Noncovalent Binding of Small Ubiquitin-related Modifier (SUMO) Protease to SUMO Is Necessary for Enzymatic Activities and Cell Growth*

Received for publication, November 20, 2006, and in revised form, February 26, 2007 Published, JBC Papers in Press, April 11, 2007, DOI 10.1074/jbc.M610723200

Motomasa Ihara1, Hirofumi Koyama1, Yasuhiro Uchimura1, Hisato Saitoh3, and Akira Kikuchi1,2

From the 1Department of Biochemistry, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima 734-8551, and the 2Department of Regulation Medicine, Institute of Molecular Embryology Genetics, Kumamoto University, Kumamoto 860-0811, Japan

SUMO proteases possess two enzymatic activities to hydrolyze the C-terminal region of SUMOs (hydrolytic activity) and to remove SUMO from SUMO-conjugated substrates (isopeptidase activity). SUMO proteases bind to SUMOs noncovalently, but the physiological roles of the binding in the functions of SUMO proteases are not well understood. In this study we found that SUMO proteases (Axam, SENP1, and yeast Ulp1) show different preferences for noncovalent binding to various SUMOS (SUMO-1, -2, -3, and yeast Smt3) and that the hydrolysis and isopeptidase activities of SUMO proteases are dependent on their binding to SUMOs through salt bridge. Expression of Smt3 suppressed the phenotype of yeast mutant lacking Smt3, which exhibits growth arrest, and the binding of Ulp1 to Smt3 was essential for this rescue activity. Although expression of an Smt3 mutant (smt3R64E(GG)), which conjugates to substrate but loses the ability to bind to Ulp1, rescued the phenotype of yeast lacking Smt3 partially, the mutant cells showed an increment in the doubling time and a delay of desumoylation of Smt3-conjugated Cdc3. These results indicate that the noncovalent binding of SUMO protease to SUMO through salt bridge is essential for the enzymatic activities and that the balance between sumoylation and desumoylation is important for cell growth.

Small ubiquitin-related modifier (SUMO) family proteins function by covalent attachment to other proteins as post-translational modifications (1, 2). Mammals contain at least three SUMO family members SUMO-1, SUMO-2, and SUMO-3. SUMO-2 and SUMO-3 share 43 and 42% identity to SUMO-1, respectively, but share greater than 96% sequence identity to each other. Saccharomyces cerevisiae (S. cerevisiae) Smt3 shares 43% amino acid identity with mammalian SUMO-1, 39% identity with SUMO-2, and 40% identity with SUMO-3. SUMO modifies many proteins that participate in diverse cellular processes, including transcriptional regulation, nuclear transport, maintenance of genome integrity, and signal transduction (1, 2). Covalent interaction between SUMO and its targets is achieved by formation of an isopeptide bond between the C-terminal glycine of SUMO and the ε-amino group of a lysine in the acceptor protein (3). This reaction is ATP-dependent and requires the SUMO-activating E1 enzyme Aos1/Uba2, the SUMO-conjugating E2 enzyme Ubc9, and a SUMO E3 ligase (4–6).

The pattern of SUMO conjugates changes dynamically during the cell cycle and in response to various stimuli (7). SUMO proteases have at least two functions in this process. They remove SUMO from proteins, making the modification reversible, and they also provide a source of free SUMO to be used for conjugation. Free SUMO is generated both from newly synthesized SUMO, which is cleaved to remove a short C-terminal peptide and expose glycine at the C terminus, and from desumoylation of existing conjugates. All known SUMO proteases belong to the ubiquitin-like protease 1 (Ulp1) cysteine protease family, which was originally found in S. cerevisiae (7). Ulp1 proteases are characterized by a C-terminal 200-amino acid core domain that contains the catalytic triad Cys-His-Asp. In addition to the catalytic domain, Ulp1 proteases contain N-terminal domains that are variable in both size and sequence. These domains are critical for intracellular localization and may also have roles in target specificity or regulation of activity (8).

Two SUMO proteases with distinct functions have been described in S. cerevisiae. Ulp1 localizes to the nuclear pore complex (NPC) and is required for cleaving both the Smt3 precursor and Smt3 conjugates to other proteins; whereas Ulp2/Smt4 localizes to the nucleus, does not cleave the precursor, and appears to desumoylate a distinct set of conjugates (7, 9, 10). At least seven genes in mammalian genomes encode proteins with the Ulp domain, which constitute the so-called Senstrin-specific protease (SENP) family (11). Among them, SENP1 mainly localizes to foci in the nucleus and the nuclear rim (12), while SENP2 (also called Axam) binds to the nucleoplasmic side of the NPC (13). Although it has been suggested that different members of the SENP family differ in their catalytic properties, the details have not yet been systematically studied.
SUMO proteases are also known to bind to SUMOs noncovalently. SMT3 IP1 (SMP3) was originally identified as a Smt3-binding protein (14). Furthermore, analyses of the x-ray structures of the complexes between Ulp1 and Smt3, between SENP2 and SUMO-1, and between SENP1 and SUMO-2 have identified some interfaces between SUMO proteases and SUMO proteins (15–17). It has been shown that six motifs of SUMO proteases recognize the SUMO/Smt3 β-sheet and C-terminal strand. Among them, the region of SENP1 that interacts with the C-terminal strand of SUMO is important for the enzymatic activity of SENP-1, whereas the region of SENP1 that recognizes the SUMO β-sheet through salt bridge plays less critical roles (17). It has been shown that mutation of the amino acid involved in the salt bridge in Ulp1 cannot rescue the ulp1 phenotype (15), indicating that the noncovalent binding is necessary for biological functions of SUMO proteases. However, the role of the noncovalent binding between SUMO proteases and SUMOs through salt bridge is not clear. In this study we analyzed the physiological significance of the salt bridge between SUMO proteases and SUMOs. Here we show that their noncovalent binding is important for the hydrolase and isopeptidase activities of SUMO proteases and is essential for cell growth.

