Yeast Ull1/Siz1 Is a Novel SUMO1/Smt3 Ligase for Septin Components and Functions as an Adaptor between Conjugating Enzyme and Substrates*

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SUMO1/Smt3, a ubiquitin-like protein modifier, is known to conjugate to other proteins and modulate their functions in various important processes. Similar to the ubiquitin conjugation system, SUMO1/Smt3 is transferred to substrate lysine residues through the thioester cascade of E1 (activating enzyme) and E2 (conjugating enzyme). In our previous report (Takahashi, Y., Toh-e, A., and Kikuchi, Y. (2001) Gene 275, 223–231), we showed that Siz1/Ull1 (YDR409w) of budding yeast, a member of the human PIAS family containing a RING-like domain, is a strong candidate for SUMO1/Smt3 ligase because the SUMO1/Smt3 modification of septin components was abolished in the all1 mutant and Ull1 associated with E2 (Ubc9) and the substrates (septin components) in immunoprecipitation experiments. Here we have developed an in vitro Smt3 conjugation system for a septin component (Cdc3) using purified recombinant proteins. In this system, Ull1 is additionally required as well as E1 (Sua1-Uba2 complex), E2 (Ubc9), and ATP. A cysteine residue of the RING-like domain was essential for the conjugation both in vivo and in vitro. Furthermore, a region containing the RING-like domain directly interacted with Ubc9 and Cdc3. Thus, this SUMO/Smt3 ligase functions as an adaptor between E2 and the target proteins.

SUMO1 (small ubiquitin-like modifier)/Smt3 is a member of a growing family of ubiquitin-related proteins and is known to be conjugated to RanGAP1, PML (promyelocytic leukemia), IκBα, p53, septins, etc. (1–4). Not only are the amino acid sequences and the three-dimensional structures similar between SUMO/Smt3 and ubiquitin, but their conjugation systems and the enzymes involved are highly related (5–9). In the ubiquitin pathway a third enzyme, ubiquitin ligase (E3), is often required for the final transfer of this modifier and plays a crucial role by recognizing target proteins and by promoting their conjugation (10). It remains unknown, however, whether any SUMO1/Smt3 ligases (E3s) are involved in this conjugation pathway.

In the ubiquitin pathway, some E3 components such as Apc11 of the anaphase-promoting complex and Rbx1 of the SCF (Skp1, cullin, F-box protein)/ubiquitin ligase complex contain a zinc-binding RING domain with an octet of ordered cysteine and histidine residues forming a cross-brace around two zinc atoms (11, 12). This type of ubiquitin ligases (E3s) has to interact with E2 and the substrate at the same time because apparently they do not form the thioester bond with ubiquitin. In the case of c-Cbl proto-oncoprotein, its RING domain interacts with UbcH7 (E2), and the tyrosine kinase binding domain, a region close to the RING domain, recognizes its substrate. Thus, c-Cbl proto-oncoprotein functions as a bridging molecule between E2 and its substrate (13).

In budding yeast, Smt3 is the only member of the SUMO family, and the Smt3 conjugation system is essential for mitotic growth. The lethality of the smt3 deletion mutant can be suppressed by expressing human SUMO1, suggesting that SUMO1 is a functional homologue of yeast Smt3 (14). As the substrate proteins in yeast, three components of septins (Cdc3, Cdc11, and Sh3) have been identified so far (14, 15). Septins are a highly conserved group of GTP-binding proteins from yeast to human and are required for the completion of cytokinesis, polar growth, and morphogenesis checkpoint control (16, 17).

In our previous work, we showed that Siz1 (YDR409w) is a member of a new family including human PIAS3 (18), containing a RING-like domain, is required for the Smt3 conjugation to septins in vivo and associates with Ubc9 and septins as assayed by immunoprecipitation experiments (19). Thereby, Siz1 could be a novel Smt3/SUMO1 ligase. In this report, we have developed an in vitro Smt3 conjugation system and demonstrate that Siz1 is a bona fide SUMO1/Smt3 ligase. Thus we propose the gene name of YDR409w as ULL1 (ubiquitin-like protein ligase). Furthermore, we show that a region containing the RING-like domain directly interacts with Ubc9 and Cdc3.

