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Human and murine serine-palmitoyl-CoA transferase

Cloning, expression and characterization of the key enzyme in sphingolipid synthesis

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Serine palmitoyltransferase (SPT, EC 2.3.1.50) is the key enzyme in sphingolipid biosynthesis. It catalyzes the pyridoxal-5′-phosphate-dependent condensation of L-serine and palmitoyl-CoA to 3-oxo-sphinganine. Human expressed-sequence-tag (EST) clones are similar to the two yeast genes for synthesis of long-chain bases, LCB1 and LCB2, which are believed to encode two subunits of SPT [Buede, R., Pinto, W. J., Lester, R. L. & Dickson, R. C. (1991) *J. Bacteriol. 173*, 4325–5332; Nagiec, M. M., Baltisberger, J. A., Wells, G. B., Lester, R. L. & Dickson, R. C. (1994) *Proc. Natl Acad. Sci. USA 91*, 7899–7902]. We have cloned and characterized two complete human and murine cDNA sequences named hLCB1 & mLCB1 and hLCB2 & mLCB2, respectively, similar to the yeast LCB1 and LCB2 genes.

Human embryonic kidney cells (HEK 293) transfected with murine sequences of LCB1 (mLCB1) and LCB2 (mLCB2) independently and in coexpression showed an overexpression of the transcripts on the mRNA and protein level. The enzymatic activity of cells expressing mLCB2 alone or coexpressed with mLCB1 was three times higher than the activity of untransfected HEK cells. mLCB1 expression was not required for the synthesis of 3-oxo-sphinganine in mammalian cells. Transcription/translation *in vitro* yielded mLCB1 (53 kDa) and mLCB2 (63 kDa). The two proteins do not contain a signal peptide nor are they glycosylated. The endogenous and overexpressed SPT activity were both sensitive to common SPT inhibitors. Labeling studies with [1-^{14}C]palmitic acid indicated that cell lines transfected with mLCB2 preferentially use the excess sphingoid bases for glucocerebroside and galactocerebroside synthesis. Our results provide conclusive genetic and biochemical evidence that the human and murine LCB2 genes described here encode serine palmitoyltransferase. Further studies will be required to unravel the function of the LCB1 gene in mammalian cells.

Keywords: serine palmitoyltransferase; sphingolipid biosynthesis; cloning; characterization; overexpression.

Sphingolipids are integral constituents of plasma and lysosomal membranes and are transiently present in the membranes of the endoplasmic reticulum. Their function as structural elements of plasma membranes remains to be elucidated. The long-chain bases (LCB) sphinganine, sphingosine and phytosphinganine form the back bone of chemically diverse sphingolipids. The unsubstituted long-chain bases in their free form are detergent-like molecules, and upon N-acyl modification with long-chain fatty acids form ceramides. Ceramides may form phosphodiesters yielding sphingomyelins or be glycosylated to form neutral and acidic glycosphingolipids (gangliosides).

Recently, intermediates of sphingolipid catabolism, e.g. sphingosine, sphingosine 1-phosphate and ceramide have been proposed as potential second messenger molecules [1–6].

The pathways of the biosynthesis and catabolism of LCB have been unravelled before [7–9]. Long-chain bases originate from the condensation of serine and palmitoyl-CoA to 3-oxo-sphinganine in a pyridoxal-5′-phosphate-dependent reaction catalyzed by the key enzyme of sphingolipid biosynthesis, serine palmitoyltransferase (SPT; palmitoyl-CoA:L-serine C-palmitoyltransferase, decarboxylating; EC 2.3.1.50), which resides in the microsomal membranes [10]. The oxo-intermediate is subsequently stereospecifically reduced to D-*erythro*-sphinganine [(2*S*,3*R*)-2-amino-1,3-dihydroxy-octadecane] [11], N-acylated and desaturated to yield ceramide with sphingenine as LCB [12–14].

Although numerous properties of the enzyme have been studied, its purification has not been achieved, mainly due to its low activity [7]. Two yeast genes LCB1 and LCB2 (21.6% identity) were isolated by rescue of SPT activity in a strain of

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Abbreviations. LCB, long-chain base; LCB1 and LCB2, yeast genes for LCB synthesis; SPT, serine palmitoyltransferase; hLCB1, mLCB1: human, murine LCB1; hLCB2, mLCB2: human, murine LCB2; HEK, human embryonic kidney; EST, expressed sequence tag; ER, endoplasmic reticulum; DMEM, Dulbecco's modified Eagle medium; RACE, rapid analysis of cDNA ends; CMV, cytomegalovirus; GST, glutathione *S*-transferase.

Enzymes. Serine palmitoyltransferse (EC 2.3.1.50); 5-aminolevulinate synthetase (EC 2.3.1.37), 2-amino-3-oxobutyrate:CoA-ligase (EC 2.3.1.29); 8-amino-7-oxononoate synthase (EC 2.3.1.47); mannosyl-glycoprotein endo-β-*N*-acetylglucosaminidase (EC 3.2.1.96).

Nomenclature. Sphinganine, (2*S*,3*R*)-2-amino-1,3-dihydroxy-octadecane (also termed dihydrosphingosine); sphingenine, (2*S*,3*R*,4*E*)-2-amino-1,3-dihydroxy-octadecene (also termed sphingosine); phytosphinganine, (2*S*,3*S*,4*R*)-2-amino-1,3,4-trihydroxy-octadecane.

Note. The novel nucleotide sequence data reported in this article has been submitted to the EMBL DNA database and is available under accession numbers X95641 for mLCB1, X95642 for mLCB2, Y08685 for hLCB1 and Y08686 for hLCB2.

Saccharomyces cerevisiae which showed SPT deficiency [15, 16]. Both gene products are similar to 5-aminolevulinate synthetase, 2-amino-3-oxobutyrate:CoA ligase and 8-amino-7-oxononoate synthase, enzymes that catalyze similar chemical reactions and, like SPT, require pyridoxal 5′-phosphate [15]. Overexpression of LCB1 and LCB2 together resulted in a 2.5-fold increase in SPT activity in yeast, an effect not seen by overexpression of either gene singly [16].

