDEED-PPAKR). A combination of structural determination, amino acid substitution studies, and biochemical analysis of the affinities have revealed a novel mechanism of recognizing target sequences by ubiquitin-like proteins and have provided the structural basis for the sequence requirement of this SBM for binding SUMO.

MATERIALS AND METHODS

Expression and Purification of Recombinant Protein and Peptide—

Expression and purification of SUMO-1 (1–97) was expressed and purified as described (22). [13C,15N]-Enriched PIASX-P peptide was made with the modified vector pET31b (Novagen), in which 5 tandem repeats of PIASX-P peptide-coding sequences were separated by methionine codon. The gelatinous solution was purified from inclusion bodies with nickel nitrilotriacetic acid affinity chromatography. The fusion protein was purified from inclusion bodies with nickel nitrilotriacetic acid affinity chromatography under denaturing conditions. The fusion protein was then precipitated by dialysis against H2O and re-dissolved in 70% formic acid. Protected from light, the fusion protein was cleaved with cyanogen bromide cleavage. BLR(De3)P-LysS cells containing the expression plasmid were grown at 37 °C in M-9 minimal media with [13C]glucose and [15N]NH4Cl (Cambridge Isotope Laboratories) and induced with 0.5 mM isopropyl-β-d-thiogalactoside. The fusion protein was purified from inclusion bodies with nickel nitrilotriacetic acid affinity chromatography under denaturing conditions. The fusion protein was then precipitated by dialysis against H2O and re-dissolved in 70% formic acid. Protected from light, the fusion protein was cleaved with cyanogen bromide in 6 ml of 70% formic acid overnight in a ventilated hood and then dried by rotary evaporation. The gelatinous material was resuspended in phosphate-buffered saline, and the pH was adjusted to 7.4. This mixture was stirred overnight, and the supernatant containing the cleaved recombinant peptide was further purified by reverse-phase HPLC and verified by mass spectrometry.

NMR Spectroscopy—PIASX-P peptide and SUMO-1 were mixed with 1:1 molar ratio in a buffer containing 20 mM phosphate buffer (pH 6.8) and 5 mM dithiothreitol in 92% H2O, 8% D2O. Two samples were made with either [13C,15N]-enriched SUMO-1 and unlabeled PIASX-P peptide or unlabeled SUMO-1 and [13C,15N]-enriched PIASX-P peptide. All NMR spectra were acquired at 22 °C on a Bruker 600 MHz NMR spectrometer equipped with a cryoprobe, processed with NMRPipe, and analyzed with NMRView (Version 5).

The backbone and side chain assignments of SUMO-1 in complex with PIASX-P peptide were obtained as described (22). Main-chain and side-chain assignments of the peptide in complex were made using three-dimensional HNCA and HNCACB spectra acquired in H2O and HCCCH-two-dimensional total correlation spectrum acquired in D2O (30). Distance restraints were obtained from the analysis of three-dimensional 15N-edited NOESY, 13C-edited NOESY, and 13C-filtered/12C-edited NOESY spectra. Main chain Φ and Ψ angles were predicted by TALOS based on Hα, Cα, Cβ and amide nitrogen chemical shifts (31).

Structure of SUMO-1 in Complex with a SUMO Binding Motif

A combination of structural determination, amino acid substitution studies, and biochemical analysis of the affinities have revealed a novel mechanism of recognizing target sequences by ubiquitin-like proteins and have provided the structural basis for the sequence requirement of this SBM for binding SUMO.

RESULTS

Structure Determination of the SBM-SUMO-1 Complex—The solution structure of SUMO-1 in complex with a peptide that contains a non-covalent SUMO binding site of PIASX was determined using NMR spectroscopy. This peptide, named PIASX-P in subsequent discussions, has the sequence KDVVDLTISSDEED-PPAKR, which contains the SBM as described previously (22). This sequence was chosen because its affinity is at least 10-fold higher than other SBM-containing peptides that we have tested, although they all bind to the same region of SUMO-1. From the 1000 structures calculated from rigid body docking, 100 structures were refined, and 10 structures with lowest intermolecular energy were retained. Structure alignments were made with INSIGHT II (MSI). Structures were analyzed using PROCHECK NMR (34).

