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Sphingosine-1-phosphate lyase SPL is an endoplasmic reticulum-resident, integral membrane protein with the pyridoxal 5′-phosphate binding domain exposed to the cytosol

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Abstract

Sphingosine-1-phosphate (S1P) is a sphingolipid metabolite that functions as a bioactive lipid molecule. S1P is degraded either by S1P lyase or by S1P phosphohydrolase. The gene encoding mammalian S1P lyase, *SPL*, has been identified. Here, we characterize the SPL protein in its expression, localization, and topology. The expression levels of the SPL protein correlated well with the dihydrosphingosine-1-phosphate (DHS1P) lyase activity in most tissues. However, liver and heart exhibited high DHS1P lyase activities compared to their SPL protein levels. The *SPL* mRNA expression was temporally regulated during mouse embryonal development. Immunofluorescence microscopy demonstrated that SPL is localized at the endoplasmic reticulum. Proteinase K digestion studies revealed that the large hydrophilic domain, containing the active site, faces the cytosol. This active site orientation is opposite to that of S1P phosphohydrolase, indicating that the degradation of S1P by two S1P-degrading enzymes occurs in spatially separated sides of the endoplasmic reticulum.

Keywords: Sphingosine-1-phosphate lyase; Sphingosine-1-phosphate; Sphingolipid; Glycerophospholipid; Endoplasmic reticulum; Pyridoxal 5′-phosphate; Platelet

The bioactive lipid molecule sphingosine-1-phosphate (S1P) regulates diverse biological processes, including proliferation, differentiation, migration, and apoptosis, by acting as both an extracellular ligand for S1P family receptors and an intracellular second messenger [1–5]. S1P is synthesized from sphingosine (Sph) by Sph kinase and is degraded either by S1P lyase to hexadecenal and phosphoethanolamine, or by S1P phosphohydrolase to Sph. Thus, the overall cellular S1P level is determined by a balance between its mediated synthesis and degradation.

The yeast gene *BST1/DPL1*, which encodes S1P lyase, was first identified during a study that isolate multicopy suppressors of a sphingosine-sensitive phenotype [6]. This enzyme was further found to be conserved among eukaryotes. The mouse S1P lyase gene *SPL* was later identified by its similarity to the *Caenorhabditis elegans* SPL gene [7].

Since the late 1960s, extensive enzymatic characterization of S1P lyase has been performed, mainly using the activity present in rat liver [8]. Studies have revealed a requirement for pyridoxal 5′-phosphate (PLP) as a cofactor and a sensitivity to heavy metal ions and detergent. More recently, the enzyme was determined to be localized in the microsomal fraction [9–12]. Moreover, it was demonstrated that S1P lyase recognizes as substrates both S1P and dihydrosphingosine-1-phosphate

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(DHS1P), a phospholipid synthesized from de novo dihydrosphingosine (DHS). Remarkably, though, this enzyme exhibits substrate specificity to only the naturally occurring D(+)-*erythro* isomers [10,13–15]. To date, however, there has been no evidence that *SPL* is the sole gene encoding a mammalian S1P lyase. Therefore, it is unclear whether the known enzymatic characteristics of the S1P lyase, determined using the activity present in rat liver, are fully attributable to the SPL protein. In the current study, we examine the tissue- and development-dependent expression of SPL, by Northern blotting, and the intracellular localization and topology, by immunoblotting. Our results provide evidence that the large hydrophilic domain of this enzyme, which contains residues involved in the active site, faces the cytosolic side of the endoplasmic reticulum (ER). Considering that the active site of S1P phosphohydrolase is localized on the luminal side of the ER, S1P is likely to be degraded in different leaflets of the ER membrane by two S1P-degrading enzymes.

Materials and methods

Cell culture and transfection. Mouse teratocarcinoma F9 cells and HeLa cells were grown in Dulbecco's modified Eagle's media D6429 or D6046, respectively (Sigma–Aldrich, St. Louis, MO), containing 10% fetal bovine serum and supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. F9 cells were grown on 0.1% gelatin-coated dishes. F9-7 cells ($SPL^{-/-}$, neomycin- and puromycin-sensitive) were generated from F9-2 cells ($SPL^{-/-}$, neomycin- and puromycin-resistant) [16]. F9-8 cells are stably transformed F9-7 cells carrying the gene for N-terminally HA-tagged mouse SPL (HA-SPL) in F9-7 cells. Details of the construction of F9-7 and F9-8 cells will be described elsewhere. Transfections were performed using Lipofectamine Plus and Lipofectamine 2000 reagents for HeLa cells and F9 cells, respectively, both from Invitrogen (Carlsbad, CA).

