Localization, Topology, and Function of the LCB1 Subunit of Serine Palmitoyltransferase in Mammalian Cells

Serine palmitoyltransferase (SPT), the enzyme catalyzing the initial step in the biosynthesis of sphingolipids, comprises two different subunits, LCB1 and LCB2. LCB1 has a single highly hydrophobic domain near the N terminus. Chinese hamster ovary cell mutant LY-B cells are defective in SPT activity because of the lack of expression of an endogenous LCB1 subunit. Stable expression of LCB1 having an epitope tag at either the N or C terminus restored SPT activity of LY-B cells, suggesting that the epitope tag did not affect the localization or topology of LCB1. Indirect immunostaining showed that the N- and C-terminal epitopes are oriented toward the luminal and cytosol side, respectively, at the endoplasmic reticulum. Interestingly, there was far less LCB2 in LY-B cells than in wild-type cells, and the amount of LCB1 in LY-B cells was restored to the wild-type level by transfection with LCB1 cDNA. In addition, overproduction of the LCB2 subunit required co-overproduction of the LCB1 subunit. These results indicated that the LCB1 subunit is most likely an integral protein having a single transmembrane domain with a luminal orientation of its N terminus in the endoplasmic reticulum and that the LCB1 subunit is indispensable for the maintenance of the LCB2 subunit in mammalian cells.

Serine palmitoyltransferase (SPT) is the enzyme catalyzing the condensation of palmitoyl coenzyme A (CoA) with L-serine to generate 3-ketodihydrosphingosine (1). In mammalian cells, SPT consists of two different subunits, LCB1 and LCB2, both of which are required for the enzymic activity (2–5). It has recently been revealed that several missense mutations in SPTLC1, the human LCB1 gene, cause hereditary sensory neuropathy type I (6, 7) and that these mutations confer dominant-negative effects on SPT activity (8, 9), supporting the notion that the LCB1 subunit plays a crucial role in the formation of an active SPT.

Comparisons of deduced amino acid sequences showed that there is about 30% identity between the mammalian LCB1 and LCB2 subunits and that both can be affiliated to a subfamily of pyridoxal phosphate (PLP)-dependent enzymes, which includes oxononanooate synthase, amino levulinate synthase, and amino ketoacetate CoA ligase (2, 5, 10–12). The subfamily members including SPT catalyze similar reactions, the condensation of carboxyl CoA thioesters with amino acids to generate α-oxoamino acids. Eukaryote SPT is a membrane-bound enzyme consisting of different subunits, whereas other members of the PLP-dependent α-oxoamine synthase (POAS) family are soluble homodimers. Curiously, although the LCB2 subunit of SPT has the PLP-binding motif conserved in the POAS family, the LCB1 subunit does not (2, 5, 10–12). In addition, the Gram-negative bacterium Sphingomonas paucimobilis produces a soluble SPT as a homodimer of a protein having the PLP-binding motif (13). These results suggest that the LCB1 subunit has a function specific for the heterosubunit type of SPT. However, it remains poorly understood why mammalian SPT comprises an LCB1 subunit, as well as the LCB2 subunit, having the PLP-binding motif.

Previous biochemical studies have shown that SPT is a membrane-bound protein enriched in the endoplasmic reticulum (ER) (14, 15). Consistently, hydrophobic profiles of deduced amino acid sequences predict that both the LCB1 and LCB2 subunits contain, at least, single transmembrane domain (TMD) motifs (2, 5, 6, 10–12). However, the number of possible TMDs depends on algorithms used for prediction, indicating the necessity of experimental approaches to determine the topology of the SPT subunits.

In the present study, we determine the topology of the LCB1 subunit experimentally. In addition, we show that the LCB1 subunit of mammalian SPT is indispensable for the maintenance of the LCB2 subunit.

**EXPERIMENTAL PROCEDURES**

**Materials**—Mouse monoclonal anti-FLAG M2 antibody, anti-α-tubulin antibody (DM1A), and digitonin were purchased from Sigma; rat monoclonal anti-HA antibody (3F10) was from Roche Applied Science; mouse monoclonal anti-KDEL antibody (10C3) was from StressGen (Victoria, British Columbia, Canada); Alexa Fluor 488 and 594 anti-mouse IgG and Alexa Fluor 488 anti-rat IgG were from Molecular Probes (Eugene, OR); Sucrose monolaurate was from Dainippon (Kumamoto, Japan). l-[3H(G)]serine (7.4 terabecquerels/mmol) was from Moravek Biochemicals (Brea, CA).

