Ubiquitin-dependent Proteolytic Control of SUMO Conjugates*

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Post translational protein modification with small ubiquitin-related modifier (SUMO) is an important regulatory mechanism implicated in many cellular processes, including several of biomedical relevance. We report that inhibition of the proteasome leads to accumulation of proteins that are simultaneously conjugated to both SUMO and ubiquitin in yeast and in human cells. A similar accumulation of such conjugates was detected in Saccharomyces cerevisiae ubc4 ubc5 cells as well as in mutants lacking two RING finger proteins, Ris1 and Hex3/Slx5-Slx8, that bind to SUMO as well as to the ubiquitin-conjugating enzyme Ubc4. In vitro, Hex3-Slx8 complexes promote Ubc4-dependent ubiquitylation. Together these data identify a previously unrecognized pathway that mediates the proteolytic down-regulation of sumoylated proteins. Formation of substrate-linked SUMO chains promotes targeting of SUMO-modified substrates for ubiquitin-mediated proteolysis. Genetic and biochemical evidence indicates that SUMO conjugation can ultimately lead to inactivation of sumoylated substrates by polysumoylation and/or ubiquitin-dependent degradation. Simultaneous inhibition of both mechanisms leads to severe phenotypic defects.

Small ubiquitin-related modifier (SUMO), which is structurally related to ubiquitin, is conjugated posttranslationally to a large number of substrates (1–5). The enzymes mediating SUMO conjugation are similar to those that catalyze the transfer of ubiquitin (3, 4). The functions of these two modifications, however, are distinct. Posttranslational modification of proteins with certain types of ubiquitin chains serves as a secondary degradation signal that targets such proteins for degradation by the 26 S proteasome (6). SUMO modification, in contrast, is not thought to result in proteolytic targeting (1–3, 7). Among the many functions of SUMO modification are regulation of transcription, nuclear transport, formation of subnuclear structures, cell cycle progression, and DNA repair (1–5, 7–9). Several substrates can be modified either by ubiquitin or SUMO (10). Modification of PCNA on a specific Lys residue by ubiquitylation mediates DNA repair (10, 11). Sumoylation of the same Lys, in contrast, mediates interaction with the Srs2 helicase, which results in inhibition of recombination during DNA replication (12, 13). In this example, sumoylation and ubiquitylation appear to direct proliferating cell nuclear antigen into distinct functions by promoting alternative interactions. Sumoylation of 1xBα on Lys21 has been proposed to prevent its ubiquitylation and subsequent degradation (14). It has also been shown that SUMO-1 modification of a pathogenic fragment of Huntingtin enhances stability of this fragment, whereby increasing neurodegeneration, whereas ubiquitylation reduces fragment stability (15).

Several recent studies suggested that, similar to ubiquitin, SUMO can form substrate-linked chains. In Saccharomyces cerevisiae, SUMO chain formation does not appear to serve an essential function (16). The reduced ability to remove SUMO chains in the ulp2Δ mutant, however, appears to be the main reason for its severe phenotypic defects (16). In mammals, SUMO-2 and SUMO-3 form chains, whereas SUMO-1, which lacks the appropriate Lys residues close to the N terminus, apparently does not (17). The physiological role of SUMO chains in dividing cells remains largely unclear. They appear to be involved in synaptonemal complex formation in meiosis (18).

The levels of SUMO-conjugated versions of substrate proteins are currently thought to be regulated by a dynamic equilibrium...
librium of conjugation and deconjugation. We report here that sumoylated proteins can, in addition, be proteolytically down-regulated by the ubiquitin/proteasome system. In S. cerevisiae, a pair of RING type ubiquitin ligases, Rsi1 and Hex3/Slx5-Slx8, cooperate with the conjugating enzymes Ubc4 and Ubc5 in the proteolytic control of sumoylated substrates.

EXPERIMENTAL PROCEDURES

Yeast Strains and Methods—Yeast strains and plasmids are listed in the supplemental tables. Yeast two-hybrid interaction cloning was carried out as described using Smt3 as a bait and yeast proteins or protein fragments expressed from a genomic DNA or cDNA libraries as prey (19, 20). Positive clones were selected on dropout medium lacking adenine. Library plasmids were isolated from positive clones and analyzed by sequencing.

Western Blot Analysis of Yeast Crude Extracts—Yeast crude extracts were prepared using an alkaline lysis/trichloroacetic acid precipitation protocol as described (21). Separating and stacking gels were analyzed by Western blotting with polyclonal rabbit anti-SUMO serum or monoclonal anti-Cdc11 antibodies as described (16, 22).

Analysis of Ubiquitin-SUMO Hybrid Conjugates—Extracts from strains transformed with pKU103 (expressing His6-ubiquitin) or a corresponding empty plasmid (p416GAL1; see supplemental Table S2) were prepared as described above and processed for pulldown experiments using nickel-nitrilotriacetic acid-agarose (Qiagen) as described (21). The data shown in Fig. 3A have been reproduced once in a similar experiment.

