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ABSTRACT

Sphingosine kinase 1 (SphK1) is an indispensable signaling molecule involved in cellular proliferative and anti-apoptotic signaling. There is a large body of evidence that SphK1 levels are elevated in hyperproliferative diseases, such as cancer and atherosclerosis, making SphK1 a novel therapeutic target for limiting aberrant cellular proliferation. Development of pharmacological inhibitors of SphK1 has been limited by the lack of understanding of the mechanisms of enzymatic activation of SphK1, which has been shown to occur in a biphasic manner. Early phase activation takes place in a matter of minutes. It is believed that following stimulation, basally active cytosolic SphK1 translocates to the plasma membrane, where it is further activated by lipid and/or protein cofactors, resulting in a transiently inducible SphK1 activation. Late phase activation occurs over the course of hours through transcriptional and translational upregulation of SphK1 expression. The goal of this study is to investigate mechanisms of early and late phase activation of SphK1.

We demonstrate that growth factor stimulation results in a transient accumulation of highly active SphK1 in a distinct plasma membrane microdomain, the membrane raft. We show that a pool of SphK1 as well as its substrate, sphingosine, reside in membrane rafts by using differential centrifugation and sucrose density gradient centrifugation techniques. This population of SphK1 exhibited a high specific catalytic activity and was depleted from membrane rafts in cells that had been treated with cholesterol binding agents that disrupt membrane raft integrity. We further show that following FBS stimulation, SphK1 protein levels at the membrane raft are increased. This increase coincided with a decrease in membrane raft sphingosine levels and an increase in sphingosine-1-phosphate (S-1-P) in the intracellular and extracellular space. We observed a colocalization of SphK1 with the membrane raft marker GM-1 by confocal microscopy, particularly at the leading edge of HEK293 cells. Together, these data provide strong evidence that membrane rafts are sites for temporally and spatially controlled induction of SphK1 activity, which has implications in compartmentalization
of S-1-P signaling at particular cellular locales, such as the leading edge of a migrating cell.

We demonstrate SphK1 is necessary for proliferation of human coronary artery smooth muscle (HCASM) cells. Our results demonstrate that PDGF stimulation results in increased SphK1 mRNA levels within 3 h, followed by an increase in SphK1 protein synthesis and enzymatic activity that is observed 8 - 24 h later. Using a panel of pharmacological inhibitors, we identified the PI3K/AKT/mTOR pathway as a key regulator of late phase SphK1 activation involving transcriptional/translational upregulation. Utilizing isoform-specific siRNA for each isoform of AKT (AKT1, AKT2, AKT3), we identified that AKT2 was necessary for an increase in SphK1 mRNA, protein, and activity levels following PDGF stimulation. In contrast, depleting AKT1 or AKT3 levels did not have an effect on abrogating the response to PDGF. These data imply that inhibition of AKT2 could limit the level of SphK1 protein produced, thus controlling cell growth rates.

Several lines of evidence suggest that SphK1 activation is downstream of protein kinase C (PKC) signaling. It has been reported that SphK1 is phosphorylated in vivo by ERK1/2 and also an unidentified kinase [1]. We show evidence that the additional kinase that phosphorylates SphK1 is PKC. We show that PMA, a direct stimulator of PKC, activates SphK1. It has previously been demonstrated that specific inhibitors of PKC (bisindolylmaleimide, calphostin C, gö 6976) inhibit SphK1 activation. We show that the PKCα isoform phosphorylates SphK1 in vitro as well as increases SphK1 activity in a dose-dependent manner. Using site-directed mutagenesis, we identified Ser371 as the most likely site of phosphorylation of SphK1 by PKC. Additional studies, including phosphoprotein analysis and kinetic analysis further suggest that Ser371 may be phosphorylated. Collectively, our data provide mechanisms that can be exploited in the development of therapeutics for hyperproliferative disease that are able to target both the acute and the transcriptional and translational phases of SphK1 activation.
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<table>
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<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>Alanine</td>
</tr>
<tr>
<td>AKT</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>Asp</td>
<td>Aspartic acid</td>
</tr>
<tr>
<td>DIM</td>
<td>Detergent insoluble membrane</td>
</tr>
<tr>
<td>DRM</td>
<td>Detergent resistant membrane</td>
</tr>
<tr>
<td>DMS</td>
<td>Dimethylsphingosine</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulphoxide</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EDG</td>
<td>Endothelial differentiation gene</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular-signal regulated kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>GEM</td>
<td>Glycolipid-enriched complex</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>Glu</td>
<td>Glutamic acid</td>
</tr>
<tr>
<td>Gly</td>
<td>Glycine</td>
</tr>
<tr>
<td>HCASM</td>
<td>Human coronary artery smooth muscle</td>
</tr>
<tr>
<td>HEK293</td>
<td>Human embryonic kidney 293</td>
</tr>
<tr>
<td>IgE</td>
<td>Immunoglobulin E</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol 1,4,5-trisphosphate</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin-1</td>
</tr>
<tr>
<td>LC/MS</td>
<td>Liquid chromatography/mass spectrometry</td>
</tr>
<tr>
<td>MALDI-TOF-MS</td>
<td>Matrix-assisted laser-desorption ionization-time-of-flight mass spectrometry</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>Sodium bicarbonate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NFkB</td>
<td>Nuclear factor-kappa B</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>OxLDL</td>
<td>Oxidized low density lipoprotein</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PDK</td>
<td>Phosphoinositide dependent kinase</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3 kinase</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphoinositol-4, 5-bisphosphate</td>
</tr>
<tr>
<td>PIP3</td>
<td>Phosphoinositol 3,4,5 trisphosphate</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate-13-acetate</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>PA</td>
<td>Phosphatidic acid</td>
</tr>
<tr>
<td>PM</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>S-1-P</td>
<td>Sphingosine-1-phosphate</td>
</tr>
<tr>
<td>S-1-P Pase</td>
<td>Sphingosine-1-phosphate phosphatase</td>
</tr>
<tr>
<td>S1PR</td>
<td>S-1-P receptor</td>
</tr>
<tr>
<td>Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>SH-5</td>
<td>AKT inhibitor II</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>Sph</td>
<td>D-erythro-sphingosine</td>
</tr>
<tr>
<td>SphK</td>
<td>Sphingosine kinase</td>
</tr>
<tr>
<td>SphK1 inhibitor</td>
<td>2-(p-Hydroxyanilino)-4-(p-chlorophenyl) thiazole</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricholoroacetic acid</td>
</tr>
<tr>
<td>Thr</td>
<td>Threonine</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>TMS</td>
<td>Trimethylsphingosine</td>
</tr>
<tr>
<td>TNFa</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VSM</td>
<td>Vascular smooth muscle</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

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Chapter 1

Introduction

With advancements in the fields of cellular and molecular biology, it is becoming increasingly apparent that dysregulated cell signaling is the cause of many disease states. In these situations, the ideal therapeutic approach is to determine exactly how an aberrant cell signal is malfunctioning. Drugs can then be developed that specifically target the faulty part of a particular cellular pathway without affecting nonspecific targets or causing undesirable toxicities. Examples of successful therapeutics developed this way include the cholesterol-lowering drug lovastatin and the antihypertension drug captopril. However, this approach is a long and arduous process. Abundant cross-talk between signaling pathways regulate the delicate balance that protects cells from premature death or excessive proliferation. Despite these complexities, new therapeutic targets are being identified. One class of signaling molecules that have recently been implicated in regulating cellular death vs. cellular growth signaling are lipid metabolites. It is a relatively new concept that lipids, and not just proteins, serve as cellular signaling molecules.

Lipid metabolites usually originate from the plasma membrane. The plasma membranes of eukaryotic cells are varied, containing cholesterol (~20%), phospholipids (~60%), sphingolipids (~15%), and glycolipids (~5%) [2]. Four major lipids predominate in the plasma membrane of many mammalian cells: phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine (PS), and sphingomyelin. The plasma membrane lipid bilayer is asymmetric, with phosphatidylcholine and sphingomyelin distributing to the outer leaflet. PS and phosphatidylinositol are concentrated in the inner leaflet, making them accessible for cleavage by activated cytosolic enzymes to form lipid second messengers. Many extracellular signals activate transmembrane receptors, resulting in the activation of signaling cascades through the action of lipid kinases and
phospholipases. Lipid metabolites generated in this fashion can then amplify signals intracellularly.

