Identification of a multifunctional binding site on Ubc9p required for Smt3p conjugation

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Running title: A multifunctional binding site on Ubc9p
SUMMARY

Ubiquitin-like proteins (ub-lps) are conjugated by a conserved enzymatic pathway, involving ATP-dependent activation at the C-terminus by an activating enzyme (E1) and formation of a thiolester intermediate with a conjugating enzyme (E2) prior to ligation to the target. Ubc9, the E2 for SUMO, synthesizes polymeric chains in the presence of its E1 and MgATP. In order to better understand conjugation of ub-lps, we have performed mutational analysis of *S. cerevisiae* Ubc9p, which conjugates the SUMO family member Smt3p. We have identified Ubc9p surfaces involved in thiolester bond and Smt3p-Smt3p chain formation. The residues involved in thiolester bond formation map to a surface we show is the E1 binding site, and E2s for other ub-lps are likely to bind to their E1s at a homologous site. We also find that this same surface binds Smt3p. A mutation that impairs binding to E1 but not Smt3p impairs thiolester bond formation, suggesting that it is the E1 interaction at this site that is crucial.

Interestingly, other E2s and their relatives also use this same surface for binding to ubiquitin, E3s and other proteins, revealing this to be a multipurpose binding site, and suggesting that the entire E1-E2-E3 pathway has coevolved for a given ub-lp.
INTRODUCTION

Post-translational covalent attachment of ubiquitin and ubiquitin-like proteins (ub-lps) has emerged as a predominant cellular regulatory mechanism (reviewed in 1) that plays an important role in a variety of biological processes including the immune response, development, endocytic trafficking, cell division (2,3) and cancer (4-6). The best understood function of these modifications is ubiquitin-mediated proteolysis, in which polymeric chains containing four or more ubiquitins, linked between K48 on the surface of one ubiquitin to the C-terminus of the next, direct proteins for degradation by the proteasome (reviewed in 7). Polyubiquitin chains with linkages via K63, rather than K48, activate the IkB kinase (8), and monoubiquitylation plays a role in processes ranging from protein trafficking to transcriptional activation (9). New ubiquitin-like proteins that are conjugated to macromolecules in vivo are being discovered at a rapid rate. For example, NEDD8 (Rub1p in budding yeast) is involved in cell cycle control (10-12), Apg8p modifies the lipid phosphatidylethanolamine to modulate membrane dynamics (13), and SUMO family members, including Smt3p in budding yeast, modify a number of proteins involved in cell division, nuclear transport, the stress response and signal transduction (reviewed in 14,15). Polymeric chains of SUMO family members have been found to modify a number of proteins in vivo and in vitro, including septins, p53, c-Jun, histone deacetylase, and RanBP2 (16-20).

All of these ub-lps are conjugated at their C-termini to protein substrates by an enzymatic cascade with common features (11,12,15,21-27). A number of ub-lps are proteolytically processed at their C-termini in order to end with the sequence Gly-Gly, required for subsequent steps of the pathway. Ub-lps are adenylated at the C-terminus by an activating
enzyme or E1. The C-terminus of the ub-lp subsequently forms a thiolester with the catalytic cysteine of E1. The ub-lp then forms a thiolester between its C-terminus and the catalytic cysteine of a conjugating enzyme, or E2. The sequences of E1s are similar, and the structures of E2s and ub-lps are conserved, suggesting that the mechanisms of activation and thiolester bond formation will be similar for all ub-lps.

There are three known mechanisms for transfer of the ub-lp to a lysine residue on the target. In many cases, transfer is facilitated by an E3 protein or protein complex that selects the target and acts as an ub-lp protein ligase (reviewed in 7,28). In the predominant mechanism of ubiquitin transfer, the E3 appears to act as a scaffold that recruits the target protein and the E2, facilitating ligation from the E2 to the target. For some E3s, ubiquitin forms a thiolester with the active site cysteine of an E3, which itself transfers ubiquitin. It is also possible for the E2 to transfer a ub-lp in the absence of an E3. For example, chimeric E2s for ubiquitin, fused to protein binding peptides, ubiquitylate the associated proteins (29), and Ubc9 binds a number of its targets directly and transfers SUMO family members to a lysine in the tetrapeptide consensus motif $\Psi$-K-x-D/E, where $\Psi$ is hydrophobic (reviewed in 30).

