The SUMO Isopeptidase Ulp2 Prevents Accumulation of SUMO Chains in Yeast*

Received for publication, July 30, 2003
Published, JBC Papers in Press, August 26, 2003, DOI 10.1074/jbc.M308357200

Gwendolyn R. Byebyl, Irina Belichenko, and Erica S. Johnson‡
From the Department of Biochemistry and Molecular Pharmacology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

SUMO is attached to many yeast proteins, and this motif is bound directly by Ubc9 (13, 14), while E3s bind other features of the substrate to enhance specificity. SUMOylation is a reversible modification, and a family of SUMO-specific isopeptidases, including the yeast proteins Ulp1 and Ulp2 (also called Smt4), removes SUMO from modified proteins. Ulp1 and Ulp2 have different subcellular localizations and are responsible for desumoylating different proteins (15–17). Ulp1 also cleaves a SUMO precursor to generate mature SUMO that can be conjugated.

In S. cerevisiae many of the genes encoding SUMO pathway proteins are essential, including SMT3, UBA2, AOS1, UBC9, and ULP1, but it is not known what the essential function of SUMO conjugation is. SUMO is attached to many yeast proteins, but only five have been characterized to date: three members of the septin family of cytoskeletal proteins, the multifunctional replication processivity factor PCNA (proliferating cell nuclear antigen) and topoisomerase II (Top2) (18–21). Mutants lacking the SUMO-activating enzyme (E1) Uba2Aos1, the SUMO-conjugating enzyme (E2) Ubc9, and several different SUMO ligases (E3a) (4–12). S. cerevisiae contains two known SUMO E3s, Siz1 and Siz2. Ubc9 and the E3s collaborate to confer substrate specificity on sumoylation: SUMO is often attached to the lysine in the short motif (I/V/L)KXE, and this motif is bound directly by Ubc9 (13, 14), while E3s bind other features of the substrate to enhance specificity.

SUMOs are ubiquitin-related proteins that function by being covalently attached to lysine residues in other proteins. Unlike ubiquitin, which is often linked to its substrates as a polyubiquitin chain, only one SUMO moiety is attached per modified site in most substrates. However, SUMO has recently been shown to form chains in vitro and in mammalian cells, with a lysine in the non-ubiquitin-like N-terminal extension serving as the major SUMO-SUMO branch site. To investigate the physiological function of SUMO chains, we generated Saccharomyces cerevisiae strains that expressed mutant SUMOs lacking various lysine residues. Otherwise wild-type strains lacking any of the nine lysines in SUMO were viable, had no obvious growth defects or stress sensitivities, and had SUMO conjugate patterns that did not differ dramatically from wild type. However, mutants lacking the SUMO-specific isopeptidase Ulp2 accumulated high molecular weight SUMO-containing species, which formed only when the N-terminal lysines of SUMO were present, suggesting that they contained SUMO chains. Furthermore SUMO branch-site mutants suppressed several of the phenotypes of ulp2Δ, consistent with the possibility that some ulp2Δ phenotypes are caused by accumulation of SUMO chains. We also found that a mutant SUMO whose non-ubiquitin-like N-terminal domain had been entirely deleted still carried out all the essential functions of SUMO. Thus, the ubiquitin-like domain of SUMO is sufficient for conjugation and all downstream functions required for yeast viability. Our data suggest that SUMO can form chains in vitro in yeast but demonstrate conclusively that chain formation is not required for the essential functions of SUMO in S. cerevisiae.

SUMO, like ubiquitin (Ub), is linked to its substrates via an amide bond between its C-terminal carboxyl group and the ε-amino group of a lysine residue in the substrate (1–3). SUMOs share only ~18% sequence identity with Ub but contain a C-terminal domain with a Ub-fold that is virtually superimposable on the structure of Ub. SUMOs also contain a ~20-residue non-Ub-related N-terminal extension. Saccharomyces cerevisiae contains a single SUMO protein encoded by the SMT3 gene, while mammals contain three different SUMOs: SUMO-2 and SUMO-3, which are 95% identical to each other, and SUMO-1. SUMO attachment is catalyzed by a three-step enzyme pathway, analogous to the Ub pathway, consisting of the heterodimeric SUMO-activating enzyme (E1) Uba2Aos1, the SUMO-conjugating enzyme (E2) Ubc9, and several different SUMO ligases (E3a) (4–12). S. cerevisiae contains two known SUMO E3s, Siz1 and Siz2. Ubc9 and the E3s collaborate to confer substrate specificity on sumoylation: SUMO is often attached to the lysine in the short motif (I/V/L)KXE, and this motif is bound directly by Ubc9 (13, 14), while E3s bind other features of the substrate to enhance specificity. SUMOylation is a reversible modification, and a family of SUMO-specific isopeptidases, including the yeast proteins Ulp1 and Ulp2 (also called Smt4), removes SUMO from modified proteins. Ulp1 and Ulp2 have different subcellular localizations and are responsible for desumoylating different proteins (15–17). Ulp1 also cleaves a SUMO precursor to generate mature SUMO that can be conjugated.

Our data suggest that SUMO can form chains in vivo and in yeast but demonstrate conclusively that chain formation is not required for the essential functions of SUMO in S. cerevisiae.

* This work was supported by National Institutes of Health Grant GM62286. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Pharmacology, Thomas Jefferson University, 233 South 10th St., BLSB 231, Philadelphia, PA 19107, Tel.: 215-503-4616; Fax: 215-503-5393; E-mail: Erica.Johnson@jefferson.edu.

