SUMO proteins are ubiquitin-related modifiers implicated in the regulation of gene transcription, cell cycle, DNA repair, and protein localization. The molecular mechanisms by which the sumoylation of target proteins regulates diverse cellular functions remain poorly understood. Here we report isolation and characterization of SUMO1- and SUMO2-binding motifs. Using yeast two-hybrid system, bioinformatics, and NMR spectroscopy we define a common SUMO-interacting motif (SIM) and map its binding surfaces on SUMO1 and SUMO2. This motif forms a β-strand that could bind in parallel or antiparallel orientation to the β3-strand of SUMO due to the environment of the hydrophobic core. A negative charge imposed by a stretch of neighboring acidic amino acids and/or phosphorylated serine residues determines its specificity in binding to distinct SUMO paralogues and can modulate the spatial orientation of SUMO-SIM interactions.

SUMO proteins are small ubiquitin (Ub)-related modifiers that become conjugated to cellular substrates and regulate diverse cellular processes including cell cycle progression, intracellular trafficking, transcription, and DNA repair (1–3). Like Ub, a SUMO protein is covalently attached to target proteins through an isopeptide bond by a mechanism similar to that of ubiquitination, which involves E1, E2, and E3 enzymes (4). In mammals, three SUMO paralogues are commonly expressed: SUMO1 shares about 45% identity to SUMO2 and SUMO3, while SUMO2 and SUMO3 are 96% identical to each other (2, 5).

In the case of TTRAP, a protein that binds SUMO2 much stronger than SUMO1 (10) and topoisomerase II with SUMO2/3 during mitosis (11). Furthermore, the distribution of the SUMO paralogues within cells seems to be different. SUMO1 is uniquely found within the nucleoli, the nuclear envelope, and cytoplasmic foci, whereas SUMO2/3 are accrued on chromosomes at an earlier point in the nuclear reformation process (12). Interestingly, there is a larger pool of free, non-conjugated SUMO2/3 than of SUMO1 (10).

In addition to targeting different substrate proteins, the functional properties of SUMO isoforms in vivo might also reflect their ability to mediate distinct protein–protein interactions. Indeed, recent studies have shown that SUMO paralogues can promote non-covalent binding to other proteins containing specific motifs that recognize SUMO paralogues. Minty and coworkers defined a Ser-Xaa-Ser motif surrounded by hydrophobic and acidic amino acids as a SUMO-interacting motif (SIM) (13). Biophysical studies of the SIM in PIAS revealed that the small hydrophobic region is an essential determinant of SUMO recognition (14). Moreover, the SUMO-binding motif was proposed as Lys-Xaa-Glu/Asp/Asn/[Val/Ile]-[Ile/Leu]-Xaa-[Asp/Glu/Gln/Asn]-[Asp/Glu]2 in yeast proteins (15).

Recent publications revealed that the hydrophobic core can bind both parallel and antiparallel to SUMO (16, 17). It is thought that sumoylated targets may control cell functions depending on their ability to interact with effectors containing SUMO-binding motifs. However, most of the SUMO interacting studies were done with the SUMO1 paralogue.

In this study we describe the identification and characterization of novel SUMO1- and SUMO2-binding partners containing a universal SIM. The molecular and structural details are presented explaining the basis for SIM binding to distinct SUMO paralogues. We show that the E3 ligase PIASxα is phosphorylated in vivo within the SIM and that phosphorylation influences its binding to SUMO1 but not to SUMO2. In the case of TTRAP, a protein that binds SUMO2 much stronger than SUMO1, and PIASxα we show that negative charged amino acids surrounding the hydrophobic core influence binding to SUMO1 but not to SUMO2.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screen—Sequences corresponding to SUMO1(DGG) and SUMO2(DGG) were subcloned in pYTH9 vector between SalI and BglII restriction site creating fusion proteins with Ga4-DNA-binding domain. Both vectors were introduced using lithium acetate/polyethylene glycol transfection with herring testis carrier DNA into Y190 yeast strain cDNA libraries were then similarly introduced and transformed cells were grown on agar plates containing a synthetic dropout medium (BD Bioscience) without leucine, tryptophan, histidine, and with 25 mM 3-amino-1,2,4-triazole. Colonies that grew on the selection medium were transferred to a filter and assayed for β-galactosidase activity with substrate X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Plasmid DNA was extracted using a glass bead disruption method and were amplified by transformation and lysis of DH5α bacteria. Plasmids were then retransformed into yeast containing the bait to confirm binding and grown on the same agar plates as described above. After X-gal test plasmids were sequenced.
SUMO Paralogue Binding Specification

To compare the strength of interaction between SUMO1 and SUMO2, 1.5 μg of each plasmid were retransformed in yeast containing SUMO1 and SUMO2 in parallel and grown on the agar plates described above. After 6 days three different colonies (if colonies grew at all) were replicated to another agar plate. After 3 days colonies were transferred to a filter to compare the growth of yeast containing SUMO1 and SUMO2 and the interacting partners.