For ubiquitin there is one E1, tens of E2s, and hundreds of E3s playing a major role in substrate conjugation, although a number of ub-lps only have one known E2. Genetic studies in *S. cerevisiae* indicate that E2s have evolved to orchestrate modification of proteins in a common pathway. For example, Rad6p and Cdc34p, which transfer ubiquitin, are involved in the DNA damage response and the G1-S transition of the cell cycle respectively (31,32). Ubc9p, which transfers Smt3p, is also involved in cell cycle control (22,33,34). Therefore, the ability of a particular E2 to accept and transfer its ub-lp is crucial to the organization of the pathway. This
involves E2 interaction with E1, thiolester bond formation with the ub-lp, interaction with the appropriate E3 and substrate, and catalysis of ub-lp transfer. Several recent structural studies have suggested the structural basis for E2 interactions with E3s (35,36), E2 interaction with ub-lps (37-39), and a recent structure has revealed the structural basis for substrate recognition by the E2 Ubc9 (40). Despite these structures, the mechanism of ub-lp transfer from E1 to E2, and from E2 to substrate is poorly understood. A particularly vexing question is how E2s form thiolester bonds with their cognate ub-lp. E1s are thought to bind tightly to E2s in order to promote thiolester bond formation (41), but the E1 binding site has not been reported for any E2. We present here an in vitro mutational study of Ubc9p where we identify residues involved in E1 binding, Smt3p thiolester bond formation, and Smt3p-Smt3p chain formation.

EXPERIMENTAL PROCEDURES

Cloning, protein expression and protein purification

The full-length wild-type clones of Smt3, Aos1 and Uba2 were obtained by PCR from yeast genomic DNA (Research Genetics). For all experiments described here, a truncated version of Smt3 terminating at G98, representing the active form of Smt3p found in yeast, was used, and is referred to as Smt3p. Two rounds of PCR were used to generate the full-length wild-type Ubc9 cDNA lacking the intron: first, the cDNA corresponding to the second exon was amplified from yeast genomic DNA (Research Genetics) with primers containing overlapping overhangs, and this PCR product was used as the template for a second PCR reaction with overlapping primers also encoding the first exon. The Ubc9 mutants were generated by PCR mutagenesis. The sequences of all clones were verified by standard
automated DNA sequencing procedures.

PCR products encoding Smt3, Ubc9 and mutants were subcloned into pGEX4T3 (Pharmacia) and expressed as GST fusions in BL21(DE3) (Novagen). The cDNAs encoding Aos1p and Uba2p were subcloned into a bicistronic vector described previously (42), and coexpressed in BL21(DE3) with Aos1p as a GST fusion. Untagged Uba2p forms a tight complex with GSTAos1p and copurifies with Aos1p over every subsequent chromatographic step in the purification. The Aos1p/Uba2p complex is referred to as E1(S). GSTSmt3p, GSTUbc9p and GSTAos1p/Uba2p were purified by glutathione affinity chromatography and the fusion proteins were cleaved overnight on ice with thrombin. Cleavage was terminated by the addition of PMSF. Smt3p and Aos1p/Uba2p were further purified by anion exchange and gel filtration chromatography, and concentrated by ultrafiltration to ~400 and ~22 µM, respectively, in 50 mM Tris-HCl, 150 mM NaCl, 5 mM DTT, pH 7.6. Ubc9p and the mutants used for enzymatic assays were further purified by cation exchange chromatography in 50 mM HEPES, 350 mM NaCl, 1 mM DTT, pH 7.0 and concentrated to 50-600 µM. The proteins used for NMR were purified in 20 mM sodium phosphate, 350 mM NaCl, 1 mM deuterated DTT, pH 7.0. All proteins were aliquotted, frozen in liquid nitrogen and stored at –80°C until further use. The Ubc9p mutants were adjusted to the same concentration for all subsequent biochemical assays in 50 mM HEPES, 350 mM NaCl, 1 mM DTT, pH 7.0.

**Thiolester bond formation assays**

Thiolester bond formation assays were performed in 50 µl volumes containing 4.5 nM
E1(S), 3.5 µM Smt3p, 2.2 µM Ubc9p or mutant, 24 mM ATP, and buffer (30 mM Tris-HCl, 10 mM MgCl2, 160 mM NaCl, 0.2 mM DTT). Reactions were incubated at room temperature for between 0 and 60 minutes as indicated in the figures. Reactions were terminated by boiling half of the reaction in nonreducing SDS sample buffer, and the other half the reaction in SDS sample buffer containing DTT. Reactions products were fractionated on 15% SDS polyacrylamide gels and visualized by Coomassie staining. The identity of the Ubc9p-Smt3p thiolester intermediate was preliminarily identified by susceptibility to reduction by DTT, and subsequently confirmed by mass spectrometry following tryptic digestion (see below).

