Sphingosine-phosphate Lyase Enhances Stress-induced Ceramide Generation and Apoptosis*

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Sphingosine-1-phosphate lyase is a widely expressed enzyme that catalyzes the essentially irreversible cleavage of the signaling molecule sphingosine 1-phosphate. To investigate whether sphingosine-1-phosphate lyase influences mammalian cell fate decisions, a recombinant human sphingosine-1-phosphate lyase fused to green fluorescent protein was expressed in HEK293 cells. The recombinant enzyme was active, localized to the endoplasmic reticulum, and reduced baseline sphingosine and sphingosine 1-phosphate levels. Stable overexpression led to diminished viability under stress, which was attributed to an increase in apoptosis and was reversible in a dose-dependent manner by exogenous sphingosine 1-phosphate. In contrast to sphingosine-1-phosphate lyase, the products of the lysis reaction had no effect on apoptosis. Lyase enzymatic activity was required to potentiate apoptosis, because cells expressing a catalytically inactive enzyme behaved like controls. Stress increased the amounts of long- and very long-chain ceramides in HEK293 cells, and this was enhanced in cells overexpressing wild type but not catalytically inactive lyase. The ceramide increases appeared to be required for apoptosis, because inhibition of ceramide synthase with fumonisin B1 decreased apoptosis in lyase-overexpressing cells. Thus, sphingosine-1-phosphate lyase overexpression in HEK293 cells decreases sphingosine and sphingosine 1-phosphate amounts but elevates stress-induced ceramide generation and apoptosis. This identifies sphingosine-1-phosphate lyase as a dual modulator of sphingosine 1-phosphate and ceramide metabolism as well as a regulator of cell fate decisions and, hence, a potential target for diseases with an imbalance in these biomodulators, such as cancer.

Sphingosine 1-phosphate (S1P)1 is a sphingolipid metabolite that regulates cell migration, survival, differentiation, angiogenesis, and development. S1P has been shown to activate an extracellular signaling pathway mediated through a family of specific G protein-coupled receptors (1, 2). However, evidence indicates that S1P also mediates effects through a receptor-mediated mechanism by functioning as a second messenger within cells (3). Intracellular S1P levels are regulated primarily by three highly conserved enzymes: sphingosine kinase (SPHK), which catalyzes the phosphorylation of sphingosine-producing S1P, S1P phosphatase (SIPP), which reverses the former reaction, and S1P lyase (SPL), a PLP-dependent enzyme that catalyzes the essentially irreversible cleavage of S1P at the C2–3 carbon–carbon bond, yielding phosphatidylcholine and a long-chain aldehyde (4–6).

One approach to uncovering roles of S1P signaling in biology has been to manipulate expression of the recently cloned genes of S1P metabolism. Toward that end, altered SPL expression in a variety of cell lines and mutant model systems has yielded pronounced effects and severe phenotypes. For example, Saccharomyces cerevisiae dpl1 null mutants, which lack SPL activity, are highly resistant to heat stress and nutrient deprivation, whereas overexpression of DPL1 can correct certain defects of endocytosis (7–11). In mouse embryonal carcinoma cells, disruption of the SPL gene enhances cellular differentiation in response to retinoic acid (12). In Drosophila melanogaster, Caenorhabditis elegans, and Dictyostelium discoideum SPL expression is required for global functions, including embryogenesis, reproduction, survival, and movement (13–15). These observations indicate that SPL has important and, in some cases, essential functions in cells and organisms. The effects of manipulating SPL expression are presumed, and in some cases have been demonstrated, to be secondary to alterations in intracellular pools of S1P and its availability to carry out downstream signaling functions. However, the specific intracellular mechanisms by which SPL exerts its effects are not well understood.

