Cloning and Initial Characterization of a New Subunit for Mammalian Serine-palmitoyltransferase*

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Serine-palmitoyltransferase (SPT) catalyzes the rate-limiting step of the de novo synthesis of sphingolipids. SPT is considered to be a heterodimer composed of two subunits, SPTLC1 and SPTLC2. Here we report the identification of a novel, third, SPT subunit (SPTLC3) that shows 68% homology to the SPTLC2 subunit. Quantitative real-time PCR revealed that SPTLC3 expression is highly variable between different human tissues and cell lines. The highest expression was observed in placenta tissue and human trophoblast cell lines. The overexpression of SPTLC3 in Hek293 cells, which otherwise have very little endogenous SPTLC3, led to a 2- to 3-fold increase in cellular SPT activity. Silencing of SPTLC3 expression in HepG2 cells or human trophoblast cells by transfecting SPTLC3-specific siRNA resulted in a significant reduction of cellular SPT activity. The expression of two SPT isoforms could be a cellular mechanism to adjust SPT activity to tissue-specific requirements of sphingolipid synthesis.

Sphingolipids are a ubiquitously distributed class of lipids that can be found in all higher organisms. Sphingoid bases confer important structural properties to membranes and to their partition into microdomains (membrane rafts) and modulate the activities of various enzymes such as protein kinases, protein phosphatases, and phospholipases in cells or cell-free systems (1). They are involved in many cellular events, including proliferation, differentiation, senescence, apoptosis, and inflammatory response (2). De novo sphingolipid biosynthesis is initiated by the condensation of L-serine with palmitoyl-CoA to generate 3-ketodihydrosphingosine. This pyridoxal 5-phosphate (PLP)-dependent reaction is catalyzed by the serine-palmitoyltransferase (SPT, EC 2.3.1.50). SPT is believed to be a heterodimer and intracellularly bound to the outer membrane of the endoplasmic reticulum (3, 4). The two SPT subunits SPTLC1 (55 kDa) and SPTLC2 (65 kDa) show a mutual similarity of ~20% and are highly conserved among species. Although both subunits seem to be required for enzyme activity, SPTLC2 is considered to be the catalytic subunit due to the presence of a PLP binding site (3).

SPT activity was detected in various tissues, including brain, lung, liver, kidney, and muscle (5) and is essential for embryonic development, because homozygous SPTLC1 and SPTLC2 knock-out mice die during embryogenesis (6).

In contrast to the membrane-bound mammalian isoform of a soluble, homodimeric SPT isoform (sSPT) was found in the sphingolipid producing prokaryote *Sphingomonas paucimobilis* (7, 8). A third 10-kDa subunit was identified in yeast, but no mammalian homologue has yet been described (9). Here we report the identification and initial characterization of a previously unknown third SPT subunit (SPTLC3) in mammalian cells.

**MATERIALS AND METHODS**

**General**—All Chemicals, unless otherwise stated, were purchased from Sigma. The direct Topo Cloning Vector pcDNA3.2 was from Invitrogen. Protein A-agarose was from Roche Applied Science. Anti-V5 tag monoclonal antibodies were from Serotec (Oxford, UK). The data base search was done with NCBI Blast (www.ncbi.nlm.nih.gov/blast), and subsequent analysis was done in the program BioEdit (version 5.0.9, Dept. of Microbiology, North Carolina State University).

**Cloning**—All constructs were amplified out of a human cDNA library (Matchmaker cDNA Library Muscle/Brain, Clontech, Mountain View, CA) by PCR using appropriate primers and the Long-Expand Mix (Roche Applied Science). The following primers were used for cloning: SPTLC1fw, 5'-caccatggcgaccgccagggcgagc-3'; SPTLC1rv, 5'-gagccagggcgagcaggtct-3'; SPTLC2fw, 5'-ccatcatgccggaggagagcagc-3'; SPTLC2rv, 5'-atctccctctctcctctctctctc-3'; and SPTLC3fw, 5'-caccatggcttcgaggtcagcagc-3' and SPTLC3rv, 5'-atctccctctctctctctctctc-3'.

