Ubch9p Is the Conjugating Enzyme for the Ubiquitin-like Protein Smt3p

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At least one essential function of Smt3p, a Saccharomyces cerevisiae ubiquitin-like protein similar to the mammalian protein SUMO-1, involves its posttranslational covalent attachment to other proteins. Using Smt3p affinity chromatography, we have isolated the second enzyme of the Smt3p conjugation pathway and have found that it is identical to Ubch9p, a previously identified protein that has extensive sequence similarity to the ubiquitin-conjugating enzymes (E2s) and that is required for yeast to progress through mitosis. A hallmark of E2s is the ability to form a thioester bond-containing covalent intermediate with ubiquitin (Ub). While we were unable to detect formation of a Ub–Ubch9p thioester, Ubch9p was found to form a thioester with Smt3p, indicating that Ubch9p is the functional analog of E2s in the Smt3p pathway and that this step is distinct from the ubiquitin pathway. Ubch9p is required for attachment of Smt3p to other proteins in vitro, suggesting that it is the only such enzyme in S. cerevisiae. These results suggest that, like ubiquitination, Smt3p conjugation may be a critical modification in cell cycle regulation.

SMT3 is an essential Saccharomyces cerevisiae gene encoding a member of a family of ubiquitin-like proteins, including the mammalian protein SUMO-1 (1) (also called GMP1, PIC1, UBL1, or sentrin (2–5)). SUMO-1 was isolated as a protein binding to GTPase-activating protein (GAP)1, and it is also conjugated to a number of other, primarily nuclear, proteins (1–3, 6). Smt3p, which is 48% identical to SUMO-1 and 17% identical to Ub, also becomes attached to several proteins posttranslationally, and at least one of its essential functions is mediated by its attachment to another protein (7).

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1 The abbreviations used are: RanGAP1, Ran-GTPase-activating protein; Ub, ubiquitin; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin-protein ligase; HF-Smt3p, His6- and FLAG-tagged mature Smt3p; DTT, dithiothreitol; NTA, nitriitol- triacetic acid; EF1a, elongation factor 1a; PAGE, polyacrylamide gel electrophoresis; MALDI-TOF, matrix-assisted laser desorption/ionization time of flight; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxyethyl)propane-1,3-diol.

Ubiquitin (Ub) conjugation is carried out by a multistep pathway culminating in formation of an isopeptide bond between the C-terminal carboxyl group of Ub and the ε-amino group of a lysine side chain in an acceptor protein (8, 9). In the initial step, Ub-activating enzyme (E1) utilizes ATP to adenylylate the Ub C terminus, which is then transferred to a conserved Cys residue in the E1, yielding an E1–Ub thioester, AMP, and pyrophosphate. Ub is transferred from the E1 to a Cys residue in a Ub-conjugating enzyme (E2). Cells contain multiple E2s (13 in yeast by sequence similarity) which are involved in ubiquitinating different proteins. In some cases Ub can be transferred directly from the E2 to the acceptor protein, but more often Ub-isopeptide bond formation is facilitated by a third heterogeneous class of proteins termed Ub-protein ligases or recognins (E3).

Several features of the Ub pathway are conserved in the early steps of the Smt3p conjugation pathway (7). Like Ub, the SMT3 translation product is proteolytically processed to expose its mature C terminus. Smt3p undergoes ATP-dependent activation by a heterodimeric activating enzyme consisting of Uba2p, a 71-kDa protein with extensive sequence similarity to the C-terminal region of E1s, including the active site Cys residue participating in the thioester (10), and Aos1p, a 40-kDa protein similar to the N termini of E1s (7). While the Smt3p- and Ub-activating enzymes are related, they do not interact with each other’s substrates, suggesting that the two pathways are distinct.

