Journal of Cellular Biochemistry 92:882–899 (2004)

Generation and Metabolism of Bioactive Sphingosine-1-Phosphate

Hervé Le Stunff,[1] Sheldon Milstien,[2] and Sarah Spiegel[3]*

[1]Laboratoire d'Activation Cellulaire et Transduction des Signaux, Institut de Biochimie et de Biophysique Moléculaire et Cellulaire, UMR CNRS 8619, Université Paris-Sud, 91405 Orsay, France
[2]Laboratory of Cellular and Molecular Regulation, National Institute of Mental Health, Bethesda, Maryland 20892
[3]Department of Biochemistry, Virginia Commonwealth University School of Medicine, Richmond, Virginia 23298

Abstract Sphingosine-1-phosphate (S1P) is a bioactive lysosphingophospholipid that has been implicated in the regulation of vital biological processes. Abundant evidence indicates that S1P acts as both an intracellular messenger and an extracellular ligand for a family of five specific G protein-coupled S1P receptors (S1PRs). Cellular levels of S1P are tightly regulated in a spatio-temporal manner through its synthesis catalyzed by sphingosine kinases (SphKs) and degradation by S1P lyase (SPL) and specific S1P phosphohydrolases. Over the past decade, the identification and cloning of genes encoding S1P metabolizing enzymes has increased rapidly. Overexpression and deletion of these enzymes has provided important insights into the intracellular and the ''inside-out'' functions of S1P. The purpose of this review is to summarize the current knowledge of S1P metabolizing enzymes, their enzymatic properties, and their roles in the control of cellular functions by S1P. J. Cell. Biochem. 92: 882–899, 2004. Published 2004 Wiley-Liss, Inc.†

Key words: sphingosine-1-phosphate; signaling; metabolism; sphingolipid; sphingosine; sphingosine kinase; S1P phosphohydrolase; S1P lyase

Sphingosine-1-phosphate (S1P) is now emerging as a vital cellular sphingolipid mediator. The importance of S1P as a regulator of cell growth was discovered in 1991 [Zhang et al., 1991]. Over the next decade, this lysosphingophospholipid metabolite was shown to mediate a wide spectrum of biological processes, including Ca^{2+} mobilization, cell growth, survival, cell motility and cytoskeleton remodeling, and immunity [Pyne and Pyne, 2002; Spiegel and Milstien, 2003]. It is now clear that this relatively simple molecule can affect such an array of diverse cellular processes because of its ability to function as both an intracellular second messenger and as a ligand for five G protein-coupled receptors (GPCRs), now named $S1P_{1-5}$ [Spiegel and Milstien, 2003; see also Hla's review in this volume], that are coupled to

Abbreviations used: AKAP, PKA anchoring protein; ATX, autotaxin; BH3, Bcl-2 homology 3 domain; DAGK, diacylglycerol kinase; DHS, dihydrosphingosine; DMS, *N,N*-dimethylsphingosine; EGF, epidermal growth factor; ER, endoplasmic reticulum; ERK, extracellular signal regulated kinase; GAP, GTPase activating protein; GPCR, G protein-coupled receptor; IL-12, interleukin-12; IL-12R, IL-12 receptor; JNK, jun N-terminal kinase; LBP, long chain base phosphate; LCB, long chain base; LPC, lysophosphatidylcholine, LPA, lysophosphatidic acid; LPP, lipid phosphate phosphohydrolase; NGF, nerve growth factor; NLS, nuclear localization sequence; PDGF, platelet derived growth factor; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D; RA, retinoic acid; RPK118, ribosomal S6 kinase-like protein; SPC; sphingosine-phosphorylcholine; S1P, sphingosine-1-phosphate; SphK, sphingosine kinase; S1PR, S1P receptor; SPL, S1P lyase; SPP, S1P phosphohydrolase; siRNA, small interfering RNA; TGFβ, transforming growth factor-β; TNFα, tumor necrosis factor-α; TRAF2, TNF receptor-associated factor 2; VEGF, vascular endothelial growth factor.

Grant sponsor: National Institutes of Health (to SS); Grant numbers: GM43880, CA61774, AI50094.

*Correspondence to: Prof. Sarah Spiegel, Department of Biochemistry, Virginia Commonwealth University School of Medicine, Richmond, Virginia. E-mail: sspiegel@vcu.edu

Received 7 November 2003; Accepted 30 December 2003

DOI 10.1002/jcb.20097

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mutiple G proteins and regulate many downstream signaling pathways.

De novo synthesis of sphingolipids occurs at the cytosolic face of the endoplasmic reticulum (ER). The initiating reaction is the condensation of serine and palmitoyl-CoA catalyzed by serine palmitoyltransferase [Hannun et al., 2001; Hannun and Obeid, 2002; Pettus et al., 2002] to form 3-ketosphinganine which is rapidly reduced to dihydrosphingosine and then *N*-acylated by ceramide synthase to form dihydroceramide. Finally, a *trans* double bond at C4–C5 is introduced by dihydroceramide desaturase, producing ceramide [Pettus et al., 2002]. Ceramide can also be released from sphingomyelin by sphingomyelinases or deacylated by ceramidases to sphingosine. More complex sphingolipids are synthesized from ceramide, which is the most common backbone found in mammalian sphingolipids. Sphingosine and other sphingoid bases can be phosphorylated by sphingosine kinases (SphKs) to form S1P (Fig. 1). Turnover of S1P is mediated either by reversible dephosphorylation to sphingosine by specific S1P phosphohydrolases (SPPs) [Le Stunff et al., 2002a] or by irreversible cleavage to ethanolamine phosphate and hexadecenal by a pyridoxal-dependent S1P lyase (SPL) [Zhou and Saba, 1998] (Fig. 1).

Because of the interconvertibility of ceramide, sphingosine, and S1P and the opposing effects on cell fate of ceramide and sphingosine (anti-growth, pro-apoptotic) compared to S1P (pro-growth, anti-apoptotic), the dynamic balance between S1P and ceramide/sphingosine has been proposed to form a "cellular rheostat" that determines cell survival or death [Spiegel and Milstien, 2002]. Accumulating evidence suggests that the sphingolipid rheostat is ultimately regulated by the relative activities of enzymes controlling the turnover of these sphingolipid metabolites, thereby underscoring the importance of understanding how these enzymes are regulated. It is not our intention to provide a comprehensive discussion of their roles in regulation of ceramide/sphingosine biosynthesis in cell signaling as several reviews of these concepts have recently been published [Cuvillier, 2002; Perry, 2002; Pettus et al., 2002]. Instead, this review is focused on highlighting recent progress in cloning and characterization of these enzymes and their importance in the biological functions of S1P.

Sphingosine Kinases

Characterization of SphKs. SphK is a ubiquitous enzyme in mammals. Little was known about its identity, properties, or mechanisms of regulation until recent years. The first mammalian SphK, SphK1 (Fig. 2), was cloned based on tryptic peptides derived from highly purified rat kidney SphK [Kohama et al., 1998]. SphK1 is most abundant in the lung, spleen, and liver. It has no recognizable signal sequences or transmembrane domains and is found predominantly in the cytosol. As expected, the naturally occurring D-erythro enantiomer of sphingosine, and to a lesser extent, D-erythro-dihydrosphingosine, are the best substrates for the SphK1. DL-*threo*-dihydrosphingosine (DHS) and *N,N*-dimethylsphingosine (DMS) are potent competitive inhibitors that have proved to be very useful for examining the functions of SphKs [Spiegel and Milstien, 2003]. On the basis of sequence homology, a second SphK isoform, SphK2, was recently cloned (Fig. 2) [Liu et al., 2000]. SphK2 is much larger than SphK1 and is expressed predominantly in the liver and the heart. Unlike SphK1, SphK2 has a slightly stronger preference for D-erythro-dihydrosphingosine compared to D-erythro-sphingosine. In addition, DMS is a potent inhibitor of SphK2, whereas DHS is not. Despite the presence of four putative transmembrane domains, overexpressed SphK2 was mostly found in the cytosol of HEK 293 cells [Liu et al., 2000]. However, recent studies suggest a plasma membrane and a nuclear localization of SphK2 [Igarashi et al., 2003; Yoshimoto et al., 2003]. A functional nuclear localization signal (NLS) with an arginine cluster (RGRRGGRRR) has been mapped to the NH2-terminal region of SphK2 that targets it into the nucleoplasm of specific cell lines [Igarashi et al., 2003] (Fig. 2). At present, the molecular mechanisms regulating the nuclear-cytoplasmic shuttling of SphK2 remain unknown. Using a yeast two hybrid screening, it has been shown that SphK2 interacts with a cytoplasmic region of the receptor for interleukin-12 receptor (IL-12R), presumably through its proline-rich domain, and the interaction modulates IL-12 signaling [Yoshimoto et al., 2003] (Fig. 2).

