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 β-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).

The structures of all three SUMO paralogues resemble the globular and compact Ub-like fold (6, 7). The differences of SUMO1 and SUMO2 are mostly found in the second β-strand and the α-helix of both proteins (7). In cells, different SUMO paralogues appear to share common properties but also have some distinct functions. For example, the promyelocytic leukemia protein is conjugated to all three SUMO paralogues (8, 9), whereas RanGAP1 is preferentially modified with 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 isomers 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-Xaa₃₋₅-[Val/Ile]-[Ile/Leu]₂-Xaa₃₋₅-[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 PIASx 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 PIASx 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 Sall 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 QuiChange (Stratagene). HA-TTRAP SIM mutant was constructed using the primer pair 5’-TTACCCCAACACGGCTGGGATGTCTGGGAGTTTTTGTTGGGCTGGTAA-3’ and 5’-CAAAGAACCAGACATCCGGACGGTTGTTTTTGTTTTGGGCAAA-3’ and 5’-TTGGGGGAAAAACACTCCCAGGCAGCCGCAGCGTTGTTGGG-3’ to introduce two further alanines and primer pair 5’-CCCAAC-AACGGCTGGGCTGGGATGTCTGGGAGTTTTTGTTGGGCTGGTAA-3’ and 5’-TTGGGGGAAAAACACTCCCAGGCAGCCGCAGCGTTGTTGGG-3’ to introduce two further alanines. GST-SUMO2 and YFP-UBC9 were kindly provided by Frauke Melchior and FLAG-PIAS1, GST-SUMO1, and FLAG-PIAS2 (PIASx) by Jorma Palvimo. GST-TTRAP was generously provided by Danny Huylebroeck.

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, GST 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 radiolabeled glucose for labeled protein. The recombinant protein was purified on a GSH resin (Amersham Biosciences) according to manufacturer’s instruction and cleaved with thrombin, leaving the extension Gly-Ser in N-term of the full length SUMO; concentrations (Amicon) were used for tag removal and concentration. The protein was lyophilized and stored at −20 °C.

NMR Spectra Acquisition and Assignment—All measurements were made at 27 °C in 25 mM Phosphate buffer at pH 7. Triple resonance and two-dimensional experiments were performed on a Varian Inova 600 equipped with shielded Z gradients. Three-dimensional NMR spectra were processed using the standard Bruker software XWINNMR. Two-dimensional NMR spectra were processed using NMRpipe (42). Analysis and visual representation of two-dimensional spectra were performed using Sparky (T. D. Goddard and D. G. Kneller, University of California, San Francisco), and three-dimensional spectra were analyzed with the program Aurelia (Bruker). Assignment of SUMO2 was generated using the spectra HNCA, HNCACB, CBCA(CO)NH, H(CO)NH, and C(CO)NH.

NMR Titration Experiments—For peptide titration experiments heteronuclear single quantum coherence (HSQC) spectra were performed on 15N-labeled SUMO1 or SUMO2 (300 mM in 25 mM phosphate buffer, pH 7). Unlabeled peptides were chemically synthesized by Thermo Electron GmbH, dissolved to a concentration of 2.7 mM in 25 mM phosphate buffer, pH 7, and titrated to the protein to reach a final peptide:SUMO ratio of 1:3.6:1. No precipitation could be observed even at the highest peptide concentrations.  

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 YTH9 bait vector and performed large scale yeast two-hybrid screens using human thymus, spleen, and kidney libraries. After transformation 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,
SUMO Paralogue Binding Specification

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. The same was done for all the clones found to bind to SUMO2. 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 transformed into the empty yeast strain. To compare the binding affinity to SUMO1 and SUMO2 we re-plated three different colonies of each retransformation on another agar plate with appropriate drop out medium. After 2 days yeast was transferred on a filter paper to compare the growth. Most of the retransformed SUMO-interacting partners bound to SUMO1 and SUMO2 with the same strength (Fig. 1B), whereas RanBP2 preferentially bound to SUMO1, an interaction that was already shown to be important for its function (33), and TTRAP (TRAF and TNF receptor-associated protein), strongly bound to SUMO2 and poorly to SUMO1 (Fig. 1B). Nothing grew on the control plates. To compare binding affinities of SUMO1 and SUMO2 with SUMO3, we did in vitro pulldown assays for PIAS1, PIASx, and UBC9 using HEK 293T cells transfected with PIAS1, PIAS2, or UBC9 and GST-SUMO1/2/3. GST alone was used as negative control. Western blots were performed with antibodies against the FLAG epitope and YFP. Levels of GST fusion proteins were determined by Ponceau S staining. TCL, total cell lysate.

