The Topology of the Lcb1p Subunit of Yeast Serine Palmitoyltransferase

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The structural organization and topology of the Lcb1p subunit of yeast and mammalian serine palmitoyltransferases (SPT) were investigated. In the yeast protein, three membrane-spanning domains were identified by insertion of glycosylation and factor Xa cleavage sites at various positions. The first domain of the yeast protein, located between residues 50 and 84, was not required for the stability, membrane association, interaction with Lcb2p, or enzymatic activity. Deletion of the comparable domain of the mammalian protein SPTLC1 also had little effect on its function, demonstrating that this region is not required for membrane localization or heterodimerization with SPTLC2. The second and third membrane-spanning domains of yeast Lcb1p, located between residues 342 and 371 and residues 425 and 457, respectively, create a luminal loop of ~60 residues. In contrast to the first membrane-spanning domain, the second and third membrane-spanning domains were both required for Lcb1p stability. In addition, mutations in the luminal loop destabilized the SPT heterodimer, indicating that this region of the protein is important for SPT function and structure. Mutations in the extreme carboxyl-terminal region of Lcb1p also disrupted heterodimer formation. Taken together, these data suggest that in contrast to other members of the α-oxoamine synthases that are soluble homodimers, the Lcb1p and Lcb2p subunits of the SPT heterodimer may interact in the cytosol, as well as within the membrane and/or the lumen of the endoplasmic reticulum.

The sphingolipids are essential components of all eukaryotic cells. They confer important structural properties to membranes and partition into microdomains that are believed to organize proteins involved in signal transduction and membrane trafficking pathways (1). In addition, sphingolipid metabolites are regulatory molecules for a variety of cellular processes (2–7). Although it is clear that the sphingolipids are crucial for the function of eukaryotic cells, very little is known about the regulation of their synthesis.

The committed step of sphingolipid synthesis, the condensation of palmitoyl-coenzyme A (CoA) and l-serine to generate 3-ketodihydrosphingosine, is catalyzed by serine palmitoyltransferase (SPT) (1). This enzyme belongs to a subfamily of pyridoxal 5’-phosphate (PLP)-dependent enzymes known as the α-oxoamine synthases (POASs) that catalyze the decarboxylative condensation between the α-carbon of an amino acid and an acyl-CoA (8). The crystal structure of one member of this family, 8-amino-7-oxopelargonic acid synthase (AONS), has been solved and its detailed mechanism determined (9).

These studies showed that AONS is a head-to-tail homodimer with two symmetrical active sites. However, although other members of the POAS family, including AONS, are soluble homodimers, SPT is a membrane-associated heterodimer composed of two subunits, which in yeast are named Lcb1p and Lcb2p and in mammalian cells are named SPTLC1 and SPTLC2. The Lcb1p and Lcb2p subunits (as well as the mammalian subunits) share a considerable degree of sequence homology but no more closely related to one another than they are to AONS. Although there is no structural information for Lcb1p or Lcb2p, sequence alignments comparing them with each other and with AONS indicate that some of the catalytic residues of AONS, including the lysine that forms a Schiff’s base with PLP, are present in Lcb2p but not in Lcb1p, whereas other catalytic residues are present in Lcb1p but not in Lcb2p. Modeling studies based on the structure of AONS suggest that SPT is likely to have a single active site that lies at the Lcb1p/Lcb2p interface with some catalytic residues contributed by Lcb1p and others by Lcb2p. In support of this model is the observation that amino acid substitutions in Lcb1p analogous to the SPTLC1 mutations that causes hereditary sensory neuropathy type I (HSN1) alter a cysteine residue (Cys-180) predicted to lie across the heterodimeric interface from the PLP-binding lysine in Lcb2p (10–12). In addition, mutations in the yeast Lcb1p protein that correspond to the HSN1 mutations abolish catalytic activity, but do prevent heterodimerization, and are dominant negative (13). Most interestingly, mutations in the PLP-binding lysine residue (Lys-366) of Lcb2p also dominantly inactivate SPT in a manner similar to the HSN1-like mutations in Lcb1p (13).

Previous studies have shown that SPT is associated with the membrane of the endoplasmic reticulum (ER) (14). Protease accessibility studies indicated that the active site is cytoplasmic (15), which is consistent with the cytoplasmic localization of its substrates, serine and palmitoyl-CoA. Based on the homology of

1 The abbreviations used are: SPT, serine palmitoyltransferase; POAS, PLP-dependent α-oxoamine synthases; AONS, 8-amino-7-oxopelargonic acid synthase; HSN, hereditary sensory neuropathy; PLP, pyridoxal 5’-phosphate; ER, endoplasmic reticulum; PHS, phytosphingosine; GC, glycosylation cassette, residues 80–133 of invertase (Suc2p); CHO, Chinese hamster ovary; GFP, green fluorescent protein; fXa, factor Xa; EndoH, endoglycosidase H; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; HA, hemagglutination.
Lcb1p Has Three Transmembrane Domains

SPT with AONS, the central regions of the Lcb1p and Lcb2p protein were predicted to associate to form a cytoplasmically oriented active site at the heterodimer interface whereas the amino and/or carboxyl-terminal extensions of Lcb1p and Lcb2p that are missing in the soluble α-oxoamine synthases were considered likely to mediate membrane association.