EXPERIMENTAL PROCEDURES

Strains and Genetic Manipulations—Escherichia coli strains DH5α and BL21 (DE3) were used for plasmid propagation and protein purification, respectively. Strains of Saccharomyces cerevisiae, T-13 (ull1::HIS3) and T-20 (ull1::HIS3 CDC3HA-TRP1), isogenic to W303-1A (MATa ade2 ura3 trp1 leu2 his3 can1 1bsd-d21), were described previously (19). PJ69-4A (MATa ura3 trp1 leu2 his3 gal4 gal80 lys2::GAL1-HIS3 GAL2-ATE2 met2::GAL7-lacZ) was used for the two-hybrid system (20). Media and genetic techniques for yeast were

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1 The abbreviations used are: SUMO, small ubiquitin-like modifier; DTT, dithiothreitol; GFP, green fluorescent protein; GST, glutathione S-transferase; HA, hemagglutinin; PIAS, protein inhibitor of activated STAT (signal transducers and activators of transcription); Smt3, suppressor of mif2; ULL1, ubiquitin-like protein ligase 1; E1, activating enzyme; E2, conjugating enzyme; E3, protein ligase; PMSF, phenylmethylsulfonyl fluoride; GAD, Gal4 activation domain.

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Plasmids—pT-17 (pTS901CL-ULL1HAA), pT-18 (pTS901CL-ULL1C377S), pT-20 (pTS904C-ULL1Imyc), pT-21 (pTS904C-ULL1myc), and pT-22 (pGDUBU-RING-like domain) were described previously. pT-23 (pGSTFastBacHT vector (Life Technologies, Inc.). About 3 μl of cell suspension was added to 200 μl of lysis buffer containing 0.01% Brij 58. The proteins were eluted by incubation with 50 mM Tris-HCl (pH 8.0) buffer containing 5 mM glutathione at 4 °C for 1 h. Preparation of Yeast Cell Lysates and Immunoblot Analysis—Cells were grown in minimal medium at 25 °C. To arrest cell growth at G2/M phase, nociodazole (15 μg/ml; Sigma) was added to cultures for 3.5 h. The complete expression system using in vitro system using recombinant proteins contains 0.5 μg of the E1 complex, 0.02 μg of E2, 0.04 μg of GST-Ull1, 0.8 μg of His- and T7-tagged Cdc3, and 1.0 μg of ubiquitin (ub, 2 μg) in 50 μl of lysis buffer. The mixtures were incubated at 25 °C for 3 h. Cell lysates were centrifuged at 15,000 × g for 15 min, and the supernatant fraction containing GST or GST-RING-like domain was incubated with glutathione-Sepharose 4B 4B beads as described above, the protein level of 5% RIPA buffer (50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1% bovine serum albumin). Bound proteins were subjected to immunoblotting analysis using anti-T7 and anti-GST.

RESULTS

Cysteine Residues in the RING-like Domain of Ull1 Are Necessary for the Smt3 Conjugation—Ull1 contains a RING-like domain in the central region (Fig. 1A). When cysteine 377 in this domain was changed to serine, the Smt3 conjugation to septins was abolished in vivo (19). To determine whether other cysteine residues in the RING-like domain are also essential for septin sumoylation, we constructed plasmids carrying mutant genes (ull1<sup>C377S</sup>, <sup>C400S</sup>, and <sup>C377S/C400S</sup>, see Fig. 1A), and those plasmids were introduced into the <sup>C377S</sup> mutant that expressed HA-tagged Cdc3, replacing the endogenous wild-type CDC3 gene. Cultures of the transformants were treated with a microtubule-depolymerizing drug, nociodazole, for 3.5 h to arrest cells at the G2/M boundary. Cell extracts were prepared and subjected to immunoblotting. Cdc3 was probed with anti-T7 (Novagen) in immunoblotting analysis.

