2009

Identification of small subunits of mammalian serine palmitoyltransferase that confer distinct acyl-CoA substrate specificities

Gongshe Han
Uniformed Services University of the Health Sciences

Sita D. Gupta
Uniformed Services University of the Health Sciences

Kenneth Gable
Uniformed Services University of the Health Sciences

Somashekarappa Niranjanakumari
Uniformed Services University of the Health Sciences

Prasun Moitra
Uniformed Services University of the Health Sciences

See next page for additional authors

Identification of small subunits of mammalian serine palmitoyltransferase that confer distinct acyl-CoA substrate specificities

Gongshe Han[a], Sita D. Gupta[a], Kenneth Gable[a], Somashekarappa Niranjanakumari[a], Prasun Moitra[b], Florian Eichler[c], Robert H. Brown, Jr.[d], Jeffrey M. Harmon[b], and Teresa M. Dunn[a,1]

Departments of [a]Biochemistry and Molecular Biology and [b]Pharmacology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814; [c]Massachusetts General Hospital Neuroscience Center, Department of Neurology, Harvard Medical School, 55 Fruit Street, Boston, MA 02114; and [d]Department of Neurology, University of Massachusetts Medical School, Lake Avenue North, Worcester, MA 01655

Edited by Alfred H. Merrill, Jr., Georgia Institute of Technology, Atlanta, GA, and accepted by the Editorial Board April 8, 2009 (received for review November 6, 2008)

Serine palmitoyltransferase (SPT) catalyzes the first committed step in sphingolipid biosynthesis. In yeast, SPT is composed of a heterodimer of 2 highly-related subunits, Lcb1p and Lcb2p, and a third subunit, Tsc3p, which increases enzyme activity markedly and is required for growth at elevated temperatures. Higher eukaryotic orthologs of Lcb1p and Lcb2p have been identified, but SPT activity is not highly correlated with coexpression of these subunits and no ortholog of Tsc3p has been identified. Here, we report the discovery of 2 proteins, ssSPTa and ssSPTb, which despite sharing no homology with Tsc3p, each substantially enhance the activity of mammalian SPT expressed in either yeast or mammalian cells and therefore define an evolutionarily conserved family of low molecular weight proteins that confer full enzyme activity. The 2 ssSPT isoforms share a conserved hydrophobic central domain predicted to reside in the membrane, and each interacts with both hLCB1 and hLCB2 as assessed by positive split ubiquitin 2-hybrid analysis. The presence of these small subunits, along with 2 hLCB2 isofoms, suggests that there are 4 distinct human SPT isozymes. When each SPT isozyme was expressed in either yeast or CHO LyB cells lacking endogenous SPT activity, characterization of their in vitro enzymatic activities, and long-chain base (LCB) profiling revealed differences in acyl-CoA preference that offer a potential explanation for the observed diversity of LCB seen in mammalian cells.

long-chain bases | sphingolipids | HSAN1 | ssSPT

Originally thought to be composed of a single subunit, genetic analysis in yeast showed that eukaryotic serine palmitoyltransferase (SPT) is a heterodimer of 2 highly-related subunits, Lcb1p and Lcb2p (1–3), associated with a third small subunit, Tsc3p, that is required for maximal enzyme activity (4). Based on the crystal structure of 8-amino-7-oxononanoate synthase (5), and the identification of key catalytic residues in other α-oxoamine synthases, we proposed that SPT contains a single cytoplasmic catalytic site at the interface of a head-to-tail heterodimer of Lcb1p and Lcb2p comprised of residues from both subunits (6). This model is supported by the recently-reported crystal structure of the homodimeric prokaryotic SPT (7). A second Lcb2p-like subunit has been identified in both *Arabidopsis* and mammalian cells (8, 9). In each case, the resulting LCB1–LCB2 heterodimers appear to catalyze the same reaction. Thus, their roles are unclear. However, whereas in *Arabidopsis* the 2 isoforms are functionally redundant (8), mouse SPTLC2 knockouts are embryonic lethal (10), suggesting that in mammalian cells the 2 LCB2 isoforms may have distinct functions.

We have shown that transfection of mammalian cells with the human LCB1 ortholog (SPTLC1) and the LCB2-like isoform, SPTLC2, produces only a modest increase in SPT activity, despite high levels of protein expression (11). The same is true for cotransfection of SPTLC1 and the other LCB2-like isoform, SPTLC3 (9). This finding suggests that, like the situation in yeast, an additional factor is required for full activity of mammalian SPT. However, homology searches identified no higher eukaryotic homologues of Tsc3p. A genetic screen was therefore devised to search for additional mammalian subunits. We report here the identification of 2 such subunits, named ssSPTa and ssSPTb, for small subunits of SPT. The discovery of these components of the committed and rate-limiting enzyme of sphingolipid synthesis provides important mechanistic insights into the biochemical basis of sphingolipid complexity.

Results

Identification of Stimulatory SPT Subunits. Because SPTLC1 is a functional ortholog of yeast Lcb1p, and SPTLC2 and SPTLC3 are orthologous to yeast Lcb2p, we hereafter refer to SPTLC1 as hLCB1, SPTLC2 as hLCB2a, and SPTLC3 as hLCB2b. To screen for additional subunits of mammalian SPT that confer increased activity, we took advantage of the fact that hLCB1–hLCB2 heterodimers support limited growth at 26 °C of a yeast *lcb1*Δ*lcb2*Δ mutant lacking endogenous SPT, but no growth at 37 °C (Fig. 1*A Top*). In contrast, the hLCB1–hLCB2b heterodimer barely supported growth even at 26 °C (Fig. 1*B Top*), without the addition of phytosphingosine (PHS), which bypasses the need for endogenous SPT. Because the hLCB1–hLCB2a heterodimer more strongly complemented the PHS requirement than the hLCB1–hLCB2b heterodimer, the yeast *lcb1*Δ*lcb2*Δ mutant expressing this heterodimer was used to screen for human Tsc3p orthologs by transforming a human cDNA expression library into this strain and selecting colonies that grew at 37 °C. From this screen, c14orf147 was identified. This gene encodes a 71-aa protein with no homology to Tsc3p (Fig. 2*A*) that allowed the hLCB1–hLCB2a heterodimer to support growth of the yeast *lcb1*Δ*lcb2*Δ mutant at 37 °C (Fig. 1*A Middle*; ssSPTa). This protein also allowed the hLCB1–hLCB2b heterodimer to support growth of cells expressing this isoform at both 26 °C and 37 °C (Fig. 1*B Middle*; ssSPTa) without the need for PHS.