Plasmids. The plasmid pcDNA3-FLAG-SPL, which encodes N-terminally FLAG-tagged mouse SPL, was constructed by cloning a 1.8 kb *Bgl*II–*Not*I fragment of pGEM-SPL [16] into the *Bam*HI–*Not*I site of pcDNA3-FLAG1 [17].

A derivative of the pCE-puro vector, pCE-puro-3×HA4, was designed for use in producing C-terminally, triple HA-tagged (3×HA) proteins. To generate pCE-puro-SPL-3×HA, the *SPL* cDNA was first amplified by PCR using primers 5′-AGATCTGCCACCATGC CCGGAACCGACCTCCTCAAG-3′ and 5′-GCTAGCGCGGGG CTTTGGAGAACCGTTC-3′ and the pGEM-SPL plasmid [16] as a template. The amplified fragment was then cloned into pGEM-T Easy (Promega, Madison, WI) to generate the pGEM-SPL-2 plasmid. The pCE-puro-SPL-3×HA was constructed by cloning a 1.8 kb *Bgl*II–*Nhe*I fragment of pGEM-SPL-2 into the *Bam*HI–*Nhe*I site of pCE-puro-3×HA4.

Measurement of DHS1P lyase activity. A DHS1P lyase assay was performed in vitro as reported [16], with minor modifications. [^{3}H]DHS1P was synthesized by incubating [4,5-^{3}H]D-*erythro*-DHS (50 Ci/mmol; American Radiolabeled Chemicals, St. Louis, MO) with purified yeast long-chain base kinase Lcb4p, in the presence of 10 mM $MgCl_2$ and 2 mM ATP, for 2 h at 37 °C. The [^{3}H]DHS1P was then purified by a two-step extraction method described previously [18] and suspended in 0.2% Triton X-100. Lysates were prepared from cultured cells or various tissues from 8-week-old C57BL/6 Cr mice, as described previously [16,19]. Briefly, cells or tissues were sonicated in buffer A (80 mM sodium phosphate (pH 7.4), 0.8 mM EDTA, 20 mM sodium fluoride, 9.6 mM β-glycerophosphate, 0.4 mM sodium pyrophosphate, 10% glycerol, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1× protease inhibitor mixture (Complete; Roche Diagnostics, Mannheim, Germany)). The resulting lysates were mixed with a 0.4-fold volume of buffer B (0.15 M sodium phosphate (pH7.4), 1.5 mM EDTA, 37.5 mM sodium fluoride, 18 mM β-glycerophosphate, 0.75 mM sodium pyrophosphate, 0.9 mM PLP, 8.9 mM sodium orthovanadate, 0.71% Triton X-100, 1 mM DTT, 1 mM PMSF, and 1× protease inhibitor mixture) and 2 μCi the [^{3}H]DHS1P. The enzyme reaction was performed by incubating the samples at 37 °C and terminated by adding 3.75 volumes of chloroform:methanol:HCl (100:200:1, v/v/v). Lipids were extracted with 1.25 volumes of chloroform and 1.25 volumes of 1% aqueous KCl. Phases were separated by centrifugation, and the organic phase was recovered, dried, and suspended in chloroform:methanol (2:1, v/v). The lipids were resolved by TLC on Silica Gel 60 high performance TLC plates (Merck, Darmstadt, Germany) with 1-butanol:acetic acid:H_2O (3:1:1, v/v/v), and were visualized by spraying the plates with the fluorographic reagent EN^{3}HANCE Spray (Perkin–Elmer, Boston, MA) and exposing them to X-ray film at −80 °C.