**Plasmids**—pSV-cLCB1 and pSV-cLCB2 are mammalian expression plasmids for hamster LCB1 and LCB2 cDNA, respectively (2). pSV-FHcLCB1 encodes a hamster LCB1 protein tagged with FLAG and hexahistidine sequences at the N terminus (4). To introduce an N-terminal hemagglutinin epitope (HA) tag into pSV-cLCB1, a PCR was performed with pSV-cLCB1 as the template, a synthetic oligonucleotide (5'-TTTAGTGACCATGTACCCATATGACGTCCCGGACTACGCCAT-3') as the reverse primer. To introduce a C-terminal HA tag into pSV-cLCB1, PCR was performed with pSV-cLCB1 as the template, an oligonucleotide (5'-ATTAGTGACCATATGAGTGAGGTC-3') as the forward primer, and an oligonucleotide (5'-TGCTCTAGACTAGGCGTAGTC-3') as the reverse primer. To introduce a C-terminal HA tag into pSV-cLCB1, PCR was performed with pSV-cLCB1 as the template, an oligonucleotide (5'-ATTAGTGACCATATGAGTGAGGTC-3') as the forward primer, and an oligonucleotide (5'-TGCTCTAGACTAGGCGTAGTC-3') as the reverse primer.
were fixed with 3.7% formaldehyde in PBS for 20 min and then incubated with 5 mM H9262 at room temperature unless noted otherwise. LY-B/cLCB1, LY-B/FHcLCB1 were established previously by us (3, 4). Transfection of CHO cells was performed with LipofectAMINE PLUS (Invitrogen), according to the manufacturer’s manual. For analysis of transfection, the transfected cells, cell lysate was prepared 48 h after the start of transfection. If necessary, after selection with G418 (400 μg/ml), stable transformants were isolated by limiting dilution. Stable transfected cells were systemically named according to the names of their parental strains and cLCB1 constructs (i.e. CHO-K1/cLCB1, LY-B/HAcLCB1, and LY-B/cLCB1/HAc).

Preparation of Cell Lysate and Membranes—All manipulations were performed at 4 °C or on ice. After being washed with phosphate-buffered saline (PBS), cells were harvested by scraping and precipitated by centrifugation (300 × g, 5 min). The precipitated cells were suspended in 10 mM Hepes-NaOH buffer (pH 7.5) containing 250 mM sucrose, 1 mM EDTA, and a protease inhibitor mixture (Roche Molecular Biochemicals) and were disrupted by sonication. If necessary, membranes were prepared from the disrupted cell lysate as described previously (16). Protein concentrations of the preparations were determined with the Pierce bicinchoninic acid protein assay kit with bovine serum albumin (BSA) as the standard.

Western Blotting—Western blot analysis of the LCB1 and LCB2 subunits of CHO cells was performed using the rabbit anti-cLCB1 73/90 and anti-cLCB2 29/45 antibodies, respectively, with an enhanced chemiluminescence system as described previously (3, 4). We routinely prepared separate blots, each of which was used for analysis of each LCB subunit, because incomplete stripping of the anti-cLCB1 antibody interfered with reprobing analysis of LCB2. For reprobing to detect LCB subunit, because incomplete stripping of the anti-cLCB1 antibody interfered with reprobing analysis of LCB2. For reprobing to detect LCB2, the chemiluminescence in blot membranes was analyzed with a LAS-1000plus lumino image analyzer (Fuji Film, Tokyo, Japan).

Assay of SPT Activity—SPT activity was assayed as described previously (17). In brief, membranes (100 μg) were incubated in 200 μl of 50 mM Hepes-NaOH buffer (pH 7.5) containing 5 mM dithiothreitol, 5 mM EDTA, 50 μM pyridoxal phosphate, 0.2 mM palmitoyl CoA, and 0.1 mM 1-[3H(G)]serine (1.85 gigabecquerels/mmol). After incubation at 37 °C for 10 min, the amount of [3H]-ketohydroxyphosphoglycerate was measured.

