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Mutations in the Yeast *LCB1* and *LCB2* Genes, Including Those Corresponding to the Hereditary Sensory Neuropathy Type I Mutations, Dominantly Inactivate Serine Palmitoyltransferase

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It was recently demonstrated that mutations in the human SPTLC1 gene, encoding the Leb1p subunit of serine palmitoyltransferase (SPT), cause hereditary sensory neuropathy type I (1, 2). As a member of the subfamily of pyridoxal 5'-phosphate enzymes known as the α-oxoamine synthases, serine palmitoyltransferase catalyzes the committed step of sphingolipid synthesis. The residues that are mutated to cause hereditary sensory neuropathy type I reside in a highly conserved region of Lcb1p that is predicted to be a catalytic domain of Lcb1p on the basis of alignments with other members of the α-oxoamine synthase family. We found that the corresponding mutations in the LCB1 gene of Saccharomyces cerevisiae reduce serine palmitoyltransferase activity. These mutations are dominant and decrease serine palmitoyltransferase activity by 50% when the wild-type and mutant LCB1 alleles are coexpressed. We also show that serine palmitoyltransferase is an Lcb1p-Lcb2p heterodimer and that the mutated Lcb1p proteins retain their ability to interact with Lcb2p. Modeling studies suggest that serine palmitoyltransferase is likely to have a single active site that lies at the Lcb1p-Lcb2p interface and that the mutations in Lcb1p reside near the lysine in Lcb2p that is expected to form the Schiff’s base with the pyridoxal 5'-phosphate cofactor. Furthermore, mutations in this lysine and in a histidine residue that is also predicted to be important for pyridoxal 5'-phosphate binding to Lcb2p also dominantly inactivate SPT similar to the hereditary sensory neuropathy type 1-like mutations in Lcb1p.

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 (3). In addition, sphingolipid metabolites are regulatory molecules for a variety of cellular processes (4–6). These bioactive sphingolipid metabolites are formed as intermediates during both the synthesis and the breakdown of the complex sphingolipids. Many human diseases that result from defects in sphingolipid catabolism have been discovered (7), but examples of defects in sphingolipid biosynthesis that cause diseases have been lacking. However, it was recently reported that hereditary sensory neuropathy type 1 (HSN1), the most common inherited peripheral neuropathy, results from mutations in the Leb1p subunit of serine palmitoyltransferase (SPT) (1, 2).

SPT catalyzes the committed step in the synthesis of sphingolipids, the condensation of serine with palmitoyl CoA (see Fig. 1 below). It is a member of the subfamily of pyridoxal 5'-phosphate (PLP) enzymes known as the α-oxoamine synthases that all catalyze the condensation of a carboxylic acid CoA thioester with the α-carbon of an amino acid. The other α-oxoamine synthases are 8-amino-7-oxononanoate synthase (AONS), which catalyzes the first step of biotin synthesis in Escherichia coli (8), 5-amino-5-deoxyhexose synthase (ALAS), which catalyzes the synthesis of 5-amino-5-deoxyhexose during heme biosynthesis (9), and 2-amino-3-ketobutyrate CoA ligase, which is involved in threonine degradation (10). The LCB1 and LCB2 genes, found to be required for SPT activity using genetic screens in Saccharomyces cerevisiae (11–14), encode proteins homologous to the α-oxoamine synthases. Homologs of the LCB1 and LCB2 genes from higher eukaryotes have been identified based on their similarity to the S. cerevisiae LCB genes (15).

The crystal structure of the AONS enzyme reveals that several functionally important residues, including those that are involved in PLP binding, are highly conserved in the α-oxoamine synthases (16, 17). Although Leb1p and Lcb2p are homologous to each other and to AONS (see Fig. 2A below), many of these conserved residues are present in Lcb2p (for example, Lys-366, which is predicted to form a Schiff’s base with PLP), but not in Leb1p. Interestingly, a soluble Lcb2p homodimeric form of SPT was recently characterized from the lipid-producing bacterium Sphingomonas paucimobilis (18). However, in eukaryotes both proteins are required for SPT activity. Thus unlike the other α-oxoamine synthases that are soluble homodimers, the eukaryotic SPT enzyme is membrane-associated, and enzyme activity requires both the Leb1p and the Lcb2p subunit. In comparison to the other members of the family, Leb1p and Lcb2p also have amino- and carboxyl-terminal extensions that may be important for membrane association.
The dominant negative mutants in the Lcb1p and Lcb2p subunits of SPT