One candidate to be the Smt3p-conjugating enzyme is Ubch9p, a member of the E2 sequence family whose Xenopus laevis homolog co-immunoprecipitates with a complex including SUMO-1-conjugated RanGAP1 (11) and whose human homolog interacts in a two-hybrid screen with SUMO-1 (4). UCBC9 is an essential gene. Conditional ubc9 mutants arrest in the cell cycle at G2/M and are impaired in proteolysis of both B-type cyclins (12) and G1 cyclins (13). However, Ubch9p does not seem to be the E2 involved in cyclin B ubiquitination (14, 15). While it has been suggested that Ubch9p functions as an E2 in the Ub pathway, there is no clear biochemical data to support this hypothesis.

We have found that Ubch9p is a Smt3p-conjugating enzyme and that it is likely to constitute the only Smt3p-conjugating activity in yeast. Furthermore, Ubch9p does not seem to be a Ub-conjugating enzyme, suggesting that the ubc9 cell cycle defect actually results from impairment of Smt3p conjugation.

EXPERIMENTAL PROCEDURES

Genetic Techniques and Strains—Standard techniques were used (16). S. cerevisiae strains used were DF5α (MATα trp1–1, ura3–52 his3–200 leu2–3,112 lys2–801) (17) and the DF5-derived strain WY0102 (MATα ubc9Δ::TRP1 leu2::ubc9Pro-Ser::LEU2) (12), which was a generous gift of S. Jentsch (Heidelberg, Germany).

Plasmid Constructs and Recombinant Proteins—PET21b or PET11a (Novagen)-based Escherichia coli expression plasmids expressing N-terminally His6- (18) and FLAG-tagged (19) Smt3p (HF-Smt3p) and His6-tagged Aos1p and Ubch9p have been described (7). PET21b-based plasmids for expressing Ubch9p-, Ubch2p-, and Pex4p-tagged C-terminally with His6 were produced by polymerase chain reaction. The Ubch2p construct expresses Ubch2p truncated after Met153, deleting most of the acidic C-terminal domain. A pET21b-based plasmid expressing N-terminally His6- and FLAG-tagged ubiquitin bearing Lys63 → Arg, Lys65 → Arg and Gly76 → Ala mutations (HF-Ub(A76)) was also produced by polymerase chain reaction. The A76 mutation alters the kinetics of thioester formation (20). Construction details are available on request.

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Proteins were expressed in *E. coli* and purified by Ni-NTA chromatography as described (7). The H-Uba2p/Aos1p complex was further purified by Smt3p affinity and gel filtration chromatography (7). Purified recombinant Ubc9p was a generous gift of V. Chau (Wayne State University School of Medicine, Detroit, MI). His6-tagged UbA1p was purified from yeast lysate of strain JD77–1A (MATa uba1Δ::HIS3) (10) bearing plasmid pJD325, which expresses H-Uba1p from PcUP1, both gifts of J. Dohmen (Heinrich-Heine Universität, Düsseldorf, Germany). UbA1p was purified by Ni-NTA chromatography as described for Uba2p (7) followed by Ub affinity chromatography (21).

**Smt3p Affinity Chromatography—**Ni-NTA-purified recombinant Aos1p and UbA2p were applied to an HF-Smt3p-Aff-Gel 15 column and washed as described (7). A few milligrams of each protein bound. Yeast nuclear extract, consisting of the soluble fractions (primarily the load) below the nuclear envelopes in a Nycodenz/sucrose flotation gradient, prepared as described (22), was a generous gift of R. Beckmann and D. Peter. Extract containing ~20 mg of protein was dialyzed against 50 mM BisTris (pH 6.5), 50 mM NaCl, 1 mM MgCl2, and 1 mM β-mercaptoethanol (β-ME), brought to final concentrations 50 mM BisTris (pH 6.5), 75 mM NaCl, 5 mM MgCl2, 2 mM ATP, and 0.5 mM β-ME and applied to the Aos1p/Uba2p-preloaded HF-Smt3p column. The column was washed with 10 column volumes of the same buffer except containing 1 M NaCl and eluted as described (7). The eluate was exchanged into 50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM MgCl2, and 1 mM β-ME by using a Biomax-10 ultrafiltration unit (Millipore), bound to 0.5 ml of Ni-NTA-Biogel (“results” and “Experimental Procedures”). The 90- and 40-kDa bands are likely to represent the endogenous Uba2p and Aos1p from the yeast lysate. The other two bands were analyzed by direct mass spectrometric analysis of a protease digestion mixture (see “Experimental Procedures”). The 50-kDa band contained the elongation factor EF1α, and the 19-kDa band contained UbC9p.