**EXPERIMENTAL PROCEDURES**

**Materials and Chemicals—**pFlag-CMV2/mouseSENP1 and pFlag-CMV2/mouseSENP1C599A, pGEX2TK/SUMO-3(GG), pT-E1E2SUMO-1(GG), and pT-E1E2SUMO-3(GG), pRS314-GAL-Myc-B5, pRS425, and pUC-HIS3 were provided by Drs. T. Nishida (Tokyo University of Pharmacy, Tokyo, Japan), K. Tanaka (Hokkaido University, Sapporo, Japan), E. Tsuchiya (Hiroshima University, Hiroshima, Japan), and M. Yukawa (Hiroshima University, Hiroshima, Japan), respectively. Yeast strains T-2 and T-9 and plasmid pT-7 were kindly given by M. Hochstrasser (Yale University) (7). SUMO-conjugated His₆-tagged Tcf-4 (273–387) (SUMO-Tcf-4) proteins were provided by Drs. Yukawa (Hiroshima University, Higashi-Hiroshima, Japan), T. Nishida (Tokyo University of Pharmacy, Tokyo, Japan), K. Yokokawa (Hiroshima University, Higashi-Hiroshima, Japan), and M. Tanaka (Hokkaido University, Sapporo, Japan), E. Tsuchiya (Hiroshima University, Higashi-Hiroshima, Japan), and M. Yukawa (Hiroshima University, Hiroshima, Japan), respectively. Yeast strains T-2 and T-9 and plasmid pT-7 were kindly given by Y. Kikuchi (University of Tokyo, Tokyo, Japan) (18, 19). HYH1321 and YCplac22-ULP1 were generously provided by M. Hochstrasser (Yale University) (7). SUMO-conjugated His₁₉-tagged Tcf-4 (273–387) (SUMO-Tcf-4) proteins were obtained by using an Escherichia coli sumoylation system (20). E. coli strain BL21(DE3), co-transformed with pET28/Tcf-4E (273–387) and pT-E1E2SUMO protein expression vectors, was used to express SUMO-Tcf-4. Glutathione S-transferase (GST) fusion proteins were purified from E. coli according to the suppliers’ instructions. Because GST-Axam was easily degraded, GST-Axam (272–588) was used as an enzyme source for Axam, and throughout this study this protein was called GST-Axam.

5-Fluoroorotic acid (5-FOA) was purchased from Wako (Osaka, Japan). Anti-FLAG (M2), anti-HA, anti-GFP, anti-His₆, anti-SUMO-1, anti-SUMO-2, and anti-SUMO-3 antibodies were purchased from Sigma-Aldrich, Inc., Covance Laboratories, Inc. (Richmond, CA), Molecular Probes, Inc. (Eugene, OR), Clontech Laboratories, Inc. (Palo Alto, CA), Alexis Biochemicals (Lausen, Switzerland), Chemicon International, Inc. (Temecula, CA), and Upstate (Lake Placid, NY), respectively. The rabbit polyclonal anti-GST antibody was made by a standard method. Other materials were from commercial sources.

**Plasmid Construction—**pCGN/SUMO-1, pCGN/SUMO-1ΔGG, pCGN/SUMO-3, pGEX2TK/SUMO-1(GG), pEGFP-C1/Axam, pEGFP-C1/AxamC547S, pGEX4T-2/Axam (72–588), pGEX4T-2/AxamC547S (72–588), and pHF-Bos-HA/hTcf-4E were constructed as described (21, 22). Standard recombinant DNA techniques were used to construct the following plasmids: pCGN/SUMO-1(GG), pCGN/SUMO-1R66E, pCGN/SUMO-1R63E(GG), pCGN/SUMO-2R58E, pCGN/SUMO-3(GG), pCGN/SUMO-3R59E, pCGN/SUMO-3R59E(GG), pGEX2T/SUMO-1-Myc, pGEX2T/SUMO-1R537E-Gal, pGEX2T/SUMO-2-Myc, pGEX2T/SUMO-2R58E-Myc, pGEX2T/SUMO-3-Myc, pGEX2T/SUMO-3R59E-Myc, pT-E1E2SUMO-1R63E(GG), pT-E1E2SUMO-2(GG), pT-E1E2SUMO-3R59E(GG), pGEX2T/MYC3, pGEX2T/smt3R64E-Myc, pGEX2T/C2/SNIP1, pGEX6p-1/SNIP1, pGEX6p-1/SNIP1C599A, pGEX6p-1/SNIP1D464N, pGEX2T/Flag-ULP1, pGEX2T/Flag-ulp1C580S, pGEX2T/Flag-ulp1D451N, pGEPF-C1/Flag-ULP1, pGEPF-C1/Flag-ulp1C580S, pGEPF-C1/Flag-ulp1D451N, pGEPF-C1/Flag-ulp1D451N, pGEPF-C1/Flag-ulp1D451N, pGEPF-C1/Flag-ulp1D451N, pGEPF-C1/Flag-ulp1D451N, pGEPF-C1/Flag-ulp1D451N, pGEPF-C1/Flag-ulp1D451N, pGEPF-C1/Flag-ulp1D451N.

**Strains and Genetic Manipulations—**S. cerevisiae strains used in this study are listed in Table 1. W303 was used as a wild-type strain. To generate YHK001, YHK002 was transformed with the ulp1::HIS3 fragment and selected a clone viable on SD-His-Ura plates and non-viable on SD-His + 5-FOA plates. The ulp1::HIS3 fragment was amplified with the primers 5’-ATTAAACAGCTTTCAGGATTGCG-3’ and 5’-CCA-

