A Six-membrane-spanning Topology for Yeast and Arabidopsis Tsc13p, the Enoyl Reductases of the Microsomal Fatty Acid Elongating System*

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The very long chain fatty acids are crucial building blocks of essential lipids, most notably the sphingolipids. These elongated fatty acids are synthesized by a system of enzymes that are organized in a complex within the endoplasmic reticulum membrane. Although several of the components of the elongase complex have recently been identified, little is known about how these proteins are organized within the membrane or about how they interact with one another during fatty acid elongation. In this study the topology of Tsc13p, the enoyl reductase of the elongase system, was investigated. The N and C termini of Tsc13p reside in the cytoplasm, and six putative membrane-spanning domains were identified by insertion of glycosylation and factor Xa cleavage sites at various positions. The N-terminal domain including the first membrane-spanning segment contains sufficient information for targeting to the endoplasmic reticulum membrane. Studies of the Arabidopsis Tsc13p protein revealed a similar topology. Highly conserved domains of the Tsc13p proteins that are likely to be important for enzymatic activity lie on the cytosolic face of the endoplasmic reticulum, possibly partially embedded within the membrane.

The endoplasmic reticulum (ER)²-associated elongase system is composed of four distinct enzymes that sequentially catalyze condensation between a CoA-esterified fatty-acyl substrate and malonyl-CoA, a 3-ketoacyl-CoA reduction, a 3-hydroxyacyl-CoA dehydration, and a final enoyl-CoA reduction to yield a fatty acid that is two carbon units longer than the primer (4). Several of the genes encoding components of the elongase system were first identified in Saccharomyces cerevisiae (5–9) and later in plants and mammals (10–16). Several studies indicate that the elongase proteins are organized in a complex within the ER (5, 7, 17). A complete understanding of the molecular mechanism and organization of the elongase complex will require structural analysis, a challenging prospect because of the intrinsic technical difficulties associated with the purification and crystallization of membrane proteins. However, in the absence of high resolution structural data, detailed topology models aid in the design and interpretation of structure-function studies of membrane proteins. Thus, as a first step toward elucidating the organization of the elongase complex we have undertaken the topological mapping of the component proteins. Here we present our studies on the topological organization of the S. cerevisiae and Arabidopsis thaliana Tsc13p proteins, the enoyl-CoA reductases of the elongase complex.

TSC13 was identified in a screen for mutants that suppress the Ca²⁺ sensitivity of csg2Δ cells (18). Csg2p is required for conversion of inositolphosphoceramide (IPC) to mannosylinositolphosphoceramide, and we discovered several years ago that the over-accumulation of IPC in the csg2Δ cells results in calcium sensitivity (18, 19). Furthermore, mutations that reduce inositolphosphoceramide (IPC) accumulation were found to suppress the Ca²⁺ sensitivity, including mutations in the genes required for the very long chain fatty acids component of IPC (18, 19). The mammalian (14) and plant (10, 15) orthologs of Tsc13p have also been identified and characterized.

In the present study we provide evidence that the N and C termini of both the yeast and Arabidopsis Tsc13p proteins reside in the cytosol and that these proteins contain six membrane-spanning domains. Based on this model, two highly con-

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2 The abbreviations used are: ER, endoplasmic reticulum; HA, hemagglutinin; GFP, green fluorescent protein; GC, glycosylation cassette; Fxa, factor Xa; TMD, transmembrane (TM) domain; Endo H, endoglycosidase H; FOA, 5-fluoroorotic acid; SC, prefix designating a S. cerevisiae gene or gene product; At, prefix designating an A. thaliana gene or gene product; SD, synthetic minimal medium with glucose.
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![Image](image-url)

FIGURE 1. Sites of insertion of the GC cassettes into the yeast and Arabidopsis Tsc13p. An alignment of the human (Human), plant (A. thaliana (At)), and yeast (S. cerevisiae (Sc)) Tsc13p was generated using ClustalW and used to predict locations within the protein that might accommodate insertion of the GC without disrupting function. The residues after which the GC was inserted are in green for functional proteins, blue for non-functional but stable proteins, or red for unstable proteins. The highly conserved residues are shaded black. The blue asterisks mark the residues identified as critical for function in this study, and the orange asterisk (Gln-81) marks a residue previously shown to be important for function (7).

served and functionally critical residues are found to be associated with the membrane, raising the possibility that the active site of the enzyme is partially embedded within the lipid bilayer.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Media**—Yeast media were prepared, and cells were grown according to standard procedures (20). The yeast *Mata ura3–52 ade his4 trp1Δ leu2Δ tsc13::TRP/pTSC13–316* strain was used in this study.