1 The abbreviations used are: Ub, ubiquitin; E1, SUMO-activating enzyme; E2, SUMO-conjugating enzyme; E3, SUMO ligase; HU, hydroxyurea; TBZ, thiabendazole; HA, hemagglutinin; Ab, antibody; DTT, dithiothreitol; GST, glutathione S-transferase; NEM, N-ethylmaleimide; wt, wild-type; YPD, yeast extract/peptone/dextrose; VSV-G, vesicular stomatitis virus glycoprotein; trSiz1, truncated Siz1.
attachment to Top2 may cause some of the phenotypes of ulp2Δ (21). The siz1Δ and siz2Δ single mutants grow well and do not have obvious phenotypes, although they are each deficient for SUMO attachment to different classes of substrates (8, 10, 22). The siz1Δ siz2Δ double mutant is also viable, although it is cold-sensitive, delays in the cell cycle at G2/M, and has very low SUMO attachment to different classes of substrates (8, 10, 22).

A critical property of Ub is its ability to form poly-Ub chains, and Lys11 in SUMO-2/3 (24, 25). These lysines are in sequences unusual. SUMO-1 does not have such a sequence but also forms yeast sequence contains a proline after the lysine, which is restricted by the activities of SUMO-specific isopeptidases.

**SUMO Chain Formation**

| Name          | Relevant genotype | Source |
|---------------|-------------------|--------|
| JD51          | MATaMATa trp1-DΔ/trp1-DΔ ura3-52/ura3-52 his3-D200/his3-D200 leu2-3,112/leu2-3,112 lys2-801/lys2-801 | Ref. 29 |
| JD52          | MATa trp1-DΔ ura3-52/ura3-52 his3-D200 leu2-3,112 lys2-801 | J. Dohmen |
| EJY332        | MATa CDC3-HA::HIS3 CDC12-HF::TRP1 | Ref. 8 |
| GBY1          | MATa smt3-R11,11,19::TRP1 | This study |
| GBY2          | MATa smt3-R27::TRP1 | This study |
| GBY3          | MATa smt3-R38,40,41::TRP1 | This study |
| GBY4          | MATa smt3-R54,58::TRP1 | This study |
| GBY5          | MATa smt3-allR::TRP1 | This study |
| I30           | MATa ulp2Δ::URA3 | This study |
| GBY6          | MATa ulp2Δ::URA3 | This study |
| GBY7          | MATa smt3-R11,15,19::TRP1 ulp2Δ::URA3 | This study |
| GBY8          | MATa smt3-R15::TRP1 ulp2Δ::URA3 | This study |
| GBY9          | MATa smt3-R27::TRP1 ulp2Δ::URA3 | This study |
| GBY10         | MATa smt3-R38,40,41::TRP1 ulp2Δ::URA3 | This study |
| GBY11         | MATa smt3-R54,58::TRP1 ulp2Δ::URA3 | This study |
| GBY12         | MATa smt3-allR::TRP1 ulp2Δ::URA3 | This study |
| GBY13         | MATa TOP2-HA::HIS3 ulp2Δ::URA3 | This study |
| GBY14         | MATa TOP2-HA::HIS3 smt3-R11,15,19::TRP1 ulp2Δ::URA3 | This study |
| GBY15         | MATa TOP2-HA::HIS3 smt3-R27::TRP1 ulp2Δ::URA3 | This study |
| GBY16         | MATa TOP2-HA::HIS3 smt3-R38,40,41::TRP1 ulp2Δ::URA3 | This study |
| GBY17         | MATa TOP2-HA::HIS3 smt3-R54,58::TRP1 ulp2Δ::URA3 | This study |
| GBY18         | MATa TOP2-HA::HIS3 smt3-allR::TRP1 ulp2Δ::URA3 | This study |
| GBY19         | MATa smt3-N21::TRP1 | This study |
| GBY20         | MATa smt3-N21::TRP1 ulp2Δ::URA3 | This study |

**EXPERIMENTAL PROCEDURES**

**Media and Genetic Techniques**—Standard techniques were used (27). Rich yeast medium containing 2% glucose (YPD) was prepared as described previously (28). Cells were arrested at G1/M in the cell cycle by incubating with 15 µg/ml nocodazole (Acros) for 3 h. Growth assays were performed by making 10-fold serial dilutions of logarithmically growing cells that had been normalized to A600 nm 1.0. A 2-µl sample of each dilution was spotted onto YPD plates or YPD plates supplemented with 0.1 M hydroxyurea (Acros); 10 mM caffeine (Acros); 1% dimethylformamide; or 75 µg/ml thiamabendazole (Sigma), 1% dimethylformamide.

**Plasmids and Yeast Strain Construction**—S. cerevisiae strains used are listed in Table I. All strains are derivatives of JD51 (29). The SUMO lysine mutant alleles (smt3-R11,15,19, smt3-R15, smt3-R27, smt3-R38,40,41, smt3-R54,58, and smt3-allR) were constructed using an overlapping PCR strategy described previously (18) to produce PCR products containing 500 bp of the SMT3 5'-flank followed by SMT3 containing Lys to Arg mutations followed by the TRP1 marker and then 500 bp of the SMT3 3'-flank. These PCR products were transformed into JD51, Trp⁺ diploids were sporulated, and the tetrads were dissected. Mutant alleles were reamplified and sequenced to confirm that they did not contain any additional mutations. smt3-(K to R) ulp2Δ double mutants were made by crossing SUMO mutants to GBY6, a MATa strain containing the ulp2Δ allele from IS30 (17), followed by sporulation and dissection of the tetrads. TOP2-HA-tagged strains were made by transforming the smt3-(K to R) ulp2Δ double mutants with a PCR product containing 500 bp of the C-terminal coding sequence of TOP2 followed by the HA epitope tag, the HIS3 marker, and 500 bp of the TOP2 3'-flank.