### TABLE 1

| Yeast strains used in this study | Genotype | Background or reference |
|---------------------------------|----------|-------------------------|
| MATa ade2-1 leu2-3,112 his3-11,15 trp1-1::ura3-1 can1-100 | W303 (this study) |
| MATa smt3Δ::TRP1 pYESHA-SMT3-URA3 (W303-1A) | W303 (18) |
| MATa CDC5-5HA::TRP1 | W303 (19) |
| MHY1321 MATa ulp1Δ::HIS3-2,112 his3-Δ200 trp1-1::ura3-52 lys2-801 | S288C (7) |
| YHK001 MATa ulp1Δ::HIS3 Ycg50-ULP1 | W303 (this study) |
| YHK002 MATa Ycg50-ULP1 | W303 (this study) |
| YHK003 MATa CDC5-5HA::HIS3 | W303 (this study) |
| YHK004 MATa smt3Δ::TRP1 pYESHA-SMT3-URA3 pYESHA-SMT3-LEU2 | W303 (this study) |
| YHK005 MATa smt3Δ::TRP1 pYESHA-SMT3-URA3 pYES-SUMO-1-LEU2 | W303 (this study) |
| YHK006 MATa smt3Δ::TRP1 pYESHA-SMT3-URA3 pYES-SUMO-3-LEU2 | W303 (this study) |
| YHK007 MATa smt3Δ::TRP1 CDC5-5HA::HIS3 pRS425-smt3R64E(GG) | W303 (this study) |
| YHK008 MATa smt3Δ::TRP1 CDC5-5HA::HIS3 pRS425-smt3R64E(GG) | W303 (this study) |
CATAGGTCGAAGACAAACTGTC-3'. MHY1321 genomic DNA was used as a template for PCR to amplify the ulp1::HIS3 fragment. To generate YHK003, YCp-CDC3-HA::HIS3 was cut with Ncol and introduced into W303, and His\(^+\) transformants were confirmed by PCR and Western blotting. Preparation of rich (yeast extract–peptone–dextrose, YPD) and synthetic complete (SC) media and standard genetic methods were as described (23).

**Interaction of SUMO Proteases and SUMO Proteins**—To determine whether SUMOs interact with deSUMoylation enzymes, COS cells (60-mm diameter dishes) transfected with pEGFP-derived plasmids or pFlag-CMV2/SENP1 were lysed in 200 μl of lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM dithiothreitol (DTT), 1% Nonidet P-40, 20 μg/ml leupeptin, 20 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride (PMSF)) and the lysates were centrifuged at 20,000 \( \times g \) for 15 min at 4 °C. The supernatant (200 μg of protein) was incubated with 1 μM GST-SUMO immobilized on glutathione-Sepharose 4B for 2 h at 4 °C. GST-SUMO was precipitated by centrifugation and washed once with lysis buffer, twice with 0.5 mM LiCl, and 100 mM Tris–HCl, pH 7.5, and once with 10 mM Tris–HCl, pH 7.5. The precipitates were probed with anti-GFP, anti-FLAG, or anti-GST antibody.

To determine whether SUMO-1-Tcf-4 interacts with Axam, 1 μM SUMO-1-Tcf-4 or SUMO-1R64E-Tcf-4 was incubated with 1 μM GST-AxamC547S immobilized on glutathione-Sepharose 4B in 50 μl of T20D1 buffer (20 mM Tris–HCl, pH 7.5 and 1 mM DTT) for 2 h on ice. GST-AxamC547S was precipitated by centrifugation, and then the precipitates were washed with T20D1 buffer three times and probed with anti-SUMO-1 antibody.

**Enzyme Assays in Vitro**—To examine the hydrolase activity of SUMO proteases in vitro, 2 μM GST-SUMOs-Myc were incubated with the indicated concentrations of GST-Axam, GST-SENP1, or GST-Flag-Ulp1 in 25 μl of reaction mixture (100 mM Tris–HCl, pH 8.0, 2 mM DTT, 1 mM EDTA, and 5% glycerol) at 30 °C for 10 min as described previously (21). After the incubation, the mixtures were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) followed by Coomassie Brilliant Blue staining. Band intensities were quantified using NIH Image (version 1.63). The \( K_m \) value was calculated by averaging the values derived from the fits for each data set.

For analysis of cleavage of SUMO-Tcf-4 by SUMO proteases in vitro, 1 μM SUMOs-Tcf-4 were incubated with the indicated concentrations of GST-Axam, GST-SENP1, or their mutants in 25 μl of reaction mixture (50 mM Tris–HCl, pH 7.5, 1 mM DTT, 20 mM EDTA, and 150 mM NaCl) for 30 min at 30 °C. After the incubation, the mixtures were subjected to SDS-PAGE followed by Coomassie Brilliant Blue staining. Band intensities were quantified using NIH Image (version 1.63). The \( K_m \) value was calculated by averaging the values derived from the fits for each data set.
and 20 mM N-ethylmaleimide (NEM)) and lysed by vigorous vortexing for 10 min with an equal volume of glass beads at 4 °C. Cdc3-HA was immunoprecipitated with anti-HA antibody. For analysis of the cleavage of Sm3-Cdc3 by Ulp1 in vitro, 20 μl of Sm3-Cdc3-HA conjugated protein A beads were incubated with the indicated amounts of GST-Ulp1 or its mutants in 25 μl of reaction mixture (50 mM Tris-HCl, pH 7.5, 1 mM DTT, 20 mM EDTA, and 150 mM NaCl) for 10 min at 30 °C. After the incubation, the mixtures were subjected to SDS-PAGE and probed with anti-HA antibody.

Fluorescence-activated Cell Sorting Analyses—W303 was grown in YPD medium and smt3Δ background strains were grown in SG-Leu-Ura medium at 28 °C. All strains were resuspended in YPD medium to 1 × 10⁶ cells/ml and incubated at 28 °C. At 0 and 12 h, 1 × 10⁷ cells were collected by centrifugation, suspended in 0.2 mM Tris-HCl, pH 7.5 and fixed with 70% (v/v) ethanol and stored overnight at −20 °C. The fixed cells were washed with 0.2 mM Tris-HCl, pH 7.5 and suspended in 200 μl of the same buffer. After a brief sonication (5 s), 100 μl of the same buffer containing RNase A (1 mg/ml) was added to the sample, and the incubation was continued for 4 h at 30 °C. The RNase A-treated cells were stained with 100 μl of propidium iodide (PI) solution (50 μg/ml) in 4 mM sodium citrate, 10 mM NaCl, and 0.1% Nonidet P-40 for 15 min on ice. The fluorescence intensities of stained cells were analyzed with a FACS Calibur (Becton Dickinson) and FlowJo (Tree Star). The morphology and nuclear localization of PI-stained cells were observed with an inverted microscope (IX-FLA; OLYMPUS, Tokyo, Japan).