**Disruption of TSC13 Gene and Construction of Tagged Yeast and Arabidopsis Tsc13p**—Construction of the tsc13Δ mutant and the pTSC13–316 and pMYC-TSC13–425 plasmids were described previously (7). The plasmid expressing the dual epitope-tagged Myc-Tsc13p-HA was generated by moving the 3×-MYC-TSC13 cassette from pMYC-TSC13–426 to pRS425, introducing an in-frame SpeI site between codon 309 and the stop codon of the TSC13 gene by QuikChange mutagenesis (Stratagene). All mutants were confirmed by DNA sequencing.

**Site-directed Mutagenesis**—The tsc13 mutant alleles with charged residues substituted by alanine were generated by QuikChange mutagenesis (Stratagene). All mutants were confirmed by DNA sequencing.

**Complementation Assay**—The various Tsc13p-GC, Tsc13p-fxa, alanine substitution mutant, and epitope-tagged Tsc13p proteins were assessed for function by determining whether their expression would complement the lethality of the tsc13Δ mutant. Plasmids expressing the modified tsc13 alleles were introduced into pTSC13–316-rescued tsc13Δ mutant cells, and the transformants were tested for their ability to lose the *URA3*+-marked pTSC13–316 rescuing plasmid. Transformants containing plasmids expressing functional Tsc13p proteins were able to lose the *URA3*+-marked plasmid and were, therefore, able to grow on 5-fluoroorotic acid (FOA)-containing plates.

**Determination of the Glycosylation Status of the Tsc13p-GC Fusion Proteins**—For analysis of the glycosylation status of the Tsc13p-GC proteins, microsomes were prepared from cells grown in minimal medium (minus leucine) to ensure maintenance of the plasmid. The cells were pelleted, washed with water, resuspended at 2 ml/g wet weight, and lysed by bead beating in 50 mM Tris, 7.5, 1 mM EGTA, 1 mM β-mercaptoethanol.
anol, 1 μM aprotinin, 1 μM phenylmethylsulfonfyl fluoride, and 1 μM leupeptin. Glass beads were added to the meniscus, and cells were lysed by four cycles (60 s each) of vortexing with cooling on ice between cycles. Unbroken cells, beads, and debris were removed by centrifugation (5000 × g for 10 min), and the low speed supernatant was centrifuged at 100,000 × g for 30 min at 4 °C to provide the microsomal pellet. The pellet was resuspended in the same buffer, repelleted at 100,000 × g for 30 min at 4 °C, and resuspended at ~10 mg/ml protein in the same buffer containing 33% glycerol. The glycosylation status of the Tsc13p-GC proteins was analyzed as described previously (21, 22) with slight modifications. In brief, 100 μg of microsomal protein was suspended in 54 μl of 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 50 mM NaCl, 5 mM Na2EDTA, 50 mM NaCl. Six μl of 10× denaturing buffer (5% SDS, 10% β-mercaptoethanol) were added, and the samples were incubated at 70 °C for 10 min. Ten μl of 0.5 M sodium citrate, pH 5.5, were added to the heated samples to bring the volume to 70 μl, and the samples were split into two aliquots. One aliquot was treated with 5000 units of endoglycosidase H (Endo H; New England Biolabs), and the other aliquot was incubated with buffer at 37 °C for 60 min and then further incubated at 70 °C for 10 min with denaturing buffer (5% SDS, 10% β-mercaptoethanol). After resolution by 12% SDS-PAGE, the proteins were analyzed by immunoblotting.

Immunoblotting—Preparation of microsomes for analyzing the fusion proteins containing the fXa cleavage sites was performed essentially as described previously (21, 22) with slight modifications. Spheroplasts were generated and lysed with lysis buffer, and the homogenates were cleared of debris. The microsomal membrane fraction was recovered by centrifugation at 100,000 × g for 30 min at 4 °C, washed once with storage buffer (20 mM Tris-HCl, pH 7.5, containing 250 mM sorbitol, 50 mM potassium acetate, and 1 mM β-mercaptoethanol), and resuspended in storage buffer at 4 mg/ml protein. The fXa protease cleavage assay was performed as described (22), and the products were separated by SDS-PAGE and detected by immunoblotting.

