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Molecular Mechanisms of Regulation of Sphingosine Kinase 1

Michael J. Pulkoski-Gross^{1,2,*,&} and Lina M. Obeid^{2,3,*}

¹Department of Pharmacological Sciences, Stony Brook University, Stony Brook, NY 11790, USA

²Department of Medicine and, The Stony Brook Cancer Center, Stony Brook University, Stony Brook, NY 11790, USA

³Northport Veterans Affairs Medical Center, Northport, NY 11768, USA

Abstract

Within the last 3 decades, there has been intense study of bioactive sphingolipids and the enzymes which metabolize those lipids. One enzyme is the critical lipid kinase sphingosine kinase 1 (SK1), which produces the potent and pleiotropic signaling lipid, sphingosine 1-phosphate (S1P). SK1 and S1P have been implicated in a host of different diseases including cancer, chronic inflammation, and metabolic diseases. However, while there is ample knowledge about the importance of these molecules in the development and progression of disease there is a dearth of knowledge of the molecular mechanisms which regulate SK1 function. In this review, we will cover some of the more recent and exciting findings about the different ways SK1 function can be regulated, from transcriptional regulation to protein stability. Finally, we will delve into recent structural insights into SK1 and how they might relate to function at cell membranes.

Keywords

Sphingolipids; Sphingosine Kinase; Sphingosine 1-phosphate; miRNA; Proteolysis; Membrane Binding

Introduction

Lipids were once thought to only play a structural role in maintaining cell membrane integrity. However, we now know that lipids can be much more impactful than just providing a barrier between the cytoplasm of the cell and the exterior environment. Sphingolipids, over the last 3 decades, have come to be recognized as a group of bioactive signaling lipids. These lipids play roles in diverse cell biologies including – cell growth, inflammation, angiogenesis, cell death, survival, and many more [1–3].

*Co-corresponding Authors: mpg1@stanford.edu; lina.obeid@stonybrookmedicine.edu.

&Current address: Department of Biochemistry, Stanford University, Stanford, CA 94305, USA

Conflict of Interest

The authors declare no conflict of interest.

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Sphingolipid metabolism is a complex network of more than 30 enzymes capable of generating hundreds of different species of sphingolipids (Figure 1). Of these lipids, the signaling molecule sphingosine-1-phosphate (S1P) has been shown to be a critical mediator in normal physiological processes such as immune cell trafficking and angiogenesis [4, 5]. On the other hand, the lipids ceramide and sphingosine are associated with cell death, senescence, and cell cycle arrest [6–8]. Sphingosine kinase 1 (SK1) sits a critical junction in the metabolic pathway (Figure 1) as it can control the levels of the pro-apoptotic lipids ceramide and sphingosine and the pro-survival lipid S1P. Given this critical role, SK1 has been implicated in a number of different disease states including – cancer, inflammation, and metabolic diseases [9–12]. SK1 has been targeted for therapeutic intervention in these diseases. However, there are currently no United States Food and Drug Administration approved drugs which target SK1 for any disease. Therefore, it is imperative to understand how SK1 is regulated in biology and dysregulated in disease. Only then can we use this knowledge for the development of better therapeutic interventions.

While there is a plethora of information about the biology of SK1 and S1P, there is a dearth of knowledge about the molecular mechanisms which regulate SK1. However, in the last several years, the field which studies SK1 has made tremendous strides in understanding the different ways SK1 can be regulated. A number of mechanisms have now been defined which underlie the regulation of SK1 in the cell.

In this review, we will explore all the different mechanisms of SK1 regulation. From gene transcription to mRNA translations to post-translational modifications of the SK1 protein. Additionally, activation and membrane binding will be discussed as they pertain to SK1 function. Finally, insight into where the field is headed and how the field can take advantage of SK1 regulation for therapeutic options will be discussed.

Transcriptional regulation of SK1

The first form of regulation of any protein can be found at the transcriptional level, which is the first decision point for whether or not a protein will be expressed. Several different transcription factors (TFs) have been described in regulating the gene expression in different cell types. In PC12 cells, a rat pheochromocytoma cell line, the transcription factor specificity protein 1 (Sp1) was shown to be responsible for *Sphk1* transcriptional induction in response to nerve-growth factor (NGF) [13]. Promoter studies revealed that there are two critical Sp1 binding sites, flanked by an AP2 binding site, 120 base pairs upstream of the transcriptional start site which are responsible for the response to NGF [13]. Additionally, Sp1 and AP2 transcription factor binding sites in the *SPHK1* promoter have been shown to be important in the upregulation of SK1 in response to glial line-derived neurotrophic factor (GDNF) in human neuroblastoma cells [14]. Similar to the rat *sphk1* gene, promoter studies of the human *SPHK1* gene show that adjacent AP2 and Sp1 binding sites, just upstream of the transcriptional start site, are critical to the transcription of *SPHK1* in response to GDNF [14].

The E2F transcription factor family members, E2F1 and E2F7, have been shown to regulate the transcription of *SPHK1* in liver and head and neck cancers, respectively [15, 16]. Lu *et al.* showed a core promoter region between –300 and +20 base pairs of the translational start

site contains an E2F1 binding site responsible for transcription in response to HULC (Highly Upregulated in Liver Cancer, which is a long non-coding RNA [lncRNA] molecule) [15]. Another lncRNA, Khps1, was found to drive expression of the *SPHK1* gene in an E2F1 dependent manner [17]. The mechanism is thought to be through the recruitment of chromatin modifiers to ensure the chromatin structure allows binding of the E2F1 transcription factor [17]. A better studied transcriptional pathway which regulates SK1 is the hypoxia response. Hypoxia-induced *SPHK1* transcriptional upregulation has been shown in several cell types including renal cancer, brain cancer, and endothelial cells [18–20].