Sphingosine kinase 1 (SphK1) is a lipid kinase that is critical in regulating the levels of bioactive metabolites of sphingomyelin. Members of the lipid kinase family that mediate cellular responses also include ceramide kinases, diacylglycerol (DAG) kinases, and inositol trisphosphate (IP3) kinases. SphK1 regulates cell growth by catalyzing the formation of promitogenic sphingosine-1-phosphate (S-1-P) at the expense of proapoptotic ceramide/sphingosine. Activation of SphK1 results in promitogenic signaling, indicating that it may be an ideal therapeutic target against hyperproliferative diseases. Supporting this, SphK1 has been demonstrated to be present in abnormally high levels in tumor cells [3, 4]. Specific inhibitors of SphK1 have been shown to prevent tumor growth [3]. There is additional evidence that cells which overexpress SphK1 have oncogenic properties [5]. These data strongly indicate that targeting SphK1 with a drug to inhibit its activity could selectively stop or slow cellular proliferation and decrease tumor growth, making SphK1 an appealing target for the next generation of cancer therapeutics.

Many studies predict that immediately following activation, SphK1 translocates to the plasma membrane, where its substrate, sphingosine, is located. The mechanism of this translocation is currently under investigation. Chapter Three of this thesis describes how a pool of activated SphK1 is present in membrane rafts, which are specialized regions of the plasma membrane that serve as platforms for signaling events. Chapter Four examines the transcriptional and translational regulation of SphK1 through the PI3K/AKT pathway following PDGF stimulation. Chapter Five describes how transient stimulation of SphK1 enzymatic activity is regulated through a post-transcriptional modification of SphK1 by protein kinase C (PKC). Understanding how SphK1 levels, activity, and cellular localization are regulated brings us closer to being able to predict how to pharmacologically prevent abnormal SphK1 signaling in hyperproliferative diseases.
Chapter 2

Literature Review

2.1 Overview of Sphingolipid Signaling

2.1.1 Sphingolipid Metabolites as Signaling Molecules

Sphingolipids have a diverse array of functions and structures. They participate in signal transduction, mediate cell-cell interactions, form specialized structures, and modulate the behavior of cellular proteins and receptors. Sphingolipids belong to a class of lipids derived from the aliphatic amino alcohol sphingosine. They are characterized by a long chain sphingoid backbone and an amide-linked fatty acid, as shown in Figure 1. The three main types of sphingolipids differ in the substituents (R) on their head group: ceramide (H), sphingomyelin (phosphocholine), and glycosphingolipid (sugar).

Ceramides consist of a fatty acid chain attached through an amide linkage to sphingosine. Sphingomyelins have a phosphocholine or phosphoethanolamine molecule esterified to the 1-hydroxy group of a ceramide. Glycosphingolipids are ceramides with one or more sugar residues joined in a β-glycosidic linkage at the 1-hydroxyl position.

Figure 1: Sphingolipids are characterized by a long chain sphingoid backbone and an amide-linked fatty acid. Source: Karol Langner.
Sphingolipids are synthesized in the endoplasmic reticulum (ER) and the Golgi apparatus, traveling between organelles via vesicles and monomeric transport in the cytosol. They constitute 20-35 molar percent of plasma membrane lipids [2]. Sphingolipids are ubiquitously found in mammalian cell membranes, where they form the mechanically stable and chemically resistant outer leaflet of the plasma membrane. Originally thought of as solely structural molecules, certain complex glycosphingolipids were found to be involved in specific functions, such as cell recognition and signaling. Recently, relatively simple sphingolipid metabolites, such as ceramide and S-1-P, have been shown to be important lipid second messengers in signaling cascades involved in apoptosis, proliferation, and stress responses [6, 7]. Sphingolipid metabolites are now believed to serve as bioactive second messengers that modulate intracellular events such as cell growth, apoptosis, differentiation, migration, and activation. They can also act as ligands to mediate extracellular signaling through G-protein coupled receptors (GPCRs).

An additional characteristic of sphingolipids is their ability to aggregate in cell membranes and form separate phases less fluid than the majority of plasma membrane phospholipids. These sphingolipid-based microdomains, or membrane rafts, are formed when the saturated acyl chains of the sphingolipids promotes their dense packing in the membrane while cholesterol fills the space between adjacent sphingolipid head groups and fatty acid chains. Membrane rafts were originally proposed to sort membrane proteins along the cellular pathways of membrane transport. More recent research focuses on the idea that membrane rafts act as signaling platforms during cellular signal transduction [8].
2.1.2 Sphingolipid Metabolism

Biosynthesis of sphingolipids and their catabolism is a dynamic process. One example is the sphingomyelin metabolic pathway, shown in Figure 2 [9-12]. Agonist-dependent activation of sphingomyelinases in response to growth factors, cytokines, cellular stress, and arachadonic acid stimulate sphingolipid metabolism by activating sphingomyelinases, which hydrolyze sphingomyelin to form ceramide [13]. At present, five sphingomyelinases have been identified, based on their pH optima, cellular localization, and cation dependence [13].

Ceramide can alternatively be produced by de novo synthesis. This process is initiated in the ER by the condensation of serine and palmitoly-CoA to form 3-oxosphinganine. This product is rapidly reduced to dihydrosphingosine (sphinganine) and N-acylated to dihydoceramide, which is then converted to ceramide by dihydroceramide desaturase. Ceramide has been shown to be important in determining apoptotic responses to stress [14].

Ceramidase is the enzyme that catalyzes the deacylation of ceramide to produce non-esterified fatty acid and sphingosine. Both an acidic ceramidase and an alkaline ceramidase have been identified. Sphingosine is rapidly phosphorylated by SphK to produce S-1-P, a promitogenic signaling molecule. The majority of this pathway is reversible. S-1-P degradation is mediated by dephosphorylation back to sphingosine by S-1-P phosphatase or by irreversible degradation by S-1-P lyase to hexadecenal and phosphoethanolamine. Ceramide can be converted back into sphingomyelin by the addition of a phosphocholine headgroup that is catalyzed by sphingomyelin synthase.
SphK is a key enzyme in this metabolic pathway. It forms an essential checkpoint that regulates the relative levels of ceramide/sphingosine and S-1-P, which have opposing downstream effects [15]. Ceramide and sphingosine inhibit cellular proliferation and promote apoptosis, whereas S-1-P stimulates cell growth and suppresses apoptosis. As ceramide/sphingosine and S-1-P are interconvertible, it has been proposed that it is the balance between the levels of these sphingolipid metabolites, not their absolute amount, which determines cell fate in response to various stimuli. Numerous studies supporting this view describe the model of a “sphingolipid metabolite rheostat” [16, 17], as illustrated in Figure 3. In the case where there is an excess of S-1-P, the cell enters a growth state. In contrast, when there is an excess of ceramide/sphingosine, the cell undergoes growth arrest or apoptosis. As SphK is the only known enzyme to convert sphingosine to S-1-P, it is critical in regulating this balance. It is not surprising, then, that basal ceramide/sphingosine and S-1-P levels in cells are low and tightly regulated [18]. This model is in agreement with the observation that overexpressing an isoform of SphK results in an accumulation of S-1-P, resulting in oncogenic effects [5]. It has also been
observed that elevated levels of ceramide have been implicated proapoptotic diseases, such as Alzheimer’s Disease [19].

2.1.3 Ceramide/Sphingosine Signaling

Ceramide was first implicated as a signal for apoptosis when patients with the genetic disorder Niemann-Pick disease were found to have certain cell types that were resistant to apoptosis [20]. Niemann-Pick disease results from a deficiency in acid sphingomyelinase. Ceramide has since been shown to be a proapoptotic molecule that can induce apoptosis in response to cytokines, antigens, anticancer drugs, or environmental stress [6]. Addition of exogenous ceramide analogues causes cells to enter apoptosis, indicating that ceramide synthase may be activated during apoptosis [21]. Ceramide has been implicated in apoptotic pathways involving mitochondrial damage leading to activation of caspases. At least two other pathways are involved in ceramide-mediated apoptotic cell death. One involves the tumor necrosis factor superfamily receptors and their specific ligands. The other is characterized by direct activation of

Figure 3: The sphingolipid rheostat model. The enzyme sphingosine kinase 1 can convert an apoptotic signal to a proliferative signal, resulting in cancer. Accumulation of ceramide is observed in an apoptotic condition, Alzheimer’s disease.
sphingomyelinase by diverse stimuli including growth factors, serum deprivation, ionizing radiation, cytokines, chemotherapy, and other cytotoxic agents. Paclitaxel, doxycycline, and tamoxifen have been shown to be effective at least partly due to their stimulation of ceramide-mediated apoptotic signaling in cancer cells.

Ceramide can be deacetylated by ceramidase to generate sphingosine. Sphingosine has also been shown to be produced during the early stages of apoptosis [21]. Exogenously added sphingosine is capable of inducing apoptosis in many cell types. The biological effects of sphingosine were originally believed to occur through an inhibition of the PKC pathway [22]. Subsequent studies have identified that sphingosine also induces apoptosis in a caspase-dependent fashion [23].