Protease Protection Assay—Right-side-out microsomes were prepared from yeast expressing the HA-TSC13-MYC fusion protein. Protease protection assays were performed as described (23), and the samples were resolved by SDS-PAGE and analyzed by immunoblotting.

Cell Fractionation—Microsomes were prepared by bead beating (17) and incubated on ice for 1 h with an equal volume of buffer containing either 1 M sodium chloride, 0.4 M sodium carbonate, or 2% Triton X-100. After centrifugation of the samples at 100,000 × g for 30 min at 4 °C, the supernatant and the pellet fractions were collected and subjected to SDS-PAGE and immunoblotting.

Immunoblotting—Proteins were resolved by 12% SDS-PAGE and transferred to nitrocellulose, and the blots were blocked in 0.1 M Tris, 7.5, 0.15 M NaCl, 0.1% Tween 20, 5% dry milk. Myc-Tsc13p was detected with horseradish peroxidase-conjugated monoclonal anti-Myc antibodies (Sigma) at 1/2500, the HA-tagged proteins were detected with horseradish peroxidase-conjugated monoclonal anti-HA antibodies (Roche Applied Science) at 1/1000, and Kar2p was detected using anti-Kar2p (1:10,000) as the primary antibody followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit antibody (Bio-Rad). The bound antibodies were detected using the ECL detection system (Amersham Biosciences).

RESULTS

Membrane Topology Predictions for Tsc13p—Tsc13p, the enoyl reductase of the fatty acid elongation system, is an integral ER membrane protein. Determination of the topology of Tsc13p is an important step toward understanding both its mechanism and its organization within the elongase complex. As is often the case, various algorithms for predicting membrane-spanning segments suggested several possible topologies for Tsc13p. SOSUI, a program that takes into account the Kyte-Doolittle hydrophathy, amphiphilicity, amino acid charge, and the length of the protein (24), predicted a single transmembrane domain (TMD) located between amino acids 262–283. In contrast, six TMDs were predicted by both Localizome (86–105, 125–143, 164–184, 204–226, 247–266, and 272–291) and HMMTOP (88–112, 145–162, 169–186, 203–220, 251–269, and 274–291). Localizome uses hnmfpam to detect the presence of Pfam domains (25) and a prediction algorithm, Phobius, to predict the TM helices. The results are combined and checked against the TM topology rules stored in a protein domain data base called LocaloDom (26). HMMTOP (Hidden Markov Model for Topology Prediction) is based on the principle that topology of TM proteins is determined by the difference in amino acid distribution in various structural parts of these proteins rather than by specific amino acid composition (27, 28). TMHMM, which is also based on a hidden Markov Model (29), predicts four TMDs (166–188, 202–222, 243–265 and 269–291). In the experiments described below, these different models for Tsc13p topology were experimentally evaluated.

Mapping the Orientation of the Tsc13p Termini by Protease Protection—To determine the orientation of the N- and C-terminal ends of Tsc13p, the N terminus was tagged by inserting a Myc epitope after the start codon, and the C terminus was tagged by inserting an HA epitope before the stop codon. To ascertain that insertion of the epitope tags did not alter the structure of Tsc13p, the function of the epitope-tagged protein was tested. TCS13 is an essential gene in S. cerevisiae (7), and thus, the plasmid carrying the wild-type TCS13 gene is required for survival of the haploid deletion strain. A LEU2-marked plasmid expressing Myc-Tsc13p-HA was transformed into the rescued tsc13A Δ. FOA-resistant colonies that had lost the URA3-marked TCS13 plasmid were recovered, indicating that insertion of the N- and C-terminal epitope tags did not disrupt the function of Tsc13p (Fig. 2A). In addition, immunofluorescence localization of the Myc-Tsc13p-HA protein revealed...
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FIGURE 2. Myc-Tsc13p-HA is functional and ER localized. A, the tsc13Δ mutant is unable to lose the pTSC13-316 plasmid and is, therefore, unable to grow on FOA. Introduction of the LEU2-marked plasmid expressing the epitope-tagged MYC-Tsc13p-HA protein (but not the empty vector) allowed the tsc13Δ mutant to lose the URA3-marked pTSC13-316 plasmid and to grow on FOA. B, indirect immunofluorescence shows that Myc-Tsc13p-HA localizes to the ER. Fixed and permeabilized cells were incubated with anti-Myc antibody, and the primary antibody was detected using Cy3-conjugated goat anti-mouse as the secondary antibody.