Here we report on the isolation and biochemical characterization of the murine and human LCB1 (hLCB1 and mLCB1) and LCB2 (hLCB2 and mLCB2) cDNAs. We characterized the gene products of mLCB1 and mLCB2 by *in vitro* transcription/translation of the respective cDNAs and by transfection experiments of human embryonic kidney cells (HEK 293). Expression of the mLCB2 cDNA was sufficient for the increase in SPT activity in HEK 293 cells. This differs from yeast where both LCB1 and LCB2 are required for the enzymatic catalysis of the serine palmitoyltransferase.

EXPERIMENTAL PROCEDURES

Materials. The following reagents were used: mouse liver cDNA library ML1035b (Clontech Laboratories), pBluescript SK vector (Stratagene GmbH), the pRc/CMV, pCDNA3 vector and TA cloning kit (pCRII vector) (Invitrogen BV), pGEX vector and EAH-Sepharose (Pharmacia Biotech), and the bicinchoninic acid protein assay (Pierce). Pyridoxal 5′-phosphate, palmitoyl-CoA, L-serine and other reagents were purchased from Sigma.

Dulbecco's modified Eagle medium (DMEM) and minimum essential medium (MEM), fetal calf serum, geneticin (G418) and other reagents for cell culture were obtained from Life Technologies/Gibco BRL; [^{35}S]methionine (37 TBq/mmol) and [U-^{14}C]serine (5.59 GBq/mmol) were from Amersham; mannosyl-glycoprotein endo-*N*-acetylglucosaminidase (EndoF) from Boehringer, Mannheim.

All molecular cloning steps were performed according to standard protocols [17] if not otherwise stated. Sequencing reactions were performed with both strands of DNA with the Cy5 autoread kit on an ALF express (Pharmacia Biotech).

RNA was prepared from cells and tissues according to Chomczynski and Sacchi [18]. Poly(A)-rich RNA was isolated by affinity chromatography with oligo(dT)-cellulose (Boehringer, Mannheim) according to the manufacturer's recommendations. The 5′ RACE was performed with the Marathon kit (Clontech Laboratories).

Cloning of the mLCB1 and mLCB2 cDNAs. Reverse-transcription PCR of HEK 293 cell mRNA was performed with the oligonucleotides hLCB2s (5′-atgaagaactagaggagcttgtagcaaggtt-3′) and hLCB2as (5′-ccagggccttcgtgtccgaggctgaccataaac-3′) as primers for the human LCB2 gene and hLCB1s (5′-tcctcttcca-gaattggttaagttaaaatac-3′) and hLCB1as (5′-ctcttccagttgtaggt-gaaaggctggag-3′) for the human LCB1 gene. These sequences were derived from human expression-sequence-tag (EST) clones (accession nos T35317, F12317, W96011, H06927 for hLCB1 and T95281 for hLCB2), which are similar to yeast LCB1 (M63674) and LCB2 (L33931, M95669), respectively. The amplification products, a 442-bp hLCB1 and a 276-bp hLCB2 fragment, were cloned into the pCRII vector and their nucleotide sequence verified by DNA sequencing.

These PCR fragments were used as hybridization probes for filter screening of the mouse liver cDNA library ML1035b. Several mLCB1 and mLCB2 cDNA clones were isolated, subcloned into the pBluescript SK vector and sequenced. The 5′ RACE was performed with RNA from mouse kidney tissue following the manufacturer's recommendations.

Plasmid constructs for stable eukaryotic expression. mLCB1 and mLCB2 cDNA inserts were excised by *Not*I/*Apa*I digestion from the respective pBluescript SK vectors and cloned in the sense direction into the eukaryotic pRc/CMV vector (pRc/CMV-mLCB1; pRc/CMV-mLCB2). A vector carrying both mLCB1 and mLCB2 (pRc/CMV-mLCB1/mLCB2) was constructed by ligating an *Eco*RI fragment harboring the mLCB1 cDNA unidirectionally into the *Eco*RI site of the pCDNA3-vector (pCDNA3-mLCB1). The complete mLCB1 cDNA insert, together with the cytomegalovirus (CMV) promoter, was then released by *Nru*I/*Sma*I digestion and cloned blunt end into the *Sma*I site of pRc/CMV-mLCB2. The resulting plasmid contained both cDNAs, each one with its own CMV promoter and bovine growth hormone poly(A) signal. Constructs were linearized by *Nru*I digestion for transfection.

Northern blot hybridization analysis. RNA was heat-denatured and size-fractionated by electrophoresis through a 1% formaldehyde/agarose gel and transferred onto a nitrocellulose membrane by capillary blotting. The baked filter (2 h at 80°C) was first hybridized for 4 h and then hybridized with a randomly ^{32}P-labeled fragment (807-bp *Ava*I fragment of mLCB1; 739-bp *Hin*dIII−*Pst*I fragment of mLCB2; 0.5×10^6 dpm/ml), washed according to the instructions of the supplier, and the radioactivity measured with a phosphor imager. The membrane was simultaneously hybridized with a randomly labeled glyceraldehyde-3-phosphate dehydrogenase (Gra*P*-DH) cDNA probe (548-bp *Hin*dIII−*Xba*I fragment).

Recombinant glutathione *S*-transferase (GST) mLCB2 and mLCB1 fusion proteins and antibody production. cDNAs coding for the C-terminal parts of mLCB1 and mLCB2 were inserted in-frame into the pGEX vector. Expression in DH5α strain of *Escherichia coli* [19] yielded the GST-mLCB1 (51 kDa) and GST-mLCB2 (67.5 kDa) fusion proteins. Lysates of induced *E. coli* cells were separated by preparative 10% SDS/PAGE. GST fusion proteins were excised and eluted from the gel matrix with elution buffer (0.1 M Tris pH 7.4, 5 mM EDTA, 5 mM mercaptoethanol, 0.05% SDS) by shaking for 4 h at 37°C. Protein concentration was determined by the bicinchoninic acid method [20] and verified by SDS/PAGE.