Our lab has previously shown a role for SK1 in clear cell renal cell carcinoma (ccRCC) cells which lack the von Hippel-Lindau (VHL) protein [20]. VHL is well-known to regulate the TFs called hypoxia-inducible factors (HIFs) and plays a role in oxygen-sensing and cancer development (reviewed in [21]). Interestingly, in 786-O cells (a ccRCC cell line with mutant VHL) *SPHK1* is upregulated due to increased HIF2 α caused through the loss of the VHL protein [20]. Furthermore, knockdown of HIF2 α by siRNA causes a consequential decrease in *SPHK1* gene and SK1 protein expression [20]. Interestingly, it has also been shown that SK1/S1P signaling can in turn regulate *HIF2 α* [22]. These two studies provide evidence for a potential feedback loop between SK1/S1P and HIFs in response to oxygen levels.

In glioma cells, hypoxia (induced via oxygen deprivation or cobalt chloride) increased expression of SK1, HIF1 α and HIF2 α [18]. Knockdown of *HIF2 α* results in decreased *SPHK1* expression. In chromatin immunoprecipitation (ChIP) assays, HIF2 α , but not HIF1 α , is bound to *SPHK1* gene in response to hypoxia [18]. In contrast to this, it was reported that SK1 regulated the expression and activity of *HIF2 α* in response to hypoxia in a number of lung and ccRCC cell lines [22]. Bouquerel *et al.* believe that this regulation of HIF is due to an SK1-dependent downregulation of the AKT/mTOR signaling pathway [22]. Whether or not there is a feedback loop involving SK1 and the HIF transcription factors remains to be seen.

Similar to the HIFs, LIM-domain-only protein 2 (LMO2) is a TF which is important in vasculogenesis and angiogenesis during remodeling events in endothelial cells. Additionally, LMO2 has been shown to promote angiogenic traits in glioma stem cells [23]. It has been shown in zebrafish, that knockdown of *Lmo2*, via morpholino, resulted in defective intersegmental vessels [24]. *Lmo2* knockdown impaired migration of human umbilical vein endothelial cells (HUVECs) [24]. Furthermore, knockdown or overexpression of LMO2 decreased or increased, respectively, SK1 protein levels [24]. Finally, LMO2 was found to bind to the *Spk1* gene using ChIP assays [24]. Insights into how SK1 is regulated transcriptionally could lead to new ways to downregulate SK1 expression in cancers where SK1 contributes to development and progression. Future research is needed to flesh out whether or not we can target TFs in cancer cells to stop transcription of genes like *SPHK1* and if it's possible to efficiently deliver lncRNA molecules to alter chromatin structure around your gene and what the consequences of these treatments would be to normal tissue.

Translational Regulation

The second step of expressing a protein is the translation of the transcribed mRNA. This allows for another level of regulation of all proteins, including SK1. Small RNA molecules

can interfere with translation of mRNA by causing the degradation of their target mRNAs. Interestingly, cancer has hijacked this regulatory system to up- or down-regulated certain proteins. Several different micro-RNA (miRNA) molecules can affect the translation of SK1 protein in different cancer models. miRNA-124 has been shown to be decreased in ovarian cancer patient samples and cell lines relative to their normal counterparts [25]. Overexpression of miRNA-124 in different ovarian cell lines results in decreased SK1 levels as well as functional outcomes including decreased migration and invasion [25]. miRNA-124 could be used as a prognostic marker or therapeutic target as it is associated with metastatic tumors and a more aggressive cellular phenotype [25]. miRNA-124 has also been implicated in SK1 downregulation in several head and neck squamous cell carcinoma (HNSCC) cell lines [26]. In HNSCC, miRNA-124 is downregulated similar to ovarian cancer cell lines and when miRNA-124 expression is rescued there are decreases in cell viability, colony formation, tumor volume, and increases in several ceramide species [26]. In bladder cancer, miRNA-125b has been implicated in SK1 regulation. Compared to normal tissue, cancerous bladder tissue has decreased expression of miRNA-125b [27]. Re-expression of miRNA-125b decreases SK1 protein levels and subsequently colony formation and migratory capacity [27]. miRNA-613 is also implicated in bladder cancer showing that it can negatively regulate SK1 expression but in cancer cell lines miRNA-613 itself is downregulated allowing SK1 expression to go unchecked [28]. Yet another miRNA which has been implicated in targeting SK1 is miRNA-659-3p. miRNA-659-3p is downregulated in colon cancer cell lines and overexpression reduces SK1 protein levels [29]. Combination of cisplatin and miRNA-659-3p expression was able to significantly reduce tumor volume in mice [27]. miRNA-506 has been implicated in hepatocellular carcinoma (HCC). Expression of miRNA-506 is negatively correlated with SK1 expression in HCC samples [30]. Additionally, conditioned media from miRNA-506 expressing HepG2 cells negatively affects the ability of HUVEC cells to form tubes in culture [30].

Interestingly, a long non-coding RNA called HULC (highly up-regulated in liver cancer) was found to be able to increase SK1 expression in HCC [15]. Increased SK1 expression resulted in increased angiogenic potential of these cells as determined by a chorioallantoic membrane (CAM) assay [15]. The mechanism of HULC was found to be sequestering of miRNA-107 allowing for the expression of E2F1 a transcription factor which can drive the expression of SK1 [15]. Regulation of SK1 expression can happen through interactions with multiple different miRNAs. These miRNAs could be used as biomarkers to determine how aggressive tumors could become or could be reintroduced into cells to stop cancer growth. However, these ideas need to be further substantiated. Aside from transcription of the *sphk1* gene and translation of the *sphk1* mRNA, the SK1 protein can be regulated in a number of ways from the different stimuli to post-translational modifications, to degradation of SK1. These methods of SK1 regulation will be discussed below.