Recently, Yasuda et al. (16) reported that the amino terminus of mammalian (CHO) SPTLC1 is located in the lumen of the ER, whereas the carboxyl terminus is cytoplasmic, demonstrating that the protein has an odd number of membrane-spanning segments. Based on hydropathy analyses and consideration of the likely position of the active site, they proposed that SPTLC1 has a single amino-terminally located membrane-spanning domain. Yeast Lcb1p protein also has a very hydrophobic amino terminus. However, mutations that replaced hydrophobic residues with charged residues or complete deletion of the putative amino-terminal membrane-spanning domain did not eliminate SPT activity, suggesting that the amino-terminal domain may not be the only region of the protein that determines membrane localization and topology.

In the present study, we provide evidence that in addition to an amino-terminal membrane-spanning domain, yeast Lcb1p protein has two additional membrane-spanning domains located in the region of the protein homologous to the PLP-binding domain of AONS. Because this region was previously predicted to participate in the formation of the heterodimeric interface with Lcb2p, these results, in combination with the finding that the carboxyl-terminal extension of Lcb1p is critical for interaction with Lcb2p, suggest that the structure of the SPT heterodimer may be more dissimilar from that of the homodimeric POAS family members than previously thought.

EXPERIMENTAL PROCEDURES

Yeast Strains and Media—Yeast strains used in this study are as follows: TTY2037, Mata ura3-52 trp1 leu2 lys2; TTY2507, Mata lcb1Δ:TRP ura3-52 trp1 leu2 lys2; TTY 2505, Mata lcb1Δ:TRP lcb2Δ:TRP ura3-52 trp1 leu2 lys2; and TTY2509, Mata lcb1Δ:TRP tsc3-2 ura3-52 trp1 leu2 lys2. Standard yeast media were prepared, and tsc3-2 ura3-52 trp1 leu2 lys2 and lcb1Δ were tested for their ability to grow on unstable media; and TDY2509, an AvrII insertion mutant, an AvrII site (underlined and in boldface) was inserted that creates a double crossover event. For this mutagenesis, a pRS315-based plasmid containing a GCTAGC by using QuikChange mutagenesis (Stratagene, La Jolla, CA) was inserted between codon 549 and 550, resulting in the formation of the likely location of the active site, they proposed that the structure of the Lcb1p Has Three Transmembrane Domains

Site-directed Mutagenesis—The lcb1 mutant alleles with charged residues for interaction with Lcb2p, these results, in combination with the finding that the carboxyl-terminal extension of Lcb1p is critical for interaction with Lcb2p, suggest that the structure of the SPT heterodimer may be more dissimilar from that of the homodimeric POAS family members than previously thought.

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two aliquots. One aliquot was treated with 5000 units of EndoH (New England Biolabs), and the other aliquot was incubated with buffer. Samples were incubated at 37 °C for 60 min, and proteins were resolved by NuPAGE gel electrophoresis (Invitrogen) and visualized by immunoblotting as described above.

Construction of LCB1-fXa Fusion Alleles—The LCB1 alleles containing factor Xa (fXa) cleavage sites were constructed by QuikChange mutagenesis. Pairs of mutagenic primers were designed to insert the sequence encoding a repeat of the fXa protease recognition site, IE-GRIEGR, after codons 4, 50, 279, 407, 416, 425, or 556 of the LCB1 gene. Each primer pair contained the sequence, GATATCGAAGGTCGAATTGAGGGACGG, flanked on either side by 18 nucleotides of homology to the LCB1 site into which the fXa cleavage site was to be inserted. An extra GAT codon on the 5′/H11032-end of the sequence encoding the fXa cleavage site was added to generate an EcoRV site that could be used to identify candidate mutants. LCB1-fXa fusion alleles were verified by DNA sequencing.

Cleavage with Factor Xa Protease—Preparation of microsomes for analyzing the fusion proteins containing the fXa cleavage site was performed essentially as described by Gilstring and Ljungdahl (18), with minor modifications. Cells were resuspended in spheroplasting buffer (20 mM Tris-HCl, pH 7.5, containing 1.2 M sorbitol, 50 mM potassium acetate, 1 mM /H9252-mercaptoethanol, and 10 mM sodium azide) at an A600 of 20. Spheroplasts were generated by treatment of the cells with 1.0 mg/ml zymolyase-100T (Seikagaku Corp., East Falmouth, MA) at 30 °C for 30 min. After centrifugation at 2000 g for 5 min at 4 °C, spheroplasts were resuspended at an A600 of 20 in lysis buffer (20 mM Tris-HCl, pH 7.5, containing 1.2 M sorbitol, 50 mM potassium acetate, 1 mM β-mercaptoethanol, and 10 mM sodium azide) at 30 °C for 30 min. After centrifugation at 2000 × g for 5 min at 4 °C, spheroplasts were resuspended at an A600 of 20 in lysis buffer (20 mM Tris-HCl, pH 7.5, containing 100 mM sorbitol, 50 mM potassium acetate, 1 mM β-mercaptoethanol, and 1 mM each of the protease inhibitors, phenylmethylsulfonyl fluoride, aprotinin, and leupeptin). Spheroplasts were lysed with 20 strokes of a Teflon pestle in a 5-ml tissue grinder followed by removal of unbroken cells by centrifugation at 10000 × g for 30 min at 4 °C. Membranes were washed once with storage buffer (20 mM Tris-HCl, pH 7.5, containing 100 mM sorbitol, 50 mM potassium acetate, and 1 mM β-mercaptoethanol), resuspended in storage buffer at

