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REVIEW ARTICLE

The compartmentalization and translocation of the sphingosine kinases: Mechanisms and functions in cell signaling and sphingolipid metabolism

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Abstract

Members of the sphingosine kinase (SK) family of lipid signaling enzymes, comprising SK1 and SK2 in humans, are receiving considerable attention for their roles in a number of physiological and pathophysiological processes. The SKs are considered signaling enzymes based on their production of the potent lipid second messenger sphingosine-1-phosphate, which is the ligand for a family of five G-protein-linked receptors. Both SK1 and SK2 are intracellular enzymes and do not possess obvious membrane anchor domains within their primary sequences. The native substrates (sphingosine and dihydrosphingosine) are lipids, as are the corresponding products, and therefore would have a propensity to be membrane associated, suggesting that specific membrane localization of the SKs could affect both access to substrate and localized production of product. Here, we consider the emerging picture of the SKs as enzymes localized to specific intracellular sites, sometimes by agonist-dependent translocation, the mechanism targeting these enzymes to those sites, and the functional consequence of that localization. Not only is the signaling output of the SKs affected by subcellular localization, but the role of these enzymes as metabolic regulators of sphingolipid metabolism may be impacted as well.

Keywords: Sphingolipid, sphingosine-1-phosphate, ceramide, lipid signaling, membrane translocation

As enzymes with hydrophobic substrates and products, it is notable that the sphingosine kinases (SKs) are soluble enzymes, lacking hydrophobic membrane-anchoring segments or lipid modifications that would tether them to membranes. They share this property with other lipid signaling enzymes such as the phospholipase A2s, and the phosphatidylinositol, diacylglycerol, and ceramide kinases. Here, we will discuss how this lack of permanent membrane anchoring may provide a means for regulated targeting of the SKs to specific intracellular sites and how this targeting may, in turn, drive selective enzymatic activation, access to specific substrate pools, and production of products at distinct locations.

There are two SK genes, *SPHK1* and *SPHK2*, which generate gene products that have high sequence similarity. These gene products, SK1 and SK2, have distinctive steady-state localizations and stimulus-dependent movement to specific intracellular sites. We will therefore discuss the two SKs separately. By and large, more is known about SK1 and therefore this will be discussed in more detail. However, the unique functions of SK2 are garnering increasing interest and details of SK2 function are beginning to emerge.

The enzymology of the SKs: Two genes, multiple splice variants

A mammalian SK was first purified from rat kidney (Olivera et al., 1998) and cloned from mouse tissues

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(Kohama et al., 1998). These initial studies set the stage for the identification of the homologous human gene (Melendez et al., 2000; Nava et al., 2000; Pitson et al., 2000). The gene product was designated as human SK1 (hSK1, gene name *SPHK1*). Subsequently, a second SK gene was identified in both mouse brain and human kidney cells that contained an extended N-terminal and an insertion of sequences in the center of the protein when compared to SK1 and this kinase was dubbed SK2 (gene name *SPHK2*) (Liu et al., 2000). As we discuss below, even though these isoenzymes share a high degree of polypeptide sequence similarity, studies continue to reveal profound differences in their tissue distribution, their subcellular localizations, both steady state and stimulus dependent, and the ways in which they are regulated.

Multiple splice variants for each isoenzyme

To date, there are three known splice variants of human SK1 and two variants of SK2 that have been functionally characterized, with the possibility of a third SK2 splice variant awaiting experimental validation (Pyne et al., 2005; Alemany et al., 2007; Shida et al., 2008; Pitson, 2011). Somewhat similar splice variants for both SK1 and SK2 also exist in the mouse and rat genomes. The human SK1 splice variants are designated SK1a, SK1b, and SK1c, and differ only in the length of their N-termini (see Table 1). SK1a, which is the shortest variant, seems to be the most abundant in a variety of human tissues. When compared to SK1a, the variants SK1b and SK1c both possess longer N-termini, having an additional 14 and 86 amino acids, respectively (reviewed in Pitson (2011)). Although the structural significance of having an extended N-terminal has not been exhaustively studied for these enzymes, recent work suggests that key residues in the N-terminus offer putative sites for post-translational modifications. Notably, mouse SK1b, which has an N-terminal extension of 10 amino acids as compared to mSK1a, has been shown to be palmitoylated on two cysteine residues located in the N-terminus (Kihara et al., 2005). This cysteine-dependent palmitoylation motif increased the membrane association of mSK1b. Additionally, mSK1b was more susceptible to ubiquitination and proteasomal degradation than the shorter mSK1a isoform, which was primarily localized to the cytosol. Post-translational modifications of this kind not only have the potential to alter the stability of variant SKs, they also offer possible clues in establishing subcellular localization as a distinct form of regulation for SKs in general. The behavior of the human splice variants has not been well studied, but there is a hint that they may share similar properties (Venkataraman et al., 2006).

Although they have not been studied as extensively as the SK1 variants, the two confirmed splice variants of human SK2 seem to arise from alternative start codons. Human SK2b (commonly referred to as SK2-long) has an additional 36 amino acids in the N-terminal region as compared to SK2a (also known as SK2-short; Okada et al., 2005; see Table 1). SK2b is expressed more abundantly than SK2a in a variety of human tissues and cultured cell lines but does not seem to be expressed in the mouse (Okada et al., 2005). As with SK1, the two splice variants of SK2 are emerging as having slightly different roles in determining cell responses (Liu et al., 2003; Okada et al., 2005). Future studies will be needed to determine how these variant SK2 constructs are differentially regulated.