Regulation of SK1 by different stimuli

Increases in the levels of SK1 activity have been attributed to a number of different stimuli, both physiological and pharmacological in nature. Physiologically, transforming growth factor β (TGF- β) has been shown to induce SK1 activity through the upregulation of expression of SK1. Interestingly, TGF- β exposure also showed a decrease in sphingosine 1-

phosphate phosphatase activity. Tumor necrosis factor α (TNF α), a soluble cytokine, has been shown to increase activity of SK1 in HUVEC cells [32]. Recently, in breast cancer, it was found that leptin can induce the expression and activation of SK1 in estrogen receptor negative cell lines including, MDA-MB-231 and BT-549 [33]. Other endogenous activators of SK1 expression include 17 β -estradiol and prolactin [34]. These were found to have biphasic effects of SK1 activity with both acute and delayed activation of SK1 activity and expression, respectively [34]. Pharmacological stimulation of leukemia cells, MEG-O1, with phorbol 12-myristate 13-acetate (PMA) was shown to induce SK1 mRNA, protein, and activity levels [35]. PMA stimulation was found to be dependent on protein kinase C (PKC) as inhibition of PKC resulted in a loss of the PMA effect on SK1 [35]. Environmental stresses, such as hypoxia, can induce SK1 expression as discussed in a previous section. Generation of reactive oxygen species inhibits the activity of SK1 and promotes an increased ratio of ceramide/S1P [36]. While increased activity by different stimuli is important, post-translational modifications (PTMs) also play a significant role in regulating SK1.

Post-translation modifications of SK1

SK1 has a single, well-known, phosphorylation site at Ser225 shown in Figure 2. This serine is located on a 40-residue long loop insertion in the kinase domain. It is conceivable that this loop gives Ser225 accessibility and flexibility, as opposed to being within a stable secondary helix, to be phosphorylated and dephosphorylated by protein kinases and phosphatases, respectively. Ser225 phosphorylation was found to be stimulated by phorbol 12-myristate acetate (PMA) and TNF α and mediated through the extracellular regulated kinase (Erk) 1 and 2 [37]. Phosphorylation of this residue has been shown to be important in SK1 localization (discussed in more detail below) and SK1 activity [37]. However, it has also been reported that translocation to membranes does not require phosphorylation of Ser225 [38–40]. To date, only one other phosphorylation site has been reported, Thr193 [41]. This phosphorylation has yet to be validated and its role in biology has not been investigated. We have predicted additional phosphorylation sites for SK1 (isoform 3) based on the primary SK1 sequence using GPS [42] (an online prediction tool, Table 1). The two aforementioned sites are predicted here as well. More complete studies of SK1 activation under different contexts and in different cell lines might validate some of these predicted phosphorylation sites on SK1.

Protein phosphatase 2A (PP2A) was found to mediate the de-phosphorylation of Ser225 in cells [43]. De-phosphorylation of SK1 by PP2A, but not the closely related PP4, resulted in decreased cellular SK1 activity [43]. It was later shown that the β -subunit of the PP2A holoenzyme plays an important role in determining whether SK1 remains phosphorylated [44]. The β' α subunit was found to interact with the proline-rich C-terminal tail of SK1 [44]. When this subunit was overexpressed the levels of phosphorylated SK1 decreased and was accompanied by a decrease in SK1 activity [44].

Another post-translational modification (PTM) which has been reported for SK1 is lysine acetylation. Acetylation is thought to occur on an identified GKGK motif (highlighted in Figure 2) which is a common motif in proteins which are acetylated [45]. It was shown that SK1 acetylation can be mediated by the p300/Creb-binding-protein acetylase [45]. It was

also implicated in this work that acetylation of SK1 was able to affect SK1's ability to become ubiquitinated [45]. Using GPS-PAIL [46], a web-based predictor of protein acetylation, we were able to predict several residues which might be acetylated, including Lys29 (Table 1). However, this work needs to be verified and corroborated and much more research is needed to understand the mechanisms which induce SK1 acetylation and how acetylation might regulate SK1.

Ubiquitination has also been shown to be a PTM of SK1 which regulates its turnover. SK1 inhibitors, whether small molecules or Sph-analogs, have been shown to induce the loss of SK1 in cells through proteasomal degradation as shown by pharmacological inhibition of the proteasome [47–49]. Furthermore, the inhibitor-induced degradation has been linked to the poly-ubiquitination of SK1 in androgen-sensitive prostate cancer cells. Ubiquitination sites for SK1 have yet to be mapped to any specific residues. Using both UbiProber [50] and UbPred [51], we have predicted five different lysine residues to be candidates for ubiquitination (Table 1). No SUMOylation sites were predicted within the SK1 sequence (Table 1). It will be interesting to see if these sites correlate with those that are ubiquitinated in cells.

Overall, PTMs potentially offer a wide variety of mechanisms which can regulate SK1. Aside from phosphorylation, we have just scratched the surface of identifying PTMs of SK1 and how they could affect SK1-dependent biologies. Protein-protein interactions (PPIs) can regulate SK1 at the protein level through direct interaction with SK1.