| LCB1 allele | Complements lcbΔ | Lcb1p stability | Stabilizes Lcb2p |
|-------------|------------------|-----------------|-----------------|
| Wild type   | Yes              | Stable          | Yes             |
| YLW (24,25,26) DRS | Yes              | Stable          | Yes             |
| YGI (66,67,68) DVK | Yes              | Stable          | Yes             |
| Δ4–50 (N-terminal ER domain) | Yes              | Stable          | Yes             |
| Δ50–85 (TMD 1) | Yes              | Stable          | Yes             |
| Δ9–85       | Yes              | Stable          | Yes             |
| Δ342–371 (TMD 2) | No               | Unstable        | No              |
| Δ371–386 (ER luminal loop) | No               | Stable          | No              |
| Δ386–416 (ER luminal loop) | No               | Unstable        | No              |
| Δ416–425(ER luminal loop) | No               | Stable          | No              |
| Δ433–458 (TMD 3) | No               | Unstable        | No              |
| IL (549,550) IASL* | Yes              | Stable          | Yes             |
| IL (549,550) AS | Yes              | Stable          | Partially       |
| IL (549,550) FPR | No               | Stable          | No              |
| IL (549,550) PR | No               | Stable          | Partially       |

* These mutated Lcb1ps have two residues inserted between 549 and 550.

**TABLE 1**

*Mutations in Yeast Lcb1p*

![Fig. 1](image-url) Sites of insertion of the GC cassettes into the yeast Lcb1p. An alignment of the mammalian (Cricetulus griseus, CHO), plant (Arabidopsis thaliana, AT), and yeast (Saccharomyces cerevisiae, SC) Lcb1ps was used to predict locations within the protein that might accommodate insertion of the GC without disrupting function. The sites into which the GC was inserted are indicated; for example, Δ4 indicates that the GC was inserted in-frame between amino acids 4 and 5 of Lcb1p. The locations of the three transmembrane domains (TMD) identified within the yeast protein are indicated by the bars.
4 mg/ml protein, and stored at −80 ºC. For assay of cleavage by FXa protease, 20 µg of microsomal protein was diluted with 20 µl of FXa buffer (20 mM Tris-HCl, pH 7.5, containing 250 mM sorbitol, 100 mM NaCl, and 1 mM EDTA). Samples were mock-digested or digested with 1.1 µg of factor Xa protease (Promega, Madison, WI) in the presence or absence of 0.2% Nonidet P-40 on ice for 3 h.

Construction of Amino-terminal Domain lcb1 Deletion Mutants with the GC at Codons 4, 416, or 425—To construct the lcb1Δ50–85 with the GC at codon 4, the SmaI fragment (extending from codon 36–221 bp downstream of the stop codon of lcb1Δ50) was isolated by gel electrophoresis from the plasmid with the GC at codon 4 and used to replace the corresponding fragment in the plasmid in which the GC had been inserted after codon 4. To construct lcb1Δ50–85 with the GC at codon 416 or at 425, a BstZ171/XbaI restriction fragment that extended from codon 144–470 bp downstream of the stop codon was generated from the plasmid with the GC at either 416 or 425 and was used to replace the corresponding fragment in the plasmid with the lcb1Δ50–85 deletion mutant allele.

All constructs were confirmed by DNA sequencing.

The CHO-SPTLC1 Deletion Mutant Alleles—The CHO-SPTLC1 gene was PCR-amplified by using pSV-clcB1 (20) (generous gift of Dr. Hanada) as template. The PCR primers, CHO1-UP, 5′-TTCGCCGGG-AATTCGTCGACATGGGCGGACCAGCATGCAAGAGCGTTCTGATCTT and its complement, CHO1-DOWN, 5′-TTCGCCGAATTCGTCGACCTAGTCACAGCAAGCAGCTGGCGGCGGCGTCGACTATG, were designed to generate a PCR fragment with multiple restriction sites on the ends. The coding sequence of CHO-SPTLC1 from the start codon through codon 7 is underlined in CHO1-UP, and the coding sequence for the last seven codons of CHO-SPTLC1 is underlined in CHO1-DOWN. Following restriction with EcoRI (site shown in boldface in CHO1-UP) and with XbaI (site shown in boldface in CHO1-down), the PCR fragment was ligated between the EcoRI and XbaI sites of pcDNA3.1(+) (Invitrogen).

For deletion of codons 3–45, primer D3F, 5′-GACCGCCTAGCTCA-GAATTGCCGGATATCTTGCAAGAGCGTTCTGATCTT and its complement, primer D3R, were used for QuikChange mutagenesis. The primer pair was designed to place an EcoRV site (underlined) at the deletion junction. The correct plasmid was identified by restriction mapping and confirmed by DNA sequencing.

CHO Culture and Transfection—The Chinese hamster ovary cell lines CHO-K1 and CHO-LY-B (RIKEN Bioresource Center, Japan) deficient in SPTLC1 expression (21) were maintained in Dulbecco’s modified Eagle’s medium containing 4.5 g/liter glucose (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS) and 2 mM glutamine (Biofluids, Camarillo, CA). Cells were plated in 10-cm tissue culture dishes and transfected at 60–80% confluency. Transfections were performed using Lipofectamine 2000 (Invitrogen) followed by the recommendations of the manufacturer and optimized for our conditions. A total of 20 µg of the pcDNA3.1(+) -based plasmids carrying the CHO-SPTLC2, CHO-SPTLC1, or the CHO-SPTLC1Δ50–85 genes and 20 µl of Lipofectamine 2000 were added to 4 ml of Opti-MEM I (Invitrogen) for each transfection. After 24 h transfection, the medium was replaced with Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum.

