An intrinsic lipid-binding interface controls sphingosine kinase 1 function

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Running Title: Novel SK1 lipid binding interface

Abbreviations: HDX-MS, Hydrogen/deuterium exchange mass spectrometry; endo-2, endophilin-A2, C17-Sph, 17-carbon sphingosine; C17-S1P, 17-carbon sphingosine-1-phosphate; Cer, ceramide; Sph, sphingosine; S1P, sphingosine-1-phosphate; SK1, sphingosine kinase 1; MβCD, methyl-β cyclohexextrin; PS, phosphatidylycerine; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine
Abstract

Sphingosine kinase 1 (SK1) is required for production of sphingosine-1-phosphate (S1P) and thereby regulates many cellular processes—including cellular growth, immune cell trafficking, and inflammation. To produce S1P, SK1 must access sphingosine directly from membranes. However, the molecular mechanisms underlying SK1’s direct membrane interactions remain unclear. We used hydrogen–deuterium exchange mass spectrometry to study interactions of SK1 with membrane vesicles. Using the CRISPR/Cas9 technique to generate HCT116 cells lacking SK1, we explored the effects of membrane interface disruption and the function of the SK1 interaction site. Disrupting the interface resulted in reduced membrane association and decreased cellular SK1 activity. Moreover, SK1-dependent signaling, including cell invasion and endocytosis, was abolished upon mutation of the membrane-binding interface. Of note, we identified a positively charged motif on SK1 that is responsible for electrostatic interactions with membranes. Furthermore, we demonstrated that SK1 uses a single contiguous interface, consisting of an electrostatic site and a hydrophobic site, to interact with membrane-associated anionic phospholipids. Altogether, these results define a composite domain in SK1 that regulates its intrinsic ability to bind membranes and indicate that this binding is critical for proper SK1 function. This work will allow for a new line of thinking for targeting SK1 in disease.

Keywords: Sphingosine Kinase 1 / sphingolipids / sphingosine-1-phosphate / hydrogen-deuterium exchange mass spectrometry / enzyme regulation / cell signaling
Introduction

Sphingosine Kinase 1 (SK1) is a critical enzyme in the sphingolipid metabolic pathway as it can modulate the balance between the pro-apoptotic lipids sphingosine (Sph) and ceramide (Cer) and the pro-survival and pro-inflammatory lipid sphingosine-1-phosphate (S1P). S1P can signal in two distinct ways, either through binding to one of five S1P-specific G-protein coupled receptors, or as an intracellular second messenger, although the latter is not a well understood mechanism. S1P and its producing enzymes have been implicated in a host of biological and pathophysiologial roles, including cancer biology, inflammation, immune responses, vascular biology, and many others [1-5].

SK1 is upregulated in several cancers including colon [6], lung [7], kidney [8], and brain cancers [9]. SK1 expression has been correlated with poor prognosis and survival in patients [9-11]. SK1 and S1P have been associated with increased proliferation, survival, and resistance to chemotherapy [12-14]. Furthermore, SK1 has been implicated in inflammatory diseases, such as inflammatory bowel disease [15, 16], which is now thought to be an underlying factor for cancer development and progression. While there is a plethora of knowledge about the biology of S1P and SK1, there is relatively little known about the molecular mechanisms which regulate its function.

SK1 has been shown to translocate to the plasma membrane after stimulation with PMA [17-19]. Additionally, SK1 has been shown to have increased activity in membrane bound fractions from cells and that it can be activated in vitro by the phospholipids phosphatidylserine (PS) and phosphatidic acid (PA) which are found in membranes [20, 21]. Additionally, it was shown that SK1 was an effector of PA in cells [22]. There are currently three different proposed mechanisms to explain SK1 translocation and interaction with membranes. Two of the
mechanisms identify certain residues that mediate membrane localization [21, 23], while the third explains SK1 localization as being dependent on another protein, calcium/integrin binding protein 1 (CIB1) [24, 25]. Interestingly, the protein-protein interaction between SK1 and CIB1 takes place at a hydrophobic site on SK1 which has been identified for membrane interaction. The residues identified by Stahelin et al., Thr54 and Asn89, were proposed to interact with PS in the membrane [21]. On the other hand, the residues identified by Shen et al. are part of a small hydrophobic patch on the surface of SK1, and these authors implicated these residues in mediating endocytosis and neurotransmission [23].

In this work, we identify a missing piece of the SK1 translocation puzzle as a small, highly positively charged, three-residue motif near the active site. Evidence is provided for a contiguous membrane interaction surface consisting of both the electrostatic and hydrophobic sites. Furthermore, the results reveal that both the previously identified hydrophobic patch and the newly identified electrostatic motif are both essential for allowing SK1 binding to membranes in vitro and in cells. The results demonstrate that this positively charged site is important for mediating SK1 signaling processes, including cell invasion and co-localization with N-bar proteins during endocytosis. Overall, we propose a dual-site mechanism along a contiguous surface interface which controls the interaction between SK1 and membranes. Importantly, disruption of either site or the combined disruption of both sites is sufficient to disrupt SK1-membrane interactions and SK1 signaling.
Results