Protein-Protein Interactions

For many basic cellular processes such as signaling, cell division, DNA replication, intracellular trafficking, etcetera, protein-protein interactions (PPIs) are critical. For SK1, many interacting proteins have been identified and fall into three categories: 1) proteins which can activate SK1, 2) proteins which can reduce SK1 activity, and 3) proteins which interact but do not have any effect on SK1 activity (overview of PPIs is provided in Table 2). Yeast two-hybrid screening has been an important technological advance as it has been used often for discovering binding partners for SK1. Mass spectrometry is also becoming an increasingly useful tool for identification of interacting partners.

One of the first SK1 interacting proteins identified was TNF receptor-associated factor 2 (TRAF2) [52]. It was identified to interact with a PPEE motif which is in the C-terminal tail of SK1. Interaction between TRAF2 and SK1 stimulated SK1 activity in response to TNF treatment but not phorbol ester treatment, suggesting there are different pathways which can activate SK1 [52]. Using a rat brain cDNA library Fujita and colleagues identified δ -Catenin/neural plakophilin-related armadillo repeat protein interacts with SK1 [53]. It was shown that δ -Catenin co-localized with SK1 in primary rat embryonic hippocampal cells and that δ -Catenin could increase SK1 activity *in vitro* and in cells [53].

SK1 interaction with Fyn and Lyn kinases implicate SK1 in signaling events proximal to triggering of the high-affinity receptor for immunoglobulin (Ig) E (Fc ϵ RI) [54, 55]. Both Fyn and Lyn kinases increase SK1 activity and SK1 plays a role in the downstream events in mast cells, such as chemotaxis and degranulation [54, 55].

Eukaryotic elongation factor 1A (eEF1A) is involved in translation of proteins but moonlights as a modulator of signaling proteins. eEF1A has been shown to interact with SK1, as well as increase the catalytic rate of SK1 and, interestingly, SK2 [56]. Importantly, when eEF1A is knocked down in cells endogenous SK1 activity is decreased [56].

Filamin A, an actin-cross-linking protein, has been shown to be responsible for activation and translocation of SK1 at lamellipodia in response to heregulin in A7 melanoma cells [57]. In A7 cells lacking Filamin A, SK1 was no longer activated or colocalizing with S1PR1 at lamellipodia in migrating cells [57]. Moreover, treating A7 cells with VPC23019, an antagonist of S1PR1 and S1PR3, resulted in the loss of enhanced colocalization of SK1 at lamellipodia [57].

While the previous set of proteins were involved in the activation of SK1, these proteins are involved in negatively regulating SK1. Sphingosine Kinase 1 Interacting Protein 1 (SKIP), a protein that is closely related to the protein kinase A anchoring proteins, was found to interact with SK1 *in vitro* through immunoprecipitation assays [58]. Overexpression of SKIP reduced basal SK1 activity and stimulated SK1 activity in HEK cells [58]. Expression of SKIP reduces the ability of SK1 to enhance cellular growth through reduction in ERK phosphorylation [58].

Platelet endothelial cell adhesion molecule 1 (PECAM-1) was found to interact with SK1 and attenuate its activity [59]. Phosphorylation of PECAM-1 reduces SK1 association but SK1 does not directly interact with the tyrosine residues on PECAM-1 which are phosphorylated [59]. This implicated a role for PECAM-1 phosphorylation in the localization and the generation of S1P. However, endogenous protein interaction remains to be determined.

Interaction of SK1 with four-and-a-half LIM domain 2 (FHL2) was identified by yeast two-hybrid screening and confirmed by pulldown assays [60]. Interaction sites were mapped to the C-terminal half of SK1 and the C-terminal 4 LIM domains of FHL2 [60]. Interaction of SK1 with FHL2 was shown to reduce SK1 activity [60]. Furthermore, the anti-apoptotic effect of SK1 is diminished in HEK cells when FHL2 is overexpressed [60]. However, when FHL2 is knocked down in cardiomyocytes, those cells become protected from apoptosis [60]. FHL2 is implicated in negatively regulating the activity of SK1 in cells and governing its anti-apoptotic effects in cardiomyocytes.

In the bovine viral diarrhea virus (BVDV), the N-terminus of the NS3 protein was shown to interact with the C-terminal domain of SK1 [61]. Interaction decreases activity and inhibition of SK1 might enhance the replication of BVDV [61]. Interestingly, the NS3 protein of the hepatitis C virus, a close relative of BVDV, does interact with SK1 but does not affect its activity [61].

A third set of interacting partners have been found which do not affect the activity of SK1. RPK118 was shown to interact with and co-localize with SK1 in pulldown experiments and cell imaging experiments respectively [62]. However, RPK118 interaction did not affect the activity of SK1 [62]. Aminoacylase 1 was shown to interact with SK1 but there is only a very small decrease in SK1 activity [63]. Furthermore, there is no change in the protective

effect of SK1 in cells in the presence of full length aminoacylase 1 [63]. Calcium-integrin binding protein 1 and 2 (CIB1/2) has been shown to affect SK1 activity through translocation of SK1 from the cytosol to the membrane [64, 65] (expanded upon in the next section). SK1 enhances the expression and activation of integrin $\alpha v \beta 3$ and promotes focal adhesion development [66]. In endothelial cells, SK1, $\alpha v \beta 3$, and CD31 form a complex which is dependent on SK1 phosphorylation at Ser225 [66]. The activity of SK1 in the presence of $\alpha v \beta 3$ was not determined. Finally, the cytosolic chaperonin CCT η (chaperonin containing t-complex polypeptide) interacts with SK1 but has no effect on SK1 activity or on SK1 activation [67]. CCT η associates with newly synthesized SK1 and is important for its maturation into an active protein [67].