Domain structure and function

Overall, SKs are evolutionarily highly conserved. In addition to their discovery in humans, mice and rats, SKs have been identified in such diverse species as *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Arabidopsis thaliana*, and *Dictyostelium discoideum* (Alemany et al., 2007). SKs have been grouped with diacylglycerol kinases (DGKs) and ceramide kinases (CERKs) to create a novel family of lipid kinases (Wattenberg et al., 2006). Their inclusion in this family is in part due to the presence of five highly conserved domains (C1–C5) in all SK isoforms that share aspects of homology with similar domains in DGKs and CERKs. The C4 domain appears to be unique to only SKs, and this unique C4 region continues to offer insights into the mechanisms of SK function and regulation. For example, in recent years this region has been shown to contain phosphorylation sites for hSK1 at Ser225 (Pitson et al., 2003) and Thr193 (Dephoure et al., 2005), to contain multiple phosphorylation sites for hSK2 (Ding et al., 2007; Hait et al., 2007; Dephoure et al., 2008), to be involved in binding sphingosine (Yokota et al., 2004), and to include the site where calcium- and integrin-binding protein 1 (CIB1) interacts with SK1 (Jarman et al., 2010; also discussed below). One site of interaction that has remained elusive is the region on SKs that binds to/interacts with acidic phospholipids. Specifically, it has been shown that phosphatidylserine (PS) and phosphatidic acid (PtOH) increase the activity of both SK1 and SK2 when added to *in vitro* assays (Liu et al., 2000; Pitson et al., 2000). Additionally, SK1 binds to both PS (Stahelin et al., 2005) and PtOH (Delon et al., 2004; also discussed in more detail below). Key amino acid residues for PS binding were identified in SK1 (Stahelin et al., 2005) and there was a recent report of a comparable PS-binding site in hSK2 that was highly homologous to the SK1 region and could be disrupted by mutating comparable residues in each isoenzyme (Weigert et al., 2010). Although this is a start, the precise binding properties and functional consequences of the interactions between SKs and acidic phospholipids need to be clarified. Considering the fact that PS and PtOH have fundamentally different roles in membrane dynamics and cell signaling, it is important to decipher the role each one plays in the regulation of SKs.

SK1 and SK2 have structural and functional differences

Even though there are highly conserved domains in all SKs, there are fundamental structural and functional

Table 1. Isoforms and enzymology of sphingosine kinases.

	Sphingosine kinase 1	Sphingosine kinase 2	References
Gene identification			
Human	*SPHK1*—8877	*SPHK2*—56,848	Liu et al., 2000 Pitson et al., 2000
Mouse	*Sphk1*—20698	*Sphk2*—56,632	Kohama et al., 1998 Liu et al., 2000
Splice variants (accession #)			
Human	SK1a (NM_001142601)[+] SK1b (NM_021972)[+] SK1c (NM_182965)[+]	SK2a/short (AF245447)[++] SK2b/long (NM_020126)[+] SK2c (EF107108)[++]	Okada et al., 2005 Alemany et al., 2007 Pyne et al., 2008 Pitson., 2010
Mouse	Sphk1a (NM_001172475)[+] Sphk1b (NM_011451)[+]	Sphk2 (NM_001172561)[+]	Kohama et al., 1998 Liu et al., 2000
Resulting polypeptides (predicted MW[+++])			
Human	SK1a—384 a.a. (42.5 kDa) SK1b—398 a.a. (43.9 kDa) SK1c—470 a.a. (51.1 kDa)	SK2a—618 a.a. (65.2 kDa) SK2b—654 a.a. (69.2 kDa) SK2c—654 a.a. (80.2 kDa)	Igarashi et al., 2003 Okada et al., 2005 Alemany et al., 2007 Pitson, 2010
Mouse	Sphk1a—381 a.a. (42.3 kDa) Sphk1b—388 a.a. (43.2 kDa)	Sphk2a—617 a.a (65.6 kDa)	Liu et al., 2000 Kihara et al., 2006
Substrate specificity (reported K_m ranges)			
	D-*Erythro*-sphingosine (2.15–14 μM) D-*Erythro*-dihydrosphingosine (20 μM)	D-*Erythro*-sphingosine (3.4–14.3 μM) D-*Erythro*-dihydrosphingosine (not reported) FTY720 (fingolimod) (18.2–24.1 μM) D,L-*Threo*-dihydrosphingosine Phytosphingosine	Kohama et al., 1998 Olivera et al., 1998 Liu et al., 2000 Melendez et al., 2000 Nava et al., 2000 Pitson et al., 2000 Billich et al., 2003
Known inhibitors*			
	N,N-dimethylsphingosine SKI-II D,L-*Threo*-dihydrosphingosine	*N,N*-dimethylsphingosine SKI-II ABC294640	Pitman et al., 2010 Tonelli et al., 2010 Antoon et al., 2010

[+], NCBI RefSeq number; [++], GenBank number; [+++], ExPASy predicted MW; a.a., amino acids.
*There is intensive research underway to identify therapeutic SK inhibitors (reviewed in Pitman et al (2010)).

differences between human SK1 and SK2 that need to be considered here. First, hSK2 is larger than hSK1 resulting from additional regions in both the N-terminus and the central part of the sequence that combine to yield a protein that is 236 amino acids longer than hSK1 (reviewed in Pitson (2011)) (see Table 1). The extended N-terminal region of hSK2 contains nuclear localization and export signals that are lacking in hSK1 (discussed below; Igarashi et al., 2003). Additionally, sulfatide-binding sites that are unique to hSK2 have been shown to be located in the N-terminus and are proposed to facilitate the membrane localization of hSK2 (Don & Rosen, 2009). Interestingly, a caspase-1 cleavage site has recently been reported in the N-terminus of hSK2, which has the functional consequence of forming a truncated form of SK2 that can be secreted from cells during apoptosis (Weigert et al., 2010). Much less is known about the functional role of the additional residues in the central part of the hSK2 sequence. In addition to being proline rich, this area of SK2 extends the proposed sphingosine-binding domain by 116 residues as compared to SK1 (reviewed in Pitson (2011)). Further research will be needed to determine whether these additional residues play a role in the ability of SK2 to phosphorylate a wider array of substrates than SK1.