Immunofluorescence Staining—All manipulations were performed at room temperature unless noted otherwise. LY-B/BcLCB1, LY-B/HAcLCB1, and LY-B/cLCB1/HAc cells, grown on glass coverslips (75 mm) were fixed with 3.7% formaldehyde in PBS for 20 min and then incubated with 0.1 mM HAcLCB1 in PBS for 20 min. After a rinse with PBS twice, the cells were incubated with 5 μg/ml of digitonin in PBS for 10 min on ice for selective permeabilization of the plasma membrane or 0.2% Triton X-100 in PBS for 15 min for permeabilization of intracellular membranes. After a wash with PBS for 5 min three times, the cells were blocked with 5% BSA in PBS for 30 min. The cells were then incubated for 1 h with one or a combination of the following primary antibodies: 1) anti-HA antibody (1 μg/ml), 2) anti-KDEL antibody (5 μg/ml), or 3) anti-α-tubulin antibody (1:500). After wash with PBS for 5 min three times, the cells were incubated with 5 μg/ml of Alexa Fluor 488 and/or 594 (1:125) and washed with PBS for 5 min three times. The specimens were observed with a fluorescence microscope (Axiovert S100TV; Carl Zeiss, Tokyo, Japan) equipped with a digital charged coupled device camera (model C4742-95-12; Hamamatsu Photonics, Hamamatsu, Japan), and pictures were taken and viewed with IP Lab 3.21J software (Scanalytics, Inc., Fairfax, VA).

For analysis of the intracellular localization of protein, the specimens were viewed with a confocal laser scanning microscope (Axiovert 100M; Carl Zeiss) equipped with a LSM510 system (Carl Zeiss).

Northern Blotting—Total RNA was prepared from CHO cells with an RNA isolation kit (ISOGEN; Nippon Gene, Toyama, Japan). After electrophoresis in an agarose gel, RNA was blotted onto a nylon membrane (Hybond N; Amersham Biosciences). The membrane was hybridized with a 32P-labeled fragment of the hamster LCB2 cDNA under stringent conditions, and hybridizing LCB2 mRNA was detected by autoradiography.

Co-immunoprecipitation of the LCB2 Subunit with a FLAG-tagged LCB1 Subunit—All manipulations were performed at 4 °C unless noted otherwise. After transfection of CHO-K1 cells with various plasmids, membranes were prepared from the cells as described above. Membranes (0.2 mg of protein) were solubilized with 1% (w/v) sucrose monolaurate in 400 μl of Buffer A (0.1 M sodium phosphate buffer (pH 8.0) containing 0.3 M NaCl and 0.1 M sucrose). After centrifugation (105 × g, 30 min), supernatant fluid (∼400 μl) was collected as the fraction of solubilized membranes. The solubilized membranes (300 μl) were incubated with anti-FLAG M2 affinity gel (25 μl of bed volume) equilibrated with Buffer A containing 0.1% sucrose monolaurate for 1 h. After precipitation of the resin (104 × g, 30 s), the supernatant was collected as the unbound fraction. Pelleted resin was washed three times with 1 ml of Buffer A containing 0.1% sucrose monolaurate. Then, the washed resin was incubated in 300 μl of Buffer EL (Buffer A supplemented with 50 mM Tris-HCl (pH 6.8), 2% (w/v) sodium dodecyl sulfate, 10% (w/v) glycerol, 50 mM dithiothreitol, and 0.1% (w/v) sucrose monolaurate) at 70 °C for 5 min. After precipitation of the resin, the supernatant was collected as the bound fraction. Collected fractions were subjected to Western blot analysis of LCB1 and LCB2 subunits as described above.