Recently, a mass spectrometry-based approach was used to identify a large set of proteins which interact differentially with the different isoforms of SK1 in breast cancer [68]. The two major transcriptional isoforms of SK1 have sets of both common and distinct interacting partners [68], adding another layer of complexity to the SK1 puzzle. These newly identified proteins need to be validated and studied in depth to understand how they affect SK1 in cells. Ultimately, mapping each of the protein-protein interactions with SK1 isoforms will allow for a deeper understanding of how these proteins can regulate SK1 activity. This will require the implementation of biochemistry and cell biology using techniques such as hydrogen-deuterium exchange mass spectrometry to map the interfaces of SK1 and its interacting partners.

Very few studies have looked into functional differences between different isoforms of SK1. These isoforms of SK1 arise from different splice variants of *sphk1* mRNA (reviewed in [69]). Unfortunately, there is a significant roadblock in studying the SK1 isoforms functional differences as all of the SK1 isoforms catalyze the same reaction. However, there have been some functional differences including stability and localization shown between SK1 isoforms from mice [70]. Another interesting study showed that SK1 isoform 1 was excreted to the extracellular matrix whereas isoforms 2 and 3 remained mostly at the membrane [71]. The field requires future studies following up on the different SK1 isoforms and how they relate to inflammatory diseases and cancer. This could be leveraged for the development of isoform specific therapies.

SK1s access to the membrane

Localization is a key component in determining the activity and ultimately the function of SK1 in a specific setting. However, SK1 is a cytosolic protein which lacks any known lipid-binding domains. How does this protein interact with membranes? There are two different ways SK1 can interact with the membrane: 1) indirect interaction mediated through protein-protein interaction and 2) direct interaction with membranes as an intrinsic property of SK1. Here we will discuss protein-protein mediated membrane localization as well as SK1-mediated membrane localization.

SK1 is activated, at least *in vitro*, in the presence of anionic phospholipids such as phosphatidic acid, phosphatidylserine, and phosphatidylinositol [72, 73]. This would suggest that SK1 is active at membranes which contain both anionic phospholipids as well as its substrate. Interestingly, it seems that there is some specificity for interaction with certain

anionic phospholipids such as phosphatidic acid and, less so, phosphatidylserine. Furthermore, SK1 has been shown to be an effector of phosphatidic acid [74]. However, SK1 does not interact with neutrally charged lipids such as phosphatidylcholine or phosphatidylethanolamine.

Several different mechanisms have been published as to how SK1 can interact with membranes. It was first published that SK1 interacted with phospholipid membranes via two residues (Thr54 and Asn89) [75]. Mutation of these residues resulted in loss of binding as measured by surface plasmon resonance [75]. However, the elucidation of the structure of SK1 [76] (reviewed in [77, 78]) revealed that these residues, Thr54 and Asn89, are involved in ATP binding and interaction between the N-terminal and C-terminal domains, respectively. A recent study has shown that membrane binding is mediated solely by a hydrophobic patch on the surface of SK1 [79]. These mechanisms are unable to explain how SK1 could discriminate between charged and neutral phospholipids. Upon closer examination of the currently published SK1 structures, Adams *et al.* proposed a mechanism by which SK1 can form dimers and interact with membranes using a single contiguous interface [78]. Our lab has recently identified a new electrostatic site on SK1 and has shown that it forms a single interface, along with the hydrophobic patch, to effectively interact with membranes [80]. This single contiguous interface is critical for the function of SK1 in cells [80]. Together the work of Pulkoski-Gross *et al.* [80], Shen *et al.* [79], and Adams *et al.* [78] provide insight into an intrinsic mechanism for SK1 interaction with membranes.

It has been proposed that SK1 dimer formation is a means of sensing membrane curvature [78]. This dimerization would occur through a “head-to-head” interaction between the N-terminal domains of two SK1 molecules (as shown in Figure 4). The evidence for this crystallographically observed dimer orientation is extremely strong as the dimer is observed in all of the structures to date, even across different crystallographic space groups. This evidence implies that this dimer structure is a crucial physiological orientation of the SK1 protein. Additionally, dimerization has been demonstrated in cells through immunoprecipitation of overexpressed SK1 proteins which have been differentially tagged [48].

Dimerization is an interesting possibility as the currently proposed dimer interface [78, 81] would allow the membrane-binding interface of each protomer to align with each other. This type of interaction would elongate the interface and strengthen the interaction between SK1 and membrane phospholipids. Our lab’s confirmation of the membrane-binding interface, first proposed by Adams *et al.*, highlights the significance of the dimeric structure of SK1 as a critical determinant of function [78]. However, further biochemical and cellular work is warranted to establish that the dimerization interface is crucial for SK1 function. There is a need for further work to understand the kinetics and dynamics which underlie SK1 dimerization and how that would affect membrane binding and function in cells.

Interestingly, membrane binding via this mechanism would allow for the loop containing Ser225 would be exposed to the cytosol for interaction with kinases and phosphatases. Additionally, it’s possible that phosphorylation of Ser225 could play a role in activation of the kinase coincidental to translocation to membranes. Adams *et al.* speculate that phospho-

Ser225 could allow for the loop to become disengaged from the kinase and allow for conformational relaxation of helices α -3 and α -4 [78]. This could allow for a more active kinase conformation as these helices are involved in substrate recognition and catalysis. NMR could be a useful tool in determining whether this is the case for SK1 as is the case for some bacteria diacylglycerol kinases.