*In silico* surface binding analysis of SK1. The structure of SK1 [26] allowed for evaluation of the previously proposed mechanisms for SK1 interaction with membranes. Thr54 which was proposed by Stahelin et al. [21] to interact with PS is in the β2-α2 loop and is important for interaction with ATP. Ans89, also proposed to interact with PS, is in the α3 helix and is involved in hydrogen bonding between the N-terminal and C-terminal domains (NTD and CTD) of SK1. The hydrophobic residues identified by Shen et al. [23] (Leu194, Phe197, and Leu198, magenta residues Figs. 1E and 1F) are located on the protein surface, and could enhance interaction with membranes, but most likely nonspecifically as this stretch does not allow SK1 to discriminate between neutral and charged lipids. Therefore, we conducted *in silico* surface electrostatic analysis using Chimera software [27] in order to reveal candidate residues that may mediate interactions with PS and PA. Electrostatic potentials were calculated using the Adaptive Poisson-Boltzman Solver in the Chimera software which were then mapped to a surface representation of SK1. On the surfaces shown in Figure 1A and 1B, red-colored areas represent negatively charged surfaces while blue-colored areas represent positively charged surfaces. A highly positively charged site composed of residues Lys27, Lys29, and Arg186 (Fig. 1A, 1B shown as blue surface) was identified on the surface of SK1. This electrostatic site (shown as blue residues in Fig 1E and 1F) is adjacent to the substrate binding sites for both ATP and Sph. Additionally, surface hydrophobicity was also analyzed using Chimera software (Fig. 1C, 1D). Hydrophobic surfaces are represented by magenta-colored areas while hydrophilic surfaces are represented by cyan-colored areas. The results showed the same hydrophobic site as that detected by Shen at al. [23], shown as magenta sticks in Figures 1E and 1F. Importantly, a novel electrostatic site has been identified which could mediate the interaction with PA and/or PS.
In vitro biochemical analysis of SK1 binding to anionic phospholipids and liposomes.

Interestingly, the identified charged patch of residues are more than 150 residues apart from each other, which makes it difficult to identify from the linear sequence. To disrupt this electrostatic site, Lys27 and Lys29 were mutated to glutamate residues and Arg186 to an aspartate residue (Triple-SK1), leaving the hydrophobic site intact. To disrupt the hydrophobic patch, Leu194 was mutated to a glutamine (L194Q-SK1) as described previously [23], leaving the electrostatic site intact. These mutants of SK1 were cloned into the pFastBac (Invitrogen) system for expression in and purification from insect cells. The mutation of these residues did not interfere with antibody (monoclonal antibody, Cell Signaling) recognition as shown in Supplemental Figure 1A. Recombinant SK1 proteins (Wild type (WT-SK1), L194Q-SK1, and Triple-SK1) were highly pure as determined in by SDS-PAGE (≥90%) as shown in Figure 2A. Furthermore, these mutants are active in vitro, albeit they are less active than WT-SK1 (Supp. Fig. 1B).

First, the ability of WT-SK1 to discriminate between different anionic phospholipids was tested by employing a lipid-protein overlay assay. The results (Supp. Figure 2) show that WT-SK1 was able to bind both PA and PS, with higher affinity for PA. WT-SK1 failed to bind to phosphatidylcholine (PC), phosphatidylglycerol (PG), or phosphatidylinositol (PI). Intriguingly, there was also weak binding to phosphatidylethanolamine (PE), which has a very similar head group to PS but with a net neutral charge (Supp. Figure 2). Next, WT-SK1 and mutant constructs were compared for their ability to bind to PA, PC, PS, or PE (Figure 2B). L194Q-SK1 had reduced ability to bind PA whereas the Triple-SK1 mutant was severely deficient in PA binding. Spot intensity for the protein-lipid overlays were quantified using ImageJ software (Figure 2C). SK1 could discriminate between different anionic phospholipids (i.e. bound to PA and PS but not PC,
PG, or PI). Furthermore, the electrostatic site was responsible for binding to PA as mutation of the electrostatic site, but not mutation of the hydrophobic site, severely reduced binding to PA.

To evaluate SK1 binding in solution, we employed liposome sedimentation assays using multilamellar liposomes composed of PC/PE/cholesterol and either PA or PS (Fig. 2D). To characterize these liposomes, we evaluated size distribution as well as pelleting efficiency (shown in Supp. Fig. 1). WT-SK1 bound strongly to liposomes containing PA (~90% bound, Fig. 2E), and similar to the lipid overlay results, there was weaker binding to PS containing liposomes (~50% bound, Fig. 2E). Very little SK1 bound to the control liposomes containing PC/PE/cholesterol (Figs. 2D and 2E). When either L194Q-SK1 or Triple-SK1 was analyzed for membrane binding, both were found to be almost exclusively in the supernatant (<10% bound, Figs. 2D and 2E). These results indicated that both sites are critical for binding to membranes containing certain anionic phospholipids and that disruption of either can be detrimental to SK1 membrane binding.

