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Extracellular and Intracellular Actions of Sphingosine-1-Phosphate

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Abstract

Sphingosine-1-phosphate (S1P) is a bioactive lipid mediator with crucial roles in a wide variety of cellular functions across a broad range of organisms. Though a simple molecule in structure, S1P functions are complex. The formation of S1P is catalyzed by one of two sphingosine kinases that have differential cellular distributions as well as both overlapping and opposing functions and which are activated by many different stimuli. S1P can act on a family of G protein-coupled receptors (S1PRs) that are also differentially expressed in different cell types, which influences the cellular responses to S1P. In addition to acting on receptors located on the plasma membrane, S1P can also function inside the cell, independently of S1PRs. It also appears that both the intracellular location and the isotype of sphingosine kinase involved are major determinants of inside-out signaling of S1P in response to many extracellular stimuli. This chapter is focused on the current literature on extracellular and intracellular actions of S1P

Keywords

sphingosine kinase; sphingosine-1-phosphate; inside-out signaling; nucleus; sphingosine; dihydrosphingosine; S1PR

1. Introduction

In just over a decade, the sphingolipid metabolite, sphingosine-1-phosphate (S1P), has emerged as a key regulator of numerous physiological functions, including cell growth and survival, angiogenesis, cell motility and migration, and lymphocyte trafficking 1. S1P promotes cell growth and inhibits apoptosis, while its precursors, ceramide and sphingosine, typically inhibit cell growth and induce apoptosis 1. Therefore, the cellular balance of these three sphingolipid metabolites, the “sphingolipid rheostat”, is of crucial importance in regulating cell fate 1. Moreover, sphingolipid metabolism has been found to be dysregulated in many human diseases, including cancer, inflammation, atherosclerosis, and asthma 2. S1P is produced intracellularly by two sphingosine kinases (SphK1 and SphK2), and is the ligand for a family of five G protein-coupled receptors, termed $S1P_{1\text{-}5}$. However, there are some actions of S1P that appear to be independent of the known S1PRs. This review will focus on the emerging evidence for S1P as a second messenger.

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2. Sphingolipid metabolism

Sphingolipids are ubiquitous components of the lipid bilayer of eukaryotic cells. Like glycerolipids, numerous agonists regulate sphingolipid metabolism to generate signaling molecules, including ceramide (N-acyl sphingosine), sphingosine and sphingosine-1-phosphate (S1P) [1, 3, 4]. Ceramide, the backbone of all sphingolipids, is produced both by *de novo* synthesis and by turnover of sphingolipids, such as hydrolysis of sphingomyelin by sphingomyelinases. De novo synthesis at the endoplasmic reticulum (ER) is initiated by condensation of serine and palmitoyl-CoA catalyzed by serine palmitoyltransferase. The 3-ketosphinganine formed is rapidly reduced to dihydrosphingosine, which is subsequently N-acylated by one of a family of six (dihydro)ceramide synthases (CerS, formerly referred to as LASS) to form dihydroceramide, with the CerSs having differing but overlapping preferences for acyl chains from 16 to 26 carbons long. Finally, a 4–5 trans double bond is introduced in the sphingoid base to produce ceramide. Ceramide is then trafficked from the ER to the Golgi, where a variety of head groups are added, forming sphingolipids. After removal of these head groups during catabolism, deacylation of ceramide by ceramidases yields sphingosine, the most common sphingoid base in mammals. It is important to note that sphingosine and dihydrosphingosine (sphinganine) are not produced de novo and are only formed by catabolism of sphingolipids. These sphingoid bases can be re-utilized for complex sphingolipid biosynthesis or phosphorylated by two sphingosine kinases (SphK1 and SphK2) to form S1P. S1P can be degraded either by reversible dephosphorylation to sphingosine by phosphatases, including lysosomal phosphatases, members of the LPP family of lipid-specific phosphatases, and two S1P-specific phosphatases, termed SPP1 and SPP2 (reviewed in 5), or degraded by irreversible cleavage to ethanolamine phosphate and hexadecenal by S1P lyase (SPL) 6.

Ceramide and sphingosine, the precursors of S1P, are important regulatory components of stress responses, typically inducing growth arrest and apoptosis [3, 4]. In contrast, S1P has been implicated in motility and cytoskeletal rearrangements, formation of adherens junctions, proliferation, survival, angiogenesis, and the trafficking of immune cells [1, 7–9]. Thus, the dynamic balance between S1P and ceramide, and the consequent regulation of opposing signaling pathways, is an important factor that determines cell fate [10]. S1P produces many of its effects by acting as a ligand for one or more of its five receptors, discussed below. However, although intracellular targets for both ceramide and sphingosine have been identified, indicating they are both bona fide second messengers, intracellular targets for S1P have remained elusive.