The PCR product was controlled for purity by agarose gel electrophoresis and cloned directly into a pcDNA3.1 Expression Vector according to the manufacturer’s instructions (Directional TOPO cloning kit, Invitrogen). The cloning was done in this way so that all constructs were expressed with a C-terminal His-V5 tag. All constructs were subsequently verified by sequencing.

**SPTLC1–3 Expression in Hek293 Cell Lines**—Hek293 cells were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Transfections were done using Metafectene (Biontex, Munich, Germany). Selection was performed in the presence of G418 according to the manufacturer’s instructions.

**siRNA Transfection**—The following sequence was used for silencing SPTLC3 expression: UAAACGUGCUUUUAUG-UUCGUCUGA. The sequence showed no homology to the
sequences of SPTLC1 and SPTLC2. The siRNA was transfected using Lipofectamine (Invitrogen) by the standard protocol. SPT activity was determined 48 h after transfection.

**Immunization and Affinity Purification of Antibodies**—Antibodies were custom made by Eurogentec (Liége, Belgium) according to their standard protocol. The following peptide sequences were used for immunization: anti-SPTLC1, FSTKYKLYKQERSDLTCT and CQLEESTGSEQDVDR; anti-SPTLC2, GGLYKRPFNEAFEC and CDRPDEVETYTED; and anti-SPTLC3, CNGKLLHKKQSGNSQ and CKSARPELYDETSFEL. All antibodies were affinity purified by the company according to the Eurogentec protocol.

The specificity of the antibodies was verified by testing the pre-immune serum and, additionally, by competing the antigen interaction with an excess of free immunization peptide. All used antibodies showed a specific binding to the target protein.

**Preparation of Human Placenta Extract**—Human placenta tissue, obtained freshly after cesarean section, was cut into small pieces and washed several times in ice-cold phosphate-buffered saline. The tissue pieces were homogenized in a blender and filtered through a gaze. The filtrate was centrifuged (2000 × g, 5 min, 4 °C), washed twice in phosphate-buffered saline, and frozen in liquid N₂.

**Quantitative Reverse Transcription-PCR**—Tissue-specific SPT mRNA expression was quantified using a normalized commercial cDNA panel for 24 human tissues (OriGene, Rockville, MD). The mRNA from cells and tissue was prepared using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH) and transcribed to cDNA using oligo(dT) primers and Superscript III (Invitrogen) according to the manufacturer’s instructions. Specific primers for the different SPT subunits were designed using the Oligo6.0 software (Molecular Biology Insights, Cascade, CO). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was used as the reference gene for quantification. LightCycler PCR was performed with a DNA SYBR Green kit following the manufacturer’s instructions (Roche Diagnostics). The following primers (0.4 μM each) were used: SPTLC1fw, 5’-aagaagccattatactactat-3’; SPTLC1rv, 5’-ggcgtgtatagaatcaatca-3’; SPTLC2fw, 5’-ggtgttagcaacagactactgct-3’; SPTLC2rv, 5’-tggctcacaagggccae-3’; SPTLC3fw, 5’-tgcaagcagatgtagctac-3’; and SPTLC3rv, 5’-gcagatgacacagagccae-3’.

Amplification was carried out for SPTLC1: 40 cycles, each consisting of 10 s at 95 °C, 10 s at 58 °C, and 20 s at 72 °C; for SPTLC2: 40 cycles, each consisting of 10 s at 95 °C, 10 s at 58 °C, and 20 s at 72 °C; and for SPTLC3: 50 cycles, each consisting of 10 s at 95 °C, 10 s at 61 °C, and 20 s at 72 °C. The linearity of the assays was determined by serial dilutions of the templates for each primer set separately.