Plasmids and Mutagenesis—HA-TTRAP-pcDNA3 plasmid was described previously (18). HA-TTRAP SUMO-binding mutants were generated with site-directed mutagenesis by PCR using QuikChange (Stratagene). HA-TTRAP SIM mutant was constructed using the primer pair 5′-TTGCCCAAAAACTCCCAGGCAGCCGCAGCGTTGTTGGG-3′ and 5′-CAAAACCTCCCAGACATCCGCAGCGGTGTTTGGTGGTAA-3′ introducing two further alanines and primer pair 5′-CCCCAC-AACGCTGCGGCTGCCTGGGAGTTTTTGGGCAAA-3′ and 5′-TTTGCCCAAAAACTCCCAGACATCCGCAGCGGTGTTTGGTGGG-3′ into two further alanines. GST-SUMO2 and YFP-UBC9 were kindly provided by Danny Huylebroeck. FLAG-PIAS3 was kindly provided by Helene Boeuf and FLAG-Sp100 —All these techniques were done as described before (19).

Acidic deletion mutants were constructed using site-directed mutagenesis. Sequences of the primers are available upon request. FLAG-PIAS3 was kindly provided by Helene Boeuf and FLAG-Sp100 and EGFP-Sp100 by Hans Will.

Cell Culture and Transfections, Cell Lysis, GD Pulldown, SDS-PAGE, and Western Blot—All these techniques were done as described before (19). The HA antibody was used from Santa Cruz Biotechnology, FLAG M2 antibody form Sigma and GFP/YFP antibody form BD Bioscience.

Protein Expression and Purification for NMR Studies—Full-length SUMO1 and SUMO2 were cloned as GST fusions into pET-41a vectors (Novagen), expressed in bacteria on either LB medium (for non-marked protein) or minimal medium with 15N-labeled SUMO1 or SUMO2 (300 mM in 25 mM phosphate buffer, pH 7). Triple resonance and dynamic acid together with the extracted peptides. The obtained solution was pipetted onto the MALDI plate avoiding to pipette gel bits. Measurements were made in reflector-positive mode, low mass gate set at 500 Da, and monitoring the 1–3-kDa range.

RESULTS

Identification of SUMO1- and SUMO2-interacting Partners—To identify proteins that non-covalently bind to SUMO1 and SUMO2, we fused SUMO1 and SUMO2 mutants lacking two C-terminal glycine residues to the Gal4-DNA-binding domain of the YTH bait vector and performed large scale yeast two-hybrid screens using human thymus, spleen, and kidney libraries. After retransformation and X-gal tests we sequenced 102 SUMO1-interacting and 77 SUMO2-interacting partners. In this collection, we subsequently identified about 20 different candidate SUMO-interacting proteins, including SUMO-conjugating enzyme (UBC9), thymine DNA glycosylase (TDG), TOPORS, four members of the PIAS family, and RanBP2, which have been previously shown to interact with SUMO1 or SUMO2 (13, 14, 26). Altogether, we identified 10 new candidate SUMO-interacting proteins for which no data about sumoylation or SUMO interaction are available (Fig. 1A).

Among these proteins there were several zinc finger-containing proteins including ZCCHC7, ZCCHC12, ZNF237, ZNF198, and ZHX1 involved in different processes like DNA repair or transcriptional repression (27, 28). Moreover, Senataxin, a newly identified helicase mutated in patients suffering from ataxia-ocular apraxia 2, was found to interact with SUMO proteins in our screens (29). So far, two helicases,

Bioinformatical Analysis—All sequence data base searches were performed with a non-redundant data set constructed from current releases of Swiss-Prot, TrEMBL, and GenPept (21, 22). Generalized profile construction (23) and searches were run locally using the pftools package, version 2.1. Generalized profiles were constructed using the BLOSUM65 substitution matrix (24) and default penalties of 2.1 for gap opening and 0.2 for gap extension. The statistical significance of profile matches was derived from the analysis of the score distribution of a randomized data base (25).
the BLM helicase and Werner’s helicase, are known to be sumoylated and play important roles in causing hereditary diseases (30, 31). The findings of new SUMO-interacting partners underline the important function of SUMO signals during transcription, DNA repair, and chromatin remodeling (32).