In this study, a recombinant human SPL-GFP fusion protein was expressed in HEK293 cells to elevate SPL activity and diminish cellular S1P. Somewhat unexpectedly, stable expression also enhanced stress-induced increases in ceramide. Overexpression of catalytically active SPL also increased apoptosis, which was reversed by the addition of exogenous S1P or the ceramide synthase inhibitor fumonisin B1. In contrast, the products of the reaction catalyzed by SPL, ethanolamine phosphate, and long-chain aldehydes, did not promote apoptosis. These studies show that SPL can affect cell fate and influence sphingolipid metabolism beyond the regulation of intracellular S1P.
EXPERIMENTAL PROCEDURES

Materials—o-erythro-Sphingosine, fumonisin B1, ethanolamine phosphate, and MITT reagent were obtained from Sigma Chemical Co. (St. Louis, MO), [4,5-3H]-o-erythro-dihydroxyphosphosine 1-phosphate (DHIS1P) was from American Radiolabeled Chemicals, Inc. (St. Louis, MO), S1P was from Avanti Polar Lipids (Alabaster, AL), ISP-1 was from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). Sequence 2.0 and [3H]-Serenine were from Amerham Pharmacia Biotech (Piscataway, NJ), and T7 primer from G. Watson, Children’s Hospital (Madison, WI). Hexadecanal and hexadecenal were synthesized essentially as described previously (16).

cDNA Cloning—BLAST search using the mouse SPL cDNA sequence against GenBank, dbEST identified two homologous human EST sequences, GenBank accession numbers T86263 and A338781 (17). T86263 was homologous to the 3’-end and A338781 to the 5’-region of mouse SPL. To clone the overlapping region of these two ESTs, primers H070F2 (Table I) and H967R2 were used for PCR amplification of the target cDNA from a human cDNA library constructed in the yeast shuttle vector pADNS (18). This PCR product was then cloned into pBluescript SK(-) at the SmaI site using the Sanger method (19). To clone the remainder of the 5’-end of the hSPL cDNA, a generic primer, ADHP2 (based on the vector’s promoter sequence), and a gene-specific primer, H967R3 (based on EST sequence T86263), were used for PCR amplification using the same cDNA library. The coding region of the hSPL cDNA was then re-cloned by RT-PCR amplification of total RNA from normal human diploid fibroblasts using N-terminal primer 5’-H967 and C-terminal primer 3’-H967. This RT-PCR product was cloned into pBluescript SK(+) at KpnI/XhoI sites to give plasmid, pBS/hSPL.

DNA Vector Constructs—The hSPL coding sequence was subcloned into pcDNA3.0 vector (Invitrogen, Carlsbad, CA) at KpnI/XhoI sites with an engineered ribosome binding site corresponding to the Kozak sequence, GCCACCATGG, for efficient expression in mammalian cells (20). This construct is referred to as pc-hSPL. A change of the coding sequence from +4 C to +4 G after the start codon ATG changes the codon for a proline to that of an alanine residue. The pc-hSPL cDNA was then re-cloned by RT-PCR amplification of total RNA from normal human diploid fibroblasts using N-terminal primer 5’-H967 and C-terminal primer 3’-H967. This RT-PCR product was cloned into pBluescript SK(+) at KpnI/XhoI sites to give plasmid, pBS/hSPL.

Cell Culture and Transfection—Human embryonic kidney cells (HEK293, American Type Culture Collection, Rockville, MD) were cultured in Dulbecco’s modified Eagle medium (Tissue Culture Facility UCSF, San Francisco, CA) containing 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 µg/ml), and amphotericin B (0.25 µg/ml), except where otherwise noted. For transfection, HEK293 cells were seeded at 1.5 × 10⁶ cells in 60-mm dishes in normal medium. After 20 h, cells were transfected with the appropriate vector construct using Effectene™ transfection reagent (Qiagen GmbH, Hilden, Germany). Transfection efficiency was determined by fluorescence microscopy by quantifying the proportion of GFP-positive cells. For transient transfection experiments, cells were harvested at 24–48 h after transfection. To obtain stably transfected cell lines, cells were exposed to selection medium containing G418 at 1 mg/ml at 48 h after transfection. Single clones of stably transfected cells were isolated and selected for evaluation according to the most intense green fluorescence indicating strongest expression of recombinant hSPL. S1P (dissolved is methanol prior to use) was added at 12 h after serum deprivation; fumonisin B1 (dissolved in PBS) was added 24 h prior to harvest. Ethanolamine phosphate was dissolved in water. Hexadecanal and hexadecenal were dissolved in ethanol and delivered in a 1:1 molar complex with bovine serum albumin.