**Identification of the Smt3p-conjugating Enzyme—**

We attempted an analogous approach to purifying the Smt3p-conjugating enzyme(s) using a column linked to His6 (18- and FLAG-tagged (19) Smt3p (HF-Smt3p) (mature processed Smt3p having C-terminal Gly96 was referred to as Smt3p). Because the Smt3p-conjugating enzyme would be predicted to bind the column by displacing UbA2p/Aos1p, which is necessary to activate the column-bound Smt3p, the Smt3p column was prebound with recombinant His6-tagged UbA2p and Aos1p to improve the efficiency of conjugating enzyme binding. Next, ATP-supplemented yeast nuclear extract was applied to the column, which was then washed and eluted with DTT. Nuclear extract was used because UbA2p has been reported to be nuclear (10) and because we had previously found a yeast cytosolic fraction to be inactive in Smt3p conjugation (data not shown). The eluate contained predominantly recombinant UbA2p and Aos1p (data not shown), but as these bore His6 tags, they were selectively removed by Ni-NTA chromatography. The resulting fraction contained four major bands of 90, 50, 40, and 19 kDa and a number of minor bands (Fig. 1).

**RESULTS**

Identification of the Smt3p-conjugating Enzyme—

E1s and E2s can be readily purified by “covalent affinity chromatography” (21) by adding ATP to a protein mixture containing these enzymes and incubating with an affinity column to which Ub has been coupled. E1s and E2s form thioester bonds with the column-bound Ub and then can be gently eluted in buffer containing a thiol reducing agent, such as DTT, which breaks the thioester bonds.

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**Fig. 1. Yeast proteins that bind an Smt3p affinity column.** ATP-supplemented yeast nuclear extract was applied to an Smt3p column that had been prebound with recombinant Aos1p and UbA2p. The DTT eluate, after removal of recombinant proteins, is shown (see “Results” and “Experimental Procedures”).

2 R. Beckmann, personal communication.
Aos1p, strengthening the analogy between this reaction and that of Ub, E1s, and E2s. This reaction also formed small amounts of a series of DTT-insensitive HF-Smt3p-containing bands, one of which ran at the same position as the HF-Smt3p–Ubc9p thioester. These bands could represent a polymer of HF-Smt3p or isopeptide-containing conjugates of Smt3p to Ubc9p.

We also asked whether Ubc9p was capable of forming thioesters with Ub as well as with Smt3p. When the yeast E2s Ubc4p, Pex4p (Pas2p/Ubc10p), or Rad6p (Ubc2p), which are involved in bulk proteolysis (25), peroxisome biogenesis (26), and DNA repair (27), respectively, were mixed with Uba1p (the yeast E1) and His6- and FLAG-tagged Ub(A76) (see "Experimental Procedures"), addition of ATP caused these E2s to shift almost quantitatively into higher molecular weight forms (Fig. 3, lanes 1–6). These products were DTT-sensitive and contained HF-Ub(A76) (data not shown), suggesting that they were the corresponding Ub–E2 thioesters. No thioester product between Ubc9p and Ub could be detected under these conditions either by Coomassie staining (Fig. 3, lane 8) or by immunoblotting with the antibody against the FLAG epitope (Fig. 3, lane 9). Conversely, extremely little or no thioester product was detected between Smt3p and either Ubc4p, Pex4p, or Rad6p (data not shown), demonstrating that the transthioleation reaction is very specific.