**Activity Assay**—A reaction mixture containing final concentrations of 25 mM Hepes (pH 8.0), 2.5 mM EDTA, 0.5 mM L-serine, 0.05 mM palmitoyl-CoA, 50 μM pyridoxal 5'-phosphate, 0.2 μCi of L-[U-14C]serine (Amersham Biosciences). The reaction was performed on ~400 μg of total extract at 37 °C for 60 min. For some assays the buffer was adjusted to 0.2% Triton X-100 (TX100) as stated in the text. The control samples included myriocin (40 μM) to specifically inhibit SPT activity. Total assay volume was 200 μL. The reaction was stopped by adding 400 μL of 0.5 N NH₄OH, followed by the addition of 2.25 ml of chloroform:methanol (1:2). Long-chain bases were extracted by adding 0.75 ml of NH₄OH and 0.75 ml of chloroform, vortexing, and centrifuging briefly. The upper aqueous layer was aspirated, and the lower layer was washed two times with 2 ml of 0.1 M KCl. The lower organic phase was removed and counted for radioactivity.

**RESULTS**

By using a systematic screen of the genomic data base for sequences that are homologous to SPTLC1 and SPTLC2 we identified a short open reading frame of 134 amino acids (NICB accession number BAC11509). This short sequence showed 65% identity (86% similarity) with the C-terminal part of SPTLC2. Three further overlapping expressed sequence tags were identified upstream (accession numbers: CF994231, AL109983, and AK075271), which revealed a continuous open reading frame of 1660 bp. This open reading frame encodes for a polypeptide with a theoretical molecular mass of 63 kDa and an overall identity to the SPTLC2 subunit of 68% (84% similarity). The identity to the SPTLC1 subunit was 21% (45% similarity). A data base scan for conserved structural elements (NCBI) assigned the relationship to the family of aminotransferases type II in accordance with the identification of a conserved PLP binding domain at position 371. Based on its homology to SPTLC2 the new protein was named SPTLC3. No overlapping expressed sequence tags were found further upstream.

The expression of the in silico identified cdna was verified by PCR using specific primer sets. Based on a human cDNA library we amplified a DNA fragment that matched to the expected size of 1.7 kbp. Subsequent DNA sequencing proofed the identity of this product with the sequence in the data base. Similar sequences were also found for other species like rat, mouse, and dog (accession numbers: AY417571.1, XM542889.1, AY417572.1, and XM230620.3). The alignment between SPTLC2 and SPTLC3 (Fig. 1a) revealed a highly homologous region within the core of the two proteins and less conserved N- and C-terminal tails. A genomic analysis showed that the SPTLC3 gene is located on chromosome 20 p12.1–12.3 and composed of 12 exons (Fig. 1b).

The mRNA expression levels of SPTLC1, -2, and -3 were analyzed by quantitative reverse transcription-PCR in a commercially available 24-human tissue cDNA panel. For each subunit a specific primer set was designed, and PCR conditions were optimized. For all primer sets a single PCR product of the expected size was seen on the agarose gel. Neither primer dimers nor unspecific amplifications were detected. The melting curve analysis showed a single peak for each primer set, which indicates specific amplifications of the particular cDNAs.

SPTLC2 was detected in all 24 tissues, whereas SPTLC1 could not be detected in small intestine. SPTLC3 was detected in most tissues except peripheral blood cells and bone marrow (Fig. 2a). To compare the relative expression of the three SPT subunits between the tissues we normalized the expression of SPTLC2 and SPTLC3 to the expression levels of SPTLC1 mRNA (Fig. 2b).

High expression levels of SPTLC3 were observed in heart, kidney, liver, uterus, and skin tissue but most strikingly in placenta where the ratio SPTLC3:SPTLC1 was 500-fold higher.
than the ratio SPTLC2:SPTLC1. For most tissues the SPTLC2:SPTLC1 ratio remained relatively constant (values between 5 and 15), whereas the ratio SPTLC3:SPTLC1 showed a much higher variability with values ranging between 2 in colon and up to 1500 in placenta. Slightly higher SPTLC2:SPTLC1 ratios were observed in those tissues that do not express SPTLC3 (peripheral blood cells and bone marrow).