3. Sphingosine kinases

Oddly, like mammalian SphKs, two SphK isoenzymes are also found in organisms as diverse as yeast, slime molds, worms, flies, and mammals [7]. In mammals, both SphKs have a broad and overlapping tissue distribution, with SphK1 predominating in lung and spleen, and SphK2 predominating in the heart, brain, and liver [11, 12]. Both SphKs are members of the diacylglycerol kinase family, containing five conserved domains responsible for substrate binding and catalytic activity [13]. Differential activation of SphKs can be determined by *in vitro* assays, as detergents stimulate SphK1 and inhibit SphK2[12]. Conversely, SphK2 is stimulated by high salt, whereas SphK1 is inhibited. SphK1 and SphK2 have similar endogenous substrate specificities, with both being able to phosphorylate d-*erythro*-dihydrosphingosine and d-*erythro*-sphingosine, the two mammalian sphingoid bases [11, 13]. Cells contain both S1P and dihydro-S1P, and both activate cell surface S1PRs with essentially the same affinity (see below). S1P predominates both in cells and plasma, and thus for the purposes of this review “S1P” will refer to all phosphorylated sphingoid bases, including phyto-S1P found in yeast and plants, unless otherwise indicated. SphK1 and SphK2 are primarily cytosolic, although their distributions are altered in different cell types and by various signals

(see below). Homozygous single knockouts of either isoenzyme are viable and there are also no obvious phenotypes in yeast [14] or mice 15, 16, suggesting that they may have redundant, overlapping, or compensatory functions. Intriguingly, there is no functional redundancy in *Drosophila*, as deletion of Sk2 but not Sk1 results in flight defects and reduced fecundity [17]. In yeast, even the double knockouts are viable [14]. However, when *Sphk1*$^{-/-}$ and *Sphk2*$^{-/-}$ mice were crossed, mice lacking 1 to 3 of the *Sphk* alleles appeared normal, but mice lacking all four alleles died *in utero* due to defective brain and cardiovascular system development [16]. This suggests that SphK1 and SphK2 are redundant in mammals, at least for viability. However, subtle yet significant phenotypic differences in *Sphk1*$^{-/-}$ and *Sphk2*$^{-/-}$ mice are emerging, and the apparent functional redundancy may not apply to a variety of pathophysiological conditions, suggesting that isozyme-specific targeting of SphKs may be an effective means of disease control or prevention.

3.1 SphK1

SphK1a is a 48 kDa splice variant that was cloned utilizing peptide sequences from SphK1 purified to homogeneity from rat kidneys [11]. Most investigations of the role of SphK1 have focused on SphK1a, the major splice form, although two N-terminal extension splice variants have been described in humans, SphK1b (+14 aa) and SphK1c (+86 aa), all with similar enzymatic properties [18]. SphK1 isoforms are predominantly cytosolic but have slightly different subcellular distributions, with SphK1b and SphK1c having greater plasma membrane localization 19. Interestingly, extracellular secretion of SphK1a, by unknown mechanisms through a Golgi-independent route has been described 19, although the biological significance of this is not yet clear. SphK1 was initially purified in part based on its ability to bind to a calcium-calmodulin column 20. Subsequently, it was shown that SphK1 has a calmodulin binding site between residues 191–206, the mutation of which blocks agonist-induced SphK1 translocation to the plasma membrane, but not its activation [21]. An important advance in understanding regulation of SphK1 was the demonstration that SphK1 is phosphorylated on Ser225 by ERK1/2[22], which was necessary but not sufficient for its targeting to the plasma membrane [23]. SphK1 has been shown to physically interact with a number of proteins that may affect its cellular localization, including RPK118[24], PECAM-1[25], Acy1[26], and δ-catenin/NPRAP [27], among others. SphK1 is activated by diverse stimuli, including hormones, growth factors, immunoglobulin receptor crosslinking, cytokines, chemokines, and lysolipids, including S1P [1]. Functionally, numerous studies have demonstrated a role for S1P produced by SphK1 in protection of cells from apoptosis, promotion of cell growth, stimulation of motility and tumorigenesis, and as an essential component of many signaling pathways, activating kinases, phospholipases, and inducing calcium release (reviewed in 1, 8, 28, 29). Indeed, many of the pro-growth and anti-apoptotic effects observed by exogenous addition of S1P can be reproduced by overexpression of SphK1.

3.2 SphK2

Much less is known about SphK2, which was cloned in mammalian cells based on its homology to SphK1[12]. SphK2 has two splice variants. The smaller, originally cloned SphK2-S, contains 618 amino acids, and the N-terminally extended SphK2-L, consists of 654 amino acids [30]. SphK2-L is expressed in human cells but not in mice, and is reported to be the predominant SphK2 mRNA in most cell lines and tissues except brain and kidney [30]. SphK1 and SphK2 have similar abilities to phosphorylate endogenous substrates, but SphK2 is mainly responsible for phosphorylation of the immunosuppressive pro-drug, FTY720[15, 31, 32]. SphK2 was found to be a two hybrid interactor with the cytoplasmic domain of the IL-12 receptor β1, and SphK2 overexpression enhanced IL-12-mediated signaling [33]. SphK2 also binds calmodulin at a site that is conserved between the SphKs, although unlike SphK1, interaction with calmodulin does not alter SphK2 localization [21]. SphK2 contains a putative BH3 domain that has been shown to contribute to the ability of overexpressed SphK2 to induce apoptosis in a variety of cell types