FIGURE 1. Yeast two-hybrid screen. A, table of the newly identified SUMO-interacting proteins with their Swiss-Prot ID and name. B, growth of yeast containing SUMO1 or SUMO2; each prey plasmid was retransformed into yeast containing either SUMO1 or SUMO2 as bait in parallel. Yeast was grown on agar plates containing the appropriate drop out medium. After 4 days three different colonies were replated on another agar plate and incubated at 30 degrees. After 3 days yeast was transferred to a filter and growth was compared. First column, Swiss-Prot ID; second column, name of the prey; third column, three replicates of yeast clones, retransformed with prey and either SUMO1 or SUMO2. C, confirmation of yeast two-hybrid results with GST pulldown assays for PIAS1, PIAS2 (isoform xa), and UBC9 using HEK 293T cells transfected with PIAS1, PIAS2, or UBC9 and GST-SUMO1/2/3. GST alone was used as negative control. Western blots were performed with antibodies against the FLAG epitope and YFP. Levels of GST fusion proteins were determined by Ponceau S staining. TCL, total cell lysate.
SUMO-Interacting Motif (SIM) (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 Ile, 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 PIASx, 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 PIASx 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 SIMpiasx are found in the β2-strand and α-helix of those proteins (Fig. 4, B and C). The KD associated with the amino acids in slow exchange is estimated to be 3 μM for SUMO1 and 2 μM 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, SIMpriasx, SIMpriasxshort) 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 $\beta$-sheet of SUMO, and the hydrophobic core of the SIM has a sequence typical of a $\beta$-strand, we propose that the hydrophobic core of the SIM binds to the $\beta$-strand of SUMO by forming an intermolecular $\beta$-sheet.

**Serine Phosphorylation in the SIM of PIASx Regulates Its Binding to SUMO1**—According to the $K_D$ values SIM_{PIAS} 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 SIM_{PIAS} have the expected shape for a 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 SIM_{PIAS} short. The titration curves of SUMO1 and SUMO2 with SIM_{PIAS} short have the classical shape, showing that the phosphorylation of the SIM is sufficient to change from one to the other binding
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 PIASx and TTRAP peptides. Amino acids in slow exchange are indicated in bold and amino acids in intermediate exchange in regular format. Average of the KD 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. β_s in purple, β_i in blue, β_l in yellow, Ψ in red, and the α-helix in green. Secondary structure elements of SUMO1 are represented in the same colors as described for C. D, binding surface analysis of SUMO1 with the SIM_PiASx peptide (left), of SUMO2 with the SIM_PiASx peptide (middle), and of SUMO2 for SIM_TTRAP 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,
those peptides have only one binding site on SUMO which conformation can be changed upon binding of the peptide (induced fit).

When SUMO1 titrations with SIM<sub>PIAS</sub>x and SIM<sub>pPIAS</sub>x are compared, it appears that the only amino acid that has a significantly different behavior in these two titrations is Lys<sup>37</sup>, which is in fast exchange with SIM<sub>pPIAS</sub>x and in fast intermediate exchange with SIM<sub>pPIAS</sub>x. This shows that the phosphate group of SIM<sub>pPIAS</sub>x is likely to bind to SUMO1 in the neighborhood of this lysine (the same difference is observed for the Lys<sup>39</sup> of SUMO2, which is equivalent to the Lys<sup>37</sup> of SUMO1, confirming this hypothesis). The Lys<sup>37</sup> of SUMO1 and the Lys<sup>34</sup> of SUMO2 are situated at the end of the β<sub>2</sub>-strand, which is much more bent in SUMO1 than in SUMO2. In close proximity to the Lys<sup>37</sup> of SUMO1 and the Lys<sup>34</sup> of SUMO1 is another lysine residue (Lys<sup>39</sup>) in SUMO1 and Lys<sup>36</sup> in SUMO2) that cannot be observed due to HSQC peaks overlap and whose conformation is different in both SUMO isoforms: the Lys<sup>39</sup> side chain of SUMO1 points into the binding site, whereas the Lys<sup>36</sup> of SUMO2 points away from the binding-site. Therefore it could be possible that the negatively charged phosphate group of SIM<sub>pPIAS</sub>x interacts with the positively charged Lys<sup>39</sup> of SUMO1 causing a conformational change favorable for SIM<sub>pPIAS</sub>x binding and strongly affecting the neighboring Lys<sup>37</sup>. The absence of a phosphate group in SIM<sub>pPIAS</sub>x would make this transition more difficult to achieve. A high SIM<sub>pPIAS</sub>x concentration would be required to maintain 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 Lys<sup>34</sup> 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 β<sub>2</sub>-strand of SUMO1 is less bent than in the free SUMO1 (6), and the Lys<sup>39</sup> side chain of SUMO1 moved away from the SIM-binding site to accommodate the peptide.