Ubc9p/E1(S) and Ubc9p/Smt3p protein-protein interaction assays

Binding was determined by a native gel mobility shift assay. Binding was performed with 3 µM E1(S), 14 µM Ubc9p and 4-32 µM Smt3p in 10 µl volumes in 75 mM Tris, 165 mM NaCl, 5% glycerol, 10 mM DTT, etc. pH 7.6 for one hour. Free and Ubc9p-bound E1(S) and Smt3p were separated on a 4.5% polyacrylamide gel (acrylamide:bis, 37.5:1) in a buffer of 90 mM Tris-borate, 2% glycerol, pH 8.0, and were visualized with Coomassie staining. Free Ubc9p does not enter the gel because of its net positive charge. The identity of components in the bands observed in native gel was confirmed by tryptic digestion followed by electrospray mass spectrometry (see below).

Smt3p conjugation assays

Formation of Smt3p-Smt3p and Ubc9p-Smt3p conjugates was observed following incubation of 45 nM E1(S), 12 µM Smt3p, 4 µM Ubc9p or mutant, 4 mM ATP, and buffer (30
mM Tris-HCl, 10 mM MgCl₂, 190 mM NaCl, pH 7.6) in a volume of 40 µl. Reactions were incubated at room temperature for between 0 and 120 minutes as indicated in the figures. Reactions were terminated by boiling half of the reaction in SDS sample buffer containing DTT. Reaction products were fractionated on 15% SDS polyacrylamide gels and visualized by Coomassie staining. The identity of proteins in the gel bands and the location of the Smt3p-Smt3p linkage was confirmed by mass spectrometry following tryptic digestion (see below).

Identification of proteins in the Ubc9p-Smt3p thiolester complex, the Ubc9p-Smt3p and Smt3p-Smt3p isopeptide complexes, and the native gel mobility shift assays by tryptic digests and MALDI-TOF or LC mass spectrometric analysis

Following electrophoresis, Coomassie-stained proteins were excised from gels, reduced, alkylated with iodoacetamide, and digested with trypsin. Samples from native gel shifts were digested in the gel first under denaturing conditions with lysyl endoproteinase in 8M urea, then diluted to reduce the urea concentration to 2M, and digested with trypsin. Peptides were extracted and subjected to mass spectrometry. For the Smt3p conjugation assays, peptide mass fingerprinting was performed by matrix-assisted laser desorption/ionization (MALDI) in a Voyager DE-RP time-of-flight (TOF) mass spectrometer from Applied Biosystems (Foster City, CA). α-cyano-4-hydroxycinnamic acid was used as the matrix. More than 10 peptides confirm the presence of Smt3p and/or Ubc9p in the conjugates. For the native gel shift assays, digests were subjected to combined capillary liquid chromatography/tandem mass spectrometry using an LCQ-Deca ion-trap mass spectrometer from ThermoFinnigan (San Jose, CA) equipped with a nanoelectrospray ion source from New Objective (Woburn, MA). Collision-induced
dissociation (CID) spectra were subjected to search using the SEQUEST program (ThermoFinnigan). Several peptides from Aos1p, Uba2p, and Ubc9p or Smt3p and Ubc9p provided positive identification of the proteins in the bands observed in the native gel.

Isopeptide bonds in the Smt3p-Smt3p and Ubc9p-Smt3p conjugates were localized by MALDI-TOF mass spectrometry using in-gel digests made with endoproteinase Glu-C or chymotrypsin. Glu-C digests of Ubc9p-Smt3p contained a peptide of average mass 2,560.9 Da that was absent from free Smt3p and Ubc9p. Further, signals due to Smt3p C-terminal peptide 91-98 (average mass 866.9 Da) and Ubc9p C-terminal peptide 144-157 (average mass 1,712.0 Da) were absent from Ubc9p-Smt3p. These data are consistent with the presence of an isopeptide linkage between the α-carboxyl group of the Smt3p C-terminal glycine residue and any of four Ubc9p lysines, 146, 147, 153 and 157. However, chymotryptic digests of Ubc9p-Smt3p provided further information. They contained a peptide of average mass 2,560.3 Da that was absent from digests of free Smt3p and Ubc9p. The signal due to Smt3p C-terminal peptide 82-98 (average mass 1,943.0 Da) was diminished in intensity in the Ubc9p-Smt3p complex. These data are consistent with the presence of Ubc9p peptide 151-155 in the isopeptide-linked species, showing that the isopeptide bond occurs through Ubc9p K153. The linkage in the Smt3p-Smt3p conjugate was established using Glu-C digests, which revealed a peptide of average mass 2,199.7 Da that was absent from free Smt3p. The signal due to peptide 10-21 (average mass 1,350.6 Da) was diminished in the dimer. This result is consistent with an isopeptide linkage between peptides 10-21 and 91-98 (average mass 866.9 Da). This was confirmed by tandem TOF/TOF mass spectrometry using an Applied Biosystems Model 4700 mass spectrometer, which further unambiguously established the involvement of K15 in the
linkage.