Isothermal Titration Calorimetry (ITC) Measurements—The wild type and mutant PIASX-N peptides (the N-terminal 10 residues of PIASX-P peptide) were synthesized by the Peptide Synthesis Core Facility at the City of Hope, purified by HPLC, and verified by mass spectrometry.

ITC measurements were performed at 30 °C using Microcal VP-ITC calorimeter. SUMO-1 and peptide samples were buffered with 20 mM phosphate (pH 7.4), 1 mM 2-mercaptoethanol and thoroughly degassed before use. The concentrations were determined by amino acid analysis. Injection of 5 μl of ~0.4 mM (for wild type peptide titrations) or ~1 mM peptide (for mutant peptide titrations) were made at 3-min intervals into the 1.4-ml sample cell containing ~0.03 mM (for wild type peptide titrations) or ~0.09 mM (for mutant peptide titrations) SUMO-1. The heat generated due to dilution of the peptides was subtracted for baseline corrections. The baseline-corrected data were fit with Microcal Origin software. Experiments were duplicated.

Pull-down Assay—Mutant and wild type RanBP2 domains and in vitro translated RanGAP1 were prepared as described before (9). Microtiter plates (Corning Costar) were coated with 5 μg of each purified protein per well overnight at 4 °C. The wells were subsequently blocked with 150 μl of blocking buffer (2% bovine serum albumin, 0.1% Tween 20, 1 mm dithiothreitol, 2 mM MgCl2, 1 mM EDTA in phosphate-buffered saline) for 1 h at room temperature. After washing with blocking buffer, 5 μl of in vitro translated RanGAP-1 protein was added with 100 μl of blocking buffer and incubated at room temperature for 1 h. The wells were then washed 3 times with 150 μl of blocking buffer followed by 4 washes with 150 μl of 0.1% Tween 20 in phosphate-buffered saline. The bound proteins were extracted with SDS-PAGE buffer. Samples were analyzed by SDS-PAGE and detected by autoradiography using PhosphorImager (Amersham Biosciences).
SUMO-1. Structural studies of this peptide in complex with SUMO-1 should yield insight into how this SBM interacts with SUMO-1 and provide an understanding regarding the higher affinity of this peptide relative to other peptides containing the same SBM.

To obtain unambiguous resonance assignments and conformational constraints, two samples were made, a 1:1 complex between 13C,15N-labeled SUMO-1 and unlabeled peptide and a 1:1 complex between 13C,15N-labeled peptide and unlabeled SUMO-1. Labeling the peptide was necessary to eliminate ambiguity in assigning the two Val, two Ile, and two Asp residues in the sequence. Complete backbone and sidechain NMR resonance assignments were achieved for both the peptide and SUMO-1 in the complex using standard triple resonance NMR experiments as described under “Materials and Methods.” Intramolecular NOEs were identified from both samples with either the peptide or SUMO-1 labeled with 13C and 15N, as shown by a representative spectrum in Fig. 1. Two three-dimensional NOESY spectra were recorded with 13C filtering in the F1 dimension and 13C editing in the F2 dimension. The NOE cross-peaks identified in these two spectra are mirror images with respect to each other and, thus, allowed for the unambiguous assignments of intermolecular NOEs.

Intramolecular conformational constraints were derived as follows. Three-dimensional 13C-edited NOESY was acquired using the 13C,15N-labeled peptide in complex with unlabeled SUMO-1. Because the peptide is small, intramolecular NOEs were easily identified when comparing this spectrum with the 13C-filtering (F1)/13C-editing (F3) NOESY spectrum taken with the same sample (Fig. 1). The SUMO-1 structure was determined twice using NMR spectroscopy and three times by x-ray crystallography (21, 35–38). The resonances of these residues are very narrow and identical to those of the unbound peptide. The sharp resonances indicate that these residues are highly flexible, and their motions are not restricted by forming the peptide-SUMO complex. Similarly, the lack of intermolecular NOEs also indicates that these residues do not form a defined structure.