Rabbits were immunized with 400 μg recombinant fusion protein in complete Freund's adjuvant, boosted three times at two-week intervals with 200 μg recombinant protein in incomplete Freund's adjuvant. The antibody titer reached 1:30000 after 6 weeks. The Ig fraction of the antiserum was purified by chromatography on DEAE-Sephacel (Pharmacia). Anti-mLCB2 antibodies of the Ig fraction were further purified by affinity chromatography on a column of EAH-Sepharose (Pharmacia) covalently linked with recombinant GST-mLCB2 fusion protein as described before [21].

Assay of serine palmitoyltransferase. Incorporation of [U-^{14}C]serine into 3-oxo-sphinganine was assayed with minor modifications as described before [22]. The assay was performed in a 0.15-ml reaction mixture containing 0.1 M Hepes, pH 8, 50 mM KCl, 5 mM dithiothreitol, 0.5 mM pyridoxal 5′-phosphate, 1 mM serine (0.37 GBq/mmol), 0.4 mM palmitoyl-CoA, 0.4% Chaps, 15 mg/ml bovine serum albumin. The reaction was started by the addition of 50−300 μg protein and incubated at 37°C for 30 min. The unstable reaction product 3-oxo-sphinganine was immediately reduced with $NaBH_4$ for 5 min at room temperature after the addition of 0.3 ml 0.5 M NaOH. Lipids were extracted twice with 0.7 ml $CHCl_3/CH_3OH$ (4:1). The combined organic phases were evaporated in a Speed Vac. Lipids were dissolved in 50 μl $CHCl_3/CH_3OH$ (4:1) and separated by TLC with the solvent system $CHCl_3/CH_3OH$/0.5 M NH_4OH (65:25:4). Radioactivity was quantified with a phosphor imager (Molecular Dynamics).

Stably expressing SPT HEK 293 cell lines. HEK 293 (human embryonal kidney) cells were grown in petri dishes at a density of 3×10^4 cells/cm^2 in DMEM (high glucose) with 10% heat-inactivated fetal calf serum and 1 mM sodium pyruvate at 37°C under 5% CO_2 in air. The medium was changed three times a week. HEK 293 cells were electroporated as follows: 5×10^6 cells were suspended in 0.8 ml DMEM with 10% fetal calf serum together with 1 μg linearized plasmid DNA, incubated for 10 min on ice, electroporated (450 V; 250 μF, 0.4-cm cuvette) in a gene pulser (Bio-Rad) and again incubated on ice for 10 min. Cells were plated after electroporation and grown in regular culture medium which was substituted by selection medium supplemented with 800 μg/ml geneticin (G418) 12 h later. G418-resistant clones were isolated and cultured under selection medium. 48 h prior to the experiments the selection medium was substituted by regular medium. SPT activity was measured from the lysate of an equal number of cells: cells were washed with NaCl/P_i (1 l contains 8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.4 g KH_2PO_4) adjusted to pH 7.4 with HCl, harvested in 1 ml NaCl/P_i with a rubber policeman, centrifuged at $1000\times g$ and resuspended in lysis buffer (0.1 M Hepes pH 8, 0.15 M NaCl, 0.4% Chaps, 2 mM EDTA), shaken slowly for 30 min at 4°C, centrifuged again at $10000\times g$ for 20 min at 4°C. The resulting supernatant contained more than 95% of the SPT activity. Protein concentration was determined by the bicinchoninic acid method [20].

Labeling of sphingolipids and lipid analysis. HEK 293 cells were grown in DMEM in 60-mm plates for 48 h. The medium of each plate was then supplemented with 50 MBq [1-^{14}C]palmitic acid for the indicated time intervals. Cells were rinsed twice with NaCl/P_i, harvested with a rubber policeman, suspended in 1 ml NaCl/P_i and transferred into a glass tube. Lipids were extracted twice with $CHCl_3$/CH_3OH (2:1). Aliquots of the organic phases were used for alkaline hydrolysis of phospholipids [23] or for acid hydrolysis [24] to measure the incorporation of labeled long-chain bases into sphingolipids. Sphingolipids were extracted as described above and separated by TLC using the solvent systems described in the figure legend. Radioactivity was quantified with a phosphor imager.

Western blotting. Protein aliquots (150 μg) of the lysate of approximately 10^7 HEK 293 cells untransfected or transfected with the vectors bearing mLCB1, mLCB2, and mLCB1/mLCB2 were separated by 12% SDS/PAGE. They were transferred to a nylon membrane by the semidry blot technique. Membranes were incubated with rabbit anti-mLCB2 and anti-mLCB1 antibodies. Specific bands were visualized with anti-(rabbit IgG) antibody conjugated with alkaline phosphatase.

Translation *in vitro* and immunoprecipitation of mLCB1 and mLCB2. Full-length mouse cDNAs of mLCB1 and mLCB2 were cloned into the pGEM3z vector, linearized and *in vitro* transcribed with SP6 polymerase. Aliquots of the two cRNAs were used for *in vitro* translation, using the reticulocyte lysate (Boehringer, Mannheim) supplemented with [^{35}S]methionine as marker. Co- and post-translational modifications were studied in the presence or absence of canine pancreatic microsomes. For immunoprecipitation with anti-mLCB1 and anti-mLCB2 antibodies, the translation mix was treated for 30 min at 4°C with 0.1% Nonidet-P40, 20 mM Tris/HCl, pH 8, 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 0.15 M NaCl and centrifuged at $100000\times g$ for 10 min. The supernatant was first treated with non-immune-serum Ig fraction for 1 h at 4°C and unspecific antigen-antibody conjugates precipitated with *Staphylococcus aureus* protein A. Anti-mLCB1 or anti-mLCB2 antibodies were added to the supernatant and the immunocomplex precipitated with *S. aureus* protein A. Pellets were resuspended in Laemmli buffer and proteins separated by 12% SDS/PAGE. Fluorography was performed as described before [21]. Radioactive bands were monitored and quantitated with a phosphor imager.

^{35}S-labeled mLCB1 and mLCB2 in the lysates of HEK 293 cells were immunoprecipitated as described for the *in vitro* synthesized products. Lysates were digested with EndoF following the manufacturer's instructions and digested lysates were subjected to immunoprecipitation.