We next investigated whether these SUMO-interacting proteins bind preferentially to SUMO1 or SUMO2 in yeast cells. We retransformed all clones found to bind to SUMO1 in yeast expressing SUMO2 and in yeast expressing SUMO1 in parallel. As a control, we transformed empty prey vector in yeast containing SUMO1 and SUMO2, while PIAS1, which was shown to interact strongly with SUMO1 and SUMO2, was transfected with PIAS1, PIASx, or UBC9 using HEK 293T cells transfected with PIAS1, PIASx, or UBC9 (Fig. 1C). As expected, SUMO3 bound with a similar affinity to these proteins as SUMO1 and SUMO2 did.

Characterization of SUMO-interacting Motif in TTRAP—TTRAP was originally found as a protein interacting with members of the tumor necrosis factor receptor (TNF-R) superfamily as well as several TRAFs (18). Since TTRAP bound more potently to SUMO2 than SUMO1 in yeast (Fig. 1B), we analyzed the interactions between SUMO1 and SUMO2 with TTRAP in more details. When cell lysates expressing HA-TTRAP were incubated with beads coupled to GST, GST-SUMO1, GST-SUMO2, or GST-SUMO3, TTRAP bound more potently to SUMO2 and SUMO3 than to SUMO1 (Fig. 2, A and B). TTRAP did not contain one of the published SUMO-binding domains. However, we could find the sequence I-V-D-V at positions 280–284, which is the inversion of the proposed V/I-X-V/I-V/I
SUMO-interacting motif (14). Recently, it was shown that this hydrophobic part could bind both parallel and antiparallel to SUMO so that these four amino acids could be a SUMO-binding domain as well (17). We mutated all four amino acids to alanine (HA-TTRAP-SIM*) and completely abolished binding to all SUMO isoforms (Fig. 2B). Since these binding assays were performed in yeast and mammalian cells, they raised a concern whether additional cellular proteins might contribute to indirect binding between SUMO and TTRAP. To verify their interaction in vitro system, full-length TTRAP and TTRAP-SIM* were expressed and purified as a GST fusion protein in bacteria and challenged with recombinant SUMO2. As shown in Fig. 2C, SUMO2 bound to GST-TTRAP but not to GST alone or GST-TTRAP-SIM*. This result confirmed that the SIM of TTRAP directly interacts with SUMO2 and that this signature motif is essential for SUMO binding to full size TTRAP.

Definition of a Universal SIM—Three different amino acid signature motifs have been proposed to mediate binding to SUMO (Fig. 3A). To identify minimal SIMs in newly cloned SUMO-interacting proteins, the sequences of the clones were subjected to bioinformatical analyses. All of the three proposed sequence motifs implicated in binding to SUMO are represented in both known and new SUMO-interacting partners pooled in our screens (Fig. 3B). Interestingly, several SUMO-interacting partners contain two SUMO-binding domains. Some SUMO-interacting partners, including TTRAP, MCAF, and ZCCHC12, do not contain the complete characteristics of any of the published domains but rather represent the inversion of motif 2 (Fig. 3B).

An alignment of all SUMO-interacting motifs revealed that they all harbored a hydrophobic core sequence consisting of stretches of three or four hydrophobic lle, Leu, or Val residues plus one acidic/polar residue at position 2 or 3. The sequences surrounding this core-binding domain are predicted to be disordered and have a net negative charge due to a stretch of acidic amino acid residues (Fig. 3C). The stretch of acidic amino acids can be either at the \( C \)- or at the N-terminal site of the hydrophobic core. Furthermore, a spacer containing a conserved threonine can separate the hydrophobic part from the acidic one. Interestingly, the majority of SIMs contains one or more serines or threonines, being potential phosphorylation sites in vivo.