To determine whether ssSPTa would enhance the activity of an hLCB1–hLCB2 heterodimer expressed in mammalian cells, it was expressed alone or in combination with the other SPT subunits in LyB cells that lack endogenous LCB1 and have negligible SPT activity (12). The results of these experiments show that c14orf147 (ssSPTa) was stably expressed in the

Author contributions: G.H., S.D.G., K.G., S.N., R.H.B., J.M.H., and T.M.D. designed research; G.H., S.D.G., K.G., S.N., and P.M. performed research; P.M. contributed new reagents/analytic tools; G.H., S.D.G., K.G., S.N., F.E., R.H.B., J.M.H., and T.M.D. analyzed data; and J.M.H. and T.M.D. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. A.H.M. is a guest editor invited by the Editorial Board.

[1]To whom correspondence should be addressed. E-mail: tdunn@usuhs.mil.

www.pnas.org/cgi/doi/10.1073/pnas.0811269106

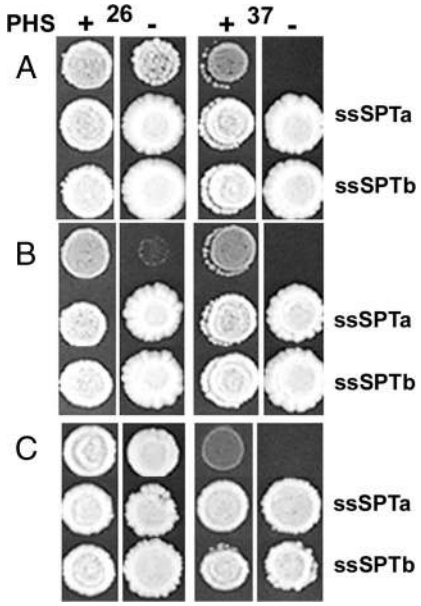

Fig. 1. ssSPTa and ssSPTb enhance the growth of yeast cells expressing human and yeast SPT heterodimers. Yeast *lcb1*Δ*/lcb2*Δ mutant cells (TDY8055) expressing hLCB1–hLCB2a (*A*), hLCB1–hLCB2b (*B*), or *tsc3–2* mutant cells, which lack Tsc3p but contain yeast Lcb1–Lcb2p (*C*), with empty vector (*Top*), ssSPTa (*Middle*), or ssSPTb (*Bottom*) were transferred to yeast extract/peptone/dextrose plates with or without PHS and incubated at 26 °C or 37 °C.

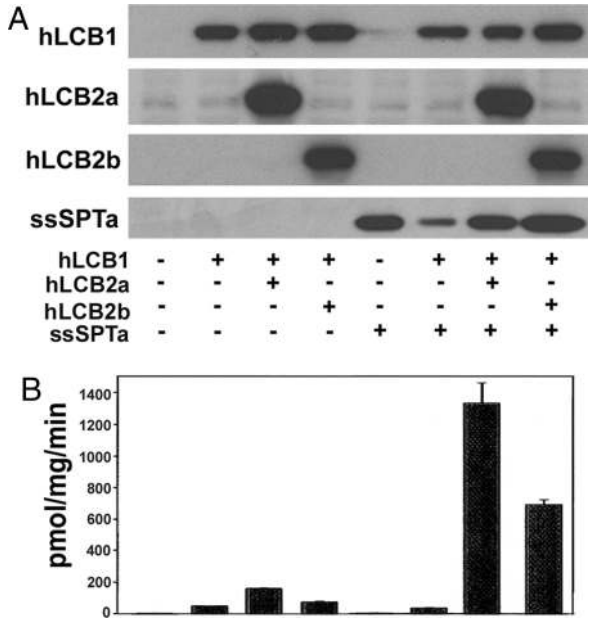

Fig. 3. ssSPTa stimulates SPT activity in mammalian cells. CHO-LyB cells were transfected with plasmids expressing hLCB1, hLCB2a, hLCB2b, or HA-tagged ssSPTa as indicated. Microsomal protein was prepared from the transfected cells. (*A*) Expression of each protein was analyzed by immunoblotting using antipeptide antibodies for detecting hLCB1, hLCB2a, and hLCB2b and anti-HA antibodies for detecting HA-ssSPTa. (*B*) SPT activity was determined as described in *Materials and Methods*.

presence or absence of any other SPT subunit and that, like Tsc3p, is present in the microsomal fraction (Fig. 3*A*). More significantly, coexpression of ssSPTa with either the hLCB1–hLCB2a or hLCB1–hLCB2b heterodimer increased in vitro activity in microsomes prepared from transfected cells by ≈10-fold (Fig. 3*B*). These results suggest that endogenous levels of ssSPTa limit SPT activity, thus providing an explanation for the lack of a simple correlation between levels of hLCB1 and hLCB2a/hLCB2b expression and SPT activity (11).