[34]. In contrast to SphK1, only a few agonists have been reported to activate SphK2, including EGF [35], IgE receptor crosslinking [36], and IL-1β and TNFα[37]. Of note, EGF activates SphK2 and induces phosphorylation on Ser351 and Thr578, both dependent on ERK1[38]. Moreover, this phosphorylation was required for SphK2-enhanced cell migration towards EGF [38]. Intriguingly, although SphK2 expression typically inhibits growth, promotes apoptosis, and chemosensitizes several cell types [30, 34, 39, 40], SphK2 protects MCF-7 breast cancer cells and HCT116 colon cancer cells from doxorubicin-induced apoptosis by a pathway mediated by p53-independent upregulation of p21[41].

3.3 SphK1 vs. SphK2

Even though mice with single knockouts of *Sphk1* and *Sphk2* are viable with few obvious phenotypes, suggesting that the SphKs have redundant functions, the kinases exhibit many differences in a variety of experimental systems. Indeed, several studies have shown that the two SphKs in yeast, Lcb3p and Lcb4p, do not complement one another (e.g. 42), although this may be due to the much higher activity of Lcb4p. Moreover, both SphKs, in yeast as well as mammals, have overlapping but distinct subcellular localizations, suggesting that they may interact with different proteins and/or lipids and utilize different sphingoid base substrate pools. The first clues that eukaryotic SphK1 and SphK2 may differ functionally came from the observations that, in contrast to many reports demonstrating a pro-growth, anti-apoptotic role for SphK1, SphK2 overexpression induced growth arrest and cell death 30, 34, 39. Consistent with these results, SphK1 decreased, while SphK2 increased, the sensitivity of several different cell lines to a variety of chemotherapeutic drugs [34, 40]. However, other studies have revealed that SphK2 knockdown reduced proliferation of glioblastoma cell lines [43]. The cell culture results demonstrating a role for SphK1 in cell growth and apoptosis are likely pathophysiologically relevant, as SphK1 is overexpressed in a number solid tumors, including breast, ovary, kidney, brain, stomach, and kidney [43, 44]. Perhaps because of its role as a promoter of apoptosis, SphK2 but not SphK1 was reported to be responsible for the secretion of S1P during apoptosis of Jurkat T cells induced by staurosporin [45]. Conversely, in hematopoietic cancer cells, it was recently shown that SphK1 produced the S1P that was secreted in response to doxorubicin-induced apoptosis [46].

The differential effects of the two SphKs on cell fate are due in part to their different roles in regulating ceramide levels. SphK1 expression decreases ceramide levels, likely by inhibiting ceramide synthases 47, 48. Conversely, SphK2 expression increases ceramide levels by increasing the salvage of sphingoid bases 47. In a pathway that is conserved in yeast [42], exogenous sphingoid bases must first be phosphorylated by a specific SphK (Lcb4p or SphK2), then dephosphorylated by a specific SPP (Lcb3p or SPP1) [49, 50]. This cycle enables cells to re-acylate sphingoid bases to ceramides and likely represents a control point that cells use to regulate the amount of ceramide and sphingolipids at the levels of *de novo* synthesis (SphK1) and salvage of sphingoid bases (SphK2). SphK1 and SphK2 also appear to have different roles in the uptake of S1P, with SphK1 but not SphK2 cooperating in another phosphorylation/dephosphorylation cycle that utilizes cell surface LPP-1 to promote sphingoid base uptake[51].

Differential effects of SphK1 and SphK2 are also apparent in transduction of signals from cell surface receptors. Perhaps because it was cloned first and thus is more well studied, SphK1-dependent formation of S1P has been found to be an important component of numerous receptor signaling pathways, but even in cases where participation of both SphK1 and SphK2 have been examined, it is still SphK1 that is typically implicated. To cite a few examples, estradiol activates SphK1, but not SphK2, ultimately leading to EGFR activation in MCF-7 cells [52]; similarly, VEGF-induced activation of ERK1/2 in T24 bladder cancer cells requires SphK1 but not SphK2[53]; moreover, targeting SphK1 but not SphK2 with siRNA also blocks TNFα-induced COX-2 induction and PGE_2 secretion [54].

Many studies have demonstrated a role for S1P and S1PRs in cell motility (reviewed in 55), and SphK1, but not SphK2, has often been reported to be the source of S1P. For example, EGF stimulates the activity and translocation of SphK1 to lamellipodia in MCF-7 cells, and overexpression of SphK1 enhances migration towards EGF 56. EGF also has been shown to stimulate both SphK1 and SphK2 in HEK 293 cells, but only SphK1 was required for EGF-induced motility 35. The lack of requirement for SphK2 in these cells is the more remarkable because SphK2 is already localized at the plasma membrane, and suggests that a SphK may need to be targeted to a specific sub-compartment of the plasma membrane, perhaps in close proximity to its substrate and specific S1PRs and/or perhaps S1P transporter(s). Intriguingly, EGF also stimulates both SphK1 and SphK2 in MDA-MB-453 cells, but in these breast cancer cells, both SphKs are required for EGF-induced motility 35.