To localize the N and C termini of Tsc13p with respect to the ER membrane, protease protection assays were conducted. Sealed right-side-out membrane vesicles were isolated by gently lysing yeast spheroplasts prepared from cells expressing Myc-Tsc13p-HA, and sensitivity of the protein to proteasome K in the presence or absence of detergent was examined. The vesicles were treated with proteasome K in the presence or absence of detergent, and the Myc-Tsc13p-HA was analyzed by immunoblotting with either anti-Myc or anti-HA antibodies. The immunoblots indicated that both the N-terminal Myc tag and the C-terminal HA tag were sensitive to proteasome K even in the absence of detergent as no protected immunoreactive protein fragments were detected in either case (Fig. 3). If the N terminus had been luminal, a protected Myc fragment should have been observed, and similarly, if the C terminus were in the ER lumen, a protected HA-tagged fragment would have been seen. This indicates that both the N and C termini of Tsc13p are cytosolic and that Tsc13p has an even number of membrane spanning domains. The integrity of the sealed right-side-out vesicles was confirmed by the observation that the ER luminal Kar2p protein was insensitive to proteasome K until the vesicles were disrupted with detergent (Fig. 3). As has been previously reported, in the presence of detergent proteasome K clipped Kar2p to a smaller size but did not completely degrade it (23). The sidedness of the vesicles prepared using this procedure was previously confirmed by demonstrating that fXa protease cleavage sites inserted into the luminal domains of the integral membrane protein, Lcb1p, were inaccessible to the fXa protease unless the vesicles were disrupted with detergent (22).

The Native Glycosylation Sites at Residues 38 and 255 of Tsc13p Are Not Modified—A well-established approach to map the topology of ER membrane proteins takes advantage of the lumen specific glycosylation machinery. The glycosylation status of the two potential N-linked glycosylation sites in the native Tsc13p sequence are not glycosylated. The first predicted TMD lies between residues 86 and 110; therefore, the lack of modification of the potential glycosylation site at 38 is consistent with the protease sensitivity results and provides further evidence that the N terminus of Tsc13p is cytosolic. The lack of glycosylation at 255 indicates that this residue is also not in a luminal loop that is accessible to the glycosylation machinery. Several of the hydropathy algorithms (discussed above) place residue 255 within a TMD, which would explain the lack of modification of this potential glycosylation site.

Analysis of Tsc13p-GC Fusion Proteins Reveals Several Membrane-associated Domains—To further investigate the topology of Tsc13p, a set of fusion proteins containing a glycosylation reporter cassette (GC) inserted in-frame at 13 positions along the length of the protein was constructed (Fig. 4A). The cassette consists of a 53-amino acid domain comprising residues 80–133 of invertase (Suc2p) that contains three NX(S/T) sites for N-linked glycosylation. These sites are located far enough from the N- and C-terminal ends of the cassette to ensure that if the GC is inserted anywhere in the luminal loop of a fusion protein, the acceptor sites will be sufficiently far from the membrane to be recognized by the glycosylation machinery (21). The locations within Tsc13p into which the cassettes were inserted were chosen based on their ability to distinguish the
The Tsc13p-GC fusion proteins were evaluated for function by testing their ability to restore viability to the \( tsc13 \) mutant. Tsc13p-GC fusions with the Suc2p domain inserted at eight different locations were functional (Fig. 4A), indicating that these proteins retained their native conformation. Tsc13p-GC fusions with the cassette inserted at position 135 or 166 resulted in unstable proteins. Although insertion of the cassette at position 159 or 170 resulted in stable proteins, these fusion proteins were not functional (Fig. 4A). Furthermore, indirect immunofluorescence revealed that these two fusion proteins failed to localize to the ER (data not shown). The Tsc13p-GC fusion protein with the cassette at position 273 was also not functional (Fig. 4A), but this protein displayed normal ER localization (data not shown).

