Recycling of Sphingosine Is Regulated by the Concerted Actions of Sphingosine-1-phosphate Phosphohydrolase 1 and Sphingosine Kinase 2*

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In yeast, the long-chain sphingoid base phosphate phosphohydrolase Lcb3p is required for efficient ceramide synthesis from exogenous sphingoid bases. Similarly, in this study, we found that incorporation of exogenous sphingosine into ceramide in mammalian cells was regulated by the homologue of Lcb3p, sphingosine-1-phosphate phosphohydrolase 1 (SPP-1), an endoplasmic reticulum resident protein. Sphingosine incorporation into endogenous long-chain ceramides was increased by SPP-1 overexpression, whereas recycling of C16:0-ceramide into long-chain ceramides was not altered. The increase in ceramide was inhibited by fumonisin B1, an inhibitor of ceramide synthase, but not by ISP-1, an inhibitor of serine palmitoyltransferase, the rate-limiting step in de novo biosynthesis of ceramide. Mass spectrometry analysis revealed that SPP-1 expression increased the incorporation of sphingosine into all ceramide acyl chain species, particularly enhancing C16:0, C18:0, and C20:0 long-chain ceramides. The increased recycling of sphingosine into ceramide was accompanied by increased hexosylceramides and, to a lesser extent, sphingomyelins. Sphingosine kinase 2, but not sphingosine kinase 1, acted in concert with SPP-1 to regulate recycling of sphingosine into ceramide. Collectively, our results suggest that an evolutionarily conserved cycle of phosphorylation-dephosphorylation regulates recycling and salvage of sphingosine to ceramide and more complex sphingolipids.

Sphingolipids are a structurally diverse family of membrane lipids. Several lines of evidence have implicated metabolites of sphingolipids such as ceramide, sphingosine, and sphingosine 1-phosphate (S1P)5 in diverse cellular processes (1, 2). Ceramide and sphingosine have been implicated in pathways involving stress responses, cell differentiation, apoptosis, and cell cycle arrest (1). Unlike ceramide and sphingosine, S1P promotes cell growth and survival and inhibits apoptosis (2, 3). Because of their inter-convertibility and opposing effects, the dynamic balance between S1P and ceramide/sphingosine has been proposed to be an important factor that determines cell fate (2). Accumulating evidence suggests that the S1P/ceramide balance is ultimately regulated by the relative activities of enzymes controlling the turnover of these sphingolipid metabolites. However, the molecular mechanisms involved in the regulation of intracellular levels of these sphingolipids are not yet fully understood.

Cellular levels of S1P are kept low by tight spatio-temporal regulation of its synthesis and degradation. Sphingosine kinases (SphKs) catalyze the synthesis of S1P by phosphorylation of sphingosine. Two distinct SphK isoforms, SphK1 and SphK2, have been cloned and characterized in mammals (2). Diverse external stimuli, particularly growth and survival factors, stimulate SphK1, generating S1P that has been implicated in their mitogenic and anti-apoptotic effects (4, 5). In contrast to SphK1, rather than promoting growth and survival, overexpression of SphK2 suppressed growth and enhanced apoptosis (6, 7), implying that they may have distinct physiological functions, likely due to their different subcellular localizations. Degradation of S1P is mediated either by irreversible cleavage to ethanolamine phosphate and hexadecenal by a pyridoxal-dependent S1P lyase or by reversible dephosphorylation to sphingosine by specific S1P phosphohydrolases (SPPs) (8). Two isoforms of mammalian S1P phosphohydrolases, designated SPP-1 (9–11) and SPP-2 (12), have been identified. Both belong to the superfamil of lipid phosphatases that also include the type 2 lipid phosphate phosphohydrolases (LPPs) (13, 14). LPPs are magnesium-independent, membrane-associated, and N-ethylmaleimide-insensitive and include three isoforms, LPP1, LPP2, and LPP3 (15, 16). Except for the conserved residues within three domains present in the active sites of LPPs (14), the two S1P phosphatases have little overall homology to other known LPPs, and in contrast to the broad specificity of the other LPPs (13), they are specific sphingoid base phosphohydrolase; PDMP, 1-phenyl-2-decanoylamino-3-morpholino-1-propanol; Sph, sphingosine; SphK, sphingosine kinase; SPP-1, S1P phosphohydrolase 1; ESI-MS/MS, electrospray ionization-tandem mass spectrometry; siRNA, short interfering RNA; SphK, sphingosine kinase.
S1P in Sphingolipid Synthesis from Exogenous Sphingosine