Immunoprecipitation of HA-tagged Protein—All manipulations were performed at 4 °C unless noted otherwise. Membranes from LY-B/cLCB1, LY-B/HAcLCB1, and LY-B/cLCB1/HAc were solubilized with 1% sucrose monolaurate as described above. The solubilized membrane fraction (200 μl) was incubated with protein G-Sepharose 4 Fast Flow (Amersham Biosciences) (25 μl of bed volume) complexed with anti-HA antibody equilibrated with Buffer A containing 0.1% sucrose monolaurate and 0.1% BSA for 3 h. After centrifugation of the resin (105 × g, 30 s), the supernatant was collected as the unbound fraction. The gel was washed with Buffer A containing 0.1% sucrose monolaurate three times and then incubated in 200 μl of Buffer EL at 70 °C for 5 min. After precipitation of the gel, the supernatant was collected as the bound fraction. The solubilized membranes and unbound fraction were used for assay of SPT activity and Western blotting. The bound fraction was used for the Western blotting.

RESULTS

Hamster LCB1 Derivatives Having the HA Epitope at N and C Termini Are Functional—The hydrophathy profile of the LCB1 sequence shows that there is a single highly hydrophobic domain (HD1) near the N-terminal region and several moderately hydrophobic domains in other regions (Fig. 1A). Some programs for predicting TMD assign HD1 as a single TMD in the LCB1 protein (1-TMD model), whereas other programs assign HD1 and HD3 as possible TMDs (2-TMD model), and still others assign all of HD1-HD4 as TMDs (4-TMD model) (Fig. 1B). To distinguish the 1-TMD model from the others experimentally, we decided to examine the orientation of the N and C termini of the LCB1 protein. For this, HACLCB1 and lCB1HAc, which are hamster LCB1 variants having an HA epitope at the N terminus and C terminus, respectively (Fig. 2A), were stably expressed in LY-B cells. The LY-B cell line is a CHO cell mutant defective in SPT activity because of a lack of expression of the LCB1 subunit of SPT (3). For fear that extreme overproduction of a membrane protein would affect its localization and/or topology, we selected stable transfectant clones (referred to as LY-B/HACLCB1 and LY-B/lCB1HAc, respectively), in which HA-tagged LCB1 levels were ~5-fold or less of the endogenous LCB1 level observed in wild-type CHO cells (Fig. 2B, upper panel), for further analysis. Importantly, SPT-activating the C-terminal region. The results obtained with the HA epitope at the N terminus are shown in Fig. 3 (lanes 2-4) and those obtained with the HA epitope at the C terminus are shown in Fig. 3 (lanes 5-7). The two HA epitopes were localized in the extracellular domain. The endogenous LCB1 level observed in wild-type CHO cells (Fig. 3B, lane 1) was almost identical to the level observed in the LY-B cells transfected with the HA-tagged LCB1 construct (Fig. 3B, lane 2). The levels of the HA-tagged LCB1 constructs were almost identical to those of the endogenous LCB1 protein. The results obtained with the HA epitope at the N terminus are shown in Fig. 3 (lanes 2-4) and those obtained with the HA epitope at the C terminus are shown in Fig. 3 (lanes 5-7). The two HA epitopes were localized in the extracellular domain. The endogenous LCB1 level observed in wild-type CHO cells (Fig. 3B, lane 1) was almost identical to the level observed in the LY-B cells transfected with the HA-tagged LCB1 construct (Fig. 3B, lane 2). The levels of the HA-tagged LCB1 constructs were almost identical to those of the endogenous LCB1 protein.

2 FLAG-tagged LCB1 constructs could not be used for cytotoxicity analysis, because substantial non-specific signal was detected in immunostaining of LY-B/cLCB1 cells with the anti-FLAG M2 antibody.
ity was restored in these LY-B transfectants to the wild-type level (Fig. 2C), indicating that both the HAcLCB1 and cLCB1HA constructs are functional. LY-B/HAcLCB1 cells unexpectedly produced an N-terminally truncated form of HA-cLCB1 at a low level, along with the full-sized protein (Fig. 2A, lane 4 of upper versus middle panels). However, when solubilized membranes from LY-B/HAcLCB1 were subjected to immunoprecipitation with anti-HA antibodies, 80% of the HAcLCB1 protein, but not its N-terminally truncated form, was immunoprecipitated (Fig. 3A, upper panel). Under such conditions, 60% of the LCB2 subunit was co-immunoprecipitated with the HAcLCB1 protein (Fig. 3A, lower panel), and 60% of SPT activity also disappeared from the nonimmunoprecipitable fraction (Fig. 3B). Neither the endogenous LCB2 subunit nor SPT activity in LY-B/cLCB1 was immunoprecipitated with anti-HA antibodies, whereas the endogenous LCB2 subunit in LY-B/cLCB1HA cells was co-immunoprecipitated with the cLCB1HA protein, confirming the specificity of the immunoprecipitation (Fig. 3A and B). These results demonstrated that the full size of HAcLCB1 is functional.