Both SphK1 and SphK2 are stimulated by acidic phospholipids [Liu et al., 2000], although neither contain consensus binding sites for this type of lipid. Because these studies were carried

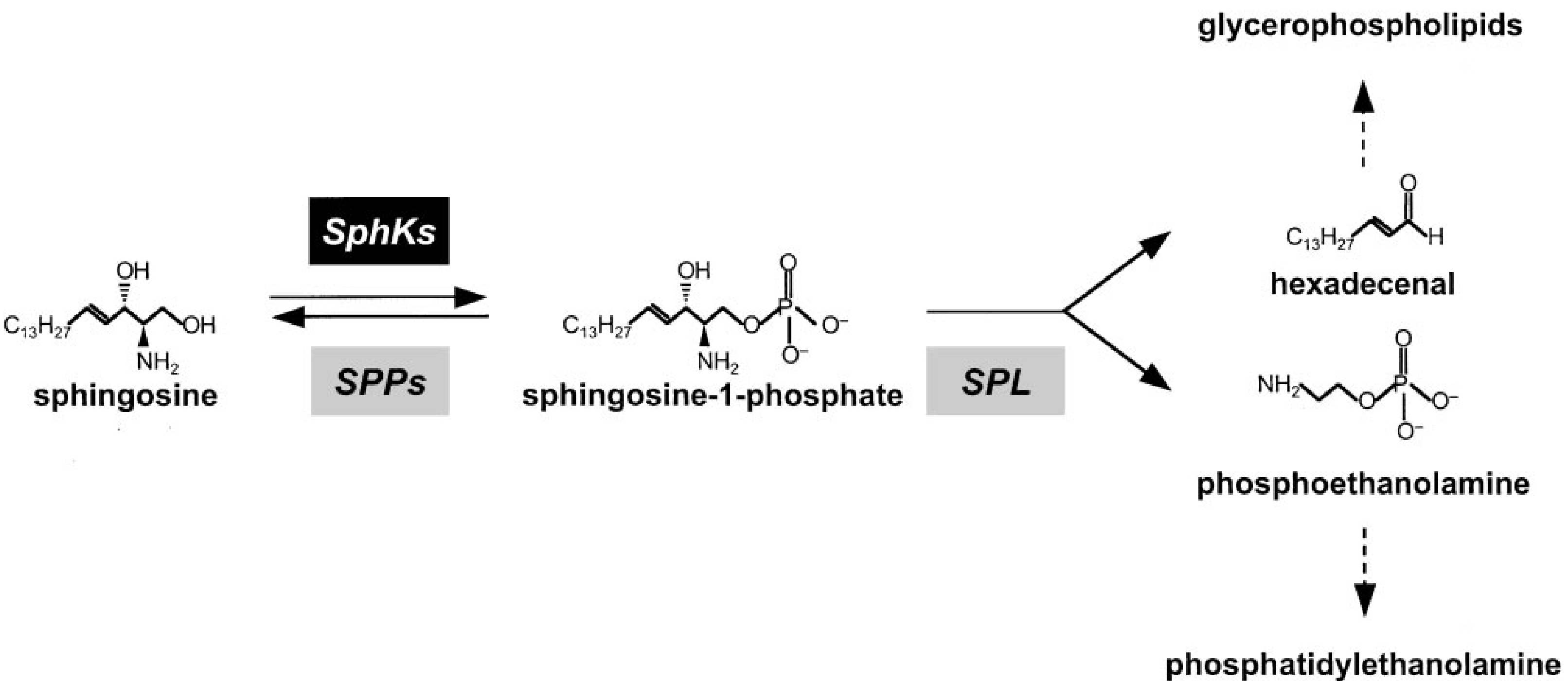
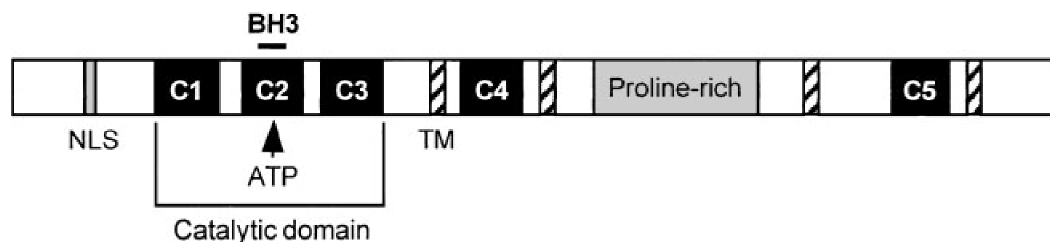
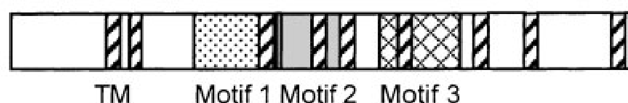


Fig. 1. Pathways of sphingosine-1-phosphate metabolism. Key enzymes for the formation and degradation of S1P are shown. S1P is produced by the phosphorylation of sphingosine by sphingosine kinase. S1P can then be metabolized by S1P lyase to phosphoethanolamine and hexadecenal, which are then further metabolized into glycerophosphlipids and phosphatidylethanolamine, respectively. Conversely, S1P phosphohydrolase regenerates sphingosine by dephosphorylating S1P. Abbreviations: SphK, sphingosine kinase; SPP, sphingosine-1-phosphate phosphohydrolase; SPL, sphingosine-1-phosphate lyase.

SPHINGOSINE KINASES



S1P PHOSPHOHYDROLASES



S1P LYASES

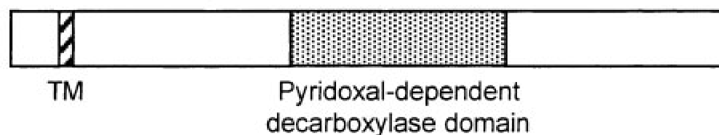


Fig. 2. Mammalian S1P metabolizing enzyme families. Two isoforms of SphK have been cloned: SphK1 and SphK2. Both are characterized by the presence of five conserved domains (C1–C5) with the unique catalytic domain contained within C1–C3. The ATP binding site [SGDGX(17-21)K] is present within C2. While hydropathy plot analysis of SphK1 does not indicate any transmembrane (TM) domains, SphK2 has four putative TM regions, a nuclear localization sequence (NLS), a Bcl-2 homology 3 (BH3) domain, and a proline-rich region. S1P can be degraded by endoplasmic reticulum (ER) localized S1P phosphohydrolases (SPPs). Two isoforms of SPPs have been cloned that contain 8–10 predicted TM domains. Both SPPs have three conserved motifs that are similar, yet distinct, to those in the lipid phosphate phosphohydrolase (LPP) family: motif 1, KDX(4)PRP; motif 2, PSXH; and motif 3, LVXX(3)RXYXHX(2)LD. S1P can also be degraded by SPL. One isoform has been cloned and is characterized by one TM near its amino terminus and a conserved pyridoxal-dependent decarboxylase domain.

out with crude lysates from cells overexpressing the SphKs, it is possible that binding of acidic phospholipids might mediate their interactions with an activator protein that then facilitates its translocation to membranes. SphK1 and SphK2 are characterized by the presence of five conserved domains, denoted C1–C5 (Fig. 2) [Liu et al., 2002]. There is considerable sequence variability outside of these domains and SphK2 contains two large unrelated sequences, one at the NH2 terminus and the other in the middle of the protein. The C1, C2, and C3 domains of SphK1 and SphK2 have high homology to the ATP-binding site of the catalytic domain of diacylglycerol kinases (DAGK), suggesting that it is part of the catalytic domain of SphKs (Fig. 2). Indeed, site-specific mutagenesis of the second glycine residue of the ATP-binding site sequence [SGDGX(17–21)K] present within the C2 domain, resulted in a catalytically inactive mutant SphK1 [Pitson et al., 2002a,c]. Despite their overall homology, SphK1 and SphK2 likely have distinct cellular functions because they have different kinetics properties, tissue distribution, temporal expression patterns during development, and additional unrelated sequences.