GST Pulldown Assay—For expression of GST or GST fusion proteins, Escherichia coli strain BL21 (DE3) (Stratagene) was transformed with the corresponding plasmids. Protein expression was induced with 1 mm isopropyl β-D-thiogalactopyranoside for 3 h at 37 °C. The cells corresponding to 10 A600 nm units were collected by centrifugation, resuspended in 600 μl of phosphate-buffered saline (21) with 0.1% Triton X-100 (PBST) supplemented with a protease inhibitor mixture (Roche Applied Science), and subjected to mechanical rupture using glass beads. The cell debris was removed by centrifugation, and the supernatants were applied to glutathione-Sepharose resin (GE Healthcare). After incubation for 2 h at 4 °C with rotation, the beads were washed three times with PBST. Yeast cell extracts, prepared by mechanical rupture using mortar and pestle in PBST, were incubated with the beads for 2 h at 4 °C with rotation. The beads were then washed four times with extraction buffer, resuspended in loading buffer containing 0.1 M dithiothreitol, and incubated at 100 °C for 5 min before SDS-PAGE. GST pulldown assays shown in Figs. 1B and 3B have been reproducibly performed with similar results.

In Vitro Ubiquitylation and SUMO Binding Assays—Hex3-Slx8-dependent ubiquitylation reactions were carried out in a volume of 60 μl and contained 2 μg of ubiquitin (Sigma), 1 μg of ubiquitin-activating enzyme (Boster Biochem), 0.04 μg purified His6-Ubc4 expressed in E. coli, 1.6 μg of purified GST-Hex3-Slx8-FLAG co-expressed in E. coli, 2 μM ATP, 2 μM dithiothreitol, 30 mM Tris-HCl, pH 7.4, 5 mM MgCl2, 100 mM NaCl, and an ATP-regenerating system (creatine phosphate/phospho-creatine kinase). The reactions were incubated for 2 h at 37 °C. Results similar to those shown in Fig. 3C have been reproduced more than three times. To assay for binding of yeast SUMO conjugates, cell pellets corresponding to 20 A600 were resuspended in 500 μl of lysis buffer (50 mM Tris-HCl, pH 7.5, 10% glycerol, 10 mM MgCl2, 1 mM EDTA, 100 mM NaCl) supplemented with a protease inhibitor mixture (Roche Applied Science), 1 mM phenylmethylsulfonyl fluoride, and 10 mM N-ethylmaleimide. After incubation at 4 °C for 30 min, the cells were lysed with glass beads. The cell debris was removed by centrifugation, and the supernatants were applied to glutathione-Sepharose-bound GST-Hex3-Slx8-FLAG. After incubation for 4 h at 4 °C with rotation, the beads were washed three times with lysis buffer. Bound material was eluted by boiling the beads in SDS/loading buffer and subjected to Western blot analysis. Results similar to those shown in Fig. 4 have been reproduced three times.

Analysis of SUMO Conjugates in HeLa Cells—HeLa B cells (ECACC 85060701) were grown in Eagle’s minimum essential medium containing 2 mM glutamine, 1% nonessential amino acids, 10% fetal bovine serum, and penicillin. Treatment with 20 μM MG132 (in Me2SO; 2 μl/1 ml of culture) was done for 8 h. The cells were washed once with PBS before extract preparation using the alkaline lysis/trichloroacetic acid precipitation procedure described above. SUMO-1 was detected with a mouse monoclonal antibody (Zhymed Laboratories Inc.), SUMO-2 and SUMO-3 were detected with a rabbit polyclonal antiserum (Abcam), and α-tubulin was detected with a mouse monoclonal antibody (Sigma). For immunoprecipitation experiments with FLAG-tagged SUMO, the cells were transiently transfected using GeneJuice (Novagen) 24 h prior to the addition of MG132. Constructs used for the transfection were pCMV-2b-SUMO1 or -SUMO3, both expressing mature FLAG-tagged versions of the respective SUMO isoforms, or the empty pCMV-2b vector. For the FLAG pulldown assays, ~5 × 106 cells were suspended and washed in PBS, resuspended in 240 μl of lysis buffer (1.85 M NaOH, 7.4% β-mercaptoethanol), and incubated on ice for 10 min. After the addition of 240 μl of 50% trichloroacetic acid and incubation on ice for 15 min, the extracts were collected by centrifugation, and the pellet was washed once with ice-cold acetone. After resuspension in 240 μl of TSG buffer (0.5 M Tris base, 6.5% SDS, 12% glycerol, 100 mM dithiothreitol), the sample was used for anti-FLAG immunoprecipitations. The immunoprecipitations were performed with 240 μl of extract diluted into 10 ml of radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, protease inhibitors (Roche Applied Science), 20 mM N-ethylmaleimide, and 50 μl of anti-FLAG resin (Sigma)) with overnight rotation at 4 °C. The resins were washed five times with radioimmune precipitation assay buffer containing 0.1% SDS, and proteins were eluted in 2× SDS loading buffer. Ubiquitylated forms of FLAG-SUMO conjugates were detected by Western blotting with anti-ubiquitin antibody (P4D1; Santa Cruz). Results confirming those shown in Fig. 5 have been reproduced two times.
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**A** Schematic representation of five proteins that were identified as two-hybrid interactors with yeast SUMO/Smt3. The position of putative SIMs (type a in blue; type b in green) and of RING domains (purple) are indicated. SIMs that were experimentally shown to be important for SUMO binding are indicated by asterisks. The sequence stretches encoded in the various two-hybrid clones are given below each protein. B, SIPs bind SUMO in vitro. GST and GST-SUMO expressed in *E. coli* were bound to glutathione-Sepharose and then incubated with crude extracts from yeast cells expressing genomically HA-tagged versions of SIPs (boxed present in the SIPs shown in A). C, alignment of putative type a SIMs (boxed) present in the SIPs shown in A (upper part), as well as in other yeast proteins and mammalian RanBP2 (lower part). Common features of these SIMs appear to be 3–4 hydrophobic residues (shown in blue) followed by several acidic ones (shown in red). D, alignment of putative type b SUMO interaction motifs (boxed) present in Hex3 and Ris1, in Rfp1 and Rfp2 from *S. pombe*, as well as in human RNF4, PIAS1, and PIASx. E, alignment of RING domains in Hex3, Ris1, and Siz1.