Plasmids for expressing Hisα-tagged Aos1, Uba2, Ubc9, and wt Smt3 and Hisα-FLAG-tagged full-length Siz1 (Siz1-HF) in Escherichia coli have been described previously (8). A truncated version of Siz1 (trSiz1), tagged with Hisα, and a vesicular stomatitis virus glycoprotein (VSV-G)-derived epitope tag, was made by ligating a PCR-amplified fragment of Siz1 encoding Met313-Arg508 and bearing the C-terminal extension YTDIEIMRKLKHSHHHHHHHHH into pET21a. Plasmids for expressing Hisα-tagged Smt3 lysine mutants in E. coli were constructed by amplifying the SMT3 sequence from genomic DNA of the corresponding yeast mutants with primers to add the N-terminal extension MASMHHH- and to produce a stop codon after Gly79. Resulting PCR products were ligated into pET21a. All plasmids were sequenced to confirm that they did not contain additional mutations. Oligo sequences and construction details are available upon request. A pGEM-based plasmid for expressing GST-Ulp2 (16) was a generous gift of Alaron Lewis and Mark Hochstrasser (Yale University, New Haven, CT).

**Antibodies and Immunoblot Analyses**—Yeast whole cell lysates were prepared as described previously (30) followed by immunoblotting and chemiluminescent detection as described previously (18). Antibodies used were a rabbit polyclonal Ab against Smt3 (18), the 16B12 mono-
clonal Ab against the HA epitope (Covance), the M2 monoclonal Ab against the FLAG epitope (Kodak Scientific Imaging Systems), a rabbit polyclonal Ab against Cdc11 (Santa Cruz Biotechnology), and a goat polyclonal Ab against Ubc9 (C-19) (Santa Cruz Biotechnology).

Affinity Purification of Epitope-tagged Proteins—His6-SUMO, Ubc9-His6, and His6-Aos1-Uba2-His6 were expressed in E. coli and purified by nickel-nitrioltriacetic acid affinity chromatography as described previously (8). VSVG-His6-trSiz1 and Siz1-HF were expressed as described for Siz1-HF (8). Cells were extracted with 5 volumes of Y-PER (Pierce) supplemented with 1 mM phenylmethylsulfonfyl fluoride. After centrifugation to remove cell debris the supernatant was diluted 10-fold with 50 mM NaPO4 (pH 8.0), 150 mM NaCl plus 0.1 mM ZnCl2 and 5 mM imidazole and bound in-batch for 2 h at 4 °C to HIS-Select HC nickel affinity gel (Sigma). Proteins were eluted with 200 mM imidazole, 45 mM HEPES (pH 8), and 0.9 mM NaCl. Sepitns were purified from EJY332, a yeast strain expressing Cdc12-HF, as described previously (8).

GST and GST-Ulp2 were expressed and purified from E. coli JM101 cells using a modification of a described protocol (16). Cells induced with 1 mM isopropyl-1-thio-D-galactopyranoside for 3 h at 37 °C were lysed under high pressure using an Avestin Emulsiflex C-5 in 1 × PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.4 mM KH2PO4, plus 0.1% Triton X-100, 1 mM phenylmethylsulfonfyl fluoride, and 1 mM DTT. After centrifugation at 35,000 × g for 30 min at 4 °C, supernatant was bound in-batch to immobilized glutathione (Sigma) at 25 °C for 30 min. Beads were washed with 1× PBS, 0.1% Triton X-100, 0.1 mM DTT and eluted with 15 mM glutathione, 60 mM Tris (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, and 0.1 mM DTT. Eluates were dialyzed against 50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM DTT, 30% glycerol for 1 h at 4 °C using mini Slide-A-Lyzer (Pierce).

In Vitro Sumoylation Assay—Sumoylation reactions were performed as described previously (8) and contained ~40 μg/ml wt or mutant Smt3, ~5 μg/ml Uba2-Aos1, 3.5 μg/ml Ubc9, and ~3 μg/ml trSiz1 or ~12 μg/ml of the full-length Siz1 preparation, which primarily contained C-terminal fragments of Siz1 and E. coli proteins.

Top2-HA Immunoprecipitations—For immunoprecipitation of Top2-HA, cells were grown to A600 ~1 in rich medium at 30 °C followed by incubation with 15 μg/ml nocodazole (Acros) for 4 h at 30 °C. Whole cell lysates were prepared as described for isolation of Smt3 conjugates (16) except that cells were lysed using an Avestin Emulsiflex C-5 instead of by sonication. Clarified lysates were bound to anti-HA-agarose (Covance) overnight at 4 °C. Beads were washed three times with 50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% SDS, 2 mM NEM and eluted by boiling in SDS sample buffer.

Ulp2 Cleavage of In Vitro Sumoylation Reactions—Sumoylation reactions containing recombinant Siz1-HF (see above) were incubated for 2.5 h at 30 °C and stopped by incubating with 25 mM glucose and 1 unit of hexokinase (Sigma) in a 26-μl reaction for 5 min at 28 °C. These samples were diluted 4-fold into new reactions to contain 50 mM Tris (pH 7.5), 12.5 mM HEPES (pH 7.0), 175 mM NaCl, 0.1% Triton X-100, 0.125 mM DTT, 2.5 mM MgCl2, 5 mM imidazole, 1.25 μm ZnCl2. Then 2.5 μg/ml GST or the GST-Ulp2 preparation, which contained primarily free GST (~90%), was added. In reactions with NEM or NEM plus DTT, the GST or GST-Ulp2 was preincubated with 10 μM NEM or 10 μM NEM plus 20 μM DTT for 15 min at 25 °C. Cleavage reactions proceeded for 30 h at 30 °C and were stopped by boiling in SDS sample buffer.