Although SphK1 and SphK2 are the only isoforms identified to date, SphK activity in various tissues has different properties and subcellular localizations than SphK1 and SphK2 when overexpressed [Banno et al., 1998; Gijsbers et al., 2001], suggesting the possible existence of additional SphKs. In agreement, immunodepletion assays of both SphK isoforms indicated that other SphKs might exist in some tissues such as spleen, small intestine and lung [Fukuda et al., 2003]. Interestingly, different splice variants of SphK1 and SphK2 have been identified which are longer at the NH2 terminus [Billich et al., 2003]. These variants do not seem to differ in substrate specificity but the additional sequences could contribute to specific mechanisms of regulation and/or subcellular localization. Moreover, one sequence homologous to the known SphKs in the human genome was detected by BLAST searching. However, this "SphK-like" protein, which contains a slightly modified ATP-binding site, is unable to phosphorylate sphingosine in vitro [Billich et al., 2003].

Activation of SphK1. There is a rapidly growing list of agonists that stimulate SphK1, the most prominent being growth and survival factors [Spiegel and Milstien, 2002]. These stimuli include agonists of receptor tyrosine kinases, such as platelet derivate growth factor (PDGF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and nerve growth factor (NGF). Ligands for GPCRs, including acetylcholine, lysophosphatidic acid (LPA), S1P, formylmethionine peptide, cytokines like tumor necrosis factor α (TNFα), and cross-linking of immunoglobulin receptors FcγRI and FcγRIII, also activate SphK1 [Spiegel and Milstien, 2002]. Moreover, several mechanisms of SphK1 activation, including protein modifications and translocation, have recently been discovered (see below).

Translocation and phosphorylation. A hint to the mechanism of stimulation of SphK1 comes from the observation that the substrate sphingosine is generated in membranes. Thus, translocation of SphK1 from the cytosol to the membrane would be expected to stimulate the phosphorylation of sphingosine. Indeed, we have shown that SphK1 is translocated to the leading edge of cells by PDGF where localized production of S1P is required for PDGF-induced migration [Rosenfeldt et al., 2001]. Moreover, long-term treatment with PDGF can induce translocation of SphK1 to the nuclear cytoplasm [Kleuser et al., 2001]. Activation of protein kinase C (PKC) by phorbol ester [Johnson et al., 2002] and VEGF [Shu et al., 2002] induced phosphorylation of SphK1 and translocation to the plasma membrane. Thus, PKC may be the link to activation of SphK1 by many agonists that stimulate phospholipase C (PLC) leading to activation of PKC. However, it is still unclear whether PKC phosphorylates SphK1 directly or activates SphK1 indirectly by phosphorylating yet other unidentified SphK1 activator proteins. PKCs are not the only protein kinases that can regulate SphK1 activity. Recently, phosphorylation of SphK1 on serine[225] by extracellular regulated kinase 1/2 (ERK1/2) has been reported during agonist-mediated SphK activation [Pitson et al., 2003]. Similar to the effect of PKC, phosphorylation of SphK1 by ERK1/2 not only increased enzyme activity, it was also necessary for translocation from the cytosol to the plasma membrane [Pitson et al., 2003].

Ca^{2+} homeostasis. Another possible mechanism for SphK activation may involve PLC-induced Ca^{2+} release. In an epithelial cell line expressing wild-type or PDGFRs with muta-

tions of specific tyrosine residues important for signaling, it was found that phosphorylation of the tyrosine responsible for binding PLCγ was necessary and sufficient for activation of SphK1 by PDGF [Olivera et al., 1999a]. In this case, stimulation of SphK1 was independent of PKC, but required an increase in intracellular Ca^{2+} concentration. Further support of Ca^{2+}-induced stimulation of SphK1 arose from studies involving purinergic P2Y2 receptors [Alemany et al., 2000]. Interestingly, calmodulin binds to SphK1 with high affinity [Olivera et al., 1998], suggesting that Ca^{2+} might modulate SphK1 activity in a calmodulin-dependent manner. However, neither Ca^{2+} nor calmodulin have any effects on SphK1 activity measured in vitro [Olivera et al., 1998; Pitson et al., 2000b]. Recently, real-time confocal imaging experiments showed that Ca^{2+}-mobilizing stimuli induced re-localization of SphK1 from the cytosol to the plasma membrane that was mediated by the Ca^{2+}-calmodulin complex [Young et al., 2003]. These results suggest that the Ca^{2+}/calmodulin complex can regulate SphK1 by affecting the subcellular localization of the enzyme.

Phospholipase D. As mentioned above, acidic phospholipids activate SphK1 and SphK2 and might facilitate membrane interactions. Indeed, stimulation of SphK1 activity by crosslinking of FcγRI has been reported to be mediated by phospholipase D (PLD), an enzyme that produces phosphatidic acid [Melendez et al., 1998]. In agreement, activation of PLD1 by FcγRI is required for increased activity of SphK1and its translocation to the plasma membrane [Melendez and Khaw, 2002]. Ras may also be a novel regulator of the SphK signaling pathway, as expression of a constitutively active form of Ras increased SphK1 activity by twofold [Xia et al., 2000].

Protein–protein interactions. A common emerging feature of signal transduction pathways is the utilization of scaffolding or binding proteins to bring relevant signaling molecules together into a large activated complex. A TNF receptor-associated factor 2 (TRAF2) binding motif on SphK1 has been identified that mediates interaction of SphK1 and TRAF2 [Xia et al., 2002]. This interaction resulted in increased SphK1 activity and led to activation of NF-κB and enhanced survival in response to TNFα. Yeast two-hybrid screening identified the ribosomal S6 kinase-like protein (RPK118) as a SphK1 binding protein [Hayashi et al., 2002]. RPK118 contains a phox homology domain and two pseudokinase domains, and binds to phosphatidylinositol 3-phosphate. The pseudokinase 2 domain mediates the interaction of SphK1 with RPK118, leading to the translocation of SphK1 to early endosomes. Thus, it is tempting to speculate that RPK118 might play a role in targeting of SphK1 to this cellular compartment where sphingosine is produced by the catabolism of complex sphingolipids. Recently, we characterized a SphK1 interacting protein with some similarities to PKA anchoring proteins (AKAP) that inhibits cellular functions of SphK1 [Lacana et al., 2002]. However, much effort is still necessary to confirm a role for these proteins in agonist-induced activation of SphK.