### 2.1.4 Sphingosine-1-Phosphate Signaling

S-1-P, the product of the SphK reaction, is an indispensable lipid second messenger that regulates diverse cellular processes. S-1-P has been identified in plants, worms, flies, slime mold, and yeast. It has been characterized as a dual function signaling molecule, with the ability to activate in a selective manner different effectors on the basis of subcellular localization. S-1-P exerts its actions extracellularly by binding to five different S-1-P receptors (S1PRs), which are differentially expressed based on tissue type [24]. The S1PRs couple to a variety of G-proteins to regulate diverse biological functions ranging from differentiation, migration, and mitogenesis to effector functions, such as proinflammatory mediator synthesis [25, 26]. Alternatively, S-1-P can act through unidentified intracellular targets, resulting in regulation of cell growth and survival [27, 28], cytoskeletal changes [29], cellular motility [30], release of intracellular calcium stores, the inflammatory response, angiogenesis, vascular maturation, heart development, and lymphocyte trafficking [31].

S-1-P levels in the cell are controlled by the balance of activity between synthesizing enzymes (SphK) and degradative enzymes (S-1-P phosphatase and S-1-P lyase) [18]. Cellular S-1-P levels have been shown to increase rapidly and transiently in response to activators of SphK1. When added exogenously, S-1-P causes DNA synthesis
and cell division, inositol trisphosphate (IP3)-independent release of calcium from internal stores, phospholipase D activation, and stimulation of the Raf/MKK/MAP kinase signaling pathway [27, 32]. S-1-P promotes cell survival in response to apoptotic stimuli, including tumor necrosis factor alpha (TNFα), Fas ligand, serum deprivation, ceramide, ionizing radiation, anticancer drugs, and withdrawal of trophic factor [33]. S-1-P suppresses the apoptotic pathway in Jurkat T lymphocytes [34]. SphK1 inhibits the mitochondrial or intrinsic death pathway, blocking the stress-activated protein kinase Jun amino terminal kinase by activating nuclear factor-kappa B (NFκB) [35]. Inhibitors of SphK1 block formation of S-1-P, selectively inhibiting proliferation induced by growth factors. SpkK1 overexpression increases intracellular S-1-P levels and enhances progression through the cell cycle [33].

S-1-P is secreted by mast cells, platelets, and monocytes and is present in high levels in serum [36]. Platelets lack S-1-P lyase and may function as storage reservoirs for S-1-P [37]. Binding of secreted S-1-P present in serum to cell surface S1PRs triggers a wide range of cellular responses including proliferation, enhanced extracellular matrix assembly, stimulation of adherent junctions, formation of actin stress fibers and inhibition of apoptosis. Additionally, S-1-P may integrate activation of downstream signals through paracrine and/or autocrine activation of S1PR1, thereby regulating directional movement triggered by non-GPCR chemoattractants, such as platelet derived growth factor (PDGF) or phorbol esters.

2.1.5 Summary

Sphingolipids and their metabolites have diverse functions, including roles as structural molecules and signaling intermediates. Specific sphingolipid metabolites in the sphingomyelin metabolic pathway, ceramide/sphingosine and S-1-P, possess opposing signaling functions. In the case where ceramide/sphingosine levels are elevated, apoptosis is favored. In contrast, when S-1-P levels are high, survival and proliferation are favored. One enzyme which controls the balance in the levels of these opposing
sphingolipid metabolites is SphK. The product of the SphK reaction, S-1-P, serves as an intracellular and extracellular second messenger involved in cell growth and proliferative signaling.

2.2 Sphingosine Kinase 1 Activation

2.2.1 The Sphingosine Kinase Reaction

SphK converts sphingosine to S-1-P by the addition of a phosphate group at its primary hydroxyl group, as illustrated in Figure 4. SphK is highly conserved from protozoa to mammals and is ubiquitous in living tissues. SphK was originally purified from rat kidney as a 49 kilodalton (kDa) protein with Km values of 5 µM and 93 µM for sphingosine and ATP, respectively [38]. Two isoforms of the enzyme have been identified, SphK1 and SphK2, which have little homology to other proteins. A double knockout of both SphK isoforms is embryonic lethal, indicating that SphK is critical for survival [39]. Although both SphK1 and SphK2 convert sphingosine to S-1-P, only SphK1 is activated in response to growth factors and other stimuli. For this reason, SphK1 is the focus of our studies. Further information on the SphK2 isoform can be found in Section 2.3.1.

Figure 4: SphK phosphorylates sphingosine, yielding sphingosine-1-phosphate. This reaction is reversible through the action of S-1-P phosphatase.
2.2.2 Stimulation of Sphingosine Kinase 1

Many factors have been shown to activate SphK1, as shown Figure 5. Stimuli of SphK1 include proinflammatory cytokines [25, 40, 41], phorbol esters [42], fetal bovine serum (FBS) [43], and ligands for GPCRs such as lysophosphatidic acid and acetylcholine [44]. Proteins that have been implicated in SphK1 activation include extracellular signal-related kinase (ERK) [1], PKC [45], and Lyn kinase [46]. SphK1 signaling downstream of plasma membrane receptors, such as PDGF, FcγRI, and FceRI receptors, results in DNA synthesis, calcium mobilization, and vesicular trafficking [47]. Interestingly, S-1-P has been shown to further activate SphK1 in a feed-forward mechanism [33]. Vitamin D inhibits apoptosis through SphK1 [48]. TNFα stimulates SphK1, resulting in antiapoptotic signals such as activation of ERK1/2 and NFκB [49]. Calcium mobilization induces SphK1 activation, which then produces S-1-P, resulting in more profound calcium mobilization [44].

Figure 5: Diverse stimuli of SphK1.
Growth and survival factors activate SphK1, such as PDGF [33], epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) [33], estrogen [50], nerve growth factor (NGF) [51] and phorbol 12-myristate-13-acetate (PMA) [16, 52, 53]. Transforming growth factor beta (TGF-β) upregulates SphK1 mRNA, protein, and activity levels, while concomitantly reducing S-1-P phosphatase activity, resulting in increased levels of S-1-P and reduced levels of ceramide/sphingosine in dermal fibroblasts [54]. Oxidized low density lipoprotein (OxLDL) activates sphingomyelinases, ceramidase, and SphK1 in vascular smooth muscle (VSM) cells, resulting in promitogenic cell signaling [55].

Many stimuli activate SphK1 in a biphasic manner. Early phase activation is believed to occur through posttranslational regulation (i.e. phosphorylation), resulting in relocalization of activated SphK1 to the plasma membrane. Late phase activation occurs through a delayed activation requiring transcription and translation. For example, PMA causes acute activation of SphK1 activity through translocation of SpkK1 to the plasma membrane [1, 45], followed by chronic induction of activity through enhanced transcription of SphK1 [56]. Estrogen also causes a biphasic SphK1 response, initially activating the enzyme within 15 minutes independent of RNA synthesis, followed by a further activation at 6 hours via a transcription requiring process [50]. Elevation of SphK1 mRNA has been illustrated in several tumors compared to adjacent normal tissue, suggesting that regulation of SphK1 by increased transcription and/or mRNA stability may be an important mechanism for driving cell proliferation [3].

### 2.2.3 Relocalization of Activated Sphingosine Kinase 1

In the unstimulated state, basally active SphK1 localizes predominantly to the cytosol. Stimulation of SphK1 is accompanied by translocation of SphK1 from the cytosol to the plasma membrane. Many signaling molecules are proposed to be involved in this translocation, including PKC [45], NGF [57], and immunoglobulin E (IgE) [46]. PMA and TNFα induce a rapid increase of SphK1 protein and activity at the plasma
membrane and an increase in S-1-P levels in the extracellular medium [45]. Calcium/calmodulin appears to be important in relocalization, but not activation, of SphK1 [58]. Membrane translocation has been demonstrated to require ERK-dependent phosphorylation of SphK1 [1] and to be mediated by PS binding [59]. Forced localization of SphK1 to the plasma membrane has been demonstrated to provide a selective growth advantage [5, 60].

SphK1 activation in the proximity of the plasma membrane may be a prerequisite for it to access its substrate, sphingosine. Sphingosine is mainly associated with the plasma membrane although it can move rapidly across and between membranes. Sphingomyelinases and ceramidases, located at the plasma membrane, are also activated by growth factors, leading to localized generation of sphingosine concomitant with SphK1 activation. S-1-P generated at the plasma membrane is positioned for cytosolic release as well as secretion into the extracellular milieu, where it can interact with S1PRs. There is speculation that SphK1 itself, once localized to the plasma membrane, could be exported extracellularly, where it could produce S-1-P in the extracellular space [61]. Translocation of activated SphK1 is reminiscent of other activated signal transduction enzymes, such as PI3K and PKC, which are recruited to membranes through protein/protein or protein/lipid interactions and are then further modulated by membrane lipid cofactors.