Western Blotting—For detection of mammalian SPL protein, an antibody was raised in rabbit against a peptide sequence comprised of 10 amino acid residues (residue #559–568, Ser-Gln-Met-Ser-Gly-Ser-Pro-Lys-Pro-His-COO-) at the C terminus of the hSPL protein (Genmed Synthesis, Inc., South San Francisco, CA). Transfected HEK293 cells were lysed by repeated freeze-thawing in 0.25 M Tris-HCl (pH 7.5) buffer. Lysates were centrifuged at 14,000 × g for 30 min. The pellets were resuspended in the same buffer. For each lane, 50 µg of protein from the pellet was separated by 8% SDS-PAGE prior to immunoblotting with the above antibody. Bands were visualized using chemiluminescent reagent, BM Chemiluminescence Western blotting kit (Roche Molecular Biochemicals GmbH, Mannheim, Germany), using horseradish peroxidase-conjugated antibody, goat anti-mouse horseradish peroxidase-conjugated antibody. Bands were visualized with chemiluminescent reagents (Pierce, Rockford, IL), were diluted 1:15,000 in blocking buffer and allowed to react with the washed membranes for 60 min followed by four washes of 5 min each in blocking buffer. Secondary antibody, goat anti-mouse horseradish peroxidase-conjugated antibodies (Pierce, Rockford, IL), were diluted 1:15,000 in blocking buffer and allowed to react with the washed membranes for 60 min followed by three washes of 5 min each in blocking buffer and a last wash in PBS alone. The antibody-antigen complexes were visualized using a SuperSignal West Pico chemiluminescence kit from Pierce following the manufacturer’s instructions.

SPL Assay—Cells were homogenized by glass bead disruption and vortexing for 15 min in extraction buffer (21). Crude membrane fraction was subjected to Western blotting indicating strong expression of human SPL. This cDNA was then re-cloned by RT-PCR amplification of total RNA from normal human diploid fibroblasts using N-terminal primer 5’-H967 and C-terminal primer 3’-H967. This RT-PCR product was cloned into pBluescript SK(+) at KpnI/XhoI sites to give plasmid, pBS/hSPL.
tions were obtained by ultracentrifugation at 100,000 × g for 60 min. Pellets were resuspended by sonication for 5-s intervals with repeats at low voltage. Protein content was determined by the Bradford method using Bio-Rad Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA). SPL activity was determined using [4,5-3H]D-erythro-DHS1P as substrate. 50 μg of protein was assayed, and reactions were carried out at 37 °C for 60 min.

### SPL-GFP Localization and Microscopy
Co-localization of recombinant SPL to the endoplasmic reticulum (ER) was performed by comparing the pattern of fluorescence from the hSPL-GFP fusion protein versus that of a red fluorescent protein containing the ER targeting sequence of calreticulin and the ER retention sequence KDEL in HEK293 cells stably expressing hSPL-GFP and transiently transfected with the mammalian expression vector pDsRed2-ER (BD Biosciences, Palo Alto, CA). Cells were grown on glass slides and visualized using a Zeiss 510 UV-visible laser scanning confocal microscope and Zeiss Plan-Apo 100×, 1.4 numerical aperture objective. The optical section thickness of both channels was equalized by adjusting the pinhole diameters of each channel. Channel one was set to one Airy; channel two was changed accordingly. The DsRed2 was excited by a 543-nm excitation light, and emitted fluorescence was collected through an LP 560 emission filter. The GFP was excited by a 488-nm excitation light, and emitted fluorescence was collected through a 505–550 BP emission filter. Image processing and visualization was performed using Bitplane Imaris software running on a Windows XP workstation.

### Sphingolipid Measurements
For sphingolipid measurements in stably transfected cell lines, sphingolipids were analyzed by liquid chromatography, electrospray ionization, and tandem mass spectrometry as described previously (22, 23). The internal standards for quantitation of the sphingolipids were obtained from Avanti Polar Lipids (Alabaster, AL).