Biophysical Parameters Underlying Binding of SIMs to Different SUMO Paralogues—We used NMR spectroscopy to gain detailed insights into the binding of different SIM motifs to SUMO1 or SUMO2. In typical NMR titrations, binding of a ligand to a protein influences the environment of the atoms of the protein, especially in the binding interface between the protein and the ligand. Such perturbations are easily observable as modification of the frequency and intensity of resonances in HSQC spectra during a titration experiment. We used the already published assignment of SUMO1 (34) and measured a set of three-dimensional spectra to assign the resonances of all atoms in the backbone and side chains of SUMO2 (supplemental Fig. 1).

To characterize the binding interface on SUMO paralogues and different SIMs we chose to study the SIMs of PIASxx, which interact with equal strength with SUMO1 and SUMO2 (Fig. 1, B and C) and contain a stretch of acidic amino acids and several putative serine phosphorylation sites. We also analyzed the SIM of TTRAP, which interacts predominantly with SUMO2 in vivo and in vitro experiments and lacks the acidic tract. Thus, three different PIASxx peptides and one TTRAP peptide were synthesized to investigate the binding characteristics of SUMO1 and SUMO2 to these peptides (Fig. 4A and supplemental Fig. 2).

Most of the amino acids in slow exchange in the titrations of SUMO1 and SUMO2 with SIMPIASxx are found in the \( \beta_2 \)-strand and \( \alpha \)-helix of these proteins (Fig. 4, B and C). The \( K_D \) associated with the amino acids in slow exchange is estimated to be 3 \( \mu M \) for SUMO1 and 2 \( \mu M \) for SUMO2, which is in good agreement with the results derived from isothermal titration calorimetry measurements (14).

The binding surface of the other peptides (SIMTTRAP, SIMPIASxx*, and SIMPIASxx short) was found to be on the same position on SUMO1 and SUMO2 showing that this surface represents a general binding surface.
for the SIM on SUMO (Fig. 4D). Since this surface is partly constituted by a side of the β-sheet of SUMO, and the hydrophobic core of the SIM has a sequence typical of a β-strand, we propose that the hydrophobic core of the SIM binds to the β₂-strand of SUMO by forming an intermolecular β-sheet.

Serine Phosphorylation in the SIM of PIASxα Regulates Its Binding to SUMO1—According to the $K_D$ values SIMPIASxα binds with similar affinity to SUMO1 and SUMO2. However, the curves of these two titrations have different shapes: Whereas the curves obtained in the titration of SUMO2 with SIMPIASxα have the expected shape for a simple 1:1 protein to peptide binding, the curves obtained in the titration of SUMO1 with this peptide have an unexpected sigmoidal shape (Fig. 5A). Such curves are also observed in the titration of SUMO1 with SIMPIASx short. The titration curves of SUMO1 and SUMO2 with SIMPIASxα short have the classical shape, showing that the phosphorylation of the SIM is sufficient to change from one to the other binding.

FIGURE 3. Definition of universal SIM. A, three published SUMO-binding motifs: “h” represents a hydrophobic amino acid and “a” an acidic one. B, summary of the clones found in all screens, their Swiss-Prot IDs, and their names and strength of interaction to SUMO1 and SUMO2 in the screen are shown. Some SUMO-interacting partners like TTRAP, MCAF, and ZCCHC12 do not contain one of the published domains. C, alignment of SUMO-interacting motifs of the yeast clones. All motifs contain a hydrophobic core (blue), acidic amino acids (red), and/or potential phosphorylation sites (brown).

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**A**

Motif 1: $h-X-[S(T)]-X-[S(T)]-a-a$

Motif 2: $[V/I]-X-[V/I]-[V/I]$

Motif 3: $K-X_{957}-[I/V]-[I/L]-[I/L]-X_{975}-[D/E/Q/N]-[D/E]-[D/E]$
FIGURE 4. Binding surface analysis. A, alignment of peptides used for SUMO titrations. Negatively charged amino acids are in red, and positively charged amino acids are in blue. Phosphorylation is represented by a circled P. B, table with amino acids of SUMO1 and SUMO2 involved in binding to PIASxα and TTRAP peptides. Amino acids in slow exchange are indicated in bold and amino acids in intermediate exchange in regular format. Average of the $K_D$ values for the amino acids in slow exchange are in the last line. C, alignment of the three SUMO isoforms with the location of secondary structure elements, which include four β-strands and one α-helix. Amino acids involved in interaction with peptides are shown in pink. β1 is shown in purple, β2 in blue, β3 in yellow, β4 in red, and the α-helix in green. Secondary structure elements of SUMO1 are represented in the same colors as described for SUMO Paralogue Binding Specification.