Moreover, Lcb4p is tightly associated with membranes and is localized to the endoplasmic reticulum (ER), where they degrade S1P to terminate its actions (11, 12, 17). These findings have far reaching implications as the ER also contains the enzymes of ceramide biosynthesis, suggesting an important role for SPPs in the regulation of ceramide levels. Indeed, we have previously found that SPP-1 functions in an unprecedented manner to enhance ceramide levels and apoptosis (17). Moreover, increased intracellular and secreted S1P due to down-regulation of SPP-1 regulates “inside-out” S1P signals through its cell surface receptors (18). In agreement, regulation of intracellular levels of S1P by SPP-1 plays an important role in epidermal growth factor-directed cell movement (19).

Biosynthetic pathways of sphingolipids are highly conserved between yeasts and mammals (20). Sphingosine is not an intermediate in the biosynthesis of ceramide and is predominantly generated in the endocytic pathway from turnover of membrane complex sphingolipids. Sphingosine can be metabolized to ceramide or S1P by ceramide synthase and SphKs, respectively. In yeast, it has been found that exogenous sphingoid bases are converted to ceramides indirectly by a cycle of phosphorylation/dephosphorylation (20). Several studies have shown previously that the yeast SPP-1 homologue, Lcb3p, is required for the incorporation of exogenous dihydrosphingosine into sphingolipids (21–23). Moreover, the major yeast sphingoid base kinase, Lcb4p, but not Lcb5p, regulates synthesis of ceramide from exogenously added dihydrosphingosine. kinase was from Calbiochem. The internal standards for quantitation of the sphingolipids by ESI-MS/MS were obtained from Avanti Polar Lipids (Alabaster, AL). Serum and medium were from Biofluids (Rockville, MD). G418 was obtained from Invitrogen. Other chemicals were from Sigma.

Cell Culture and Plasmids—Human embryonic kidney cells (HEK 293, ATCC CRL-1573) were cultured in high glucose Dulbecco’s modified Eagle’s medium containing 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mm l-glutamine supplemented with 10% fetal bovine serum (17). Mammalian expression vectors for SPP-1, SphK1, SphK1-G82D, SphK2, and SphK2-G213E were described previously (7, 17, 25). Cells were transfected with Lipofectamine Plus (Invitrogen) as described (17) and stable transfectants were selected in medium containing 1 g/liter G418.

siRNA Transfection—Cells were transfected with 50 nmol of sequence-specific, ON-TARGETplus SMARTpool siRNA against SPP-1 SphK2, and control siRNA from Dharmacon (Lafayette, CO) using Oligofectamine (Invitrogen) as described previously (26). Total RNA was isolated with TRIzol reagent (Invitrogen) and reverse-transcribed with Superscript II (Invitrogen). Quantitative PCR was performed with premixed primer-probe sets using the ABI 7800 (Applied Biosystems, Foster City, CA).

Measurement of Mass Levels of Ceramide—HEK 293 cells (5 × 10⁴) were seeded in 6-well polyl-lysine-coated plates. Mass levels of sphingosine and ceramide and total phospholip-
**S1P in Sphingolipid Synthesis from Exogenous Sphingosine**

**RESULTS**

Expression of SPP-1 Increases the Incorporation of Exogenous Sphingosine into Long-chain Ceramides—As was previously found in A549 lung carcinoma cells (28), treatment of HEK 293 cells with C<sub>e</sub>-ceramide caused a significant increase in endogenous long-chain ceramide levels (Fig. 1A). However, this increase was not affected by expression of SPP-1 (Fig. 1A). In contrast, increased ceramide due to sphingosine treatment was drastically further elevated by overexpression of SPP-1 (Fig. 1A). The increase in ceramide levels in SPP-1-transfected cells was dependent on the concentration of added sphingosine (Fig. 1B) and was detected within 30 min (Fig. 1C), reaching a maximum after...
2 h and remaining elevated for at least 6 h (Fig. 1C). One h of treatment with exogenous sphingosine followed by incubation in its absence reduced the increase in ceramide levels by 20 and 76% at 6 and 24 h, respectively (Fig. 1D).