We also observed that there was far lower LCB2 in LY-B cells than in wild-type CHO cells and that the amount of the LCB2 subunit in LY-B cells was restored to the wild-type level by stable transfection with the cLCB1 or HA-tagged cLCB1 constructs (Fig. 2B, lower panel, lane 2 versus other lanes). The LCB1-dependent alteration of LCB2 will be further analyzed later in this paper.

**Topology of the LCB1 Subunit at the ER**—The orientation of the HA-tagged N and C termini of the LCB1 protein was determined by antibody accessibility in appropriately permeabilized cells. Digitonin treatment of fixed cells selectively permeabilizes the plasma membrane, whereas Triton X-100 treatment permeabilizes all intracellular membranes (18, 19). Indeed, when immunostained with an antibody against α-tubulin, a cytosolic protein, signal was observed in digitonin-treated cells, as well as in Triton X-100-treated cells (Fig. 4, C, F, I, and L). In contrast, immunostaining of Grp78 (or BiP) and Grp94, both of which reside in the ER lumen (20), with anti-KDEL antibodies was observed in Triton X-100-treated cells but not in digitonin-treated cells (Fig. 4, C, F, I, and L). Signal for the HA epitope in LY-B/HAcLCB1 cells was detected after Triton X-100, but not digitonin, treatment (Fig. 4, A versus D), whereas signal in LY-B/cLCB1HA was observed under both conditions almost equally (Fig. 4, G and J). No signal was observed in LY-B/cLCB1 cells expressing non-tagged cLCB1.

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3 The N-terminally truncated form is probably because of translation from the second ATG codon in the HAcLCB1 construct, because the nucleotide sequence around the second ATG, rather than the first ATG, in the HAcLCB1 construct matches the Kozak consensus for efficient translational initiation.
Fig. 3. Co-immunoprecipitation of LCB2 subunit and SPT activity with HAcLCB1 and cLCB1HA. Solubilized membranes (Sol. M.) were incubated with anti-HA antibody bound to protein G-Sepharose as described under “Experimental Procedures.” After centrifugation of the resin, the supernatant was collected as unbound fraction. The resin was washed with a wash buffer and incubated in an SDS-sample buffer. After precipitation of the resin, the supernatant was collected as the bound fraction. A, Western blotting of the solubilized membranes and unbound and bound fractions. Lanes 1, 4, and 7, samples from LY-B/cLCB1 cells; lanes 2, 5, and 8, samples from LY-B/HAcLCB1 cells; lanes 3, 6, and 9, samples from LY-B/cLCB1HA cells. The faint band (marked by asterisk) reacted with anti-LCB2 antibody might represent a modified form of LCB2, although identity of the faint band remains unclear. B, non-immunoprecipitable SPT activity. SPT activity in the unbound fraction is represented as the percentage of the activity in the solubilized membranes. The data are shown as means ± S.D. from triplicate experiments.

Fig. 4. The orientation of the HA-tagged N and C termini of cLCB1 protein in appropriately permeabilized cells. LY-B/HAcLCB1 (A–F), LY-B/cLCB1HA (G–L), and LY-B/cLCB1 (M) cells grown on glass coverslips were fixed and treated with 5 μg/ml of digitonin (A–C and G–I) or 0.2% Triton X-100 (D–F and J–M). The cells were immunostained with anti-HA (A, D, G, J, and M), anti-KDEL (B, E, H, and K), or anti-α-tubulin (C, F, I, and L) primary antibody and then with Alexa Fluor-coupled secondary antibodies. The immunostained cells were observed by fluorescence microscopy. Scale bar, 10 μm.

(Fig. 4M), indicating the specificity of the immunostaining for the HA epitope.