Translocation of SK1 to the plasma membrane has also been attributed to interaction with calcium/integrin binding protein 1 (CIB1) [64, 65]. Removal of CIB1 from cells inhibits SK1 translocation to membrane [64, 65]. Furthermore, in Ras-dependent cancers CIB1 has been shown to contribute to oncogenic signaling through SK1 translocation [65]. Interestingly, CIB2, a close relative of CIB1, negatively regulates the translocation of SK1 in ovarian cancer [82]. Interaction between SK1 and the CIB proteins takes place at the hydrophobic patch which has been shown to be imperative to direct membrane binding. Therefore, it is possible that CIB interacts with SK1 only after it is at the membrane as association before could preclude it from membrane interaction. It is also possible that it acts as a 'molecular shepherd' bringing SK1 near the membrane but not affecting direct interaction. It is also possible that CIB1 could co-translocate and serve to open the α -8 helix to allow SK1 to bind sphingosine once at the membrane, as suggested by Adams *et al.* [78]. Further research is needed to fully comprehend the interplay between SK1 and CIB during SK1 translocation events.

Other proteins have been attributed with the ability to stimulate the translocation of SK1 to membranes as well. G-protein coupled receptors (GPCRs) have been shown to induce the translocation of SK1 to cellular membrane. Two GPCRs, muscarinic M3 receptor and the bradykinin receptor, which are both coupled to the Gq G-protein, induce SK1 translocation to the plasma membrane [39, 40]. The molecular mechanisms which underly these GPCR-induced translocation events remain unknown. Interestingly, phosphorylation of SK1 is not required for the translocation of SK1 after Gq-coupled receptors. Additionally, phosphorylation-independent translocation to phagosomal membranes has been noted previously in macrophages infected with *Mycobacterium tuberculosis* [38]. The role and timing of phosphorylation of SK1 in cellular responses to different stimuli, and how that relates to translocation of SK1 to the membrane, will be an interesting avenue of future work. The last way SK1 can be regulated is via its removal from the cell, as discussed below.

Degradation of SK1

Finally, SK1 is regulated, like many other proteins, through its degradation. Interestingly, many of the inhibitors which target SK1 have been shown to degrade SK1 at the protein level [47–49, 83–87]. Some of these inhibitors have been linked to proteasomal degradation as well as ubiquitination as mentioned in the previous section. One inhibitor, 2-(p-hydroxyanilino)-4-(p-chlorophenyl) thiazole (SKI II), was found to induce degradation through a lysosomal pathway as inhibition of the lysosome with chloroquine reversed the degradation of SK1 in SKI II treated cells [84]. The exact mechanism of how these inhibitors can induce the degradation of SK1 remains to be resolved.

Aside from the SK1 inhibitor-induced degradation of SK1, it has been observed that induction of cellular DNA damage response causes the degradation of SK1. Upon treatment

with DNA-damaging agents, including Actinomycin D, Doxorubicin, Etoposide, or γ -irradiation, SK1 at the protein level was reduced in the acute lymphoblastic leukemia cell line MOLT-4 [88]. ZVAD, a pan-caspase inhibitor, and CA-074, a cathepsin B inhibitor, were able to reverse the effect of Actinomycin D on SK1 protein levels [88]. It was further shown that SK1 degradation was dependent on the tumor suppressor p53 [88]. TNF α has been shown to induce the loss of SK1 protein with prolonged treatment (36 hours) [89]. Loss of cathepsin B by siRNA knock-down rescues the TNF-induced SK1 loss implying this process is dependent on cathepsin B [89].

Whether through the proteasome or through the lysosome, the degradation of SK1 ultimately results in loss of the pro-survival lipid S1P. This is important in treatment of cancer as many approved chemotherapies induce DNA damage in tumor cells. Inhibition/degradation of SK1 will stop the production of S1P not allowing cancer cells to have the pro-growth signaling of S1P. This allows for potential combinatorial approaches utilizing SK1 inhibition in cancer treatments.

Conclusions

Given the critical point at which SK1 sits within the sphingolipid metabolic pathway, it is imperative to understand how SK1 is regulated in cells. The sphingolipid field has made significant strides in understanding how the regulation of SK1 occurs. However, there is work that needs to be completed in order to have a more complete picture of the molecular mechanisms of SK1 regulation.

Continuing work to understand how post-translational modifications affect SK1 activity will be critical. It is possible that the current list of known/validated phosphorylation sites of SK1 is incomplete. Thr193 has been identified as a phospho-threonine residue, yet it has not been validated or investigated as to whether it is critical for SK1-mediated biology. Additionally, we have predicted a number of additional potential phosphorylation sites based on the primary sequence of SK1.

Using a similar method, we have predicted other PTMs including lysine acetylation, ubiquitination, and arginine methylation. While some of these PTMs are annotated in the literature [45, 47], we provide predictions for the most likely candidate residues for ubiquitination and acetylation. Furthermore, research into the actual function of these PTMs will reveal what role they have in regulating SK1 biology. Finally, understanding which ubiquitin ligases, acetyl transferases, and arginine methylases are responsible for these modifications will provide insight into alternative methods of disrupting SK1-driven biology in cells.

Studying SK1 interacting proteins provides an interesting platform for understanding where SK1 might fit into different signaling pathways. Research of this type would provide information on how SK1 activity can be modulated through protein-protein interaction. Very few studies have identified specific interaction sites between SK1 and the interacting partner. Yet, these sites offer the potential for disruption by small molecules in order to modulate SK1 activity in cells.