### Labeling and Isolation of Cellular Sphingolipids and Phospholipids
Biosynthesis of sphingolipids was determined by following incorporation of L-[14C]serine into newly synthesized sphingolipids as previously described (24). Briefly, cells were seeded at 5 × 10^6 cells per dish in 100-mm dishes and grown under standard conditions in medium supplemented with 10% fetal calf serum for 24 h. Medium was then replaced with serum-free medium containing 1 μCi/ml L-[14C]-serine (specific activity, 57 mCi/mmol). After 24 h, cells were harvested and lipids extracted from cell pellets by incubating with chloroform/metha-
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RESULTS

Cloning and Characterization of Recombinant Human SPL—We previously reported the identification of SPL cDNA from *Mus musculus* (17). Using the BLAST search program we compared the mouse SPL cDNA sequence against the GenBank EST database and identified two homologous human EST sequences. Using this EST sequence information and a PCR cloning strategy as described under “Experimental Procedures,” we cloned human SPL (hSPL) cDNA from a human cDNA library. The coding region of hSPL was then re-cloned by RT-PCR from normal human diploid fibroblast RNA, and resequenced on both strands. This full-length open reading frame of hSPL contains 1707 nucleotides (GenBank accession number AF144638) encoding 568 amino acids with a predicted molecular mass of 63,482 Da. This sequence is consistent with that recently published by others (27). To confirm that the cloned sequence encoded human SPL, it was expressed in the *S. cerevisiae* Δdpl1 mutant strain, which is devoid of SPL enzyme activity and is especially sensitive to the toxic effects of exogenous *d*-erythro-sphingosine. Expression of hSPL in this background using the pYES2 yeast expression vector (Invitrogen) resulted in correction of sphingosine hypersensitivity and restoration of SPL enzyme activity (data not shown).

Overexpression of Recombinant hSPL in HEK293 Cells—HEK293 cells were transiently transfected with hSPL using the pcDNA3.0 expression system. Western blotting with an antibody recognizing mouse and human SPL demonstrated a large increase in hSPL protein in transiently transfected HEK293 cells. The endogenous SPL protein, however, was not detectable under these immunoblotting conditions (Fig. 1). As shown in Table II, the majority of both the endogenous and recombinant hSPL activity resides in the membrane fraction. Furthermore, SPL activity in the membrane fraction of extracts from hSPL-transfected HEK293 cells was more than 100-fold higher than vector transfected cells, indicating that the recombinant protein is active. A fusion protein constructed to contain hSPL and GFP, pc-hSPL-GFP, localized to the ER (Fig. 2) and demonstrated similar activity to recombinant hSPL, as shown in Fig. 3.

Stable Overexpression of hSPL-GFP Leads to Diminished Viability under Stress Conditions—SPL has been shown to affect metazoan organ development and mammalian cell differentiation. To determine whether human SPL expression directly affects cell fate decisions, cell lines stably expressing the hSPL-GFP fusion protein were generated. In all experiments, pooled stable transfectants were employed, to avoid potential mutations or artifacts associated with the selection and propagation of individual clones from single transfected cells. The hSPL-GFP-overexpressing cells demonstrated a 19-fold increase in SPL activity compared with cells stably transfected with a GFP control construct (Fig. 3). Under normal growth conditions, cells overexpressing hSPL-GFP appeared morphologically similar to the control cells; growth characteristics, as determined by cell number and MTT assay, were indistinguishable (Fig. 4). However, when deprived of serum, the two groups behaved differently. Whereas the control cells exhibited a leveling off of the growth curve, the hSPL-GFP-expressing cells demonstrated a marked reduction in cell