In Vitro Binding Assay—Transformants of <i>E. coli</i> BL21 (DE3) with pT-40 (pGEX-KG-RING-like domain), pGEX-KG, pT-42 (pET21a-UBC9), pT-36 (pET21b-CDC3), or pET21a were grown to midlog phase, and each recombinant protein was added by incubation with 50 mM Tris-HCl (pH 7.4), 5 mM ATP, 5 mM MgCl<sub>2</sub>, and 2 mM DTT, or 0.5 μg Smt3 (mature form) was added in place of GST-Smt3gg. In a negative control, GST (1.0 μg) was added in place of GST-Smt3gg. The tagged U11<sup>C377S</sup> mutant protein (0.04 μg) was added in place of GST-Ull1. The mixtures were incubated at 25 °C for 40 min. Cdc3 was probed with anti-T7 (Novagen) in immunoblotting analysis.
Identification of a Novel Yeast SUMO1/Smt3 Ligase

...wild-type Ull1 protein (Fig. 1C). Even when a Myc-tagged mutant protein (C377S) was expressed from a multi-copy plasmid and the protein level was higher than the wild-type level, it did not fully recover the Smt3 conjugation to HA-tagged Cdc3, although the mutant protein may still retain some activity (Fig. 1D). Thus we conclude that those cysteine residues in the RING-like domain of Ull1 are important for the Smt3 conjugation.

Ull1 is phosphorylated especially in the M phase (19). There is one potential cyclin-dependent kinase target site (SPXK) near the RING-like domain. We changed this serine 460 to cysteine, but the septin sumoylation was not impaired (Fig. 1B).

**Ull1 Promotes Septin Sumoylation in Vitro**—To demonstrate that Ull1 is a bona fide Smt3/SUMO ligase (E3), we have developed an in vitro system for septin sumoylation. As a substrate, T7- and His-tagged Cdc3 was purified from E. coli lysate. The mature form of GST-tagged Smt3 was purified from E. coli lysate. The mature form of untagged Smt3 was prepared from GST-Smt3 by treatment with thrombin. As E1 and E2 enzymes, we used GST-tagged human Sua1-His-tagged Uba2 complex purified from SF-9 cells and T7-tagged human Ubc9 purified from E. coli lysate, respectively. These E1 and E2 enzymes successfully promoted the in vitro SUMO1 conjugation to RanGAP1 (24). GST-tagged Ull1 or Ull1(C377S) mutant protein was expressed and purified by the baculovirus protein expression system. The purified recombinant proteins were subjected to SDS-polyacrylamide gel electrophoresis, and the gels were stained with Coomassie Brilliant Blue as shown in Fig. 2A. The Smt3 fraction contained a breakdown product of Smt3 since the band of the smaller size was also stained with anti-Smt3 (data not shown). In the lane of the E1 complex fraction, the band of Uba2 was very weak since we purified the E1 fraction of GST-Sua1/Uba2 heterodimer through glutathione-Sepharose 4B resin. Also in this fraction, GST (marked by an asterisk) from SF-9 cells was co-purified.

For an in vitro conjugation assay, the various reaction mixtures shown in Fig. 2B were incubated with 3.3 mM ATP and 2 mM DTT at 25 °C for 40 min and subjected to immunoblotting. Cdc3 was probed with anti-T7 antibody. A new band corresponding to Cdc3 modified with GST-Smt3 was detected in the complete reaction mixture in lane 6. Appearance of this new band depended on the presence of E1 (lane 1), E2 (lane 2), GST-Ull1 (lane 3), or GST-Smt3 (lane 5). Higher molecular weight bands detected by the antibody were derived from the T7-Cdc3-His fraction since these bands were missing in lane 4 and were present even without other components (lanes 9 and 12). Whether those are Cdc3 polymers or unrelated proteins that are reactive to this antibody remains unknown.

When untagged Smt3 was used in place of GST-Smt3, a new band of a smaller size was detected in lane 7. The size difference between these bands corresponds to the size of GST. Furthermore, production of the new band was abolished in the presence of 5 mM N-ethylmaleimide (Fig. 2C), which is consistent with a notion that thioester bond formation should be involved in these reactions. This is the first demonstration that sumoylation requires an additional factor besides E1 and E2.

**Fig. 1.** The RING-like domain of Ull1 is essential for septin sumoylation. A, schematic structure of Ull1 and amino acid sequence of its RING-like domain. The RING-like domain (from amino acids 352 to 409) and a potential cyclin-dependent kinase target site (marked by an asterisk) of Ull1 are shown. Cysteine 361, 377, or 400 within the RING-like domain of Ull1 are important for the Smt3 conjugation. Thus we conclude that those cysteine residues in the RING-like domain of Ull1 are important for the Smt3 conjugation.