Sumoylation and Desumoylation Assay in Intact Cells—COS or 293T cells (35-mm diameter dishes) were transfected with pCGN-, pEGFP-, pEF-BOS-, or pFlag-CMV2-derived plasmids to examine the sumoylation and desumoylation in intact mammalian cells as described (22).

To examine the desumoylation of the Sm3 modification of Cdc3-HA in intact yeast cells, the T-9 strain was arrested at G₂/M with 15 μM nocodazole for 4 h. At the indicated time after nocodazole was removed, cells were lysed in lysis buffer (50 mM Tris-HCl, pH 8.0, 300 mM mannitol, 100 mM KCl, and 1 mM EGTA) containing protease inhibitor mixture (20 μg/ml apro tinin and leupeptin, 10 μg/ml pepstatin A, and 10 mM PMSF) and 10 mM NEM. After the lysates (200 μg of protein) were immunoprecipitated with anti-HA antibody, the precipitates and the samples were further incubated with the indicated amounts of GST-Flag-Ulp1, GST-Flag-ulp1C580S, or GST-Flag-ulp1D451N in 25 μl of reaction mixture for 30 min at 30 °C. After incubation, the mixtures were subjected to SDS-PAGE and probed with the anti-HA or anti-GST antibody.

Other Assays—The immunofluorescence study was done as described (22, 24).
SUMO diglycine motif by SUMO protease may be specific.

is dependent on the salt bridge and that the recognition of the specificity of the binding between SUMO protease and SUMO

**FIGURE 3. Isopeptidase activity of Axam and SENP1.** A, SUMO-conjugated His$_6$-Tcf-4 (SUMO-Tcf-4) (0.1 μg of each) used in the experiments was subjected to SDS-PAGE and probed with anti-His$_6$ antibody. Ab, antibody; IB, immunoblotting; WT, wild type. B, 1 μM SUMO-3-Tcf-4 was incubated with the indicated concentrations of GST-Axam for 30 min at 30 °C. GG, SUMO-3-(1–97). C, 1 μM SUMO-1-Tcf-4 ( ), SUMO-1R63E-Tcf-4 ( ), SUMO-2-Tcf-4 ( ), SUMO-3-Tcf-4 ( ), or SUMO-3R59E-Tcf-4 ( ) was incubated with the indicated concentrations of GST-Axam for 30 min at 30 °C. The results shown are means ± S.D. of five independent experiments. D, 1 μM SUMO-1-Tcf-4 or SUMO-1R63E-Tcf-4 was incubated with 1 μM GST-AxamC547S. GST-AxamC547S was precipitated by centrifugation, and then the precipitates were probed with anti-SUMO-1 antibody. Ab, antibody; IB, immunoblotting; WT, wild type; CS, C547S; R63E, SUMO-1R63E. E, 1 μM SUMO-1-Tcf-4 was incubated with the indicated concentrations of GST-SENP1 for 30 min at 30 °C. GG, SUMO-3-(1–97). F, 1 μM SUMO-1-Tcf-4 ( ), SUMO-2-Tcf-4 ( ), SUMO-SENP1-Tcf-4 ( ), SUMO-3-Tcf-4 ( ), or SUMO-3R59E-Tcf-4 ( ) was incubated with the indicated concentrations of GST-SENP1 for 30 min at 30 °C.

**Specificity of Hydrolyase Activity of Axam and SENP1**—To clarify the role of the salt bridge to SUMO in the actions of SUMO proteases, we analyzed the hydrolyase activity using purified proteins. Axam hydrolyzed SUMO-3-Myc efficiently to produce SUMO-3(GG) in a dose-dependent manner (Fig. 2B). Axam did not act on SUMO-1-Myc (Fig. 2B). Axam did not hydrolyze SUMO-3R59E-Myc (Fig. 2C), and AxamD412N did not act on SUMO-3-Myc (Fig. 2B). When a large amount of Axam (1 μM) was incubated with SUMO-1 and SUMO-2 for 3 h, hydrolysis of SUMO-1 and SUMO-2 was observed. Even under these conditions, Axam could not hydrolyze SUMO-1R63E or SUMO-2R58E (Fig. 2C).

SENP1 hydrolyzed SUMO-1-Myc and SUMO-3-Myc, and less effectively hydrolyzed SUMO-2-Myc (Fig. 2, D and E). SENP1 did not hydrolyze SUMO-1R63E-Myc, SUMO-2R58E-Myc, and SUMO-3R59E-Myc (Fig. 2F). SENP1D464N did not act on SUMO-1 (Fig. 2F). These results indicate that the binding of SUMO proteases and SUMOs is necessary but not sufficient for the hydrolyase reaction.

**Specificity of Isopeptidase Activity of Axam and SENP1**—Next we examined the role of the salt bridge between SUMO proteases and SUMOs in isopeptidase activity. We previously showed that Axam cleaves SUMO-1 from SUMO-1-conjugated Tcf-4 (22, 24). Because Tcf-4 could not be modified with SUMO-1 efficiently in *in vitro* reconstitutive assays, we overexpressed SUMO-conjugated Tcf-4 in *E. coli* (20) to generate high concentrations of SUMO-1-Tcf-4 that would be suitable for enzymatic analyses. Approximately 50% of His$_6$-Tcf-4 was modified with SUMO-1, -3, or their mutants in *E. coli* and purified them to use as substrates (Fig. 3A). His$_6$-Tcf-4 was also modified with SUMO-2 and its mutant (data not shown). Axam catalyzed the deconjugation of SUMO from SUMO-3-Tcf-4 and SUMO-2-Tcf-4 at nearly equivalent rates in a dose-dependent manner (Fig. 3B, C and F). Furthermore, Axam was able to deconjugate SUMO-1-Tcf-4 although it could not hydrolyze SUMO-1. However, Axam did not show isopeptidase activity for SUMO-3R59E-Tcf-4 and SUMO-1R63E-Tcf-4 (Fig. 3C). Interestingly, GST-AxamC547S, which was used to prevent desumoylation of SUMO-1-Tcf-4, directly bound to SUMO-1-Tcf-4 but not SUMO-1R63E-Tcf-4 (Fig. 3D). Similarly, SENP1 deconjugated