A second gene, identified as androgen-down-regulated in mouse prostate (ADMP) (13), ADMP/c3orf57, encodes a 76-aa protein with high homology (45% identity, 68% similarity) to ssSPTa. Because ADMP is both structurally and functionally related to ssSPTa, it is hereafter referred to as ssSPTb. Both proteins contain 2 predicted membrane-spanning domains and are highly conserved throughout evolution (Fig. 2 *B* and *C*). However, there are distinct differences, particularly in their C termini; the C-terminal ends of the ssSPTa family members are highly conserved and different from the less highly-conserved C-terminal ends of the ssSPTb family members. Despite these differences, ssSPTb also enhances growth of *lcb1*Δ *lcb2*Δ mutant yeast cells expressing either hLCB1–hLCB2a or hLCB1–hLCB2b heterodimers (Fig. 1 *A* and *B Bottom*; ssSPTb). Most remarkably, although neither ssSPT isoform is homologous to yeast Tsc3p or any other proteins in *Saccharomyces cerevisiae*, both reverse the temperature sensitivity of a *tsc3* mutant (Fig. 1*C*), demonstrating that although they are unrelated evolution-

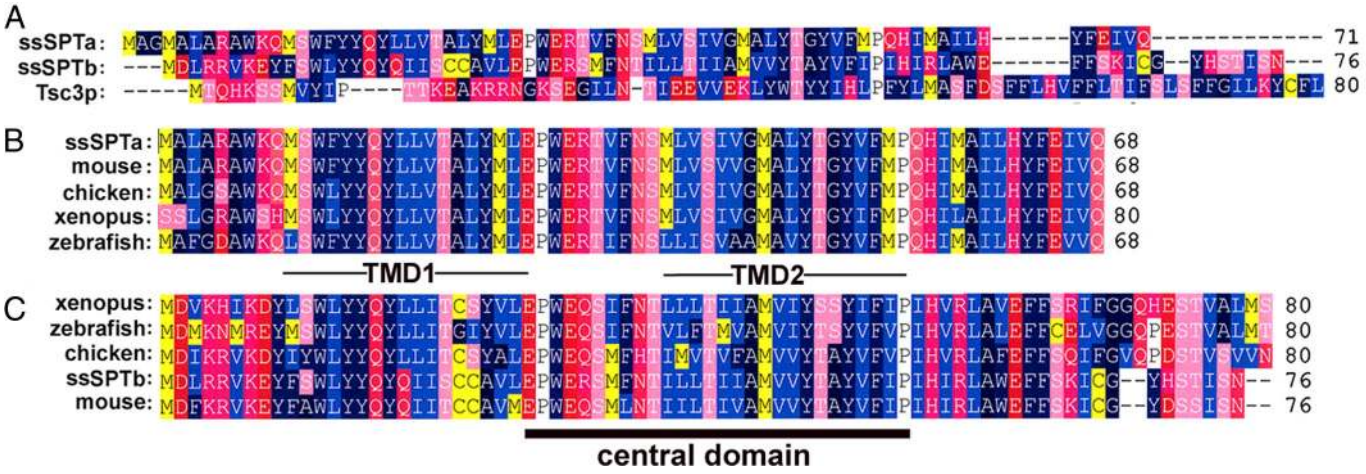

Fig. 2. *ssSPTa* and *ssSPTb* define a new gene family. (*A*) The ssSPT proteins are homologous to each other but share no homology with yeast Tsc3p. (*B* and *C*) The ssSPTa (*B*) and ssSPTb (*C*) subfamilies contain a highly-conserved central domain that extends from just after the first predicted membrane spanning domain (TMD1) through TMD2.

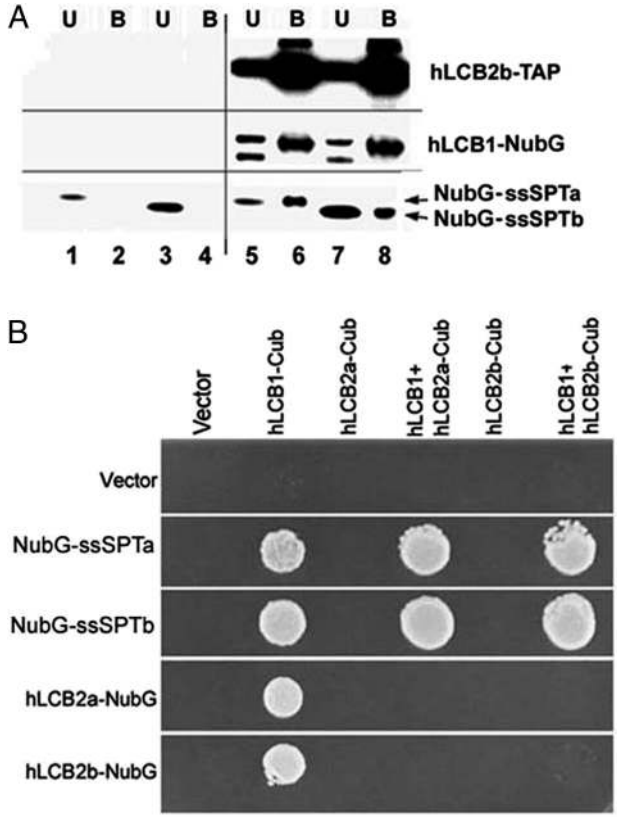

Fig. 4. Interactions between human SPT subunits. (*A*) Solubilized yeast microsomes prepared from cells expressing hLCB2b–TAP, hLCB1-HA–NubG and NubG-HA–ssSPTa (lanes 5 and 6) or NubG-HA–ssSPTb (lanes 7 and 8) or cells expressing only NubG-HA–ssSPTa (lanes 1 and 2) or NubG-HA–ssSPTb (lanes 3 and 4) were incubated with IgG-conjugated Sepharose, and unbound (U) and bound (B) proteins were detected by immunoblotting. The smaller of the 2 hLCB1-HA–NubG bands seen in the unbound lanes appears to be a proteolytic fragment not present in the holoenzyme. (*B*) Split-ubiquitin 2-hybrid interactions between hLCB1 and the other SPT subunits were identified by using hLCB1–Cub and C-terminally Nub-tagged hLCB2 isoforms or N-terminally Nub-tagged ssSPT isoforms. Interactions between hLCB2 isoforms and ssSPT isoforms were determined by using N-terminally Cub-tagged hLCB2s and N-terminally Nub-tagged ssSPTs in the presence or absence of untagged hLCB1. Potential interactions between hLCB2 isoforms were investigated by using C-terminally Nub-tagged and N-terminally Cub-tagged proteins. Positive interactions were detected by growth on medium lacking histidine and containing 50 mM aminotriazole.