**Mapping of SK1/Liposome Interface with Hydrogen/Deuterium Exchange Mass Spectrometry.** To characterize the interactions of SK1 with membrane vesicles, we employed hydrogen deuterium exchange mass spectrometry (HDX-MS) (Fig. 3). This is a powerful analytical technique that measures the exchange rate of amide hydrogens with solvent. Protection from amide exchange is mediated by involvement in secondary structure, and measuring exchange rates allows determination of protein conformational dynamics. This technique has been deployed to probe the interaction of lipid modifying enzymes with membranes and membrane proteins [28-32]. SK1 was incubated in the presence and absence of membrane vesicles composed of 40% PC, 35% PE, 20% PA, and 5% cholesterol. HDX-MS experiments were carried out for three timepoints (3, 30, and 300 s) for both conditions. All peptides identified and analyzed for all experiments, along with their deuteration states, are listed in Supplemental Figure 3.
Large decreases in H/D exchange were apparent in a single contiguous surface of both the NTD and CTD of SK1 (Fig. 3A). SK1, in the absence of any membranes, showed no protection in the α7 and α8 helices indicating they contain either no or very transient secondary structure. However, the largest decreases in exchange upon membrane binding, by far, were apparent in the same region stretching from amino acids 167-197 (encompassing the α7 and α8 helices, Fig. 3B). This is indicative of a disorder-order transition upon membrane binding. Large conformational changes upon lipid binding had been discovered in this region using X-ray crystallography [26], and is consistent with the proposed model of α7 and α8 acting as a lid over the active site [33]. Importantly, this region contains Arg186 of the electrostatic patch and Leu194 of hydrophobic patch. There were also large decreases in exchange in the α9 helix (peptides 289-299, 303-313, Fig. 3B) upon membrane binding, consistent with the previous annotation of this region as participating in lipid binding. There were also smaller, but significant, decreases in exchange in the NTD, with decreases at the β1 α1 elements (peptide 18-33, Fig. 3B), as well as at α5 (peptide 117-125, Fig. 3B). The β1 α1 region (containing Lys27 and Lys29 of the electrostatic motif, Fig. 3A) is located on the putative membrane binding face of the NTD, and is located directly opposite the α7 α8 region. Furthermore, there was a decrease in exchange at the C-terminal section of the α5 helix. Additionally, a decrease in exchange at the C-terminus and β17 (peptide 360-385, with the majority of the difference localized in the region from 360-365, Fig. 3B and Supp. Fig. 3) region was also observed. Structurally, the C-terminus lies directly over the α5 helix and may contribute in the regulation of SK as previously noted [33]. Membrane binding induced changes in deuterium incorporation into SK1 peptides including peptides spanning the electrostatic and hydrophobic sites. These two sites align to form a single contiguous membrane binding interface.
Loss of membrane binding in cells. In order to test these mutants in cells, it became important to express them in the absence of endogenous WT-SK1. Therefore, we generated HCT116 CRISPR SK1 cells (HCT116 crSK1) using CRISPR/Cas9 technology. These cells were probed by western blot revealing that there was no detectable SK1 protein compared to the vector control cells (Fig 4A). Furthermore, we used a genomic cleavage assay where DNA is cleaved at insertion/deletion (indel) regions (using the GeneArt Genomic modification detection assay, LifeTechnologies). In the HCT116crSK1 cells, smaller DNA fragments were observed indicating that genomic modification, specifically indels, has occurred in these cells and not in the vector control cells (Fig 4B). Finally, we functionally confirmed that SK1 was removed by measuring endogenous sphingolipids of the HCT116crSK1 cells compared to the vector control cells. Both Sph and dhSph levels were elevated in the crSK1 cells compared to the vector control, and S1P and dhS1P levels were decreased (Figs 4C and D).

Using the HCT116crSK1 cells, SK1 association with membranes was evaluated. We overexpressed either WT, L194Q, Triple, or a combination of both the Triple and L194Q mutants (T+L) constructs and fractionated the cells. When probed for SK1 using the monoclonal antibody (Cell Signaling) which recognizes all the mutants, the results showed that there was less SK1 associated at the membrane in the L194Q- and Triple-SK1 transfected cells compared to the WT in serum starved conditions (Fig. 5A). The most striking loss of membrane association occurred when the two mutated sites were in the same construct, the T+L-SK1 mutation, resulting in more than a 67% decrease in the ratio of membrane-bound SK1 to cytosolic SK1 (Fig. 5A).

Next, it became important to determine the effects of membrane binding on SK1 activity in cells. SK activity in cells can be monitored by mass spectrometry via the conversion of 17-carbon sphingosine (17C-Sph) to 17C-S1P [34]. When WT-SK1 was over-expressed, there was a
significant increase in C17-S1P as compared to HCT116crSK1 cells transfected with empty vector (EV) (Fig. 5B). However, in the case of the over-expression of membrane binding mutants, the increase observed in the WT was diminished. When the two mutations were combined, this loss of C17-S1P generation was decreased further (Fig. 5B). The residual C17-S1P activity is likely due to the presence of SK2 in these cells. The overexpression of these mutants did not affect uptake of C17-Sph as shown by the cellular levels of C17-Sph (Fig. 5B). These data indicate that SK1 activity requires membrane binding, mediated by the two identified sites.

SK1 localization at the plasma membrane in HEK293 cells was also evaluated using confocal microscopy in HEK293 cells expressing GFP-tagged SK1 constructs (Fig. 5C-K). In cells expressing WT-SK1, SK1 was localized at the plasma membrane after stimulation with media containing 10% FBS. However, under the same conditions, the L194Q- and Triple-SK1 mutations were unable to associate with the plasma membrane with serum stimulation. Therefore, mutation of either of the two sites can disrupt SK1 association with membranes.

**Requirement of SK1 membrane binding for SK1 signaling and biology.** To determine if mutation of SK1’s membrane binding interface influenced SK1 signaling processes we used methyl-β cyclodextrin (MβCD) to sequester cholesterol and induce endocytosis. Shen et al. showed that WT-SK1 could co-localize with the N-bar protein endophilin-2 in response to membrane perturbations with either MβCD or sphingomyelinase treatment [23]. In agreement with previous data, GFP-tagged WT-SK1 co-localized with Ruby-tagged endophilin-2 at membrane invaginations upon treatment with MβCD (Fig. 6A). Importantly, mutation of either the electrostatic site or the hydrophobic site was sufficient to disrupt this co-localization of SK1 and endophilin-2 (Fig. 6B and Fig. 6C). Combination of the two sites did not enhance the observed
loss of co-localization (Fig. 6D) indicating that both sites are necessary for membrane binding in a biological setting.