_NMR Spectroscopy_

$^1$H Nuclear Magnetic Resonance (NMR) data were acquired with a Varian Inova 600-MHz spectrometer. One-dimensional spectra were acquired at 20°C with a spectral width of 10,000 Hz, 4096 complex points, 512 transients and a recycle delay of 1.3 seconds. The water peak was suppressed by Watergate. Chemical shifts were referenced to the residual water peak at 4.75 ppm. Resonances from residual glycerine from the Centricon (Millipore) membranes used for concentration also appear in the spectra.

RESULTS

_Identification of surfaces of Ubc9p_

To identify the surfaces on Ubc9p available for mediating protein-protein interactions and catalysis, we performed structural analysis. Although the structure of _S. cerevisiae_ Ubc9p has not been determined, three crystal structures are available of the human ortholog (40,43,44), whose sequence is 56% identical to the yeast protein, with most of the differences being conservative substitutions. We first analyzed the crystal structures of human Ubc9, and compared them with the crystal structures of human UbcH7 (35,36), which transfers ubiquitin. We reasoned that the residues involved in binding to E1 and SUMO family members should be conserved among Ubc9 family members, but not among E2s that transfer ubiquitin, and residues involved in common features of catalysis should be conserved around the active sites of both types of E2s. We identified 62 residues that cover most protein-protein interaction sites on the
surface, and mutated these to either alanine or the corresponding residue in E2s that transfer ubiquitin and not Smt3p. Our goal was to make mutations that would disrupt protein-protein interactions or catalysis, without significantly disrupting the structure. One of the greatest structural differences between Ubc9 and E2s that transfer ubiquitin is a 5-residue insertion in the loop between β-strand 1 and β-strand 2 (43,44), so some deletion mutants were also made that truncate this loop to the size of that found in E2s for ubiquitin to test the function of this loop. The location of amino acids that were mutated in this study is shown on the structure of human Ubc9 in Figure 1.

*Surfaces involved in thiolester bond formation*

In order to identify regions of Ubc9p other than the active site cysteine required for activity, we developed an *in vitro* assay using purified recombinant Ubc9p, Smt3p and the heterodimeric Aos1p/Uba2p complex, which is the E1 for Smt3p, hereafter referred to as E1(S). This assay is similar to that described previously for human Ubc9, with reactants and products separated by SDS-PAGE and visualized by Coomassie staining (18). The Ubc9p-Smt3p thiolester-linked complex migrates more slowly than either Ubc9p or Smt3p alone, and is susceptible to reduction by DTT. At later time points, or at higher protein concentration, Ubc9p automodifies and forms a nonreducible isopeptide linkage with Smt3p, as is found for a number of other E2s (39,45,46; see below). We assayed the time-course of thiolester bond formation for 36 mutants of Ubc9p, with a total of 62 residues mutated either individually or in combination, and the results are shown in Figure 2. Only one variant with these mutations close to the active site, E99S, D100S, D102S, showed accelerated automodifying activity, forming a nonreducible
linkage by the 60-minute time point.

Mutations most strongly hindering thiolester bond formation map to a distinct surface of Ubc9p, involving the C-terminal portion of the N-terminal helix and the loop between the first and second β-strands. This includes the following mutants: K14E, K14E/R17A/K18A, V29A/K30D/32-34Δ, 32-34Δ, and 34Δ37Δ, where Δ stands for a deletion. Although R17A and K18A have no effect on their own, these mutations further reduce the ability of the K14E mutant to form a thiolester bond. These mutations all map to a distinct surface distal from the active site.

Because of the essential role that activating enzymes play in transferring ub-lps to E2s, we wanted to know whether this surface is involved in binding to E1(S). In addition, previous yeast 2-hybrid and NMR studies have shown that Ubc9 family members interact noncovalently with SUMO family members (38,47-49), and this region overlaps with the region of human Ubc9 previously identified by NMR as the SUMO-1 binding site (38). Therefore, we tested whether these or other mutations in Ubc9p impair noncovalent binding to either E1(S) or to Smt3p.