Several other mechanisms have been elucidated. Transcriptional regulators have been identified as important in development (LMO2) and in response to hypoxic stress (HIFs). These transcription factors are important for vessel growth making them particularly interesting as cancer therapeutic targets. Targeting these transcription factors could provide an alternative to targeting SK1. Targeting chromatin structure, like the lncRNA Khps1 can do, might also lead to new ways of targeting SK1 upregulation in diseases such as inflammation and cancer.

We now know that SK1 has an intrinsic ability to bind to membranes which allows access to sphingosine and production of S1P. Furthermore, it has been shown that membrane binding can be facilitated by proteins. These in-depth studies have allowed a new line of thought for pharmacological inhibition of SK1 catalytic activity. Successful SK1 inhibitors might be able to be designed to inhibit interaction with membranes, rather than target the active site. These types of compounds would not be competing with sphingosine binding but would deny SK1 access to sphingosine in the first place.

While there has been tremendous success in understanding fundamental biology and regulation of SK1, there remains much to be discovered. There is a need to dive deeper into the molecular mechanisms which govern SK1 function in cell biology and different diseases. These new discoveries will fuel future research and promote the identification of new molecules to add to the arsenal for targeting SK1.

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Highlights

- SK1 is a highly targeted candidate for drug development in different disease types including cancer and inflammation.
- There have been significant developments in understanding the molecular events regulating SK1 expression and activity.
- New opportunities are arising for better ways to target SK1 for example inhibition of membrane binding/dimerization or targeting small RNA molecules responsible for SK1 expression levels.

Figure 1: Sphingolipid metabolic pathway.

De novo synthesis of sphingolipids starts with the condensation of serine and palmitoyl-CoA via the serine palmitoyltransferase (SPT) to generate 3-ketodihydrosphingosine. This is reduced to dihydrosphingosine by 3-ketodihydrosphingosine reductase (3KHDR). This becomes acylated by ceramide synthases (CerS) to dihydroceramides. Ceramide is generated through desaturation by the 4-dihydroceramide desaturase (DES). Ceramide can be phosphorylated by ceramide kinase (CerK) and dephosphorylated by lipid phosphate phosphatases (LPPs). Ceramide is converted to sphingomyelin by sphingomyelin synthases

(SMS) and converted back to ceramide by sphingomyelinases (SMases). Ceramide can also be acylated by a complex of diacylglycerol acyl transferase (DGAT) and acyl-CoA long chain synthase (ACLS). Ceramide can also be glycosylated by glucosylceramide synthase (GCS). Ceramide can also be broken down to sphingosine by ceramidases (CDases). Sphingosine can either be phosphorylated by sphingosine kinases (SKs) or acylated by CerS. Sphingosine-1-phosphate is either dephosphorylated by sphingosine-1-phosphate phosphatases (S1PPs) or broken down to hexadecanal and ethanolamine phosphate by sphingosine phosphate lyase (SPL) to exit the sphingolipid metabolic pathway. The great diversity of sphingolipids comes from the addition of different chain length fatty acids as well as modification to the primary hydroxyl of ceramide. This headgroup complexity is most evident in the glycosphingolipids (reviewed in [90]).

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Figure 2: Protein-protein interactions and post-translational modifications mapped to SK1. Primary sequence of human SK1 (SK1a, Isoform 3, Q9NYA1–1) showing secondary structure. Blue and magenta residues represent the membrane binding interface which is important for SK1 function. Post-translational modifications (PTMs) are shown in ovals and circles underneath the primary sequence. Acetylation is not confirmed and ubiquitination has been shown to happen but is not mapped to any residue(s). Only a few protein-protein interactions have been mapped and they localize to the C-terminal tail of SK1 except for CIB1/2. For a full list of predicted PTMs see Table 1.

Figure 3: Mechanisms of sphingosine kinase 1 regulation in cells.

A single post-translational modification of SK1 is well known, the phosphorylation of Ser225 by ERK1/2 kinases. Phospholipase D which generates the lipid phosphatidic acid can activate SK1 in response to growth stimuli. SK1 can be degraded through the lysosome via cathepsins as a part of the DNA-Damage Response (DDR) and downstream of the tumor suppressor p53 or it can be proteosomally degraded after treatment with pharmacological inhibitors. Several different micro-RNAs or long non-coding RNAs (miRNAs or lncRNAs) can suppress the expression of SK1 in certain systems. Many protein-protein interactions with SK1 have been shown to increase or decrease activity of SK1. As a part of angiogenesis in response to stimuli or during development transcription of SK1 can be regulated by different transcription factors.

Figure 4: Potential membrane binding mechanisms.

There is a growing literature of evidence which would suggest that SK1 is a functional dimer in cells. Furthermore, it has been suggested that this dimerization would allow for SK1 to sense high membrane curvature. Interestingly, SK1 has been observed to translocate to the plasma membrane which lacks high curvature. When and how does SK1 decide to become a dimer? Does SK1 membrane binding require dimerization? Is the context of membrane binding the determining factor for whether SK1 can utilize its intrinsic interface or whether SK1 needs assistance from other proteins? These questions will require further insight into the dynamics and kinetics of the SK1 protein in cells.

Table 1:
Prediction of post-translational modifications to SK1.

This table shows a number of different predicted post translational modifications (PTMs) of SK1. The primary sequence of SK1 was used to predict possible PTMs and only those residues which were solvent exposed based on the structure of SK1 are shown.