Incorporation of exogenous [3H]sphingosine into long-chain ceramides (C16–C18), and to a lesser extent into very long-chain ceramides (C24–C26), was increased in SPP-1-expressing cells compared with vector transfectants (Fig. 2A). Moreover, sphingosine addition to SPP-1-transfected cells predominantly increased levels of long-chain ceramide species as determined by conversion to ceramide 1-phosphates with diacylglycerol kinase (Fig. 2B). In agreement with our previous studies (17), very little incorporation of [3H]palmitate, a substrate for serine palmitoyltransferase, into ceramide was detected in the absence of exogenous sphingosine. However, sphingosine significantly increased incorporation into [3H]-labeled C16-ceramide after 6 and 24 h, which was markedly enhanced by overexpression of SPP-1 (Fig. 2C). These results

**FIGURE 4.** Fumonisin B1, but not ISP1, reduces incorporation of sphingosine into ceramide in SPP-1-expressing cells. Vector- or SPP-1-transfected HEK 293 cells were treated without (Vehicle) or with 5 μM Sph in the absence or presence of 10 μM ISP1 (A) or 25 μM fumonisin B1 (B) for 24 h. Ceramide (Cer) was determined as described under “Experimental Procedures.” Data are the means ± S.E. of three independent experiments. *, p ≤ 0.05. Cells were incubated in the presence of 10 μCi/ml [3H]palmitic acid, 25 μM FB, or 10 μM ISP1 without or with 5 μM Sph for 24 h. Labeled lipids were extracted, separated by TLC, and visualized by autoradiography. Representative results from three independent experiments are shown. *, p ≤ 0.05, indicates significant difference in cells treated with FB1.

**FIGURE 5.** Effect of SPP-1 expression on sphingolipids species. A, vector- or SPP-1-transfected HEK 293 cells were incubated without or with 5 μM Sph in the presence of 10 μCi/ml [3H]palmitic acid for the indicated times. Labeled sphingolipids were extracted, and glycerolipids were removed by mild saponification, separated by TLC in a solvent system of chloroform, methanol, 20 mM CaCl2 (20:10:4, v/v), and visualized by autoradiography. SM, sphingomyelin; HexCer, hexosylceramide. Similar results were obtained in three independent experiments. B and C, duplicate cultures were treated without (Vehicle) or with 5 μM Sph for 24 h. Lipids were extracted, and hexosylceramide (B) and sphingomyelin (C) species were determined by ESI-MS/MS. Data are expressed as pmol of lipid/mg of protein. Numbers indicate chain length followed by the number of double bonds in the fatty acid. Similar results were found in two additional experiments.
S1P in Sphingolipid Synthesis from Exogenous Sphingosine

**FIGURE 6. Inhibition of glucosylceramide synthase enhances incorporation of sphingosine into ceramide in SPP-1-expressing cells.** A, vector- and SPP-1-transfected HEK 293 cells were incubated without or with 5 μM Sph in the absence or presence of 10 μM PDMP for 24 h. Ceramide (Cer) levels were determined by the DAG kinase assay. Data are the means ± S.E. of three independent experiments. B and C, cells were incubated with 10 μCi/ml [3H]palmitic acid in the absence or presence of 10 μM PDMP or 5 μM Sph for 24 h as indicated. Labeled lipids were extracted and [3H]-labeled ceramide (B) or sphingolipids (C) analyzed by TLC. D, duplicate cultures were incubated in the presence of 10 μCi/ml [3H]palmitic acid, without or with 25 μM FB1, and 5 μM Sph as indicated. For all panels, representative results from three independent experiments are shown. *, p ≤ 0.05. HexCer, hexosylceramide.

indicate that SPP-1, like its yeast counterpart Lcb3p (21–23), may be involved in the incorporation of exogenous sphingosine into ceramide.