The human homolog of the LCB1 gene, SPTLC1, is mutated at cysteine 133 (C133W or C133Y) or at valine 144 (V144D) in HSN1 patients (1, 2). Because the HSN1 mutations reside in a region of Lcb1p that has been conserved throughout evolution (Fig. 2) (13, 15), we characterized the corresponding mutations in S. cerevisiae to gain insight into how the HSN1 mutations in Lcb1p might affect SPT activity. In the studies reported here, we show that the HSN1 mutations in yeast Lcb1p dominantly inactivate SPT. We also demonstrate that SPT is an Lcb1p-Lcb2p heterodimer. On the basis of modeling studies, we predicted that the mutated cysteine in Lcb1p resides in close proximity to residues in Lcb2p that are involved in PLP binding. We mutated two residues in Lcb2p that are predicted to be important for PLP binding and found that these mutant proteins also dominantly inactivate SPT.

EXPERIMENTAL PROCEDURES

Yeast Methods—Yeast cells were grown according to standard procedures (19). The wild-type yeast strain was TDY2037 (Mata uro3-52 leu2 trp1 lys2) and its complement. Following centrifugation at 4 °C, the beads were washed three times, and the immunoprecipitated protein was subjected to 8% SDS-PAGE electrophoresis. The methods used for immunoblotting and for detection of Lcb1p and Lcb2p antibodies were previously described (20).

RESULTS

The HSN1 Mutations in Yeast Lcb1p Dominantly Inactivate SPT—The LCB1 gene is essential for viability, but lcb1Δ yeast cells will grow if phytosphingosine (PHS) is provided in the medium (Fig. 1). To determine the effect of the HSN1 mutations on SPT activity, we made the corresponding mutations (C180W, C180Y, and V191D) in the yeast LCB1 gene (Fig. 2). The HSN1-LCB1 alleles did not complement the PHS-requiring phenotype of the lcb1Δ mutant. Transformants carrying centromeric plasmids harboring the LCB1-C180W allele were unable to grow without PHS under any conditions, whereas those carrying the LCB1-C180Y or the LCB1-V191D alleles grew poorly at 26 °C and failed to grow at 37 °C (data not shown). Based on prior characterization of the growth phenotypes of SPT mutants (20–22) these results indicated that the HSN1 mutations reduced SPT activity. Assays of microsomal SPT activity confirmed this conclusion (Fig. 3A), because there was no detectable SPT activity in microsomes prepared from the LCB1-C180W and LCB1-C180Y mutant cells and about 10% of wild-type SPT activity in microsomes prepared from LCB1-V191D cells. These results demonstrated that the HSN1 mutations in Lcb1p severely compromise SPT activity in yeast. The yeast HSN1-LCB1 alleles are loss-of-function mutations. Assuming that the corresponding mutations also inactivate human SPT, the dominant HSN1 disorder could result from haploinsufficiency or the mutations could be dominant-negative. We tested whether the HSN1-LCB1 alleles were dominant by introducing them into haploid yeast cells. An LCB1+ allele was borne on a 20% gradient gel. Molecular markers were used for the SPT assay. Cells lacking the CSG2 gene failed to mannosylate inositolphosphorylceramide (IPC) and therefore accumulate high levels of IPC (Fig. 1), which leads to Ca2+ sensitivity (12). Mutations that reduce IPC, including those that reduce SPT activity, suppress the Ca2+ sensitivity of the csg2 mutant (12, 20–22). Introduction of the plasmid-borne LCB1-C180W, LCB1-C180Y, or LCB1-V191D allele into the LCB1+ csg2Δ haploid strain suppressed the Ca2+ sensitivity indicating that the mutant alleles dominantly inactivated SPT activity in vivo (Fig. 4A). Microsomal SPT activity measurements confirmed this, revealing a 2-fold reduction in enzymatic activity (Fig. 3B). These experiments demonstrated that the HSN1-LCB1 alleles were dominant inactivating mutations that reduced in vitro SPT activity about 50% when they were coexpressed with a wild-type LCB1 allele, and that they also reduced in vivo SPT activity.