To assess whether membrane binding effected SK1-dependent cell biology, we employed two different assays. We had previously shown that the phosphorylation of the Ezrin-Radixin-Moesin (ERM) family of proteins upon treatment with exogenous Sph was dependent on SK1 [35]. Indeed, using the CRISPR-mediated deletion of SK1, we now show that there was no significant ERM protein phosphorylation after treatment with exogenous Sph in the HCT116crSK1 cells transfected with empty vector (EV) (Fig 6E). On the other hand, the response to S1P, which bypasses the requirement for SK1, was maintained. When WT-SK1 was over-expressed, it rescued the phenotype showing that exogenous sphingosine addition induced phosphorylation of ERM proteins. Disruption of either the electrostatic or hydrophobic sites individually did not result in a dramatic loss of ERM phosphorylation (Fig. 6E). Interestingly, when the two mutants were combined (both sites mutated in the same construct), there was almost complete loss of ERM phosphorylation (Fig. 6A). These data suggest that disruption of the membrane interface can disrupt S1P-induced ERM phosphorylation.

Since ERM proteins are involved in cell motility, migration, and invasion, we evaluated invasion of the HCT116 crSK1 cells using a matrigel-coated transwell assay. The results showed that these HCT116crSK1 cells invaded through the matrigel more efficiently when WT-SK1 was over-expressed, compared to EV. Strikingly, overexpressing either the electrostatic mutant, hydrophobic mutant, or the two mutants combined, invasion was completely reduced to EV levels (Fig. 6F). This suggests that SK1’s membrane binding is important for enhancing invasion and that disruption of membrane binding can have negative consequences for the functions of SK1.
Discussion

For more than two decades, SK1, and its pleiotropic lipid product S1P, have been studied and implicated in cancer [1], inflammation [3], and development [36] yet, only recently have strides been made in understanding SK1 structure, function, and regulation. Even so, the mechanisms underlying SK1 interactions with membranes, where it can access Sph and produce S1P, have remained unclear.

Here we identify a new electrostatic motif of SK1 responsible for binding to certain anionic phospholipids. Furthermore, this electrostatic site works in conjunction with a previously identified hydrophobic patch to bind to membranes. To map the membrane binding interface, we used HDX-MS to show that these two sites comprise a single contiguous interface. Cellular studies demonstrate that mutation of the two sites causes decreased membrane association and SK1 activity. Importantly, disrupting this interface results in loss of function for SK1-dependent cell invasion and SK1’s role in endocytosis.

Our data suggest that SK1 uses an intrinsic single contiguous interaction interface (Fig. 7C) to associate with membranes. The contiguous site consists of two adjacent regions on the surface of SK1, an electrostatic region (colored blue in Figs. 7A, 7B, and 7C) and a hydrophobic region (colored magenta in Figs. 7A, 7B, and 7C). Our data show that the newly identified electrostatic site is equally responsible for membrane association as the hydrophobic site, which indicates that SK1 requires both sites to be intact for optimal membrane binding. We speculate that this interaction might result in the partial insertion of the helices into the membrane (Fig. 7D). We thought about a different mechanism, where SK1 might partially extract lipids from the membrane but this seems to be the more unlikely scenario. Interestingly, membrane interaction along this contiguous surface leaves the 40-residue loop insertion in the catalytic domain which
contains Ser225 (Fig. 7D, red residue) exposed to the cytosol. Ser225 is known to be phosphorylated [19] and dephosphorylated [37, 38] making its exposure important for access by kinases and/or phosphatases. It is not clear how SK1 might exchange ATP when bound to the membrane as membrane binding would mostly occlude the ATP binding site. Molecular dynamics simulations of SK1 and membranes would be beneficial to further our understanding of the effect of membrane binding on the catalytic cycle for SK1. Disruption of either of these two sites or the combined disruption of both sites was sufficient to result in loss of in vitro binding and cellular function when looking at SK1-mediated signaling processes.

SK1 has been shown to be activated in the presence of certain anionic phospholipids such as PS and PA [20] and has been further defined as an effector of PA [22]. The electrostatic site identified in this work provides a means by which SK1 can discriminate between negatively charged lipids, such as PA, from neutral lipids including PC and PE. Furthermore, residues Thr54 and Asn89 which have been previously implicated in PS binding [21] showed no significant changes upon membrane binding in the HDX-MS experiments. This indicated that these residues do not play a direct role in the interaction with membranes, but it is possible that they play an indirect role. Furthermore, SK1 would potentially be able to extract Sph through membrane interaction with the helices (α7, α8, α9) previously identified as important for membrane/lipid binding via assessing crystallographic data [26]. Helices α7 and α8 have been shown to change position (via comparison of crystal structures) after lipid binding to the Sph binding pocket [26]. Helices α9 and α10 (peptides 288-299 and 303-319) also showed protection from deuterium incorporation upon membrane binding. This result suggests a stabilization in their secondary structure after lipid binding or that there is a potential role for membrane interaction for substrate
extraction, however, this remains to be seen. We speculate that interaction with cell membranes could induce the movement of these helices to promote binding of substrate and release of product.