The immunofluorescence staining also enabled us to explore the intracellular distribution of the HA-tagged LCB1 at the confocal microscopic level. When Triton X-100-treated LY-B/HAcLCB1 and LY-B/cLCB1HA cells were doubly immunostained with anti-KDEL and anti-HA antibodies, both signals were distributed to the reticular structure residing throughout the cytoplasm and largely co-localized (Fig. 5), demonstrating that the HA-tagged LCB1 proteins predominantly reside in the ER. It might also be noteworthy that, when compared with the signal distribution of Grp78 and/or Grp94, signal for the HA-tagged LCB1 proteins was denser in the perinuclear region than in other regions of the cytoplasmic reticular membranes (see green signal in Fig. 5, C and F). Collectively, these results indicated that the N-terminal HA epitope of the LCB1 subunit
is oriented into the lumen of the ER and that the C-terminal epitope is oriented toward the cytosol. This experimentally determined orientation of the N and C termini of the HA-tagged LCB1 proteins is compatible with the N-in-C-out type of the 1-TMD model but not with other models shown in Fig. 1B.

**The Lack of the LCB1 Subunit Causes the Reduction in the Level of LCB2 in CHO Cells**—To determine whether a lack of the LCB1 subunit affected the level of LCB2, we examined the levels of both subunits in various CHO cell strains by Western blotting. The level of LCB1 in LY-B cells was less than 10% of that in wild-type CHO-K1 cells (Fig. 6A, lane 1 versus lane 2), consistent with our previous studies (3, 4). Interestingly, the level of the LCB2 subunit in LY-B cells was only 10% of the wild-type (Fig. 6A, lane 1 versus lane 2). LCB2 levels in LY-B cells were restored to the wild-type level when cDNA of hamster LCB1 (cLCB1) or its FLAG- and hexahistidine-tagged version (FHcLCB1) was introduced into LY-B cells by stable transfection (Fig. 6A, lanes 3 and 4). We also introduced cLCB1 to CHO-K1 cells and obtained a stable transformant named CHO-K1/cLCB1. Although LCB1 levels in LY-B/cLCB1 and CHO-K1/cLCB1 cells were 5-fold the wild-type level, such overproduction of LCB1 did not increase the amount of LCB2 beyond the wild-type level (Fig. 6A). Reprobing of the blots with the anti-KDEL antibody showed that the levels of Grp94 and Grp78 were virtually equal among the cell types examined, eliminating the possibility that the reduced levels of both LCB1 and LCB2 in LY-B cells were because of nonspecific protein degradation. Similar results were observed when LY-B cells were transfected with HA-tagged cLCB1 constructs (Fig. 2B). It should also be noted that there was no difference in the LCB2 mRNA level between CHO-K1 and LY-B cells (Fig. 6B). These results demonstrated that the lack of LCB1 reduces the steady state level of LCB2 in CHO cells, suggesting that, when LCB1 is missing, LCB2 cannot exist stably in mammalian cells.

**Overproduction of the LCB2 Subunit Requires Co-overproduction of the LCB1 Subunit**—To further examine whether the
LCB1 subunit played a crucial role in the maintenance of the LCB2 subunit, we analyzed the levels of the two after the transient transfection of CHO-K1 cells with various plasmids. Mock transfection with the empty pSV vector affected neither LCB1 nor LCB2 (Fig. 7, lane 1 versus lane 2). On transfection with pSV-cLCB1, the LCB1 subunit was overproduced to \(50\)-fold or more of the wild-type level regardless of co-transfection with pSV-cLCB2 (Fig. 7, lane 1 versus lanes 3 and 5). In contrast, transfection with pSV-cLCB2 without pSV-cLCB1 caused only an \(\sim 2\)-fold increase in LCB2, compared with the non-transfected control (Fig. 7, lane 1 versus lane 4). However, when cells were co-transfected with pSV-cLCB1 and pSV-cLCB2, the level of overproduction of the LCB2 subunit became comparable with that of the LCB1 subunit (Fig. 7, lane 5). Reprobing of the blots with the anti-KDEL antibody confirmed loading of equal protein amount. These results indicated that overproduction of the LCB2 subunit requires co-overproduction of the LCB1 subunit.