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**FIG. 5.** Stress-induced apoptosis is enhanced in cells overexpressing human SPL. Caspase-3 protease enzyme activity was measured in HEK293 cells stably transfected with either empty vector, pc-hSPL-GFP, or pc-hSPL(K353L)-GFP, a mutant SPL-GFP fusion in which the predicted cofactor binding lysine is replaced with leucine. Cells were treated as described in Fig. 4; aliquots were then removed at 36 and 48 h after reseeding, and apoptosis was quantified using the caspase-3 activity assay. Data are representative of six separate experiments. *, for pc-hSPL-GFP versus empty vector (48 h samples), *p < 0.001. **, for pc-hSPL-GFP versus pc-hSPL(K353L)-GFP, *p < 0.001.
The Effect of SPL on Growth Is Attributable to an Increase in Apoptosis—S1P metabolism has been shown to affect survival, proliferation, and apoptosis in many cell types, including HEK293 cells (28–30). To investigate whether overexpression of hSPL-GFP affected cell growth through an apoptotic mechanism, activation of caspase-3, one of the central effector caspasases was measured. Caspase-3 activity was significantly elevated in cells overexpressing hSPL-GFP in response to serum deprivation for 48 h. In contrast, negligible caspase activation was observed in control cells under identical conditions (Fig. 5).

SPL Enzyme Activity Is Required to Induce Apoptosis—To determine whether the biological effects observed in association with hSPL-GFP overexpression require SPL enzyme activity, a mutant SPL with no enzymatic activity was created. The SPL amino acid sequences of S. cerevisiae, C. elegans, Homo sapiens, M. musculus, and D. melanogaster reveal several regions of high homology, including a central 24-amino acid stretch (human SPL amino acids 343–366). Contained within this region are a predicted protein kinase C phosphorylation site (amino acids 351–353) and a predicted PLP binding site (Lys135), based on comparison to the sequences of other PLP-dependent enzymes. A mutant hSPL-GFP with a single (K353L) amino acid substitution at the predicted cofactor-binding site was generated by site-specific mutagenesis of the hSPL-GFP protein. Like the wild type hSPL-GFP, mutant hSPL-GFP is highly expressed and localizes properly to the ER, as determined by fluorescence microscopy (data not shown). However, transient and stable expression of pc-hSPL(K353L)-GFP indicated that the mutant protein is devoid of significant SPL activity (Fig. 5). When stable hSPL(K353L)-GFP expressing cells were evaluated under serum deprivation conditions, they responded similarly to control cells, indicating that SPL activity is required to promote apoptosis (Fig. 5).

Baseline S1P and Sphingosine Levels Are Diminished in SPL-overexpressing Cells—We were interested in determining whether the cellular sphingolipid profile is affected by continuous SPL overexpression. Toward that end, phosphorylated and unphosphorylated long-chain bases, ceramides, sphingomyelin, and higher order sphingolipids were quantified using mass spectrometry in HEK293 cells grown under standard conditions. As shown in Fig. 6 (A and B), C16 ceramide, S1P, and sphingosine levels were substantially diminished under baseline conditions in cells overexpressing wild type but not mutant SPL, whereas the content of most other sphingolipids was not appreciably changed.

Accumulation of Long- and Very Long-chain Ceramides Occurs in SPL-overexpressing Cells under Stress Conditions—To determine the effects of SPL overexpression on sphingolipid mass and subspecies, cellular sphingolipids were analyzed by mass spectrometry. Because serum contains sphingolipids (including S1P), these analyses were conducted in serum-free medium. Under these conditions, the amounts of S1P and sphingosine were not diminished in the cells stably overexpressing SPL (Fig. 7A), and some subspecies of ceramide (C16, C18, C24, and C26-ceramides) were significantly elevated. In most cases, this difference was evident early and was sustained throughout the time course. In contrast, the amounts of sphingomyelin and other (glyco)sphingolipids (data not shown) were not significantly affected by SPL overexpression. The finding of increased amounts of ceramide in SPL-overexpressing cells compared with control cells under these conditions was unexpected and suggested that a direct substrate availability relationship between sphingosine and ceramide does not exist when SPL is overexpressed.