Microsome Preparation and Immunoblotting of CHO SPTLC1 and CHO SPTLC2—Cells were harvested and microsomes were prepared 48 h after transfection. The cells were washed with PBSSE (phosphate-buffered saline with 1 mM EGTA, 1 mM EDTA) and harvested by scraping into 4 ml of PBSSE buffer. The cells were transferred into 15-ml conical tubes and centrifuged at 1000 × g for 5 min. The cell pellets were resuspended in MEM buffer (20 mM Tris-HCl, pH 7.5, containing 1 mM EGTA, 1 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, 1 mM aprotinin, and 1 mM peptatin A). Following cell lysis by sonication (three 6-s pulses at a setting of 5), the lysate was centrifuged at 8000 × g for 10 min. Microsomes were recovered from the supernatant by centrifugation at 100,000 × g for 30 min, and the microsomal pellet was washed and resuspended in 100 µl MEM buffer containing 15% glycerol.

For detection of CHO SPTLC1, 2 µg of microsomal protein were resolved on 4–12% Bis-Tris NuPAGE and transferred to nitrocellulose as described above. A monoclonal anti-CHO SPTLC1 antibody (BD Biosciences) was used at a dilution of 1/12,000. A horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad) was used as the secondary antibody, and detection was with an enhanced chemiluminescence kit (Amersham Biosciences). For detection of CHO SPTLC2, 5 µg of microsomal protein were resolved by NuPAGE, transferred to nitrocellulose, and visualized using rabbit anti-CHO SPTLC2 anti-peptide antibodies raised against two combined peptides, CGKYSRHRHVLPLLDRPF (residues 538–552) and CGDRPFDETTYEETED (residues 548–561) of SPTLC2; these peptides had been used previously for generating anti-SPTLC2 antibodies by Uhlinger et al. (22). Antisera were prepared by Sigma Genoress (The Woodlands, TX), and specific antibodies were purified by affinity chromatography using peptides coupled to CNBr-activated Sepharose 6MB (Amersham Biosciences). Anti-Lcb2p antibodies were used at a dilution of 1/5,000, and a horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) was used as the secondary antibody. Detection was as for CHO-SPTLC1.

SPT Assays—Microsomal protein was purified as described above. SPT was assayed as described previously (19) except that 0.2 µg of microsomal protein and 0.05 mM palmitoyl-CoA were used. Background incorporation of [1-3H]serine was measured without the addition of palmitoyl-CoA and subtracted. Each assay was conducted in triplicate, and the average SPT activity was reported.

**RESULTS**

**Hydropathic Profile Analysis Is Not Informative for Predicting the Topology of Lcb1p**—The Lcb1p subunit of serine palmitoyltransferase (SPT) is a 558-amino acid protein. Although Lcb1p has biochemical properties consistent with it being an integral membrane protein, various algorithms for predicting membrane-spanning segments suggest vastly different topologies for the yeast Lcb1p protein; hydropathic profile analyses predict anywhere from 0 to 5 potential membrane-spanning domains in the protein (Table II). The inability of these analyses to predict a single model for the topology of mammalian SPTLC1 was noted previously and motivated a study of the topology of the mammalian protein by Hanada and co-workers (16), who demonstrated that the amino- and carboxyl-terminal ends of the mammalian SPTLC1 are on opposite sides of the membrane. Based on these results, they suggested that there is a single membrane-spanning domain composed of the hydrophobic domain in the amino terminus of the protein (16).

**The Amino-terminal Hydrophobic Domain of the Yeast Lcb1p Protein Is Not Essential for Function**—Unlike the case for mammalian SPTLC1, three of the hydropathy analyses predicted two membrane-spanning segments in the amino terminus of the yeast Lcb1p, located from residues 10–30 and from 50–70 (Table II). To investigate the role of these hydrophobic domains, two mutant Lcb1pS with charged amino acids substituted for hydrophobic ones, YLV 24–26 DRS and YGI 66–68 DVK, were generated and tested for their ability to restore...
growth to an lcb1Δ mutant unable to grow in the absence of exogenously provided PHS (Table I). Both mutant Lcb1p proteins supported PHS-independent growth, demonstrating that the introduction of charged residues into either of the potential membrane-spanning segments did not abolish Lcb1p function (Table I). In addition, mutants in which either one or both putative membrane-spanning domains were deleted also complemented the lcb1Δ mutant (Table I), demonstrating that the amino-terminal domain is not required for SPT function.

Immunoblot analysis after crude cellular fractionation demonstrated the ∆4–50, the ∆50–85, and the ∆9–85 mutant Lcb1p proteins were all membrane-associated (Fig. 2A). This was confirmed by fluorescence microscopy of cells containing wild-type Lcb1p or any of the three deletion mutants tagged with GFP in their carboxyl termini; all four proteins complemented the lcb1Δ mutant (data not shown), and all three amino-terminal deletion mutants displayed nuclear-peripheral ER fluorescence similar to that seen for wild-type Lcb1p (Fig. 2B). Thus, the amino-terminal domain of yeast Lcb1p is not required for its correct membrane localization, indicating that other regions of the protein contain the information necessary to specify its localization. In addition, immunoblot analysis of Lcb2p revealed that its membrane localization and stability were not affected by the deletion of the amino-terminal domain of Lcb1p (Fig. 2A). This result indicates that not only is the localization of Lcb1p independent of its amino-terminal domain but that its association with Lcb2p is similarly independent. Thus, the localization and topology of Lcb1p are most likely influenced by other regions of the protein.