To examine whether the overproduced LCB2 subunit was associated with the LCB1 subunit, we carried out co-immunoprecipitation experiments using a FLAG epitope-tagged LCB1. Co-transfection of CHO-K1 cells with pSV-cLCB2 and pSV-FHcLCB1 resulted in overproduction of the LCB2 subunit (Fig. 8, lane 2 versus lane 4), similar to the case of co-transfection with pSV-cLCB1 alone (see Fig. 7 and Fig. 8, lane 3). When the solubilized membrane fraction of the cells was incubated with an anti-FLAG antibody-coupled resin and then fractions bound and bound to the resin were obtained as described 

**FIG. 7.** Overproduction of the LCB2 subunit requires co-overproduction of the LCB1 subunit. Subconfluent CHO-K1 cells grown in 35-mm dishes were transfected with pSV-cLCB1, pSV-cLCB2, and/or the empty vector in the indicated combinations. Lysate (7.5 \(\mu\)g of protein per well) prepared from the transfected cells was subjected to Western blot analysis of LCB1 and LCB2 subunits. Then, the blots used for analysis of the LCB proteins were reprobed with the anti-KDEL antibody to examine the levels of Grp94 and Grp78 as loading controls. The reprobed pattern is presented below the pattern of each LCB blot.

**FIG. 8.** Co-immunoprecipitation of the LCB2 subunit with a FLAG-tagged LCB1 subunit. CHO-K1 cells were transfected, and membranes were prepared from the cells. After solubilization with 1% sucrose monolaurate, the solubilized membrane fraction was incubated with an anti-FLAG antibody-coupled resin, and then fractions unbound and bound to the resin were obtained as described under “Experimental Procedures.” Equivalent volume of these fractions was loaded into each lane and analyzed by Western blotting for LCB1 and LCB2 subunits. Lane 1, 5, and 9, transfected with pSV-cLCB1 alone; lane 2, 6, and 10, transfected with pSV-FHcLCB1 alone; lane 3, 7, and 11, co-transfected with pSV-cLCB1 and pSV-cLCB2; lane 4, 8, and 12, co-transfected with pSV-FHcLCB1 and pSV-cLCB2. Sol. M., solubilized membrane fraction; Unbnd., unbound fraction; Bound, bound fraction.
DISCUSSION

Although hydrophobic profiles of the primary structure of membrane-bound proteins predict possible TMDs and also their membrane topology, different algorithms sometimes give different predictions (21, 22). Therefore, for the elucidation of topology of membrane proteins, experimental approaches remain indispensable. In the present study, we determined the orientation of the N and C termini of the hamster LCB1 subunit by using epitope-tagged constructs (Fig. 4) and also showed the predominant distribution of this protein in the ER at the confocal microscopic level (Fig. 5). The ER localization of the LCB1 subunit is consistent with previous studies showing that SPT activity is enriched in the ER fraction among various subcellular fractions (14, 15). From the hydrophytropy profile of the LCB1 protein, together with our experimental results shown above, we conclude that hamster LCB1 is an integral protein having a single TMD with a luminal orientation of its N terminus in the ER. One might imagine other possible models, in which the protein had three or five TDMs, because LCB1 has several moderately hydrophobic regions (Fig. 1A). However, such regions (including HD2-4 in Fig. 1A) show significant similarities to corresponding regions of soluble enzymes of the POAS family in sequence alignments. In contrast, the highly hydrophobic domain of the LCB1 protein (HD1 in Fig. 1A) has no similarity to any other member of the family. Bioinformatic analyses strongly suggest the moderately hydrophobic regions of the LCB1 protein serve as internal domains of a globular part of the protein, not as TMDs, although further studies would be needed to prove this prediction.

The topological assignment we made above is most likely valid in LCB1 proteins of other organisms, because hamster LCB1 has ~95% identity to human and mouse LCB1 and 35% identity to yeast Lcb1p at the amino acid level (2, 5). From the topological model for the LCB1 subunit, the catalytic site of SPT can be deduced to be oriented to the cytosolic space at the ER, assuming that the catalytic site of mammalian SPT is formed at the interface of the LCB1 and LCB2 subunits as discussed below. This deduction is consistent with a previous study (15) showing that SPT bound to isolated intact microsomes is inactivated by externally added proteases. The cytosolic orientation of the catalytic site of SPT is probably relevant to the accessibility of enzyme substrates, because the cytosol is the major pool of serine and palmitoyl CoA in cells.