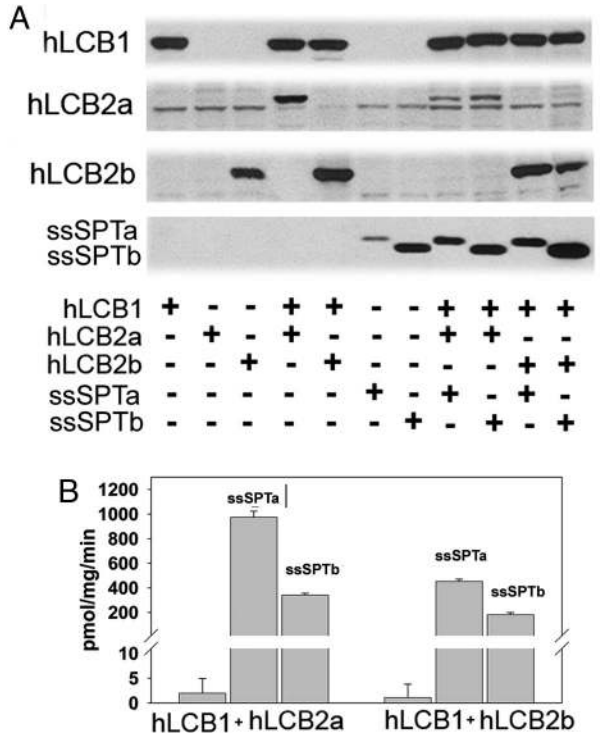

Fig. 5. Reconstitution of human SPT activity in yeast. Human SPT subunits were expressed in *lcb1*Δ*lcb2*Δ double mutant yeast cells in various combinations as indicated. Protein expression was verified by immunoblotting as in Fig. 3 (*A*) and microsomal SPT activity assayed with [^{3}H]serine and palmitoyl-CoA (*B*).

arily to Tsc3p, they serve a common function and potentially recognize a common structural feature in the yeast and mammalian heterodimers.

Analysis of SPT Subunit Interactions. The results described above suggest that both ssSPTa and ssSPTb interact with canonical SPT heterodimers. This idea was confirmed by showing that hLCB1–HA-NubG and NubG-HA-tagged ssSPTa/ssSPTb copurified with hLCB2b-TAP (Fig. 4*A*) when adsorbed to IgG Sepharose. These results do not address the stoichiometry of the complex because the HA epitopes in hLCB1–HA-NubG and NubG-HA–ssSPTa/b may not be equally accessible and the 2 proteins may transfer with different efficiencies. To explore subunit interactions further, a split ubiquitin 2-hybrid system was used (14). As expected, the hLCB1–C-terminal domain of ubiquitin (Cub) showed a positive interaction with either hLCB2a–NubG or hLCB2b–NubG (Fig. 4*B*). hLCB1–Cub also showed a positive interaction with NubG–ssSPTa and NubG–ssSPTb. Thus, these proteins interact directly with hLCB1, even in the absence of an hLCB2 subunit. Neither NubG–ssSPTa nor NubG–ssSPTb appeared to interact directly with hLCB2a–Cub or hLCB2b–Cub unless hLCB1 was present. However, these results need to be interpreted with care, because hLCB2a cannot be stably expressed in the absence of hLCB1 (Fig. 5*A*) and hLCB2b–GFP showed aberrant localization in the absence of hLCB1 in both yeast and mammalian cells. Nonetheless, it is clear that when coexpressed with hLCB1, the Cub domains of the hLCB2–Cub proteins interact with the NubG domains of the NubG–ssSPT proteins (Fig. 4*B*). Combined with the interaction between hLCB1–Cub and either NubG–ssSPT isoform, these data strongly suggest that the N termini of the ssSPTs are in close proximity to the C-terminal ends of both hLCB1 and the hLCB2s when the latter are present as heterodimers.

The 2-hybrid analysis also allowed us to investigate the reported presence of higher-order complexes containing both hLCB2 isoforms (15). Examination of cells expressing hLCB1, hLCB2a–Cub, and hLCB2b–NubG or hLCB1, hLCB2b–Cub, and hLCB2a–NubG failed to provide any evidence for an interaction between the C-terminal ends of hLCB2a and hLCB2b. Nor, in the presence of hLCB1, was there any interaction of hLCB2a–Cub with hLCB2a–NubG or hLCB2b–Cub with hLCB2b–NubG (Fig. 4*B*). Thus, if higher-order complexes exist, their organization is such that the C-terminal ends of the 2 hLCB2 isoforms are not topologically positioned to report a 2-hybrid interaction.

Both ssSPT Orthologs Activate the Human SPT Heterodimeric Isoforms in Yeast. Transient transfection of LyB cells with ssSPTa stimulated SPT activity ≈10-fold (Fig. 3). However, in these experiments only a fraction of the cells express all 3 proteins, and endogenous levels of the ssSPT subunits are unknown. The ability to reconstitute mammalian SPT in yeast afforded the

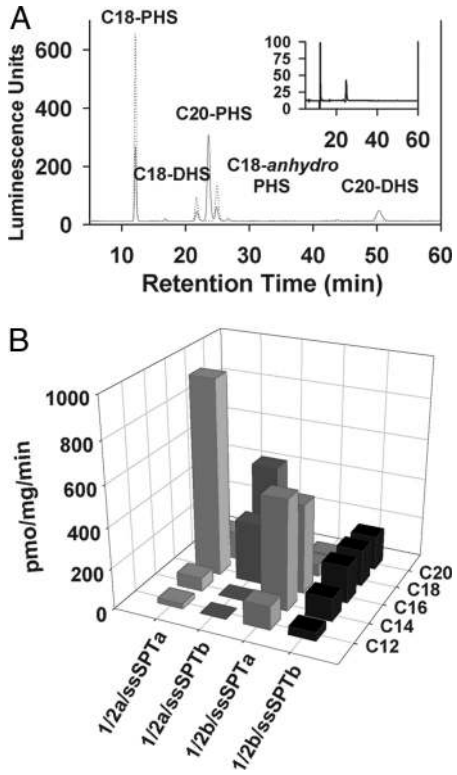

Fig. 6. Human SPT isozymes synthesize different LCBs. (*A*) LCBs were extracted from yeast expressing the hLCB1–hLCB2a heterodimer alone (*Inset*) or coexpressed with ssSPTa (dotted line) or ssSPTb (solid line) and analyzed by HPLC. Note that cells expressing only the heterodimer (*Inset*) accumulate significantly less LCBs than those coexpressing either ssSPTa or ssSPTb. (*B*) SPT activity was measured in microsomes prepared from yeast expressing each of the 4 isozymes using [^{3}H]serine and acyl-CoAs of the indicated (C12–C20) chain lengths.