Cellular functions of SphKs

Cell growth. Much of our understanding of SphK1 activation has relied on the use of pharmacological inhibitors to block agonist-induced cellular processes. However, in many studies, the site of action of S1P—intracellular targets versus interaction with its cell surface receptors—was not established. Cloning of SphKs has provided molecular tools to investigate the role of SphKs/S1P in biological responses. For example, overexpression of SphK1 increased the proportion of cells in S phase of the cell cycle, promoted the G_1–S phase, and reduced the doubling time, suggesting that SphK1 regulates DNA synthesis (Fig. 3) [Olivera et al., 1999b]. Furthermore, a recent study suggests that VEGF stimulates SphK1 and this has ramifications for its mitogenicity [Shu et al., 2002]. Using dominant-negative SphK1 or SphK2 mutants and small interfering RNA (siRNA) targeted specifically against each isozyme, Broek et al. showed that SphK1, but not SphK2, was responsible for VEGF-induced accumulation of active Ras [Shu et al., 2002]. They proposed that endogenous sphingosine attenuated basal Ras activity by stimulating Ras GTPase-activating proteins (Ras-GAP) and that VEGF decreased sphingosine levels by stimulating SphK1, thereby increasing activated Ras levels. Pathophysiological significance of this model emerged from the finding that VEGF, through its effects on SphK1, regulates Ras-GAP activity that plays an important role in the survival and proliferation of bladder tumor cells [Wu et al., 2003]. In addition, expression of SphK1 has been correlated with the acquisition of a transformed cell phenotype,

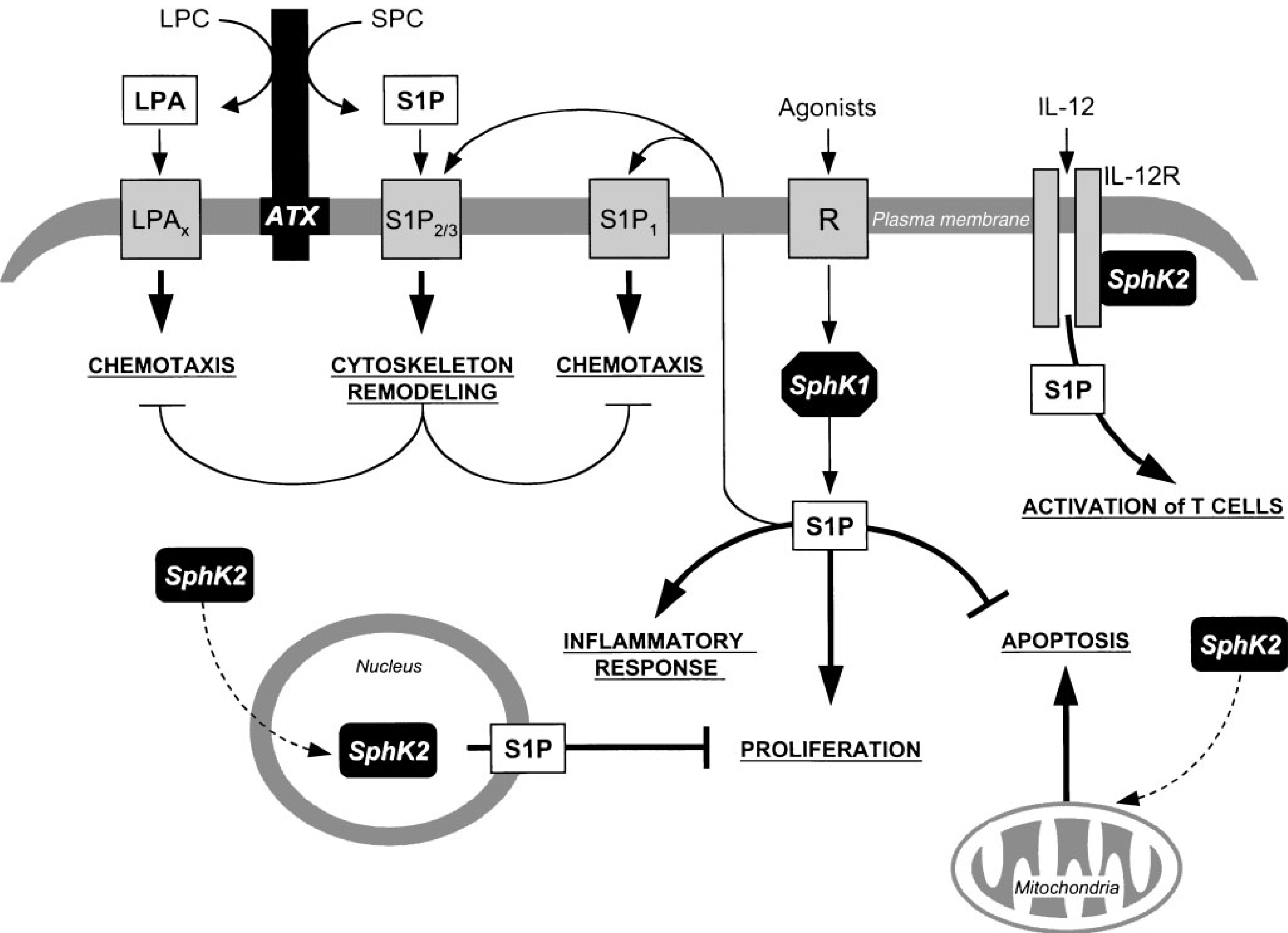


Fig. 3.

as determined by assays of foci formation, colony growth in soft agar, and the ability to form tumors in mice [Xia et al., 2000; Nava et al., 2002], suggesting an oncogenic function. Interestingly, it was found that SphK1 contributes to cell transformation by oncogenic H-Ras, plausibly via Ras and ERK signaling. The recent engineering of mice null for several S1PRs provided important models to investigate the role of S1PRs in the regulation of cell proliferation. Thus, using cells from knockout mice, we were able to show that $S1P_1$ was not involved in PDGF-induced cell growth [Rosenfeldt et al., 2001] (Fig. 3). More recently, by blocking signaling of the heterotrimeric G-proteins that S1PRs couple to and signal through, we have found that SphK1-induced cell growth is independent of S1PR signaling [Olivera et al., 2003] (Fig. 3). Moreover, in contrast to exogenous S1P, intracellular S1P formed by overexpression of SphK1, promoted cell growth in mouse embryonic fibroblasts (MEFs) null for $S1P_2$ and $S1P_3$ to the same extent as in wild-type MEFs [Olivera et al., 2003]. These results support the idea that cell growth regulated by intracellular S1P is independent of inside-out signaling. Intracellular S1P binding proteins/targets, which remain to be identified, may determine the fate of cell.

In contrast to the proliferative effect of overexpressed SphK1 [Olivera et al., 1999b; Liu et al., 2003], SphK2 has been shown to inhibit of DNA synthesis [Igarashi et al., 2003; Liu et al., 2003] (Fig. 3). In one study, nuclear localization of SphK2 was correlated with inhibition of cell proliferation since mutation of a NLS abolished its inhibitory effect on cell growth [Igarashi et al., 2003]. Moreover, when the NLS sequence from SphK2 was fused to SphK1, it also caused an inhibitory effect on DNA synthesis. Interestingly, we have reported that a significant fraction of total SphK1 resides in the nuclei of Swiss 3T3 cells [Kleuser et al., 2001]. In addition, PDGF was found to translocate SphK1 to the nucleoplasm of these cells. Intriguingly, two functional nuclear export signal sequences in SphK1 have recently been identified suggesting an active nuclear export of SphK1 [Inagaki et al., 2003].