(Arg$^{59}$), Axam (Asp$^{112}$), and SENP1 (Asp$^{464}$). None of the SUMO mutants in which Arg is changed to Glu could precipitate Axam or SENP1 (Fig. 1B). AxamD412N and SENP1D464N, in which Asp is changed to Asn, did not form a complex with GST-SUMO-3 (Fig. 1C). Furthermore, AxamD412N did not bind to GST-SUMO-2 either, and SENP1D464N interacted with neither GST-SUMO-1 nor GST-SUMO-2 (data not shown). To examine whether the C-terminal region of SUMO is required for the binding to SUMO protease, we generated SUMO mutants in which the C-terminal region after the diglycine motif (GG) or the C-terminal region, including the diglycine motif (DG), is deleted. Axam bound to GST-SUMO-3 (GG) and GST-SUMO-3 (DG) with the similar efficiency to its binding with GST-SUMO-3 (WT) (Fig. 1D, left panel). SENP1 interacted with GST-SUMO-1(GG) or GST-SUMO-3(GG) but not with GST-SUMO-1(DG) or GST-SUMO-3(DG) (Fig. 1D, right panel). These results indicate that the specificity of the binding between SUMO protease and SUMO is dependent on the salt bridge and that the recognition of the SUMO diglycine motif by SUMO protease may be specific.
Noncovalent Binding of SUMO Protease and SUMO

FIGURE 4. Hydrolysis of and sumoylation by SUMO mutants in intact cells. A, HeLaS3 cells expressing HA-SUMO-1 or its mutants were stained with anti-HA antibody. The nuclei were stained with DAPI. The results shown are representative of three independent experiments. WT, wild type; R63E, SUMO-1R63E; ΔGG, SUMO-1-(1–97); GG, SUMO-1-(1–97); R63E(GG), SUMO-1R63E(GG). B, COS cells expressing the indicated proteins were treated with 10% trichloroacetic acid. The precipitates were probed with anti-HA antibody. C, 293T cells expressing the indicated proteins were treated with 10% trichloroacetic acid. The precipitates were probed with anti-Tcf-3/4 antibody.

The Binding of SUMO Proteases to SUMOs Regulates Sumoylation Status in Intact Cells—To show how the salt bridge of SUMOs affects sumoylation in intact cells, we expressed various SUMO-1 mutants in HeLa S3 cells and observed their intracellular localization (Fig. 4A). HA-SUMO-1(GG) and HA-SUMO-1R63E(GG) showed diffuse nuclear localization with a few small dots. In contrast, HA-SUMO-1R63E and HA-SUMO-1(ΔGG) showed diffuse distribution in the cytoplasm and nucleus. Similar results were obtained with HA-SUMO-3 mutants (data not shown).

To see whether these SUMO mutants were present as monomer forms or protein-conjugated forms, lysates of cells expressing various SUMO-1 mutants were analyzed by Western blotting (Fig. 4B). When HA-SUMO-1 was expressed, both the monomer form of SUMO-1 and SUMO-conjugated proteins were observed. Expression of HA-SUMO-1(GG) and HA-SUMO-1(ΔGG) mainly produced SUMO-conjugated proteins and the monomer form of SUMO-1, respectively. Although the monomer form was observed in lysates of cells by expressing HA-SUMO-1R63E, SUMO-conjugated proteins were mainly detected by the expression of HA-SUMO-1R63E(GG). Consistent with these results, Tcf-4 was sumoylated efficiently when SUMO-1R63E(GG) was expressed (Fig. 4C).

Taken together with the data from the biochemical analyses, these results indicate that the SUMO Arg mutants in which the C-terminal region is cleaved are used for sumoylation and that the SUMO Arg mutant-conjugated proteins are not substrates for SUMO proteases in intact cells. Sumoylation of proteins is critical for various cell functions, but it is not clear whether desumoylation itself is necessary. We thought that SUMO-1R63E(GG), SUMO-2R58E(GG), and SUMO-3R59E(GG) would be good tools for analyzing the roles of the salt bridge between SUMO proteases and SUMOs in this point. However, it was hard to effectively knockdown SUMO-1, SUMO-2, SUMO-3, SENP1, and Axam by RNA interference in mammalian cells. Therefore, we used smt3 and/or ulp1 mutant of yeasts.

The Binding of Smt3 and Ulp1 Is Essential for Cell Growth—To test the importance of the salt bridge between Ulp1 and Smt3 in yeast biochemically, the lysates of yeast expressing GFP-Flag-Ulp1 were incubated with various GST-SUMOs. GFP-Flag-Ulp1 formed a complex with GST-Smt3 but not with GST-smt3R64E (Fig. 5A). Furthermore, it formed a complex with GST-SUMO-1 with lower efficiency as compared with GST-Smt3, and did not bind to GST-SUMO-2 or GST-SUMO-3 (Fig. 5A). GFP-Flagulp1C580S but not GFP-Flag-ulp1D451N bound to GST-Smt3 (Fig. 5A). GFP-SENP1 but not GFP-Axam showed binding activity for GST-Smt3, and GFP-SENP1 did not bind to GST-smt3R64E (data not shown). GST-Smt3 and GST-smt3(GG) bound to GFP-Flag-Ulp1 at the similar efficiency, but GST-smt3R64E(GG) did not (Fig. 5B). As is the case with the relationship between mammalian SUMO proteases and SUMOs, Ulp1 hydrolyzed the C-terminal region of Smt3 but not that of smt3R64E (Fig. 5C, left panel). In addition to Smt3, Ulp1 cleaved the C-terminal region of SUMO-1 less efficiently, and did not hydrolyze SUMO-2 or SUMO-3 (Fig. 5C, left panel). ulp1D451N did not show hydrolase activity as well as ulp1C580S (Fig. 5C, right panel). Furthermore, SENP1 but
not Axam exhibited hydrolase activity for smt3R64E (data not shown).