SPT Is an Lcb1p-Lcb2p Heterodimer—We next investigated the subunit structure of SPT. As mentioned above, the other \( \alpha \)-oxoamine synthases, including a bacterial SPT enzyme (18), are soluble homodimers. However, eukaryotic SPT is a membrane-associated enzyme comprised of two homologous subunits with molecular weights of 57,000...
Dominant Negative Mutations in the Lcb1p and Lcb2p Subunits of SPT

The sphingolipid synthesis pathway in yeast. Serine palmitoyltransferase (SPT) catalyzes the decarboxylative condensation of serine with palmitoyl CoA to form 3-KS in the first step of the long-chain-base synthesis pathway. 3-KS is reduced to DHS by Tsc10p (22) and DHS is hydroxylated to PHS by Sur2p (28,29). Addition of long-chain-base synthesis pathway. 3-KS is reduced to DHS by Tsc10p

Mutations in the Predicted PLP-binding Residues of Lcb2p—On the basis of the crystal structure of AONS and the alignment between AONS and Lcb1p, the residue in AONS Gly-74 that is analogous to Cys-180 of yeast (Cys-133 of human) Lcb1p resides at the interface of the PLP-binding site at the interface between the monomeric structure of AONS and the alignment between AONS and Lcb1p. This was confirmed by showing that the mutant Lcb1p proteins coimmunoprecipitate with Lcb2p (data not shown).

The HSN1 Lcb1p Mutant Proteins Are Stable and Heterodimerize with Lcb2p—We found that the HSN1 mutant Lcb1p proteins were stable (Fig. 6A, lanes 3–5). Furthermore, although Lcb2p is unstable in the absence of Lcb1p (20) (Fig. 6A, lane 1), Lcb2p is present in each of the mutants indicating that the mutant Lcb1p proteins form stable heterodimers with Lcb2p. This was confirmed by showing that the mutant Lcb1p proteins coimmunoprecipitate with Lcb2p (data not shown).

Mutations in the Predicted PLP-binding Residues of Lcb2p Also Dominantly Inactivate SPT—On the basis of the crystal structure of AONS and the alignment between AONS and Lcb1p, the residue in AONS Gly-74 that is analogous to Cys-133 of human Lcb1p resides at the interface between the subunits of the AONS homodimer. It is in close proximity to residues in the opposite subunit that are involved in catalysis, including the lysine residue of AONS that forms the Schiff's base with PLP (Fig. 2B). This suggested that the mutations in Lcb1p might be affecting PLP binding. Indeed, Alexeev and coworkers (17) noted that mutations in this region of AONS might alter the dimer structure and affect the geometry of the PLP binding site at the interface between the monomers to produce a loss of enzymatic activity. The AONS-V85 (Lcb1p-V191) residue is further from the interface, but mutations at this site may also alter the geometry of the PLP binding site by a long-range perturbation.