Substrate specificity

The main lipid substrates for both hSK1 and hSK2 are D-*erythro*-sphingosine and D-*erythro*-dihydrosphingosine. hSK1 exhibits a higher catalytic efficiency with both of these sphingolipids when compared to hSK2 (Liu et al., 2000; Pitson et al., 2000; see Table 1). SK2 seems to have traded catalytic efficiency for a broader substrate specificity. SK2 can phosphorylate D,L-*threo*-dihydrosphingosine and phytosphingosine, albeit with much less efficiency than either sphingosine or dihydrosphingsoine, whereas SK1 exhibits no measurable activity with these sphingolipids (Liu et al., 2000). Importantly, SK2 has a much higher affinity for FTY720 (fingolimod), a sphingosine analogue, than does SK1 (Billich et al., 2003) and seems to be the main enzyme responsible for the phosphorylation of this compound. FTY720, which is currently of intense clinical interest as an immune suppressive, acts by functional antagonism of cell surface receptors for S1P, and must be phosphorylated by SK to be active.

The changeable localizations of SK1 and SK2: Impact on signaling and metabolic functions

SK1 and SK2 have distinct functions and localizations. Here, we will discuss the details of their localization and translocation separately, but will conclude by discussing the overall concepts that govern the role of localization of the function of these enzymes.

SK1: Localization and translocation are determined by protein and lipid interactions

SK1 is mostly a cytosolic protein in unstimulated cells but has some associations with intracellular organelles

When measured using enzymatic assays or immunoblotting, most, but not all of SK1 is found in the soluble, cytosolic fraction of cell and tissue lysates. This is true of both endogenous enzyme in cells and tissues and ectopically expressed SK1. Between 60 and 80% of both endogenous and ectopically expressed SK1 activity is found in the cytosol (Kohama et al., 1998). The membrane-bound portion is very tenuously associated with the membranes and is easily washed off (our unpublished observations). A twist to this story is the identification of the mouse SK1b splice isoform. As discussed above, this isoform is palmitoylated on amino-terminal cysteines. Palmitoylation of this SK isoform appears to drive constitutive membrane association. However, this isoform has a short half-life when membrane associated, which results in a low steady-state abundance of this isoform on membranes (Kihara et al., 2005). This presumably accounts for both the low measured levels of membrane-associated SK activity and, potentially, the variability in which the extent of SK membrane-association has been reported, at least in mouse tissues. There is a suggestion that membrane-associated SK is localized into cholesterol/sphingomyelin-enriched microdomains (rafts; Hengst et al., 2009). This would certainly make sense as this is where the substrate sphingosine would be expected to be concentrated; however, this finding has yet to be confirmed. In tissues, total cytosolic SK activity, which combines SK1 and SK2 activity, ranges from a low of 30% (kidney) to almost 80% (brain and liver; Gijsbers et al., 2001). The nature of the association with the membrane in these tissues has not been explored.

By immunofluorescence, ectopically expressed, GFP-tagged SK1 can be seen to associate with punctate structures in the perinuclear region of fibroblasts (Delon et al., 2004). Whether endogenous SK1 also has this distribution has yet to be determined. A striking association with centrosomes has also been noted for SK1 (and SK2) in HEK293 cells (Gillies et al., 2009). This was demonstrated both biochemically and by immunofluorescence with endogenous SK1.

Together, these data suggest that even without stimulus, SK1 has some association with intracellular structures. In addition, considering that the physiological substrate, sphingosine, is membrane associated, SK1 must at least transiently encounter sphingosine-containing membranes, although this association seems to be of sufficiently low stability that it cannot be detected by standard membrane fractionation techniques. The challenge for the future will be to demonstrate that the specific associations with these intracellular structures have functional significance.

SK1 translocates to the plasma membrane, cytoskeleton, and intracellular organelles when stimulated

Stimulation of cells with a variety of agonists leads to the translocation of SK1 to the plasma membrane (reviewed in Wattenberg et al. (2006)). In the majority of these studies the translocation appears to involve a relatively minor proportion of total SK (the exact proportion is difficult to discern because by and large quantitation is lacking). It should be noted, however, that these data are derived from studies using overexpressed SK1. Notably, however, when HEK293 cells transfected with the M_3 muscarinic receptor are treated with the muscarinic agonist carbachol, approximately 50% of ectopically expressed SK translocates to the plasma membrane (ter Braak et al., 2009). Translocation is very rapid, with a half-time of 3–5 s. In this system, the plasma membrane translocation persists for hours after stimulation, but is rapidly reversed if a muscarinic antagonist is applied. This suggests that continuous signaling is required to maintain SK1 at the plasma membrane and that the retention mechanism is labile. Whether SK1 translocates to specific sites within the plasma membrane is a matter of controversy. As noted above, there is some evidence for localization to lipid rafts. In addition, Spiegel et al., noted a heregulin-induced translocation of SK1 to lamellipodia of melanoma cells. A phorbol 12-myristate 13-acetate (PMA)-induced reorganization of SK1 to the acrosomal region of sperm has also been observed (Suhaiman et al., 2010).