We next examined the effects of SPP-1 on ceramide mass levels by high performance liquid chromatography ESI-MS/MS, as this is the method of choice to simultaneously determine the degree of saturation of the sphingoid base and the chain length of the fatty acids (29). Treatment with sphingosine increased total ceramide mass in vector- and SPP-1-transfected cells by 1.6- and 3.5-fold, respectively. In agreement with previous studies (7, 30–33), the most abundant ceramide species in HEK 293 cells was 16:0, followed by 24:1, 24:0, and 18:0 (Fig. 3A). Exogenous sphingosine increased the mass of all ceramide acyl chain species, most obviously enhancing C16- and C18-ceramides (Fig. 3A). It is also evident that when the subspecies distributions are compared in “donut” diagrams, treatment with sphingosine increased the proportion of long-chain ceramides, particularly C16 and C19, and reduced the proportion of very long-chain ceramides (C24:0 and C24:1). Cells overexpressing SPP-1 increased the incorporation of sphingosine into all ceramide acyl chain species, most notably enhancing the C16-0-, C18:0-, and C20:0-ceramides (Fig. 3, A and B). Cellular sphingosine levels measured by ESI-MS/MS were unaffected by expression of SPP-1, indicating that uptake of sphingosine was not altered (Fig. 3C). Moreover, as expected, when cells were treated with sphingosine, there were also increases in S1P mass (Fig. 3D). However, although uptake of sphingosine was unaffected by expression of SPP-1, levels of S1P were markedly decreased by its expression (Fig. 3D), consistent with its enzymatic activity.

Involvement of Ceramide Synthase(s) in Ceramide Accumulation Induced by SPP-1—To determine whether the increase in long-chain ceramides results from enhanced de novo biosynthesis or salvage and recycling of sphingosine, cells were pretreated with myriocin (ISP1), a specific inhibitor of serine palmitoyl transferase, the rate-limiting step in de novo synthesis or with fumonisin B1 (FB1), an inhibitor of dihydroceramide/ceramide synthases that acylate sphingoid bases. In SPP-1-expressing cells, ISP1 had no effect or even slightly increased incorporation of added sphingosine into ceramide (Fig. 4A), whereas FB1 significantly reduced the increase in ceramide levels resulting from treatment with exogenous sphingosine (Fig. 4B). Similar results were obtained when incorporation of sphingosine into ceramide was examined by [3H]palmitate labeling (Fig. 4C). The elevation of C16-ceramide was significantly attenuated by FB1 but not by ISP1 (Fig. 4C).

**Effect of SPP-1 on Ceramide Metabolism**—There was an increase in the incorporation of [3H]palmitate into long-chain hexosylceramides and to a much lesser extent into sphingomyelin in vector- and SPP-1-transfected cells treated with sphingosine (Fig. 5A). Expression of SPP-1 potentiated the increase in labeled long-chain hexosylceramides (Fig. 5A). There was also an increase in total mass of hexosylceramides in SPP-1-transfected cells treated with sphingosine as determined by ESI-MS/MS. Analysis of subspecies of complex sphingolipids revealed that the increase was mainly in C16:0-, C18:0-, and C20:0-hexosylceramides (Fig. 5B). Because the total amount of sphingomyelin in cells is much greater than hexosylceramide or ceramide, overexpression of SPP-1 caused only small changes in sphingomyelin mass levels, as expected (Fig. 5C).

To further substantiate that SPP-1 is involved in salvage and recycling of sphingosine back to ceramide and glycosphingolipids, cells were treated with (R,R)-(α-threo)-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), an analogue of glucosylceramide and a potent inhibitor of glucosylceramide synthase (34). PDMP augmented the elevated ceramide accumulation induced by sphingosine in SPP-1 transfectants (Fig.
SphK2 Acts in Concert with SPP-1 to Recycle Sphingosine to Ceramide—In yeast, a cycle of phosphorylation and subsequent dephosphorylation is required for conversion of exogenous sphingoid bases to ceramide (24). To determine whether reutilization of sphingosine in mammalian cells proceeds similarly, the effects of SphK1 or SphK2 on SPP-1-mediated conversion of sphingosine to ceramide were examined. When SphK2 was co-expressed with SPP-1, basal ceramide levels were increased by 2-fold (Fig. 7A). In addition, co-expression of SphK2 and SPP-1 greatly increased the incorporation of exogenous sphingosine into ceramide, whereas co-expression with SphK1 did not potentiate the effects of SPP-1 (Fig. 7A). We next determined if the catalytic activity of SphK2 was required for these effects. Single point mutation of the second conserved glycine residue of the ATP-binding sequence of both SphKs resulted in a complete loss of sphingosine phosphorylating activity (11, 35, 36). The catalytically inactive G82D-SphK1 and G231E-SphK2 mutants have been shown to block various functions of these enzymes (11, 26, 36). Expression of G82D-SphK1 or G231E-SphK2 did not significantly affect the increased ceramide levels induced by sphingosine in SPP-1-expressing cells (Fig. 7B), suggesting that incorporation of exogenous sphingosine mediated by SPP-1 requires enzymatically active SphK2. SphK2 expression has been shown to increase the levels of C16:0-ceramide determined by ESI-MS/MS (35). In agreement, SphK2 also increased incorporation of \([3H]palmmitate\) into long-chain ceramides, especially in the presence of exogenous sphingosine (Fig. 7C). SphK2 also enhanced incorporation of exogenous sphingosine into ceramide stimulated by SPP-1 expression (Fig. 7, A and C). Moreover, transient expression of SphK2 also up-regulated the incorporation of \([3H]palmmitate\) into hexosylceramides, and to a lesser extent into sphingomyelin, in SPP-1-transfected cells treated with sphingosine (Fig. 7D).