Cell survival. S1P has also been implicated as an important second messenger in suppression of apoptosis. A central finding is that the dynamic balance between the intracellular concentration of S1P and ceramide, referred to as the "sphingolipid rheostat" plays an essential role in determining the survival or death of mammalian cells [Spiegel and Milstien, 2003]. SphK plays a crucial role in regulating this balance and consequently, the equilibrium between survival and death of cells. Indeed, blocking S1P formation in response to TNFα by DMS enhanced ceramide-mediated apoptosis [Xia et al., 1999]. In support of an anti-apoptotic role of SphK, overexpression of SphK1 in several cell types resulted in protection from apoptosis, particularly cell death induced by growth factor withdrawal and exogenous ceramides (Fig. 3) [Olivera et al., 1999b; Edsall et al., 2001]. S1P, generated by overexpression of SphK1, protects against apoptosis by activating signaling pathways involved in cell survival, including activation of ERK1/2, Akt [Spiegel and Milstien, 2003], and the nuclear factor NFκB [Xia et al., 2002]. S1P also inhibits signaling cascades implicated in apoptosis, such as cytocrome c release from mitochondria, activation of caspases, and activation of the stress activated protein kinase, Jun amino-terminal kinase (JNK) [Spiegel and Milstien, 2003]. How overexpression of SphK1, through intracellular S1P production, is able to protect against apoptosis is not completely understood but it appears to be independent of binding to its cell surface receptors (Fig. 3). Using MEFs null for S1PRs, we have found that overexpression of SphK1 increased cell survival independently of its GPCRs [Olivera et al., 2003].

Surprisingly in contrast to the protective effect of SphK1 overexpression, we recently found that SphK2 induces apoptosis of various cell lines [Liu et al., 2003] (Fig. 3). Cell death

Fig. 3. Cellular localization and function of S1P synthesizing enzymes. Modulation of the activity of key enzymes involved in formation of S1P by various agonists has been linked to several biological processes. SphK1 has been implicated in the stimulation of cell growth and inflammatory responses, reorganization of the cytoskeleton, promotion of cell migration, and inhibition of apoptosis. In contrast, SphK2 has been involved in the inhibition of cell growth, stimulation of apoptosis and immune responses. ATX is a novel lysophospholipase D that can induce cell migration by LPC hydrolysis leading to LPA formation and inhibit chemotaxis by catalyzing S1P formation from sphingosinephosphorylcholine. Abbreviations: ATX, autotaxin; IL-12; interleukin-12; IL-12R, IL-12 receptor; LPC, lysophosphatidylcholine; LPA, lysophosphatidic acid; LPA_x, LPA receptors; R, receptor, S1P, sphingosine-1-phosphate; $S1P_1$, S1P receptor-1; $S1P_{2/3}$, S1P receptor-2/3; SPC, sphingosine-phosphorylcholine; SphK, sphingosine kinase.

induced by SphK2 was mediated through the intrinsic mitochondrial death pathway [Liu et al., 2003]. Similar to the pro-survival effect of SphK1, S1PRs were dispensable for the apoptotic effect of SphK2. Interestingly, sequence analysis revealed that SphK2, but not SphK1, contains a 9 amino acid sequence reminiscent of the Bcl-2 homology 3 domain (BH3) [Liu et al., 2003] (Fig. 2), which plays an important role in cell death triggered by a subgroup of pro-apoptotic Bcl-2 family members [Huang and Strasser, 2000; Cheng et al., 2001]. The BH3 domain of SphK2 interacted with Bcl-$_{XL}$ and site-directed mutagenesis of the critical upstream leucine residue at position 229 of this domain decreased drastically SphK2-induced apoptosis. These results suggest that SphK2 may have a bona fide BH3 domain. However, since a NH2 terminal fragment of SphK2, which contains the BH3 domain, induced apoptosis to a lesser extend than the holo protein, it is possible that additional unknown mechanisms are involved in apoptosis mediated by SphK2.

Ca^{2+} homeostasis. The sphingolipid rheostat has also been implicated in several other biological processes including Ca^{2+} homeostasis [Young and Nahorski, 2001]. Sphingosine blocks the store-operated Ca^{2+} release-activated Ca^{2+} current (I_{CRAC}), which is important for replenishing Ca^{2+} stores [Mathes et al., 1998]. In contrast, more than a decade ago, it was shown that S1P mobilizes Ca^{2+} from internal stores in an inositol 1,4,5-trisphosphate-independent manner [Ghosh et al., 1990, 1994; Mattie et al., 1994]. Over the ensuing years, many external stimuli that regulate cytosolic Ca^{2+} concentrations without activation of the PLC pathway, have been shown to stimulate SphK [Choi et al., 1996; Melendez et al., 1998]. SphK1 was recently implicated in Ca^{2+} mobilization induced by immunoglobulin receptors (Fig. 3) [Melendez and Khaw, 2002]. Interestingly, Meyer zu Heringdorf et al. [2003] have recently provided evidence that intracellular S1P mobilizes thapisgargin-sensitive stores of Ca^{2+} independently of S1P receptors (S1PRs) in several cell lines. However, these results do not exclude the possibility that S1P may also induce Ca^{2+} influx. Indeed, it has recently been suggested that intracellular S1P is a diffusible Ca^{2+} influx factor linking Ca^{2+} store depletion to downstream store-operated Ca^{2+} entry [Itagaki and Hauser, 2003]. The authors proposed that synthesis of S1P by SphK downstream of GPCR activation and upstream of channel activation, is a necessary step in Ca^{2+} influx. Although several lines of evidence strongly support a role for SphK in Ca^{2+} mobilization, the intracellular targets of S1P and/or Ca^{2+} channels regulated by S1P remain to be identified. These intracellular actions of S1P are likely to be different than the mechanisms by which exogenous S1P, by activation of its receptors, regulates Ca^{2+} mobilization through PLC stimulation [Meacci et al., 2002].

Immune responses. The balance between S1P and sphingosine and regulation of downstream signals, such as Ca^{2+} mobilization, seems to play an important role in the immune system, and particularly during the inflammatory response [Baumruker and Prieschl, 2002]. At higher concentrations, sphingosine markedly inhibited leukotriene synthesis and cytokine production in response to immunoglobulin in mast cells primed with antigen [Prieschl et al., 1999]. In contrast, an increase in the intracellular concentration of S1P was involved in the secretion of various cytokines [Prieschl et al., 1999]. It was recently found that SphK2 is associated with the cytoplasmic region of IL-12R [Yoshimoto et al., 2003] (Fig. 3). This protein–protein interaction was functional since SphK2 activity contributed to the differentiation of naïve $CD4^+$ T cells into the Th1 subset of T cells mediated by the immunoregulatory cytokine IL-12 (Fig. 3).

SphK1 has also been found to be a major player in responses induced by immune receptors. FcεRI, a high affinity receptor for IgE, triggered mast cell degranulation primarly through the consecutive activation of PLD1 and SphK1 [Melendez and Khaw, 2002]. The SphK1/S1P pathway has also been involved in the inflammatory response mediated by TNFα [Pettus et al., 2003] (Fig. 3). Cyclooxygenase-2 (COX-2) and prostaglandin E_2 (PGE_2) production induced by TNFα in L929 cells and adenocarcinoma A549 cells were dependent on SphK1 activity. It is possible that this could involve TRAF2, an adaptor of the TNF receptor, which interacts with and regulates SphK1 [Xia et al., 2002].

An additional role for SphK in the immune system was highlighted by studies with the immunomodulator, FTY720 [Brinkmann et al., 2002; Mandala et al., 2002]. Two independent studies showed that the phosphorylated form of FTY720, an analog of sphingosine,

was the pharmacologically active component. Phosphorylated FTY720 was able to elicit lymphopenia, which resulted from a reversible redistribution of lymphocytes from the circulation to secondary lymphoid tissues, thereby reducing the availability of immune cells for immune responses. In addition, phosphorylated FTY720 activated four of five S1PRs, suggesting that this compound mediates its effect through S1PR signaling pathways [Brinkmann et al., 2002; Mandala et al., 2002]. S1P was less potent in inducing lymphopenia than phosphorylated FTY720, perhaps due to reduced degradation by endogenous SPPs and/or SPL. Although, initially it was suggested that SphK1 phosphorylated FTY720 [Brinkmann et al., 2002], it has recently been shown that recombinant SphK2 was more potent than SphK1 in phosphorylating FTY720 [Billich et al., 2003; Paugh et al., 2003; Sanchez et al., 2003]. However, the phosphorylation of FTY720 was much less efficient than that of the natural substrate sphingosine [Fukuda et al., 2003; Paugh et al., 2003; Sanchez et al., 2003].