In addition to the plasma membrane, stimulus-dependent translocation of SK1 has been observed to several other intracellular sites. Thompson et al. (2005) find that induction of phagocytosis in macrophages triggers a concentration of SK1 to an area around the phagosome, but not specifically to the limiting membrane of the phagosome. This localization is transient, reversing within 30 min. This group also reports that SK1 binds to the actin cytoskeleton in macrophages (Kusner et al., 2007), and it may be that SK1 is binding to the concentration of actin surrounding the phagosomes rather than membrane structures in this situation.

As will be discussed below, SK1 localization to membranes may involve binding to PtOH, generated from phosphatidylcholine by phospholipase D (PLD). Induction of PLD activity drives ectopically expressed SK1 to an unidentified perinuclear structure and peripheral punctate structures (Delon et al., 2004), which may represent the Golgi and endosomes. This localization is enhanced by the protein kinase C (PKC) activator PMA. This system utilized both overexpressed SK1 and PLD, so it is not clear whether this is a physiological site of translocation. However, considering that PLD is known to be

an important regulator of Golgi function (Riebeling et al., 2009), a physiological role for PLD in translocation of SK1 to the Golgi would not be surprising.

An interesting relocalizaton of SK1 has been proposed in which SK1 is secreted outside of the cell. This goes against the dogmatic grain as SK1 does not have a signal sequence and so is not secreted through the canonical vesicular route through the secretory pathway to the plasma membrane. However, Venkataraman et al. (2006) suggest that there is a constitutive event, which results in the extracellular generation of S1P in serum. In addition, the extracellular release of SK1 in microvesicles has been observed (Rigogliuso et al., 2010). SK1 appears to be released by monocytes treated with oxidized low-density lipoprotein immune complexes (Hammad et al., 2006). Ultimately, by determining the molecular mechanisms that control these release mechanisms it should be possible to test the contribution that extracellular SK makes to the generation of extracellular S1P.

The membrane translocation of SK1 involves interaction with acidic phospholipids, SK1-binding proteins, and phosphorylation of SK1

Role of lipids in translocation Acidic phospholipids mediate the binding of SK1 to membranes. Both PS and PtOH enhance SK1 enzymatic activity in mixed micelle and liposome-based cell-free assays (Pitson et al., 2000; Stahelin et al., 2005). However, which of these lipids is a driver of SK1 membrane association in cells is not yet clear. Stahelin et al. (2005) find that recombinant SK1 binding to artificial membranes is dependent on PS content. Surprisingly PtOH does not enhance binding. This contrasts with lipid-binding studies by Delon et al. (2004). They find that SK1 binds strongly to PtOH bound to beads. They indirectly tested PS binding by looking for competition of PS for SK1 binding to bead-immobilized PtOH, and did not detect a role for PS by this measure. This group also elegantly demonstrated that the generation of PtOH in biological membranes drives SK1 membrane association in a cell-free system. The resolution of the apparently divergent findings on PS versus PtOH function could be attributed to the methodologies used. This is, however, unsatisfying in addressing the physiological role of these lipids. It is critically important to understand which lipids drive SK1 membrane association in a biological setting because this would determine the temporal and spatial activation of SK1 signaling and metabolic functions. It may well be that both lipids have roles under different conditions. PS is relatively abundant on the inner surface of the plasma membrane and therefore may serve as a constitutive platform for the binding of SK1 when other required activation steps have been triggered. PtOH is transiently produced by the actions of PLD and diacylglycerol kinases. Therefore, the production of PtOH may more precisely control the timing of SK1 translocation at specific sites of PLD activation.

Role of SK1 phosphorylation in membrane translocation Phosphorylation of SK1 at Serine225 (S225 in the human sequence) is required for SK1 translocation under many, but not all, conditions (reviewed in Wattenberg et al. (2006)). Mutation of S225 to alanine blocks translocation of SK1 to the plasma membrane in response to activation of the MAP kinase pathway (Pitson et al., 2003). How does this phosphorylation lead to translocation? Phosphorylation at S225 is required for the PS-mediated binding of SK to artificial membranes detected by Stahelin et al. (2005), indicating that a conformational change induced by phosphorylation of SK1 exposes a lipid-binding domain. It is unknown whether the binding to PtOH also requires S225 phosphorylation. This is an important gap in our understanding.

Not all membrane translocation of SK1 requires S225 phosphorylation. The localization of SK1 to nascent phagosomes is independent of S225 phosphorylation (Thompson et al., 2005). Another clear example is the robust translocation of SK1 induced by muscarinic receptor activation (ter Braak et al., 2009). This is mediated by the Gq G-protein. However, the downstream pathway that triggers SK1 translocation in this system remains a mystery. Two downstream effectors of Gq, PKC and elevated calcium, were ruled out as important mediators of translocation of SK1. Similarly, activation of PLD does not seem to be involved. One possibility is that an effector downstream of Gq mediates a conformational change in SK1 which exposes a PS-binding site.

SK1 protein-binding partners involved in translocation SK1 was initially identified as a calmodulin-binding protein (Olivera et al., 1998; Pitson et al., 2000; Sutherland et al., 2006). However, the actual binding partner is not calmodulin itself, but a closely related family member, CIB1 (Jarman et al., 2010). CIB1 binds to a specific calmodulin-binding site in SK1 (Sutherland et al., 2006) and is required for PMA-stimulated, S225 phosphorylation-dependent, translocation of SK1 to the plasma membrane. In contrast to Gq-mediated translocation, PMA-induced translocation requires calcium (Jarman et al., 2010). This calcium appears to be required for the binding of CIB1 to the plasma membrane mediated by exposure of a covalently bound, amino-terminal myristic acid. Binding of CIB1 to SK1 does not depend on S225 phosphorylation, so the details of this dependence have yet to be worked out. Jarman et al. (2010) suggest that the calcium-mediated binding of CIB1 to the plasma membrane may initially target SK1 to that site and that S225 phosphorylation may retain it thereby enabling the binding of SK1 to PS.