### 2.2.4 Sphingosine Kinase 1 and Disease

SphK1 is a potent promitogenic signaling molecule implicated in the pathogenesis of various diseases. Abnormally high levels of SphK1 protein and activity have been correlated with proliferative diseases, including cancer and vascular diseases. Mycobacterium tuberculosis blocks calcium signaling and phagosome maturation in human macrophages by inhibiting SphK1 [62]. S-1-P was observed to be increased in the airways of asthmatics [63]. A stimulatory effect of SphK1 on monocytes, neutrophils, mast cells and epithelial cells suggest a role for S-1-P in acute inflammation. The immunosuppressant drug FTY720 acts as an S-1-P agonist when it is phosphorylated...
to FTY720-P [64]. FTY720-P targets S1PRs, implicating SphK1 signaling in the immune response [65]. Respiratory syncytial virus stimulated SphK1 in lung epithelial cells, resulting in increased proliferation of virally infected cells [66].

SphK1 activation is implicated in the pathogenesis of atherosclerosis. SphK1 induction regulates vascular smooth muscle (VSM) cell proliferation, migration, and vascular maturation. S-1-P increases the expression of several proteins, including COX-2, in VSM cells, which contributes to atherosclerosis [67]. OxLDL, a major risk factor for atherosclerosis, can sequentially induce sphingomyelinase, ceramidase and SphK in VSM cells, resulting in S-1-P production and enhanced mitogenesis [55]. Basic fibroblast growth factor induces hyperproliferation of VSM cells through SphK1 activation [68]. In endothelial cells, TNFα was shown to induce ERK and NFkB activities and adhesion molecule expression through SphK activation [49]. Activated SphK1 results in high serum S-1-P levels, which has been identified as a significant predictor of obstructive coronary artery disease [69].

Many studies have shown that SphK1 has oncogenic properties. SphK1 overexpression in itself can enhance growth of cells even without extracellular stimulation. SphK1 overexpression induced cell proliferation by promoting G1 to S phase transition and increasing DNA synthesis [33]. SphK1 overexpression was sufficient to promote foci formation and cell growth in soft agar and formed tumors in NOD/SCID mice [5]. Overexpression of SphK1 in NIH 3T3 fibroblasts, human embryonic kidney 293 (HEK293) fibroblasts, Jurkat T-cells, or neuronal PC-12 cells significantly increased the rates of cellular proliferation and/or protected the cells from apoptosis [33, 70]. In breast adenocarcinoma MCF-7 cells, overexpression of SphK1 conferred a growth advantage and blocked induced cell death, indicating that SphK1 has a role in estrogen-dependent breast tumor cell growth and survival [71]. Activation of SphK1 appears necessary for mediating downstream estrogen effects including ERK activation, colony growth on soft agar, and foci formation [50]. Increased activity of SphK1 has been correlated with the resistance of tumor cells to death-inducing signals such as ceramide and Fas ligand [71, 72]. Transformation of NIH 3T3 cells by H-Ras was partially blocked by overexpression of a catalytically inactive dominant negative
SphK1 [5]. Administration of inhibitors of SphK1 inhibited the growth and metastasis of tumor cells in nude mice models [73]. S-1-P has been detected in the ascites fluid of ovarian cancer patients [74]. Targeting SphK1 to the plasma membrane, a common mechanism of activation by growth agonists, enhanced foci formation and growth in soft agar [60].

Endogenous SphK1 gene and protein expression are upregulated in a variety of human cancer types [3]. We have observed higher SphK1 mRNA and protein levels in multiple breast tumor tissues compared to normal matched mammary tissues (manuscript submitted to *J. Histochem Cytochem*). SphK1 message and protein levels were reported to be increased in human colon cancer tissues [4]. Elevated SphK1 expression correlated with poor survival of patients with glioblastoma multiforme [75]. In breast adenocarcinoma cells, enforced expression of SphK1 conferred a growth advantage and blocked the apoptosis induced by anticancer drugs, sphingosine, and TNFα [71]. Specific inhibition of SphK1 was antiproliferative and proapoptotic to several tumor cell lines and reduced tumors in mouse models [3] as well as enhancing the sensitivity of cancer cells to chemotherapy [76]. Collectively, these data indicate that limiting the activity of SphK1 would result in reduced proliferation of cancer cells.

### 2.2.5 Summary

SphK1 is an indispensable signaling molecule involved in cell growth and proliferative signaling pathways. A multitude of stimuli, such as growth factors and tumor promoting factors, stimulate SphK1. Activated SphK1 translocates from the cytosol to the plasma membrane. Elevated activity of SphK1 may contribute to clinical pathologies, such as atherosclerosis and autoimmune diseases. SphK1 exhibits higher expression and activity levels in cancer tissues, making it a potential target for cancer therapeutics. Novel drugs that block the activity of SphK1 specifically at a tumor site should force ceramide accumulation and favor apoptosis.
2.3 Sphingosine Kinase Structure

2.3.1 Sphingosine Kinase Isoforms

There are two known isoforms of SphK, SphK1 and SphK2. SphK1 (chromosome 17q25.2) is a cytosolic protein that localizes to the plasma membrane following growth factor stimulation and is involved in promitogenic signaling. Alternative splice variants of human SphK1 have been reported, consisting of 384, 398 and 470 amino acids, which all contain nuclear export sequences. In contrast, SphK2 (chromosome 19q13.2) is composed of 618 amino acids and has a nuclear localization sequence. SphK2 has a low basal activity that does not respond to stimuli. It is likely associated with sphingolipid biogenesis. Nuclear localization of SphK2 has been shown to cause inhibition of DNA synthesis leading to cell cycle arrest [77]. Unlike SphK1, overexpressing SpkK2 causes apoptosis [78]. Both SphK isoforms are inhibited by the substrate analogue dimethylsphingosine (DMS) and are stimulated by PS [79]. Triton X-100 stimulates SphK1 activity, yet suppresses SphK2 activity [80].

The different physiological functions of SphK1 and SphK2 are likely based on their different kinetic properties and enzymatic activities. Homogenized mouse tissue exhibited tenfold higher SphK1 than SphK2 activity. SphK1 activity was detected to be the highest in lung, spleen and blood. SphK2 activities were the highest in blood, with detectable levels in the spleen, brain, lung, and lymph node. SphK1 mRNA expression was shown to be highest in liver, lung and spleen. SphK2 mRNA levels were highest in the liver and heart [81].

Neither the mechanism of activation nor the crystal structure of either SphK isoform has been elucidated to date. The majority of SphK studies have focused on the SphK1 isoform (Figure 6), as this is the isoform that responds to extracellular signals and is involved in promitogenic signaling. Sequence analysis reveals that both SphK1 and SphK2 have five motifs (C1-C5) that are evolutionarily conserved in humans, mice, yeast, and plants. These motifs may be important in substrate recognition. Both isoforms contain a putative catalytic domain which has 38% identity with the catalytic domain of
DAG kinase, whose three dimensional structure is also unknown. Database searches for putative binding domains involved in protein-protein interactions with SphK1 have identified calcium/calmodulin binding motifs, TRAF binding sites, putative PKC phosphorylation sites and a putative casein kinase II phosphorylation site [82].

2.3.2 Mutational Studies

The lack of structural information about the SphK isoforms has made it difficult to target these enzymes with specific inhibitors. What is known about their structure has been inferred from modeling and mutagenesis studies. Most of these studies have focused on SphK1. Figure 7 shows a map of the amino acids that compose the 384 amino acid isoform of human SphK1. Predicted putative posttranslational modification sites include N-myristoylation sites (indicated at residues 5-10, 111-116, 113-118, 293-298), an N-glycosylation site (N137), calmodulin binding sites (residues 133-153; 289-305), and a TRAF2 binding motif. Conserved domains are underlined and labeled C1-C5. The C1-C3 domains reside within the putative catalytic domain of the enzymes and share homology with DAG and ceramide kinases [83]. Homology to the DAG catalytic domain is highlighted in light purple. The predicted catalytic domain (residues 16-153) spans C1-C3, which includes the ATP binding site (residues 82-103, dark purple italics) and two glycine residues (residues 82, 113) that are critical for catalytic activity [84]. A
point mutation of Gly82 resulted in a dominant negative SphK1 protein [85]. The sphingosine binding site was reported to involve a conserved aspartate in the C4 domain [86]. The four putative PKC phosphorylation sites are in red and are examined in detail in Chapter 5. The ERK1/2 phosphorylation site (Ser225) is in green and is described in Section 2.4.1.

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**Figure 7:** Protein sequence of human SphK1. Predicted N-myristolation, calmodulin binding, and TRAF2 binding motifs are indicated. Conserved domains are underlined and labeled C1-C5. Homology to the DAG catalytic domain is highlighted in light purple. The proposed ATP binding site is in dark purple italics. Putative PKC phosphorylation sites are in red. The ERK1/2 phosphorylation site (Ser225) is in green. Modified from Taha et. al. [82].