The roles of S1P in the immune system are complex and deciphering which SphK isozyme is involved is a challenging task. It has been shown that SphK1, but not SphK2, is required for degranulation of rat RBL-2H3 mast cells in response to IgE receptor crosslinking [57]. In contrast, in mouse bone marrow-derived mast cells, both SphK1 and SphK2 are activated by IgE receptor crosslinking in a Fyn-dependent manner [36]. Interestingly, SphK1 and SphK2 have different requirements for effectors downstream of Fyn, suggesting other levels of regulation of SphK activation. These authors also showed that exogenous S1P could only partially restore degranulation to *Fyn*$^{-/-}$ mice, hinting at an intracellular role for S1P (discussed below). This group later took advantage of *Sphk1*$^{-/-}$ and *Sphk2*$^{-/-}$ double knockout mice to demonstrate that in fetal liver-derived and bone marrow-derived mast cells, only SphK2 was responsible for IgE receptor triggered degranulation and cytokine release [58]. However, in a passive systemic anaphylaxis model, they found that *Sphk2*$^{-/-}$ mice fared as well or slightly worse than wild-type mice in terms of increased plasma histamine levels. Conversely, *Sphk1*$^{-/-}$ mice had reduced plasma histamine levels. *Sphk1*$^{-/-}$ mice also had reduced plasma S1P levels compared to wild type and *Sphk2*$^{-/-}$ mice, and the intensity of the histamine released positively correlated with circulating S1P levels. The triple allele knockout *Sphk1*$^{+/-}$*Sphk2*$^{-/-}$ mice had the lowest histamine responses and had plasma S1P levels as low as the *Sphk1*$^{-/-}$ mice. Thus, mast cell function in mice is determined both by SphK2 in mast cells (intrinsic S1P) and circulating S1P levels determined by non-mast cell SphK1 (extrinsic S1P). In contrast, in both human LAD2 mast cells and human umbilical cord blood-derived mast cells, knockdown of SphK1 expression decreased degranulation, cytokine release, and motility in response to IgE/antigen [59]. Conversely, SphK2 was dispensable for antigen-induced degranulation, motility, or release of most cytokines 59. S1P likely also plays important roles in other types of immune cells, although neutrophil function in cells isolated from *Sphk1*$^{-/-}$ or *Sphk2*$^{-/-}$ mice, or even in the whole animals themselves, showed little observable differences between the knockouts and wild type. The *Sphk2*$^{-/-}$ mice did have increased disease progression in a lung infection model [60]. Differences between mouse and human immune systems remain to be resolved, but will likely require SphK isozyme-specific inhibitors to elucidate the roles for the SphK1 and SphK2 in mast cell functions and development.

4. S1P Receptors

S1P is a ligand for five specific GPCRs, $S1P_{1\text{-}5}$, formerly called endothelial differentiation gene (EDG) receptors, which are differentially expressed in different tissues. The cell type specific expression of S1PRs, as well as their differential coupling to different G proteins, explains the diverse signaling of S1P 61. As mentioned above, many stimuli, including hormones, immunoglobulin receptor ligation, growth factors, and cytokines, activate cytosolic SphKs and the production of S1P that is required for the full activity of these agonists. In many cases, the S1P produced activates cell surface S1PRs in a paracrine and/or autocrine manner (reviewed in 62). Indeed, many of the downstream effects of these stimuli require transactivation of one or more S1PRs, also called “inside-out” signaling. For example, in

MCF-7 cells, estradiol stimulates ERK1/2 though a mechanism that requires at least two autocrine signaling loops 52. In the first loop, estradiol stimulates SphK1 and formation of S1P leading to activation of $S1P_3$. In the second loop, $S1P_3$ activates the metalloproteinase MMP-9, which in turn releases EGF from the EGF-heparin binding protein and activates EGFR, finally leading to ERK1/2 phosphorylation [52]. How transactivation of S1PRs is accomplished is an intriguing puzzle as the SphKs and S1P production are both on the cytosolic side of the plasma membrane and S1PRs bind S1P on the exoplasmic side. While it has been suggested that SphK proteins themselves may be secreted and produce S1P extracellularly [19], it has been convincingly shown that the ABCC1 transporter mediates secretion of intracellularly produced S1P from mast cells [63]. Moreover, S1PR transactivation is not only regulated by S1P secretion, cellular levels of S1P are also important, as decreasing levels by overexpression of SPP1 inhibits transactivation [64], while decreasing S1P degradation by inhibition of SPL promotes it [65]. Such inside-out transactivation loops may be a general phenomenon, as chemotactic signals for neutrophils acting through their receptors induce secretion of ATP that then locally activates cell surface nucleotide receptors to coordinate directed cell migration [66].