RESULTS

N-terminal Lysines Are Required for SUMO Chain Formation in Vitro—To identify the branch site(s) in SUMO chains that had been assembled in in vitro reactions containing Siz1, high molecular weight SUMO-containing species were excised from the top of an SDS-polyacrylamide resolving gel (much like Fig. 2B, lane 2), digested with trypsin and endoprotease Lys-C, and analyzed by surface-enhanced laser desorption/ionization-time of flight mass spectrometry (data not shown). A major peak was detected that was the correct size to contain the C-terminal tryptic peptide of SUMO covalently linked to the N-terminal peptide of SUMO up to Lys27. This experiment suggested that SUMO was attached to Lys11, Lys15, and/or Lys19 of Siz1 but did not distinguish among them. Neither trypsin nor Lys-C cleaved after any of these lysine residues, which are all followd by prolines (Fig. 1). However, a similar experiment, using chains that were assembled by Uba2-Aos1 and Ubc9 alone and then cleaved with endoprotease Glu-C, was published recently showing the major branch site to be at Lys15 (25).

Yeast SUMO has a total of nine lysines that fall into four groups (Fig. 1). Three of these, Lys11, Lys15, and Lys19 are in the N-terminal non-Ub-like region and are part of a repeat sequence containing three tetrapeptide units resembling SUMO attachment consensus motifs. We decided to generate mutants lacking each of the nine lysines in yeast SUMO because preliminary experiments indicated that mutating only the N-terminal lysines, which were identified as potential branch sites by mass spectrometry, did not prevent formation of extremely high molecular weight SUMO conjugates in vitro (see below). Also we wanted to test whether SUMO chains with different branch sites might have different functions in vivo. A series of SUMO mutants was constructed in which one group, all but one group, or all four groups of lysines were mutated to arginine. These mutants were expressed in E. coli, purified, and assayed in sumoylation reactions containing recombinant Aos1-Uba2 (E1), Ubc9 (E2), and trSiz1 (Fig. 2A). trSiz1 was used because full-length Siz1 was itself sumoylated and generated large amounts of high molecular weight conjugates that complicated analysis. Because these reactions did not contain any additional substrate, all SUMO-containing species larger than free SUMO contained SUMO attached either to itself (SUMO chains) or to the enzymes in the reaction. Wild-type SUMO formed a ladder of bands corresponding to SUMO chains (Fig. 2A, lane 3). This ladder was absent in all reactions containing SUMO mutants lacking Lys11, Lys15, and Lys19 (Fig. 2A, lanes 4 and 9–12). These results confirm that SUMO forms chains in vitro primarily via the N terminus (25, 31). Other groups have examined SUMO chain formation in reactions that lack Siz proteins and depend only on the E1 and Ubc9 (25, 31), but under our reaction conditions, SUMO chain formation requires trSiz. Thus, these results show that trSiz also does not promote formation of SUMO chains with linkages outside the N-terminal domain of SUMO.

![Diagram of lysine residues in SUMO. A, there are nine lysines in Smt3 that fall into four groups. Mutant SUMOs were constructed by mutating one, all but one, or all groups of lysines to arginines. B, alignment of the N terminal of Smt3, SUMO-1, SUMO-2, SUMO-3, and Ub. Lysines are highlighted. The arrow above Smt3 designates the start position for the smt3-N237 allele.](image-url)
SUMO Chain Formation

Fig. 2. N-terminal lysines are required for formation of SUMO chains in vitro. A, wild-type (lanes 1–3) and mutant (lanes 4–12) SUMOs were incubated for 10 min at 30 °C with 5 mM ATP (lanes 2–12), Aos1, Uba2, Ubc9, and truncated Siz1 (lanes 1 and 3–12). Reactions were analyzed by SDS-PAGE and immunoblotting with an Ab against SUMO. Bands corresponding to free SUMO are indicated by arrows. Smt3-R11,15,19 proteins had increased gel mobility. B, wild-type (lanes 1 and 2) and mutant (lanes 3–11) SUMO was incubated for 1 h at 30 °C with 5 mM ATP (lanes 2–11), Aos1, Uba2, Ubc9, and full-length recombinant Siz1. Septins, derived from yeast EJY332, were also added in the experiment shown in the bottom panel. Reactions were analyzed by SDS-PAGE and immunoblotting with an AB against SUMO (top panel), Ubc9 (middle panel), and Cdc11 (bottom panel). Unmodified SUMO, Ubc9, and Cdc11 are indicated. An asterisk designates the SUMO-Ubc9 conjugate. IB, immunoblot.

We also tested the mutant SUMOs in reactions containing full-length Siz1 (Fig. 2B). Interestingly even SUMO mutants lacking N-terminal lysines were incorporated into very high molecular weight conjugates in these reactions (Fig. 2B, top panel, lanes 3 and 8–11), although the fraction of these mutant SUMOs incorporated was much lower than for wt SUMO, which was completely depleted (Fig. 2B, lane 2). These high molecular weight species formed even with the Smt3-allR mutant (Fig. 2B, lane 11) in which all lysines were mutated, indicating that these species did not contain lysine-linked chains. Furthermore immunoblotting against Ubc9 revealed that SUMO mutants lacking Lys11, Lys15, and Lys19 were attached to Ubc9 at higher levels than were the mutants that contained these lysines (Fig. 2B, middle panel). We also tested the mutants for conjugation to septins and found that all of the Lys mutants were capable of modifying the septin Cdc11 (Fig. 2B, bottom panel). As with Ubc9, when mutant SUMOs lacking the N-terminal lysines were used in the reaction, more high molecular weight forms of Cdc11 were formed, suggesting that Cdc11 was modified at additional sites that were not modified in vivo or in vitro with wt SUMO. This enhanced attachment of branch-site mutant SUMOs to Ubc9 and Cdc11 suggested that the specificity of conjugation was relaxed in reactions containing SUMO mutants unable to form chains, possibly because chain formation normally competes with nonspecific sites for attachment of activated SUMO.