**Materials and Methods**

**Immunoblotting** was performed using anti-HA. A nonspecific band is marked by an asterisk. Equal loading of each sample was confirmed by anti-PSTAIRE staining (lower panel). A nonspecific band is marked by an asterisk. Equal loading of each sample was confirmed by anti-HA and anti-Myc, respectively. Equal loading of each sample was confirmed by anti-PSTAIRE staining. SE, short exposure; LE, long exposure.

For an in vitro conjugation assay, the various reaction mixtures shown in Fig. 2B were incubated with 3.3 mM ATP and 2 mM DTT at 25 °C for 40 min and subjected to immunoblotting. Cdc3 was probed with anti-T7 antibody. A new band corresponding to Cdc3 modified with GST-Smt3 was detected in the complete reaction mixture in lane 6. Appearance of this new band depended on the presence of E1 (lane 1), E2 (lane 2), GST-Ull1 (lane 3), or GST-Smt3 (lane 5). Higher molecular weight bands detected by the antibody were derived from the T7-Cdc3-His fraction since these bands were missing in lane 4 and were present even without other components (lanes 9 and 12). Whether those are Cdc3 polymers or unrelated proteins that are reactive to this antibody remains unknown.
Fig. 2. Ull1 is required for in vitro Smt3 conjugation to Cdc3. A, preparation of recombinant proteins for in vitro assay. The proteins prepared for the in vitro system were subjected to SDS-polyacrylamide gel electrophoresis, and the gels were stained with Coomassie Brilliant Blue. Loaded samples are 6.3 µg of GST, 3.9 µg of GST-Smt3, 0.4 µg of Smt3 (+) indicates a breakdown product of Smt3), 1.2 µg of the E1 complex (His-Uba2 and GST-Sua1; *) indicates GST copurified from Sf-9 cells), 1.0 µg of E2 (T7-hUbc9), 0.8 µg of T7-Cdc3-His, 0.08 µg of GST-Ull1, and 0.08 µg of GST-Ull1(C377S). Each component is marked by an arrowhead. WT, wild type. B, the Smt3 conjugation to Cdc3 requires Ull1 in vitro, and cysteine 377 of Ull1 is essential for the modification. The complete reaction mixture contains 0.5 µg of the E1 complex (GST-Sua1-His-hUba2), 0.02 µg of E2 (T7-hUbc9), 0.04 µg of GST-Ull1, 0.8 µg of T7-Cdc3-His, and 1.0 µg of GST-Smt3. (+) indicates absence of the factor; *, absence of the factor. G, 1.0 µg of GST was added instead of GST-Smt3; S, 0.5 µg of Smt3 was added instead of GST-Smt3; M, 0.04 µg of GST-Ull1(C377S) mutant protein was added instead of GST-Ull1. Various reaction mixtures as indicated were incubated with 3.3 mM ATP and 2 mM DTT at 25 °C for 40 min and were subjected to immunoblotting. Cdc3 was probed with anti-T7. The right panel shows various control experiments with (+) or without (−) incubation in the buffer containing ATP. Molecular weight markers are shown in the left side of the figure. Non-specific bands are marked by asterisks. C, the conjugation is N-ethylmaleimide-sensitive. The reaction mixtures contained all the components with (+) or without (−) 2 mM DTT or 5 mM N-ethylmaleimide (NEM) as indicated.

Taken together with our previous study (19), we conclude that Ull1 is a SUMO1/Smt3 ligase for septin components.

When the Ull1(C377S) mutant protein was added to this in vitro system, the production of the Smt3 conjugates decreased at least severalfold (Fig. 2B, lane 8). Thus cysteine 377 in the RING-like domain is essential for this conjugation in vitro as well as in vivo.

The RING-like Domain Interacts with Ubc9 and Cdc3—We previously showed that the region (from amino acids 327 to 465) containing the RING-like domain of Ull1 interacted with Cdc3 in the two-hybrid system (19). This region also interacted with human SUMO1 and mouse Ubc9 (Fig. 3A) as well as yeast Smt3 and Ubc9 in the two-hybrid system (Fig. 3B). The C377S mutation within the RING-like domain impaired the interaction both with Ubc9 and Smt3 (Fig. 3B).