4.1 $S1P_1$

$S1P_1$ is ubiquitously expressed, with high levels in brain, lung, spleen, cardiovascular system, and kidney. It was originally identified as an orphan GPCR involved in differentiation of endothelial cells [67]. Since its discovery, many of the important physiological functions of S1P have been attributed to ligation of this receptor. It is now known that $S1P_1$ plays a key role in angiogenesis, because its deletion in mice is embryonic lethal due to hemorrhage resulting from incomplete vascular maturation as smooth muscle cells and pericytes fail to migrate and envelop nascent endothelial tubes [68]. Endothelial cell conditional $S1P_1$ knockout mice have been generated using the Cre/Lox system, and these mice display the same vascular deficiencies, suggesting that $S1P_1$ receptors on endothelial cells are also responsible for vessel coverage by smooth muscle cells [69]. $S1P_1$ also plays an important role in maintenance of endothelial and epithelial barrier integrity by functioning in conjunction with $S1P_2$ and $S1P_3$ to increase vascular integrity [70–72]. Disruption of endothelial barriers leads to increased vascular permeability, often found in tumors and in inflammation. Silencing of $S1P_1$ expression with siRNA blocks barrier enhancement, determined by transendothelial monolayer electrical resistance, while silencing of $S1P_3$ inhibits vascular disruption [71]. These experiments also identified the downstream signaling molecules Akt and Rac as effectors of $S1P_1$ actions on vascular integrity [71]. A role for $S1P_1$ in vascular integrity has also been demonstrated *in vivo* [73–76], and experiments using a $S1P_1$ selective antagonist demonstrated that $S1P_1$ is crucial in maintaining vascular tone [77]. Of particular interest are the conditional SphK1/SphK2 double knockout mice, which have normal vascular integrity despite having over 100-fold lower plasma S1P levels than wild type mice [78], suggesting that even a very low level of S1P is sufficient as long as $S1P_1$ expression is normal.

$S1P_1$ is also intimately involved in immune cell function. In particular, its expression is required for lymphocyte egress from lymph nodes. Resting T and B cells express primarily $S1P_1$, and its downregulation or deletion results in lymphopenia due to the inability of lymphocytes to exit from the lymph nodes [15, 79]. Additionally, transplantation of $S1P_1$ deficient thymocytes and lymphocytes into normal mice results in their sequestration in lymph nodes and Peyer's patches. Moreover, T-cells overexpressing $S1P_1$ preferentially distribute into blood [80]. These results confirm that $S1P_1$ controls lymphocyte recirculation.

4.2 $S1P_2$

$S1P_2$ is also widely expressed in a variety of different cell types. Unlike $S1P_1$ knockout mice, newborn $S1P_2$ deficient mice do not demonstrate any striking abnormalities, although they have been reported to develop sporadic seizures between 3–7 weeks of age [81]. Neocortical

pyramidal cells from these mice also display an increase in excitability [81]. In addition, $S1P_2^{-/-}$ mice are deaf indicating that $S1P_2$ is required for proper development of the auditory and vestibular systems [82, 83]. $S1P_2$ is also required for proper degranulation of mast cells [57]. $S1P_2$ is generally considered to be a repellant receptor as its activation inhibits cell migration and appears to work in opposition to $S1P_1$ and $S1P_3$, which both enhance cell migration [84]. Similarly, activation of $S1P_2$ activates ROCK/Rho and leads to increases in vascular permeability [72]. Finally, although the visceral organs of $S1P_2^{-/-}$ mice develop normally, expression of $S1P_2$ promotes liver tissue remodeling in response to acute injury [85].

4.3 $S1P_3$

Much like the lack of phenotypic effects of deletion of $S1P_2$, deletion of $S1P_3$ in mice does not generate any obvious phenotype. $S1P_3$ is expressed in the cardiovascular system, lungs, kidney, intestines, spleen, and cartilage [86]. Knockouts of both $S1P_2$ and $S1P_3$ increases perinatal lethality, but not to a great extent [87]. However, the triple knockout of $S1P_{1\text{-}3}$ leads to embryonic lethality due to massive vascular deficiencies perhaps even worse than those resulting from knockout of $S1P_1$ alone [88]. $S1P_3$ is also an important regulator of vascular permeability signaling through the downstream effectors ROCK and Rho [72]. A clear role for $S1P_3$ has also been demonstrated in the regulation of heart rate [89], as $S1P_3$ expression is localized to myocytes and perivascular smooth muscle cells, and its activation results in bradycardia and hypertension.

4.2 $S1P_4$ and $S1P_5$

$S1P_{4\text{-}5}$ have much narrower patterns of expression than the dominant $S1P_{1\text{-}3}$ receptors, localizing in human leukocytes, NK cells, airway smooth muscle cells and white matter of CNS tracts [90–93]. $S1P_4$ is primarily expressed in lymphoid tissues, including the thymus, spleen, bone marrow, appendix, and peripheral leukocytes [94]. $S1P_4$ directly couples to $G\alpha_i$ and $G\alpha_{12/13}$ subunits of trimeric G proteins, and Jurkat cells overexpressing $S1P_4$ display enhanced pertussis toxin-sensitive cell motility in the absence of S1P [95]. $S1P_4$ stimulation also activates the mitogen activated-protein kinases ERK1/2, activates phospholipase C, and modulates the opening of intracellular calcium stores [96, 97]. Stimulation of $S1P_4$ ectopically expressed on CHO-K1 cells induced cytoskeletal rearrangements and cell rounding, as well as its internalization following S1P stimulation [95]. Whether or not $S1P_4$ has a role in cell motility remains unclear. In D10G4.1 mouse Th2 cells and EL4.IL-2 mouse T cells lacking endogenous S1P receptors but transfected with $S1P_4$, its activation failed to transduce chemotactic responses [98]. These cells also displayed enhanced secretion of IL-10 and decreased proliferation in response to S1P [98].