Immunoblotting and immunofluorescence microscopy. Cell lysates were prepared from cultured cells or various mouse tissues, as described previously [16,19]. Immunoblotting was performed as reported [16] using ECL or ECL plus Western blotting detection reagents (Amersham Biosciences, Piscataway, NJ). Primary antibodies used in the assay included our affinity-purified anti-SPL antibody [16], the anti-calnexin antibody H-70 (0.2 μg/ml), the anti-HA antibody Y-11 (0.2 μg/ml), and the anti-CYP1A1 antibody H-70 (0.2 μg/ml), all from Santa Cruz Biotechnology (Santa Cruz, CA), anti-GM130 antibody (1 μg/ml; BD Biosciences, Palo Alto, CA), and anti-KDEL antibody (2.5 μg/ml; Stressgen Biotechnologies, San Diego, CA). Peroxidase-labeled donkey anti-rabbit Ig F(ab′)$_2$ (diluted 1:10,000) and donkey anti-mouse Ig F(ab′)$_2$ (diluted 1:10,000), both from Amersham Biosciences, were used as secondary antibodies.

Immunofluorescence microscopic analysis was performed as described previously [20] using anti-FLAG M5 (5 μg/ml; Sigma) and anti-calreticulin (5 μg/ml; Alexis, Lausen, Switzerland) antibodies. Alexa 488-conjugated anti-mouse IgG (3.3 μg/ml), and Alexa 594-conjugated anti-rabbit IgG (5 μg/ml) antibodies, both from Molecular Probes (Eugene, OR) were used as secondary antibodies. Cells were observed under an LSM510 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany) with a 63× lens (Carl Zeiss).

Preparation of smooth and rough endoplasmic reticulum. Liver was isolated from an adult C57BL/6 J mouse, washed with cold phosphate-buffered saline (PBS), and lysed, using an electric Potter homogenizer for 10 strokes, in buffer C (10 mM Tris–HCl (pH 7.5), 0.25 M sucrose, 5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, and 1× protease inhibitor mixture). After one centrifugation at 8000*g* at 4 °C for 10 min, the supernatant was further centrifuged at 105,000*g* at 4 °C for 1 h. The resulting pellet (microsomal fraction) was suspended in 1.67 ml buffer C and then mixed with 2.51 ml of a 2.1 M sucrose solution in buffer D (50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, and 1× protease inhibitor mixture). This mixture (final concentration 1.35 M sucrose) was layered onto 3 ml of a solution of 2.1 M sucrose in buffer D, and covered with 3 ml of a 0.8 M sucrose solution in buffer D, thereby providing a sucrose gradient. Samples were centrifuged at 105,000*g* at 4 °C for 6 h. The smooth ER (SER) at the 0.8 M/1.35 M sucrose interface and the rough ER (RER) at the 1.35 M/2.1 M sucrose interface were collected.

Preparation of soluble and membrane fractions. F9-8 cells were washed twice with PBS, suspended in buffer E (20 mM Hepes–NaOH (pH 7.5), 0.25 M sucrose, 1 mM DTT, 1 mM PMSF, and 1× protease inhibitor mixture), and sonicated. After centrifugation at 300*g* for 3 min at 4 °C, the resulting supernatant (total lysate) was treated with an equal volume of buffer E, 0.2 M Na_2CO_3 (pH 11.5), 0.2 M NaOH, 5 M urea, or 2 M NaCl. After a 1 h incubation at 4 °C, the mixtures

were centrifuged at 100,000*g* for 1 h at 4 °C to separate soluble and membrane fractions.

Proteinase K digestion. F9-7 cells transfected with pCE-puro-SPL-3×HA were washed twice with PBS, suspended in buffer E, and lysed using an electric Potter homogenizer for 10 strokes. After removal of cell debris by centrifuging at 300*g* for 3 min at 4 °C, the supernatant was subjected to centrifugation at 100,000*g* for 1 h at 4 °C. The resulting pellet was suspended in buffer E (without protease inhibitors) and treated with 0.5 mg/ml proteinase K on ice for 1 h, in the presence or absence of 1% Triton X-100. After termination of the digestion with 1 mM PMSF, total proteins were precipitated with ice-cold 5% trichloroacetic acid for 20 min. Protein precipitates were washed with 5% trichloroacetic acid and with acetone, and were then suspended in SDS sample buffer (62.5 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, and a trace amount of bromophenol blue) containing 1 mM PMSF. Proteins were separated on a 10% polyacrylamide gel by SDS–PAGE and subjected to immunoblotting as above.