D, binding surface analysis of SUMO1 with the SIMPIASxα peptide (left), of SUMO2 with the SIMPIASxα peptide (middle), and of SUMO2 for SIMTTRAP peptide (right). Amino acids in slow exchange at saturation are in fuschia, and amino acids in intermediate exchange are in blue.
mode. Three explanations can be invoked for the sigmoid shape of the titration curves of SUMO1 with SIMPIASx/H9251. The first possibility is that these peptides could have two binding interfaces on SUMO. However, our results show no evidence for a second binding site on SUMO, and the small size of SIMPIASx/H9251 short makes it unlikely that it binds SUMO through two different sites. The second possibility is that SIMPIASx binds in different orientations to SUMO1, which can be ruled out according to the underlying calculations. Alternatively,

![Graph showing titration curves of SUMO1 with SIMPIASx/H9251 and SIMPIASx short](image)

**FIGURE 5.** Phosphorylation and negatively charged amino acids within the SIM alter binding to SUMO isoforms. **A,** comparison of titration curves obtained for SUMO1 or SUMO2 with SIMPIASx, SIMPIASx short, or SIMPIASx short. The peaks of the path are in arbitrary units resulting from normalization of $^1$H and $^{15}$N shifts. The values for SUMO1-SIMPIASx short have been multiplied by 10 for the sake of eligibility. **B,** binding surface analysis of SUMO1 for SIMPIASx. **C,** binding surface analysis of SUMO1 for phosphorylated SIMPIASx. Lys 37 is shown in yellow. **D,** binding surface analysis of SUMO2 for SIMPIASx. **E,** list of PIAS tryptic fragments containing the SIM and detected in a phosphorylated and/or unphosphorylated form by MALDI spectrometry. **Bottom,** schematic diagram of the domain structure of PIASxα.
SUMO Paralogue Binding Specification

those peptides have only one binding site on SUMO which confor-
mation can be changed upon binding of the peptide (induced fit).

When SUMO1 titrations with SIMpPIASx and SIMpPIASx are com-
pared, it appears that the only amino acid that has a significantly differ-
ent behavior in these two titrations is Lys34, which is in fast exchange
with SIMpPIASx and in fast intermediate exchange with SIMpPIASx.
This shows that the phosphate group of SIMpPIASx is likely to bind to
SUMO1 in the neighborhood of this lysine (the same difference is
observed for the Lys34 of SUMO2, which is equivalent to the Lys37 of
SUMO1, confirming this hypothesis). The Lys37 of SUMO1 and the
Lys38 of SUMO2 are situated at the end of the /3-strand, which is much
more bent in SUMO1 than in SUMO2. In close proximity to the Lys37
of SUMO1 and the Lys34 of SUMO1 is another lysine residue (Lys39 in
SUMO1 and Lys36 in SUMO2) that cannot be observed due to HSQC
peaks overlap and whose conformation is different in both SUMO iso-
forms: the Lys39 side chain of SUMO1 points into the binding site,
whereas the Lys36 of SUMO2 points away from the SIM-binding site.
Therefore it could be possible that the negatively charged phosphate
group of SIMpPIASx interacts with the positively charged Lys39 of
SUMO1 causing a conformational change favorable for SIMpPIASx
binding and strongly affecting the neighboring Lys37. The absence of
a phosphate group in SIMpPIASx would make this transition more dif-
ficult to achieve. A high SIMpPIASx concentration would be required to main-
tain SUMO1 in the binding-favorable conformation, producing the
observed two-step titration curve. Whether phosphorylation is present
or not does not influence the binding of PIAS peptides to SUMO2 much,
since the conformation of Lys34 is already favorable. The recently
published structures of SUMO1 in complex with a PIAS derived peptide
(17) and with a RanBP2 derived peptide (16) confirm this hypothesis.
In both structures the end of the /3-strand of SUMO1 is less bent than in
the free SUMO1 (6), and the Lys39 side chain of SUMO1 moved away
from the SIM-binding site to accommodate the peptide.

We note that the interaction between the Lys37 of SUMO1 and the
phosphate group of SIMpPIASx indicates the orientation in which the
SIM binds to SUMO: to take place while having the /3-strand of SUMO
binding the hydrophobic core of the SIM, those two elements must be
parallel to each other. This has been recently verified by the structure
published by Song et al. (17).