We examined the role of the salt bridge between Smt3 and Ulp1 in isopeptidase activity. Smt3 is attached to the septins Cdc3, Cdc11, and Sbs1/Sept7 during mitosis, with conjugates appearing shortly before anaphase onset and disappearing abruptly at cytokinesis (25). GST-Flag-Ulp1 catalyzed the deconjugation of Smt3 from Smt3-conjugated Cdc3-HA, which were immunoprecipitated from the T-9 (CDC3-HA) strain arrested with nocodazole in a dose-dependent manner (Fig. 5D). However, GST-Flag-ulp1D451N catalyzed the deconjugation less efficiently and GST-Flag-ulp1C580S did not show isopeptidase activity for Smt3-conjugated Cdc3-HA (Fig. 5D). These results indicate that the hydrolase and isopeptidase activities of Ulp1 are dependent on its salt bridge with Smt3.

To gain further insights into the biological significance of the salt bridge between SUMO proteases and SUMOs, we constructed yeast strains in which the authentic SMT3 gene is deleted and SMT3(YHK004) or its mutants or human SUMOs (YHK005 and YHK006) are instead expressed under the control of the GAL1 promoter. Consistent with the previous observations (18), the smt3-null strain expressing SMT3 under the GAL1 promoter (T-2) grew on a galactose-containing plate but not on a glucose-containing plate with 5-fluoro-orotic acid (5-FOA), which selects for cells that have lost the URA3-marked plasmid (pYESHA-SMT3-URA3) (Fig. 6A). Various smt3 mutants were expressed in the T-2 strain, and the growth on the glucose-containing plate was examined. Although multicopies of smt3R64E and smt3(GG) both failed to rescue the smt3-null strain from the lethality, multicopies of smt3R64E(GG) and smt3(GG) were able to restore the cell growth of the smt3-null mutants comparably to SMT3 (Fig. 6A). Consistent with the results that Ulp1 hydrolyzes SUMO-1 but not SUMO-3 in vitro, overexpressed SUMO-1 but not SUMO-3 could suppress the lethality of the T-2 strain on 5-FOA containing plates (data not shown). Therefore, SUMO-1 is able to substitute for Smt3 in yeast.
Noncovalent Binding of SUMO Protease and SUMO

The ulp1-null mutant has also been reported to be inviable (7). To test whether SENP1 and Axam are able to rescue the ulp1-null mutants from the lethality with a LIRA3-marked plasmid (Ycp50-ULP1) on a 5-FOA containing plates, we constructed plasmids which could express SENP1 or Axam under the GAL1 promoter. SENP1 but not Axam restored cell growth under the conditions in which ULP1 restored cell growth of the ulp1 strains (Fig. 6B). However, none of ulp1D451N, SENP1D464N, and AxamD412N could rescue these strains (Fig. 6B). Therefore, the specificity of the salt bridge between SUMO proteases and SUMOs determines their ability to restore the cell growth in the smt3- or ulp1-null mutants.

Failure of Desumoylation Delays Cell Growth—The smt3-null cells expressing smt3 mutants were grown for 12 h in liquid culture containing glucose. The smt3-null cells transformed with vector alone exhibited slow growth, with an increase of the cells with 1N DNA as compared with the wild type and smt3-null cells expressing SMT3 or smt3(GG) showed intermediate phenotypes: the proportions of cells in G1 phase were decreased to 28.9% and that in G2/M phase was increased to 29.4%. One remarkable aspect of the phenotype was the increase in the cells in which a dividing nucleus was detected in the mother-daughter neck of the dividing cells.

Cells were also analyzed by fluorescence-activated cell sorting (FACS). There was no significant difference in DNA content between the wild-type and smt3-null cells expressing SMT3 or smt3(GG) after 12 h of growth in liquid culture containing glucose, whereas the smt3-null cells expressing smt3R64E or smt3R64E(GG) exhibited an enrichment of cells with 2N DNA content, which is consistent with the observations that these cells arrest in G2/M phase (Fig. 7C). Furthermore, the smt3R64E(GG)-expressing cells showed an increase of the cells with 1N DNA as compared with the smt3-null cells.

Cdc3 is known to be modified with Smt3 during mitosis (25). We expressed CDC3-HA with SMT3 or smt3R64E(GG) in the smt3-null cells and tested the sumoylation status of Cdc3-3HA. The cells were arrested at the G2/M phase by nocodazole and then released to allow them to enter M phase. At 0 time, Cdc3-HA was released to allow them to enter M phase. At 0 time, Cdc3-HA was reproducibly obtained the same fluorescence-activated cell sorting results showing that desumoylation of Cdc3 occurs more efficiently in the cells expressing SMT3 than those expressing smt3R64E(GG). Therefore, desumoylation is impaired in yeasts by expressing smt3R64E(GG).

**DISCUSSION**

X-ray analyses have revealed that Ulp1, SENP2 (Axam), and SENP1 form a complex with Smt3, SUMO-1, and SUMO-2, were in G2/M phase. However, ~43% of the smt3-null cells expressing smt3R64E exhibited large- or elongated-budded morphology and remained in G2/M phase. Staining of nuclei with propidium iodide (PI) revealed a single nucleus near the bud neck in most cells, indicating that the cells had accumulated at a stage before anaphase. The smt3-null cells expressing smt3R64E(GG) showed intermediate phenotypes: the proportions of cells in G1 phase was decreased to 28.9% and that in G2/M phase was increased to 29.4%. One remarkable aspect of the phenotype was the increase in the cells in which a dividing nucleus was detected in the mother-daughter neck of the dividing cells.
respectively (15–17). Structural analyses predicted that Asp451 in Ulp1, Asp412 in Axam, and Asp464 in SENP1 participate in salt bridges with Arg64 in Smt3, Arg63 in SUMO-1, and Arg58 in SUMO-2, respectively. Mutations in either the Asp residue in SUMO proteases or the Arg residue in SUMOs indeed disrupted the SUMO protease-SUMO binding when biochemical binding assays were performed at the submicromolar concentrations. Thus, these amino acids are necessary for noncovalent binding between SUMO proteases and SUMOs. However, in the case of Axam and SUMO-1, or Ulp1 and SUMO-2 or SUMO-3, these amino acids were not sufficient for the interaction. Therefore, our results are not consistent with the findings about the crystal structures of SENP2 (Axam) and SUMO-1 (16). This discrepancy might be due to the differences of the concentrations used between the two studies. Crystallization requires more than millimolar concentrations, while our binding assay was done at less than micromolar concentrations.