RESULTS

Murine and human LCB1 and LCB2 cDNA and derived protein structure. Oligonucleotides derived from human EST clones similar to the yeast LCB1 (accession no. M63674) and LCB2 (accession no. L33931) genes [15, 16] were used as primers in the reverse-transcription PCR with RNA prepared from HEK 293 cells. A 442-bp hLCB1 and a 276-bp hLCB2 fragment were isolated and used as hybridization probes for the screening of a mouse liver cDNA library.

Several mLCB1- and mLCB2-specific clones were isolated and completely sequenced. The 5′ region of the mLCB1 and mLCB2 cDNAs were further analyzed by 5′ RACE. The most 5′ ATGs are used as translation start sites. Therefore the LCB2 cDNAs of mouse and yeast differ only slightly in their molecular masses (Fig. 1). This was further supported by similarity to a genomic sequence of *Caenorhabditis elegans* (accession no. U50307) and to the LCB2 sequences of *Schizosaccharomyces pombe* (U15645) and *Kluyveromyces lactis* (U15646). The mLCB1 cDNA revealed an ORF of 1419 bp (473 amino acids, 52490 Da) and the mLCB2 cDNA had an ORF of 1680 bp (560 amino acids, 62981 Da).

The hLCB1 cDNA was cloned by overlapping 3′ and 5′ RACE with RNA prepared from HEK 293 cells using the oligonucleotide primers hLCB1s and hLCB1as. The hLCB2 cDNA was obtained as a human EST clone (accession no. AA074413) from the IMAGE consortium. This EST clone contained the complete human LCB2 (hLCB2) cDNA. The hLCB1 cDNA revealed an ORF of 1419 bp (473 amino acids, 52743 Da) and the hLCB2 cDNA had an ORF of 1686 bp encoding 562 amino acids (62923 Da).

The four newly isolated cDNA sequences and the two yeast cDNAs were translated and aligned as in Fig. 1.

Serine palmitoyltransferase is a highly conserved protein. The identity of hLCB1 and mLCB1 on the protein level is 92% and of hLCB2 and mLCB2 96%. hLCB1 and hLCB2 show 29% identity and 52% similarity (Fig. 1). The hydrophobicity pattern [25] suggests a 20-amino-acid transmembrane segment in the N-terminus of h-LCB2 and mLCB2 (Met66 to Leu85 of mLCB2) and a 17-residue segment of hLCB1 and mLCB1 (Ala20 to Ile36), sufficient to span the lipid bilayer of the endoplasmic reticulum (ER) membrane. The hLCB2 and mLCB2 sequences contain a putative pyridoxal-5-phosphate-binding site around Lys377 (Lys379 in hLCB2). Neither hLCB1 and mLCB1 nor hLCB2 and mLCB2 reveal a signal peptide sequence. One putative glycosylation site is present in LCB2 (at Asn155 in the mLCB2 sequence; Asn157 in hLCB2). The predictions derived from the amino acid sequence deduced from the cDNA sequence have been examined by *in vitro* transcription/translation and *in vivo* experiments.

Expression of mLCB1 and mLCB2 in different tissues of the Mouse. Poly(A)-rich RNA was isolated from different tissues of three-month-old mice and subjected to northern blot hybridization analysis to determine the size of *mLCB1* and *mLCB2* mRNAs (2.9 kb and 2.3 kb, respectively) and their tissue distribution (Fig. 2). The ratio of the amounts of the two transcripts