$S1P_5$ is highly expressed in oligodendrocytes [92]; however, silencing of $S1P_5$ expression does not inhibit myelination or produce any other obvious phenotype in these cells [99]. Binding of S1P to $S1P_5$ induces phosphatase-dependent inhibition of ERK1/2, resulting in an anti-proliferative phenotype [91, 100, 101]. In addition, stimulation of rat oligodendrocytes with PDGF increases $S1P_1$ expression with a concomitant downregulation of $S1P_5$, resulting in an amplified mitogenic response [102]. A recent report emonstrated that $S1P_5$ is present in natural killer cells (NK). Mice deficient in $S1P_5$ display aberrant NK cell homing and mobilization of NK cells to inflamed organs [93]. Finally, despite a multitude of studies focusing on physiological functions of S1PRs, some actions of S1P resulting from activation of SphKs are independent of S1PRs.

5. Evidence for intracellular targets of S1P

As discussed above, cellular levels of S1P are controlled both by its synthesis and by its degradation. S1P can be degraded either by dephosphorylation back to sphingosine or

irreversibly degraded by SPL to ethanolamine phosphate and fatty aldehyde. In fact, the latter is the only pathway in eukaryotic cells for degradation of sphingoid bases. Thus, S1P formation and subsequent degradation by SPL is one means for decreasing sphingolipid levels within the cell. Intriguingly, S1P is also an intermediate in the formation of sphingolipids from salvaged sphingoid bases. Though these pathways regulating sphingolipid levels demonstrate a central role for S1P, emerging evidence from yeast, plants, and mammals points to S1P as a classical, intracellular second messenger.

5.1 S1P in Saccharomyces cerevisiaei

The yeast genome does not encode a recognizable cell surface receptor for S1P and exogenous S1P does not affect yeast growth [103]. Thus, any role for S1P in yeast physiology must therefore be intracellular. While no direct target for S1P has been found, S1P does indeed have intracellular functions in yeast. First, it has been demonstrated that yeast cells deleted of S1P phosphatase (*lcb3/ysr2/lbp1*) and SPL (*dpl1*) accumulate large amounts of S1P and are non-viable or very slow growing. This growth arrest can be can be rescued if the major yeast SphK (*lcb4*) is deleted, indicating that intracellular S1P suppresses yeast cell growth [104, 105]. Indeed, sphingosine itself induces growth arrest in yeast, and this effect can be blocked by mutational inactivation of SphK [103]. The growth inhibition may be due to elevated levels cytosolic calcium, as S1P has been shown to increase intracellular calcium levels [106]. In contrast, while heat shock-induced cell cycle arrest is mediated by the sphingoid base itself [107], Heat shock also increases SphK activity [103] and S1P accumulation [108], suggesting a functional role for S1P in heat shock responses. Preventing S1P metabolism by deletion of either the S1P phosphatase Lcb3p [109, 110] or SPL [108] led to enhanced levels of S1P and increased heat shock tolerance. Moreover, mutational inactivation of SphK reversed the protective effect of SPL deletion [103]. That deletion of either an S1P phosphatase or SPL promotes heat tolerance indicates that S1P has direct actions, rather than merely functioning as a metabolic intermediate. Identification of molecular targets of S1P in yeast would aid this quest in mammalian cells.

5.2 S1P in Arabidopsis thaliana

Abscisic acid is a plant hormone responsible for mediating responses to drought conditions such as closure of stomata to prevent water loss. It was recently demonstrated that drought increased S1P levels in plants and that abscisic acid-induced stomatal closure was reduced by SphK inhibitors [111]. Subsequently, it was shown that abscisic acid activated SphK in *Arabidopsis* [112]. Intriguingly, these authors showed that the effects of S1P on stomatal openings were dependent on the single canonical G_α protein in plants, GPA1. This finding suggests that S1P might be acting through plant GPCRs. However, *Arabidopsis* has only one GPCR-like protein, designated GCR1, which is not homologous to the known S1PRs, does not bind phosphorylated sphingoid bases, and GCR1 mutants are hypersensitive to S1P-induced stomatal closure [113]. Thus, S1P likely regulates stomatal apertures and drought responses intracellularly in plants.