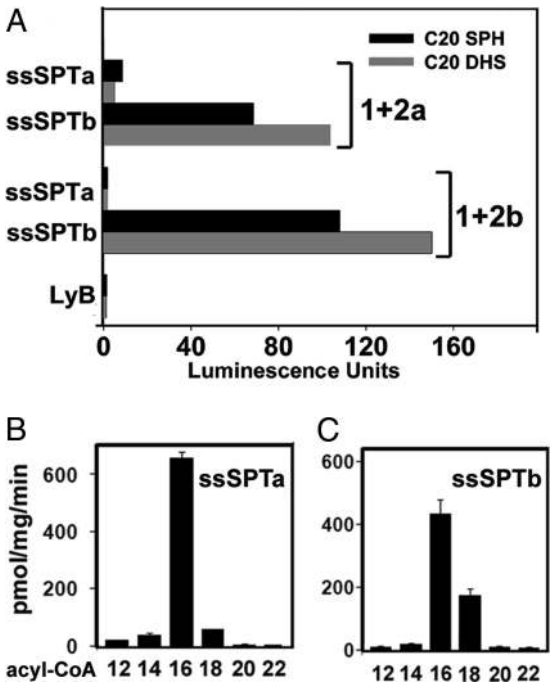

Fig. 7. Expression of ssSPTb-containing isozymes in CHO LyB cells increases C20–LCBs. (*A*) LCBs were extracted from LyB cells expressing each of the 4 human SPT isozymes and levels of C20–SPH and C20–DHS were quantified. (*B* and *C*) Microsomes were prepared from LyB cells expressing hLCB1 and hLCB2a with either ssSPTa (*B*) or ssSPTb (*C*), and SPT activity was measured by using [^{3}H]serine and acyl-CoAs of the indicated (C12–C22) chain lengths.

opportunity to examine each potential multimeric SPT complex in the absence of any endogenous SPT subunits or activity. All 5 human SPT subunits could be stably expressed in yeast, although as has been reported (11, 16), hLCB2a expression required coexpression of hLCB1 (Fig. 5*A*). Consistent with the results in Fig. 1, both the hLCB1–hLCB2a and the hLCB1–hLCB2b heterodimers had extremely low basal SPT activity (Fig. 5*B*). In contrast, when expressed with the ssSPTs, all 4 isozymes had dramatically higher SPT activity, with the hLCB1–hLCB2a–ssSPTa combination being the most active. Although the basal activity of the hLCB1–hLCB2b heterodimer was lower, ssSPTa stimulated it to the same extent as it did the hLCB1–hLCB2a heterodimer. These experiments also demonstrate that ssSPTb increases the activity of both heterodimers, albeit to a lesser extent.

Four SPT Isozymes Have Distinct Acyl-CoA Preferences. Surprisingly, the LCB profiles of yeast cells expressing each of the human SPT isozymes differed, most strikingly in that cells expressing ssSPTb accumulate significant levels of C20-PHS and C20-dihydrosphinganine (DHS) (Fig. 6*A*, solid line), not seen in cells expressing ssSPTa (Fig. 6*A*, dotted line). The profiles shown are for the hLCB1–hLCB2a heterodimer, but a similar result was seen for the hLCB1–hLCB2b heterodimer. This observation, along with the presence of structurally diverse LCBs predicted to be derived from acyl-CoAs other than palmitoyl-CoA in mammalian cells (17), and our previous discovery that a viral fusion SPT prefers myristoyl-CoA as substrate (18), suggested that the different isozymes have different acyl-CoA preferences. This hypothesis was confirmed by assaying each of the SPT isozymes with a panel of acyl-CoA substrates. The most active and selective isozyme was hLCB1–hLCB2a–ssSPTa showing a strong preference for C16–CoA (Fig. 6*B*). In contrast, the hLCB1–hLCB2b–ssSPTa isozyme used C14–CoA as well, if not slightly better than, C16–CoA (Fig. 6*B*). Remarkably, isozymes containing the ssSPTb subunit preferred longer acyl-CoAs. The hLCB1–hLCB2a–ssSPTb enzyme showed a clear preference for C18–CoA, whereas the hLCB1–hLCB2b–ssSPTb multimer displayed an ability to use a broader range of acyl-CoAs, without apparent preference (Fig. 6*B*). Despite their different acyl-CoA preferences, the K_M for serine for each of the 4 isozymes was ≈1–2 mM when assayed with the preferred acyl-CoA acceptor. These results suggest that a heretofore-unappreciated diversity of SPT enzymes is responsible for the synthesis of a complex family of LCBs with potentially unique biological properties.

The ability of the ssSPT subunits to modulate the chain lengths of LCBs in mammalian cells was investigated by comparing the amounts of C20–LCBs in LyB cells expressing the 4 combinations of SPT subunits. The results showed that, as observed in yeast (Fig. 6*A*), coexpression of ssSPTa with either the hLCB1–LCB2a or hLCB1–LCB2b heterodimer did not produce significant amounts of either C20–DHS or C20–sphingosine (SPH). In contrast, coexpression of either heterodimer with ssSPTb resulted in a substantial increase in the levels of both C20–DHS and C20–SPH (Fig. 7*A*). This difference was also seen in in vitro assays using microsomes prepared from LyB cells transfected with hLCB1, hLCB2a, and either ssSPT isoform. Microsomes prepared from cells transfected with ssSPTa were highly selective for C16–CoA; little activity was observed with shorter or longer acyl-CoA substrates. However, microsomes prepared from cells transfected with ssSPTb had substantial activity when C18–CoA was presented as the substrate (Fig. 7*B*). In these experiments it is important to note that

BIOCHEMISTRY

SPT multimers containing endogenous ssSPTs contribute to overall activities measured. Thus, although the results with ssSPTb appear less dramatic than those seen in yeast (Fig. 6*B*), they almost certainly underestimate the selectivity conferred by the ssSPTb isoform. In any case, they clearly confirm that the ssSPT subunits alter the acyl-CoA selectivity of SPT in mammalian cells.