**Construction of Lcb1p Topology Reporter Proteins**—To experimentally determine the topology of the yeast Lcb1p protein, a set of fusion proteins containing a glycosylation reporter cassette (GC) inserted in-frame at 17 positions along the length of Lcb1p was constructed. The cassette consists of a 53-amino acid domain comprising residues 80–133 of invertebrate Suc2p that contains three NX(S/T) sites for asparagine-linked glycosylation. These sites are known to be glycosylated in the mature Suc2p protein. In addition, the first and last NX(S/T) glycosylation acceptor sites are located far enough from the amino- and carboxyl-terminal ends of the cassette, respectively, 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 efficiently recognized by the glycosylation machinery (18). The locations within Lcb1p into which the GC was inserted were chosen based on their ability to distinguish the various models predicted by the hydrophathy analyses, and the likelihood that insertion of a 53-amino peptide would not disrupt protein function. The latter consideration was based on alignment of Lcb1p proteins from different species to identify potentially flexible sites with low conservation across evolution, and where there are extra amino acids in the yeast Lcb1p (Fig. 1). Although insertion of a 53-amino glycosylation domain might be expected to interfere with the function of the Lcb1p protein or alter its topology, this reporter cassette has been used in determining the topology of other membrane proteins (18), and its insertion into the luminal loops between membrane-spanning domains has not been observed to interfere with their insertion or orientation.

Immunoblot analysis demonstrated that all 17 Lcb1p-Suc2p fusion proteins were stably expressed in yeast cells (Fig. 3). In addition, two functional properties of the fusion proteins were used to assess whether they accurately report the native Lcb1p topology. Initially, each fusion protein was tested for its ability to support the growth of the lcb1Δ mutant in the absence of PHS. Eleven of the 17 fusion proteins supported such growth and were therefore assumed to be catalytically active and to have been inserted in their native topology (Fig. 3). Based on our previous demonstration that the stability of Lcb2p is dependent on its association with Lcb1p (29), the ability of each Lcb1p-Suc2p fusion protein to heterodimerize with and thereby stabilize Lcb2p was also tested. As expected, immunoblot analysis of Lcb2p expression revealed that all of the Lcb1p-Suc2p fusion proteins that complemented the lcb1Δ mutant also stabilized Lcb2p (Fig. 3). In addition, the noncomplementing Lcb1p-Suc2p fusion proteins with the GC cassette inserted after residues 342, 371, and 416 appeared to partially stabilize the Lcb2p subunit, indicating that although these proteins are not catalytically active, they can form heterodimers with Lcb2p and therefore also appear to adopt their native topology.

**Analysis of the GC-tagged Lcb1p Fusion Proteins Reveals Additional Membrane-associated Domains in Lcb1p**—Support for a Three-membrane-spanning Model—The mobilities of Lcb1p-Suc2p fusion proteins in which the GC was inserted into the amino-terminal domain were reduced after treatment with EndoH, demonstrating that in these proteins the GC cassette...
had been glycosylated. Furthermore, the Lcb1-Suc2p was not glycosylated when the GC was inserted after residue 84. These results are consistent with those reported previously for mammalian Lcb1p and localize the yeast amino-terminal membrane-spanning domain between residues 50 and 84. The mobilities of the Lcb1p-Suc2p fusion proteins in which the GC cassette was inserted after residues 371, 386, 407, 416, and 425 were also reduced after EndoH treatment (Fig. 3), showing that these GCs had also been glycosylated. Thus, Lcb1p appears to contain two additional membrane-spanning domains, located between residues 342 and 371 and between residues 425 and 457, that allow the placement of a second, centrally located region of Lcb1p in the lumen of the ER (Fig. 1). This interpretation is consistent with the prediction of both TMPred and MEMSAT, although each of these programs predicted at least one additional membrane-spanning domain not identified by insertion of GCs.

Curiously, the insertion of the 53-amino acid GC cassette at different positions along the length of Lcb1p had different effects on the mobility of the fusion protein (Fig. 3). We have observed this phenomenon previously when inserting the HA epitope tag at different positions along the protein. Nevertheless, the differences in electrophoretic mobility between different Lcb1-Suc2p fusion proteins did not complicate the interpretation of these experiments.

An Alternative Method for Assigning Topology Is Consistent with the Three Membrane-spanning Model for Lcb1p—To confirm the presence of the additional membrane-associated domains in Lcb1p, tandem factor Xa (fXa) protease cleavage sites (IEGRIGERG) were inserted at several diagnostic positions along Lcb1p (24), and their accessibility to fXa protease, which cleaves proteins containing the IEGR tetrapeptide on the carboxy-terminal side of the arginine, in sealed right side-out membrane vesicles was assessed. A tandem recognition sequence was inserted to increase the probability of cleavage.