5.3 S1P in mammalian cells

The observation that expression of SphK1, but not SphK2, decreases ceramide levels and increases dihydrosphingosine levels [47] suggests that S1P produced by SphK1 may negatively regulate one or more of the six (dihydro)ceramide synthases (CerS), leading to accumulation of its substrate, dihydrosphingosine. Subsequently, using lysates from cells over-expressing individual CerSs, S1P was shown to be a non-competitive *in vitro* inhibitor of CerS2, but not of other CerSs [48]. *In silico* analysis identified two domains in CerS2 with predicted homology to S1PRs, and mutation of one arginine to alanine in each of these two domains removed S1P inhibition without altering CerS activity [48]. The authors concluded that S1P directly binds to,

and inhibits, CerS2. However, it is also possible that S1P acts through intermediate proteins in cell lysates to inhibit CerS2. Moreover, CerS2 does not use C16 or C18 acyl CoAs as substrates, yet it is C16 and C18 ceramides that are most affected by expression of SphK1[47]. Additionally, S1P produced by SphK1 reverses CerS1-induced chemosensitivity, suggesting that S1P directly affects CerS2[40]. Finally, it is not yet clear whether inhibition of CerS2 by S1P is a signal-mediated effect or whether it is simply feedback product inhibition. Further work is needed to confirm that S1P binds to and inhibits CerS2.

As mentioned previously, autocrine and/or paracrine transactivation of S1PRs is typically accompanied by translocation of SphK1 to the plasma membrane. However, both SphK1 and SphK2 have been shown to translocate to other cellular compartments, suggesting that S1P may be produced locally in these compartments to act on specific intracellular targets. For example, SphK1 is targeted to internal membranes through interaction with RPK118[24] and Acy1[26]. SphK1 translocated to nascent phagosomes promotes maturation into mature phagolysosomes [114]. SphK1 has also been shown to translocate to the nuclear envelope during S-phase [115], and this translocation may play a role in the ability of SphK1 overexpression to promote the G_1/S transition [116]. Conversely, SphK2 has been shown to reside in the nucleoplasm in certain cells, where it functions to arrest cells [30, 39]. Moreover, SphK2 has a nuclear export signal sequence that is activated by in G_1 phorbol ester-induced phosphorylation, likely through protein kinase D [117]. Whether the nuclear export signal serves to promote SphK2/S1P signaling in the cytosol or to decrease SphK2/S1P signaling in the nucleus is unclear. In some cells, SphK2 has been shown to translocate to the ER under stress conditions and promote apoptosis [47]. Indeed, targeting SphK1 to the ER induced apoptosis [34], suggesting that S1P produced at the ER has specific targets.

A direct target for S1P in the ER has not conclusively been identified, although strong evidence indicates that S1P can activate thapsigargin-sensitive calcium channels, likely in the ER. S1P induced inositol trisphosphate receptor-independent release of calcium from permeablized cells [118] and from cell fractions rich in rough, but not smooth, ER [119]. Consistent with these results, cells overexpressing SphK2, which localizes in part to the ER, were also shown to have elevated intracellular calcium [47]. Conversely, fetal liver derived-mast cells from *Sphk2*$^{-/-}$ mice have a defect in calcium mobilization in response to IgE receptor crosslinking that cannot be restored with exogenous S1P [58], again suggesting an internal S1P target. Exogenous S1P increases calcium in HEK293 cells, a response that can be inhibited with pertussis toxin. However, microinjection of S1P increases calcium bypassing a pertussis toxin block, again supporting a role for intracellular S1P in calcium release [120]. Similarly, caged S1P can elicit calcium mobilization in cells that do not respond to exogenous S1P [121]. Likewise, UTP stimulates calcium mobilization in a SphK-dependent manner, but this effect is not mimicked by exogenous S1P [122]. Finally, exogenous S1P itself can stimulate SphK, and SphK inhibitors reduced calcium release induced by S1P [123]. Intracellular calcium release by S1P may be an evolutionarily ancient pathway, as it has been shown that S1P can increase calcium in yeast [106]. In sum, these results strongly suggest that S1P is a second messenger that can activate calcium channels.

Finally, there are other effects of S1P that cannot be explained by activation of the known S1PRs. For example, overexpression of SphK1 promotes survival of endothelial cells in part through PECAM-1 expression and activation of Akt, an effect that is not reproduced by exogenous S1P [124]. Dihydro-S1P and S1P bind and activate S1PRs with similar affinities, but dihydro-S1P cannot recapitulate all of the effects of S1P [125–128]. For example, S1P, but not dihydro-S1P, protects male germ cells from apoptosis, an effect that was linked to inhibition of NF-κB and activation of Akt [128]. A similar protection pattern was observed for HL-60 and PC-12 cells [125]. Conversely, S1P-phosphonate, which does not bind to S1PRs, also protects these cells from apoptosis [125], suggesting an intracellular action. Other studies using embryonic