By analogy to AONS, two residues in Lcb2p (Lys-366 and His-334) that are predicted to participate in PLP binding are expected to lie across the subunit interface from Cys-180 of Lcb1p. This is shown in a model of the central catalytic domain of the Lcb1p-Lcb2p heterodimer that was generated using the crystal coordinates of the highly homologous AONS enzyme (Fig. 2C). The Lys-366 residue of Lcb2p (predicted to form the Schiff's base with PLP) was replaced with threonine and His...
FIG. 2. Location of the dominant inactivating mutations in the Lcb1p and Lcb2p subunits of SPT. A, the yeast and human Lcb1p and Lcb2p each consist of variable amino- and carboxyl-terminal sequences flanking a central domain that is conserved among the α-oxoamine synthases. A BOXSHADE alignment (generated using the output from ClustalW) of the central domain of the human and yeast Lcb1p and Lcb2p proteins with the bacterial AONS protein (the only α-oxoamine synthase for which a crystal structure is available (17)) is shown. The numbers on the left indicate the amino acid positions, the dark boxes indicate identical residues, and the shaded boxes represent conserved residues. The asterisks mark the positions of the mutations (C180W, C180Y, and V191D) in the yeast Lcb1p, and the triangles mark the positions of the mutations (H334F and K366T) in the yeast Lcb2p. B, the symmetrical AONS homodimer backbone (17) is shown with the residues of AONS (Gly-74 and Val-85) that correspond to the mutations in yeast Lcb1p (Cys-180 and Val-191) and the residues of AONS (His-207 and Lys-236) that correspond to the mutations in yeast Lcb2p (His-334 and Lys-366) indicated by space-filling models. The two identical monomers are distinguished by the line thickness of the backbone. C, a backbone model for the structure of the Lcb1p-Lcb2p heterodimer is presented with the positions of the dominant inactivating mutations in Lcb1p (Cys-180 and Val-191) and in Lcb2p (His-334 and Lys-366) indicated by space-filling models. The alignment of Lcb1p and Lcb2p with AONS (Fig. 1A) was used to build a three-dimensional model of the SPT heterodimer by fitting the Lcb1p and Lcb2p sequences to the known three-dimensional structure of AONS (17) using Modeler (33). The stereo three-dimensional structures were drawn using Rasmol (34). Heavy lines represent two or more consecutive residues that are conserved between the yeast and human Lcb1p and Lcb2p proteins.
334 (predicted to hydrogen-bond with O3' of PLP) was replaced with phenylalanine, because these are the corresponding amino acids in Lcb1p (Fig. 2A). We found that these mutations in Lcb2p also dominantly inactivated SPT activity similar to the HSN1-like mutations in Lcb1p. Centromeric plasmids carrying either the LCB2-K366T or the LCB2-H334F allele failed to rescue the PHS-requiring phenotype of an lcb2/H9004 mutant (data not shown). Assays of the microsomal SPT activity from these haploids confirmed that the mutations in LCB2 abolished SPT activity (Fig. 3C). Introduction of the mutant LCB2 alleles into a haploid csg2ΔLcb1pLcb2p haploid strain, and the purified transformants were streaked onto YPD plates ± 50 mM CaCl2. The plates were incubated at 26 °C for 3 days.

Therefore, substitutions in the Lcb2p subunit of SPT that may disrupt PLP binding, but do not eliminate heterodimer formation, were also found to be dominant inactivating mutations.

**DISCUSSION**

These studies clearly demonstrate that the HSN1-like substitutions in yeast Lcb1p catalytically inactivate SPT. Furthermore, mutations in predicted catalytic residues of the Lcb2p subunit that conferred similar phenotypes were also identified. The mutant Lcb1p and Lcb2p proteins retained their ability to associate with their partners to form catalytically inactive heterodimers. This suggests that there is no mechanism to upregulate LCB1 and LCB2 expression when SPT activity is low, because increased expression of the proteins would be expected to restore 100% SPT activity even if half of the heterodimers were inactive. However, it should be pointed out that it is not known whether the levels of Lcb1p and/or Lcb2p normally limit...
SPT activity. If a third protein limits SPT activity, then the dominant inactivation could result from its binding to the catalytically inactive heterodimers making it unavailable to optimally activate the functional heterodimers. In yeast the recently discovered Tsc3p protein that associates with Lcb1p-Lcb2p and stimulates SPT activity severalfold (20) is a candidate for a third protein that might limit SPT activity.

The Lcb1p subunit lacks several of the catalytic residues that are conserved among the other \( \alpha \)-oxoamine synthases, and this has led to the notion that Lcb1p might function exclusively as a regulatory subunit. On the other hand, it is well established that eukaryotic cells that lack Lcb1p have no SPT activity (24).

The recent identification of a bacterial SPT enzyme that is an Lcb2p homodimer (18) raised further questions about the role of Lcb1p and about the oligomeric structure of the eukaryotic enzyme. Although mammalian SPT was shown to consist of Lcb1p and Lcb2p in a 1:1 stoichiometry (23), it remained possible that the oligomeric structure of the enzyme was higher order, for example a tetramer of Lcb2p and Lcb1p homodimers. The experiments presented here demonstrate that the native yeast enzyme is a heterodimer.