Lcb1p-fXa fusion proteins were expressed in lcb1Δ mutant cells, sealed right side-out vesicles were prepared, and the sensitivity to fXa protease cleavage was assayed in the absence or presence of Nonidet P-40 (Fig. 4). The luminal location of the amino-terminal region of Lcb1p was confirmed by the detergent dependence of fXa protease cleavage when the cleavage site was inserted after residue 50 (Fig. 4); virtually no cleavage was observed in the absence of Nonidet P-40, a result that also demonstrates that the vesicles were right side out and sealed. Given its proximity to the amino terminus of the protease, there was no observable change in mobility after fXa protease cleavage when the fXa cleavage site was inserted after residue 4 (Fig. 4). However, when the fXa cleavage site was inserted at position 4 in an amino-terminally HA-tagged Lcb1p, fXa treatment released the HA tag only after treatment of microsomes with Nonidet P-40, providing additional evidence that residue 4 is in the lumen of the ER (data not shown).

In contrast, when the fXa cleavage site was inserted at position 279 in Lcb1p, it was equally accessible to the protease in the presence or absence of detergent. This result is entirely consistent with the lack of glycosylation of the GC cassette inserted at the same position and confirms the cytoplasmic orientation of this region of the protein. Most important, when the fXa cleavage site was inserted after residue 425, it was found to be resistant to cleavage in the absence of detergent, showing fXa protease does not have access to this domain of Lcb1p in intact right side-out vesicles. This confirms the presence of an additional luminal domain in Lcb1p. However, when fXa cleavage sites were inserted after positions 407 and 416, the fusion proteins were refractory to cleavage, even in the presence of detergent. Thus, as has been reported for yeast Sec61p, not every site where a fXa cleavage site is inserted is protease-sensitive (24). Nevertheless, there is no discordance between the two methods of analysis, and the results of both are most consistent with the presence of three membrane-spanning domains in Lcb1p.

**Lcb1p Topology Is Not Affected by Phytosphingosine**—The experiments described above were performed using lcb1Δ mutant cells grown in medium supplemented with 15 μM PHS. Because the topology of some membrane proteins can be affected by membrane lipid composition (25), it was necessary to demonstrate that this addition had no effect on the topology of Lcb1p. Accordingly, cells expressing each of the 11 Lcb1p-Suc2p fusion proteins that complemented the lcb1Δ mutation were grown in the presence or absence of PHS, and the glycosylation status of the GC was determined. The results showed that growth in PHS did not affect the glycosylation status of the insert (a subset is shown in Fig. 5A). Thus, the topology of Lcb1p is not influenced by the presence of PHS in the growth medium.

**Lcb1p Adopts Its Native Topology in the Absence of Either Lcb2p or Tsc3p**—We have shown previously that Lcb2p is unstable in the absence of Lcb1p and that this effect is not the result of an altered rate of LCB2 transcription (19). Conversely, Lcb1p stability is unaffected by the absence of Lcb2p. However, it has not been determined whether the correct localization and topology of Lcb1p are dependent upon the presence of Lcb2p. To address this question, Lcb1-Suc2p fusions were expressed in lcb1Δ/lcb2Δ double mutants lacking both native SPT subunits, and the glycosylation status of the inserts was determined. In every case examined, the Lcb1-Suc2p fusion proteins that were glycosylated in the presence of Lcb2p were also found to be glycosylated in the absence of Lcb2p (Fig. 5B).

A third protein, Tsc3p, associated with the Lcb1p-Lcb2p heterodimer and that stimulates its activity severalfold has also been identified (19). It was therefore of interest to determine whether Tsc3p influences the topology of Lcb1p. Accordingly, Lcb1-Suc2p fusion proteins were also expressed in a mutant that lacked Tsc3p and analyzed for glycosylation status. The data indicated that Tsc3p does not influence the topology of Lcb1p (Fig. 5C). Although there appears to be reduced glycosylation of the GCs in the amino-terminal domain of Lcb1p in cells lacking either Lcb2p or Tsc3p, taken as a whole these results suggest that the information necessary for the native topology of Lcb1p resides solely within Lcb1p.

**Formation of the Central Luminal Domain Is Independent of the Amino-terminal Hydrophobic Domain**—To determine which regions of Lcb1p were necessary and/or sufficient to form
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Identification of Regions of Lcb1p That Influence the Stability of Lcb2p—The data presented in Fig. 3 indicate that insertion of GCs at some positions in Lcb1p results in loss of Lcb2p stability, presumably as a result of perturbation of the interaction between the two subunits. Specifically, insertions after residues 120, 239, 342, 416, and 549 resulted in the nearly complete loss of Lcb2p immunoreactivity in microsomal membranes. Destabilization of Lcb2p by insertions at 120, 239, and 342 was not surprising given our assignment of this region of Lcb1p to the cytosol where the active site of SPT is believed to reside. However, it was surprising that the insertion at 416 abolished association with Lcb2p because the results obtained with GC and Xa cleavage site insertion suggest that this region of Lcb1p is in the lumen of the ER (Figs. 3 and 4). To further investigate the role of the central luminal domain of Lcb1p in the stabilization of Lcb2p, deletion mutants that eliminated either end as well as the central portion of this domain were constructed, and the cells expressing these mutants were analyzed for membrane-associated Lcb2p immunoreactivity (Table I). The results showed that although both the Lcb1pΔ371–386 and the Lcb1pΔ416–425 deletion mutant proteins were stable, they failed to stabilize Lcb2p (Table I). These data provide further evidence that the centrally located luminal domain of Lcb1p is important for interaction with and stabilization of Lcb2p.