We have previously characterized domains that are involved in SphK1 catalytic activation. The minimal required domains which retain wild type kinase activity encompass residues 11-367, indicating that the residues at the amino and carboxyl termini may be involved in regulation of kinase catalytic activity. Truncation before
residue 11 at the amino terminus or further truncation beyond residue 363 abrogates SphK1 activity. However, a SphK1 mutant truncated at residue 363 exhibited higher than wild type activity and showed a strong relocalization from the cytosol to the membrane compared to wild type, acting as a constitutively active SphK1 (manuscript submitted to FEBS Letters). Although SphK1 lacks a known activation loop, it is possible that either terminus is necessary for recruitment of cofactors that are critical for SphK1 catalytic activation.

2.3.3 Summary

The two SphK isoforms, SphK1 and SphK2, have opposing phenotypes when they are overexpressed. SphK1 is promitogenic and responds to stimuli, whereas SphK2 is proapoptotic and is not inducible. For this reason, SphK1 has been the subject of the majority of SphK studies. The lack of a crystal structure and absence of homology to another enzyme make it difficult to predict global changes in SphK1 structure following its activation. The majority of structural information known about SphK1 is implied from sequence analysis and mutagenesis studies that predict the importance of particular motifs and putative protein-protein interactions.

2.4 Signaling Pathways Associated with Sphingosine Kinase 1

SphK1 has been implicated in many promitogenic signaling pathways including ERK1/2 [1], PKC [45, 56], PI3K/AKT [87], IP3 [44], and NfxB [49]. Whether these proteins interact directly with SphK1 or if there is signaling cross-talk between pathways at the level of SphK1 is unknown. It is not clear at this time how many other signaling molecules are upstream or downstream of SphK1. It is likely that there is cross-talk between multiple signaling cascades that are involved in activation and downregulation of SphK1.
Attempts have been made to identify protein/protein interactions involving SphK1. A study using a yeast two hybrid system identified SphK1 interactions with TRAF2, PKA anchoring protein related protein, and RPK118 [88]. An antibody array identified Sam68, Bin-1, clathrin, Lyn tyrosine kinase, Fyn, and Lck as SphK1 interacting proteins [89]. Other proteins that are believed to interact with SphK1 are PECAM-1 [90], aminoacylase 1 [91], and SphK1-interaction protein (SKIP) [92]. It is uncertain how these identified interactions of SphK1 with other proteins modulate SphK1 translocation, activation, and downstream signaling pathways.

2.4.1 ERK1/2

One of the most well characterized interactions of SphK1 with another protein is with ERK1/2. ERK belongs to the mitogen-activated protein kinases (MAPK) family of serine/threonine protein kinases. The ERK signaling pathway is activated in response to growth factors and is involved in regulation of cellular proliferation and differentiation. ERK1/2 has been demonstrated to coimmunoprecipitate with endogenous human SphK1 [1]. ERK1/2 pathway inhibitors blocked stimuli-induced increases in SphK1 phosphorylation, activity, and plasma membrane translocation. SphK1 is necessary for ERK1/2 activation by TNFα and VEGF, which activate SphK1, resulting in further ERK1/2 activation that likely involves S1PR engagement following S-1-P release. ERK1/2 has been demonstrated to activate SphK1 through phosphorylation at Ser225, resulting in increased SphK1 catalytic activity [1]. This phosphorylation was shown to be necessary for translocation of activated SphK1 to the plasma membrane. The oncogenic effects of overexpressed SphK1 (Section 2.2.4) were blocked by a Ser225Ala SphK1 mutant. Overexpression of the Ser225Ala mutant was also sufficient to reduce Ras-induced cell transformation, indicating that the mutant acted as a SphK1 dominant negative, despite possessing catalytic activity [60].
2.4.2 Protein Kinase C

SphK1 activation has also been linked to PKC. The PKC family of serine/threonine kinases contains at least ten isoforms that are involved in development, memory, differentiation, proliferation and carcinogenesis. The isoforms are grouped into one of three classes: conventional, novel and atypical, each having different tissue distribution and requiring different cofactors (i.e. calcium, DAG) for their catalytic activity. Similar to SphK1, PKC enzymes translocate to the plasma membrane upon activation by growth factors.

Stimulating cells with PMA, a direct activator of PKC, resulted in relocalization of cytoplasmic SphK1 to the plasma membrane. Following translocation, the membrane-associated SphK1 showed a higher catalytic activity in HEK293 cells [45]. Cells treated with PKC inhibitors caused an inhibition of SphK1 activity and translocation, indicating that PKC is upstream of SphK1 activation [45, 51]. Ischemic preconditioning has been observed to active SphK1 in a PKC dependent manner [93]. Similar to other activators described in Section 2.4.3, PKC activates SphK1 in a biphasic manner. Initially, it causes SphK1 to translocate to the plasma membrane [45, 56]. Late term activation occurs through stimulation of SphK1 promoter activity [56, 94]. Metabolic labeling experiments showed that immunoprecipitated GFP-SphK1 was phosphorylated following PMA treatment, indicating that SphK1 is phosphorylated upon PKC stimulation [45]. Whether PKC is the kinase responsible for this event is the topic of Chapter 5.

2.4.3 PI3K/AKT

AKT (protein kinase B), a serine/threonine kinase, has emerged as a critical enzyme in several signal transduction pathways involved in cell proliferation [95], apoptosis [96], angiogenesis [97], and diabetes [98]. Three isoforms of AKT have been reported in mammals (AKT1, AKT2, and AKT3) that exhibit a high degree of homology, but differ slightly in the localization of their regulatory phosphorylation sites. The principal role of AKT is to facilitate growth factor-mediated cell survival and to block...
apoptotic cell death. AKT achieves this by phosphorylating a variety of substrates, such as Bad, caspase-9, and Forkhead transcription factors [99]. High activity of AKT has been detected in many types of human cancers [100], hence it is a target in cancer chemotherapy.

Activation of AKT involves growth factor binding to a receptor tyrosine kinase and activation of phosphoinositide 3 kinase (PI3K), which phosphorylates the membrane bound phosphoinositol-4, 5-bisphosphate (PIP2) to generate phosphoinositol 3,4,5 trisphosphate (PIP3). The binding of PIP3 anchors AKT to the plasma membrane and allows its phosphorylation and activation by 3-phosphoinositide-dependent kinase-1 (PDK1). AKT is fully activated following its phosphorylation at two regulatory residues, a threonine residue on the kinase domain and a serine residue on the hydrophobic motif. AKT deactivation is believed to be regulated through the tumor suppressor PTEN, which dephosphorylates PIP3, removing the membrane-localization factor from the AKT signaling pathway and shutting off signaling amplification.

Many studies have linked AKT activation to SphK1. It has been reported that OxLDL-induced VSM cell proliferation requires the concomitant activation of SphK and AKT [55, 101]. Another study in VSM cells showed that S-1-P induced COX-2 gene expression through activation of AKT [67]. Overexpression of SphK1 in endothelial cells mediated survival through activation of PI3K and AKT. Inhibition of PI3K was shown to block S-1-P induced AKT phosphorylation [102]. TNFα-stimulated cell proliferation was reported to be mediated through SphK-dependent AKT activation [87]. Phospholipase D (PLD) was shown to link S-1-P signaling to stimulation of the PI3K/AKT pathway in CHO cells [103]. S-1-P treatment of aortic endothelial cells increased eNOS enzyme activity through the PI3K/AKT pathway in endothelial cells [104]. S-1-P treatment of ovarian cancer cells induced AKT activation and decreased the efficacy of the anticancer drug paclitaxel [105]. S-1-P induced the phosphorylation of AKT through PI3K signaling in osteoblasts [106] and promoted mouse melanocyte survival via AKT activation [107]. Interestingly, suppression of AKT is one of the mechanisms by which sphingosine induces apoptosis in hepatoma cells [96].
2.4.4 Summary

Many attempts have been made to identify signaling pathways and proteins that are directly involved in SphK1 activation and downstream signaling. The only protein that has been identified to definitively modulate SphK1 through a phosphorylation event is ERK1/2. ERK-dependent phosphorylation of SphK1 at Ser225 has been shown to facilitate plasma membrane association of SphK1 without enhancing its intrinsic enzymatic activity. This plasma membrane association of SphK1 appears to drive its oncogenic effects. Many other promitogenic signaling pathways have been implicated in SphK1 relocalization, activation, and signaling, including PKC and PI3K/AKT.
Chapter 3

Sphingosine Kinase 1 Localization at Membrane Rafts

3.1 Introduction

It is well established in the literature that SphK1 translocates to the plasma membrane following stimulation (Section 2.2.3). This chapter examines the localization of SphK1 specifically to plasma membrane rafts. Membrane rafts are transient phase separations in the fluid lipid bilayer (~70 nm in diameter) that are rich in both sphingolipids and cholesterol. They were first proposed in 1988, describing cell membrane microdomains enriched with cholesterol, glycolipids, and sphingolipids [108]. Membrane rafts were first isolated by adding non-ionic detergent to cells at low temperatures, causing the fluid membrane to dissolve while the membrane rafts remained intact and could be extracted. Due to their composition and detergent resistance, membrane rafts are also referred to as lipid rafts, detergent insoluble membranes (DIMs), glycolipid-enriched complexes (GEMs), and detergent resistant membranes (DRMs). Recently the term “membrane raft” has been agreed upon by experts in the field [109].