In the present study, we obtained evidence that the LCB1 subunit is required for the maintenance of the LCB2 subunit in mammalian cells. First, the amount of LCB2 in CHO mutant cells lacking the endogenous LCB1 subunit was found to be far lower than the wild-type level (see Fig. 2B and Fig. 6A). When the LCB1 subunit was expressed in the mutant cells by transfection, the LCB2 level was restored to the wild-type level (see Fig. 2B and Fig. 6A). In addition, overproduction of the LCB2 subunit in CHO cells required overproduction of the LCB1 subunit (see Figs. 7 and 8). The requirement of LCB1 expression for the maintenance of the LCB2 subunit was unlikely because of a possible transcriptional regulation of the LCB2 gene by the level of the LCB1 subunit, because the LCB2 mRNA level in LY-B cells is similar to that in CHO-K1 cells (Fig. 6B). In addition, LCB1-dependent alteration of the LCB2 subunit was observed even when the expression of cLCB1 and cLCB2 was driven by an SV40-derived promoter (see Figs. 7 and 8). Probably, post-transcriptional events cause the alteration of the LCB2 subunit. Taking account of the fact that almost all of the overproduced LCB2 protein molecules are associated with the LCB1 subunit (Fig. 8), we suggest that the LCB2 subunit is unstable unless it is associated with the LCB1 subunit.

The nature of the LCB2 subunit, which requires the LCB1 subunit for maintenance, may be conserved among eukaryotes, because a lack of Lcb1p also causes a reduction in Lcb2p in the yeast Saccharomyces cerevisiae (23). By contrast, the LCB1 subunit can be highly overproduced without co-overproduction of the LCB2 subunit (see Figs. 7 and 8). These results suggest that, if a pool of free LCB1 subunit is available, cells can regulate the level of LCB1/LCB2 complex through transcriptional regulation of the LCB2 gene even without parallel regulation of LCB1. This scenario might account for the previously reported observation that the change in SPT activity in response to ultraviolet irradiation parallels the change in the LCB2 mRNA level but not the LCB1 mRNA level in cultured human keratinocytes (24). The nature of the LCB2 subunit could also serve to prevent harmful side reactions by free LCB2 having a latently reactive PLP. The LCB1 subunit has no PLP-binding motif and, therefore, is theoretically inert. Thus, free LCB1 might not be severely toxic.

We previously demonstrated that hamster LCB1 forms a complex with hamster LCB2 at a molecular ratio of 1:1 (4). In the present study, we further showed that, when expressed in wild-type CHO cells, a FLAG-tagged LCB1 was co-immunoprecipitated with the endogenous (and also co-overproduced) LCB2 but not any endogenous LCB1 (Fig. 8). These results demonstrate that two LCB1 molecules are not simultaneously integrated into one LCB1/LCB2 complex, consistent with the results observed in a yeast system (9). Although additional gene product, Tsc3p, is required for the optimum activity of SPT in yeast cells (23), no mammalian homolog of Tsc3p has been found even in the human genome data base. Moreover, no proteins except for LCB1 and LCB2 proteins were detected in silver staining patterns of purified hamster SPT (4). Collectively, it is most likely that mammalian SPT is a heterodimer of the LCB1 and LCB2 subunits, although as yet unknown factors might associate with the LCB1/LCB2 complex to regulate the activity of SPT in vivo.

Analysis of the tertiary structure of bacterial oxonioanonte synthase, a member of the POAS family, has revealed that the catalytic site forms at the interface of each subunit of this homodimer enzyme (25, 26). By analogy, SPT is suggested to form its catalytic site at the interface of the subunits in the LCB1/LCB2 heterodimer. If so, the LCB1 subunit may play crucial roles in the formation of the enzyme catalytic site, besides the role in the maintenance of the LCB2 subunit. This is supported by recent studies showing that several specific missense mutations in LCB1 confer dominant negative effects on SPT activity without affecting the steady state level of LCB2 subunit in yeast (9) and mammalian cells (8).

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