The structure of the PIAST-P peptide in complex with SUMO-1 was calculated using the conformational constraints described above and using the program HADDOCK (32). In the initial stages of the calculation, the complex was first docked using the intramolecular and with an isopeptidase, and in a covalent complex with E2–25K. Despite complexing with three different proteins, a comparison of the three SUMO-1 x-ray structures showed highly similar conformations with a root mean square deviation (r.m.s.d.) of 0.4–0.6 Å among backbone atoms excluding the N- and C-terminal residues that are flexible in solution. Because chemical shift perturbation does not suggest any global conformational changes in SUMO-1 upon binding PIASX-P, we decided not to obtain the complete intramolecular NOE constraints of SUMO-1. Instead, we derived backbone conformational constraints of SUMO-1 in the complex and used the crystal structure of SUMO-1 in the covalent complex with E2–25K (21) as the starting point in calculating the structure of the complex. In this structure SUMO-1 has minimal contact with E2–25K, and the interface with E2–25K only involves the C terminus of SUMO-1 (21), which is not required for binding the PIAST-P peptide.

Fifty-seven intermolecular NOEs were identified between residues 2 and 8 of the PIAST-P peptide and SUMO-1. In addition, 24 inter-residue NOEs were identified within the peptide. Consistent with the previous finding (22), the SSS sequence of PIAST-P and all residues C-terminal to the SSS sequence do not show inter-residue NOEs. The resonances of these residues are very narrow and identical to those of the unbound peptide. The sharp resonances indicate that these residues are highly flexible, and their motions are not restricted by forming the peptide-SUMO complex. Similarly, the lack of intermolecular NOEs also indicates that these residues do not form a defined structure.

The structure of the PIAST-P peptide in complex with SUMO-1 was calculated using the conformational constraints described above and using the program HADDOCK (32). In the initial stages of the calculation, the complex was first docked using the intramolecular and
intramolecular NOE constraints with the backbone conformation of SUMO-1 fixed, whereas the side-chain conformations of residues at the binding interface, as indicated by chemical shift perturbation, were allowed to be flexible. All other side chains of SUMO-1 were fixed. The peptide was allowed to be fully flexible. Two cycles of simulated annealing and energy minimization were performed. In the third simulated annealing cycle, both the backbone and side chains of SUMO-1 residues at the interface were allowed to move, but backbone dihedral restraints were applied to SUMO-1. Although the intramolecular distance constraints were not applied to SUMO side chains, the structures were well converged, as indicated by the small r.m.s.d. of the backbone and side-chain atoms from the average structure (TABLE ONE). This demonstrates that the extensive intermolecular NOEs and NOEs within the peptide were able to define the side-chain orientations at the interface of SUMO-1. No conformational constraints were identified for residues 9–23 of the peptide, and their structures were completely random. The randomness of these unconstrained residues is a good indication of the sufficient sampling of conformational space by the calculation protocol.

**Description of the Overall Structure**—An ensemble of 10 structures of SUMO-1 in complex with PIASX-P is shown in Fig. 2A. The ribbon diagram of a representative structure is shown in Fig. 2B. The structures satisfy all input conformational restraints with no distance or torsion angle violations greater than 0.3 Å and 5°, respectively. The structures also do not violate covalent geometry, have favorable Ramachandran statistics (TABLE ONE), and have favorable van der Waals energies.

Residues 2–8 of the PIASX-P peptide embed, as an extended conformation, into a groove located between the α-helix and a β-strand of SUMO-1. These residues appear to extend the β-sheet and continue the twist of the main β-sheet of SUMO-1 (Fig. 2). However, analysis of the NMR data reveals that only residues 2–6 have the backbone conformation of a β-strand. Residue 7, which is a key residue in the SBM as described below (under “Essential Residues of the SBM”), as well as residue 8 do not have the backbone dihedral angles close to those of a standard β-strand, as indicated by backbone chemical shifts (31). Additionally, hydrogen-deuterium exchange experiments show that the peptide lacks slowly exchanging backbone amide protons that are often observed for β-sheet residues (data not shown).