Changes in Ceramide Composition Occur in SPL-overexpressing Cells—Backbone composition changes were evident when the subspecies distributions at 48 h are compared with “donut” diagrams (Fig. 7B) (similar trends were seen at other time points, data not shown). These show that hSPL-overexpressing cells have proportionately less C16-dihydroceramide (16:0DH) (as well as less C16-ceramide overall) and a larger fraction of the 24:0 fatty acid species versus C24:1. The latter appears not to be due to SPL activity, because it is also seen in the cells with mutant (K→L) hSPL, suggesting that SPL may exert some effects on cells through properties that are independent of its catalytic function. The ceramide backbones of glucosylceramide also displayed the shift in 24-carbon ceramides in wild type and mutant hSPL-overexpressing cells (Fig. 7B), and the hSPL-transfected cells showed a somewhat lower fraction of the backbones as C16 species. These results show that overexpression of SPL leads to shifts in ceramide composition that include changes in backbone composition of the ceramides, as well as some shifts in fatty acid profile. Although these changes are subtle, they demonstrate that transfection with hSPL alters both the total amount and structure of ceramides and that, in some cases such the C24 species, these changes may occur independent of catalytic function.

![Fig. 6. Baseline sphingolipid levels in control cells and cells overexpressing wild type or mutant human SPL.](Image) HEK293 cells stably expressing either empty vector (hatched bars), pc-hSPL-GFP (solid bars), or the mutant pc-hSPL(K353L)-GFP (dotted bars) were grown in medium containing 10% serum and analyzed for sphingolipid content by mass spectrometry as described under "Experimental Procedures." Data are presented as averages of triplicate measurements. All measurements are in picomoles per 1 million cells. A, sphingomyelin (SM), glucosylceramide (Glc-cer), and lactosylceramide (Lac-cer); B, free ceramides (C16 cer, C18 cer, C24 cer and C26 cer), S1P, and sphingosine (Sph).
FIG. 7. Sphingolipid profile under stress conditions in control cells and cells overexpressing wild type or mutant human SPL. In A (includes panels A–H): HEK293 cells stably expressing either empty vector (hatched bars), pc-hSPL-GFP (solid bars), or the mutant pc-hSPL(K353L)-GFP (dotted bars) were serum-deprived as described in Fig. 5 and analyzed for sphingolipid content by mass spectrometry as described under “Experimental Procedures.” Data are presented as averages of triplicate measurements. All measurements are in picomoles per 1 million cells. Sph, sphingosine; S1P, sphingosine 1-phosphate; SM, sphingomyelin; C16 Cer, C16 ceramide; Total Cer, total ceramide; C18 Cer, C18 ceramide; C24 Cer, C24 ceramide; C26 Cer, C26 ceramide. Shown are unsaturated C24 ceramide and C26 ceramide levels; comparison of the combined C24:0 plus C24:1 (and similarly C26:0 plus C26:1) ceramides yielded similar results (data not shown). In B: fatty acid and dihydro-
The de Novo Synthesis of Sphingolipids Is Increased in Cells Overexpressing hSPL—We were interested in the reason for ceramide elevation in cells overexpressing SPL. We considered that long-term S1P depletion might not be well tolerated by the cell and could induce a cellular response to increase sphingolipid synthesis. In fact, when sphingolipid biosynthesis was measured by the incorporation of L-[14C]serine into new sphingolipids, a 32 ± 2% increase in incorporation into sphingomyelin and a 47 ± 12.7% increase in incorporation into total sphingolipids were observed in cells overexpressing wild type but not mutant SPL. These findings suggest that sphingolipid biosynthesis may be enhanced in response to expression of an enzymatically active SPL. To investigate whether sphingolipid biosynthesis was required for SPL-induced apoptosis, de novo biosynthesis was blocked by incubating cells with ISP-1, which inhibits serine palmitoyltransferase. Although treatment of SPL-overexpressing cells with 5 nM ISP-1 led to a 10–20% reduction in stress-induced apoptosis (data not shown), ISP-1-induced apoptosis in control cells at doses greater than 5 nM.