Membrane rafts have been implicated in many cellular processes and systems, including polarization, viral entry, migration, vesicular trafficking, as illustrated in Figure 8 [109-112]. Proteins associated with cellular signaling processes have been shown to associate with membrane rafts, including glycosylphosphatidylinositol (GPI)-anchored proteins [113], dually acylated tyrosine kinases of the Src family, and transmembrane proteins. This association can be partially attributed to the acylated, saturated tails of both the tyrosine kinases and the GPI-anchored proteins [114]. While the aforementioned proteins tend to continuously be present in membrane rafts, there are other proteins that associate with rafts only when the protein is activated, such as B and T cell receptors, GPCRs, and Ras [115, 116]. Other proteins are always excluded from rafts, such as the transferrin receptor and calnexin. Membrane raft associations can be
characterized by disrupting the rafts by removing the cholesterol from the membrane, using chemicals such as methyl-β-cyclodextrin or filipin.

There is some preliminary evidence that activated SphK1 may specifically translocate to membrane rafts. SphK1 has been demonstrated to be detergent insoluble [117], which is a characteristic of membrane raft resident proteins. A study in activated mast cells reported an interaction of SphK1 with Lyn kinase at membrane rafts in correlation with an increase in SphK1 activity in these domains [89]. Lyn is known to move into rafts following stimulation [118] and is speculated to recruit SphK1 to the membrane raft domain. PS, an allosteric activator that retains SphK1 at the plasma membrane [59], is enriched in membrane rafts [119], suggesting that SphK1 may be recruited to these domains through protein/lipid interactions. It is believed that signaling molecules are targeted to the monolayer of membrane rafts by myristoylation or palmitoylation [120]. Targeting SphK1 to membranes by tagging a myristoylation signal sequence enhanced cell growth and was sufficient for cell transformation [121]. Although these data suggest that SphK1 signaling within membrane rafts is important,
direct evidence for the presence of SphK1 protein, activity, and its substrate at plasma membrane rafts has not yet been shown.

3.2 Hypothesis

Many mechanisms of targeting activated SphK1 to the plasma membrane have been proposed, however, it is unclear how this membrane association is regulated in a spatial and temporal manner. We hypothesize that SphK1 protein and activity are present at plasma membrane raft microdomains. As membrane rafts are known to be involved in cell signaling, we expect increased SphK1 activity in rafts following stimulation with FBS. We further postulate that the substrate of SphK1, sphingosine, is present in membrane rafts and would be depleted following SphK1 stimulation. We expect that compartmentalization of SphK1 signaling in membrane rafts would make S-1-P locally available for downstream signaling effects.

3.3 Methods

3.3.1 Cell Culture

HEK293 cells (CRL-1573) and NIH 3T3 mouse fibroblasts (CRL-1658) were purchased from ATCC (Manassas, VA). The cells were cultured in DMEM (Gibco, Grand Island, NY) with 10% FBS for HEK293 cells, or 5% FCS for NIH 3T3 cells, and penicillin/streptomycin at 37°C in a humidified atmosphere of 5% carbon dioxide. Transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Stable lines were generated by selection of antibiotic resistant cells in the presence of 500 µg/mL geneticin (Calbiochem, San Diego, CA).
3.3.2 Cloning of Human Sphingosine Kinase 1

Human SphK1 (GenBank™ accession number AF200328) was cloned from HEK293 cells. Total RNA was isolated using Trizol (Molecular Research Center, Cincinnati, OH) and treated with DNase (Ambion, Austin, TX). First strand cDNA synthesis was accomplished with Oligo dT(12-18) primers (Invitrogen) and Omniscript reverse transcriptase enzyme (Qiagen, Valencia, CA). Primers specific for the hSphK1 cDNA (5’CCCAGGAATTCCACCATGGATCCAGCGGGC) and (3’ATGATCTGCGCCGCTTTAAGG GTTCTTCTGG) were used to obtain PCR products using Platinum Pfx high fidelity DNA polymerase (Invitrogen). The hSphK1 cDNA was directionally cloned into the Eco RI (5’) and Not I (3’) sites of pcDNA3.1/HIS B (Invitrogen) for mammalian expression and sequence verified. To create the GFP-hSphK1 construct, the His<sub>6</sub>-hSphK1 vector was digested with EcoRI/XbaI and subcloned into the EcoRI/XbaI sites of EGFPC1 (Clontech, Mountain View, CA). The GFP-hSphK1_1-220 truncation mutant was PCR amplified using a 3’ primer (5’ ATGATCTGCGCCGCTAGGAACCCACTTC) designed to truncate and introduce a stop codon into the coding region of SK1. The product was directionally cloned into pcDNA 3.1/HIS B as described above. The His<sub>6</sub>-hSphK1Gly82Asp mutant was generated by mutagenesis of the wild-type SphK1 cDNA using the Quikchange Site-Directed Mutagenesis system (Stratagene, La Jolla, CA) according to the manufacturer's recommendations.

3.3.3 Membrane and Cytoplasmic Fraction Isolation

HEK293 cells were seeded at 2 x 10<sup>5</sup> cells/mL into six well culture plates in DMEM containing 2% FBS. After 18 h, cells were stimulated with 10% FBS for 0, 5, 15, and 60 minutes. The cells were then washed with cold PBS and snap frozen in liquid nitrogen. Thawed cells were scraped in 200 µL lysis buffer (50 mM HEPES, 50 mM MgCl<sub>2</sub>, 10 mM KCl, 10 mM NaF, 2 mM NaVO<sub>3</sub>, 500 µM 4-deoxypyridoxine, 0.05% Triton X-100, pH 7.4) and probe sonicated for 15 sec on ice. The lysates were
centrifuged at 100,000 x g for 30 min at 4°C. The supernatant (cytosolic fraction) was removed and 50 μL was used for Western blotting. The pellet (membrane fraction) was washed and resuspended by brief probe sonication in 150 μL lysis buffer. The remaining volume of supernatant and resuspended pellet (150 μL each) was used in their entirety for determination of SphK1 activity.

3.3.4 Western Blot Analysis

Samples from subcellular fractions were separated on a 10% SDS-PAGE gel (Invitrogen), and transferred to a PVDF membrane (Invitrogen). Membranes were blocked with 5% milk in TBS-T (25 mM Tris-HCl, pH 8.0; 125 mM NaCl; 0.1% Tween 20) for at least 1 h and then incubated with one of the following primary antibodies for at least 1 h: anti-caveolin-1, anti-GAPDH, and anti-GFP (Santa Cruz, Santa Cruz, CA); anti-HIS6X (BD Biosciences, San Jose, CA), anti-transferrin receptor (Zymed, San Francisco, CA), anti-flotillin-1 (BD Biosciences), anti-calnexin (Stressgen, Victoria, BC), anti-SphK1 (Exalpha, Maynard, MA). The membranes were washed and incubated with horseradish peroxidase conjugated anti-rabbit or anti-mouse IgG antibody (Amersham, Piscataway, NJ) for at least 1 h. The bands were visualized either using the Fuji Film Intelligent Dark Box II or on film using ECL (Amersham) or Super Signal West Dura (Pierce, Rockford, IL) reagents.

3.3.5 Differential Centrifugation

Two 10 cm culture dishes were seeded with HEK293 cells stably overexpressing His6x-hSphK1 at 4x10^5 cells/mL. After 24 h, the cells were washed in cold PBS and snap frozen in liquid nitrogen. The cells were thawed in 400 μL TBS buffer (1x TBS with protease inhibitors, 1 mM PMSF, 5 mM Na3VO4, and 1 mM glycerophosphatase). The post-nuclear supernatants were isolated (200 x g for 5 min at 4°C) and further sedimented at 100,000 x g for 30 min at 4°C. The resulting supernatants were separated (cytosolic
fractions) and the pellets were washed with 500 µL TBS buffer. Separate pellets were resuspended in either 400 µL TBS buffer or 500 mM NaHCO3, pH 11. Following centrifugation at 100,000 x g, the supernatants were removed (soluble fraction) and the pellets were washed in TBS buffer. Subsequently, each pellet was resuspended in 400 µL TBS buffer containing 1% Triton X-100 and incubated on ice for 30 min with periodic inversion. A final centrifugation at 100,000 x g separated the Triton X-100 soluble fraction (supernatant) from the Triton X-100 insoluble fraction (pellet) fraction. The pellet was washed in TBS buffer and solubilized in 400 µL NuPAGE LDS sample buffer (Invitrogen) prior to Western blot analysis.