**Description of the Interface**—Extensive side-chain contacts occur between the PIASX-P peptide and SUMO-1 (Fig. 3, A and B). The residues from the PIASX-P peptide that contact SUMO-1 include Val-2, Asp-3, Val-4, Ile-5, Leu-7, and Thr-8. Except for residue Asp-3, all of these residues have much broader NMR resonances in the complex than in the unbound peptide, indicating that complex formation has significantly restricted their motions. The side chain of residue Asp-3 is considerably mobile, as indicated by its narrower resonances and its much fewer NOE relative to neighboring residues. The Asp-6 side chain is also not restricted, as no inter-residue NOEs were observed involving this residue and the line widths of its side-chain resonances were very narrow.

The peptide binding surface of SUMO-1 forms a deep groove that is “lined” with hydrophobic and aromatic patches consisting of residues His-35, Ile-34, Tyr-51, Phe-36, Leu-47, and Val-38 (Fig. 3B). Interestingly, this is the only concave surface on SUMO-1 that is lined with hydrophobic patches. These residues exhibit many intermolecular NOEs with the PIASX-P peptide and, therefore, have extensive contacts with the peptide. The Val-2 side chain of the PIASX-P peptide contacts the residues Glu-33, Ile-34, and His-35 of SUMO-1. Asp-3 also contacts Ile-34, whereas Val-4 interacts with His-35 of SUMO-1. Ile-5 and Leu-7 insert into a pocket defined by residues Tyr-51, Phe-36, Leu-47, and Val-38 of SUMO-1. Finally, Thr-8 interacts with the side chain of Tyr-21 of SUMO-1. The interface covers an area of ~1300 Å², which is consistent with the affinity in the micromolar range (22).

To test the importance of the residues forming the hydrophobic SBM binding groove on SUMO-1 (Fig. 3A), Phe-36 of SUMO-1 was mutated to alanine, and the binding of the mutant protein to the wild type PIASX-P peptide was measured by ITC (Fig. 4). The mutation of Phe-36 of SUMO-1 did not alter the overall structural integrity of SUMO-1, as judged by an NMR spectrum (data not shown), but it reduced the affinity for PIASX-P to such an extent that no significant heat changes were detected by ITC upon complex formation. Consistent with the structural study, Ile-34, Val-38, and their equivalent residues in SUMO-2
have been demonstrated to be important in SUMO-mediated transcriptional inhibition (28).

The Essential Residues of the SBM—The residues from the peptide that make extensive contact with SUMO-1 were substituted to make a series of synthetic peptides, as listed in Fig. 4B, to evaluate their importance in binding SUMO. Specifically, we substituted residues Val-2, Val-4, Ile-5, Leu-7, and Thr-8 individually with Ala. ITC experiments were performed to evaluate the affinities of the mutated peptides with SUMO-1. All substitutions significantly reduced the binding affinity for SUMO-1.

Among all the residues that make significant contact with SUMO-1 in solution, three residues, Val-4, Ile-5, and Leu-7, are shown to be essential (Fig. 4B). A single amino acid substitution of any of these residues reduced the affinity so dramatically that no significant heat changes associated with the complex formation were detected by ITC. This result suggests that these residues are the most critical for binding SUMO-1.

Two other residues, Val-2 and Thr-8, also make significant contributions to the affinity. Substitution of Val-2 with an Ala reduced the binding affinity for SUMO-1 by a factor of 3. Although Thr-8 is not a conserved residue in the SBM, the substitution of Thr-8 by an Ala reduced the affinity by ~10-fold. Thr-8 contacts residue Tyr-21 of SUMO-1, as indicated by the NOEs between the two residues. The contribution of this residue to SUMO recognition is well correlated with the differences observed in NMR chemical shift perturbations and the 10-fold higher affinities of PIASX-P for SUMO-1 than other peptides (22). Low affinities also result in higher uncertainties in the ITC affinity measurement.