Identification of the E1(S) binding site on Ubc9p

To address whether mutations that impair thiolester bond formation are important for Ubc9p binding to E1(S) or Smt3p, we developed a nondenaturing gel mobility shift assay to examine protein-protein interactions between E1(S) and Ubc9p, and Smt3p and Ubc9p. Briefly, E1(S) and Smt3p are acidic, so they enter a native gel at pH 8. However, the pI of Ubc9p and all of the mutants in this study is greater than 8, so they do not enter a pH 8 gel. Binding between
E1(S) and Ubc9p, or Smt3p and Ubc9p, is reflected by a slower mobility in the gel (Fig. 3A and B, respectively). We tested the ability of all of the Ubc9p mutants to bind to E1(S) and Smt3p (summarized in Table 1). Using this approach we identified the E1(S) binding site on Ubc9p to correspond to the surface important for Smt3p thiolester bond formation, involving the N-terminal helix and the loop between β-strands 1 and 2. To our surprise, the binding sites for E1(S) and Smt3p partially overlap each other, as this region is also involved in noncovalent interactions with Smt3p, consistent with a previous NMR study of human Ubc9 and SUMO-1 (38). To exclude the possibility that mutations such as K14E, which impair thiolester bond formation and binding to both E1(S) and Smt3p, lead to a protein folding defect, we examined the one-dimensional 1H NMR spectrum of the K14E mutant (Fig. 4). The spectrum contains extensive chemical shift dispersion, including resonances shifted upfield of 0 ppm and downfield of 10 ppm that resemble those of the wild-type protein. These features indicate proper folding.

_Smt3p and E1(S) compete for binding to Ubc9p_

Because the binding sites for E1(S) and Smt3p are partially overlapping, we tested whether Smt3p can compete with E1(S) for binding to Ubc9p. We first tested whether Smt3p interacts with E1(S) using our native gel shift assay and gel filtration chromatography (data not shown), and we see no evidence for interaction in the absence of MgATP, consistent with classic findings for the E1 for ubiquitin (50,51). We mixed 3 µM E1(S) with 14 µM Ubc9p and observed complex formation by native gel. We then titrated increasing amounts of Smt3p into the mixture (4, 8, 16 and 32 µM), and observed two different noncovalent complexes by native gel: Ubc9p/E1(S) and Ubc9p/Smt3p. With increasing concentrations of Smt3p, the E1(S)/Ubc9p
complex is displaced and replaced by the Ubc9p/Smt3p complex (Fig. 5). This demonstrates that Smt3p and E1(S) bind to overlapping sites on Ubc9p.

*Ubc9p binding to E1 is important for thiolester bond formation*

Because the binding sites for Smt3p and E1(S) and Smt3p are partially overlapping, we wanted to determine which interaction is important for thiolester bond formation. We could distinguish between the importance of interacting with Smt3p and the importance of interacting with E1(S) with two mutants that show distinct binding behavior: the F22A/G23Q/Y25S mutant binds to E1(S) but not Smt3p, and the -32-34Δ and 34, 37Δ mutants bind to Smt3p but not E1(S). Even though the 32-34Δ and 34, 37Δ mutants show wild-type binding to Smt3p, they are severely impaired for their ability to form thiolester bonds with Smt3p. This contrasts with the F22A/G23Q/Y24S mutant, which shows wild-type levels of thiolester bond formation, even though it does not bind noncovalently to Smt3p. Thus, noncovalent E1(S)-binding by this region, and not Smt3p binding, is crucial for Ubc9p-Smt3p thiolester bond formation.

*Residues near the active site are involved in Smt3p conjugation*

Recently, human SUMO family members SUMO-2 and SUMO-3 have been shown to form polymeric chains *in vitro* simply in the presence of human E1(S) and Ubc9, and the modification is linked to K11 (18), the only lysine in the sequence that is part of an essential SUMO modification consensus sequence ψKXE (52). Inspection of the sequence of *S. cerevisiae* Smt3p reveals a homologous site at K15, although there are two other lysines that are part of consensus sequences that are not conserved in other SUMO family members. Therefore,
we tested whether Smt3p forms polymeric chains *in vitro* simply in the presence of Ubc9p, E1(S) and MgATP (Fig. 6A), and find that Smt3p also forms polymeric chains *in vitro* under similar conditions to those described for the human proteins. Ubc9p is also automodified via an isopeptide linkage with Smt3p, and this same modification has been observed *in vivo* (Pamela Meluh, personal communication). We isolated the Smt3p-Smt3p species from the SDS-PAGE gel and determined the isopeptide linkage to be through K15 by a combination of proteolysis and mass spectrometry (see Experimental Procedures). To confirm that K15 is the site of the Smt3p-Smt3p linkage, we tested the ability of K to R mutants to form the Smt3p-Smt3p conjugates. Both the K15R mutation and the triple mutation of all three possible sites severely impaired for formation of the Smt3p-Smt3p conjugate, without affecting formation of the Ubc9p-Smt3p complex (Fig. 6B). There is no effect of mutating the other sites (Fig. 6B), consistent with the identification of K15 as the site of Smt3p-Smt3p isopeptide bond formation. This suggests that the mechanism of polymeric chain formation will be conserved among SUMO family members.