In the AONS homodimer there are two symmetrical active sites that lie at the interface between the two monomers. In particular, the signature lysine-containing PLP-binding domain of one AONS monomer lies across the interface from the glycine-rich domain of the other monomer at each symmetrical active site. Because Lcb2p has the lysine-containing predicted PLP-binding domain whereas Lcb1p does not, it is likely that the Lcb1p-Lcb2p heterodimer has a single active site. Modeling of the central catalytic domain of the Lcb1p-Lcb2p heterodimer suggests that the HSN1 substitutions in Lcb1p lie across the subunit interface from predicted PLP-binding residues of Lcb2p and may affect PLP binding. The cysteine residue lies in the conserved glycine-rich sequence that has been extensively mutagenized in the ALAS enzyme. On the basis of these studies, Ferreira and coworkers (25) concluded that this region of the ALAS enzyme was important for PLP binding and catalysis. As mentioned above, the crystal structure of AONS also provided evidence that this region is important in PLP binding (12). If, as the modeling studies suggest, the active site of SPT lies at the interface between the Lcb1p and Lcb2p subunits with residues in Lcb1p influencing PLP binding, then Lcb1p is not simply a regulatory subunit.

Our characterization of the mutant proteins shows that the mutations do not grossly perturb the structures of the Lcb1p or Lcb2p subunits. The proteins are stable and retain their association with their partners. Bejaoui et al. (1) pointed out that the cysteine residue in human Lcb1p is in a potential consensus site for N-myristoylation and raised the possibility that the HSN1 mutations disrupt membrane association. However, this cysteine residue of yeast Lcb1p is not required for membrane association, because the mutant Lcb1p protein retains its association with the membranes. It would be surprising if the mutations in human Lcb1p were affecting membrane association, because they lie in the central domain of Lcb1p within a region that is conserved with the other soluble \( \alpha \)-oxoamine synthases. Although this cysteine residue appears to be important for catalysis, it is unlikely that the sulfhydryl group participates directly in the catalytic chemistry, because the corresponding residue is a glycine in both AONS and ALAS.

That the HSN1-like mutations in yeast Lcb1p are dominant inactivating mutations raises the possibility that the pathology associated with the HSN1 neuropathy might result from reduced rather than increased SPT activity. Because HSN1 is a dominant disease and lymphocytes from patients displayed increased levels of glucosylceramides, it was suggested that the mutations activate SPT and thereby increase ceramide, which in turn triggers apoptosis (2). Although it is possible that the HSN1 mutations have opposite effects on yeast and human SPT activity, it seems unlikely, because the residues that are altered are in a particularly well-conserved region of Lcb1p and in a region of the protein that is known to be important for catalysis in other \( \alpha \)-oxoamine synthases. It will be important to directly test the effect of expression of the HSN1-mutant Lcb1p proteins on SPT activity in mammalian cells to resolve this issue. If the HSN1 mutations do prove to dominantly inactivate mammalian SPT, the increased glucosylceramides could reflect...
an up-regulation of the glucosyltransferases in response to reduced SPT activity.

All eukaryotic cells have sphingolipids, although the abundance and types of sphingolipids vary in different cell types. There is apparently a single LCB1 gene in humans. Therefore, regardless of whether the human mutations increase or decrease SPT activity, the HSN1 patients could have abnormal SPT activity in all their cells. However, little is known about the levels of SPT activity in different tissues and about how the enzyme is regulated. It may be that optimal SPT activity is not required for normal development and function of all types of cells and tissues. For example, the SPT deficiency may be most acute in neuronal cells, because they are especially dependent on normal sphingolipid synthesis. Furthermore, it remains to be determined whether the pathology of HSN1 results from altered lipid composition of the neuronal membranes that renders them more susceptible to damage, or whether the altered sphingolipid synthesis leads to an aberrant signaling process. It will be of interest to compare SPT activity and the lipid composition in various tissues from unaffected and HSN1-affected individuals.

HSN1 is genetically heterogeneous with at least two loci (26, 27), and studies are underway to determine whether HSN1 families that do not have mutations in LCB1 have mutations in LCB2 (2). Based on the results reported here, mutations that catalytically inactivate Lcb2p without compromising its ability to heterodimerize with Lcb1p, similar to those identified in this study, are likely to be found in HSN1 patients. The potential to alter SPT activity using the dominant mutant alleles of LCB1 and LCB2 should be useful for probing the functions of sphingolipids in higher eukaryotes. Studies in cultured cells and transgenic animals will provide the opportunity to establish how altered SPT activity influences the neural degeneration that accompanies HSN1.

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