Discussion

Although coexpression of hLCB1 with either hLCB2 subunit in mammalian cells increases SPT activity, the increased activity does not correlate well with the levels of subunit expression (11). In addition, we have recently coexpressed the LCB1 and LCB2 subunits from a variety of higher eukaryotes in *lcb1*Δ *lcb2*Δ yeast and found none conferred activity comparable to that measured in the organisms of origin (8, 19). Thus, it seemed reasonable to expect that an additional component is required to achieve optimal SPT activity. Indeed, we discovered such a component, Tsc3p, in yeast (4). However, homology searches failed to identify candidates from higher eukaryotes, necessitating the use of a functional screening assay for their identification. This screen allowed us to identify 2 short membrane proteins that, by a variety of criteria, appear to be the functional orthologs of yeast Tsc3p. Although SPT activity is apparently not highly regulated, a variety of stimuli have been reported to modulate activity a few fold without altering the levels of the LCB1 or LCB2 mRNAs or proteins (20–22). While it is possible that the ssSPTs contribute to regulation of enzyme activity, given the observation that all 3 proteins copurify it seems more likely that they are integral subunits of the SPT holoenzymes, a conclusion entirely consistent with the extremely small increase in SPT activity observed when the hLCB1 and hLCB2 subunits are overexpressed by themselves. Nonetheless, because the 2 subunits confer different acyl-CoA selectivities, they do have the potential to regulate the species of LCBs generated. Interestingly, Cowart and Hannun (23) reported a Tsc3p-dependent increase in C20–LCBs in yeast after transient heat shock.

The split-ubiquitin assay used N-terminally NubG-HA-tagged ssSPT isoforms, allowing us to conclude that their N termini are cytoplasmic. The location of the C terminus has not been experimentally determined. Because the 28-aa central domain is the most highly-conserved region of the ssSPTs, it seems likely that it is this domain that interacts with the hLCB1–hLCB2 heterodimers. Hydropathy analysis predicts that most of this domain resides in the membrane, suggesting that the interaction of the ssSPT isoforms with hLCB1 and hLCB2 is in the membrane. The N- and C-terminal ends of ssSPTa and ssSPTb are divergent from each other, but conserved within the ssSPTa and ssSPTb subfamilies. Protease sensitivity (16, 24) and topological analysis (11) place the catalytic domain of SPT in the cytoplasm. Thus, it is tempting to speculate that the differences in acyl-CoA preferences conferred by the ssSPT proteins reflect an interaction between their N- and/or C-terminal regions with the active site of the heterodimer. However, even within the central domain there are clear differences between the ssSPTa and ssSPTb families that could also contribute to acyl-CoA selectivity. In addition, we cannot exclude the possibility that the SPT holoenzyme contains >1 ssSPT subunit, a configuration that would increase the potential complexity of the acyl-CoA preferences.

Although no function has previously been assigned to ssSPTa, "gene atlas" (25) expression data indicate that it is ubiquitously expressed, albeit at different levels, with highest expression seen in the adrenal gland, lung, salivary gland, prostate, and placenta. In contrast, a mouse ortholog of ssSPTb was originally described as an ADMP in mouse prostate (13). In hypogonadal mice expression was restricted to the prostate, whereas in normal mice there was little, if any, expression in the prostate but robust expression in kidney and brain. These results suggest that androgens could regulate the LCB base composition in androgen target tissues. Indeed, it has been observed in *Drosophila* that sperm production is reduced in mutants lacking the LCB-desaturase required for sphingosine synthesis (26). Moreover, mice overexpressing the hereditary sensory autonomic neuropathy (HSAN) mutant LCB1 subunit showed clear evidence of aspermatogenesis (27).

There are numerous reports of novel LCBs (17), including some with unusual chain lengths that appear to be derived from substrates other than palmitoyl-CoA. The data presented here provide direct evidence for the presence of SPT isozymes with distinct acyl-CoA preferences. We have described a virally-encoded single-chain fusion SPT with a preference for myristoyl-CoA (18). In contrast, mammalian SPT isozymes containing ssSPTb prefer longer acyl-CoAs. It is known that different ceramide synthases have preferences for different fatty acids. It will therefore be interesting to determine whether they also exhibit a preference for LCBs of different lengths.

HSAN1 is the result of dominant mutations in hLCB1 (28, 29). It has been presumed that these mutations lead to a 50% decrease in SPT activity in all tissues. However, the fact that nonsense or frameshift mutations have not been identified in HSAN patients and that mutation of only 2 residues has been conclusively linked to the disease suggests that a simple haploinsufficiency cannot explain the phenotype. The discovery that ssSPT subunits can alter substrate specificity, combined with the location of the mutations near the active site of the enzyme, suggests alternative explanations that will need to be experimentally tested.

Materials and Methods

Yeast Strains. The yeast strain TDY6049 (*Mat*a *lcb1ΔKAN lcb2ΔKAN leu2 ura3 his3 met 15 lys2*) was constructed by crossing haploid *lcb1ΔKAN* and *lcb2ΔKAN* mutants derived from heterozygous knockout strains (OpenBiosystems) followed by tetrad dissection. The *TRP1* gene was then replaced with *HIS3* to create TDY8055 in which hLCB1–hLCB2a and hLCB1–hLCB2b heterodimers were expressed for the functional complementation screens. A *ura3* mutant of strain L40 (*Mat*a, *trp1–901 leu2–3,112 his3Δ200 lys2–801 ade2 ura3 LYS2*::*lexA-HIS3 ura3*::*lexA-lacZ*) (30) was selected on 5-fluoroorotic acid for the split ubiquitin yeast 2-hybrid study. The *tsc3–2* mutant has been described (4).