Traf2, a downstream effector of the tumor necrosis factor-α (TNFα) receptor, is also a binding partner for SK1 (Xia et al., 2002). It is possible that Traf2 bound to the TNFα receptor mediates a degree of SK1 translocation to the plasma membrane. This has yet to be directly tested.

SK2 shuttles between the cytosol and the nucleus.

There has been considerable confusion about the subcellular localization of SK2. Initially, based on overexpressions studies, SK2 was reported as predominantly a soluble, cytoplasmic protein (Liu et al., 2000). The overexpressed protein was also reported to be associated with the endoplasmic reticulum in a serum-dependent manner (Maceyka et al., 2005). However, ectopically expressed SK2 was also reported to be a nuclear protein (Igarashi et al., 2003; Okada et al., 2005). Both nuclear import (Igarashi et al., 2003) and nuclear export signals (NESs; Ding et al., 2007) have been identified in the SK2 sequence. More recent studies in which the endogenous protein was examined have determined that SK2 is, indeed, found in both the nucleus and the cytoplasm and shuttles between these two sites (Ding et al., 2007; Sankala et al., 2007; Hait et al., 2009). The steady-state distribution of SK2 between cytosol and nucleus seems to depend on both the cell type and culture conditions. In some cells, such as the MCF-7 breast cancer cell line, the nuclear/cytoplasmic ratio is very high (Sankala et al., 2007). Regulation of the nuclear/cytoplasmic ratio is accomplished by modulating the activity of the NES by phosphorylation. Phosphorylation of the NES enhances nuclear export of SK2 and therefore decreases the nuclear/cytoplasmic ratio (Ding et al., 2007).

Recently Weigert et al. (2010) reported that SK2 can be released from apoptotic cells by a caspase-mediated cleavage at the amino terminus. They find that this release is coupled to the PS-binding properties of SK2. They suggest that the externalization of PS during apoptosis facilitates the flipping of SK2 onto the extracytoplasmic face of the plasma membrane.

Strub et al. (2011) have observed that SK2 is also found in the mitochondrial matrix. This is a provocative assertion, which rests most directly on fractionation data of endogenous SK2 from heart tissue. This will be an extremely interesting story to follow as it is developed.

The function of SK localization and translocation to specific sites: S1P secretion, S1P delivery to intracellular effectors, access to substrate and SK modulation of sphingolipid metabolism

Secretion of S1P

One potential role of SK1 translocation to the plasma membrane is to enhance the secretion of S1P outside of the cell. There has been a spirited discussion about the relative contributions of intracellular versus extracellular S1P toward the biological effects of SK activity (reviewed in Strub et al. (2010)). The identification of G-protein-linked receptors for which S1P is a potent ligand (reviewed in Rosen et al. (2009), Strub et al. (2010)) clarified the extracellular role of S1P. As SK is a cytosolic protein (with the possible exception of SK released outside the cell as discussed above) S1P must be exported from the cell to reach the extracellular milieu. The identification of the S1P plasma membrane transporter is somewhat controversial. Several members of the ABC transporter family have been implicated in S1P secretion (reviewed in Kim et al. (2009)). A novel transporter, Spns2 (Kawahara et al., 2009; Hisano et al., 2011) also has been demonstrated to be an S1P transporter. Work still needs to be done to establish the relative importance of these two transport systems. The ABC transporters are relatively promiscuous with respect to their transport substrates, so it would not be surprising if members of the ABC transporter family export S1P when levels of the more specific S1P transporter, Spns2, are low.

Translocation of SK1 to the plasma membrane does indeed lead to enhanced secretion of S1P (Johnson et al., 2002; Pitson et al., 2003). The enhanced secretion is relatively modest, increasing the proportion of S1P secreted from the cell by approximately 3-fold. However, translocation of SK1 in these systems is only partial. Therefore, the portion of SK1 that does translocate to the plasma membrane is considerably more active than cytosolic SK1 in promoting S1P secretion.

S1P delivery to specific effectors

As more details are established about intracellular effectors of S1P action, the notion has emerged that SK may be targeted in proximity to those effectors. For example, S1P is an essential cofactor for the ubiquitin ligase activity of Traf2 (Alvarez et al., 2010). As SK1 binds to Traf2 (Xia et al., 2002), it seems reasonable to presume that the interaction SK1 with Traf2 ensures that S1P is generated close to its target. This raises a number of interesting questions, the most important of which is why it is necessary to generate a high local concentration of S1P to satisfy the binding requirements of Traf2? Below we will discuss the movement of S1P in cells and the implications for delivery to intracellular effectors and downstream S1P metabolic enzymes.

The recent identification of S1P as a negative regulator of histone deacetylases-1 and -2 (HDAC1 and 2) rationalizes the nuclear localization of SK2 (Hait et al., 2009). Furthermore, in these studies SK2 was found to associate with the histones themselves, bringing SK2 into close association with the HDACs. It will be fascinating to see whether this association is regulated to fine-tune histone deacetylation under specific conditions.