Cell motility. S1P regulates cell migration by interacting with cell surface S1PRs, whose downstream signaling controls factors important for cell locomotion [Spiegel and Milstien, 2003]. PDGF is an important mediator of cell migration and is a potent activator of SphK. Surprisingly, we found that in contrast to more well known types of receptor cross-talk or transactivation in which activation of GPCRs results in transactivation of growth factor tyrosine kinase receptors, the PDGF tyrosine kinase receptor transactivated $S1P_1$ by stimulating SphK1 and production of S1P [Hobson et al., 2001]. This reciprocal transactivation was essential for PDGF-directed cell movement and suggests that intracellularly generated S1P signals "inside-out" through its cell surface receptors to modulate chemotaxis (Fig. 3) [Hobson et al., 2001].

However, the effects of S1P on cellular motility are complex and depend on which S1PRs are expressed on the surface of the cell. In many cell types, activation of $S1P_1$ or $S1P_3$ by S1P stimulates chemotaxis, whereas activation of $S1P_2$ has inhibitory effects [Kluk and Hla, 2002]. We have recently shown that PDGF-induced chemotaxis was dysregulated in NIH 3T3 fibroblasts overexpressing SphK1 [Olivera et al., 2003]. In NIH 3T3 cells, which endogenously express $S1P_2$ and $S1P_3$ but not $S1P_1$, formation of intracellular S1P by SphK1 impaired focal adhesion remodeling and its turnover leading to aberrant cell migration. The S1P effect was mediated through inside-out signaling, where the intracellularly generated S1P activated a S1PR linked to a heterotrimeric $G\alpha_{12/13}$ protein (Fig. 3), leading to Rho activation and stress fiber formation which inhibits cell locomotion [Olivera et al., 2003].

Interestingly, the potent immunosuppressive agent FTY720 has unappreciated actions as a profound regulator of endothelial cell functions, including chemotaxis. Moreover, phosphorylated FTY720, similar to S1P, induced adherens junction assembly in endothelial cells by stimulation of VE-cadherin and β-catenin translocation. Recruitment of cell–cell junction molecules by phosphorylated FTY720 potently blocked VEGF-induced trans-cellular permeability in vitro and in vivo [Sanchez et al., 2003]. These results suggest that in addition to its function in transplantation medicine [Brinkmann and Lynch, 2002], FTY720 has potential utility in vascular permeability disorders.

SphK signaling in plants. S1P is also a signaling molecule in plants where it is involved in regulation of guard cell turgor by the phytohormone abscisic acid (ABA) [Ng et al., 2001]. Recently, we showed that SphK in *Arabidopsis thaliana* is activated by ABA, and mediates both ABA inhibition of stomatal opening and promotion of stomatal closure [Coursol et al., 2003]. Consistent with this observation, inhibition of SphK attenuated ABA regulation of guard cell inward K^+ channels and slow anion channels, which are involved in the regulation of stomatal pore size. Surprisingly, S1P regulated stomatal apertures and guard cell ion channel activities in wild-type plants, but not in knockout lines of the sole prototypical heterotrimeric G protein α-subunit gene, *GPA1*. Our results implicate heterotrimeric G proteins as downstream elements in the S1P signaling pathway that mediates ABA regulation of stomatal function, and suggest that the interaction between S1P and heterotrimeric G proteins is an evolutionarily conserved signaling mechanism [Coursol et al., 2003].

Autotaxin

Characterization of autotaxin. Autotaxin (ATX) is a member of the nucleotide pyrophosphatase/phosphodiesterase family of

ecto/exoenzymes [Murata et al., 1994; see also Tokumura's review in this volume]. ATX has multiple biological activities ranging from stimulation of chemotaxis to tumorigenic potential. Of importance to sphingolipid and lysophospholipid signaling is the recent discovery that ATX has lyso-phospholipase D (LysoPLD) activity [Moolenaar, 2002; Tokumura et al., 2002; Umezu-Goto et al., 2003]. ATX can hydrolyze lysophosphatidylcholine (LPC) to produce LPA. Its substrate specificity has been subsequently extended to several other glycerolysophospholipids, including lysophosphatidylinositol, lysophosphatidylserine, and lysophosphotidylethanolamine [Aoki et al., 2002]. Interestingly, the novel lipid sphingosinephosphorylcholine (SPC) has recently been added to the list of substrates for ATX, which cleaves it to form S1P [Clair et al., 2003] (Fig. 3). However, the catalytic efficiency for SPC hydrolysis to S1P is 4.5-fold lower than for the hydrolysis of LPC to LPA and the importance of these provocative findings remains to be established.

Cellular functions of ATX. The tumor motility-stimulating protein ATX possesses enzymatic activity that is necessary for its stimulation of migration and invasion [Lee et al., 1996; Nam et al., 2000]. The hydrolysis of lyso-phospholipids catalyzed by ATX [Umezu-Goto et al., 2003] appears to mediate many of the documented effect of ATX [Moolenaar, 2002]. Indeed, hydrolysis of LPC by ATX produces LPA, a potent mitogen and motogen that acts through the LPA receptor family [Chun et al., 2002; see also Chun's review in this volume] (Fig. 3). Surprisingly, ATX in the presence of exogenous SPC, which produces S1P, inhibited cell migration-induced by LPA but not by PDGF [Clair et al., 2003] (Fig. 3). This is the first report showing inhibition of chemotaxis by an enzymatic product of ATX. Interestingly, similarly to the mechanism of inhibition of chemotaxis by overexpressed SphK1 [Olivera et al., 2003], dysregulation of cell locomotion by ATX in the presence of SPC was correlated with high levels of activated Rho [Clair et al., 2003]. However, in this case, S1P mediated "outside-in" signaling activation of a S1PR.

Because ATX stimulates motility of various cell lines [Umezu-Goto et al., 2003], it is likely that this is mediated by production of LPA. However, as substrate specificity and availability can vary locally, the in vivo effects of ATX are likely to depend on its microenvironment. Moreover, both LPA and S1P stimulate cell growth and angiogenesis and protect against apoptosis [Tigyi, 2001; Spiegel and Milstien, 2003]. Therefore, ATX may play a complex regulatory role depending on the availability of lipid substrates and receptors.

BIODEGRADATION OF S1P

S1P Phosphohydrolases

Characterization of S1P phosphohydrolases. S1P is degraded to sphingosine by dephosphorylation catalyzed by specific SPPs [Le Stunff et al., 2002a] and/or members of the broad specificity lipid phosphate phosphohydrolase family (LPPs) [Brindley et al., 2002]. LPPs belong to a phosphohydrolase superfamily, which is characterized by the presence of three highly conserved motifs: KDX(4)PRP; PSXH; and LVX(3)RXYXHX(2)LD [Brindley et al., 2002]. Mutational analysis of LPPs showed that conserved residues in each domain function in coordination to form the catalytic site.