An interesting component of the mechanism of many proteins which can bind membranes is their ability to sense curvature. It has been suggested that SK1 can sense the negative curvature of membranes [23]. A mechanism has also been proposed in which SK1 can dimerize through interactions of the NTD of two protomers making a head-to-head homodimer complex [33]. A recent study used computational analysis to better understand how SK1 might dimerize. Bayraktar and colleagues [39] suggest a putative dimerization interface which somewhat overlaps with the proposed mechanism of Adams et al. [33]. Interestingly, a dimerization through the NTDs of two SK1 protomers would allow for the alignment of the interface identified in this work. This dimerization would have two implications, first, dimerization could allow for curvature sensing of SK1 and, second, dimerization would allow for the strengthening of the interaction between SK1 and membranes. However, this putative dimer interface has yet to be confirmed biochemically or biophysically and requires further investigation.

Membrane localization of SK1 has also been attributed to interaction with CIB1 [24, 25], which acts as a calcium-myristoyl switch. Interestingly, this CIB1 interaction site F197/L198 [24] overlaps with the hydrophobic patch that has been shown to be important for direct membrane binding in a previous study [23] and this work. As suggested by Jarman et al. [24], it is possible that CIB1 acts as a “molecular shepherd” to bring SK1 close enough to the membrane but does not influence SK1’s intrinsic mechanism for binding to membranes. However, it is also likely that CIB1 can “override” the intrinsic mechanism of SK1 membrane binding and force it to the membrane as a function of the myristoyl-switch. It is also possible that interaction with CIB1 is dependent on SK1 membrane interaction and mutation of the hydrophobic patch disrupts SK1 membrane
binding and subsequently CIB1 interaction. Future work should be aimed at fully understanding the relationship between CIB1 and SK1 in SK1s translocation process.

Activity of membrane-associated SK1 has been documented in several studies [17, 40, 41]. The C-terminal tail of SK1 has been previously shown to be important in regulating the activity of SK1 [42]. It has since been postulated that the C-terminal tail of SK1 (specifically, residues 364-367) can act as a cap for the \( \alpha_5 \) helix which extends into the catalytic site of SK1 [33]. Our HDX-MS data show protection from exchange in the C-terminal tail of SK1 (mostly in residues 360-365) as well as the \( \alpha_5 \) helix upon membrane binding. It is possible that changes in the conformation of the C-terminal tail upon membrane binding could affect the capping/positioning of the catalytic residue, Asp81, indirectly through repositioning of the \( \alpha_5 \) helix ultimately regulating SK1; however, this will require further study.

The role of SK1/S1P in cell biology and in disease has been well documented and are reviewed in [1, 3, 43-45]. We show that SK1’s innate ability to bind to membranes is critical for the proper function of SK1 in invasion and its role in endocytosis. Our results also indicate that SK1 prefers to bind to PA in accordance with previous literature showing SK1 as an effector of PA in humans [22] and in Arabidopsis thaliana [46], and possibly suggesting conservation of SK1-PA binding. PA can be generated by the action of two different families of enzymes, phospholipase Ds (PLDs) or by diacylglycerol kinases (DGKs). A number of stimuli that activate PLD, such as platelet-derived growth factor [47], epidermal growth factor [48] and phorbol esters [49], are also known to activate and/or induce translocation of SK1 [17, 50, 51]. Furthermore, PLD had been implicated in both clathrin-mediated and clathrin-independent endocytosis (reviewed in [52]). Therefore, it will be interesting to fully comprehend the dynamic crosstalk between PA generation by PLD and/or diacylglycerol kinase and SK1 membrane association.
Overall, these data provide the first evidence of a complete interaction interface responsible for SK1 binding to membranes. It is also shown that membrane binding is critical for SK1-mediated biologies including cell invasion and SK1’s role in endocytosis. This work also opens new opportunities for studying allosteric mechanisms which might regulate SK1 function. Targeting this newly identified PA/PS binding motif might provide interesting avenues for inhibition of SK1 activity as a therapeutic option in cancer and/or inflammatory diseases.
Materials and Methods

**Materials.** Lipids including 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphate (DOPA), 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS), 1,2-dioleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DOPG), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-sn-glycero-3-phospho-(1′-myo-inositol) (DOPI), Sphingosine, and Sphingosine-1-phosphate were purchased from Avanti Polar lipids (Alabaster, AL, USA). Monoclonal SK1, Na/K ATPase, phospho-ERM, and t-Ezrin antibodies were purchased from Cell Signaling Technologies (Danvers, MA, USA). Tubulin antibody was purchased from Sigma-Aldrich (St. Louis, MO, USA).

**In silico analysis of Sphingosine Kinase 1.** Hydrophobicity and electrostatic potential maps were plotted on a surface representation (PDB ID: 3VZB) using Chimera Software [27]. The molecule was prepared by adding hydrogen atoms and assigning atomic charge and radii through the PDB2PQR tool [53, 54] in Chimera which uses parameters optimized for Poisson-Boltzmann calculations. Electrostatic and hydrophobic potentials were calculated with the Adaptive Poisson-Boltzman Solver (APBS) [55] through the web service provided by the National Biomedical Computational Resource and the Kyte-Doolittle scale of hydrophobicity [56], respectively. Chimera software were used to generate cartoon representations of SK1.