Complementation Cloning of ssSPTa. A NubG-HA-X human library prepared from whole brain was purchased from Dualsystems Biotech and transformed into TDY8055 expressing hLCB1 and hLCB2a. From $\approx 5 \times 10^5$ transformants, 2 colonies that grew well at 37 °C were selected for further analysis. Plasmids were purified by passage through *Escherichia coli* and their ability to complement confirmed upon reintroduction into TDY8055/hLCB1-hLCB2a. The plasmids (pPR3N-NubG-HA-ssSPTa) recovered from the 2 yeast transformants were identical.

Mammalian Expression of SPT Subunits. LyB CHO cells (12) were grown and transfected as described (11). pCMV6-based plasmids expressing hLCB1 or hLCB2a were purchased from OriGene. hLCB2b cDNA was prepared by RT-PCR using HEK mRNA by standard methods and inserted into pcDNA. The NubG-HA–ssSPTa insert from pPR3N–NubG-HA–ssSPTa was excised by digestion with XhoI and SpeI and ligated into pcDNA to generate pcDNA–NubG-HA–ssSPTa. The pcDNA–NubG-HA–ssSPTb plasmid was constructed by the same strategy.

Yeast Expression of SPT Subunits. A yeast expression vector, pGH316, was constructed by inserting a fragment of the yeast *LCB2* gene including 680 bp of upstream and 345 bp of downstream flanking sequence into pRS316 followed by replacement of the entire coding sequence with an NheI site. For expression under control of the yeast LCB2 promoter, PCR fragments containing hLCB1, hLCB2a, or hLCB2b were inserted into the NheI site of pGH316. Expression plasmids with *LEU2* substituted for the *URA3* marker were also constructed to allow for pairwise expression of the human SPT subunits. To generate a plasmid for expression of ssSPTb in yeast, an SfiI-ended PCR fragment containing the open-reading frame of ssSPTb was substituted for the ssSPTa open-reading frame in pPR3N–NubG-HA–ssSPTa.

Measurement of SPT Activity. Microsomal proteins were prepared from LyB or yeast cells expressing the human SPT subunits, and hLCB1 and hLCB2a were detected by immunoblotting as described (11). For detection of hLCB2b, antibodies against C-terminal peptides of hLCB2b were generated (Covance). The NubG–ssSPTs, containing an HA tag after the Nub domain, were detected by using anti-HA antibodies. For determination of SPT activity, a reaction containing 50 mM Hepes (pH 8.1), 50 μM pyridoxyl phosphate, 2 mM NADPH, 2 mM NADH, 10 mM L- [G-^{3}H]serine (10 μCi/mL), 75 or 100 μM acyl-CoA, and 0.2–0.4 mg of microsomal membranes was incubated at 37 °C for 10 min. The reaction was stopped and the products were extracted and quantified as described (4).

Affinity Purification of SPT. hLCB2b–TAP was constructed by inserting an SpeI-ended TAP cassette into an NheI site introduced by Quikchange mutagenesis before the stop codon of hLCB2b. Microsomes prepared from yeast coexpressing hLCB1-HA–NubG, hLCB2b–TAP, NubG-HA–ssSPTa, or NubG-HA–ssSPTb were solubilized with 0.1% sucrose monolaurate and incubated with IgG-conjugated Sepharose beads for 14 h at 4 °C. After washing 4 times with 50 mM Tris·HCl, pH 7.5, 1 mM EGTA, the beads were boiled in SDS sample buffer and proteins were resolved by SDS/PAGE and visualized by immunoblotting with anti-HA antibodies that recognize the NubG-HA subunits, and a secondary antibody that recognizes the primary antibody and the protein A epitope in hLCB2–TAP.

LCB Extraction and Analysis. LCBs were extracted, derivatized for detection, and resolved by HPLC according to published procedures (31).

Split-Ubiquitin 2-Hybrid Constructs. For C-terminal Cub tagging of SPT subunits, a Cub-LexA-VP16 cassette was inserted in-frame before the stop codon of hLCB1, hLCB2a, or hLCB2b. For C-terminal Nub tagging of SPT subunits, a NubG-HA cassette was inserted before the stop codon of hLCB2a and hLCB2b. The NubG-HA—ssSPT-expressing plasmids are described above. Potentially interacting Nub and Cub-tagged subunits were coexpressed in the *ura3*$^-$ derivative of L40 described above and interactions were identified by the ability of the transformed cells to grow in the absence of histidine in the presence of 50 mM 3-aminotriazole.

ACKNOWLEDGMENTS. We thank Kentaro Hanada (Department of Biochemistry and Cell Biology, National Institute of Infectious Diseases, Tokyo, Japan) for the CHO LyB cells. This work was supported by United States Public Health Service Grant NS47717 awarded by National Institute of Neurological Disorders and Stroke, National Institutes of Health.