PIASx Is Phosphorylated within the SIM in Vivo—These biophysi-
cal findings indicate that serine phosphorylation of the SIM may be
relevant for in vivo interactions between SUMO1 and the SIMpPIASx.
Accordingly, mutations of corresponding serines to alanines in the
SIMpPIASx, revealed that these serines are required for its binding to
SUMO1 in yeast cells (13). We therefore investigated the phosphory-
lation pattern of the SIM of PIASx in cells. MALDI fingerprinting
was used to verify in vivo phosphorylation of PIASx at the putative
phosphorylation site within the SIM. Masses corresponding to dif-
f erent phosphorylated fragments upon trypsin digestion contained in
the SIM were observed in several spectra, in addition to less fre-
quently observed masses corresponding the same fragments without
phosphate incorporated (Fig. 5E; a more detailed table is given in
the supplemental Fig. 3). This shows that the PIASx proteins are phos-
phorylated within the SIM in vivo and indicates that this modifica-
tion may be of functional importance for binding of SUMO1 to SIM
in PIAS proteins.

We next tested whether phosphorylation of SIM is essential for bind-
ing to SUMO isoforms in GST pulldown assays. In contrast to previ-
ously published data whereby mutations of the corresponding serines to
alanines in the SIM of PM-ScI75 blocked their binding to SUMO1 in
yeast cells (13), we have not observed a significant decrease upon muta-
tion of all three serine residues to alanine within the SIM motif of
PIASx (data not shown). This could be explained by a compensa-
tory interaction between the negatively charged amino acid tract of
SIMpPIASx and SIMpPIASx with Lys37 of SUMO1 (Figs. 5, B and C).
This interaction results in the similar affinity of non-phosphorylated
and phosphorylated PIAS peptides binding to SUMO1 and SUMO2 (see
below). Therefore, it is possible that PIAS phosphorylation in vivo may
modulate the spatial orientation rather than affinities of PIAS binding
to its sumoylated targets.

Contribution of Acidic Amino Acids in SIMs for SUMO1 and SUMO2
Binding—A number of negatively charged amino acids (Glu, Asp)
are present in the SIM of most proteins found in the yeast two-hybrid
screens, which indicates that they may play a regulatory role in bind-
ing to SUMO. To investigate their role, we studied the binding of SUMO
to SIMpPIASx short (Fig. 4A), a variant of the PIASx peptide lacking the
negatively charged amino acids tract. This peptide binds to SUMO1
with much lower affinity than SIMpPIASx (no resonance of any amino
acid in the SIM-binding site is observed to be in the slow exchange
regime) (Fig. 4B). However, the resonance of amino acids Ile32, Phe36,
Val38, Lys39, Lys45, and Ser50 are in intermediate exchange. The K_D
value can be estimated to be higher than for SIMpPIASx and SIMpPIASx
and lower than 0.2 mm, a value obtained from titration curves of amino acids
of SUMO outside the SIM-binding site. When studying the titration
of SUMO1 with SIMpPIASx and with SIMpPIASx, the resonance of Lys37
(situated in the loop between the /3a- and /3b-strands) was in slow
exchange, whereas it was not in the SIMpPIASx short titration experi-
ment. This further supports the notion of an interaction between the nega-
tively charged amino acid tract of SIMpPIASx and SIMpPIASx with Lys38
of SUMO1 (Fig. 5, B and C). This interaction results in the above
described higher affinity of SUMO1 for SIMpPIASx and SIMpPIASx than
for SIMpPIASx short. In contrast, results obtained in the titration
of SUMO2 with SIMpPIASx short are fairly similar to those obtained in
the titration of SUMO2 with SIMpPIASx and SIMpPIASx. Furthermore,
SIMTTRAP, which has no tract of negatively charged amino acids, binds
to SUMO2 than to SUMO1. Taken together, these data show that
negatively charged residues in the SIM make an important contribu-
tion to binding to SUMO1 but are only little involved in SUMO2 binding.