Expression of SPTLC1–3 in Human Cell Lines—The mRNA expression of SPTLC1, SPTLC2, and SPTLC3 was tested in Hek293 and HepG2 cells. Because the tissue panel showed a high SPTLC3 expression in placenta tissue we also determined the expression in two human trophoblastic cell lines (JEG3 and JAR) and in human placenta tissue, which we obtained freshly after a cesarean section (Fig. 3a). As seen before, the SPTLC1 and SPTLC2 expression levels were similar in the tested cell lines, whereas the SPTLC3 expression showed notable differences. Hek293 cells expressed SPTLC3 only in minute amounts, whereas HepG2 cells showed a significantly higher SPTLC3 expression. The expression of SPTLC3 in the trophoblast cell lines JEG3 and JAR was 7- to 8-fold higher than in HepG2 cells. In agreement with the tissue panel data we found the highest expression in the primary placenta sample.

We verified these results at the protein level by generating polyclonal peptide antibodies against the N- and C-terminal tails of the three subunits SPTLC1–3 (see “Material and Methods”). The pepptide antibodies were purified from the respective antisera, using a peptide affinity column with the covalently coupled immunization peptides. All antibodies specifically detected proteins in the expected size on a Western blot (Fig. 3b). SPTLC1 was detected at 55 kDa and SPTLC2 at 65 kDa, whereas SPTLC3 migrated to a size of 63 kDa. The specificity of the antibodies was verified by probing the blots with preimmune serum and, secondly, by competing the antigen binding with an excess of free immunization peptide. In both cases no signal was observed (data not shown).

Although all generated antibodies specifically recognized the targeted proteins the sensitivity of the antibodies against SPTLC1 and SPTLC2 were higher than those against SPTLC3. This resulted in a generally weaker signal for SPTLC3 on the blot compared with SPTLC1 or SPTLC2, although the amount of SPTLC3 mRNA in these cells was comparable or even higher. In agreement with the mRNA expression data the cell lines showed a variable expression of the SPTLC3 subunit on the protein level (Fig. 3b). No signal for SPTLC3 was seen in Hek293 cells, which also express only negligible levels of SPTLC3 mRNA. HepG2 cells show a faint band and a moderate mRNA expression. The trophoblastic cells JEG3 and JAR showed a strong signal for SPTLC3, and in agreement with the reverse transcription-PCR data we observed the strongest signal in placenta tissue. In contrast to SPTLC3, SPTLC1 and SPTLC2 were expressed in rather constant amounts in the different cell lines.

SPTLC3 Overexpression Increases SPT Activity 2- to 3-Fold—To test whether SPTLC3 influences SPT activity, we cloned the cDNAs for SPTLC1, SPTLC2, and SPTLC3 into a mammalian expression vector (pcDNA3.1) together with a C-terminal V5-His tag extension. Each of the three subunits was transfected and overexpressed in Hek293 cells. To avoid differences in the expression levels due to variations in the transfection efficiency we generated stable cell lines for all the constructs. By using an anti-V5 antibody all three subunits were specifically detected at similar levels. On SDS-
PAGE the expressed proteins separated according to their expected size (including the mass addition of 5 kDa due to the V5-His tag) and revealed no signs of degradation (Fig. 4a).

SPT activity (Fig. 4b) was determined with the help of an established assay using 14C-labeled serine as the substrate (10). No increase in SPT activity was seen in the SPTLC1-overexpressing cells. The overexpression of SPTLC2 and SPTLC3 resulted in a 2- to 3-fold higher SPT activity compared with the control, whereas the increase was a bit less pronounced in the SPTLC3-overexpressing cells compared with the SPTLC2-overexpressing cells. This result shows that the newly identified SPTLC3 subunit, like SPTLC2, plays a functional role in the SPT reaction.

Silencing of SPTLC3 by siRNA Decreases SPT Activity—Because the overexpression of SPTLC3 increased SPT activity, we were interested if silencing the expression of this subunit has the opposite effect. We transfected Hek293, HepG2, JEG3, and JAR cells with SPTLC3-specific siRNA. The SPTLC3 siRNA sequence had no similarity to the sequences of SPTLC1 and SPTLC2 to exclude any accidental down-regulation of these subunits. The transfection of the siRNA resulted in a 60–70% reduction of SPTLC3 mRNA expression compared with the mock transfected controls (Fig. 5a). The reduced expression of SPTLC3 mRNA also led to reduced protein expression as seen on the Western blot.