Two Opposite-bound Orientations—Our previous studies have identified three critical residues in the SBM of RanBP2 for binding SUMO, which are Val-2632, Ile-2634, and Val-2635, and have suggested (V/I)(V/I) or (V/I)(V/I) sequence as the key sequence requirement for binding SUMO (22). The three key residues in the SBM of PIASX, Val-4, Ile-5, and Leu-7, do not exactly resemble such a consensus sequence, although both the SBMs of RanBP2 and PIASX bind to the same site of SUMO-1 (22). Pull-down assays after a systematic mutagenesis were performed to further evaluate the role of every residue of the SBM in RanBP2 (Fig. 4, C and D). These studies again indicated that Val-2632, Ile-2634, and Val-2635 are critical for binding sumoylated RanGAP1. In addition, Leu-2633 plays some role in the affinity, but it is not as important as the other three residues.

A recent x-ray crystal structure of sumoylated RanGAP1 in complex with a RanBP2 fragment that contains the SBM and Ubc9 has shown the interaction between SUMO-1 and the SBM in RanBP2 (PDB code 1ZS5) (39). The SBM of RanBP2 contacts SUMO-1 in a similar manner as that of PIASX-P; however, it binds in the opposite orientation from that of PIASX-P. The previously identified key residues of the SBM of RanBP2, Val-2632, Ile-2634, and Val-2635, occupy the equivalent positions of Leu-7, Ile-5, and Val-4 of PIASX-P, respectively (Fig. 4E), which are also most critical for SUMO recognition of PIASX-P. Leu-2633 is in an analogous position as Asp-6 in PIASX-P, and the position is consistent with the less significant role in binding as shown by the pull-down experiments after mutation to an Ala or Lys (Fig. 4, C and D). Taken together, the combination of amino acid substitutions, affinity measurements, and the structure-based sequence alignment indicates that the SBM can bind to the same site of SUMO-1 in two different orientations opposite from each other, depending on whether the sequence is (V/I)X(V/I) or (V/I)(V/I)X(V/I/L) (Fig. 4E).

Role of Electrostatic Interactions—The surface of SUMO-1 binding with the peptide has a positive electrostatic potential (Fig. 3C). The two negatively charged Asp residues are placed strategically, one next to Arg-54 and another one next to Lys-37 of SUMO-1. They likely contribute to the affinity of the PIASX-P peptide for binding SUMO-1. The amino acid substitution of the equivalent residue of Asp-3 in RanBP2 significantly reduced the binding affinity (22). However, the acidic residues are unlikely to be critical for the binding specificity due to their minimal contacts with SUMO-1, as the two Asp residues are not conserved in other SUMO binding amino acid segments, such as that in the homeodomain-interacting kinase PKM (VSVITISSDT).

Yeast SUMO Homologues—The residues at the SBM binding interface are conserved between yeast and human SUMO proteins. This result suggests that the SBM is also conserved between yeasts and human. We performed an ITC measurement of the complex formation between the yeast SUMO homologue, Smt3p, and the PIASX-P peptide. The affinity of the
yeast homologue Smt3p to PIASX-P is similar to that of SUMO-1 (Fig. 4A). This result is consistent with the fact that the SBM identified in yeast proteins are similar to those identified in human proteins (13).