3.3.6 Membrane Raft Isolation by Sucrose Gradient

Cells were seeded at 4x10^5 cells/mL in 15 cm dishes. For FBS stimulation experiments, cells were plated in DMEM containing 2% FBS for 18 h prior to stimulation with 10% FBS. Cells were washed with cold PBS and kept on ice thereafter. The cells were scraped in 1 mL of either TNE buffer using the method of Popik et al [122] (25 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, pH 7.5, 1 mM PMSF, 5 mM Na3VO4, 1 mM glycerophosphatase, protease inhibitors) or high pH/carbonate buffer using the method of Song et al [123] (500 mM NaHCO3, pH 11, 1 mM PMSF, 5 mM Na3VO4, 1 mM glycerophosphatase, protease inhibitors). Cell suspensions were subjected to 30 strokes in a Dounce homogenizer followed by probe sonication for 10 sec. For detergent solubilization of the cell lysates, Triton X-100 was added to the TNE buffer to a final optimized concentration of 0.8% (Figure 10), and incubated on ice for 30 min with periodic inversion. Sucrose solution prepared in TNE for the Triton X-100 preparation, or MBS (25 mM MES, pH 6.5, 0.15 M NaCl) for the NaHCO3 preparation, was added to adjust the final sucrose concentration to 40%. This solution (2 mL) was overlain with 4 mL of sucrose (35% w/v for high pH/carbonate, 30% w/v for TNE), followed by 3 mL of 5% w/v sucrose to form a 5-40% discontinuous sucrose gradient. The samples were centrifuged for 18 h at 200,000 x g at 4°C using a Beckman SW-41 rotor. The 9 mL
sample was subsequently fractionated into 1 mL fractions, starting at the top, which were used for SphK1 activity or Western blot analysis.

Trichloroacetic acid (TCA) precipitation (1%) was used to concentrate protein prior to Western blot analysis. Protein concentrations were determined using the Bradford assay (BioRad, Hercules, CA). For cholesterol depletion studies, cell lysates were incubated in 20 mM methyl-β-cyclodextrin or 15 µg/mL filipin for 30 min, followed by membrane raft isolation using the Triton X-100 preparation.

### 3.3.7 Sphingosine Kinase Activity Assay

The SphK1 activity assay was performed as described by Olivera et al. with minor modifications [124]. Briefly, 50 µM D-erythro-sphingosine (Avanti Polar Lipids, Alabaster, AL) was combined with 200 µM ATP containing 2 µCi [γ32P] ATP (ICN Radiochemicals, Costa Mesa, CA) in 100 µL final volume of SphK1 Activity Assay Buffer (50 mM HEPES, 50 mM MgCl2, 10 mM KCl, 10 mM NaF, 2 mM NaVO3, 500 µM 4-deoxypyridoxine, 0.05% Triton X-100, pH 7.4). The reaction was initiated by addition of enzyme. The reaction was agitated for 20 min at 37°C and reactions were terminated by the addition of 10 µL 6N HCl. Labeled lipids were extracted by the addition of 250 µL of chloroform:methanol:HCl 100:200:1 v/v/v, 125 µL chloroform, and 125 µL 1M KCl. The organic phase, which contained radiolabeled S-1-P, was removed and scintillation counted.

### 3.3.8 Membrane Raft Labeling and Confocal Microscopy

HEK293 cells stably overexpressing empty vector (GFP), GFP-hSphK1, GFP-hSphK1Gly82Asp, or GFP-hSphK1_1-220 were plated at 2x10^5 cells/mL into 4 well Lab-Tek chamber slides (Nalge Nunc, Rochester, NY) that had been coated with polylysine (Sigma, St. Louis, MO). After 24 h, membrane rafts were labeled with the
Vybrant Lipid Raft Labeling Kit (Molecular Probes, Eugene, OR). Confocal images were obtained using a Leica TCS SP2 AOBS microscope (Microscopy Imaging Core Facility, Penn State College of Medicine). All pictures were taken using a 10x eyepiece, 63x objective. Excitation for GFP-SphK1 was 488 nm and for the Alexa Fluor was 594 nm. The single scan images, taken at a pixel resolution of 1024 x 1024, were overlain to determine colocalization (yellow) of GFP-SphK1 (green) and membrane rafts (red). All images were collected and processed identically.

3.3.9 Subcellular Fractionation Following [³H] Sphingosine Incorporation

HEK293 cells were plated into 6 well dishes at 2 x 10⁵ cells/mL. After 24 h, the culture media was changed to serum free for 1.5 h. D-erythro-sphingosine (2 µM, Avanti Polar Lipids) and 0.4 µCi [³H] sphingosine (ARC, St. Louis, MO) complexed with 4 mg/mL BSA in DMEM was added for 5, 30, and 60 min. Immediately before cell lysis, the media was centrifuged at 1,000 x g for 5 min to pellet unattached cells, and the supernatant was removed for scintillation counting. The cells were washed in cold PBS and then snap frozen in liquid nitrogen. The cells were thawed in 200 µL Lysis Buffer (20 mM Tris, pH 7.4, 20% glycerol, 1 mM β-mercaptoethanol, 5 mM EDTA, 2 mM EGTA, 1 mM Na₃VO₄, 40 mM beta glycerophosphate, 15 mM NaF, protease inhibitors, 1 mM PMSF, 0.5 mM 4-deoxypyroxine) and centrifuged at 100,000 x g for 30 min. The supernatant (cytosolic fraction) was removed and the pellet (total membrane fraction) was rinsed in cold PBS and resuspended in 300 µL Lysis Buffer by brief probe sonication. Scintillation counting was used to trace the [³H] sphingosine that was present in the media, cytosolic, and membrane fractions.

3.3.10 Thin Layer Chromatography

HEK293 cells were seeded at 4x10⁵ cells/mL in 15 cm culture dishes. After 18 h, the media was replaced with serum free DMEM for 1.5 h. A mixture of 2 µM D-erythro-
sphingosine (Avanti Polar Lipids) and 0.4 μCi [³H] sphingosine (ARC, St. Louis, MO) complexed with 4 mg/mL BSA in DMEM was added 30 min prior to stimulation with 10% delipidated FBS (Cocalico Biologicals, Reamstown, PA). At 5, 30, 60, or 120 min, cell lysates were subjected to sucrose gradient centrifugation using the high pH/carbonate method. Lipids were extracted from each 1 mL fraction using the Bligh and Dyer [125] method with minor modifications. Samples were acidified with 50 μL 6N HCl. Chloroform:methanol (3.75 mL, 1:2 v/v) was then added to each fraction, followed by 1.25 mL chloroform and 1.25 mL water. The samples were centrifuged at 2,000 x g for 5 min and the organic phase was dried down. If observable sucrose had precipitated into the organic phase, the extraction was repeated at half scale. Samples were then resuspended in 30 μL 1:1 chloroform:methanol and applied to a Partisil LK60 TLC plate (Whatman, Florham Park, NJ). Standards included sphingosine, ceramide, sphinganine, and sphingomyelin (Avanti Polar Lipids). The chloroform:methanol:water:acetic acid, 30:30:2:5 v/v/v, solvent system was used and the data was confirmed using additional solvent systems (butanol:water:acetic acid 3:1:1 v/v/v; and chloroform:methanol:ammonium hydroxide 80:20:2 v/v/v). The plates were developed in an iodine chamber. Bands corresponding to each standard were scraped and scintillation counted.

### 3.3.11 LC/MS/MS Analysis of Membrane Raft Fractions

HEK293 cells were plated at 4x10⁵ cells/mL in 10 cm culture dishes in DMEM containing 2% FBS. After 24 h, the media was replaced with DMEM containing 2 μM D-erythro-sphingosine complexed with 4 mg/mL BSA. After 30 min, the cells were stimulated with 10% delipidated FBS. At 5, 15, 30, or 60 min the media was removed for lipid extraction. The cells were harvested in 1 mL pH 11/carbonate buffer and sucrose gradient centrifugation was performed. The resulting fractions 3, 4 and 5 were pooled as the “raft” sample. Fractions 7, 8, 9 were pooled as the “dense” sample. Pooled fractions were acidified with 80 μL 6N HCl. Lipids were separated and
quantified using HPLC in conjunction with tandem mass spectrometry as described by Sullards et al [126] with minor modifications. Briefly, 10 µL of internal standards (25 µM C17 sphingosine and 25 µM C17 S-1-P, Avanti Polar Lipids) were added to each 3 mL pooled sample. The addition of 3 mL chloroform partitioned the sucrose to the aqueous phase. After centrifugation at 2,000 x g for 5 min, the organic phase was removed and dried under a nitrogen stream. Lipids were reconstituted in 250 µL PBS in combination with 250 µL of the original aqueous phase (to enhance S-1-P recovery) and re-extracted by addition of 500 µL methanol and then 250 µL chloroform. The mixture was bath sonicated for 30 sec, followed by 3 h incubation at 50°C. Subsequently, 75 µL 1 M KOH was added, followed by 30 sec sonication and 2 h incubation at 37°C. The aqueous phase (600 µL) was dried down under a stream of nitrogen, resuspended in positive ion MS spray solution (methanol: acetic acid, 99:1 v/v, containing 5 mM ammonium acetate), and filtered through 0.22 µm filters (Millipore, Bedford, MA) into 96 well plates.