fibroblasts from S1PR knockout mice ruled out S1PR involvement, demonstrating that SphK1 overexpression stimulated growth and survival in wild type and in S1PR negative cells [129]. In the $APC^{min/+}$ model of intestinal tumorigenesis, it was shown that $Sphk1^{-/-}$ mice, but not $S1p_1^{-/+}$, $S1p_2^{-/-}$, or $S1p_3^{-/-}$ mice, had reduced tumor progression and size 130, suggesting an intracellular role for S1P. Moreover, SphK1 null mice had elevated levels of sphingosine but not S1P, so it is possible that reduced tumor progression and size was due to inhibitory effects of sphingosine rather than to the absence of S1P stimulation. S1PR knockouts were similarly used to demonstrate that SphK2-induced apoptosis was S1PR independent 34. Consistent with a role for SphK2 in apoptosis, the FTY720 analog AAL(R), which is phosphorylated by SphK2 but only poorly by SphK1, induces apoptosis in Jurkat cells and primary splenocytes [131]. The authors showed that AAL(R) had to be phosphorylated to affect cells as the non-phosphorylated AAL(R) isomer did not induce apoptosis in cells from $Sphk2^{-/-}$ mice. However, exogenous addition of phosphorylated AAL(R) did not induce apoptosis, suggesting both that phospho-AAL(R) must be generated at or near its site of action and that S1PRs are not involved in SphK2-induced apoptosis.

Intracellular S1P has also been linked to regulation of inflammatory responses. $CD4^+$ T cells from SphK2 knockout mice displayed a hyperactivated phenotype, increased proliferation, and enhanced secretion of cytokines and STAT5 activation in response to IL-2[132]. This phenotype was physiologically relevant, as T cells from SphK2 knockout mice induced a much more rapid response than T cells from wild type littermates in an adoptive transfer model of inflammatory bowel disease. The hyperresponsiveness to IL-2 could not be reversed with exogenous S1P, indicating that internal S1P normally suppresses IL-2-induced inflammatory responses. Interestingly, activation of the T cell receptor in Th1 and Th2 cells leads to increased expression of SphK1, but not SphK2, and SphK1 negatively regulates chemokine expression, although the authors did not examine whether exogenous S1P reproduced chemokine suppression [133]. Similarly, it has been shown that in primary umbilical vein endothelial cells, the pro-inflammatory cytokine TNFα greatly increased expression of the S1P phosphatase SPP2[134]. Induction of SPP2 was required for the TNFα-induced production of IL-1β and IL-8. SPP2 is an integral membrane protein of the ER and its requirement for induction of inflammatory responses suggests that it acts at the ER to remove an inhibitory S1P signal. Moreover, downregulation of SPP1 demonstrated that it played no role in TNFα-induced cytokine secretion. As SPP1 is also an ER resident protein, these results suggest a role for S1P at a specific subcompartment of the ER. Moreover, parallel findings have been reported in yeast, where the S1P-specific phosphatases, YSR2 and YSR3, are both localized to the ER and have overlapping but distinct functions [5].

Many studies have utilized non-isozyme specific SphK inhibitors to implicate S1P in various signaling pathways (e.g. 135, 136), leading to the conclusion that S1P was acting intracellularly in these systems, but S1P release was not detected. Still, care should be used in interpreting these results as S1P release may have been below the limits of detection. Moreover, exogenous S1P can activate all surface S1PRs, perhaps resulting in net opposing effects. It is also possible that signaling events localize secretion of S1P to regions of the plasma membrane where specific receptors to be activated are localized. Thus, lack of detectable S1P secretion does not definitively show that S1PRs are not involved in a particular signaling pathway.

Furthermore, studies in endothelial cells using a pan sphingosine kinase inhibitor demonstrated that endogenously generated S1P functions as a positive modulator of calcium entry via store operated channels (SOC), whereas exogenously administered S1P initiated calcium release from the ER similar to histamine and decreased endothelial cell permeability [137]. These results suggest that the production of intracellular S1P, and not the secretion of S1P to act on other immunoregulatory cells, is the primary determinant of the inflammatory response.

Recently, a new study demonstrated that the vascular permeability inhibitor Ang-1 stimulated SphK1 and led to increases in intracellular S1P and decreases in vascular permeability. This function remained undisturbed even when expression of $S1P_{1-3}$ were downregulated [138]. Exogenous S1P also decreased vascular permeability, but not in cells where S1PRs were downregulated [138]. These results suggest that Ang-1/SphK1 actions on vascular permeability are mediated via an intracellular mechanism.

6. Implications and future directions

It is now clear that the bioactive lipid mediator S1P exerts effects both intracellularly and extracellularly. In addition, SphK1 and SphK2 are not only distributed differently throughout tissues and within cells, S1P produced by these kinases can have different, sometimes opposite, downstream effects. How can one simple molecule like S1P have such a wide range of effects? It seems that in the case of S1P, the location of its production may be a major determinant of the resulting phenotype. The key to unlocking this riddle will be the inevitable discovery of intracellular S1P binding partners, but to date, none have been unequivocally identified. Extensive studies of proteins in the nucleus, particularly those involved in the transcriptional machinery and the regulation of the cell cycle, are clearly indicated to elucidate the mechanism by which S1P exerts its effects there. Additionally, the consequences of activation or inhibition SphK1 vs. SphK2 must be studied with more scrutiny, as it is apparent that despite catalyzing the same reaction, they have different functions. In addition, because the intracellular actions of S1P involve many functions related to cancer and other diseases, a full understanding of both the extracellular and intracellular actions of S1P will be needed to best design clinical therapies targeting SphKs and S1P production as well as its receptors.

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