**RESULTS**

Properties of SUMO-interacting Proteins (SIPs) in *S. cerevisiae*—Using the yeast two-hybrid system with *S. cerevisiae* SUMO (Smt3) as bait, we identified five strongly interacting SIPs, some of which were represented in multiple overlapping clones (Fig. 1A). One clone encoded a peptide of Siz1, a known SUMO ligase (23). To test whether interaction of SUMO with the other four identified SIPs, Nis1, Jip1 (24), Fir1 (25), Hex3/Slx5 (26, 27), and Ris1/Dis1 (28), could also be detected with their full-length versions and whether the interaction was noncovalent, the respective SIP-encoding genes were chromosomally tagged to express HA epitope-marked versions. In pull-down assays, these four SIPs specifically bound to GST-SUMO expressed in *E. coli*, but not to GST, confirming that they where able to bind SUMO (Fig. 1B). Because the binding assays were performed under conditions (on ice and without addition of ATP) that precluded in vitro SUMO conjugation, the interactions between these SIPs and SUMO were not a consequence of covalent modification of SIPs by SUMO but were apparently noncovalent in nature. Profile-based sequence comparisons (22) of the peptides encoded by these two-hybrid clones allowed us to identify a short (~10 residue) peptide motif that is encoded by all clones. In these putative SUMO interaction motifs (termed a “type a” SIM), a patch of 3–4 hydrophobic residues, is followed by a stretch of 3–4 acidic residues (Fig. 1C). All two-hybrid clones derived from Siz1 and Hex3 contain two type a SIMs (Fig. 1, A and C). Ris1, Nis1, Fir1, and Hex3 were also identified as two-hybrid interactors of SUMO in other studies (29–31). One of these studies (31) predicted some of the same putative SUMO interacting motifs that were identified by our analysis. Because of different criteria including a lysine residue upstream of the hydrophobic patch (shown in green in Fig. 1C), these authors, however, predicted only the SIMs in Nis1 and Fir1 as well as SIM1 of Ris1. The functional significance of these motifs and the role of these SIPs in the SUMO conjugation system remained unknown. Several studies on mammalian proteins that interacted with SUMO-1, however, suggested that SIMs related to the ones described here represent functionally conserved SUMO-binding motifs (32–35). A related but distinct SUMO-binding motif has previously been detected in RanBP2 (33). In this case the motif