The importance of the salt bridge between SUMO proteases and SUMOs have not yet been studied systematically, rather it has been shown that the interaction plays a less critical role in hydrolase and isopeptidase activities in SENP1 (17). Therefore, we analyzed the significance of the salt bridge in enzymatic activities. Axam did not bind to or hydrolyze SUMO-1, and Ulp1 did not interact with or hydrolyze SUMO-2 and SUMO-3. Taken together with the observation that disruption of the salt bridge causes loss of the hydrolysis activity of SUMO proteases for SUMOs, the noncovalent binding through the salt bridge is essential for removing a short C-terminal peptide of SUMOs. Several groups, including ours, showed that Axam hydrolyzes SUMO-1 (21, 26). When a large amount of Axam was incubated with SUMO-1 for a long time, hydrolysis of SUMO-1 was indeed observed in vitro. It is possible that the salt bridge between SUMO proteases and SUMOs is related to affinity. Therefore, under physiological conditions in intact cells, Axam would neither bind to nor hydrolyze SUMO-1. It has been reported that the amino acid sequence after the C-terminal diglycine motif determines the specificity of hydrolysis of SUMOs by SUMO proteases, as indicated by the finding that replacement of the C-terminal fragment of SUMOs changed the efficiency of hydrolysis of SUMOs by SENP1 and Axam (16, 27). The reason why Axam cannot hydrolyze SUMO-2 irrespective of its binding is thought to be due to the C-terminal sequence of SUMO-2. It is interesting to speculate that SUMOs undergo local rearrangements in

FIGURE 7. Effect of wild type or mutant SMT3 on morphology and cell cycle of yeast. A, W303 and T-2 strains transformed with pRS425, pRS425-SMT3, pRS425-smt3R64E, and pRS425-smt3R64E(GG) were grown in YPD or SG-Leu-Ura medium and were shifted to YPD medium at 28 °C. After 12 h of incubation, cells were fixed with 70% ethanol and stained with PI. B, morphology of the cells was classified into the following patterns: (a) unbudded cells; (b) small budded cells; (c) large-budded cells prior to mitosis; (d) elongated (abnormal) cells; (e), mitotic cells; (f), post-mitotic cells. C, after incubation for 12 h in YPD medium, FACS analyses of wild-type cells or smt3 mutants were performed. D, CDC3-HA smt3Δ strain expressing SMT3 or smt3R64E(GG) was arrested with 15 μg/ml nocodazole and released. Left panel, the cells were collected at the indicated times after release and whole cell extracts were prepared. The indicated amounts of samples were applied to an SDS-PAGE gel and Cdc3-HA was probed with anti-HA antibody. AS, asynchronized cells. Right panel, the Smt3- and smt3R64E(GG)-conjugated form of Cdc3-HA at each time (10 μl) were analyzed by the NIH Image and expressed as the percentage of time 0. The results shown are means ± S.D. of four independent experiments.
response to the binding to SUMO proteases, resulting in the interaction of the catalytic domain of SUMO proteases with the SUMO C-terminal sequence.

Our results also showed that the salt bridge is important for the isopeptidase activity of SUMO proteases. We used SUMO-conjugated Tcf-4 as a substrate. As expected, SENP1 deconjugated SUMO-1, SUMO-2, and SUMO-3 from the substrates in vitro. As a result with mutation of either the Arg residues in SUMOs or the Asp residue in SENP1, the deconjugation reaction was completely suppressed. Consistent with the results of these in vitro studies, Tcf-4 was highly sumoylated by expressing SUMO-1R63E(GG) in intact cells. Therefore, SUMO Arg mutants may be a good tool to modify substrates with SUMO efficiently. Although Axam could not show hydrolysis activity for SUMO-1, it deconjugated SUMO-1 from Tcf-4. The isopeptidase activity was completely dependent on the salt bridge between Axam and SUMO-1-Tcf-4, which was demonstrated by the biochemical binding assay. Axam also showed the isopeptidase activity for SUMO-1-p53 (data not shown). Therefore, conjugation with substrates may change the structure of SUMO-1 or increase the affinity of SUMO-1 to Axam, resulting in its interaction with Axam through the salt bridge. It is notable that 10-fold lower concentrations of SENP1 and Axam were required for the removal of SUMOs from SUMO-conjugated Tcf-4 than the hydrolysis of the C-terminal region of SUMO, suggesting that both proteases interact more readily with SUMO-conjugated substrates. However, the apparent lack of substrate specificity toward SUMO conjugates of Axam and SENP1 compared with SUMO monomer proteins suggests some difference of the mechanisms between the recognition of a monomer form of SUMO and a protein-conjugated SUMO by SUMO proteases.

To show that our in vitro findings using purified proteins are important in intact cells, we used a yeast genetics approach. The smt3-null mutant showed the phenotypes of the ulp1-null mutant, producing elongated cells and failing to undergo cytokinesis. Consistent with the previously reported observations (15), ulp1D451N, which cannot hydrolyze Smt3, did not suppress the lethality of the ulp1-null mutants. Furthermore, SUMO-1 but not SUMO-3 recovered the growth of the smt3-null mutants, and SENP1 but not Axam restored the growth of the ulp1-null mutant, consistent with the in vitro results that Ulp1 binds to and hydrolyzes SUMO-1 but not SUMO-3, and that SENP1 but not Axam binds to and hydrolyzes Smt3. These results clearly indicate that the SUMO-cleaving activity of SUMO proteases based on the salt bridge between SUMO proteases and SUMOs correlates with their ability to regulate cell growth. The importance of this noncovalent binding is conserved in other ubiquitin-like modifier and protease systems. Atg8, a member of a novel ubiquitin-like protein family, is an essential component of the autophagic machinery in yeast and is recognized by Atg4, a cysteine protease (28). It has been reported that the conserved Phe residue in the same region as Smt3R64 is essential for the binding of Atg8 to Atg4, cleavage by Atg4 activity, and autophagic pathways (29).