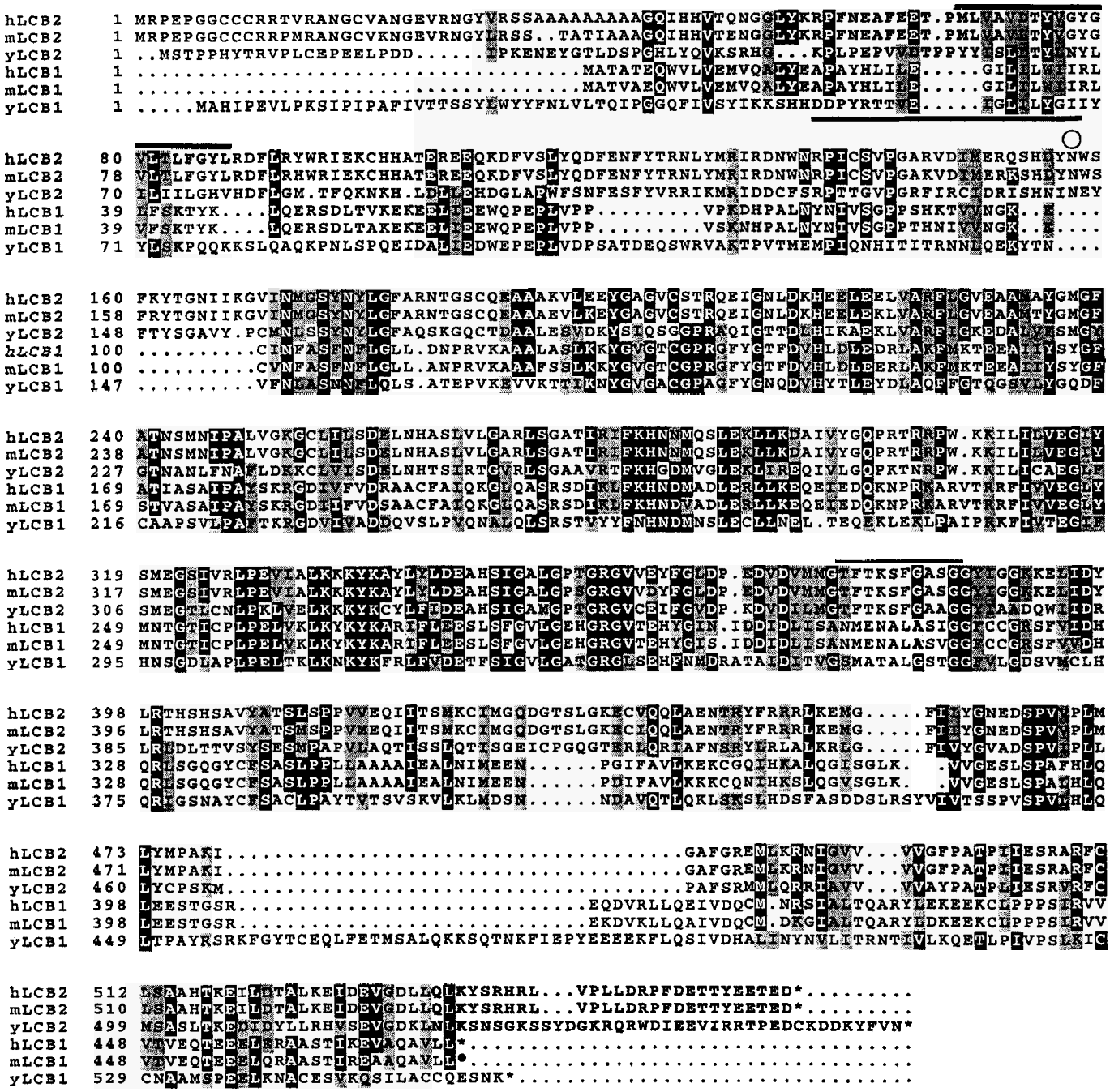


Fig. 1. Alignment of cDNA-derived amino acid sequences of human, murine and yeast LCB1 and LCB2. Identities between murine and human proteins are 92% (LCB2) and 96% (LCB1). hLCB1 and hLCB2 show 29% identity. Putative transmembrane domains in the N-terminal regions are marked above hLCB2 (from Met66 to Leu85 of mLCB2) and below the sequence of yLCB1 (from Ala20 to Ile36 of mLCB1). The putative motif for pyridoxal-5′-phosphate-binding site (T[LIVMFYW][STAG]K[SAG][LIVMFYWR][SAG]⟨2⟩[SAG]) in the LCB2 amino acid sequences is marked by a thin line. Members of the LCB1 subfamily lack this motif. The putative N-glycosylation site of hLCB2 and mLCB2 at Asn155 is marked with an open circle.

remains approximately constant throughout all tissues except in brain where the mLCB1 mRNA is more highly expressed. The tissue distribution of mLCB2 mRNA is in accordance with the distribution of SPT activity described previously [26].

Transcription/translation *in vitro* show no signal peptide cleavage or N-glycosylation. We studied posttranslational modifications of the mLCB1 and mLCB2 by *in vitro* transcription/translation in the absence and presence of dog pancreas microsomal membranes. The apparent molecular masses of mLCB1 (53 kDa) and mLCB2 (63 kDa) protein remained unaltered as indicated by their mobilities in SDS/PAGE (Fig. 3). Therefore neither primary translation product carries a cleavable signal peptide for targeting the ER membrane. Futhermore the putative glycosylation site at Asn155 of mLCB2 remains unglycosylated. This latter finding was further confirmed by EndoF digestion of [^{35}S]methionine-labeled proteins in mLCB2-expressing HEK cells. After immunoprecipitation and separation by SDS/PAGE, the molecular masses of mLCB2 in the EndoF-digested and in the control lysates did not differ (data not shown).

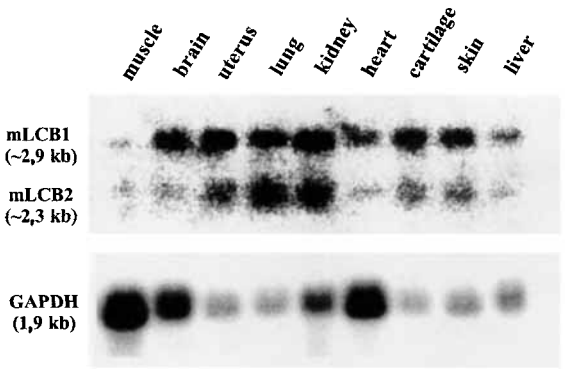


Fig. 2. Tissue distribution in mouse of mLCB2 and mLCB1 mRNA analyzed by northern blot analysis. Poly(A)-rich RNA from different tissues of three-month-old mice was prepared, subjected to formaldehyde/agarose electrophoresis (5 µg each lane) and analyzed by northern blot hybridization with randomly labeled fragments of mLCB1 (807-bp *Ava*I) and mLCB2 (739-bp *Hin*dIII−*Pst*I). For comparison, the blot was also hybridized with a Gra*P*-DH probe. The blot was hybridized at 42 °C with the labeled fragments. Autoradiography was performed with a phosphor imager.

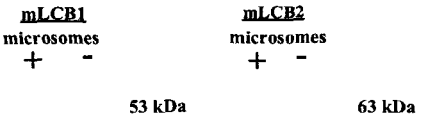
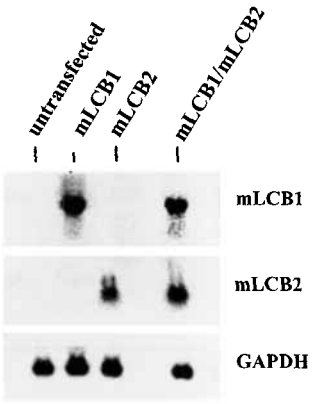


Fig. 3. Translation *in vitro* of mLCB1- and mLCB2-specific cRNAs using the reticulocyte lysate supplemented with [^{35}S]methionine in the presence (+) and absence (−) of canine pancreatic microsomal membranes. Labeled proteins were immunoprecipitated with anti-mLCB1 and anti-mLCB2 antibodies bound to *S. aureus* protein A. For purification they were separated by 12% SDS/PAGE and visualized by fluorography on a phosphor imager. mLCB2 and mLCB1 are not glycosylated and have no cleavable signal peptide sequence.

Functional analysis of heterologously expressed mLCB1 and mLCB2. The functions of the products of mLCB1 and mLCB2 cDNAS were studied in HEK 293 cells. mLCB1, mLCB2, and the construct carrying the mLCB1 and mLCB2 cDNAs were used for transfection and stable expression in HEK 293 cells. The mLCB1 and mLCB2 cDNAs are transcribed under the control of the strong CMV promoter.