To examine further the concerted actions of SphK2 and SPP-1, we first utilized DMS, which inhibits both SphK1 and SphK2. Although DMS only slightly but significantly decreased mass levels of ceramide induced by addition of sphingosine in SPP-1-overexpressing cells (Fig. 8A), it markedly decreased incorporation of sphingosine into ceramide determined by \([3H]palmmitate\) labeling (Fig. 8B). The elevation of \(C_{16}\)-ceramide was significantly attenuated by DMS in vector control cells and in SPP-1 overexpressors (Fig. 8B). Moreover, DMS also reduced incorporation of \([3H]palmmitate\) into hexosylceramides and, to a lesser extent into sphingomyelin, in SPP-1-transfected cells treated with sphingosine (Fig. 8C).

To examine more specifically the biological significance of SPP-1 and SphK2 in sphingosine recycling, their levels were down-regulated with specific siRNAs. Transfection with siRNA for SPP-1 and SphK2 markedly reduced their expression by 70 and 80% (Fig. 9A), respectively, without affecting expression of each other or their isozymes (data not shown). In agreement with recent studies (7), down-regulation of SphK2 markedly reduced incorporation of sphingosine into ceramide by almost 40% (Fig. 9B). Down-regulation of SPP-1 also significantly reduced incorporation of sphingosine into ceramide measured by \([3H]palmmitate\) labeling, albeit to a lesser extent than knocking down SphK2 (Fig. 9B). Moreover, down-regulation of SphK2 also drastically reduced
overexpressed SPP-1-mediated incorporation of sphingosine into ceramide (Fig. 9C). Taken together, these data indicate that, similar to yeast, a cycle of phosphorylation/dephosphorylation catalyzed by the concerted action of SphK2 and SPP-1 plays a role in recycling of sphingosine in mammalian cells.

DISCUSSION

It has been shown previously that exogenous C₆-sphingosine induces generation of endogenous long-chain ceramides in mammalian cells by recycling of the sphingosine backbone via deacylation/reacylation reactions (28, 37, 38). A different recycling pathway has been described in yeast where phosphorylation and subsequent dephosphorylation of the long-chain sphingoid base is required for efficient ceramide formation (21–24). We report here that a similar phosphorylation-dephosphorylation cycle is involved in re-utilization of sphingosine in mammalian cells. Increased incorporation of exogenous sphingosine into ceramide by SPP-1 expression suggests that this sphingosine-recycling pathway takes place in the ER where SPP-1 resides (17, 18). In contrast, incorporation of short-chain ceramide into endogenous long-chain ceramides, which has been shown previously to require an intact Golgi apparatus (28, 38), was not affected by SPP-1 overexpression. Six ceramide synthases, which are located in the ER and utilize a relatively restricted subset of fatty acyl-CoAs, have been cloned and characterized (39). Interestingly, although SPP-1 increased incorporation of sphingosine into all ceramide species, the most pronounced increase was in long-chain ceramides (C₁₆:0, C₁₈:0, and C₂₀:0). This might reflect the relative concentrations of different fatty acyl-CoA species. Alternatively, it is possible that SPP-1 is co-localized with specific ceramide synthases.

Whereas deletion of the yeast SPP-1 homologue, Lcb3p, completely prevents the incorporation of exogenous dihydrosphingosine into sphingolipids (21–23), down-regulation of mammalian SPP-1 only partially reduces added sphingosine into ceramide, suggesting that mammalian cells possess redundant pathways for reutilization of sphingosine.