1. Buede R, Rinker-Schaffer C, Pinto WJ, Lester RL, Dickson RC (1991) Cloning and characterization of *LCB1*, a *Saccharomyces* gene required for biosynthesis of the long-chain base component of sphingolipids. *J Bacteriol* 173:4325–4332 and erratum (1993) 175:919.
2. Nagiec MM, Baltisberger JA, Wells GB, Lester RL, Dickson RC (1994) The LCB2 gene of *Saccharomyces* and the related *LCB1* gene encode subunits of serine palmitoyltransferase, the initial enzyme in sphingolipid synthesis. *Proc Natl Acad Sci USA* 91:7899–7902.
3. Zhao C, Beeler T, Dunn T (1994) Suppressors of the Ca^{2+}-sensitive yeast mutant (*csg2*) identify genes involved in sphingolipid biosynthesis. Cloning and characterization of *SCS1*, a gene required for serine palmitoyltransferase activity. *J Biol Chem* 269:21480–21488.
4. Gable K, Slife H, Bacikova D, Monaghan E, Dunn TM (2000) Tsc3p is an 80-amino acid protein associated with serine palmitoyltransferase and required for optimal enzyme activity. *J Biol Chem* 275:7597–7603.
5. Alexeev D, et al. (1998) The crystal structure of 8-amino-7-oxononanoate synthase: A bacterial PLP-dependent, acyl-CoA-condensing enzyme. *J Mol Biol* 284:401–419.
6. Gable K, et al. (2002) Mutations in the yeast LCB1 and LCB2 genes, including those corresponding to the hereditary sensory neuropathy type I mutations, dominantly inactivate serine palmitoyltransferase. *J Biol Chem* 277:10194–10200.
7. Yard BA, et al. (2007) The structure of serine palmitoyltransferase; gateway to sphingolipid biosynthesis. *J Mol Biol* 370:870–886.
8. Dietrich CR, et al. (2008) Loss-of-function mutations and inducible RNAi suppression of *Arabidopsis LCB2* genes reveal the critical role of sphingolipids in gametophytic and sporophytic cell viability. *Plant J* 54:284–298.
9. Hornemann T, Richard S, Rutti MF, Wei Y, von Eckardstein A (2006) Cloning and initial characterization of a new subunit for mammalian serine-palmitoyltransferase. *J Biol Chem* 281:37275–37281.
10. Hojjati MR, Li Z, Jiang XC (2005) Serine palmitoyl-CoA transferase (SPT) deficiency and sphingolipid levels in mice. *Biochim Biophys Acta* 1737:44–51.
11. Han G, et al. (2004) The topology of the Lcb1p subunit of yeast serine palmitoyltransferase. *J Biol Chem* 279:53707–53716.
12. Hanada K, et al. (1998) Mammalian cell mutants resistant to a sphingomyelin-directed cytolysin. Genetic and biochemical evidence for complex formation of the LCB1 protein with the LCB2 protein for serine palmitoyltransferase. *J Biol Chem* 273:33787–33794.
13. Singh J, Young L, Handelsman DJ, Dong Q (2005) Molecular cloning and characterization of a novel androgen repressible gene expressed in the prostate epithelium. *Gene* 348:55–63.
14. Johnsson N, Varshavsky A (1994) Split ubiquitin as a sensor of protein interactions in vivo. *Proc Natl Acad Sci USA* 91:10340–10344.
15. Hornemann T, Wei Y, von Eckardstein A (2007) Is the mammalian serine palmitoyltransferase a high-molecular-mass complex? *Biochem J* 405:157–164.
16. Hanada K (2003) Serine palmitoyltransferase, a key enzyme of sphingolipid metabolism. *Biochim Biophys Acta* 1632:16–30.
17. Pruett ST, et al. (2008) Biodiversity of sphingoid bases (''sphingosines'') and related amino alcohols. *J Lipid Res* 49:1621–1639.
18. Han G, et al. (2006) Expression of a novel marine viral single-chain serine palmitoyltransferase and construction of yeast and mammalian single-chain chimera. *J Biol Chem* 281:39935–39942.
19. Chen M, Han G, Dietrich CR, Dunn TM, Cahoon EB (2006) The essential nature of sphingolipids in plants as revealed by the functional identification and characterization of the *Arabidopsis LCB1* subunit of serine palmitoyltransferase. *Plant Cell* 18:3576–3593.
20. Gómez del Pulgar T, Velasco G, Sánchez C, Haro A, Guzmán M (2002) De novo-synthesized ceramide is involved in cannabinoid-induced apoptosis. *Biochem J* 363:183–188.
21. Herget T, et al. (2000) Production of ceramides causes apoptosis during early neural differentiation in vitro. *J Biol Chem* 275:30344–30354.
22. Perry DK, et al. (2000) Serine palmitoyltransferase regulates de novo ceramide generation during etoposide-induced apoptosis. *J Biol Chem* 275:9078–9084.
23. Cowart LA, Hannun YA (2007) Selective substrate supply in the regulation of yeast de novo sphingolipid synthesis. *J Biol Chem* 282:12330–12340.
24. Mandon EC, Ehses I, Rother J, van Echten G, Sandhoff K (1992) Subcellular localization and membrane topology of serine palmitoyltransferase, 3-dehydrosphinganine reductase, and sphinganine *N*-acyltransferase in mouse liver. *J Biol Chem* 267:11144–11148.
25. Su AI, et al. (2004) A gene atlas of the mouse and human protein-encoding transcriptomes. *Proc Natl Acad Sciences USA* 101:6062–6067.
26. Ternes P, Franke S, Zahringer U, Sperling P, Heinz E (2002) Identification and characterization of a sphingolipid delta 4-desaturase family. *J Biol Chem* 277:25512–25518.
27. McCampbell A, et al. (2005) Mutant *SPTLC1* dominantly inhibits serine palmitoyltransferase activity in vivo and confers an age-dependent neuropathy. *Hum Mol Genet* 14:3507–3521.
28. Bejaoui K, et al. (2001) SPTLC1 is mutated in hereditary sensory neuropathy type 1. *Nat Genet* 27:261–262.
29. Nicholson GA, et al. (1996) The gene for hereditary sensory neuropathy type I (HSN-I) maps to chromosome 9q22.1-q22.3. *Nat Genet* 13:101–104.
30. Vojtek AB, Hollenberg SM, Cooper JA (1993) Mammalian Ras interacts directly with the serine/threonine kinase Raf. *Cell* 74:205–214.
31. Tsegaye Y, et al. (2007) *Arabidopsis* mutants lacking long chain base phosphate lyase are fumonisin-sensitive and accumulate trihydroxy-18:1 long chain base phosphate. *J Biol Chem* 282:28195–28206.

Corrections

BIOCHEMISTRY

Correction for "Identification of small subunits of mammalian serine palmitoyltransferase that confer distinct acyl-CoA substrate specificities," by Gongshe Han, Sita D. Gupta, Kenneth Gable, Somashekarappa Niranjanakumari, Prasun Moitra, Florian Eichler, Robert H. Brown, Jr., Jeffrey M. Harmon, and Teresa M. Dunn, which appeared in issue 20, May 19, 2009, of *Proc Natl Acad Sci USA* (106:8186–8191; first published May 5, 2009; 10.1073/pnas.0811269106).

The authors note that the reference numbers throughout the article appeared incorrectly and that four references were inadvertently omitted from the reference list. The online version has been corrected.

www.pnas.org/cgi/doi/10.1073/pnas.0905301106