Experiments using tissue homogenates and whole cells have long suggested the existence of a phosphohydrolase highly specific for S1P [Van Veldhoven and Mannaerts, 1994; De Ceuster et al., 1995]. A mammalian S1P-specific phosphohydrolase (SPP-1) (Fig. 2) was cloned based on its homology with the yeast long chain base phosphatases (LBP) [Mandala et al., 2000; Le Stunff et al., 2002b]. SPP-1 shows high homology to the conserved amino acid domain predicted to be the active site of LPPs, indicating that it is a member of the phosphohydrolase superfamily. However, some differences exist in the spacing between conserved domains 1 and 2, suggesting that SPP-1 evolved separately from the LPPs [Le Stunff et al., 2002a]. In comparison to mammalian LPPs, which have broad substrate specificity, SPP-1 is highly specific for sphingoid base phosphate esters, including S1P, dihydrosphingosine-1-phosphate (dihydroS1P) and phytosphingosine-1-phosphate (phytoS1P) [Mandala et al., 2000; Le Stunff et al., 2002b]. SPP-1 activity was exclusively expressed in membrane fractions, consistent with its hydrophobicity profile suggesting the presence of 8–10 membrane-spanning helices (Fig. 2). While SPP-1 activity shares some characteristics with the S1P phosphohydrolase activity in crude rat liver preparations, northern analysis showed a lack of correlation between SPP-1 expression and the relative S1P

dephosphorylating activity measured in rat tissues [De Ceuster et al., 1995; Le Stunff et al., 2002b]. Indeed, SPP-1 mRNA levels are highest in liver, kidney, and placenta [Mandala et al., 2000], whereas the highest level of S1P dephosphorylating activity is in brain extracts, followed by spleen and intestinal mucosa [De Ceuster et al., 1995]. These differences could be due to the presence of additional SPPs. Interestingly, a second SPP, SPP-2 (Fig. 2), has recently been identified [Ogawa et al., 2003]. Although the NH_2-terminal of SPP-2 is shorter than that of SPP-1, the transmembrane structure of this enzyme appears to be similar and it is predicted to have as many as 9 membrane-spanning segments (Fig. 2). While SPP-2 is also highly specific for sphingoid base phosphate esters, it has different properties than SPP-1, including sensitivity to various phosphohydrolase inhibitors. Whereas LPPs are expressed mostly on the plasma membrane and function as so-called ecto-phosphohydrolases, both SPP-1 and SPP-2, similar to their yeast counterparts, are localized to the ER [Le Stunff et al., 2002c; Ogawa et al., 2003]. Additionally, unlike SPP-1, expression of SPP-2 is rather tissue-specific and is highest in kidney and heart and not detectable in placenta or liver [Ogawa et al., 2003]. Despite their overall homologies, their distinct tissue distribution indicates that they may carry out distinct cellular functions and be regulated differently.

Cellular functions of S1P phosphohydrolases

Sphingolipid metabolism. The ER localization of SPP-1 and SPP-2 suggests that they function to dephosphorylate intracellular S1P, thereby modulating intracellular actions of S1P. Analogous to the role of LBP1 in yeast [Le Stunff et al., 2002a], SPP-1 expression altered the levels of S1P in mammalian cells [Mandala et al., 2000]. The presence of SPPs, which produce sphingosine, the precursor of ceramide synthesis in the ER where all of the enzymes of de novo ceramide synthesis reside, suggests that SPPs could also have an important role in regulating ceramide biosynthesis. In agreement, expression of SPP-1 increased ceramide levels in mammalian cells (Fig. 4) [Mandala et al., 2000; Le Stunff et al., 2002c]. Surprisingly, sphingosine levels were not significantly affected, reflecting a rapid conversion into ceramide. Whereas exogenous S1P induced ceramide biosynthesis in SPP-1 transfectants, dihydroS1P had no effect on ceramide, even though it is as good a substrate for SPP-1 as S1P (Fig. 4) [Le Stunff et al., 2002c]. Thus, it appears that the presence or the absence of the *trans* double bond in the sphingoid base dictates its function in the biosynthesis of ceramide and also suggests that dihydroceramide might be more efficiently used for complex sphingolipids synthesis than ceramide (Fig. 4). Alternatively, translocation of dihydroceramide to the Golgi apparatus could be more rapid than ceramide. As such, the biosynthetic trafficking of ceramide and dihydroceramide may be differentially regulated, contributing to specific cellular functions.

Apoptosis. Divergence in the regulation of ceramide and dihydroceramide biosynthesis by SPP-1 has important implications, not only for sphingolipid metabolism, but also for their distinct roles in apoptosis. It is well known that dihydroceramide is much less potent than ceramide in the induction of cell death. Interestingly, in SPP1-transfectants, S1P, but not dihydroS1P, induced accumulation of C16-ceramide, resulting in enhanced cell death (Fig. 4) [Le Stunff et al., 2002c]. Since sites of contact have often been observed in the membranes of the ER and mitochondria [Marsh et al., 2001], it is possible that these could be responsible for the delivery of apoptotic C16-ceramide, but not dihydroceramide, to the mitochondria (Fig. 4). Alternatively, sphingosine but not dihydrosphingosine, formed at the ER by SPP-1, might be transported to the mitochondria where it could serve as a substrate for a mitochondrial ceramide synthase or for a mitochondrial ceramidase with reciprocal activity [El Bawab et al., 2002]. Interestingly, knockdown of SPP-1 by siRNA technology endowed resistance to TNFα and the chemotherapeutic agent daunorubicin [Johnson et al., 2003], in support of a role for endogenous SPP-1, through the regulation of intracellular S1P levels, in the control of apoptosis.

Cell signaling. In addition to the regulation of the sphingolipid rheostat by SPPs, it has recently been shown that expression of yeast LBP1 potentiates transforming growth factor-β (TGFβ) signaling in mammalian cells, in particular, regulation of extracellular matrix gene expression [Sato et al., 2003]. In contrast, SphK1 inhibits TGFβ-induction of the collagen promoter, suggesting that ceramide, and not sphingosine or S1P, is a positive regulator of collagen expression. Interestingly, TGFβ receptor

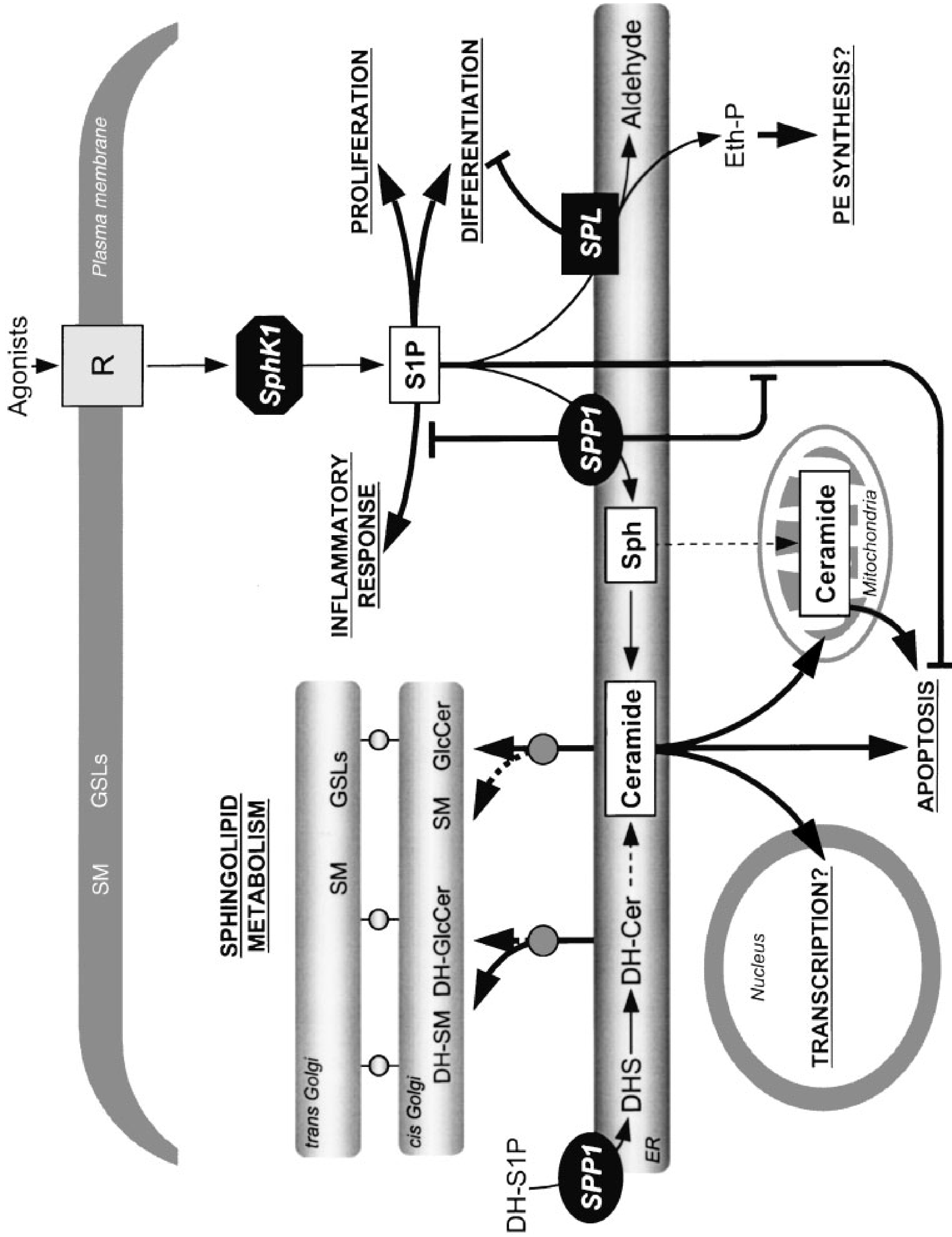


Fig. 4.