Characterization of SUMO Lysine Mutants in Vivo—To determine whether SUMO forms chains in vivo, we constructed yeast strains where the genomic SMT3 locus was replaced with mutant versions lacking various lysines. All mutants were viable and did not show notable sensitivity to caffeine, hydroxyurea (HU), the microtubule-destabilizing reagent thiamethoxam (TBZ), methyl methanesulfonate, or high or low temperatures (data not shown). However, the mutant lacking all lysines (smt3-allR) did grow somewhat slowly, contained aberrant cells, and showed a synthetic growth defect with siz1Δ (data not shown). We next looked at SUMO conjugate patterns from whole cell lysates of the mutants to determine whether there were any differences that might be attributed to absence of SUMO chains (Fig. 3). A strain unable to form chains should either be missing one or more bands, representing conjugates bearing chains, or should have bands of increased intensity, representing monosumoylated conjugates of proteins that would normally be attached to a chain. No significant differences in conjugate patterns were observed between wt and any of the single group Lys mutants, although the smt3-allR mutant had reduced amounts of conjugates at ~33 and ~43 kDa. All mutants modified Cdc11 as in wt (Fig. 3, lower panel). These results show that the most abundant SUMO conjugates do not contain SUMO chains. However, there may be changes that were not detected because the majority of SUMO-modified proteins migrated at very high molecular weights and were not well resolved.

We next examined the possibility that SUMO chains might not accumulate in wt yeast because of the action of SUMO
isopeptidases. Yeast contain two genes for SUMO-specific isopeptidases: ULP1, which is essential for viability, and ULP2, whose mutants exhibit a variety of phenotypes including slow growth and sensitivity to heat, HU, caffeine, methyl methanesulfonate, and microtubule-depolymerizing drugs such as TBZ (15–17). To test for genetic interactions between ulp2Δ and the SUMO lysine mutants, double mutants were assessed for growth at 30 °C, at 37 °C, and in the presence of 0.1 M HU (Fig. 4). Strikingly the smt3-R11,15,19 and smt3-allR alleles completely suppressed the temperature sensitivity of the ulp2Δ strain. The smt3-R15 mutant also permitted growth at 37 °C although not as well as the smt3-R11,15,19 and smt3-allR mutations. Oddly all of the other lysine mutations also suppressed the ulp2Δ temperature sensitivity slightly with the smt3-R54,58 mutant being most effective. This suppression was not a marker effect as the ulp2Δ strain with wt SUMO also contained a TRP1 marker at the 3′-end of the SMT3 gene. The smt3-allR mutation also strongly suppressed the HU sensitivity of the ulp2Δ strain, while the smt3-R11,15,19 and smt3-R54,58 suppressed to a lesser degree. The smt3-R15 mutant had little effect on HU sensitivity. These results are consistent with the possibility that accumulation of SUMO chains contributes to the temperature sensitivity of the ulp2Δ mutant.

Reducing SUMO attachment to Top2 by elimination of the major SUMO attachment sites in Top2 also suppresses ulp2Δ temperature sensitivity (21). To test whether the SUMO mutants might be suppressing ulp2Δ phenotypes by altering levels of SUMO attachment to Top2, we introduced an HA epitope tag into the genomic copy of TOP2 in the ulp2Δ smt3-(K to R) double mutants. SUMO-Top2-HA conjugates were detected in anti-HA immunoprecipitates by immunoblotting with antibodies against SUMO (Fig. 5). Levels of SUMO-modified Top2 in most ulp2Δ SUMO lysine mutants were similar to those in the ulp2Δ mutant alone both in the fraction of Top2 modified and the number of SUMO moieties attached. Most importantly, the smt3-R11,15,19 mutant, which strongly suppressed ulp2Δ temperature sensitivity, contained levels of Top2-SUMO conjugates that were comparable to those in ulp2Δ alone and in the double mutants containing SUMO mutants that did not strongly suppress the phenotype (Fig. 5, lanes 3 versus lanes 2 and 4–6). This result suggests that suppression of ulp2Δ by smt3-R11,15,19 does not result from an effect on the amount of SUMO attached to Top2. However, the ulp2Δ smt3-allR strain did contain ~5-fold lower levels of Top2-SUMO conjugates (Fig. 5, lane 7), suggesting that lower levels of SUMO-Top2 conjugates may contribute to the suppression of ulp2Δ phenotypes by this mutant.

We also examined the pattern of total SUMO conjugates in ulp2Δ strains containing each of the SUMO mutants (Fig. 6). The patterns of SUMO conjugates were somewhat difficult to compare because of the faster gel mobility of mutants where Lys11, Lys15, and Lys19 were mutated and the intermediate mobility of the smt3-R15 mutant. However, the smt3-R11,15,19 and smt3-allR mutants appeared to contain greater quantities of conjugates at ~24, 28, and 58 kDa, consistent with the possibility that these bands represent monosumoylated versions of proteins that would normally be multiply sumoylated. However, the smt3-R15 mutant did not accumulate these bands. More strikingly, the ulp2Δ single mutant accumulated extremely high molecular weight SUMO conjugates that were
Fig. 7. SUMO conjugate patterns in suppressors of ulp2Δ temperature sensitivity. A, strains of the indicated genotypes were streaked onto YPD plates and grown for 2–3 days at 30°C (middle panel) or 37°C (bottom panel). B, whole cell lysates prepared from cells growing logarithmically at 30°C were analyzed by SDS-PAGE and immunoblotting with an Ab against SUMO (top and middle panels) or Cdc11 as a loading control (bottom panel). The top panel is an overexposure of the stacking gel portion of the gel shown in the middle panel. Unmodified Cdc11 is indicated. IB, immunoblot.

retained in the stacking gel on SDS-PAGE (Fig. 6, top panel, lane 2). These species were eliminated in the strains containing smt3-R11,15,19 or smt3-allR and were dramatically reduced in the smt3-R15 mutant (Fig. 6, lanes 3, 4, and 8), suggesting that the ulp2Δ mutant may be accumulating extremely long SUMO chains with branch sites at the N-terminal lysines, primarily Lys15. Consistent with this interpretation, other SUMO mutants that did not affect the N-terminal lysines had minimal effects on these high molecular weight conjugates.