Northern blotting. Total RNA blots containing 20 μg RNA from several mouse embryo stages were purchased from Seegene (Seoul, Korea). A probe for the *SPL* mRNA was prepared using a 0.53-kb DNA fragment corresponding to the middle region of the *SPL* ORF. This fragment was amplified using primers 5′-GGCGTTAGAGA AGGGGATCAAAACTCC-3′ and 5′-CCAGGCCGTGAGCCAGCT ATGCTTGG-3′ from pGEM-SPL plasmid [16]. The amplified fragments were then gel-purified and labeled with [α-^{32}P]dCTP (3000 Ci/mmol; Perkin–Elmer Life and Analytical Sciences, Boston, MA) by random priming, using Random Primer DNA Labeling Kit Ver.2 (Takara Bio, Shiga, Japan). After hybridization with ExpressHyb buffer (BD Biosciences Clontech, Palo Alto, CA) at 68 °C for 2 h, the membrane was washed and exposed to X-ray film at −80 °C.

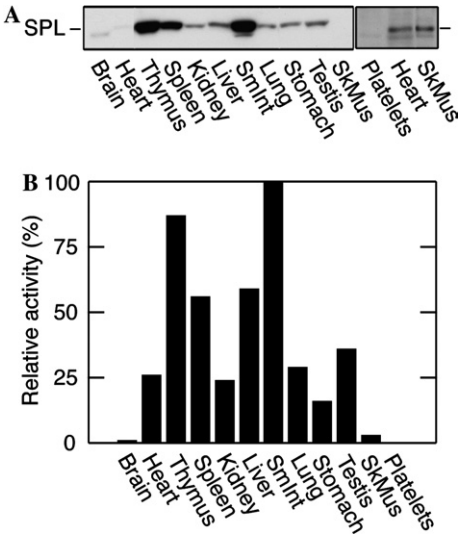

Fig. 1. Tissue distribution patterns of SPL. (A) Immunoblot of the SPL protein in mouse tissues. Total lysates (20 μg protein per lane) prepared from 12 different mouse tissues were separated by SDS–PAGE, transferred to a polyvinylidene fluoride membrane, and detected by immunoblotting with anti-SPL antibodies. Left panel was detected using ECL reagent and exposing to film for 5 min, whereas right panel was detected using ECL plus reagent and exposing to film for 20 min. (B) DHS1P lyase activity in mouse tissues. Samples (300 μg protein) from the same total lysates used in (A) were incubated with 2 μCi [^{3}H]DHS1P at 37 °C for 1 h. Lipids were extracted and separated by TLC with 1-butanol:acetic acid:H_2O (3:1:1, v/v/v). Values shown are relative to the DHS1P lyase activity exhibited by small intestine. Two independent experiments gave similar results, and a representative result is shown. SmInt, small intestine. SkMus, skeletal muscle.

Results

Tissue distribution of SPL

Gene expression of mouse SPL has been analyzed only by Northern blotting in a limited number of tissues [7]. To investigate the tissue-specific expression of the SPL protein more extensively, immunoblotting was performed on samples from 12 different mouse tissues. As shown in Fig. 1A, expression levels of the SPL protein were highest in small intestine and thymus. Spleen also expressed high levels of the SPL protein, whereas liver, kidney, lung, stomach, and testis expressed only moderate levels, and brain, heart, and skeletal muscle barely detectable amounts (left panel). SPL was also not detected in platelets, the only tissue known to have no S1P lyase activity [21]. Even after a longer film exposure, which aided detection in heart and skeletal muscle (right panel), no expression was observed in platelets.

We also measured in vitro the DHS1P lyase activities in the tissue lysates. As shown in Fig. 1B, the highest activity was detected in small intestine and thymus, followed by liver and spleen. Most of these high activities correlated well with the high protein levels of SPL, except in liver, which expressed only intermediate levels of SPL. Testis, heart, lung, kidney, and stomach all displayed moderate activity, whereas brain and skeletal muscle exhibited low activity, and platelets no activity. Again, the relatively high DHS1P lyase activity in heart did not correlate with the very low expression of SPL.