The Tsc13p-GC fusion proteins with the cassette inserted at 117, 126, 198, or 273 were glycosylated as indicated by increased electrophoretic mobility after Endo H treatment, and therefore, these regions of Tsc13p are localized in the lumen of the ER. Moreover, the electrophoretic mobilities of the fusion proteins with the GC located at position 7, 69, 226, 236, or 309 were not altered after treatment with Endo H, demonstrating that these regions of Tsc13p are located in the cytosol (Fig. 4A). Although the fusion proteins with the GC at 159 and 170 were not glycosylated because they were not functional and did not localize properly, it is not possible to definitively conclude that they are normally cytosolic. Although these analyses clearly reveal the presence of at least two luminal loops in Tsc13p, it is not clear from these data whether the segment between 126 and 198 forms a single large luminal loop or whether there are additional membrane-associated domains within this region of Tsc13p.

**Analysis of Tsc13p Topology by fXa Insertion**—To further investigate the topology of Tsc13p, tandem fXa protease cleavage sites (IEGRfXGR) were inserted at several positions (31), and their accessibility to fXa protease in sealed right-side-out membrane vesicles was assessed. A tandem recognition sequence was inserted to increase the probability of cleavage by the fXa protease, which cuts on the C-terminal side of the arginine in the IEGR tetrapeptide. The Tsc13p-fXa fusion proteins were expressed in \( tsc13 \) mutant cells, sealed right-side-out vesicles were prepared, and the sensitivity to fXa protease cleavage was assayed in the absence or presence of detergent. The integrity of the vesicle preparations was verified by showing that the lumenal ER protein, Kar2p, was accessible to proteinase K only in the presence of detergent (discussed above). The fXa sites inserted at positions 69, 226, or 236 were accessible to fXa protease whether or not Nonidet P-40 (Nonidet P-40) was present (Fig. 4B). This result is consistent with the lack of glycosylation of the GC cassettes inserted at the same positions and confirms the cytoplasmic orientation of these regions of the protein. Furthermore, the different topological models predicted by the hydrophathy analyses. In addition, alignments of Tsc13p proteins from different species were used in an attempt to identify sites with low conservation across evolution that might be sufficiently flexible to tolerate an insertion without disrupting function (Fig. 1). This reporter cassette has been successfully used in determining the topology of other membrane proteins (21, 22, 30), and its insertion into luminal loops between membrane-spanning domains has not been observed to interfere with their insertion or orientation.

The Tsc13p-GC fusion proteins were evaluated for function by testing their ability to restore viability to the \( tsc13 \) mutant. Tsc13p-GC fusions with the Suc2p domain inserted at eight different locations were functional (Fig. 4A), indicating that these proteins retained their native conformation. Tsc13p-GC fusions with the cassette inserted at position 135 or 166 resulted in unstable proteins. Although insertion of the cassette at position 159 or 170 resulted in stable proteins, these fusion proteins were not functional (Fig. 4A). Furthermore, indirect immunoblotting with anti-Myc antibodies revealed the presence of at least two membrane-spanning domains in Tsc13p. A schematic model of Tsc13p according to the results of the glycosylation and factor Xa protease susceptibility experiments. The cassette insertion sites are denoted by circles. The locations of the experimentally determined TMDs are denoted and marked 1–4. The dashed line indicates the region of Tsc13p whose topology (whether lumenal or membrane-associated) cannot be resolved from the results in panels A and B.