SPL-induced Apoptosis Is Reversed by Addition of Exogenous S1P—S1P has been shown to afford protection against apoptosis induced by ceramide and other noxious stimuli (31–34). To assess whether SPL-induced apoptosis could be abrogated by S1P, we incubated cells with increasing amounts of exogenous S1P after introduction into serum-free medium. As shown in Fig. 8 (A and B), SPL-induced apoptosis was reversed by addition of S1P in a dose-dependent manner.

Products of the SPL Reaction Do Not Contribute to SPL-induced Apoptosis in HEK293 Cells—To determine whether the effects of SPL on promoting apoptosis might be due to the increased formation of the products of the reaction catalyzed by this enzyme, cells stably expressing a vector control plasmid were incubated with ethanolamine phosphate, hexadecenal, or hexadecanal, the products of the lyase-catalyzed cleavage of S1P or DHS1P, respectively. As shown in Fig. 8A, incubation of cells with doses of up to 50 μM of these three molecules for 48 h did not elicit an apoptotic response, as measured by caspase-3 activity.

Enhanced Apoptosis in Cells Overexpressing SPL Requires the Generation of Ceramide—To determine whether the elevation in long- and very long-chain ceramides contributed to the enhanced apoptosis exhibited by cells overexpressing SPL, cells were treated with the ceramide synthase inhibitor fumonisin

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ceramide distribution of sphingomyelin (SM), ceramide (Cer), and glucosylceramide (GlcCer) from HEK293 cells transfected with only the vector GFP (upper), the active hSPL (middle) and the mutant, inactive hSPL (lower) for 48 h in serum-free medium. The fatty acids are identified beside the respective region of the "donut" diagrams by the carbon chain length followed by the number of double bonds in the fatty acid; C₁₆-ceramides with a sphinganine backbone (dihydroceramide) are designated "16:0DH" and highlighted in solid black.
Importantly, fumonisin B1 treatment of cells overexpressing SPL reduced stress-induced apoptosis to the level of stressed vector-transfected control cells. This suggests that the increase in apoptosis observed in this cell line requires the generation of ceramide.

Cytochrome c Release in Cells Overexpressing Wild Type or Mutant SPL Proteins—To correlate ceramide elevation with earlier events in the apoptotic pathway, we evaluated the release of cytochrome c into the cytosol of cells expressing wild type or mutant SPL during serum deprivation. As shown in Fig. 10, early (by 12 h) and sustained (through 48 h) cytochrome c release into the cytosol was observed in cells overexpressing catalytically active SPL. In contrast, wild type cells did not release appreciable amounts of cytochrome c into the cytosol until 48 h. Interestingly, cytochrome c was observed transiently in the cytosol of cells overexpressing mutant SPL. Because expression of mutant SPL did not induce either caspase activation or ceramide elevation, these results suggest that ceramide elevation may contribute to the increased apoptosis in SPL-overexpressing cells, but that cytochrome c release alone is either not sufficient or must be sustained to promote apoptosis.

**DISCUSSION**

The fundamental role of S1P in the regulation of cell growth, survival, differentiation, and movement and its contribution to the process of angiogenesis are becoming increasingly evident. The production of S1P by SPHK, which is activated by numer-
ous agonists and through different molecular mechanisms, has garnered considerable attention, even placing it in the limelight as a potential therapeutic target in the treatment of cancer (35, 36). Some attention has also been given to phosphatases that dephosphorylate S1P (SIPP) (5, 37); however, SLP has been generally regarded as having a “housekeeper” function despite being the only enzyme that terminally degrades S1P. This view is changing, because recent studies in genetically tractable metazoan models and in mammalian cells suggest that SLP may play a more substantial role in biology than previously recognized, as a mediator of differentiation and animal development (12–15). Thus, it is not surprising to find that, in addition to SPHK and S1PP, SLP can regulate human cell fate decisions.