HEK 293 cells were electroporated with these three constructs and, for each, six G418-resistant clones were selected. The expression of mLCB1 and mLCB2 in transfected cell clones was examined on the transcriptional level by northern blot hybridization, and on the translational level by western blotting and immunoprecipitation of [^{35}S]methionine-labeled gene products. Stably transfected cell clones overexpressing SPT were assayed for enzyme activity as described in Experimental Procedures. The analytical data from one representative clone for each transfection are shown in Figs 4−7.

The northern blot analysis of untransfected and stably transfected HEK 293 cell lines expressing mLCB1, mLCB2 and coexpressing mLCB1 and mLCB2 was performed and a representative blot is shown in Fig. 4. Endogenous hLCB1 and hLCB2 messages were undetectable even when hybridized with human-specific probes. Where clones overexpressed mLCB1 or mLCB2 either singly or together, the levels of the corresponding mRNAs were similar. Further, the levels of mLCB1 and mLCB2 mRNA expression were comparable in the co-overexpressing clones (detailed in lower part of Fig. 4)



Probe	Total mRNA from			
	untransfected	mLCB1	mLCB2	mLCB1/mLCB2
	counts/32 h			
mLCB1	–	1250689	–	325678
mLCB2	–	–	357138	372296
Gra*P*-DH	653553	830018	735088	568390
mLCB1 (normal)	–	850468	–	325678
mLCB2 (normal)	–	–	276148	372296

Fig. 4. Northern blot analysis of total RNA from untransfected HEK 293 cells and representative HEK 293 cell clones stably expressing mLCB1, mLCB2, and mLCB1/mLCB2 with mLCB1, mLCB2 and Gra*P*-DH-specific probes. More details are given in the legend to Fig. 2. The lower part of the figure tabulates the levels of mRNA expression, measured as counts ^{35}S/32-h exposure period of phosphor imaging, normalized to Gra*P*-DH signal intensities as internal standard.

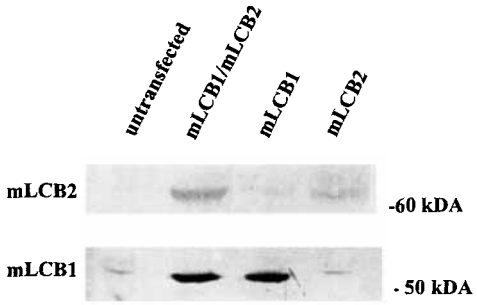
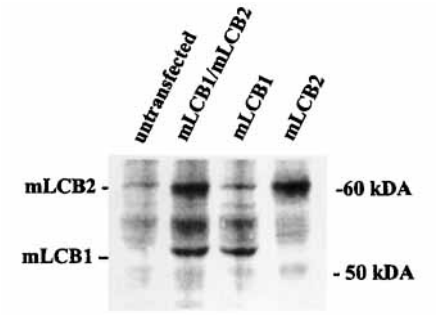


Fig. 5. Western blot of lysates of untransfected and mLCB1, mLCB2 and doubly transfected cells. Lysates of the HEK 293 cell clones were separated by SDS/PAGE and blotted onto a nitrocellulose membrane. Incubation with anti-mLCB2 and anti-mLCB1 antibodies revealed that mLCB1 and mLCB2 are overexpressed compared to untransfected cell clones.

In western blot analysis the anti-mLCB1 and anti-mLCB2 antibodies recognized the expected 53-kDa band for LCB1 and the 63-kDa band for LCB2 in the lysates of transfected and untransfected cell lines (Fig. 5). Untransfected cell clones or those overexpressing mLCB1 alone had barely detectable levels of LCB2 protein. LCB2 was readily detectable in lysates from the clones transfected with either of the LCB2 expression vectors. Similar results were seen for LCB1. However signal intensities when probed for mLCB1 were much stronger than that of mLCB2.



Probe	Total mRNA from			
	untransfected	mLCB1/mLCB2	mLCB1	mLCB2
	counts/14 h			
mLCB2	65253	378110	75689	366649
mLCB1	background	(171198×3 = 513594)	(201544×3 = 604632)	background

Fig. 6. Immunoprecipitation of [^{35}S]methionine-labeled mLCB1 and mLCB2 from lysates of untransfected and transfected HEK 293 cells. Lysates were treated with anti-mLCB1 and anti-mLCB2 antibodies and the antigen/antibody conjugates precipitated with *S. aureus* protein A. The lower part of the figure tabulates the levels of mRNA expression, as described in Fig. 4 but with a 14-h exposure period. The intensity of the mLCB2 protein band is about three times higher than that of the mLCB1 protein.

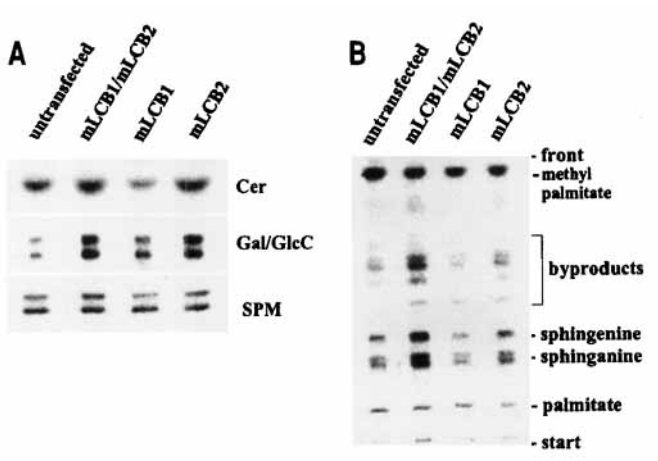
Table 1. Serine palmitoyltransferase activity from different untransfected and transfected HEK 293 cell clones. Specific activities were determined in triplicate by the *in vitro* assay as described under Experimental Procedures.