**DISCUSSION**

**A Novel Mechanism of Binding Ubiquitin-like Modules**—The three-dimensional structure of SUMO-1 in complex with a SBM has revealed a novel mechanism as to how SUMO interacts with short amino acid sequence motifs. The interaction between SUMO-1 and SBM is likely to be important in SUMO-dependent signaling and targeting mechanisms, as demonstrated by recent studies (22, 28). The SBM-containing PIASX-P peptide forms an extended conformation and embeds into a hydrophobic groove of SUMO-1 between a β-hairpin structure and the α-helix. Surprisingly, the SBM can bind with the same surface of SUMO in two different orientations. This is very different from ubiquitin recognition by known ubiquitin binding motifs. All identified ubiquitin-binding motifs...
domains, such as CUE (40), UIM (41), UBA (42), NZF (43), and UEV (44), form helices that pack onto the conserved hydrophobic β-sheet that contains residue Ile-44 in ubiquitin. Therefore, this study has revealed a novel mechanism of binding target amino acid sequences by ubiquitin-like proteins.

Sequence Requirement of the SBM—This study has allowed a structure-based alignment between two SBMs. In combination with amino acid substitutions and affinity measurements described here and before (22), the key residues of the SBM in binding SUMO-1 include three residues, which are Val, Leu, or Ile. They can be arranged as either (V/I)(V/I)(V/I) or (V/I)(V/I)(X/V/I/L), and the two different sequences bind to the same site of SUMO in two orientations that are opposite from each other. Other residues that flank these sequences can contribute to the binding affinity. For example, Thr-8 in PIASX-P increases the affinity for SUMO-1 by a factor of 10. Acidic residues flanking the key (V/I)(V/I)(V/I) or (V/I)(V/I)(X/V/I/L) sequences also enhance the affinity as demonstrated previously (22). Such a consensus sequence occurs in nearly all SUMO binding segments or consensus sequences described in the Introduction.

A recent structural study of sumoylated DNA glycosylase has shown that a segment of DNA glycosylase forms a β-strand and binds to SUMO-1 in a manner similar to that of the SBM (45). This contact appears to be further stabilized by the other interactions within the DNA glycosylase. Such non-covalent interactions with SUMO in sumoylated proteins would prevent the SBM-mediated protein-protein interactions.

A Reversal of the Bound Orientation—This study unexpectedly reveals that the orientations of the SBM bound to SUMO can reverse. In protein-peptide interactions, short peptides often bind in an extended conformation into concave surfaces or grooves on their cognate protein targets. Examples of this type of complexes include SH2, SH3, and PDZ domains with their target sequence motifs in signal transductions and binding target peptides to major histocompatibility complex molecules in the immune response. In all of these complexes, hydrophobic and aromatic residues are important in providing the specificity of the interaction. The groove of SUMO-1 interacts with the SBM and lined with hydrophobic and aromatic amino acid residues (Fig. 3B) and, thus, ideal for binding short sequences. However, reversal of the bound orientations is not common and only observed in SH3-peptide recognition (46). In the structure of SUMO-1 in complex with its cognate E1 enzyme, the N-terminal extension of SUMO-folds back and points toward the same groove of SUMO-1 where the SBM binds. However, the N terminus of SUMO-1 does not extend into this groove to contact the key residues that interact with the key SBM sequence, such as Phe-36, Leu-47, and Tyr-51. This is consistent with the lack of an SBM at the N terminus of SUMO-1.

An important implication of this study is that the SBM sequences containing symmetry, such as VVVI in the protein PML, may bind SUMO in either orientation and the specific-bound direction depends on the additional contacts that stabilize one of the orientations. These additional contacts include asymmetrically located polar or charged residues, as shown here. Most SBM-containing proteins that have been implicated in SUMO-dependent processes have asymmetrically located charged or polar residues in the surrounding sequences, which would likely stabilize one of the bound orientations. Similar phenomena have been observed in SH3-peptide interactions (46).

In conclusion, the non-covalent interaction between SUMO-1 and a SUMO binding motif represents a novel mechanism of binding target sequences by ubiquitin-like proteins. An unusual feature of the SBM is that it can reverse its orientation for binding with the same site of SUMO. The structural and biochemical studies described here provide a basis for identifying SUMO-binding proteins and for understanding the molecular basis of SUMO-dependent protein interactions.
Structure of SUMO-1 in Complex with a SUMO Binding Motif

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