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**FIGURE 1.** SIPs. A. Schematic representation of five proteins that were identified as two-hybrid interactors with yeast SUMO/Smt3. The position of putative SIMs (type a in blue; type b in green) and of RING domains (purple) are indicated. SIMs that were experimentally shown to be important for SUMO binding are indicated by asterisks. The sequence stretches encoded in the various two-hybrid clones are given below each protein. B, SIPs bind SUMO in vitro. GST and GST-SUMO expressed in *E. coli* were bound to glutathione-Sepharose and then incubated with crude extracts from yeast cells expressing genomically HA-tagged versions of SIPs (boxed present in the SIPs shown in A). C, alignment of putative type a SIMs (boxed) present in the SIPs shown in A (upper part), as well as in other yeast proteins and mammalian RanBP2 (lower part). Common features of these SIMs appear to be 3–4 hydrophobic residues (shown in blue) followed by several acidic ones (shown in red). D, alignment of putative type b SUMO interaction motifs (boxed) present in Hex3 and Ris1, in Rfp1 and Rfp2 from *S. pombe*, as well as in human RNF4, PIAS1, and PIASx. E, alignment of RING domains in Hex3, Ris1, and Siz1. The relevant Cys and His residues are shown in purple and a larger font. Hex3 and Siz8 are known to be detected with their full-length versions and whether the interaction was noncovalent, the respective SIP-encoding genes were chromosomally tagged to express HA epitope-marked versions. In pull-down assays, these four SIPs specifically bound to GST-SUMO expressed in *E. coli*, but not to GST, confirming that they where able to bind SUMO (Fig. 1B). Because the binding assays were performed under conditions (on ice and without addition of ATP) that precluded in vitro SUMO conjugation, the interactions between these SIPs and SUMO were not a consequence of covalent modification of SIPs by SUMO but were apparently noncovalent in nature. Profile-based sequence comparisons (22) of the peptides encoded by these two-hybrid clones allowed us to identify a short (~10 residue) peptide motif that is encoded by all clones. In these putative SUMO interaction motifs (termed a “type a” SIM), a patch of 3–4 hydrophobic residues, is followed by a stretch of 3–4 acidic residues (Fig. 1C). All two-hybrid clones derived from Siz1 and Hex3 contain two type a SIMs (Fig. 1, A and C). Ris1, Nis1, Fir1, and Hex3 were also identified as two-hybrid interactors of SUMO in other studies (29–31). One of these studies (31) predicted some of the same putative SUMO interacting motifs that were identified by our analysis. Because of different criteria including a lysine residue upstream of the hydrophobic patch (shown in green in Fig. 1C), these authors, however, predicted only the SIMs in Nis1 and Fir1 as well as SIM1 of Ris1. The functional significance of these motifs and the role of these SIPs in the SUMO conjugation system remained unknown. Several studies on mammalian proteins that interacted with SUMO-1, however, suggested that SIMs related to the ones described here represent functionally conserved SUMO-binding motifs (32–35). A related but distinct SUMO-binding motif has previously been detected in RanBP2 (33). In this case the motif
appears to be in an inversed orientation, i.e. the acidic residues precede a hydrophobic stretch. Using profile-based data base searches, we found related motifs in Slx8, a protein that forms a functional complex with Hex3 (see above), the Uba2 protein, a subunit of SUMO activating enzyme (36), and Wss1, a protein that was previously linked to the SUMO system, although the molecular details of this connection are unknown (37) (Fig. 1C). For Nis1 we obtained in vivo evidence for an interaction between the inferred SIMs and SUMO conjugates (supplemental Figs. S1 and S2). Residues both of the hydrophobic stretch as well as of the acidic patch are important determinants within this motif. The SUMO binding properties of Hex3-Slx8 and Ris1 appear to be more complex. Similar mutations as for Nis1, when introduced into the two putative type a SIMs of Ris1, had no detectable effect (data not shown). We used the two-hybrid assay to investigate the SUMO binding properties of Hex3 and Slx8 in more detail. We could not detect any two-hybrid interaction with SUMO for Slx8 (data not shown). The SUMO interaction of the Hex3 segment found in the two-hybrid screen was only slightly affected by mutation of the type a SIM2 but strongly reduced by mutation of SIM1. The double mutant behaved similarly to the SIM1 single mutant (supplemental Fig. S3). These results indicated that Ris1 and Hex3 contain multiple SUMO-binding sites or possibly interact with other SUMO-binding proteins. A study from Boddy and colleagues (38) identified Schizosaccharomyces pombe proteins that appear to be functionally related to the S. cerevisiae Hex3-Slx8 complex. S. pombe Rfp1 and Rfp2 are redundant proteins that interact with S. pombe Slx8. No apparent sequence homologue of Hex3/Slx5 is encoded in the S. pombe genome. DNA damage hypersensitivity of double mutants lacking Rfp1 and Rfp2 is complemented by the human Protein RNF4, which is related in sequence to Rfp1 and Rfp2 (39). None of these proteins displays extensive sequence similarity to Hex3. A small sequence motif (consensus: (I/V)DL(T/D)), which occurs twice each in Rfp1, Rfp2, and RNF4, however, is also present once in Hex3 and twice in Ris1. This motif resembles the core sequence of the SIM in PIAS proteins (Fig. 1D) (33). We therefore asked whether this motif, which we tentatively termed “type b” SIM, contributed to the SUMO binding property of Hex3. To test this, we mutated the VDLD motif to AAAD and tested how this mutation affected SUMO binding in the two-hybrid assay. Although the “type b” SIM mutation in Hex3 alone had only a small effect on SUMO binding, it led to a loss of detectable interaction when combined with mutation of type a SIM1 (supplemental Fig. S3). Together these data indicated that both types of SIMs in Hex3 contribute to SUMO binding. The complex arrangement of SIMs in Hex3 is likely to underline the preference for binding to multiply sumoylated proteins (see below). In addition to SIMs, Hex3, Slx8, and Ris1, share a RING domain (Fig. 1, A and D) similar to those found in many ubiquitin ligases (40).