**FIGURE 4**. **Topology reporters indicate the presence of several membrane-spanning domains in Tsc13p.** A, the glycosylation status of the topological reporter Tsc13p-GC fusion proteins reveals the presence of at least four membrane-spanning domains. With the exception of the fusion proteins with the GC cassette inserted after amino acid 135, 159, 166, 170, and 273, the Tsc13p-GC proteins complemented the \( tsc13 \) mutant (indicated by growth on FOA). Microsomes were prepared from the \( tsc13 \) mutant yeast expressing the Tsc13p-GC fusion proteins, 10 \( \mu \)g of microsomal protein (with or without Endo H treatment) was resolved by 12% SDS-PAGE, and the fusion proteins were detected using anti-Myc. All the samples were run in the same experiment except for the sample expressing the fusion with the GC inserted at position 159, which was constructed and analyzed later. V, vector; WT, wild type. B, factor Xa protease cleavage of Tsc13p-fXa fusion proteins confirms the topology of Tsc13p. Sealed right-side-out microsomal vesicles were prepared from cells expressing Tsc13p-fXa proteins with a tandem repeat of the factor Xa cleavage sites inserted at the indicated positions. The microsomes were mock-digested or digested with factor Xa protease in the absence or presence of Nonidet P-40 on ice for 3 h. 10 \( \mu \)g of protein was resolved by 12% SDS-PAGE, and the Myc-tagged full-length or N-terminal fragments of Tsc13p were detected by immunoblotting with anti-Myc antibodies. C, a schematic model of Tsc13p according to the results of the glycosylation and factor Xa protease susceptibility experiments. The cassette insertion sites are denoted by circles. The locations of the experimentally determined TMDs are denoted and marked 1–4. The dashed line indicates the region of Tsc13p whose topology (whether lumenal or membrane-associated) cannot be resolved from the results in panels A and B.
protease recognition sites at 117, 198, or 273 were only accessible to the protease when Nonidet P-40 was present (Fig. 4B), a result that is also consistent with the glycosylation experiments. Taken together, the glycosylation and fXa protease cleavage results suggest that Tsc13p contains at least four membrane spanning domains located between amino acids 69–117, 198–
226, 236–273, and 273–309 and that the N and C termini are cytosolic (Fig. 4C). This agrees with the Localizome and HMMTOP algorithms, which predicted the presence of TMDs between amino acids 88–108, 204–224, 248–268, and 270–290. It is likely that there are two additional TMDs located between 126 and 198, but this could not be demonstrated using these methods because the insertion of GC and fXa recognition sequences into this region of Tsc13p resulted in unstable, non-functional, and/or mislocalized proteins. The possibility that there are additional membrane domains between 126 and 198 is addressed below.

**Evidence for Additional TMDs between Residues 126 and 198 of Tsc13p**—To determine whether the segment of Tsc13p between residues 126 and 198 is a large lumenal domain or whether it contains additional membrane associated segments, fXa cleavage sites were inserted after residue 117 and after residue 200 such that this fragment could be cut out of Tsc13p and its membrane association could be assessed. The fXa-flanked segment of Tsc13p also had an HA tag inserted at 198 for immunodetection (Fig. 5A). This HA-tagged Tsc13p with the two fXa cleavage sites retained the ability to rescue the tsc13Δ mutant, and therefore, it was assumed to adopt its native topology. Microsomes were prepared from the tsc13Δ mutant cells expressing the tagged Tsc13p using a bead beating procedure that generates inverted vesicles. The segment between residues 117 and 200 was liberated from the protein with fXa protease, the microsomes were subjected to high speed centrifugation, and the pellets and supernatants were analyzed for the presence of the HA-tagged fragment (Fig. 5B). Four distinct bands were detected with the anti-HA antibody, the sizes of which were consistent with the full-length tagged Tsc13p, the products from cleavage at one or the other of the fXa sites, and the 117–200-amino acid fragment derived from cleavage of both fXa sites. After high speed centrifugation, the liberated 117–200 fragment of Tsc13p was found exclusively in the pellet. Furthermore, the 117–200 fragment of Tsc13p was found to be integrally associated with the membrane because 1% Triton X-100, but not 0.5 M NaCl or 0.1 M sodium carbonate, solubilized it (Fig. 5B). These results clearly showed that the 117–200 fragment of Tsc13p is not a luminal loop, but rather, that it contains additional membrane-associated domains. Taken together, the data are, therefore, most consistent with the presence of six-membrane-spanning domains in Tsc13p (Fig. 5C).

**The N-terminal Membrane-spanning Domain of Tsc13p Is Sufficient for ER Localization**—Polytopic membrane proteins are initially targeted to the ER by a signal sequence that may include the first TMD, and subsequent TMDs often contain information that directs insertion and contributes to the topogenesis (32). To determine whether the first hydrophobic segment of Tsc13p contains sufficient information to direct membrane association, a chimeric protein with the N terminus including TMD1 of Tsc13p (amino acids 1–117, with a Myc tag after the first codon) was fused to GFP. When expressed in yeast, this chimera revealed the presence of six membrane-spanning domains in Tsc13p (Fig. 5C). These results are entirely consistent with the presence of six membrane-spanning domains in AtTSC13 and, thus, indicate that the Arabidopsis and yeast proteins have similar topologies (Figs. 1, 5C, and 7B).