Smt3R64E(GG) indeed overcame the cell cycle abnormality in the smt3-null cells, but the cells still exhibited some defects. The ratio of the cells in which a nucleus was observed on both sides of the bud neck increased in the smt3R64E(GG)-expressing cells compared with the smt3R64E-expressing cells. Although we did not examine all of the sumoylated proteins, at least desumoylation of Cdc3 was suppressed in the smt3R64E(GG)-expressing cells compared with wild-type cells. Therefore, impairment of desumoylation of proteins including Cdc3 may be involved in the delays of cytokinesis. Thus, the balance between the sumoylation and desumoylation of the SUMO target proteins is important for cell cycle control. However, inhibition of desumoylation of smt3R64E(GG)-conjugated Cdc3 was less prominent than expected from the results with in vitro experiments. It has been shown that SUMO-1-modified RanGAP1 is protected from SENP2 activity when complexed with Nup358 and Ubc9 (13). By analogy with this observation, Smt3-conjugated Cdc3 may bind to protein(s) in yeast, thereby it is not easily accessible to endogenous Ulp1. This might be the reason why the difference between desumoylation of Smt3-conjugated Cdc3 and Smt3R64E(GG)-conjugated Cdc3 is small.

Although PIAS family members, SUMO E3 ligases, are also known to bind to SUMO or SUMO-conjugated proteins (24, 30), the physiological roles of such binding are not clear. Clarifying the biological significance of the noncovalent interaction between SUMOs and enzymes that regulate sumoylation would be necessary to understand the molecular mechanisms of the determination of the substrate specificity of sumoylation and the subcellular localization of SUMO-conjugated proteins.

Acknowledgments—We thank Drs. T. Nishida, K. Tanaka, E. Tsuchiya, and M. Yukawa, M. Hochstrasser, and Y. Kikuchi for helpful discussions and technical assistance.

REFERENCES
1. Johnson, E. S. (2004) Annu. Rev. Biochem. 73, 355–382
2. Melchior, F., Schergaut, M., and Pichler, A. (2003) Trends Biochem. Sci. 28, 612–618
3. Schwartz, D. C., and Hochstrasser, M. (2003) Trends Biochem. Sci. 28, 321–328
4. Matunis, M. J., Coutavas, E., and Blobel, G. (1996) J. Cell Biol. 135, 1457–1470
5. Saitoh, H., Sparrow, D. B., Shiomi, T., Pu, R. T., Nishimoto, T., Mohun, T. J., and Dasso, M. (1998) Curr. Biol. 8, 121–124
6. Muller, S., Ledl, A., and Schmidt, D. (2004) Oncogene 23, 1998–2008
7. Li, S. J., and Hochstrasser, M. (1999) Nature 398, 246–251
8. Li, S. J., and Hochstrasser, M. (2003) J. Cell Biol. 160, 1089–1081
9. Panse, V. G., Kuster, B., Gerstberger, T., and Hurt, E. (2003) Nat. Cell Biol. 5, 21–27
10. Li, S. J., and Hochstrasser, M. (2000) Mol. Cell. Biol. 20, 2367–2377
11. Yeh, E. T., Gong, L., and Kamitani, T. (2000) Gene (Amst.) 248, 1–14
12. Bailey, D., and O’Hare, P. (2002) J. Gen. Virol. 83, 2951–2964
13. Zhang, H., Saitoh, H., and Matunis, M. J. (2002) Mol. Cell. Biol. 22, 6498–6508
14. Nishida, T., Tanaka, H., and Yasuda, H. (2000) Eur. J. Biochem. 267, 6423–6427
15. Mosessova, E., and Lima, C. D. (2000) Mol. Cell 5, 865–876
16. Reverter, D., and Lima, C. D. (2004) Structure 12, 1519–1531
17. Shen, L. N., Dong, C., Liu, H., Naismith, J. H., and Hay, R. T. (2006) Biochem. J. 397, 279–288
Noncovalent Binding of SUMO Protease and SUMO

18. Takahashi, Y., Iwase, M., Konishi, M., Tanaka, M., Toh-e, A., and Kikuchi, Y. (1999) Biochem. Biophys. Res. Commun. 259, 582–587
19. Takahashi, Y., Mizoi, J., Toh-E, A., and Kikuchi, Y. (2000) J. Biochem. 128, 723–725
20. Uchimura, Y., Nakao, M., and Saitoh, H. (2004) FEBS Lett. 564, 85–90
21. Kadoya, T., Yamamoto, H., Suzuki, T., Yukita, A., Fukui, A., Michiue, T., Asahara, T., Tanaka, K., Asashima, M., and Kikuchi, A. (2002) Mol. Cell. Biol. 22, 3803–3819
22. Yamamoto, H., Ihara, M., Matsuura, Y., and Kikuchi, A. (2003) EMBO J. 22, 2047–2059
23. Rose, M. D., Winston, F., and Hieter, P. (1990) Methods in Yeast Genetics Cold Spring Harbor Laboratory Press, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
24. Ihara, M., Yamamoto, H., and Kikuchi, A. (2005) Mol. Cell. Biol. 25, 3506–3518
25. Johnson, E. S., and Blobel, G. (1999) J. Cell Biol. 147, 981–994
26. Nishida, T., Kaneko, F. M. K., and Yasuda, H. (2001) J. Biol. Chem. 276, 39060–39066
27. Xu, Z., and Au, S. W. (2005) Biochem. J. 386, 325–330
28. Ohsumi, Y., and Mizushima, N. (2004) Semin. Cell Dev. Biol. 15, 231–236
29. Amar, N., Lustig, G., Ichimura, Y., Ohsumi, Y., and Elazar, Z. (2006) EMBO Rep. 7, 635–642
30. Kotaja, N., Karvonen, U., Janne, O. A., and Palvimo, J. J. (2002) Mol. Cell. Biol. 22, 5222–5234