Looking for evidence of functional roles of the identified SIPs in the SUMO system, we asked whether overexpression of their Gal4 activation domain (GAD) fusions that were obtained in the two-hybrid screen would have an effect on the pattern of SUMO conjugates in the cell. Expression of GAD-Ris1 or GAD-Nis1, and to a lesser extent of GAD-Siz1, GAD-Fir1 or GAD-Hex3, led to accumulation of high molecular weight SUMO conjugates (HMW-SC) (supplemental Fig. S1). It was reported recently that S. cerevisiae SUMO forms HMW-SC in vivo, some of which apparently bear substrate-attached SUMO chains (16). Such polysumoylated proteins accumulated in mutants that lacked the SUMO deconjugating enzyme Ulp2. Similar to the conjugates observed upon overexpression of SIPs, these conjugates in the ulp2Δ mutant were most prominently detected upon anti-SUMO Western blotting on the top of separating SDS-polyacrylamide gels and even more strikingly so upon blotting of the corresponding stacking gels (16). Identical effects as with the two-hybrid constructs were also obtained when we overexpressed native full-length Nis1 (supplemental Figs. S2 and S4). Together these results suggested that these SIPs bind and, upon overexpression, stabilize SUMO chains. Accumulation of HMW-SC in these experiments depended on the presence of lysines residues in SUMO (Lys11, Lys15, and Lys19) that have been implicated in formation of SUMO chains (supplemental Fig. S4) (16).

Although the effects of overexpressed SIM-containing fragments of SIPs on SUMO conjugate stability described above provided independent in vivo evidence for their SUMO binding property, the physiological function of these proteins remained unclear. It should be noted in this context that the GAD-Ris1 and GAD-Hex3 constructs used in these experiments lacked the RING domains (supplemental Fig. S1). We therefore asked whether deletion of SIP-encoding genes had an effect on SUMO conjugate patterns. Significantly increased amounts of HMW-SC were detected in extracts of the ris1Δ mutant and in the hex3Δ strain, and even more strikingly so in the double mutant (Fig. 2A). Deletion of NIS1 or FIR1, in contrast, had no detectable effects on SUMO patterns. These results together with the presence of RING domains in both proteins suggested that Ris1 and Hex3 might be ubiquitin ligases (E3s) that control the levels of SUMO conjugates. Hex3 was described to be in a complex with Slx8, another protein containing a RING finger domain (26, 41, 42). Hex3 and slx8 null mutants have similar phenotypes and effects on SUMO conjugate accumulation (data not shown), suggesting that the two proteins function as a heterodimer similar to other RING finger ubiquitin ligases such as BRCA1/BARD1 (27, 43).

**Degradation of SUMO Conjugates by the UPS**—The results obtained with the ris1Δ and hex3Δ mutants prompted us to ask whether the UPS is involved in a control of SUMO conjugates. Consistent with a role of Ris1- and Hex3-dependent ubiquitin conjugation in controlling the level of SUMO conjugates, we observed a similar accumulation of HMW-SC in a mutant (ubc1-1ts) carrying a temperature-sensitive ubiquitin-activating (E1) enzyme (22) (Fig. 2B). We went on to test various ubc mutants (ubc1Δ, ubc2Δ, ubc4Δ, ubc5Δ, ubc4Δ ubc5Δ, ubc6Δ ubc7Δ, ubc8Δ, ubc10Δ, and ubc13Δ) lacking ubiquitin-conjugating (E2) enzymes. The ubc6Δ ubc5Δ strain lacking the redundant Ubc4 and Ubc5 enzymes (44) displayed a striking accumulation of HMW-SC, suggesting that these enzymes mediate a proteolytic control of such conjugates (Fig. 2B). The patterns detected in the other ubc mutants, in contrast, were similar to those in the wild type (data not shown). Consistent with a role for the proteasome in the control of SUMO conju-
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Ubiquitin-conjugating enzymes (Ubc4 and Ubc5) are involved in the control of SUMO conjugate levels. When these enzymes are mutated, SUMO conjugates are stabilized in the proteasome-deficient rpt6/cim3 mutant (45). Independent evidence for the importance of the proteasome in controlling SUMO conjugates was obtained with the pdr5Δ mutant, which is particularly sensitive to proteasome inhibitors (46). Treatment of this mutant with MG132 resulted in a striking accumulation of HMW-SC (Fig. 2B). These results suggested that Hex3 and Ris1 together with Ubc4/5 mediate a proteolytic control of SUMO conjugates in targeting them for degradation by the proteasome.