We next tested the Ubc9p mutants for their ability to form Smt3p-Smt3p conjugates (Fig. 7). Three mutations impair formation of Smt3p-Smt3p conjugates, without affecting thiolester bond formation, P88S/S89A/T91A, N124A and N140A/A142E/E143K. The crystal structures of Ubc9 show that the first two of these variants contain mutations directly around the active site, and the mutations in the third variant are located on the outside of a helix that packs against the loop containing N124, so these mutations may affect packing around N124 (40,43,44). S89 and T91 are conserved as polar or charged residues among E2s in general, and previous mutational studies have implicated this part of the structure as being involved in transfer of ub-lps. A previous mutational study of human Ubc9 has also shown a role for S89 and T91 in
SUMOylation of a number of substrates (40), and a mutation in this region of Ubc13 plays a role in recognition of the acceptor ubiquitin molecule in polyubiquitin chain assembly (53). The N124A and N140A/A142E/E143K mutants are more severely impaired for Smt3p transfer, as they are also defective in Ubc9p-Smt3p isopeptide bond formation (data not shown). Interestingly, N124 is highly conserved as either an Asn or a Gln in the sequences of other E2s, suggesting that it may play a general role in catalysis of transfer of ub-lps.

**DISCUSSION**

*Specificity of the E1-E2 interaction*

Several previous findings have raised the question of how a given E2 selects its cognate ub-lp. First, the sequences of some ubiquitin conjugating E2s are closer to the sequences of both Ubc9 and Ubc12, which transfer SUMO and NEDD8, respectively, than to other ubiquitin conjugating E2s. Second, the three-dimensional structure of human Ubc9 is very similar to the structure of ubiquitin conjugating enzymes (43,44). These observations suggest that the mechanism of conjugation is conserved for all E2s. Our finding that the residues involved in Smt3p-Smt3p conjugate formation map to the region around the active site, which is generally conserved among E2s, is consistent with this notion. Third, NMR studies have revealed few contacts between ubiquitin and ubiquitin conjugating enzymes in thiolester or analogous ester complexes formed by catalytic cysteine to serine mutants of E2s (37,39). In addition, even the
ester complexes are labile (37). Therefore it has been suggested that the E1 may play a major role in bringing the E2 together with the correct ub-lp (7). This hypothesis is supported by the kinetics of ubiquitin transfer, which are consistent with high affinity interaction between E1 and E2 (41). Also, a mutant form of NEDD8 activated by the E1 for ubiquitin was found to form a thiolester intermediate with an E2 for ubiquitin (54). Here we provide direct support for this hypothesis, by finding that the mutations disrupting Ubc9p-Smt3p thiolester bond formation map to the binding site for E1(S). Together, these findings suggest that binding to the appropriate E1 by an E2 is involved selection of the appropriate ub-lp.

We have mapped the E1(S) binding site on Ubc9p to involve the N-terminal helix and the loop between the first and second \( \beta \)-strands. Interestingly, in Ubc9 family members, this loop contains an insertion not found in the sequences of E2s for Ub or other Ub-lps (43,44). This region protrudes away from the Ubc core and is one of the most prominent differences between the structure of Ubc9 and the structures of E2s for Ub (43,44). In addition, K14, in the N-terminal helix, is often replaced by an acidic or polar residue in E2s for ubiquitin. Thus, this surface does contain differences between E2s for different ub-lps that could serve as the basis for specificity in E1 binding.