The ceramide elevation was accompanied by an increase in the mass of long-chain hexosylceramides of corresponding chain lengths (C₁₆:0 and C₁₈:0), suggesting that one of the functions of the phosphorylation-dephosphorylation cycle might be to funnel excess sphingosine through ceramide back into complex sphingolipids, with a bias for glycosphingolipids over sphingomyelin. Intriguingly, the ceramide transfer protein CERT delivers ceramide for sphingomyelin but not glycosphingolipid synthesis (40). Thus, it is possible that ceramide formed by SPP-1-mediated recycling is not transported to the Golgi by CERT. A certain pool of ceramide molecules might be selectively translocated to a Golgi sub-fraction for glucosylceramide synthesis by a vesicular and non-CERT pathway (41). Another possibility is that ceramide is converted to hexosylceramides in the ER without inter-organelle transfer, as galactosylceramide synthase is located in the ER, and it has been suggested that de novo synthesis of glucosylceramide takes place not only in the cis-Golgi but also in a sub-region of the ER (42–44). Interestingly, it has been shown previously that glycosphingolipids are synthesized predominantly from sphingosine salvaged from the lysosomal pathway in slowly dividing cells, whereas in rapidly dividing cells, the need for increased synthesis is met by up-regulation of the de novo pathway (45). Moreover, both sphingosine and dihydrosphingosine are present in human plasma and serum, and their levels are elevated in patients with type 2 dia-
S1P in Sphingolipid Synthesis from Exogenous Sphingosine

This study provides new insights into the synthesis of glycosphingolipids through sphingosine recycling, which requires phosphorylation of sphingosine and then dephosphorylation of S1P by ER resident SPP-1 and finally acylation to ceramide. We found that SphK2, but not SphK1, acts in concert with SPP-1 to recycle sphingosine into ceramide. Previous studies demonstrated that SphK1 expression markedly increases levels of dihydro-S1P, and to a lesser extent S1P (35, 47, 48), with a parallel decrease of ceramide levels. Intriguingly, although dihydrophosphosine levels were also significantly increased, there were no changes in dihydroceramide levels (35, 47). Thus, it is possible that SphK1 could reduce ceramide levels by routing sphingosine to phosphorylation and subsequent irreversible degradation by the lyase (47). Alternatively, it is also possible that the dihydro-S1P formed by SphK1 may negatively regulate de novo ceramide biosynthesis either by inhibition of serine palmitoyltransferase (49) or by inhibiting (dihydro)ceramide synthase (35).

SphK1 might regulate de novo ceramide biosynthesis (35, 47), yet in contrast to SphK2, it does not influence sphingosine recycling through the salvage pathway. SphK2 expression did not significantly alter S1P or dihydro-S1P levels, whereas it increased basal levels of ceramide (35, 47) and increased incorporation of free sphingosine into ceramide, suggesting a role for SphK2 in the reutilization of sphingosine and its conversion to ceramide. It has been suggested previously that during serum starvation, SphK2 is translocated to the ER (35). There, it can act in concert with SPP-1 to convert sphingosine into a specific pool of ceramides by ceramide synthase(s). Interestingly, the

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Recently, a novel pathway for intracellular generation of S1P in human lung endothelial cells was described, which involves the conversion of extracellular S1P to sphingosine by the ecto lipid phosphate phosphatase 1 (LPP-1), followed by intracellular phosphorylation to S1P by SphK1 (52). It was suggested that LPP-1 acts in concert with SphK1 but not with SphK2 to modify the balance of S1P signaling by decreasing extracellular S1P and lowering activation of S1P receptors and by promoting formation of intracellular S1P and its intracellular signaling (52). Our work revealed that a different phosphohydrolase, SPP-1, acts in concert with SphK2 to regulate levels of ceramide, another potent bioactive sphingolipid metabolite. Although it is unclear why yeast as well as mammalian cells use coupled phosphorylation by SphK2 and dephosphorylation by SPP-1 to recycle sphingoid bases to ceramide for reutilization, it seems likely that this is an important mechanism that sorts dihydrophosphosine/sphingosine arising from different pools, for regulation of glycosphingolipid synthesis, and/or signaling.

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S1P in Sphingolipid Synthesis from Exogenous Sphingosine

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