The localization of SK to regions of the forming phagosomes in macrophages may be related to the ability of S1P to induce release of calcium from intracellular stores (Ghosh et al., 1990; Ghosh et al. 1994). Indeed, *Mycobacterium tuberculosis* inhibits phagocytosis by inhibiting SK activity (Thompson et al., 2005). In this case, localized release of calcium may be required for phagosome formation. Perhaps the generation of S1P in proximity to elements of the endoplasmic reticulum near the forming phagosome generates a localized elevation of calcium required for the process.

The observation that SK1 is localized to membrane ruffles at the leading edge of migrating cells may be connected to activation of the p21-activated protein kinase 1 (PAK1) a downstream effector of Rac and CDC24, which have well known roles in cytoskeletal rearrangements. S1P activates PAK1 *in vitro* at micromolar S1P levels

(Maceyka et al., 2008). The physiological relevance of this activation has yet to be confirmed. However, because a high concentration of S1P is required for activation of PAK1, localized production of S1P may be required to drive S1P-dependent PAK1 activation.

Access of SK to sphingosine

Early studies of partially purified SK demonstrated that SK observes surface dilution kinetics, meaning that enzyme activity is sensitive to the concentration of sphingosine within the membrane that it encounters rather than on the bulk concentration of substrate (Buehrer & Bell, 1992). The implication is that when SK contacts a membrane surface it resides there for a considerable length of time searching for substrate. Consequently, SK localization relative to the concentration of sphingosine at a particular membrane site may have a profound effect on S1P production. Unfortunately, there is a paucity of data on the distribution of sphingosine within the cell. Sphingosine is produced solely through ceramide degradation by one of several ceramidases. Acid ceramidase is a lysosomal enzyme, neutral ceramidase is found at the plasma membrane, and the alkaline ceramidases are localized to the Golgi apparatus and the endoplasmic reticulum (reviewed in Mao and Obeid (2008)). In the absence of further transport of sphingosine, these might be expected to be the sites of sphingosine concentration. However, based on its physical–chemical properties, sphingosine should transfer fairly easily between membranes through the aqueous phase (Garmy et al., 2005b). This would suggest that regardless of where it is generated, sphingosine would redistribute to diverse intracellular membranes and be accessible to SK at many sites. However, there is indirect evidence that sphingosine is compartmentalized. When sphingosine is added externally to the cell, despite its rapid uptake it cannot be used directly for ceramide synthesis, which takes place in the ER (reviewed in Stiban et al. (2010)). Instead, externally added sphingosine has to be activated by the combined action of SK and an S1P phosphatase. This activation might involve transport of sphingosine from the plasma membrane pool to the ER pool, although direct evidence is lacking. Further complicating the issue of access of SK to sphingosine is the fact that sphingosine interacts with cholesterol and therefore its chemical activity as a substrate may depend on the lipid composition of the membrane in which it is found (Garmy et al., 2005a). Consequently, although it is likely that SK localization to specific sites may dictate access to sphingosine, this has yet to be definitively established and is an important area for further study.

Access of SK to dihydrosphingosine and consequences of SK localization for sphingolipid metabolism

SK localization has been shown to affect access to an alternate substrate, dihydrosphingosine. Dihydrosphingosine is produced by the *de novo* pathway of ceramide biosynthesis, *via* the concerted action of serine palmitoyl transferase and 3-ketosphingosine reductase (Figure 1). When cellular SK is elevated, high levels of dihydrosphingosine-1-phosphate (DHS1P) are produced (Berdyshev et al., 2006; Siow et al., 2010). This increase in DHS1P is produced by either cytosolic SK1 or SK1 artificially anchored to the ER. However, SK targeted to the plasma membrane does not produce DHS1P (Siow et al., 2010). This result is understandable because the enzymes producing dihydrosphingosine are localized to the ER (Mandon et al., 1992). Steady-state levels of dihydrosphingosine are extremely low (Berdyshev et al., 2006; Siow et al., 2010), indicating that under normal circumstances dihydrosphingosine is efficiently utilized by the ceramide synthases, which are also in the ER (reviewed in Stiban et al. (2010)) and probably does not have time to redistribute to non-ER membranes. Therefore, only when SK has access to the ER can dihydrosphingosine be utilized as a substrate. The regulated generation of DHS1P could have two important consequences. First, S1P and DHS1P have distinct biological profiles and so the balance between SK access to sphingosine and dihydrosphingosine could affect SK signaling (Bu et al., 2005; Bu et al., 2008). Possibly more importantly, the diversion of dihydrosphingosine from the ceramide biosynthetic pathway by SK may modulate ceramide biosynthesis (Figure 1). Indeed, depletion of SK1 results in elevated ceramide levels (Taha et al., 2005; Siow et al., 2010). The idea that SK, by consuming sphingosine and dihydrosphingosine, can modulate ceramide levels is attractive. It is notable that by producing S1P and DHS1P SK provides the substrates for the S1P lyase, the only cellular enzyme that can degrade the sphingosine backbone (Figure 1). Theoretically, this places SK at a key juncture in sphingolipid metabolism. It should be kept in mind that steady-state levels of S1P are minor compared to levels of ceramide (for example see Berdyshev et al. (2006), Siow et al. (2010)). However, the flux of sphingosine/dihydrosphingosine through this pathway has yet to be measured and while the steady-state levels of S1P/DHS1P may be relatively low, the rate of flux through them may be sufficient to have a significant role on the production of ceramide by the *de novo* and salvage pathways.