**Conservation of the E1 binding location**

The overall location of the E1 binding site, the surface containing the N-terminal helix and the loop between the first two \( \beta \)-strands, is probably conserved among E2s for other ub-lps. Two previous mutational studies of E2s for ubiquitin have shown that the N-terminal helix is important for thiolester bond formation (55,56), and we have found that the loop between the
first two β-strands is important for thiolester bond formation for another E2 (data not shown). Although these studies did not directly examine E1 binding, they led Cook and colleagues to propose a role for the E2 N-terminus in E1 binding in their description of the first crystal structure of an E2 (57). If this region is involved in E1 binding in general, then other protein-protein interactions that block access to this surface would be expected to impair E2-ubiquitin thiolester bond formation. This result has recently been reported for human Ubc13. Two recent crystal structures show that MMS2 binding blocks access to the loop between the first two β-strands of Ubc13 (53,58), and Ubc13-ubiquitin thiolester bond formation has recently been reported to be inhibited by the binding of MMS2 (45).

A conserved multifunctional binding site on E2

We have demonstrated that the E1(S) binding site on Ubc9p is also the binding site for Smt3p, and that Smt3p competes with E1(S) for binding to this site. While binding to E1 is important for thiolester bond formation, the function of noncovalent binding of Ubc9 by SUMO family members remains a mystery. Interestingly, tight noncovalent interactions with E2s have not been reported for other ub-lps, so this may be a unique feature of SUMO family members. A previous NMR study revealed that the SUMO-1 C-terminus, which is the site of conjugation, is not involved in the noncovalent interaction with Ubc9, suggesting that at least the terminal SUMO in a conjugated polySUMO chain could interact with Ubc9 (38). If this were the case, then thiolester-linked SUMO-Ubc9 complexes could bind to polySUMO chains to promote chain elongation, although our mutational analysis suggests that this interaction is not essential for Smt3p-Smt3p chain assembly.
The region corresponding to the E1(S) and Smt3p binding site on Ubc9p also serves as a protein-protein interaction surface on other E2s and their relatives. Several noncatalytic variants of E2s also use this region as a binding site. The Ubiquitin E2 Variant (UEV) domain of Tsg101 uses the region corresponding to the Ubc9 E1(S)/Smt3 binding site to interact noncovalently with ubiquitin, although the function of this interaction is unknown (59). Another UEV family member, MMS2 uses this surface to interact noncovalently with the Ub E2 Ubc13, and this interaction is required for Ubc13-mediated assembly of K63-linked polyubiquitin chains required for Ikb kinase activation (58). Perhaps most interestingly, the N-terminal helix and loop between the first two β-strands from UbcH7 is involved in binding to E3s. This region of UbcH7 contributes ~30% of the interaction surface for the complex with E6AP (35), and ~60% of the interaction surface for the complex with c-Cbl (36), and although it is partially exposed in the complex with E6AP (35), it is completely buried in the complex with c-Cbl (36).

**Coevolution of the E1-E2-E3 pathway**

Because it is not known whether E1 and E3 bind to E2 simultaneously, it is possible that E1 and E3 bind to the same site on E2. Our finding that the corresponding Ubc9p binding site for E1(S) is completely buried in the UbcH7/c-Cbl complex (36) suggests that the E2 is a central coordinator in the pathway, and raises the possibility that E1, E2 and E3 have coevolved as a given ub-lp as a method of ensuring that the correct ub-lp is directed to the correct target.

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FIGURE LEGENDS

Figure 1: Summary of the mutational analysis of Ubc9p. Mutated sites are shown schematically on the structure of human Ubc9. The active site cysteine is labeled C93, the first two $\beta$-strands are indicated as $\beta_1$ and $\beta_2$ and the N- and C-termini are labeled N and C, respectively.

Figure 2: Smt3p thiolester bond formation with Ubc9p mutants. Thiolester intermediates between Ubc9p and Smt3p were assayed at 0, 15, 30 and 60 minutes as indicated and resolved by SDS PAGE as described in Experimental Procedures. Only the relevant portion of each gel is shown for clarity. The identity of the thiolester bond is confirmed by susceptibility to reduction by DTT at the final time point, labeled “60+DTT”. Wild-type and mutants enzymes are denoted above each lane by “WT” or mutation (e.g., “K14E” refers to mutation of lysine 14 to glutamate). Lanes where mutations significantly impede thiolester bond formation are indicated.
with asterisks.

**Figure 3: E1(S) and Smt3p interaction with Ubc9p mutants.** Noncovalent complexes between 14 µM Ubc9p and (A) 3 µM E1(S) and (B) 20 µM Smt3p were resolved by nondenaturing polyacrylamide gel mobility shift assays. Migration of E1(S) and Smt3p is retarded by complex formation with Ubc9p, with complexes labeled “Ubc9p/E1(S)” or “Ubc9p/Smt3p”, respectively. Proteins mixed in each lane are indicated at the top of each gel. Proteins were visualized by Coomassie blue staining.