A prediction of the above model was that SUMO conjugates should be ubiquitylated prior to their degradation. To test this prediction, we expressed His
tagged ubiquitin (His
t-Ub) in wild-type cells as well as in various mutant strains. Extracts from these cells were produced under denaturing conditions followed by affinity purification of His
t-Ub conjugates. SUMO conjugates were detected when His
t-Ub pulldown assays were performed with extracts from wild-type cells, and particularly so with extracts from the pdr5Δ mutant treated with proteasome inhibitor (Fig. 3A). In contrast, ubiquitylated forms of SUMO conjugates were absent from extracts derived from the ubc4 ubc5 or the hex3Δ ris1Δ mutants. Another interesting finding of this analysis was that particularly strong signals of ubiquitylated HMW-SC were detected in pulldown assays from ulp2Δ extracts. Because Ulp2 has been shown to counteract SUMO chain formation (16), we asked whether inhibition of SUMO chain formation would affect the formation of ubiquitin-SUMO hybrid conjugates. To test this, we used a strain (ulp2Δ smt3-R11,15,19) in which formation of SUMO chains was impaired because of the mutations of lysine residues (to arginines) in three SUMO attachment sites within the N-terminal domain of.
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SUMO. In contrast to the congenic ulp2Δ strain, this mutant did not yield appreciable amounts of ubiquitin-SUMO hybrid conjugates (Fig. 3A). Together these in vivo data indicated that Hex3 and Ris1 recognize sumoylated proteins and together with Ubc4,5 mediate their ubiquitylation and that SUMO chains that are stabilized in ulp2Δ promote formation of HMW ubiquitin-SUMO hybrid conjugates.

RING finger type ubiquitin ligases recognize and bind their substrates, either directly or via substrate binding ancillary factors, and bring them into close contact with their cognate E2s, which are bound to the RING domains (47, 48). We therefore asked whether Hex3 and Ris1 present in yeast extracts could bind Ubc4,5. Pulldown experiments with GST-Ubc4 expressed in E. coli confirmed that both Hex3 and Ris1 bind to Ubc4,5 (Fig. 3B). Experiments described above suggested that Hex3 and Ris1 bind sumoylated substrates via complex SUMO-binding sites and to the Ubc4,5 E2 enzymes via their RING domains. To inactivate the RING domains of Hex3 and Ris1, we mutated the respective second cysteine residues to serine residues (Fig. 1D). Strains expressing versions of Ris1 or Hex3 carrying these mutations in their RING domain instead of their wild-type counterparts accumulated HMW-SUMO conjugates in similar extents as ris1Δ or hex3Δ, respectively, indicating that the RING domains are essential for the observed function of these proteins in the proteolytic control of SUMO conjugates (data not shown).

Ubiquitin Ligase Activity of Hex3-Slx8 in Vitro—To obtain biochemical evidence, beyond their interaction with the Ubc4 enzyme, for a function of Ris1 and Hex3-Slx8 as ubiquitin ligases, we expressed HA-Ris1 and expressed GST-Hex3 together with Slx8-FLAG in E. coli. While HA-Ris1 was expressed only poorly and remained insoluble, we were able to recover active GST-Hex3-Slx8-FLAG heterodimer from E. coli extracts. As shown in Fig. 3C, Hex3-Slx8 promoted the formation of ubiquitin conjugates in vitro. Similar to autoubiquitylation reactions that have been observed for other RING type ligases (49), these ubiquitin chains were attached to a large extend to Hex3 itself (data not shown). We went on to ask whether the GST-Hex3-Slx8 complex would be able to bind to SUMO conjugates present in wild type, ulp2Δ, or hex3Δ ris1Δ. In these experiments, we found a selective enrichment of HMW-SUMO, because they are particularly accumulating in ulp2Δ or hex3Δ ris1Δ, among those retained on the GST-Hex3-Slx8 matrix (Fig. 4). These findings were consistent with the idea that HMW-SUMO are prefer substrates of the Hex3-Slx8 ubiquitin ligase.

Multi-level Control of SUMO Conjugates—Mutants lacking Hex3 and/or Ris1, as well as other mutants deficient in the UPS, accumulated HMW-SUMO, which at first glance appeared to be similar to those observed in ulp2Δ mutants (16) (Fig. 2). For ulp2Δ it was concluded that formation of such conjugates of low electrophoretic mobility required SUMO chain formation (polysumoylation) because they were undetectable in strains that expressed a mutant SUMO lacking lysine residues (Lys11, Lys15, and Lys19) critical for linking SUMO to SUMO (16). We therefore asked whether it is a function of proteolytic control to prevent a toxic accumulation of polysumoylated proteins. To investigate this question, we replaced the genomic SMT3 gene encoding SUMO by a mutant gene expressing SUMO, in which Lys11, Lys15, and Lys19 were replaced by arginine residues (referred to as SUMO-R11,15,19) in wild type, ulp2Δ, hex3Δ and ubc4Δ ubc5Δ (Fig. 2, C and D). Consistent with previous data, SUMO-R11,15,19 expression had little effect on the growth properties of wild-type cells but resulted in a striking suppression of the ulp2Δ growth defects (16) (Fig. 2D). Surprisingly, however, we found that SUMO-R11,15,19 expression resulted in a severe synthetic growth inhibition when combined with hex3Δ ris1Δ and also when combined with ubc4Δ ubc5Δ (Fig. 2D). Anti-SUMO Western blot analysis revealed that elimination of the branch sites in SUMO resulted in a drastic reduction of HMW-SUMO not only in ulp2Δ but also in the hex3Δ ris1Δ and ubc4Δ ubc5Δ mutants (Fig. 2C). Together our data suggest that formation of SUMO chain formation and degradation of SUMO conjugates may have overlapping functions.