**Alanine Substitutions of Conserved Charged Residues in or near the TMDs of Tsc13p**—A comparison of the amino acid sequences of Tsc13p homologs from Arabidopsis, Homo sapiens, and S. cerevisiae revealed the presence of several highly conserved amino acids including several charged residues near or within the predicted TMDs (Figs. 1 and 5C). To examine the physiological significance of several of these amino acids, alanine substitution mutants were generated and tested for their ability to complement tsc13Δ.

Despite their high conservation, several of these residues were not critical for function since the alanine substitution mutants, K76A, D77A, Y103A, H137A, E144A, H149A, and...
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**FIGURE 7.** Membrane topology of the A. thaliana Tsc13p (ATSC13). A, the glycosylation status of several ATSC13-GC fusion proteins was assessed as described above for the yeast Tsc13p-GC fusion proteins. Briefly, microsomes were prepared from yeast expressing the indicated ATSC13p-Suc2p fusion proteins, 10 μg of microsomal protein (with or without Endo H treatment) were resolved by 12% SDS-PAGE, and Tsc13p proteins were detected using anti-HA antibodies. V, vector; WT, wild type. B, proposed six-membrane-spanning topology model for the A. thaliana TSC13 ortholog. The circles represent the amino acids after which the GC was inserted.

**FIGURE 8.** Mutational analysis of yeast Tsc13p. A, several conserved charged residues (indicated in Fig. 5C) were mutated to alanine, and the mutant Tsc13p proteins were tested for function by the complementation assay (see “Experimental Procedures”). WT, wild type. B, the glycosylation status of the Tsc13p-GC fusion proteins carrying the K140A and R141A mutations was assayed. Microsomes were prepared from yeast harboring the indicated AtTSC13p-Suc2p fusion proteins, 10 μg of microsomal protein (with or without Endo H treatment) were resolved by 12% SDS-PAGE, and Tsc13p proteins were detected using anti-HA antibodies. V, vector; WT, wild type. B, proposed six-membrane-spanning topology model for the A. thaliana TSC13 ortholog. The circles represent the amino acids after which the GC was inserted.

**E259A** (Fig. 8A) and E91A (data not shown) were able to complement the tsc13Δ mutant. On the other hand, the Y138A, K140A, and R141A mutants were not functional (Fig. 8A). Immunoblot analysis revealed that the Y138A mutant protein was unstable, but the K140A and R141A mutant proteins were present at similar levels to wild-type Tsc13p (data not shown).

The Lys-140 and Arg-141 residues are critical for function, they are not required for interaction with other appropriately tagged elongase proteins. Such an interaction is reflected by cleavage of the reconstituted ubiquitin by a cytosolic protease and, therefore, further confirms the C terminus of Tsc13p is in the cytosol.

Our glycosylation and factor Xa results provide strong evidence that the regions of Tsc13p comprised of amino acids 85–110, 202–222, 243–269, and 274–291 contain membrane
spanning domains. Because the majority of the topological reporter cassette-containing fusion proteins were capable of complementing the tsc13Δ mutant, it is likely that these proteins are adopting the native Tsc13p topology. Moreover, the cytosolically oriented N-terminal 85-amino acid tail together with the first predicted membrane-spanning domain located between residues 85 and 110 contains sufficient information to target a cytosolic GFP to the ER, providing clear evidence for the presence of a transmembrane domain, TMD1, in this region.

Because all insertions between residues 126 and 198 resulted in mislocalization and/or disruption of function, it was unclear whether this segment of Tsc13p represented a long luminal domain or whether, as predicted by several algorithms, there were additional membrane-spanning domains in this region. Because there is one TMD before residue 126 and three after residue 198 and both ends of Tsc13p are cytosolic, the presence of any additional TMDs in this region would require an even number, most likely two. We provided evidence for additional membrane-associated segments within this region by proteolytically cleaving it from the full-length Tsc13p and showing that the released fragment remained associated with the membrane fraction. The membrane association of this liberated fragment is apparently not a result of interaction with other membrane proteins because it was stable to high salt and pH but could be solubilized with detergent.