We note that the interaction between the Lys<sup>37</sup> of SUMO1 and the phosphate group of SIM<sub>pPIAS</sub>x indicates the orientation in which the SIM binds to SUMO: to take place while having the β<sub>2</sub>-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 biophysical findings indicate that serine phosphorylation of the SIM may be relevant for in vivo interactions between SUMO1 and the SIM<sub>pPIAS</sub>x. Accordingly, mutations of corresponding serines to alanines in the SIM<sub>pPIAS</sub>x revealed that these serines are required for its binding to SUMO1 in yeast cells (13). We therefore investigated the phosphorylation pattern of the SIM of PIASx in cells. MALDI fingerprinting was used to verify in vivo phosphorylation of SIM<sub>pPIAS</sub>x at the putative phosphorylation site within the SIM. Masses corresponding to different phosphorylated fragments upon trypsin digestion contained in the SIM were observed in several spectra, in addition to less frequently observed masses corresponding to 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 phosphorylated within the SIM in vivo and indicates that this modification may be of functional importance for binding of SUMO1 to SIM in PIAS proteins.

We next tested whether phosphorylation of SIM is essential for binding to SUMO isoforms in GST pulldown assays. In contrast to previously published data whereby mutations of the corresponding serines to alanines in the SIM of PM-Sc175 blocked their binding to SUMO1 in yeast cells (13), we have not observed a significant decrease upon mutation of all three serine residues to alanine within the SIM motif of PIASx (data not shown). This could be explained by a compensatory interaction between the negatively charged amino acid tracts of SIM<sub>pPIAS</sub>x and SIM<sub>pPIAS</sub>x with Lys<sup>38</sup> 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 binding to SUMO. To investigate their role, we studied the binding of SUMO to SIM<sub>pPIAS</sub>x short (Fig. 4A), a variant of the PIASx peptide lacking the negatively charged amino acids tracts. This peptide binds to SUMO1 with much lower affinity than SIM<sub>pPIAS</sub>x (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 Ile<sup>42</sup>, Phe<sup>43</sup>, Val<sup>45</sup>, Lys<sup>49</sup>, Lys<sup>43</sup> and Ser<sup>50</sup> are in intermediate exchange. The K<sub>d</sub> value can be estimated to be higher than for SIM<sub>pPIAS</sub>x and SIM<sub>pPIAS</sub>x 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 SIM<sub>pPIAS</sub>x and with SIM<sub>pPIAS</sub>x the resonance of Lys<sup>38</sup> (situated in the loop between the β<sub>2</sub>- and β<sub>3</sub>-strands) was in slow exchange, whereas it was not in the SIM<sub>pPIAS</sub>x short titration experiment. This further supports the notion of an interaction between the negatively charged amino acid trac of SIM<sub>pPIAS</sub>x and SIM<sub>pPIAS</sub>x with Lys<sup>38</sup> of SUMO1 (Fig. 5, B and C). This interaction results in the above described higher affinity of SUMO1 for SIM<sub>pPIAS</sub>x and SIM<sub>pPIAS</sub>x than for SIM<sub>pPIAS</sub>x short. In contrast, results obtained in the titration of SUMO2 with SIM<sub>pPIAS</sub>x are fairly similar to those obtained in the titration of SUMO2 with SIM<sub>pPIAS</sub>x and SIM<sub>pPIAS</sub>x. Furthermore, SIM<sub>TTRAP</sub>, which has no tract of negatively charged amino acids, binds better to SUMO2 than to SUMO1. Taken together, these data show that negatively charged residues in the SIM make an important contribution 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 peptide was created (Fig. 6A). These mutants were transformed into yeast or were used for GST pulldown assays. The yeast deletion mutants (Sp100, 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 deletion 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 (Sp100, 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-

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**SUMO Parologue Binding Specification**

VOLUME 281 • NUMBER 23 • JUNE 9, 2006

16124 JOURNAL OF BIOLOGICAL CHEMISTRY
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 SUMO2/3.

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...
that SUMO-SIM recognition is much more specific than Ub/NEDD8/FAT10 association with UBPs. In contrast to the formation of an intermolecular SIM-SUMO β-sheet, the interaction between UBPs and Ub is defined by a hydrophobic surface containing Ile 44 (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 Ile 44, which are exposed to the surface, can serve as key elements defining the specificity of UBPs. 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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