Silencing of SPTLC3 expression resulted in a significant decrease in SPT activity (Fig. 5b). The activity reduction correlated well with the residual amount of endogenously expressed SPTLC3 mRNA. No silencing effect was seen in Hek293 cells, which however express virtually no SPTLC3, whereas a slight reduction could be observed in HepG2 cells. A 30–40% reduction was seen in JAR and JEG3 cells, which also express the highest levels of SPTLC3. These results further support the notion that SPTLC3 is a functional factor contributing to cellular SPT activity.

Triton X-100 Interferes with the SPTLC3 but Not SPTLC2 Reaction—We observed that the addition of 0.2% TX100 to the activity assay increased SPT activity ~10-fold in Hek293 cells (Fig. 6a). This increase in activity could be caused by an improved solubilization of the membrane-bound SPT complex, which subsequently results in a higher specific SPT activity. On the other hand, SPT activity in placenta extract was ~30% reduced in the presence of TX100. Furthermore, the enzymatic activity of the soluble SPT isofrom from S. paucimobilis (sSPT) is almost completely abolished in the presence of TX100. To better understand the effect of TX100 on SPT activity, we compared the activity in the SPTLC1–3-overexpressing cells in the presence or absence of 0.2% TX100. Surprisingly, the presence of TX100 had almost no effect on the SPTLC2-overexpressing cells, whereas the stimulating effect of SPTLC3 overexpression was completely abolished (Fig. 6b). No change in activity was observed in SPTLC1-expressing cells. This indicates that the SPTLC3-mediated reaction differs from the SPTLC2-mediated reaction. It can also explain why TX100 leads to a 10-fold activation in Hek293 cells but to a 30% decrease in placenta tissue. Due to the lack of SPTLC3 expression in Hek293 cells (Fig. 3a), SPT activity depends here mainly on the SPTLC2-mediated reaction (Fig. 2), which is activated by TX100. In contrast, placenta tissue expresses strongly SPTLC3 but only little SPTLC2. The addition of TX100 leads to an inhibition of the SPTLC3 and in parallel to an activation of the SPTLC2-mediated reaction. Therefore we observe in this tissue a net reduction of 30% in the presence of TX100.
Four missense mutations in the SPTLC1 gene (C133W, C133Y, V144D, and G387A) were reported to be responsible for the development of an inherited sensory neuropathy (hereditary sensory neuropathy type I (HSN1)) (11, 12). HSN1 is clinically defined by a severe sensory loss, which leads to painless injuries, chronic skin ulcers, and distal amputations, distinguishing it from other dominantly inherited sensory-motoric neuropathies such as Charcot-Marie-Tooth type 2 syndromes (13). Although SPT activity is essential for embryogenesis and cell survival (6), the pathological effects of these mutations are restricted to the peripheral nerve and do not commonly involve other sphingolipid-rich tissues like central nervous system, lung, or kidney. This indicates variable susceptibility of the different tissues for an impaired SPT activity. One explanation of this issue could be the existence of different SPT isoforms (6). In agreement with these results silencing of SPTLC3 expression by transfecting a SPTLC3-specific siRNA significantly decreased cellular SPT activity. The activity reduction showed a close correlation to the amount of endogenously expressed SPTLC3 mRNA in the various cell lines.

Although SPTLC3 and SPTLC2 show similar enzymatic functions, we also unraveled some different biochemical properties between the two subunits. Most notably TX100 inhibits the SPTLC3-based SPT reaction but activates the SPTLC2-mediated reaction. We hypothesize that TX100 interferes with substrate binding or the active site in SPTLC3, because the inhibitory effect of TX100 was also observed for the soluble SPT isoform of S. paucimobilis.