**Ubc9p Is Essential for Smt3p Conjugation**—When HF-Smt3p and ATP were incubated with whole yeast cell lysate from wild-type cells, a series of DTT-resistant high molecular weight HF-Smt3p-containing bands formed (7) (Fig. 4, lane 2). The pattern of Smt3p-containing bands was similar but not identical to that seen in vitro, with a greater proportion of HF-Smt3p found in very high molecular mass bands >200 kDa (7). The ubc9–1 mutant, which has a Ser residue in place of Pro69, is temperature-sensitive by virtue of the fact that Ubc9p(P69S) is rapidly degraded at 37 °C (28). When a yeast lysate made from this mutant strain after incubation at 37 °C was used in the same reaction, no Smt3p conjugation was detected even on long exposure. (Fig. 4, lane 3). Addition of recombinant Ubc9p restored the Smt3p conjugation activity (Fig. 4, lane 4). These results suggest a direct requirement for Ubc9p in HF-Smt3p conjugation to other proteins in these lysates. Thus, Ubc9p is likely to be the only Smt3p-conjugating enzyme in yeast, at least that is expressed under normal growth conditions.

**DISCUSSION**

Our results show that the Smt3p conjugation pathway is distinct from the Ub pathway, at least up to formation of the thioester with the conjugating enzymes. However, sequence comparisons of of the Smt3p-conjugating enzyme Ubc9p to the Ub-conjugating enzymes Rad6p (Ubc2p), Ubc4p, and Pex4p (Pas2p/Ubc10p) do not reveal any large continuous regions of dissimilarity, and the degree of sequence similarity between Ubc9p and any of these three E2s is comparable with that among the E2s. Ubc4p is 37% identical to Ubc2p, 35% identical to Pex4p, and 34% identical to Ubc9p. Yet the transthioleation reaction involving Aos1p/Uba2p and Smt3p is extremely selective for Ubc9p, and the reaction involving Uba1p and Ub selects strongly against Ubc9p. One possible factor in this discrimination may be that Ubc9p is much more basic than the other proteins, with a pI of 9.2 as compared with 6.3 for Ubc4p,
5.4 for Pex4p, or 4.0 for Rad6p. How this specificity is generated depends on whether conjugating enzymes interact directly with the activating enzyme, the Ub-like protein, or both.

Although our inability to detect Ub–Ubc9p thiostereos does not prove that Ubc9p never participates in Ub conjugation in vivo, it is unlikely that the metabolic stabilization of cyclins observed in ubc9 mutants (12, 13) results directly from reduced ubiquitination of these proteins by Ubc9p, both because of our results and because the E2s that participate in cyclin B ubiquitination have been isolated and do not include Ubc9p (14, 15). It is also very unlikely that Smt3p conjugation substitutes for ubiquitination in targeting cyclins for proteolysis, as cyclins have been heavily studied and never found to be Smt3p-conjugated. Furthermore, Smt3p, which is only 17% identical to Ub, probably does not target its substrates for proteasome-dependent proteolysis. Smt3p conjugation could affect cyclin proteolysis by activating some component of the ubiquitination/proteolysis machinery, or its effect could be several steps removed from ubiquitination. It also has not been excluded that Ubc9p could be required for the conjugation of a different Ub-like protein, which could mediate the cell cycle effect. Smt3p, Aos1p, and Uba2p are all essential genes (7, 10), which is consistent with their being required for cells to transit mitosis, but the arrest phenotypes of conditional alleles of these genes have not been characterized. Ubc9p, Smt3p, and SUMO-1 interact genetically or in the two-hybrid system with a variety of DNA-binding proteins, including centromere-binding proteins (29, 30), proteins involved in recombination (4, 31), and transcription factors (4, 32–34), any of which might affect cell cycle progression. Identification of the targets of Smt3p conjugation should address these questions.

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