SPL expression not only influences the metabolism of both S1P and ceramide but also induces cell death under certain conditions. Our observations that caspase activation in SPL-overexpressing cells is inhibited by supplying cells with exogenous S1P or by blocking ceramide generation suggest that both these bioactive lipids affect apoptosis induced by SLP. The depletion of S1P by SPL and, conversely, the addition of exogenous S1P to cells depleted of this lipid might affect cell survival through either extracellular or intracellular mechanisms. To gain insight into how SPL might enhance apoptosis in serum-deprived cells, we explored the release of cytochrome c from the outer mitochondrial membrane into the cytosol, an important molecular event upstream of caspase-3 activation in the apoptotic pathway. The long- and very long-chain ceramides species that accumulate in stressed SPL-overexpressing cells have been shown recently to contribute coordinately to both induction and amplification of the apoptotic response (38). Therefore, ceramide accumulation and/or S1P depletion in cells overexpressing catalytically active SPL might contribute to the sustained cytochrome c release necessary for apoptosis to proceed. Our observation that overexpression of a catalytically inactive SPL mutant leads to a transient release of cytochrome c (although caspase 3 activation is not enhanced in this cell line) raises the intriguing possibility that non-catalytic functions of SPL might exist.

It was somewhat surprising that ceramide amounts were not reduced by SPL overexpression, because elevated lyase activity might be predicted to divert cellular sphingoid bases toward degradation rather than acylation. This might be due to an increase in de novo sphingolipid synthesis, an explanation consistent with our labeling study and with studies by van Echten-Deckert et al. (24, 39) that have suggested that phosphorylated long-chain bases may reduce serine palmitoyltransferase activity and de novo sphingolipid biosynthesis. It was interesting also that cells overexpressing SPL differ in ceramide composition. They contain lower proportions of dihydroceramides, which might indicate that additional enzymes of this pathway (e.g., the desaturases that convert dihydroceramides to ceramides) are also induced. The other change in ceramide composition (a shift in the portions of C24:0 versus C24:1 fatty acids) was not due to SPL activity, because the shift was seen in cells overexpressing the mutant hSPL, raising the possibility that the lyase polypeptide may influence the types of ceramides that are made or turned over. Although we do not fully understand why these changes in ceramide composition have occurred, they may indicate a higher order regulation of this pathway (for example, perhaps through interaction of some of the proteins in a macromolecular complex).

These findings are important on several levels. First, SPL expression regulates not only intracellular S1P and sphingosine but also appears to increase ceramide generation under stress conditions. This observation underscores the fact that sphingolipid metabolism is a highly complex network and that manipulation of a single enzyme in this pathway can give rise to distant and sometimes paradoxical effects, through feedback inhibition and other mechanisms of regulation. Furthermore, recent studies have shown that SPL is involved in regulation of sterol regulatory element binding protein processing through its influence on sphingolipid metabolism (40). Thus, SPL exerts global effects on lipid metabolism in eukaryotic cells. Second, the ability of SPL to regulate cell fate decisions may provide insights into its role in metazoan development and is consistent with observations of dysregulated developmental apoptosis in Drosophila SPL mutants (13). Third, the ability of SPL to affect cell fate leads to the prediction that loss of SPL expression could contribute to cancer cell survival. In fact, deletions of the 10q21 chromosomal region where the human SPL locus resides have been demonstrated in a variety of cancers, including leukemia and solid tumors of thyroid, colon, and brain (41–53). Thus, mutations in the SPL locus of cancer cells could potentially have diagnostic and predictive value. Lastly, our findings indicate that SPL may be a unique target for pharmacological agonists in the treatment of cancer. It has been suggested that the effective deployment of ceramide-induced apoptosis as a cancer therapy must take into account the fact that increased ceramide production eventaully also gives rise to increased S1P production, limiting the desired effect on the cancer cell (54). The generation of ceramide analogs that do not give rise to S1P or the simultaneous generation of ceramide and inhibition of SPHK are schemes proposed to circumvent this problem. However, SPL activation causes both ceramide accumulation and S1P depletion, efficiently accomplishing this goal in one step. The manipulation of SPL for therapeutic purposes may not be far-fetched, because temporal, spatial and differentiation-induced regulation of SPL expression has recently been demonstrated in metazoan models and mammalian cells. The identification of specific factors involved in SPL gene regulation should facilitate our ability to manipulate SPL for therapeutic purposes in the future.

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