**Protein expression and purification.** Insect cells (2-3x10^6 cells/mL) were infected with bacculovirus expressing His_6-tagged SK1 constructs made using the Bac-to-Bac protocol (Invitrogen). After 72 hrs, cells were harvested and resuspended in lysis buffer (50 mM Tris pH 8.5, 5 mM β-mercaptoethanol, 100 mM NaCl, 5mM phenylmethyl sulfonyl fluoride, 1% Tween20, protease inhibitor cocktail tablet (Roche)). Cells were sonicated and spun down at 22,000 RPM for 1 hr at 4°C. Supernatant was run over an Ni^{2+}-NTA column. The column was washed with 10
column volumes (CV) of Buffer A (20mM Tris pH 8.5, 5mM beta-mercaptoethanol, 250mM NaCl, 40mM Imidazole pH 8, 0.1% Tween 20, 10% glycerol), washed with 2 CV Buffer B (20mM Tris pH 8.5, 5mM β-mercaptoethanol, 1M NaCl, 0.1% Tween 20, 10% glycerol), and eluted in 5 CV of Buffer C (20mM Tris pH 8.5, 5mM β-mercaptoethanol, 100mM NaCl, 500mM Imidazole pH 8, 0.1% Tween 20, 10% glycerol). Fractions with SK1 protein were pooled and run over a size exclusion column in SEC Buffer (20mM Tris pH 8.5, 5mM beta-mercaptoethanol, 100mM sodium chloride, 0.1% Tween 20, 10% glycerol). Protein was concentrated to >1mg/mL using a spin column with a 10,000 Da molecular weight cutoff. Protein purified for hydrogen-deuterium exchange experiments was purified as described above but in Tris buffer pH 7.0.

**Lipid Protein Overlay.** Five µg of lipid (in chloroform) was spotted onto nitrocellulose membrane and blocked for 1 hr in 3% fatty acid free BSA (Akron Biotech, Boca Raton, FL, USA) in phosphate buffered saline with Tween20 (PBST). WT or mutant protein (at a concentration of 1 µg/mL) in PBST was incubated overnight with shaking at 4°C. Primary and secondary antibodies (in 3% fatty acid free BSA in PBST) were incubated for 1 hr each with 1 twenty-minute wash in PBST in between each incubation and after the last incubation. For specificity overlays, different amounts of lipids were spotted onto nitrocellulose membranes and the same protocol was followed as above. Membranes were developed using standard chemiluminescence-based western blotting.

**Sphingosine kinase activity assay.** Activity was measured by the production of a fluorescent sphingosine-1-phosphate as previously described [57, 58].

**Liposome generation and sedimentation assays.** Liposomes were made by 3 rounds of freeze-thaw (−80° to room temperature) with agitation. This was followed by sonication in a water bath until a homogenous solution was achieved (~5 minutes) which generates small unilamellar liposomes [59]. The mean sizes of these liposomes were estimated by nanoparticle tracking
analysis with a ZetaView (Particle Metrix, Germany) with a diameter of ~70 nm. The pelleting efficiency of these liposomes was calculated by measuring fluorescence of NBD-PE labelled liposomes before and after centrifugation and was found to be 87% ± 6%. Liposomes were composed of 40% DOPC, 35% DOPE, 20% DOPA or DOPS and 5% cholesterol at a final concentration of 1 mM in buffer containing 100 mM NaCl, 50 mM HEPES pH 7.5, and 150 mM sucrose. 40 µL of liposomes were mixed with 50 µL of 0.1 ug/uL protein (in 50 mM HEPES pH 7.5 and 100 mM NaCl) and incubated at room temperature for 10 minutes. Liposomes were sedimented at 100,000 x g for 1 hr and supernatants were removed, pellets were solubilized in buffer and samples were analyzed by SDS-PAGE. Proteins were visualized by Coomassie Brilliant Blue staining. Image analysis was performed with Image J.

**Generation of HCT116crSK1 cells using Crispr/Cas9 technology.** To knockout SK1 from HC116 cells, we used the Lenticrispr v2.0 system [60, 61]. The LentiCRISPR v2 plasmid was a gift from Feng Zhang (Addgene plasmid # 52961). Briefly, guide RNAs targeting the SK1 gene were designed using the ChopChop algorithm (http://chopchop.cbu.uib.no/) and cloned into the plasmid as described previously [60, 61]. To generate lentivirus, plasmids were co-transfected with VSV-G and dVPR into 293T cells and virus-containing media was harvested and filtered (0.22uM PVDF membrane) after 72h. HCT116 cells (100K) were infected with 1ml virus in the presence of polybrene (8ug/ml). After 48h, cells were selected in puromycin (2ug/ml) for 7 days. Subsequently, cells were maintained in normal growth medium. Validation of genetic modification was performed with the GeneArt Genomic Cleavage Detection kit (Thermoscientific) according to manufacturer instructions.

**Cell Lines and Transfection.** HCT116 Vec and HCT116crSK1 cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies) containing 10% fetal bovine
serum and HeLa cells were maintained in RPMI 1640 (Life Technologies) containing 10% fetal bovine serum. Medium without serum was used for serum starvation. Cells were checked for mycoplasma contamination every two months using the Mycoplasma Detection Kit (Lonza, Basel, Switzerland) as per the manufacturer’s instructions. Transfections were carried out using Xtreme Gene 9 (Roche, Basel, Switzerland) (3uL of reagent per transfection) and 500 ng of each DNA construct. Transfections were allowed to incubate for 24 hours before changing media.

**17 Carbon Sphingosine (C17-Sph) Labeling.** Cells were transfected for 24 hrs with SK1 constructs using XtremeGene (Roche). At 24 hrs media was changed followed by 15 min incubation with C17-Sph (0.5 µM final concentration). Briefly, cells were harvested by scraping directly into ice-cold PBS. Cells were subsequently pelleted and the PBS was removed. Cells were resuspended in 2 mL of cell extraction buffer (70% isopropanol:Ethyl Acetate; 2:3) and vortexed. Extraction and analysis were performed as previously described [34].