The inability to detect a luminal loop between residues 126 and 198 leaves open the possibility that there are membrane-embedded rather than membrane-spanning domains in this region of Tsc13p. However, because the analysis of the Arabidopsis ortholog of Tsc13p provides evidence for the presence of a luminal loop in the region analogous to the yeast 126 to 198 segment (discussed below), we favor a topological model with six membrane-spanning domains (Fig. 5C). This topology is consistent with the predictions of the Localizome and HMMTOP prediction programs, although these programs differ in their prediction of the precise location of TMD2. Localizome places it between residues 125 and 143, and HMMTOP places it between residues 145 and 162. We have indicated the uncertainty about the location of TMD2 using the dashed line in Fig. 5C. However, it seems likely that TMD2 is located between 125 and 143 because the corresponding regions of the plant and mammalian orthologs are also predicted to contain a membrane-spanning domain. Furthermore, this topology would place the highly conserved domain that contains the functionally important Lys-140 and Arg-141 residues identified in this study near the cytosolic face of TMD2 where it could act in conjunction with the conserved cytosolic domain immediately preceding TMD1. This later domain contains the Gln-81 residue that when mutated to lysine reduces enoyl-reductase activity (7).

The plant and mammalian Tsc13p orthologs have hydrophilicity profiles that are similar to that of the yeast protein. As mentioned above, HMMTOP predicts six TMDs in Arabidopsis Tsc13p located in similar relative positions and with the same orientation as those in yeast except that the fifth predicted TMD of AtTSC13 is more N-terminal, suggesting that the third cytoplasmic loop is shorter and the third luminal loop longer in comparison to yeast Tsc13p. Our topological analysis of the AtTSC13 protein expressed in yeast is fully consistent with this model. HMMTOP also predicts six TMDs and a very similar overall topology for the human Tsc13p ortholog.

Several recent studies have indicated that membrane spanning segments of proteins are often flanked by tryptophan residues because of their propensity to localize to the interface between the polar and hydrophobic layers of the phospholipid bilayer (35). It is, therefore, of interest to note that several of the predicted membrane-spanning domains of Tsc13p are flanked by tryptophan residues (purple circles, Fig. 5C). Although in many cases the Arabidopsis and mammalian Tsc13p orthologs lack a similarly positioned tryptophan residue (Fig. 1), there are often either tyrosines or phenylalanines (which also often flank membrane-spanning domains) located in equivalent positions near the ends of the predicted membrane-spanning domains.

Based on our topology model of Tsc13p, the three cysteine residues in the protein all lay near the cytosolic side of the membrane (yellow circles, Fig. 5C). We tested whether any of the cysteines were critical for function by substituting them with serine. Each of the single mutants and the triple cysteine-less mutant was functional, suggesting that these residues do not participate directly in catalysis. However, it should be emphasized that the assay for function was the ability to complement the tsc13Δ mutant, and thus, we cannot conclude that these residues are not required for optimal activity of Tsc13p.

Little is known about the mechanism of Tsc13p or about its active site, and it bears no similarity with the well characterized enoyl-ACP reductase of the soluble fatty acid synthase system. Furthermore, although the C terminus of Tsc13p shares significant homology with steroid-5α reductase and both enzymes catalyze the reduction of a double bond that is α,β to a carbonyl group, Tsc13p lacks the predicted NADPH binding site that is present in steroid-5α reductase. In this study several conserved charged residues present in highly conserved regions (blue shading in Fig. 5C) as well as some conserved charged residues that are predicted to lie in the TMDs were substituted with alanine. We identified two residues, Lys-140 and Arg-141, that are critical for function but not for stability or topogenesis, whereas the Y138A mutation rendered Tsc13p unstable. Our topology studies suggest that these residues lie in or at the cytosolic face of TMD2. It is also worth pointing out that several of the insertions into the 126–198 region of Tsc13p abolished function. Although it is tempting to speculate that these two highly conserved domains contribute to the formation of an active site at the cytosolic face of the ER membrane or possibly extending into the membrane, the precise functions of these highly conserved domains of the trans-2,3-enoyl-CoA reductases remain to be determined. In conclusion, our topology study has established the foundation for further structure-function studies on Tsc13p.

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