Protein Modification	Predicted Residue	Sequence	Modification Status	Web Server Prediction Software
Myristoylation	None Predicted	N/A	N/A	NMT Server http://mendel.imp.ac.at/myristate/SUPLpredictor.htm
Prenylation	None Predicted	N/A	N/A	PrePS [91]
SUMOylation	None Predicted	N/A	N/A	pSUMO-CD [92]
Palmitoylation	Residue	Sequence	Modification Status	CSS-Palm 2.0 [93]
	Cys 15	GVLPRP-C-RVLVLL	Prediction only	
Ubiquitination	Residue	Sequence	Modification Status	UbiProber [50] and UbPred [51]
	Lys 29	PRGGKG-K-ALQLFR	Prediction only	
	Lys 183	VDLESE-K-YRRLGE	Prediction only	
	Lys 221	VGRVGS-K-TPASPV	Prediction only	
	Lys 335	LEPKDG-K-GVFAVD	Prediction only	
	Lys 373	EPPPSW-K-PQMPMP	Prediction only	
Lysine Acetylation	Residue	Sequence	Modification Status	GPS-PAIL [46]
	Lys 27	LNPRGG-K-GKALQL	Identified but not validated [45]	
	Lys 29	PRGGKG-K-ALQLFR	Identified but not validated [45]	
	Lys 221	VGRVGS-K-TPASPV	Prediction only	
	Lys 373	EPPPSW-K-PQMPMP	Prediction only	
Arginine Methylation	Residue	Sequence	Modification Status	PMes [94]
	Arg 24	LVLLNP-R-GGKGKA	Prediction only	
	Arg 186	ESEKYR-R-LGEMRF	Prediction only	
	Arg 191	RRLGEM-R-FTLGTF	Prediction only	
	Arg 199	TLGTFL-R-LAALRT	Prediction only	
	Arg 207	AALRTY-R-GRLAYL	Prediction only	
	Arg 296	VRAGVS-R-AMLLRL	Prediction only	
	Arg 301	SRAMLL-R-LFLAME	Prediction only	
Phosphorylation (Serine, Threonine, Tyrosine)	Residue	Sequence	Modification Status	GPS [42]
	Ser 36	ALQLFR-S-HVQPLL	Prediction only	
	Ser 48	LAEAEI-S-FTLMLT	Prediction only	

Protein Modification	Predicted Residue	Sequence	Modification Status	Web Server Prediction Software
	Ser 66	ARELVR-S-EELGRW	Prediction only	
	Ser 148	LCRRLL-S-PMNLLS	Prediction only	
	Ser 159	LSLHTA-S-GLRLF	Prediction only	
	Ser 220	PVGRVG-S-KTPASP	Prediction only	
	Ser 225	GSKTPA-S-PVVVQQ	Validated [37, 44, 95]	
	Ser 247	LEEPVP-S-HWTVVP	Prediction only	
	Ser 295	YVRAGV-S-RAMLLR	Prediction only	
	Ser 347	DGELMV-S-EAVQQQ	Prediction only	
	Ser 363	NYFWMV-S-GCVEPP	Prediction only	
	Ser 371	CVEPPP-S-WKPQQM	Prediction only	
	Thr 50	EAEISF-T-LMLTERR	Prediction only	
	Thr 99	ERPDWE-T-AIQKPL	Prediction only	
	Thr 136	TNEDLL-T-NCTL	Prediction only	
	Thr 157	NLLSLH-T-ASGLRL	Prediction only	
	Thr 193	LGEMRF-T-LGTFLR	Identified but not validated [41]	
	Thr 205	RLAALR-T-YRGRLA	Prediction only	
	Thr 222	GRVGSK-T-PASPVV	Prediction only	
	Tyr 126	LNHYAG-Y-EQVTNE	Prediction only	
	Tyr 184	DLESEK-Y-RRLGEM	Prediction only	
	Tyr 314	KGRHME-Y-ECPYLV	Prediction only	
	Tyr 318	MEYECY-Y-LVYVPV	Prediction only	
	Tyr 358	GQVHPN-Y-FWMVSG	Prediction only	

Table 2:
Interacting partners of Sphingosine Kinase 1.

Interacting partners of SK1 are listed in this table along with the effect they have on SK1 activity, and interaction sites on SK1 if they are known. Mechanisms of their effect on SK1 activity remains to be resolved.

Interacting Protein	Effect on SK1	Interaction Site	Reference
TRAF2	Stimulates SK1 activity	C-terminal tail	[52]
δ -Catenin/neural plakophilin-related armadillo repeat	Stimulates SK1 activity	Unknown	[53]
Fyn kinase	Stimulates SK1 activity	Unknown	[55]
Lyn kinase	Stimulates SK1 activity	Unknown	[54]
Eukaryotic elongation factor 1A	Stimulates SK1 activity	Unknown	[56]
Filamin A	Stimulates SK1 activity	Unknown	[57]
Extracellular regulated kinase 1/2	Stimulates SK1 activity	Loop containing Ser225	[37]
Interacting Protein	Effect on SK1	Interaction Site	Reference
Sphingosine kinase 1 Interacting Protein	Decreases SK1 activity	Unknown	[58]
Platlet endothelial cell adhesion molecule 1`	Decreases SK1 activity	Unknown	[59]
Four-and -a-half LIM domain 2	Decreases SK1 activity	Unknown	[60]
NS3 protein of bovine diarrhea virus	Decreases SK1 activity	Unknown	[61]
Protein phosphatase 2A	Decreases SK1 activity	C-terminal tail	[43]
Interacting Protein	Effect on SK1	Interaction Site	Reference
RPK118	No effect	Unknown	[62]
Aminoacylase 1	No effect	Unknown	[63]
Calcium Integrin Binding Proteins 1/2	No effect	F197/L198	[64, 65, 82]
Integrin α v β 3/CD31	Not determined	Unknown	[66]
CCT η (chaperonin containing t-complex polypeptide)	No effect	Unknown	[67]