Proteolytic Control of SUMO Conjugates in Human Cells—The observed degradation of SUMO conjugates in S. cerevisiae prompted us to ask whether such a mechanism is also detectable in mammals, whose SUMO conjugation system is more complex. In mammals, SUMO-2 and SUMO-3, which are 95% identical (therefore called SUMO-2/3 from hereon), appear to
form SUMO chains, whereas SUMO-1 does not (see Introduction). We analyzed SUMO-1 as well as SUMO-2/3 conjugates in HeLa cells after treatment with proteasome inhibitor (Fig. 5, A and B). Inhibition of the proteasome with MG132 resulted in a dramatic accumulation of high molecular weight SUMO-2/3 conjugates (Fig. 5B), similar to what we had observed in a comparable experiment with yeast cells. Detection with monoclonal antibody specific for SUMO-1, in contrast, showed that patterns of SUMO-1 conjugates were less affected by proteasome inhibition (Fig. 5A). Using HeLa cells that were transfected to express FLAG-tagged SUMO-1 or SUMO-3, we detected SUMO-ubiquitin hybrid conjugates after treatment with proteasome inhibitor and FLAG pulldown (Fig. 5C). High molecular weight ubiquitylated forms were observed both for SUMO-1 and SUMO-3 conjugates. Because of the stronger accumulation of SUMO-3 conjugates upon treatment with proteasome inhibitor, however, much higher amounts of ubiquitylated SUMO-3 conjugates were detected. Together these data indicated that in particular conjugates formed by the SUMO-2/3 isoform are subject to a proteolytic control by the UPS in human cells, similar to what we observed for SUMO conjugates in yeast. The fact that this mechanism appears not to apply to SUMO-1 conjugates to the same extent, however, suggested that a preferential proteolytic control of SUMO-2/3 conjugates provides a functional distinction from SUMO-1 in humans. These findings raised the possibility that SUMO-2/3 chain formation may contribute to proteolytic control of conjugates of these SUMO paralogues.

DISCUSSION

Contrary to what was believed previously, we have discovered that SUMO modification can serve as a targeting signal in the ubiquitin/proteasome system (Fig. 6). We show that two RING finger proteins with SUMO interaction motifs, Ris1 and Hex3-Slx8, are required to ubiquitylate SUMO conjugates in S. cerevisiae cells. In vitro experiments (Fig. 3C) confirmed that the Hex3-Slx8 heterodimer produced in E. coli possesses ubiquitin ligase activity. A parallel study by Hochstrasser and co-

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**FIGURE 4.** Preferential binding of high molecular mass SUMO conjugates to Hex3-Slx8. Glass bead extracts from cells with the indicated genotypes were analyzed in a GST pulldown assay either with GST or with GST-Hex3-Slx8-FLAG produced in E. coli. In lanes 1–3, samples (amounts correspond to those used for GST pulldown assays) of the extracts were loaded. In lanes 4–9, samples of the unbound material, and in lanes 10–15, samples of the material bound to the GST matrix were loaded. The samples were analyzed by SDS-PAGE and anti-SUMO Western blotting. The separating and the stacking parts of the gel were simultaneously blotted in this experiment. The positions of the prestained molecular mass markers are indicated. wt, wild type.

**FIGURE 5.** Ubiquitylation and proteasome-mediated degradation of SUMO conjugates in human cells. HeLa cells were incubated for 8 h either without any addition, with the addition of Me2SO (DMSO), or with the addition of Me2SO + MG132 (final concentration, 20 μM). A, whole cell extracts were analyzed by SDS-PAGE and anti-SUMO-1 Western blotting. To compare loading, the blot was reprobed with anti-tubulin antibody. B, same as in A, but with anti-SUMO-2/3 antibody. C, detection of hybrid SUMO-ubiquitin conjugates in HeLa cells. Extracts from cells transiently transfected to express FLAG-SUMO-1 or FLAG-SUMO-3 and treated for 8 h with MG132 were subjected to anti-FLAG pulldown followed by anti-ubiquitin and anti-FLAG Western blotting. wt, wild type; IP, immunoprecipitation.
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FIGURE 6. Proteolytic control of SUMO conjugates in S. cerevisiae. Shown is a model of the role of SUMO chain formation and SUMO conjugate degradation. Substrates marked either by multi-sumoylation (upper branch) or by polysumoylation (SUMO chain formation, lower branch; link between upper and lower branch) are recognized and ubiquitylated by Uls1,2 (Uls1/Ris1 and Uls2/Hex3-Slx8) together with the conjugating enzymes Ubc4 and Ubc5. SUMO chains apparently enhance targeting, which is indicated by the thick arrow. SUMO chain formation is counteracted by the activity of the Ulp2 desumoylating enzyme. SUMO chain formation and SUMO conjugate degradation are two alternative mechanisms to inactivate mono- or multi-sumoylated substrates.