It was also surprising that insertion of the GC after residue 549 resulted in destabilization of Lcb2p (Fig. 3), because this portion of Lcb1p is in a region of the protein for which there is no counterpart in the other POAS family members. To investigate whether this is a trivial consequence of the bulky insertion, or whether it reflects an important site of interaction between the two proteins, we analyzed several COOH-terminal missense and insertion mutations. Specifically, Lcb1p mutants with the Ile/Leu residues at 549/550 changed to either Ala/Ser (IL → AS) or to Pro/Arg (IL → PR), and those with an AS or PR insertion between residues 549 and 550, IL → IASL or IL → IPRL, were evaluated for their effect on the stability and function of Lcb1p and on the stability of Lcb2p (Table I). Insertion of the AS dipeptide between residues 549 and 550 had virtually no effect on the growth of cells containing this mutation (Fig. 7) or the stability of the Lcb1p and Lcb2p proteins (Fig. 7). However, replacing the IL dipeptide with AS resulted in marked reduction of growth of an lcb1Δ mutant at 37 °C. In addition, immunoblot analysis revealed that in cells expressing this mutant, Lcb2p was unstable (Fig. 7). Insertion of the dipeptide PR between residues 549 and 550, or creation of the double mutant I549P/L550R, also resulted in Lcb1p proteins that failed to stabilize Lcb2p and had even greater effects on cell growth, completely abolishing growth of an lcb1Δ mutant at temperatures above 22 °C. As it seems unlikely that the 2-amino acid substitutions or insertions into the cytosolic tail would alter the membrane topology of Lcb1p, these data suggest that destabilization of Lcb2p by the insertion of the 53 residue GC between residues 549 and 550 is not the result of the bulky substitution but rather may reflect a specific interaction of the carboxyl-terminal tail of Lcb1p with Lcb2p.

The Amino-terminal Membrane-spanning Domain of CHO SPTLC1 Is Not Required for Stability, Membrane Association, or Stabilization of CHO SPTLC2—It was proposed previously that the CHO SPTLC1 contains a single membrane-spanning domain located in the amino-terminal region of the protein (16). Given the evidence that this region of the yeast protein is...
not essential for enzymatic activity, membrane localization, or stabilization of Lcb2p, it was therefore of interest to evaluate the role of the amino-terminal region of the mammalian protein. Accordingly, CHO-K1 cells were transfected with a gene encoding either the wild-type CHO SPTLC1 protein or a deletion mutant lacking the putative amino-terminal membrane-spanning domain, with or without co-transfection of a gene encoding SPTLC2 (Fig. 8, lanes 1–6). As reported previously, immunoblot analysis with anti-SPTLC1 and anti-SPTLC2 antibodies demonstrated that in the absence of transfected SPTLC1, transfection of the SPTLC2 gene did not result in stable overexpression of the SPTLC2 protein (16) (Fig. 8A, lanes 3 and 4). However, overexpression of either full-length CHO SPTLC1 or the Δ3–45 mutant was able to stabilize the expression of SPTLC2 (Fig. 8A, lanes 4 and 6), suggesting that the amino-terminal membrane-spanning domain is not involved in stabilization of SPTLC2. The same result was obtained when SPTLC1 and SPTLC2 were transfected into CHO LY-B cells, which lack SPT activity and detectable expression of the endogenous SPTLC1 gene (16, 21), thus eliminating the possibility that the stabilization of SPTLC2 in cells co-transfected with CHO SPTLC1Δ3–45 is the result of low level expression of the endogenous wild-type protein (Fig. 8A, lanes 9 and 10). Most importantly, measurement of SPT activity in microsomes prepared from CHO LY-B cells transiently transfected with either the wild-type SPTLC1 gene (Fig. 8B, lane 2) or the SPTLC1Δ3–45 mutant gene (Fig. 8B, lane 4) revealed that both proteins were equally effective in reconstituting enzymatic activity. Indeed, SPT activity in microsomes prepared from cells expressing either form of SPTLC1 was ~40% of that seen in CHO-K1 wild-type cells (Fig. 8B, lane 5). Given that transient transfection is not 100% efficient, these results therefore suggest that the amino-terminal membrane-spanning domain of yeast Lcb1p, the amino-terminal domain of the mammalian protein is not essential for protein localization or enzymatic activity.

DISCUSSION

The functional organization of the yeast Lcb1p subunit of SPT and its interaction with the Lcb2p subunit were investigated. Deletion mutants were used to study the role of the amino-terminal region of the protein in membrane localization, enzymatic activity, and stabilization of its heterodimeric partner, Lcb2p. Insertion of either a 53-amino acid glycosylation cassette from invertase (Suc2p), containing three potential glycosylation sites, or a dimer of the 4-amino acid factor Xa protease cleavage sequence was used to probe the transmembrane topology of the protein. Our results are most consistent with a model in which yeast Lcb1p contains three membrane-spanning domains resulting in the localization of the amino terminus, and the region of protein between residues 371 and 425 in the lumen of the ER. In addition, they suggest that both the central luminal domain and the carboxyl-terminal cytoplasmic domain are important for stabilization of Lcb2p and formation of an active enzymatic complex.