Acidic Amino Acids Influence Binding to SUMO1 but Not to SUMO2
in Vitro and in Vivo—To examine whether the observation that acidic
amino acids of SIMs influence binding to SUMO paralogues is a general
phenomenon, we performed mutation studies and analyzed SUMO
binding in both yeast and pulldown assays. We deleted the acidic part of
several SUMO-interacting partners in a similar way as PIAS short pep-
tide was created (Fig. 6A). These mutants were transformed into yeast
or were used for GST pulldown assays. The yeast deletion mutants
(Sc100, Senataxin, PIAS4, TOPORS) were transformed into yeast
expressing SUMO1 and SUMO2 in parallel with the corresponding wild
type SUMO-interacting partners. To compare the growth of yeast three
different colonies of each retransformation were replated on a fresh agar
plate with the same dropout medium. After 2 days the yeast was
transferred to a filter paper, and the growth of yeast expressing SUMO1 or
SUMO2 and either wild type or mutant constructs was compared (Fig.
6B). In all cases yeast containing SUMO1 and the acidic deletion
mutants grew much slower than yeast containing SUMO2 and the dele-
tion mutants, while yeast transformed with the wild type clones grew
with equal speed in both cases. Furthermore we deleted acidic amino
acids of the SIM in several SUMO-interacting partners (Sc100, PIAS1,
PIAS3) and expressed them in mammalian cells. In GST pulldown
assays with GST-SUMO1, GST-SUMO2, and GST-SUMO3, the results
were similar to those obtained with the yeast two-hybrid system: bind-
ing to GST-SUMO1 was reduced or even abolished, whereas binding to SUMO2/3 was unaltered (Fig. 6C). Taken together, these results show that acidic amino acids and negative charges are important for binding to SUMO1 but not to SUMO2.

The SIM of TTRAP does not contain a negative amino acid tract (Fig. 3C) and was shown to preferentially bind to SUMO2 compared with SUMO1 both in vitro and in vivo (Figs. 1B, 2A, and 4B). We were further interested in analyzing whether addition of acidic charged amino acids in TTRAP would switch its binding preference toward SUMO1 in the context of full protein. The amino acids Leu286, Gly287, and Lys288 were mutated to the negatively charged acidic amino acids Glu, Asp, and Glu, respectively. In GST pulldown assays of TTRAP wild type and TTRAP mutant, we found that the negatively charged SIM of the TTRAP mutant gains ability to bind to SUMO1, whereas the binding to SUMO2 decreased compared with the binding between TTRAP wild type and SUMO2 (Fig. 6D). Thus, in this case introduction of negatively charged amino acids shifts the interaction from preferential SUMO2 to SUMO1 binding.

**DISCUSSION**

Protein modifications mediated by conjugation of SUMO to target proteins represent an emerging mechanism by which cells control distinct cellular functions (35). While our understanding of the mechanisms of protein conjugation by SUMO are quite advanced (4, 16, 33, 36), much remains to be understood on how these modifications are translated into different biological responses. It has already been described that SIMs bind to SUMO1 via their hydrophobic core. In this report we describe molecular and biophysical parameters underlying the interactions between SUMO1 and SUMO2 and SIMs in more detail.

Several recent publications nicely describe the binding of the hydrophobic core to be the main mediator of SUMO binding. Our mutation studies in TTRAP support these results indicating that few hydrophobic amino acids are sufficient to mediate binding to SUMO. However, our in vivo studies in yeast show a completely different picture: only 1 out of 20 different yeast clones exclusively contains the hydrophobic core. All the others additionally contain acidic amino acids and sometimes putative phosphorylation sites. This suggests that amino acids surrounding the hydrophobic core also influence binding to SUMO.

Three different SUMO isoforms are expressed in eukaryotes, which differ mostly in the amino acid composition of the β-strand and the α-helix (7), exactly the regions that we found to mediate binding to SIMs. This surface of SUMO can thus be regarded as a “code of specificity” of SUMO isoforms for the SUMO-SIM interaction. Interestingly, this region has been shown to be critical for the transcriptional inhibitory properties of SUMO (37). As the signature of the SIM motif is limited to the short β-strand forming motif, it should be possible to find the intermolecular strand either in parallel or antiparallel orientation, as long as backbone hydrogen binding can occur and the hydrophobic side chains are arranged in an inverse (reverse) manner. Indeed, we and others (16, 17) found the PIASxx peptides bound parallel to the

![FIGURE 6. Deletion of the acidic amino acids of the SIM influences SUMO1 much stronger than SUMO2 binding. A, acidic amino acids were deleted in several SUMO-interacting partners according to the creation of the PIAS short peptide described before. The table shows the deleted part and amino acids. B, wild type and mutants of SUMO-interacting partners were retransformed in yeast containing SUMO1 and SUMO2 in parallel. Growth was analyzed as described before (Fig. 1B). C, lysates from HEK 293T cells expressing wild type FLAG-SP100, FLAG-PIAS1, or FLAG-PIAS3 and their corresponding mutants lacking the acidic amino acids of the SIM (EGFP-SP100 MUT, FLAG-PIAS1 MUT, or FLAG-PIAS3 MUT) were incubated with GST alone or GST-SUMO1/2/3 and analyzed by SDS-PAGE and subsequent Western blotting with indicated antibodies. Levels of GST fusion proteins were determined by Ponceau S staining. TCL, total cell lysate. D, GST pulldown of HA-TTRAP wild type and HA-TTRAP mutant containing additional amino acids. The mutant with acidic amino acids binds stronger to SUMO1 but not to SUMO2, which is opposite from the wild type protein.]