types I and II form homodimers in the ER, consistent with their potential interaction with the ER-resident LBP1. Recently, mammalian SPP-1 has also been involved in TNFα signaling [Pettus et al., 2003] (Fig. 4). Indeed, downregulation of SPP-1 mRNA enhances the ability of TNFα to induce COX-2 expression and PGE_2 production. This result also demonstrates that SPP-1 can serve to attenuate the effect of intracellular formed S1P induced by various agonists.

S1P Lyase

Characterization of SPL. Another route of S1P degradation is through cleavage at the C2–C3 bond by SPL to produce hexadecenal and phosphoethanolamine, which can then be further metabolized to glycerol and phospholipids [Van Veldhoven and Mannaerts, 1994]. SPL also degrades dihydroS1P and phytoS1P. The first mammalian SPL was identified based on sequence homology to yeast SPL [Zhou and Saba, 1998; Van Veldhoven et al., 2000]. SPL is a pyridoxal 5′-phosphate-dependent enzyme with a conserved pyridoxal-dependent decarboxylase domain positioned at the middle of the protein (Fig. 2) [Kihara et al., 2003]. SPL expression has been detected in diverse species and in many mammalian tissues, with the highest abundance in liver, followed by kidney, lung, heart, and brain [Zhou and Saba, 1998; Van Veldhoven et al., 2000]. Interestingly, SPL is not found in platelets, which may explain their high levels of S1P. In addition, SPL has been shown to localized in the ER [Reiss et al., 2004], where all of the enzymes of ceramide biosynthesis are located.

Cellular functions of SPL. Mutation studies have demonstrated that yeast strains lacking SPL are resistant to heat stress and deregulate proliferation upon approaching stationary phase [Gottlieb et al., 1999; Skrzypek et al., 1999]. Moreover, disruption of the *SPL* gene in *Dictyostelium discoideum* affects many stages of development, including the cytoskeleton architecture of aggregating cells, the ability to form migrating 'slugs,' and terminal spore differentiation [Li et al., 2001]. Thus, SPL may be a key determinant of multi-cellular development. Moreover, differentiation of SPL-null mammalian F9 cells into primitive endoderm by retinoic acid was accelerated. In contrast, expression of SphK1 in F9 cells potentiated RA-induced differentiation [Kihara et al., 2003], suggesting that intracellular S1P has a role in primitive endoderm differentiation and that SPL may be involved in the regulation of intracellular S1P levels during differentiation (Fig. 4). Recently, mammalian SPL has also been involved in TNFα signaling [Pettus et al., 2003]. Thus, downregulation of SPP-1 expression enhanced the ability of TNFα to induce COX-2 expression and PGE_2 production. Recently, by analogy with the action of SPL on sphingolipipid metabolism and apoptosis, it has been shown that overexpression of SPL in mammalian cells increases apoptosis through ceramide generation [Reiss et al., 2004]. Interestingly, the increase in ceramide levels mediated by both SPP-1 and SPL is sensitive to potent inhibitors of ceramide synthase [Le Stunff et al., 2002c; Reiss et al., 2004], suggesting that S1P may play a negative regulatory role on the ceramide biosynthetic machinery. Together, these latter results indicate that both S1P catabolizing enzymes are dual modulator of S1P and ceramide metabolism, which play an important role in cell fate decisions.

In another species, *Caenorhabditis elegans*, inhibition of SPL expression causes accumulation of phosphorylated and unphosphorylated long chain sphingoid bases and leads to poor feeding, delayed growth, reproductive abnormalities and intestinal damage [Mendel et al., 2003]. SPL null *Drosophila* mutants also accumulate phosphorylated and unphosphorylated sphingoid bases and exhibit semi-lethality, increased apoptosis of developing embryos, diminished egg-laying, and gross pattern abnormalities in dorsal longitudinal flight muscles [Herr et al., 2003]. These results suggest

Fig. 4. Cellular localization and function of S1P degrading enzymes. Modulation of the activity of key enzymes involved in degradation of S1P may be linked to biological processes. SPP-1, which is localized to the ER and transforms S1P to ceramide through the production of sphingosine, has been involved in the induction of apoptosis, differential regulation of sphingolipid metabolism, inhibition of inflammatory responses, and potentially in gene expression. SPL, which degrades S1P into ethanolamine-1-phosphate and palmitaldehyde, and SphK1 have been implicated in the regulation of endoderm differentiation in early mouse embryogenesis. Abbreviations: DH, dihydro; DHS, dihydrosphingosine; Eth-P, ethanolamine-1-phosphate; GlcCer, glucosylceramide; GSls, glycosphingolipids; PE, phosphatidylethanolamine; R, receptor; SM, sphingomyelin; Sph, sphingosine; S1P, sphingosine-1-phosphate; SphK1, sphingosine kinase-1; SPP1, S1P phosphohydrolase-1; SPL, S1P lyase.

that the sphingolipid degradative pathway and SPL play a role in regulating animal development. Future studies with SPL knockout mice should provide additional clues to the functions of this enzyme.

SUMMARY AND PERSPECTIVES

Research on the sphingolipid metabolite S1P has expanded tremendously in the past few years. This is partly due to the discovery of genes known to be involved in phosphorylated sphingoid base metabolism. Overexpression and deletion of some of these enzymes has provided important insights into the functions of S1P and other sphingolipid metabolites. Nevertheless, much remains to be learned about their regulation, and about the mechanisms by which extracellular stimuli orchestrate the S1P arm of the sphingolipid rheostat. Generation of null mutants by gene targeting can help to elucidate the in vivo role of enzymes involved in S1P metabolism. In addition to the regulatory mechanisms leading to the activation of S1P metabolizing enzymes (e.g., translocation and post-translational modifications), studies at the transcriptional level may also provide important clues to the biological functions of these enzymes. Indeed, phorbol esters increase SphK1 activity by protein translocation/phosphorylation [Johnson et al., 2002], and also increase protein expression by enhancing promoter activity of the *SphK1* gene [Nakade et al., 2004]. Additionally, new techniques, such as DNA microarray technology and proteomic approaches, may prove to be useful for identifying the cellular functions of these enzymes. Results from research with yeast, plants, zebrafish, and *Drosophila*, are likely to reveal novel mechanisms of S1P signaling [Birchwood et al., 2001; Ng et al., 2001; Coursol et al., 2003; Herr et al., 2003]. Finally, greater knowledge of S1P metabolizing enzymes and their roles in regulating signal transduction may provide the basis for the development of novel therapeutics to control diseases in which dysregulated S1P homeostasis could be an underlying contributory determinant.

ACKNOWLEDGMENTS

We thank the many members of the Spiegel lab for their contributions to the studies that were quoted in this review. We apologize to those authors whose work could not be cited owing to space limitations. Hervé Le Stunff received a postdoctoral fellowship from Association de Recherche contre le Cancer (France).

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