Clone	Serine palmitoyltransferase specific activity in			
	untransfected	mLCB1/mLCB2	mLCB1	mLCB2
	pmol · min^{-1} · mg^{-1}			
1	18.3 ± 1.9	52.3 ± 4.9	15.6 ± 2.1	30.5 ± 5.8
2		57.4 ± 1.8	17.8 ± 1.1	55.0 ± 3.8
3		40.2 ± 3.2	20.1 ± 2.3	46.3 ± 4.8
4		45.3 ± 9.9	14.0 ± 1.1	50.2 ± 4.2
5		30.2 ± 2.2	15.8 ± 1.3	25.2 ± 2.1
6		58.1 ± 4.8	12.8 ± 3.3	56.8 ± 3.9
Average	18.3	47.3	16.0	44.0

Immunoprecipitation of [^{35}S]methionine-labeled mLCB1 and mLCB2 protein from these cell lines agrees well with the results of western blot analysis except that no mLCB1 is detectable in untransfected nor mLCB2 in transfected cells (Fig. 6).

We assayed the SPT activity in the lysate of the transfected and of untransfected cells (Table 1). All mLCB2 and mLCB1/mLCB2 overexpressing cell lines showed an approximately threefold increase of SPT activity which was constantly observed in all cell clones analyzed. Transfection with mLCB1 alone did not enhance SPT activity.

These observations were expanded by labeling experiments which addressed the question of the further metabolism of the newly synthesized sphinganine. Cell clones stably expressing mLCB1, mLCB2 and mLCB1/mLCB2 and untransfected cells were labeled with [^{14}C]palmitate for 30 h. Labeled lipids were extracted and analyzed after alkaline hydrolysis by TLC (Fig. 7A). Cells expressing mLCB2 and mLCB2 in coexpression with mLCB1 synthesized elevated concentrations of labeled glucocerebrosides and galactocerebrosides (2–3-fold) due to the enhanced synthesis of 3-oxo-sphinganine, the precursor of the parent long-chain sphingosine bases. The accumulation of labeled sphingosine and sphinganine in ceramide monohexosides is shown in the chromatogram of the acid hydrolysate of the labeled sphingolipids (Fig. 7B).



A LCB	RNA expression in			
	untransfected	mLCB1/mLCB2	mLCB1	mLCB2
	counts/72 h			
Ceramide	194442	244041	161759	235071
Gal/Glc ceramide	111176	276813	156437	277401
Sphingenine	93271	396768	163692	308069
Sphingomyelin	171235	197196	153286	154835

B LCB	RNA expression in			
	untransfected	mLCB1/mLCB2	mLCB1	mLCB2
	counts/72 h			
Methylpalmitate	575567	454328 (313192)	310602 (88336)	264512 (141579)
Sphinganine	168669	597573 (474265)	245670 (132795)	463638 (213069)

Fig. 7. TLC of ^{14}C-labeled unsaponifiable lipids of transfected HEK cell lines grown in medium supplemented with [1-^{14}C]palmitate. (A) HEK 293 cells overexpressing mLCB2 and mLCB1 (lane 2), and mLCB2 (lane 4) strongly incorporate [1-^{14}C]palmitate into ceramide monohexosides (Gal/GlcC). Ceramide (Cer) and sphingomyelin (SPM) labeling remains unaffected. Sphingolipids were separated in the solvent system $CHCl_3$/MeOH (15:1) for the detection of ceramides; SPM and Gal/GlcC were separated in solvent system $CHCl_3$/MeOH/H_2O (65:25:4). (B) ^{14}C-labeled sphinganine or sphingosine were released by acid hydrolysis of total lipids. mLCB2-expressing cells synthesize increased amounts of long-chain bases. Unlabeled sphingomyelin and cerebrosides were subjected to acid hydrolysis for comparison. The lower parts of the figure tabulate the levels of mRNA expression as described in Fig. 4. In B, counts were normalized to methyl palmitate as internal standard; values in brackets are counts before normalization.

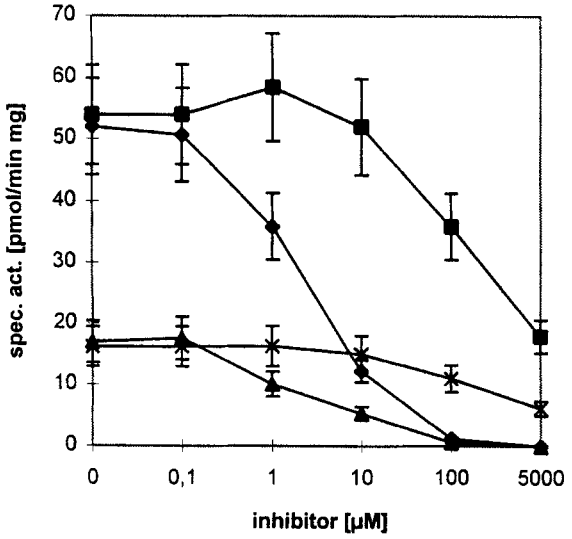


Fig. 8. Inhibition of SPT activity in HEK 293 untransfected and transfected with the pRc/CMV-mLCB1/mLCB2 expression vector. Cells were lysed with 0.5% Chaps and lysates incubated prior to the *in vitro* assay for 15 min at 4°C with the concentrations of inhibitors L-cycloserine and β-chloro-L-alanine indicated in the figure. The data are given as the mean ± SD of three experiments: (▲) untransfected cells (L-cycloserine); (×) untransfected cells (β-chloro-L-alanine); (◆) mLCB1/mLCB2-expressing cells (L-cycloserine); (■) mLCB1/mLCB2-expressing cells (β-chloro-L-alanine).

Inhibition of SPT by L-cycloserine and β-chloro-L-alanine. SPT activity is inhibited effectively by several drugs, e.g. β-chloro-L-alanine [27], L-cycloserine [27–30], sphingofungins [31], myriocin (ISP1) [32] and lipoxamycin [33], the latter being structurally related to sphingosine bases. We tested the inhibition of endogenous SPT in the lysate of untransfected HEK 293 cells and of SPT in the lysate of overexpressing cell clones *in vitro* by two of its most common inhibitors, L-cycloserine and β-chloro-L-alanine (summarized in Fig. 8). Both overexpressed and endogenous SPT could be inhibited in the same dose-dependent manner.