The fact that the individually expressed SPTLC2 and SPTLC3 subunits increase SPT activity indicates that both subunits can act independently. This raises the question on the mechanistic role of the different SPT subunits. It was shown in yeast that the SPTLC1 subunit contains two transmembrane domains and is bound to the outer membrane of the endoplasmic reticulum (4, 15). The overexpression of SPTLC1 did not
increase SPT activity (Fig. 4b), suggesting that SPTLC1 is not directly involved in the catalytic reaction. Instead, SPTLC1 may rather act as an anchor that targets SPTLC2 and SPTLC3 to the endoplasmic reticulum membrane. However, the situation seems to be more complex. The tissue expression pattern showed that, by contrast to the ratio of SPTLC2:SPTLC1, the ratio of SPTLC3:SPTLC1 expression is highly variable between various tissues. Of special note are the very high expression levels in placenta and the trophoblast cell lines. This argues for a tissue-specific demand of SPTLC3 expression, possibly dependent of the physiological need, for instance, a highly active sphingolipid metabolism.

Myriocin, a specific inhibitor for SPT, shows immunosuppressive activity, and several other findings indicate a link between SPT activity and immune response (16–18). In this regard it is interesting that SPTLC3 is not or only little expressed in tissues that are functionally connected to erythropoiesis or immune response like peripheral blood cells, bone marrow, and spleen. Instead, the lack of SPTLC3 expression seems to be compensated by increased expression of SPTLC2 in these tissues.

The highly variable SPTLC3 expression in various tissues raises further questions about the structure and stoichiometry of the SPT enzyme. SPT is reported to be a dimer composed of SPTLC1 and SPTLC2. Due to the similarity between SPTLC2 and SPTLC3 it is likely that SPTLC3 also forms dimers with SPTLC1.

However, the composition of the enzyme is an interesting issue especially in the context of the highly variable ratios of SPTLC3 to SPTLC1 mRNA expression. Because SPTLC3 shows an expression of up to 1500-fold higher on the mRNA level than SPTLC1, it is unlikely that all SPTLC3 subunits can bind to SPTLC1 in a 1:1 ratio. Also the mRNA of SPTLC2 shows generally higher expression levels than the SPTLC1 mRNA. One possible explanation for this issue would be that the unbound SPTLC2 and SPTLC3 subunits are rapidly degraded within the cell. This concept is supported by the observation that an SPT activity-deficient Chinese hamster ovary cell line (LY-B) (19), which shows no expression of SPTLC1, also has a very low expression of SPTLC2, although the mRNA levels for SPTLC2 are normal. Stable transfection of LY-B cells with SPTLC1 cDNA restores both SPT activity and SPTLC2 expression levels (15), thus indicating that the stability of SPTLC2 depends on the presence of SPTLC1. On the other hand, the fact that overexpression of either SPTLC2 or SPTLC3 results in a 2- to 3-fold increase in SPT activity indicates that not SPTLC1
expression but rather SPTLC2 and SPTLC3 are the limiting factors for SPT activity. Another explanation could be that the functional SPT is not a dimer but a higher organized complex that consists of several subunits. This hypothesis was put forward recently by Hojjati and colleagues (6). However, further investigations are necessary to unravel the structure of the SPT enzyme and the role of SPTLC1 in this context.

It will also be interesting to investigate if the reported HSN1 mutations in the SPTLC1 gene have the same impact on the SPTLC2- and SPTLC3-mediated reaction. The observation that SPTLC2 and SPTLC3 have similar functions but different tissue-expression patterns could provide an explanation why certain tissues are more susceptible for the HSN1 mutations than others. Up to now, four mutations in the SPTLC1 gene were identified as the elicitor of HSN1 in several patients. However, there are further cases of HSN1 where no mutation was found in either the SPTLC1 gene or the SPTLC2 gene (20). It would be interesting, therefore, to see if the SPTLC3 gene shows mutations in those unresolved HSN1 cases.

In conclusion, our results show that SPT is organized in a more complex way than earlier believed. The existence of a third subunit raises new questions as to the biochemical function and cellular role of this enzyme. Further studies are therefore necessary to get a better knowledge on the organization and cellular role of this enzyme.

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