Subcellular localization of SPL

We investigated the intracellular localization of SPL in detail. Indirect immunofluorescent microscopic analysis demonstrated that FLAG-tagged SPL expressed in HeLa cells was localized at the ER, as its fluorescence completely merged with that of calreticulin, an ER protein (Fig. 2A). The ER is categorized into two subcompartments, the rough ER (RER) and smooth ER (SER), by the presence or absence of ribosomes. The SER is abundant only in certain cell types including steroid-synthesizing cells and liver cells, and it plays an important role in lipid metabolism. Although most membrane proteins are shared between the RER and SER, some proteins, especially those involved in lipid metabolism such as cytochrome P-450, are enriched only in the SER [22]. We investigated the distribution of SPL between the RER and SER in mouse liver extracts using microsomes prepared by sucrose-density gradient centrifugation. SPL, together with ER chaperones such as calnexin, Grp94, and Grp78, was recovered nearly equally from

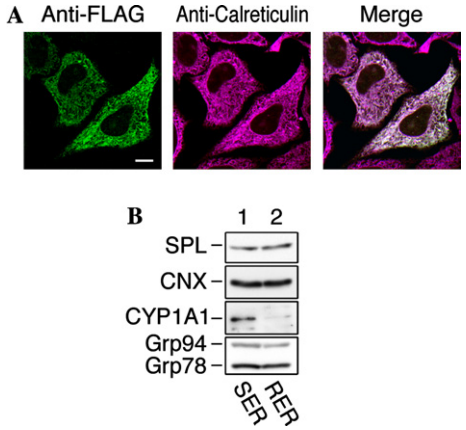

Fig. 2. SPL is localized at the ER. (A) HeLa cells transiently transfected with pcDNA3-Flag-SPL were fixed with 3.7% formaldehyde, permeabilized with 0.5% Triton X-100, and subjected to immunostaining with anti-FLAG (left panel) and anti-calreticulin (middle panel) antibodies, and the images merged (right panel). Bar, 10 μm. (B) The microsomal fraction prepared from mouse liver was separated into SER (lane 1) and RER (lane 2) by sucrose-density gradient centrifugation, and these were subjected to immunoblotting with anti-SPL, anti-calnexin, anti-CYP1A1, or anti-KDEL (to detect Grp94 and Grp78) antibody.

the SER and RER, in contrast to the cytochrome P-450 CYP1A1, which was localized predominantly in the SER (Fig. 2B).

Membrane topology of SPL

We also examined the membrane association of SPL under various conditions using F9-8 cells, which stably express HA-tagged SPL. The association was not disturbed by alkaline, urea, or high salt treatment (Fig. 3, upper panel). These results were similar to those for the integral membrane protein marker calnexin (Fig. 3, middle panel), but contrasted sharply with those for the peripheral membrane protein marker GM130, which

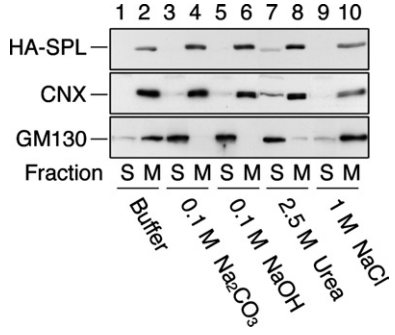

Fig. 3. SPL is a membrane-spanning protein. Total lysates prepared from F9-8 cells ($SPL^{-/-}$/*HA-SPL*) were treated with buffer, 0.1 M Na_2CO_3, 0.1 M NaOH, 2.5 M urea, or 1 M NaCl, separated by centrifugation at 100,000*g* for 1 h into soluble (S) and membrane (M) fractions, and examined by immunoblotting with an anti-HA, anti-calnexin, or anti-GM130 antibody. CNX, calnexin.

was dissociated from the membrane by alkaline or urea treatment (Fig. 3, lower panel). These data indicated that SPL is an integral membrane protein, which is consistent with the prediction that SPL contains a transmembrane segment at the N-terminus.