**Figure 4: ^1H NMR spectra of Ubc9p (top panel) and the K14E mutant (bottom panel).** Spectra were obtained of ~150 µM protein at 20°C, pH 7.0 as described in Experimental Procedures. The sharp peaks around 3.5 ppm correspond to glycerine from the membranes used during protein concentration.

**Figure 5: Smt3p competes with E1(S) for noncovalent interaction with Ubc9p.** Competition experiments were performed where 14 µM Ubc9p was incubated with either 3 µM E1(S) or 8 µM Smt3p alone, or with E1(S) in the presence of increasing amounts of Smt3p (4, 18, 16 or 32 µM) as described in Experimental Procedures. E1(S) alone, Smt3p alone, the E1(S)/Ubc9p noncovalent complex and the Smt3p/Ubc9p noncovalent complex were readily identified by their different mobilities on a Coomassie stained nondenaturing polyacrylamide gel and are indicated to the right of the figure.
Figure 6: Smt3p forms polymeric chains via lysine 15. (A) The Smt3p conjugation assay depends on the presence of E1(S), Ubc9p, Smt3p, and MgATP, as absence of any of these components prevents formation of Smt3p conjugates. Reactions were carried out for 120 minutes, boiled in the presence of DTT as described in Experimental Procedures, and either contained (+) or lacked (-) components indicated above each lane. Smt3p and Ubc9p alone, as well as Smt3p conjugates formed with Smt3p (“Smt3p-Smt3p”) and Ubc9p (“Ubc9p-Smt3p”) were resolved by SDS-PAGE. Proteins were visualized by Coomassie staining and the identity of each band was confirmed by mass spectrometry. (B) Smt3p-Smt3p conjugates are linked through lysine 15. Smt3p conjugation assays were performed as in (A) with Smt3p mutants containing arginine substitutions for lysines 11, 15 and/or 19, which are part of SUMO consensus motifs. The identity of Smt3p variant used for the reaction is indicated above each lane.

Figure 7: Smt3p conjugation by Ubc9p mutants. Smt3p-Smt3p conjugates were assayed at 0, 30, 60 and 120 minutes, boiled in the presence of DTT, and resolved by SDS PAGE and visualized by Coomassie staining as described in Experimental Procedures. Only the relevant portion of each gel is shown for clarity. Wild-type and mutants enzymes are denoted above each lane by “WT” or mutation (e.g., “K14E” refers to mutation of lysine 14 to glutamate). Lanes where mutations significantly impede Smt3p-Smt3p conjugate formation without affecting thiolester bond formation are indicated with an X.
Table 1: E1(S) and Smt3p binding by Ubc9p mutants

| Ubc9p                  | E1(S) | Smt3p | Ubc9p                  | E1(S) | Smt3p |
|------------------------|-------|-------|------------------------|-------|-------|
| WT                     | +++   | +++   | N98A                   | +++   | +++   |
| K14E                   | -     | -     | Q101A                  | +++   | +++   |
| R17A                   | +     | -     | E99SD100SD102S         | +++   | ND    |
| K18A                   | +     | +     | K110SQ111K             | +++   | +++   |
| F22AG23QY25S           | +++   | -     | D118AS122A             | +++   | +++   |
| V29AK30D               | -     | -     | N124A                  | +++   | +++   |
| V29AK30D -A32,D33,G34  | -     | -     | N126A                  | +++   | +++   |
| -A32,D33,G34           | +     | +++   | S127A                  | +++   | +++   |
| -G34, D37              | -     | ++    | W134AS136AR139S        | +++   | +++   |
| Q39AK40AE42A           | +++   | +++   | N140AA142EE143K        | +++   | +++   |
| K48AE49A               | +++   | +++   | D145AK146AK147N        | +++   | +++   |
| A54EG55D               | +++   | +++   | L149EL150EQ154A        | +++   | +++   |
| P59AT61AE63A           | +++   | ++    | R104SP105AA106R        | +++   | +++   |
| P65AN66A               | +++   | +++   | K14ER17AK18A           | -     | -     |
| K74AK76A               | +++   | +++   | S70G                   | +++   | +++   |
| P78TA79T               | +++   | +++   | S70F                   | +++   | +++   |
| Y87F                   | +++   | +++   | Q7R                    | +++   | +++   |
| P88SS89AT91A           | +++   | +++   | E11K                   | +++   | ++    |
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Identification of a multifunctional binding site on Ubc9p required for Smt3p conjugation
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