DISCUSSION

We have isolated the human and mouse cDNAs, hLCB1 & mLCB1 and hLCB2 & mLCB2, as homologues of two yeast genes, LCB1 and LCB2, which had previously been reported [15, 16] and shown to be responsible for serine palmitoyltransferase (SPT) activity [34, 35]. SPT activity could only be restored in yeast by coexpression of both the yeast LCB genes [16].

We studied the function of the murine LCB cDNAs by stable transfection of human embryonic kidney cells (HEK 293) with the murine LCB1 and LCB2 cDNAs, singly and in coexpression, and found that, unlike the yeast system, mLCB2 alone was sufficient for an increase in SPT activity. mLCB1 and mLCB2 were highly expressed on the mRNA level in HEK cells. The strongly elevated mLCB1 mRNA concentration leads to a similar increase in mLCB1 protein. Surprisingly, despite the high overexpression of LCB2 mRNA there was an approximately fourfold increase in the LCB2 protein. However, this was associated with a threefold elevation in serine palmitoyltransferase activity. Cell clones coexpressing mLCB1 and mLCB2 did not differ significantly in serine palmitoyltransferase activity from cells expressing the mLCB2 cDNA alone. These results indicate that mLCB2 cDNA encodes serine palmitoyltransferase, and that the LCB1 gene is not required for catalytic activity of SPT in mammalian cells. The SPT activity in *S. cerevisiae* increased 2.5-fold when transformed with a multicopy vector bearing both yeast genes, LCB1 and LCB2, whereas strains transformed with the individual plasmids carrying single LCB genes showed no elevated SPT activity [16]. The strong difference between mRNA and protein concentration of mLCB2 could be explained by cotranslational down-regulation or posttranslational degradation of mLCB2 protein.

Degradation of overexpressed mLCB2 protein might be caused by rapid proteolysis. The discrepancy between different signal intensities of mLCB1 and mLCB2 in the western blot analysis but similar amounts of mLCB1 and mLCB2 seen in the immunoprecipitation could indicate comparable translation rates for both proteins but a higher degradation rate for mLCB2.

In the light of the suggested functions of sphingoid bases as second messengers affecting many cellular functions [1–6], high activities of SPT might lead to non-permissible concentrations of free sphingoid bases or ceramides [36]. Previous reports discuss a strong regulation of SPT on translational or posttranslational levels either by inhibition of enzyme biosynthesis or by increase in its degradation [37]. Our expression experiments reported here are in support of this hypothesis. In spite of the high mRNA levels of mLCB2, the newly synthesized mLCB2 protein, and accordingly SPT activity, is only moderately elevated in our stably transfected HEK cell lines.

Labeling studies with [^{14}C]palmitate showed increased concentrations (threefold) of cerebrosides in cell clones transfected with mLCB1/mLCB2 or mLCB2 alone compared to untransfected or mLCB1 transfected cell lines. Ceramide levels were not significantly elevated and the sphingomyelin fraction remained unaffected. Free 3-oxo-sphinganine and sphinganine were not detected in this study. These results indicate that, even when LCB2 is overexpressed, free sphingoid bases do not accumulate appreciably but are rapidly transformed to ceramides. However, as the cellular ceramide pool remains constant, excess ceramide seems to be immediately shuttled into the cerebroside (galactocerebroside and glucocerebroside) biosynthesis.

Structures of mLCB1 and mLCB2. Endogenous and overexpressed mLCB1 and mLCB2 protein were found in the microsomal fraction of HEK 293 cells (data not shown). The intracellular location of SPT has been previously assigned to the ER membranes [10]. The cDNA-derived polypeptide sequences of mammalian LCB1 and LCB2 protein lack putative signal peptide sequences characteristic for proteins residing in the ER membranes. Translation *in vitro* of mLCB1 and mLCB2 cRNAs in the presence or absence of microsomal membranes proved that neither mLCB1 (53 kDa) nor mLCB2 (63 kDa) changed their molecular masses (Fig. 3). These findings and the results of the EndoF digestion excluded the existence of a signal peptide sequence or N-glycosylation of the putative site at Asn155 of mLCB2. A single N-glycosylation of the protein would increase its molecular mass on average by 2–3 kDa.

The high degree of similarity to other pyridoxal-5′-phosphate-dependent enzymes led to the assumption of a putative pyridoxal-5′-phosphate-binding site of LCB2 [15]. In 2-amino-3-oxobutyrate:CoA ligase Lys244 had been identified to bind pyridoxal 5′-phosphate [38] and in mouse erythroid 5-aminolevulinate synthase, Lys313 has been detected as the residue involved in the Schiff base linkage with this coenzyme [39]. Mutation of Lys313 to Ala, His or Gly abolished 5-aminolevulinate synthase enzyme activity [40]. Mutation of Lys377 to Gln in the mLCB2 product similarly abolished SPT activity when transfected into HEK 293 cells. Thus, Lys377 of the mLCB2 gene product appears to be essential for catalytic activity of SPT.

While this work was in progress the murine (accession no. U27455) and a partial sequence of the human (accession no. U15555) LCB2 cDNA were reported [41]. The authors proved the identity of the partial human cDNA sequence as that of the LCB2 gene by chimeric constructs in which human LCB2 sequences were inserted into the corresponding yeast LCB2 gene and tested for their ability to complement the long-chain base requirement of a LCB2-deleted *S. cerevisiae* strain.

Our data, as well as the genetic data of others [16, 41], prove that the hLCB2 and mLCB2 sequences encode serine palmitoyltransferase, the enzyme catalyzing the initial step of sphingolipid biosynthesis. Our experiments do not support a regulatory function of LCB1 in the mammalian cell as suggested for LCB1 in yeast [16]. The unknown function of mammalian LCB1 awaits further analysis.

The availability of mouse and human SPT cDNA, as well as monospecific antibodies, might prove useful in the study of sphingolipid metabolism, the regulation of sphinganine synthesis and proposed functions of sphingolipid metabolites in signal transduction.

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