It might be expected that localization of SK would affect the downstream metabolism of S1P (Figure 1). S1P is dephosphorylated by two S1P-specific phosphatases (SPP1-2) and, potentially, three less specific lipid phosphatases (LPP1-3) (Le Stunff et al., 2002; Pyne and Pyne, 2002; Ogawa et al., 2003). The SPPs are localized to the ER whereas the LPPs are localized both to the ER and the PM. S1P lyase, the only cellular enzyme that can irreversibly degrade the sphingosine backbone, is also localized to the ER (Reiss et al., 2003; Ikeda et al., 2004). Surprisingly, however, regardless of whether the site of S1P production is at the ER or the PM, the rates of degradation of S1P by these enzymes is the same (Siow et al., 2010). The most obvious explanation for this observation is that S1P is rapidly transported throughout the cell. This is supported directly by pulse-labeling experiments followed by subcellular fractionation (Siow et al., 2010). The mechanism of intracellular movement of S1P is unknown. S1P may move by diffusion through the aqueous space from membrane to membrane or S1P may, like many other lipids, be transported by a specific protein-mediated process.

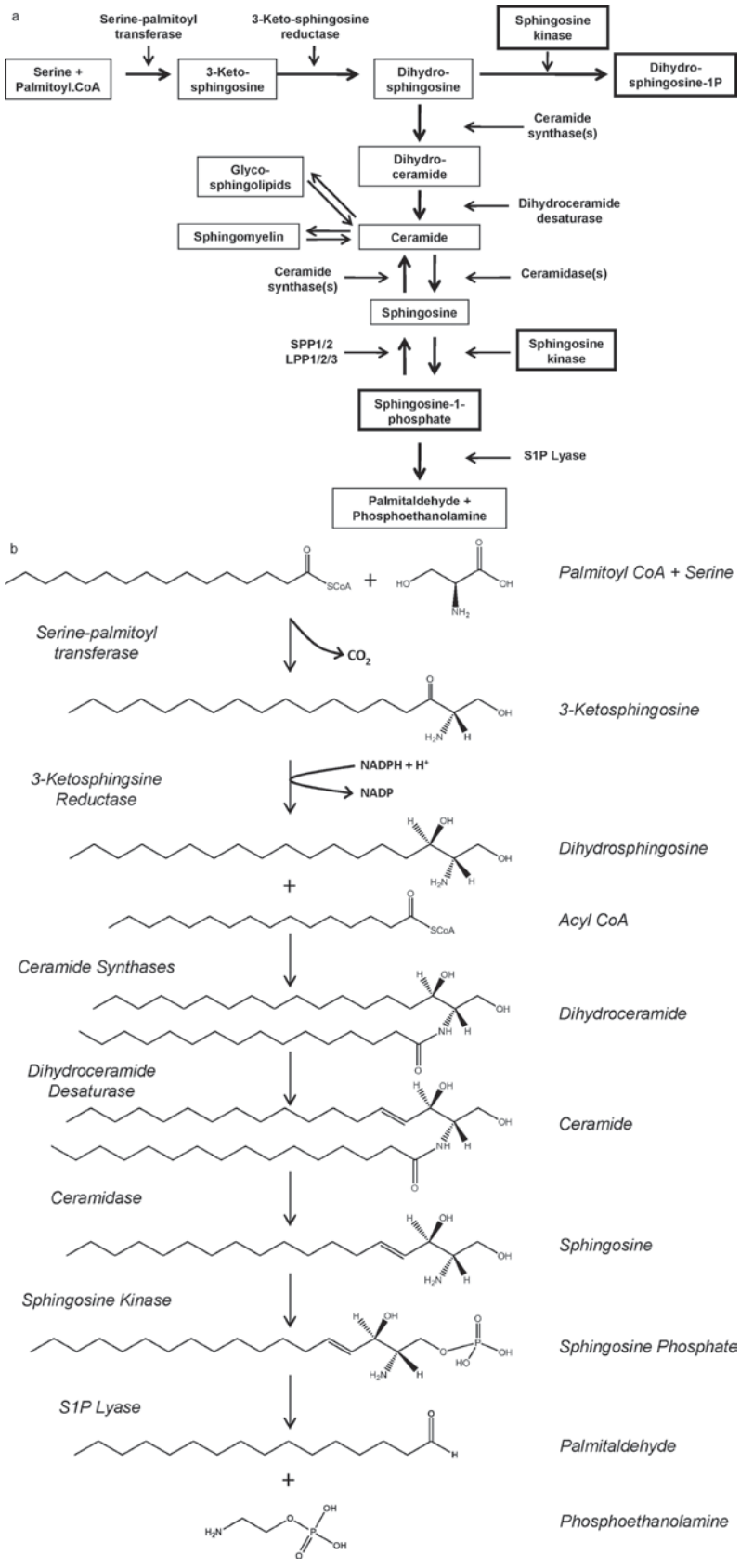

Figure 1. (A) Sphingolipid metabolism. Note that the *de novo* biosynthesis of sphingolipids begins with the condensation of serine and palmitoyl CoA and that the sole enzyme that can accomplish the degradation of the sphingosine backbone is S1P lyase. Note also that sphingosine kinase can utilize dihydrosphingosine generated during *de novo* sphingolipid synthesis or sphingosine released from ceramide by ceramidases. (B) Structures of sphingolipid intermediates. Note that the double 4,5 double bond in sphingosine is only introduced in the context of dihydroceramide. Therefore, all dihydrosphingosine-1-phosphate must be derived from dihydrosphingosine diverted from ceramide biosynthesis. (Modified from Siow et al. (2011). Adv. Enzyme Reg. and Wattenberg (2011). World J. Biochem).