A variety of programs designed to identify potential transmembrane regions, as well as analysis of the mammalian
SPTLC1 protein, suggest the presence of a membrane-spanning domain in the amino-terminal region of the protein. Based on these results, and the absence of an amino-terminal hydrophobic extension in other homodimeric members of the POAS family that are soluble, it seemed reasonable to hypothesize that the amino-terminal domain of Lcb1p was responsible for localizing the protein to the ER. This was supported by analysis of Lcb1p-Suc2p fusion proteins in which a 53-amino acid glycosylation cassette was inserted after residues 4, 50, or 84. Insertions at the first two positions were found to glycosylated when expressed in yeast cells, whereas the insert after residue 84 was not, providing strong evidence that there is a membrane-spanning domain between residues 57 and 73. However, immunoblot analysis of microsomal membranes prepared from cells expressing mutants lacking either the amino terminus of the protein, the putative membrane-spanning domain, or both regions showed the same membrane localization as full-length protein. In addition, direct examination of cells expressing GFP-tagged Lcb1p mutants demonstrated the same pattern of nuclear- peripheral ER fluorescence as wild-type protein, and all of the mutants were capable of complementing an lcb1Δ mutant. Thus, it seems unlikely that the amino terminus of the protein or amino-terminal membrane-spanning domain contains all of the information responsible for targeting Lcb1p to the membrane.

The presence of two additional membrane-spanning domains, located between residues 343 and 371 and residues 425 and 457, was inferred from analysis of Lcb1p-Suc2p fusion proteins. Insertion of the Suc2p glycosylation sequence at any of five positions between residues 371 and 426 resulted in EndoH-sensitive glycosylation of the chimeric protein, suggesting that this region of the protein is in the lumen of the ER. One possible explanation for this result is that although this region of Lcb1p is not normally in the lumen of the ER, insertion of the 53-amino acid GC increases its partitioning into the lumen. Several lines of evidence suggest that this is not the case. First, three of the insertions did not disrupt the ability of the fusion protein to complement an lcb1Δ mutant, demonstrating that they retain enzymatic activity. In addition, each of these fusion proteins stabilized Lcb2p expression. It seems unlikely that an insertion capable of substantially altering the topology of Lcb1p would leave these functions intact. Most importantly, however, is the result obtained when a factor Xa protease cleavage site was inserted in the putative centrally located luminal domain; the cleavage site at position 425 was only protease-sensitive after detergent treatment of right side-out vesicles. This result confirms that this region of the protein is in the lumen of the ER.

Each of the programs used to predict potential membrane-spanning domains identified TMD1. In contrast, there was no consensus prediction for additional membrane-spanning domains between residues 345 and 454. However, three of the programs predicted a membrane-spanning domain between residues 349 and 373 of the yeast protein. These programs also predicted a membrane-spanning domain between residues 377 and 397. Although both regions are capable of forming α-helices, the glycosylation of GCs inserted after residues 377 and 397 and the fact that Lcb1pA3432–371 is unstable, whereas Lcb1pA371–386 is stable, suggest that TMD2 is located between residues 345 and 367. The position of TMD3 was inferred from the glycosylation of the GC inserted after residue 425, but not of the GC inserted after residue 457, and was predicted by two of the three programs that correctly identified TMD2.

The presence of additional membrane-spanning domains in the central region of yeast Lcb1p was unexpected, in part because the three-dimensional structure of AONS, another member of the POAS family, predicts that residues in this region of Lcb1p should be involved in the formation of the heterodimeric interface with Lcb2p and therefore located in the cytosol. However, whereas AONS is a head to tail homodimer containing two catalytic sites, the fact that the critical lysine residue is conserved in only the Lcb2p subunit has led to the presumption that SPT has only one catalytic site. The residue in Lcb1p corresponding to Lys-236 in AONS is Thr-356, which we predict is in TMD2. Indeed, although there is substantial homology between AONS and Lcb2p in the regions surrounding the critical lysine residue, there is far less homology with Lcb1p. Thus, it is not clear how much of the structural information determined for AONS can be extended to Lcb1p or the Lcb1p/Lcb2p heterodimer.

The ability to associate with the membrane and the formation of the central luminal loop appear to be independent of information in the amino-terminal membrane-spanning domain, because its deletion has no effect on the association of Lcb1p with the membrane or the glycosylation of GCs inserted after residues 416 or 424. However, insertion of a GC after residue 549 resulted in a protein that did not complement an lcb1Δ mutant and did not stabilize Lcb2p. This was not simply the result of a bulky insertion because insertion of the dipeptide PR between 549 and 550 or even the mutation of IL (549, 550) to PR or AS had essentially the same effect. The fact that the mutated Lcb1p proteins are stable but fail to stabilize Lcb2p strongly suggest that the carboxyl terminus of Lcb1p plays an important role in forming the Lcb1p/Lcb2p heterodimer. Moreover, these results provide additional justification for reevaluating the structural similarity between AONS and SPT.

It has been suggested previously that CHO SPTLC1 has a single amino-terminally located membrane-spanning domain. This hypothesis was based on the demonstration that the amino terminus was in the ER lumen, whereas the carboxyl terminus was cytosolic. Whereas hydrophathy analyses predicted the possibility of additional membrane-spanning domains, their presence was discounted because they were in regions of the protein expected to be involved in heterodimerization with SPTLC2 (16). The data presented here show that the amino-terminal hydrophobic domain of CHO SPTLC1 that corresponds to TMD1 of yeast Lcb1p is not required for membrane association, stabilization of CHO SPTLC2, or enzymatic activity. Thus, it is possible that the information required for correct localization of the mammalian protein is contained in other regions of the protein. Indeed, the sequence of the luminal loop identified in yeast Lcb1p is conserved in both the mammalian and plant homologs. Although there are significant differences between the overall sequences of the yeast and mammalian proteins, it is possible that mammalian SPTLC1 also contains additional membrane-spanning domains that form a centrally located luminal loop. Further experiments will be required to address this possibility.

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