We attempted to ask whether these high molecular weight species contained other proteins attached to the potential SUMO chains by immunoblotting the stacking gel of ulp2Δ samples with antibodies against the septin Cdc11 or against the HA epitope in TOP2-HA samples. Neither of these proteins were detected in the stacking gel (data not shown), but it is unlikely that this assay would have detected these species if they had been present. Our anti-Smt3 antibody is very sensitive, and these species, which contain dozens of copies of SUMO, are still much fainter than many lower molecular weight sumoylated species. Other epitopes present at single copy probably would not be detected.

To gain further insight into the nature and physiological relevance of these high molecular weight species, we looked for similar species in other mutants that also suppress the phenotypes of ulp2Δ. Previous work has shown that a temperature-sensitive mutation in ulp1 strongly suppresses the phenotypes of the ulp2Δ mutant (16, 17), and we have also found that the ulp2Δ siz1Δ siz2Δ strain grows somewhat better at high temperature than does ulp2Δ or either of the ulp2Δ siz double mutants (Fig. 7A). Samples from both the ulp2Δ ulp1ts and the ulp2Δ siz1Δ siz2Δ mutants also had greatly reduced levels of sumoylated species in the stacking gel as did the ulp2Δ siz2Δ double mutant (Fig. 7B). The correlation between suppression of the ulp2Δ phenotypes and reduction or elimination of these high molecular weight species supports the hypothesis that one cause of the temperature sensitivity of the ulp2Δ mutant could be accumulation of SUMO chains.

This model rests on the assumption that Ulp2 has SUMO chain cleaving activity. To test this, we formed high molecular weight SUMO chains in vitro using full-length Siz1 to drive all the SUMO in the reaction into extremely high molecular weight species. Adding GST-Ulp2 to these reactions released free SUMO and what appeared to be short chains containing two or three SUMO moieties (Fig. 8). This result suggested that Ulp2 is capable of cleaving SUMO chains. Since longer incubations did not cleave SUMO chains to completion and since the configuration of the original conjugates in this reaction, which contain some Siz1, is not known, it cannot be stated absolutely conclusively that Ulp2 is cleaving SUMO-SUMO bonds rather than exclusively SUMO-Siz1 bonds. However, it is unlikely that this substrate consists of mono-, di-, and tri-SUMO attached to a multitude of different lysines in Siz1 especially since the sample in Fig. 8, lane 4, appears to contain a uniform ladder of bands containing up to ~10 SUMO moieties. The modest activity of Ulp2 against this substrate is consistent with the weak activity of Ulp2 against other substrates in vitro (16). Ulp1, in contrast, rapidly cleaves any sumoylated protein in vitro, including the substrate used here (data not shown).

The N-terminal Extension of SUMO Is Dispensable in Yeast—One major structural difference between SUMO and ubiquitin is the N-terminal extension of SUMO. A large fraction of the extension in yeast SUMO is taken up by the repeat containing Lys1, Lys15, and Lys19, and since the lysines in this repeat were dispensable for the essential functions of SUMO, we asked what the effect would be of deleting the entire extension. A strain in which the genomic SMT3 locus was replaced with a truncated version lacking the 21 N-terminal amino acids (Fig. 1) grew well and was not notably sensitive to high or low temperatures or to HU, methyl methanesulfonate, or TBZ (data not shown). This strain appeared to have reduced overall
SUMO Chain Formation

Fig. 8. Ulp2 cleaves SUMO chains in vitro. Reactions containing SUMO, Aos1, Uba2, Ubc9, and recombinant full-length Siz1 were incubated with 5 mM ATP (lanes 2–8) for 2.5 h to form high molecular weight SUMO chains and stopped by the addition of hexokinase and glucose. Products of these reactions were then incubated at 30 °C for 3 h with GST (lanes 3, 5, and 7) or GST-Ulp2 (lanes 4, 6, and 8) that had been preincubated with 10 mM NEM (lanes 5 and 6) or 10 mM NEM plus 20 mM DTT (lanes 7 and 8). The stacking gel is indicated by the bar. Unmodified SUMO as well as bands corresponding to di- and tri-SUMO are marked.

Fig. 9. The N-terminal extension of SUMO is not essential for viability. A, whole cell lysates from log phase (lanes 1 and 3) and nocodazole-arrested (lanes 2 and 4) wild-type (lanes 1 and 2) and smt3-NΔ21 (lanes 3 and 4) cells were analyzed by SDS-PAGE and immunoblotting with an Ab against SUMO (top panel) or Cdc11 (bottom panel). Unmodified Cdc11 is indicated. B, strains of the indicated genotypes were grown to log phase, serially diluted, and spotted out onto YPD; YPD + 0.1 M HU; YPD + 10 mM caffeine; YPD + 1% dimethylformamide (DMF); or YPD + 75 μg/ml TBZ, 1% dimethylformamide and grown for 2–3 days at 30 °C or 37 °C as indicated. IB, immunoblot; NOC, nocodazole.