To examine whether the RING-like domain interacts with E2 and the substrate directly, an in vitro binding assay was performed. GST-tagged RING-like domain (from amino acids 327 to 465) of Ull1 and GST was expressed in E. coli and bound to glutathione-Sepharose 4B beads. T7-tagged Ubc9 and T7-Cdc3 were separately expressed in E. coli, and each of those cell lysates was mixed with the beads bound with GST-RING-like domain or GST. After washing, bound proteins were subjected to immunoblotting. As shown in Fig. 3C, both Ubc9 and Cdc3 were bound to the GST-RING-like domain. In contrast, neither Ubc9 nor Cdc3 interacted with GST. Thus both Ubc9 (E2) and Cdc3 (substrate) directly and specifically interact with the region containing the RING-like domain of Ull1.

Discussion

In our previous study, we showed that the SUMO1/Smt3 conjugation to Cdc3 depends on Ull1 in vivo and that Ull1 interacts with E2 and the substrates in immunoprecipitation analysis (19). In the present study, we have developed an in vitro system where Ull1 is required for the Smt3 modification of Cdc3 in addition to E1 and E2 enzymes (Fig. 2). These results exclude a possibility that the absence of septin sumoylation in the ull1 mutant is due to a defect in expression of hypothetical factors in the Smt3 conjugation pathway in the mutant. Although it has been published that E3 is not required in the SUMO1/Smt3 conjugation pathway (24), we suspect that it may be an in vitro artifact because only E1 and E2 enzymes promoted the modification in our system when a large amount of E2 was added to the reaction.2

Human SUMO1 rescues the lethality of the yeast smt3 deletion mutant (14), and both SUMO1 and mouse Ubc9 interacted with yeast Ull1 in the two-hybrid system (Fig. 3A). Furthermore,

2 Y. Takahashi, T. Kahyo, A. Toh-e, H. Yasuda, and Y. Kikuchi, unpublished results.
human E1 and E2 enzymes successfully promoted the in vitro yeast Smt3 conjugation to the septin component (Fig. 2). These results indicate that enzymes in the SUMO1/Smt3 conjugation pathway are well conserved from yeast to human.

Certain ubiquitin ligases are known to carry a zinc-binding RING-finger domain that often interacts with ubiquitin-conjugating enzymes in vivo. In accordance with these facts, Ull1 as a SUMO1/Smt3 ligase contains a RING-like domain, and the conserved cysteine residues in the domain are important for the Smt3-protein conjugation in vivo (Ref. 19 and Fig. 1) and in vitro (Fig. 2). It is not known, however, whether the structure of this RING-like domain is similar to the authentic RING-finger domain of a bracelet structure containing two zinc atoms. This issue remains to be elucidated.

The region containing the RING-like domain (from amino acids 327 to 465) of Ull1 interacted with Ubc9 in the two-hybrid system (Fig. 3B) and in vitro binding assay (Fig. 3C). On the other hand, the Ull1C377S mutant protein did not interact with Ubc9 in the two-hybrid system (Fig. 3B). Also, this domain directly interacted with Cdc3 (Fig. 3C). Among ubiquitin ligases, only the HECT proteins are known to bind a ubiquitin molecule through thioester bond formation. The other ubiquitin ligases have to interact with E2 and the substrate at the same time because those ubiquitin ligases apparently do not form the thioester bond with ubiquitin and function as bridging molecules between E2 and the substrates. In the case of c-Cbl proto-oncoprotein, the RING domain interacts with UbcH7 (E2), and the region (tyrosine kinase binding domain) close to the RING domain is known to be a recognition site for its substrate (13). Just like this c-Cbl proto-oncoprotein, the region containing the RING-like domain of Ull1 binds to Ubc9 (E2) and Cdc3 (substrate) (Fig. 3). Taken together, Ull1 should be qualified as an E3 (SUMO1/Smt3 ligase) in the SUMO1/Smt3 conjugation pathway, and enzymes both in the SUMO1/Smt3 and ubiquitin conjugation pathways are conserved.

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