**Membrane fractionation.** HCT116crSK1 cells transfected with SK1 constructs were scraped in 500 µL fractionation buffer (250mM sucrose, 20mM HEPES pH 7.4, 10mM potassium chloride, 1.5mM magnesium chloride, and 1mM EDTA, protease inhibitor cocktail (Sigma)) at 75-80% confluency. Cells were lysed by 20 passes through an 27G needle. Lysates underwent differential centrifugations: 3,000 RPM for 5 minutes to remove nuclei, followed by centrifugation at 10,000 RPM for 10 minutes to remove mitochondria, followed by a final spin at 47,000 RPM (100,000 x g) for 1 hr at 4°C to separate cytosol from membranes. The pellet present after the final was the membrane fraction. The pellet was resuspended in 1/10th the starting volume and solubilized by sonication. Protein concentration was determined by BCA (Pierce) and proteins were separated by SDS-PAGE and analyzed by western blot.
Membrane localization. HEK293 cells were transfected with GFP-tagged SK1 constructs for 24 hrs using XtremeGene (Invitrogen). After 24 hrs cells were serum starved for 4-6h and stimulated with serum for 30 minutes. After serum stimulation cells were washed with PBS and fixed in 3.7% paraformaldehyde for 10 minutes. After fixation cells were washed for 5 min in PBS 3 times followed by addition of mounting media containing the nuclear stain DAPI. Imaging was performed on a Leica TCS SP8 scanning-laser confocal.

Cell invasion Assays. Twenty-four-well Matrigel coated transwell invasion plates (Corning Life Sciences, Corning, NY) were used to assess the invasive capacity of HCT116crSK1 cells. Cells were transfected with pcDNA3 empty vector or pcDNA3 containing SK1 constructs for 24 hrs. Cell were then serum starved for 4 hrs prior to seeding in 24-well transwell plates. 500 µL of full serum (10%) media was placed in the bottom chamber as a chemoattractant while 75,000 cells per well were seeded into the top chamber in serum-free media. Plates were incubated for 48 hrs at 37°C and 5% CO₂. At 48 hrs cells that had invaded to the bottom of the transwell were stained with CalceinAM (Invitrogen) and fluorescence was read in a SpectraMax plate reader.

Methyl β Cyclodextrin (MβCD) treatment. HeLa cells were co-transfected with GFP-tagged SK1 constructs and with Endophilin A2-Ruby construct (a generous gift from Dr. Pietro De Camilli, Yale University). 24 hrs after transfection cells were serum starved overnight followed by treatment with 1X MβCD (a 5X starting stock of 66 mg/mL MβCD in imaging buffer [10mM HEPES pH 7.5, 120mM sodium chloride, 2mM calcium chloride, 2mM magnesium chloride, and 3mM potassium chloride]). After a two-minute treatment, cells were immediately washed with PBS and fixed in 3.7% paraformaldehyde for 10 minutes. Mounting media was added and cells were imaged. Imaging was performed on a Leica TCS SP8 scanning-laser confocal.
**Hydrogen/Deuterium Exchange Mass Spectrometry.** HDX reactions were prepared with 20pmol Sphingosine Kinase 1 in either a lipid buffer (20mM TRIS pH 7.0, 100mM NaCl, 10% glycerol, 0.1% Tween20, 5mM BME) or a liposome containing buffer (40% DOPC, 35% DOPE, 20% DOPA, 5% Cholesterol) at a final concentration of 0.69mg/ml. Liposomes for exchange experiments were made via extrusion through 0.1µm polycarbonate filter after 3 freeze-thaw cycles. Exchange was initiated by the addition of 39.8µL of D₂O Buffer Solution (10 mM HEPES pH 7.5, 50 mM NaCl, 97% D₂O), to give a final concentration of 77% D₂O, following the incubation of protein with either the lipid containing buffer or the liposome containing buffer. Exchange was carried out for 3s, 30s, and 300s, and exchange was terminated by the addition of a quench buffer (final concentration 0.6 M guanidine-HCl, 0.8% formic acid). Samples were rapidly frozen in liquid nitrogen and stored at −80°C until mass analysis.

Protein samples were rapidly thawed and injected onto a UPLC system at 2°C according to previously published protocols [32]. The protein was run over two immobilized pepsin columns (Applied Biosystems; porosyme, 2-3131-00) at 10°C and 2°C at 200 µL/min for 3 minutes, and peptides were collected onto a VanGuard precolumn trap (Waters). The trap was subsequently eluted in line with an Acquity 1.7 µm particle, 100 × 1 mm2 C18 UPLC column (Waters), using a gradient of 5-36% B (buffer A 0.1% formic acid, buffer B 100% acetonitrile) over 16 minutes. Mass spectrometry experiments were performed on an Impact II TOF (Bruker) acquiring over a mass range from 150 to 2200 m/z using an electrospray ionization source operated at a temperature of 200°C and a spray voltage of 4.5 kV. Peptides were identified using data-dependent acquisition methods following tandem MS/MS experiments (0.5 s precursor scan from 150-2200 m/z; twelve 0.25 s fragment scans from 150-2200 m/z). MS/MS datasets were analyzed using PEAKS7.
(PEAKS), and a false discovery rate was set at 1% using a database of purified proteins and known contaminants.

HD-Examiner Software (Sierra Analytics) was used to automatically calculate the level of deuterium incorporation into each peptide. All peptides were manually inspected for correct charge state and presence of overlapping peptides. Deuteration levels were calculated using the centroid of the experimental isotope clusters. Changes in deuterium incorporation were considered significant for all changes between conditions >0.5 Da and 5% deuteration incorporation with p<0.05 between triplicate samples.