We further investigated the orientation within the ER membrane of the large hydrophilic domain in SPL that follows the putative N-terminal transmembrane segment. For this purpose, intact organelles prepared from F9-7 cells transiently transfected with the pCE-puro SPL-3×HA plasmid were treated with proteinase K in the presence or absence of Triton X-100. Calnexin, a type I membrane protein with an N-terminal large domain facing towards the lumen of the ER [23], was used as a control for preparing the intact ER. Upon treatment with proteinase K in the absence of Triton X-100, the C-terminal tail of calnexin was digested, generating calnexinΔC (Fig. 4A, lane 8). On the other hand, no similarly protected band for the SPL protein was detected by immunoblotting with either an anti-SPL or anti-HA antibody (Fig. 4A, lanes 2 and 5, repectively).

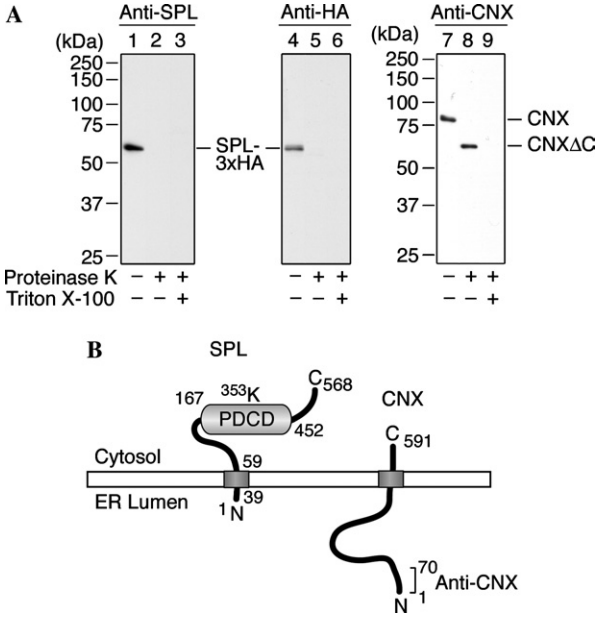

Fig. 4. Determination of the membrane topology of the SPL protein. (A) Total membrane fractions were prepared from F9-7 ($SPL^{-/-}$) cells transfected with pCE-puro-SPL-3×HA and treated without or with 0.5 mg/ml proteinase K, on ice for 1 h in the absence or presence of 1% Triton X-100. Proteins were separated by SDS–PAGE and transferred to polyvinylidene fluoride membranes, followed by immunoblotting with an anti-SPL (lanes 1–3), anti-HA (lanes 4–6), or anti-calnexin (lanes 7–9) antibody. The anti-calnexin antibody recognizes the N-terminal region of calnexin (amino acid residues 1–70). (B) A model depicting the proposed topology of SPL, and the established topology of calnexin [23]. SPL is an integral membrane protein with one transmembrane domain at the N-terminus. Its N-terminus is located in the lumen of the ER, whereas the large hydrophilic domain containing the active site is located in the cytosol. CNX, calnexin. PDCD, pyridoxal-dependent decarboxylase conserved domain.

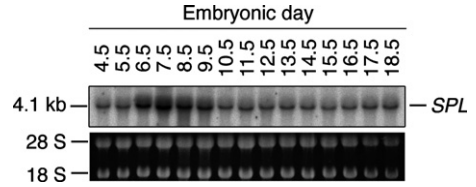

Fig. 5. The expression of *SPL* mRNA is developmentally regulated. (upper panel) A ^{32}P-labeled *SPL* probe was hybridized to 20 μg of total RNA from each stage of mouse embryo. (lower panel) 28S and 18S ribosomal RNAs were stained with ethidium bromide to demonstrate uniform RNA loading.

These results indicated that the large hydrophilic domain of SPL is exposed to the cytosol.

SPL expression during mouse embryogenesis

Recently, it was reported that SPL expression was developmentally regulated in *Dictyostelium discoideum*, *C. elegans*, and *Drosophila melanogaster* [24–26]. We also previously demonstrated that SPL expression is increased during in vitro primitive endoderm differentiation using embryonic carcinoma (EC) F9 cells [16]. However, whether, how, and to what extent expression of SPL is regulated during mouse development remains unclear. Therefore, we examined the developmental stage specific expression pattern of *SPL* mRNA by Northern blotting. *SPL* mRNA was expressed throughout mouse embryogenesis. Additionally, a transient increase was observed from embryonic day (E) 5.5 to E 7.5 (Fig. 5).