A | SUMO interacting partners | Deletion part | Deleted amino acids |
--- | --- | --- | --- |
Senataxin | 1020-1026 | DDDDDDE |
PIAS3 | 446-452 | DEED |
PIAS4 | 476-493 | EDEEEEEEEDDEE |
Sp100 | 329-333 | ED9E |
TOPORS | 911-916 | DSSD DSSD |
PIAS1 | 464-474 | DSSD DSSD |

B | SUMO1 | SUMO2 |
--- | --- | --- |
Sp100 wt | | |
mut | | |
Senataxin wt | | |
mut | | |

C | | WB: Anti-FLAG | TLC | GST | GST-SUMO1 | GST-SUMO2 | GST-SUMO3 |
--- | --- | --- | --- | --- | --- | --- | --- |
SP100 WT | Ponceau S staining | Ponceau S staining | Ponceau S staining |
SP100 MUT | Ponceau S staining | Ponceau S staining |
PIAS3 WT | Ponceau S staining | Ponceau S staining |
PIAS3 MUT | Ponceau S staining |

D | | WB: Anti-FLAG | TLC | GST | GST-SUMO1 | GST-SUMO2 | GST-SUMO3 |
--- | --- | --- | --- | --- | --- | --- | --- |
Acidic mutant HA-TTRAP | Wild type HA-TTRAP | Ponceau S staining | Ponceau S staining | Ponceau S staining | Ponceau S staining |

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SUMO Paralogue Binding Specification

The presented concept of surrounding charges determining the orientation of a peptide binding to SUMO resembles the binding mode of polypeptide stretches when interacting with SH3 domains. The polypeptide type II helices bind to SH3 domains in both N-C and C-N directions while forming similar hydrogen bond networks. In analogy to the basic amino acid tract flanking PXXP motifs, a negatively charged tract assists binding in the case of SIM motifs. The salt bridge formed between a positively charged residue in the flanking region of the polypeptide type II helix to a negative residue in the SH3 domain resembles the strong binding between Lys\(^am\) of SUMO1 and the negatively charged residues following the hydrophobic core of the SIM of PIASx. The similarity found in the binding of polypeptide stretches and SH3 to the SIM-SUMO interaction entails us to define SIM ligands binding in antiparallel orientation to the β\(_2\)-strand of SUMO as class I ligands (RanBP2) and those binding in parallel orientation as class II ligands (PIASx).

Although the structures of SUMO and Ub are very similar, the binding surfaces of SUMO-SIM versus Ub-binding domains (UBDs) are clearly different (Fig. 7). At present, all currently tested SIMs show exclusive binding to SUMO isoforms but not to Ub or other Ub-like molecules. Although two other Ub-like proteins (NEDD8 and FAT10) have been implicated to bind to UBDs (38, 39), no known Ub-binding domain was shown to associate with SUMO isoforms, thus implicating that SUMO-SIM recognition is much more specific than Ub/NEDD8/FAT10 association with UBDs. In contrast to the formation of an intermolecular SIM-SUMO β-sheet, the interaction between UBDs and Ub is defined by a hydrophobic surface containing Ile44 (40). This hydrophobic surface would correspond to the β\(_2\)-strand of SUMO, which is located to the opposite side of the molecule in relation to the β\(_2\)-strand where the SIM binds (Fig. 6). In principle all amino acids in the close vicinity of Ile44, which are exposed to the surface, can serve as key elements defining the specificity of UBDs. Despite these structural differences, the SUMO-SIM and the Ub-UBD interactions serve the same purpose: they are signaling pairs that transmit intracellular signals and regulate numerous cell functions.

In conclusion, this study describes the molecular details of specific interactions between SUMO1 and SUMO2 paralogues and SIMs and indicates the rational for their functional differences in vivo.

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REFERENCES