**Statistics.** One-way or two-way analysis of variance (ANOVA) with Bonferroni post-hoc tests or Student’s t-test were used to assess statistical significance using GraphPad Prism 4 (La Jolla, CA, USA) as appropriate. Statistical significance was defined as P ≤ 0.05.
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Author Contributions

MJPG, JEB, YAH and LMO designed research. MJPG, MLJ, JPT, MFS, and CJC, performed experiments. MJPG, MLJ, JEB, YAH, and LMO analyzed data. MJPG, MLJ, JEB, LMO wrote the first draft of the manuscript. All authors edited and revised the manuscript.
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Figures

A

B

90°

C

D

90°

E

F

90°
**Figure 1: In silico surface binding analysis of SK1.** A) Electrostatic potential maps were generated using Chimera software using the Adaptive Poisson-Boltzman Solver. The scale represents kcal·mol\(^{-1}\) where blue represents positively charged areas and red represents negatively charged areas. B) Kyte-Doolittle scale of hydrophobicity was used to predict hydrophobicity and was mapped to the surface of SK1 where cyan represents hydrophilic areas and magenta represents hydrophobic areas. C) Cartoon representation of SK1 with residues of the hydrophobic patch and electrostatic patch represented as magenta and blue sticks, respectively. PDB ID 3VZB [26] used for all analyses.
Figure 2: *In vitro* binding analysis of SK1 wild type and SK1 mutants. A) SDS-PAGE analysis of purified SK1 proteins. B) Protein-lipid overlay comparing the ability of WT vs. mutant to bind to phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylserine (PS), or phosphatidylethanolamine (PE). C) Quantification of B) using densitometry (ImageJ) n=3 ± S.D. **p<0.01 as per one-way ANOVA. D) Liposome sedimentation assay with liposomes containing PC, PE, cholesterol (Chol), and either PA or PS, representative image of n=3. E) Quantification of D) using Image J. Data represent mean of n=3 ± S.D. **p<0.01; ***p<0.001 as per a two-way ANOVA.
Figure 3: HDX-MS characterizes changes induced by SK1 membrane binding. A) Peptides which showed deuterium exchange differences greater than 5% and 0.5 Da between apo state and membrane bound states of SK1 are mapped according the legend on the crystal structure of SK1 [PDB ID: 3VZB] [26]. Residues mutated in this study are shown as sticks and labeled. Secondary structure annotations are labeled on the structure. B) Time course of deuterium incorporation for a selection of peptides which showed significant differences in percent deuteration upon the addition of membrane (error shown as S.D.; n=3). The full set of deuterium incorporation data is shown in supplemental figure 3.
Figure 4: Validation of HCT116 SK1 CRISPR cells. A) Immunoblot analysis of SK1 protein levels in vector control and CRISPER SK1 (crSK1) cells. B) GeneArt genomic cleavage detection assay (LifeTechnologies) used to detect small insertions and deletions (INDELS) in the genome of the cells with CRISPR for SK1. Black arrow indicates the PCR product and the red arrowheads denote cleavage products. C) Decreased Sphingosine 1-phosphate (Sph-1P) and dihydro Sph-1P (dhSph-1P) measurements in the CRISPR cells. D) Sphingosine (Sph) and dihydroSph (dhSph) measurements of CRISPR and control cells increase in both species of lipids. Lipid measurements are n=3 ± S.D. *p<0.05; **p<0.01; ***p<0.001 as per paired Student’s t-test.
Figure 5: Effects of SK1 membrane binding mutants in cells. A) Biochemical cell fractionation of HCT116 crSK1 cells overexpressing SK1 constructs. Cytosol was separated from membranes using ultracentrifugation. Quantification of the membrane to cytosolic ratio using Image J. Data represent mean ± S.D. for 3 independent replicates. *p<0.05 as per one-way ANOVA. B) C17-Sph labeling of HCT116 crSK1 cells expressing empty vector (EV), WT,
L194Q, Triple, or T+L mutants. Data represent the mean of 3 independent experiments ± S.D.

*p<0.05; **p<0.01. C-K) HEK293 cells expressing GFP-tagged SK1 (WT, L194Q, or Triple) stimulated with 10% fetal bovine serum. The nuclei of the cells were stained with DAPI. Representative images of three independent experiments.
Figure 6: Biological consequences of loss of membrane binding for SK1. A-D)

Representative images of HeLa cells co-expressing GFP-SK1 (green) constructs and Endophilin2-Ruby (red). Colocalization was assessed after 2 minutes of treatment with methyl-β-cyclodextrin. All experiments are n=3. E) Effect of SK1 mutation on ERM phosphorylation upon addition of exogenous Sph (2.0 µM) or S1P (200 nM). F) Effect of SK1 mutation of the ability of HCT116 crSK1 cells to invade through matrigel. Cells were transfected with either empty vector (EV), or
SK1 constructs and allowed to invade through Matrigel coated membranes (8.0 µm pore size).

*p<0.05; **p<0.01; as per one-way ANOVA; n=3; error bars represent SD.
Figure 7: Model of SK1 binding to Membranes. A) Cartoon representations of SK1 showing the residues, as sticks, from the electrostatic patch (colored blue) and the hydrophobic patch (colored magenta). B) Surface representation of SK1 in the same orientation as A). The
surface is colored according to A). **C)** Surface representation which is rotated approximately 90° relative to B) and shows that these sites line up to form a single contiguous surface (dashed lines). **D)** Possible positioning of SK1 at a membrane interface to engage the single contiguous interaction interface (partially embedded in membrane) leaving Ser225 (red residue) accessible to cytosolic kinases/phosphatases.