Levels of SUMO conjugates compared with wt (Fig. 9A, upper panel), although it is possible that the polyclonal antibody used here has significant reactivity against the N-terminal extension so that the observed reduction in SUMO conjugate levels may actually reflect reduced binding of the antibody to the same amounts of conjugates. The septin Cdc11 was modified to similar levels by wt and N-terminally truncated SUMO (Fig. 9A, lower panel). Like the smt3-R11,15,19 mutant, smt3-NΔ21 suppressed the temperature, caffeine, and TBZ sensitivities of the ulp2Δ mutant (Fig. 9B). Unlike smt3-R11,15,19, smt3-NΔ21 suppressed HU sensitivity very poorly. These results indicate that the N-terminal extension of SUMO is not required for the enzymatic activation or conjugation of SUMO or for the role of SUMO in yeast viability.

DISCUSSION

Previous studies have shown that SUMO, like ubiquitin, is capable of forming chains in vitro and have found evidence that some mammalian proteins are modified by SUMO chains in vivo (8, 24–26). However, the biological function of SUMO chain formation is not known. We have investigated the function of chain formation by SUMO in vivo in the yeast S. cerevisiae. The major result of these studies is the negative result that chain formation is not an essential feature of the role of SUMO in yeast. Strains expressing mutant SUMO either lacking Lys15, the major in vitro sumoylation site, or lacking all three lysines in the N-terminal non-ubiquitin-like domain were indistinguishable from wt both in their levels of the most abundant SUMO conjugates and in their growth rates and sensitivities to stress conditions. Strains lacking any of the other lysines in SUMO were similarly unremarkable both phenotypically and in their SUMO conjugation patterns, indicating that SUMO chains formed at other lysines also are not involved in any of the presently detectable functions of SUMO. Furthermore a strain in which all nine SUMO lysines were mutated to arginine was also viable, although it had some growth abnormalities and was conjugated to some proteins at lower levels than was wt SUMO. These differences probably reflect decreased overall efficiency of conjugation of the multiple mutant Smt3-allR protein rather than an effect on chain formation. For example, all SUMO conjugates to Top2 were present at reduced levels in the smt3-allR strain, including the monosumoylated form, which should not be affected by differences in chain formation.

The other major result of this study is that ulp2Δ mutants accumulated SUMO chains and that SUMO branch-site mutants suppressed several of the phenotypes of ulp2Δ. The evidence that these high molecular weight species were covalent conjugates containing SUMO chains was that they 1) required the N-terminal branch-site lysines of SUMO, 2) accumulated SUMO branch-site mutants suppressed several of the phenotypes of ulp2Δ, 3) were formed in the absence of the SUMO-specific protease Ulp2 whose function is to cleave covalent SUMO conjugates, 3) were formed only in the presence of Siz1 or Siz2, which have been shown to accumulate SUMO chains and that SUMO branch-site mutants suppressed the temperature, caffeine, and TBZ sensitivities of the ulp2Δ mutant (Fig. 9B). Unlike smt3-R11,15,19, smt3-NΔ21 suppressed HU sensitivity very poorly. These results indicate that the N-terminal extension of SUMO is not required for the enzymatic activation or conjugation of SUMO or for the role of SUMO in yeast viability.
non-covalently and sometimes have similar biological activity even when their active sites have been mutated (16, 32). However, the preponderance of the evidence favors the presence of SUMO chains.

Our data are also consistent with the possibility that accumulation of SUMO chains is one of the causes of the various phenotypes displayed by ulp2ΔΔ cells. The reduction of high molecular weight species correlated well with suppression of the ulp2ΔΔ phenotypes as smt3-R11,15,19, smt3-R15, smt3-allR, smt3-NΔ21, ulp1ts, and siz1Δ siz2 all reduced or eliminated the high molecular weight species and suppressed at least some of the ulp2ΔΔ phenotypes. However, the siz2 Δ ulp2 mutant also had reduced levels of high molecular weight species but did not suppress ulp2ΔΔ temperature sensitivity. In theory, the phenotypes of ulp2ΔΔ mutants could result from toxicity of uncleaved SUMO conjugates, possibly chains, from depletion of the pool of free SUMO or from a disrupted flux of SUMO from one substrate to the next. However, overexpression of free SUMO is actually toxic to ulp2ΔΔ mutants, suggesting that its phenotypes do not stem from a reduction in free SUMO (17). The possibility that specific SUMO conjugates can inhibit growth is supported by the observation that elimination of the major SUMO attachment sites in Top2 suppresses ulp2ΔΔ phenotypes (21); Top2 conjugates do not make up a significant enough fraction of SUMO conjugates to have an effect on the concentration of free SUMO. We tested whether suppression by SUMO N-terminal mutants was linked to an effect on SUMO-Top2 and found that the smt3-R11,15,19 mutant, which strongly suppressed the temperature sensitivity of ulp2ΔΔ, did not significantly reduce SUMO conjugation to Top2. This observation suggests that smt3-R11,15,19 suppresses ulp2ΔΔ phenotypes by a mechanism not involving Top2, although it remains possible that the growth-inhibitory Top2 conjugates are extremely high molecular weight ones that were not detected or that the activity of the SUMO-Top2 conjugates in this mutant is changed so that they are less toxic.

Our work focused primarily on the interaction between SUMO chains and Ulp2, but Ulp1 may also reduce levels of SUMO chains in yeast. Ulp1 cleaves chains in vitro (data not shown), and the ulp1 ts mutant accumulated high molecular weight SUMO-containing species when grown at the permissive temperature (at the restrictive temperature the SUMO precursor was not cleaved) (Fig. 7 and data not shown). It remains to be tested whether these species require the branch-site lysines of SUMO. Since Ulp1 and Ulp2 cleave SUMO from different proteins and localize to different parts of the cell, it is possible that they would cleave chains attached to different proteins.