The concept that S1P rapidly moves between membranes is puzzling in light of the suggestions noted above that SK is localized in proximity to certain effectors. One possibility is that flow of S1P is facile between the PM and ER but there are other, less accessible sites within the cell. One obvious example is that S1P may not have access from the cytosol to the nucleus, thus necessitating the localization of SK2 to the nucleus to supply S1P to nuclear effectors.

Summary and directions for the future

SK1 and SK2 are membrane active, but soluble enzymes, and clearly can have distinct, and changeable, sites of intracellular concentration. Both the substrates and products of the SKs are lipids and it is to be expected that localizing the SKs to a particular site will impact on the utilization of substrate and the localized production of product (Figure 2). Localization can determine access of SKs to sphingosine and dihydrosphingosine, and can therefore affect both the level and the type of signaling lipid product that is produced. As dihydrosphingosine and sphingosine are the precursors of ceramide synthesis through the *de novo* and salvage pathways, respectively, regulation of SK access to these substrates could also affect levels of ceramide and downstream ceramide metabolites such as sphingomyelin and glycosphingolipids (Figure 2). There are hints that SK localization may be used to load specific downstream S1P effectors with S1P in a spacially restricted way. SK translocation to the plasma membrane seems to enhance the secretion of S1P to the extracellular milieu where it can encounter members of the S1P receptor family and therefore may have a profound effect on autocrine and paracrine signaling function. Surprisingly, the site of S1P production seems to have a limited effect on the degradation of S1P, despite the fact that the enzymes of degradation are, for the most part, localized to the endoplasmic reticulum. This suggests that there is a robust mechanism for redistributing S1P throughout the cell. Establishing the mechanism and routes of intracellular S1P movement will be important for tying together SK localization and S1P signaling function.

Studies to localize the SKs have relied heavily on immunofluorescence and, to a limited extent, on subcellular fractionation. These techniques, although informative, may be missing important, but more transient, associations of SKs with specific membrane sites. More sophisticated fluorescent techniques such as FRET and correlation spectroscopy will be required to detect such interactions. The mechanisms governing localization of SKs to specific sites are still being uncovered. Understanding these mechanisms in detail will provide the necessary tools for determining the regulation of SK localization during cellular responses and generate the means to experimentally

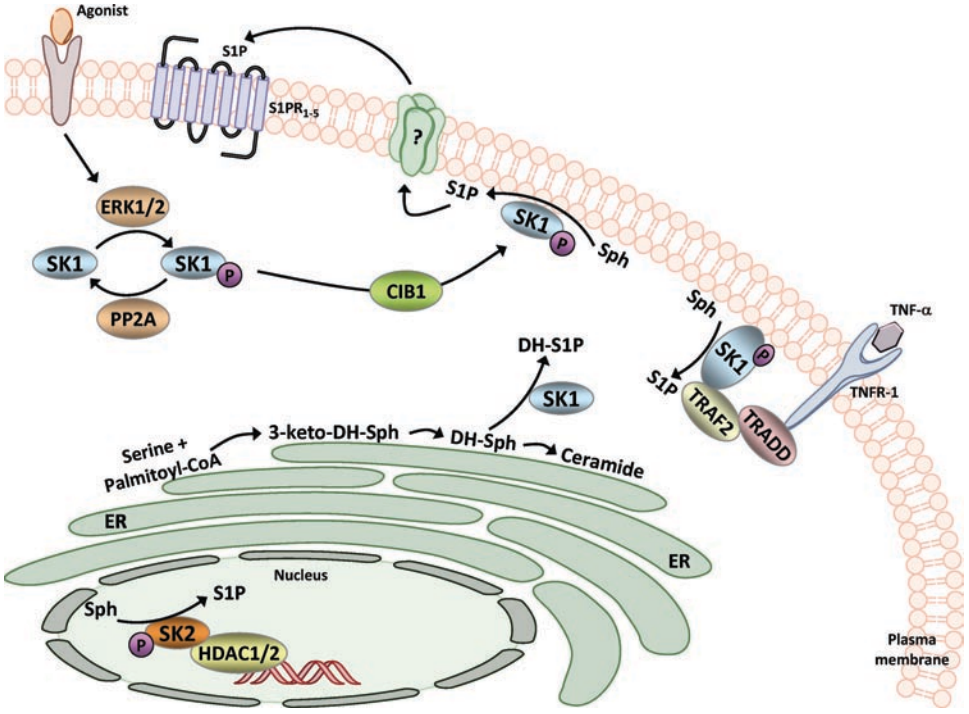

Figure 2. A model of the role of sphingosine kinase localization on substrate utilization and delivery of S1P to specific sites for secretion or loading of intracellular effectors. During agonist stimulation SK1 is phosphorylated by ERK1/2, or a related kinase, resulting in translocation to the plasma membrane (PM). This translocation is mediated by the CIB1 protein and binding to acidic phospholipids. Translocation to the PM enhances secretion of S1P, and ultimately S1P binds to a member of the S1P receptor (S1PR) family. Additionally, SK1 binds in part to TRAF2 and this may load S1P onto TRAF2 to activate the ubiquitin ligase activity of TRAF2. Similarly, the nuclear localization of SK2 is essential for its binding to histones, which may optimize the loading of S1P onto histone deacetylases-1 or -2 (HDAC1/2) and the subsequent inhibition of histone deacetylase activity. Membrane binding of SK1 may specify whether the lipid substrate is sphingosine (Sph; PM) or dihydrosphingosine (DH-Sph; endoplasmic reticulum, ER). Localization of SK1 at the ER diverts dihydrosphingosine from the ceramide biosynthetic pathway.

test the role of SK localization in signaling and metabolism of sphingolipids.

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Declaration of interest

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