workers (50) provided independent in vitro evidence for a stimulation of Hex3-Slx8-mediated substrate ubiquitylation by sumoylation of the substrate. Together these data confirm the conclusion derived from our in vivo analysis that this novel ubiquitin ligase mediates targeting of sumoylated proteins to the proteasome. A similar activity remains to be confirmed in vitro for Ris1, which was insoluble upon expression in E. coli. Similar properties of Ris1 and Hex3-Slx8, which include the presence of RING domains, binding of SUMO conjugates and Ubc4, accumulation of SUMO conjugates in the null mutants, and the synthetic effects of the ris1 hex3 double mutant, suggest that Ris1, similar to Hex3-Slx8, can function as a SUMO-dependent ubiquitin ligase. We therefore propose the terms Uls1 and Uls2 (ubiquitin ligases for SUMO conjugates 1 and 2) as alternative names for these proteins to indicate this function (Fig. 6). In S. pombe, a complex of Slx8 with either Rfp1 or Rfp2, two proteins with weak sequence similarity to human RNF4, apparently perform functions similar to Hex3-Slx8 in S. cerevisiae (38). S. pombe rfp1 rfp2 mutants were complemented by human RNF4, indicating that this small RING domain protein, which was previously shown to bind SUMO and to auto-ubiquitylate in vitro (51, 52), may have a function similar to the S. pombe Rfp proteins in human cells (39). Consistent with this notion, we found that the human RNF4, but not PML, another RING finger protein that binds to SUMO, efficiently complements both hex3Δ and slx8Δ mutations (supplemental Fig. S5). These data indicate that ubiquitylation of SUMO conjugates is conserved from yeast to humans. Consistent with this notion, we observed an accumulation of ubiquitylated SUMO-2/3 conjugates in human cells upon inhibition of the proteasome, suggesting that a proteolytic control of SUMO conjugates is operating in humans as well. SUMO-1 conjugates, in contrast, were much less affected. A possible explanation for this difference between conjugates of these isoforms is that SUMO-1, because of a lack of appropriate attachment sites in the N-terminal domain, does not efficiently form chains, whereas SUMO-2/3 does (17). In this context it is noteworthy that higher levels of SUMO-2/3 conjugates are detected after subjecting cells to stresses such as heat (53). This indicates that under these conditions either higher conjugation rates are induced, or turnover of SUMO-2/3 substrates is slowed down because of a stress-related overload of the UPS. If the latter effect is the cause for the stress induction of SUMO-2/3 conjugates, these data resemble the ones obtained after inhibition of the proteasome using MG132 (Fig. 5B). If a given substrate can be modified either by SUMO-1 or by SUMO-2/3, then modification with the former may lead to stabilization whereas modification with the latter may lead to a destabilization. Noteworthy in this context is the observation that the amount of amyloid β peptide (Aβ), which is generated from amyloid precursor protein and plays a prominent role in Alzheimer’s disease, drops upon overexpression of SUMO-2. Overexpression of SUMO-2(K11R), in which SUMO chain formation is blocked, in contrast, results in increased amounts of Aβ (54). Even though no sumoylated forms of amyloid precursor protein were detected in this study, its SUMO-1 modification was detected in a later proteomic study (5, 55). Together with our results, these data are consistent with a role for SUMO-2/3 chains in regulating Aβ formation via controlling stability of amyloid precursor protein. Interestingly, inhibition of the proteasome was also reported to enhance Aβ formation (56).

Similar to SUMO-2/3 in mammals, yeast SUMO was reported to form chains, but the functional significance remained unclear (16). Our data indicate that formation of SUMO chains (polysumoylation) promotes ubiquitin-mediated targeting. A requirement for multiple SUMO moieties in efficient targeting is consistent with the observation that Hex3-Slx8 and Ris1 contain multiple putative SUMO interaction motifs (Fig. 1).

Protein modification by SUMO in many cases is a transient and cell cycle-controlled process that is critically controlled by conjugation and deconjugation (21, 57–60). SUMO modification has been shown to mediate specific interactions, for example between SUMO-PCNA and Srs2 (12, 13). Our current data suggest that several mechanisms, including desumoylation, polysumoylation, and degradation, may be employed to terminate such transient interactions.

It is also possible that for certain substrates the primary function of SUMO attachment is to target them for ubiquitin-dependent proteolysis. In this case SUMO attachment could provide a second level of control in the regulation of protein destruction. The mechanism discovered in this study may provide a plausible explanation for the observed induction of turnover of certain mammalian proteins following their sumoylation (61–63).

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Addendum—While this paper was in press, another study by Sun et al. provided evidence for a SUMO-dependent ubiquitin ligase activity of Rfp1,2-Slx8 and of RNF4 (64).

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