Discussion

S1P lyase activity had already been detected by the late 1960s [27–32], and its enzymatic characteristics, including substrate specificity, localization, and tissue distribution, were investigated extensively. However, identification of the mammalian gene (*SPL*) encoding S1P lyase occurred only recently in 1998 [7] and direct studies regarding gene expression have been few. We investigated the tissue distribution patterns of the SPL protein and the DHS1P lyase activity in detail and found some inconsistencies (Fig. 1). Our results for the DHS1P lyase activity of various mouse tissues were similar to previous results for several rat tissues [8], and most activities correlated with the expression levels of the protein (Fig. 1). However, although the SPL protein levels in liver were equivalent to those in lung, stomach, testis, and kidney, the activity in liver was approximately 2-fold higher than activities in the other tissues. Furthermore, the amount of SPL in liver was much less than that in spleen, yet the activities in these tissues were nearly equal (Fig. 1). In addition, SPL was barely detectable in heart, while the activity in this tissue was comparable to that in kidney or lung (Fig. 1). It is possible that another S1P lyase exists, and that liver and heart express the second enzyme in high amounts. Alternatively, the activities of *SPL* may be up-regulated in liver and heart. We prefer the former possibility, since the *SPL*-null cells (F9-7) still possess weak but significant S1P lyase activity (M. Ikeda, A. Kihara, Y. Kariya, and Y. Igarashi, unpublished results).

S1P functions as a bioactive lipid molecule not only extracellularly but also intracellularly. Its intracellular levels are maintained by a balance in the activities of the synthesizing enzyme, Sph kinase, and the degrading enzymes, S1P lyase and S1P phosphohydrolase. In addition to their contributions to signaling, these enzymes seem to play important roles in regulating the cellular amounts of sphingolipids and glycerophospholipids, since some tissues, such as small intestine and thymus, possess high activities for both Sph kinase [19] and S1P lyase (Fig. 1B). Small intestine might actively convert sphingolipids absorbed from food into glycerophospholipids.

Immunofluorescence microscopic analysis indicated that mouse SPL is localized at the ER (Fig. 2A), which is consistent with a recent report regarding human SPL [33]. Separation of the SER and RER by sucrose density gradient centrifugation demonstrated that SPL is equally distributed between the SER and RER (Fig. 2B). The membrane association of SPL was resistant to alkaline, urea, and high salt treatments, suggesting that SPL is an integral membrane protein (Fig. 3). Proteinase K digestion assays revealed that most of the SPL protein is exposed to the cytosol (Fig. 4A). From these results and predictions deduced from its sequence, we propose a structural model for the SPL protein as illustrated in Fig. 4B. In this model, SPL has one transmembrane segment at its N-terminus, with its large C-terminal domain facing the cytosol. SPL is a PLP-dependent enzyme, and its Lys residue (Lys-353) is an active site that forms an internal Schiff base with PLP. The pyridoxal-dependent decarboxylase conserved domain which contains Lys-353 is located in the C-terminal domain, suggesting that S1P is cleaved to hexadecenal and phosphoethanolamine on the cytosolic side of the ER membrane. The subcellular localization and topology of SPL revealed here are in agreement with previous enzymatic analyses of a S1P lyase [12]. This membrane topology is reasonable since glycerophospholipid synthesis also takes place in the cytoplasmic leaflet of the ER membrane bilayer. Interestingly, though, the active site of another S1P-degrading enzyme, S1P phosphohydrolase, is exposed to the opposite side of the ER membrane, the ER lumen [34]. Thus, the location at which these two enzymes degrade S1P differs.

Using F9 cells, we recently demonstrated that SPL is regulated in its expression during EC to primitive endoderm differentiation and that it is involved in this differentiation [16]. In the study presented here, we revealed that

expression of SPL is also regulated during embryogenesis. The expression was transiently increased during E 5.5 to E 7.5 (Fig. 5). E 7.5 is the approximate beginning of gastrulation, in which pronounced differentiation and rearrangement are observed. Therefore, it is possible that SPL has a role in mouse development, although further studies using SPL knock-out mice are required.

Acknowledgments

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