Another result from this study was that SUMO mutants lacking the N-terminal branch sites were attached to other proteins more promiscuously in vitro. In our in vitro reactions, which contain large amounts of free SUMO in addition to other possible substrates, the chain formation reaction appears to compete with sumoylation of other proteins. Peptides conforming to the SUMO attachment site motif competed well, while nonspecific lysines in the substrates and enzymes in the reaction competed poorly. Thus, when the branch site was not present, sites that were poor substrates were modified to a greater extent. It seems unlikely that this is a major mechanism for controlling substrate specificity of SUMO conjugation.

In vivo simply because the levels of free SUMO are quite low relative to all the potential nonspecific substrates having surface-exposed lysine residues.

In these studies we have examined the effects of mutating or deleting the N-terminal extension of yeast SUMO, which contains the branch site for formation of SUMO chains. These experiments have shown that SUMO chain formation is not a major feature of SUMO function in yeast either for production of the most abundant conjugates or for the activities that are required for yeast viability and stress resistance. However, it is likely that SUMO chains do form in wt yeast but that SUMO proteases limit their steady-state quantities. The functions of these chains and the identities of substrates to which they are attached are topics for future studies.

Acknowledgments—We thank Alan Lewis, Mark Hochstrasser, Jürgen Dohmen, Ingrid Schwienhorst, and Chris Lima for strains and plasmids; Denise Hollman for constructing the original Top2-HA strain; and Nishant Gandhi for technical assistance. We also thank Alison Reindel for comments on the manuscript.

REFERENCES

1. Melchior, F. (2000) Annu. Rev. Cell Dev. Biol. 16, 591–626
2. Kim, K. I., Baek, S. H., and Chung, C. H. (2002) J. Cell Physiol. 191, 257–268
3. Schwartz, D. C., and Hochstrasser, M. (2003) Trends Biochem. Sci. 28, 321–328
4. Johnson, E. S., Schwienhorst, I., Dohmen, R. J., and Blobel, G. (1997) EMBO J. 16, 5509–5519
5. Ohtani, T., Honda, R., Ichikawa, G., Tsunemari, N., and Yasuda, H. (1999) Biochem. Biophys. Res. Commun. 254, 693–698
6. Johnson, E. S., and Blobel, G. (1997) J. Biol. Chem. 272, 26799–26802
7. Desterrro, J. M., Thomson, J., and Hay, R. T. (1997) FEBS Lett. 417, 297–300
8. Johnson, E. S., and Gupta, A. A. (2001) Cell 106, 735–744
9. Kahyo, T., Nishida, T., and Yasuda, H. (2001) Mol. Cell 8, 713–718
10. Takahashi, Y., Kahyo, T., Toh-e, A., Yasuda, H., and Kikuchi, Y. (2001) J. Biol. Chem. 276, 4873–4877
11. Pichler, A., Gast, A., Seeler, J. S., Dejean, A., and Melchior, F. (2002) Cell 108, 109–120
12. Kagey, M. H., Melhuish, T. A., and Wotton, D. (2003) Cell 114, 253–264
13. Sampson, D. A., Wang, M., and Matunis, M. J. (2001) J. Biol. Chem. 276, 21664–21669
14. Bernier-Villamor, V., Sampson, D. A., Matunis, M. J., and Lima, C. D. (2002) Cell 108, 345–356
15. Li, S. J., and Hochstrasser, M. (1999) Nature 398, 246–251
16. Li, S. J., and Hochstrasser, M. (2000) Mol. Cell. Biol. 20, 2367–2377
17. Schwienhorst, I., Johnson, E. S., and Dohmen, R. J. (2000) Mol. Gen. Genet. 263, 771–786
18. Johnson, E. S., and Blobel, G. (1999) J. Cell Biol. 147, 981–994
19. Takahashi, Y., Iwase, M., Kinomochi, M., Tanaka, M., Toh-e, A., and Kikuchi, Y. (1999) Biochem. Biophys. Res. Commun. 259, 582–587
20. Hoeger, C., Pfander, B., Moldovan, G. L., Pyrowolakis, G., and Jentsch, S. (2002) Nature 419, 135–141
21. Bachant, J., Alcasabas, A., Blat, Y., Kieckner, N., and Elledge, S. J. (2002) Mol. Cell 9, 1169–1182
22. Strunnikov, A. V., Arwind, L., and Koonin, E. V. (2001) Genetics 158, 95–107
23. Johnson, E. S. (2002) Nat. Cell Biol. 4, E285–E288
24. Tatham, M. H., Jaffray, E., Vaughan, O. A., Desterro, J. M., Botting, C. H., Naimish, J. H., and Hay, R. T. (2001) J. Biol. Chem. 276, 35368–35374
25. Benoist, K. P., Podgorski, M. S., Pagala, V. R., Slaughter, C. A., and Schulman, B. A. (2002) J. Biol. Chem. 277, 47938–47945
26. Li, Y., Wang, H., Wang, S., Quon, D., Liu, Y. W., and Cordell, B. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 259–264
27. Aeschbacher, K., Breit, R., Kingston, R. E., Moore, D. D., Smith, J. A., Seidman, J. G., and Struhl, K. (2000) Current Protocols in Molecular Biology, Wiley-Interscience, New York
28. Sherman, F., Fink, G. R., and Hicks, J. B. (1986) Methods in Yeast Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
29. Dohmen, R. J., Stappen, R., McGrath, J. P., Forrova, H., Kolarov, J., Goffeau, A., and Varshavsky, A. (1995) J. Biol. Chem. 270, 18099–18109
30. Yaffe, M. P., and Schatz, G. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 4819–4823
31. Takahashi, Y., Toh-e, A., and Kikuchi, Y. (2003) J. Biochem. (Tokyo) 133, 415–422
32. Best, L. J., Ganiatias, S., Agarwal, S., Changou, A., Salomoni, P., Shirihai, O., Meluh, P. B., Pandolfi, P. P., and Zon, L. I. (2002) Mol. Cell 10, 843–855