Quantitation by LC/MS/MS using multiple reaction monitoring was performed as previously described [126]. We used a 4000 Q trap LC/MS/MS system (Applied Biosystems, Foster City, CA) with an Agilent 1100 Series binary pump (Palo Alto, CA) and the HTS PAL autosampler from CTC Analytics AG (Zwingen, Switzerland).

3.3.12 Data Analysis

Results are presented as means of at least three independent experiments. Statistical significance was assessed by the Student t-test for paired samples, unequal variance. Values were considered to be significant if p < 0.05. The optical density of SphK1 bands from Western blots and RT-PCR gels were quantified using Image J [127] or Image Gauge V4.0 (Fujifilm) software.
3.4 Results

3.4.1 Inducible Sphingosine Kinase 1 Activity is Found in the Membrane Fraction

The current model of SphK1 activation involves agonist-induced translocation of basally active SphK1 from the cytoplasm to the plasma membrane, where it is fully catalytically active (Section 2.2.3). Previous studies have shown that membrane associated SphK1 activity is responsive to PMA stimulation [117]. In order to determine whether membrane associated SphK1 is activated by FBS in HEK293 cells, cells were stimulated with 10% FBS over a 60 min timecourse. The cell lysates were then separated into cytosolic and total membrane fractions by ultracentrifugation. Each fraction was assayed for SphK1 activity (Figure 9). Cytosolic GAPDH levels were monitored as an indicator of equal total protein between samples. The total membrane fraction (black bars) exhibited approximately 50% higher SphK1 activity than the total cytosolic fraction (gray bars). We do not believe that this difference is due to higher levels of SphK1 being present in membrane fractions, but rather that the membrane environment is more suitable for SphK1 activity, perhaps by the presence of activating factors or favorable protein/protein or protein/lipid interactions. Supporting this, we found that SphK1 activity in the cytosolic fraction did not respond to FBS stimulation. In contrast, SphK1 activity in the total membrane fraction increased 1.3 fold after 5 min of FBS stimulation. SphK1 activity in this fraction remained elevated at 15 min (1.2 fold) and 60 min (1.4 fold). This small but significant increase in activity is consistent with typical levels of SphK1 activation [117]. We conclude that inducible SphK1 activity is specific to the total membrane fraction.
3.4.2. Sphingosine Kinase 1 Protein is Found in Membrane Raft Fractions

We further characterized the association of SphK1 with the plasma membrane. To determine whether SphK1 is present specifically in the membrane rafts, we began with a crude raft preparation using two differential centrifugation approaches. The first involved extraction with 1% Triton X-100 followed by separation of the detergent-resistant membrane fraction. Because of the possible introduction of artifacts through the use of a detergent method, we secondly used a detergent-free high-pH/carbonate membrane raft preparation. A flow chart representing the design of this experiment is shown in Figure 10A. HEK293 cells overexpressing vector only (control) or His<sub>6x</sub>-hSphK1 were lysed in TBS buffer. Cytosolic proteins were isolated by centrifugation. Duplicate pellets were resuspended in either TBS buffer (blank) or high-pH/carbonate buffer and centrifuged. The supernatant (soluble) was not expected to contain any protein in the case of the TBS buffer resuspension, but contained membrane nonraft...
associated proteins in the case of the high-pH/carbonate buffer resuspension. The resulting pellets were resuspended, incubated in cold 1% Triton X-100, and centrifuged. The supernatant (triton soluble) contained membrane nonraft associated proteins. The pellet (triton insoluble) contained membrane raft resident proteins.

Figure 10: Differential centrifugation. A: Schematic describing the differential centrifugation method used to demonstrate that SphK1 is present in membrane rafts. B: HEK293 cells that were stably overexpressing empty vector (odd numbers) or His<sub>6x</sub>-hSphK1 (even numbers) were fractionated into a cytosolic fraction (lanes 1-4) and a pellet. The pellet was further solubilized in TBS buffer (blank) or in high pH/carbonate buffer and separated into a soluble fraction (lanes 5-8) and a pellet. These pellets were incubated in Triton X-100 and separated into Triton X-100 soluble (lanes 9-12) and Triton X-100 insoluble (lanes 13-16) fractions. A band corresponding to His<sub>6x</sub>-SphK1 was seen in the cytosolic fractions (lanes 2 and 4). Both the detergent (Triton X-100) and detergent-free (high pH/carbonate) methods solubilized membrane associated SphK1 (lanes 8 and 10). Irrespective of preparation method, His<sub>6x</sub>-SphK1 was also present in the membrane raft fraction (lanes 14 and 16).

We identified three distinct pools of SphK1 protein (Figure 10B). The odd numbered lanes contain samples from HEK293 cells overexpressing empty vector. The even numbered lanes contain samples from HEK293 cells overexpressing His<sub>6x</sub>-hSphK1. The majority of SphK1 was found in the cytosolic fraction (lanes 1-4), as has been
previously described [117]. We also identified a membrane-associated pool of SphK1, which was extracted from membranes in both the detergent (Triton X-100) method (lane 10) and the detergent-free (high-pH/carbonate) method (lane 8). Only a minimal amount of the high-pH/carbonate sample was further solubilized by detergent (lane 12), indicating that the membrane-associated fraction released by high-pH/carbonate (lane 8) is essentially equivalent to the detergent soluble membrane fraction (lane 10). The third pool of SphK1 was present in the Triton X-100 insoluble fractions (lanes 14 and 16), which is indicative of the membrane raft fraction. Importantly, we observed this membrane raft pool of SphK1 irrespective of preparation method.

3.4.3 Preliminary Sucrose Gradient Experiments

We next optimized the detergent method of sucrose density gradient centrifugation in HEK293 cells. This method is based on the principle that membrane rafts, extracted in cold detergent (i.e. Triton X-100), will remain detergent insoluble and float to an area of low density during sucrose gradient centrifugation [128]. To optimize the concentration of Triton X-100 that is sufficient to extract membrane associated proteins without disrupting the membrane raft architecture, we examined the distribution of several membrane proteins after 0%, 0.8%, and 1.5% Triton X-100 solubilization of HEK293 cell lysates (Figure 11). Known membrane raft markers (caveolin-1 and flotillin-1) as well as membrane non-raft markers (transferrin receptor and calnexin) were present in the membrane raft fractions when cells were incubated in buffer alone (0% Triton X-100). In contrast, incubation in 1.5% Triton X-100 resulted in all four markers being identified in the densest fraction. We adopted 0.8% Triton X-100 as our standard protocol, which partitioned caveolin-1 and flotillin-1 to the raft fraction, and the transferrin receptor and calnexin to the densest fraction.
3.4.4 Identification of Sphingosine Kinase 1 in Membrane Rafts by Sucrose Gradient Centrifugation

We used sucrose gradient centrifugation to verify the presence of SphK1 in membrane rafts. As a positive control, we identified the membrane raft markers caveolin-1 and flotillin-1 in the low density membrane raft fractions in samples prepared in Triton X-100 (Figure 12a and b, fractions 4 and 5). As a negative control, we identified the membrane non-raft markers transferrin receptor and calnexin in the dense fractions (Figure 12c and d, fractions 8 and 9). We next examined the localization of His<sub>6</sub>-hSphK1, which was present in the membrane raft fractions, while the empty vector was not (Figure 12e and f). A small pool of endogenous SphK1 was also identified in the membrane raft fraction (Figure 12h). To confirm these findings in a detergent-free system, we repeated the sucrose gradients using the high-pH/carbonate method with similar results (Figure 12g). Overall, we identified the majority of SphK1 in the densest fractions when using either detergent or detergent-free sucrose density gradient methods. Importantly, there was also a separate pool of membrane raft associated SphK1.
We next examined the SphK1 enzymatic activity per sucrose gradient fraction (Figure 13, bar graph) as compared to total protein per fraction (line graph) in NIH 3T3 cells. We found that the majority of total protein (98.5%) was observed in the densest fractions of a sucrose gradient (fractions 8 and 9). A small, yet significant, fraction of total protein (0.21%) was observed in the membrane raft containing fraction (fraction 4). Consistent with this observation, the majority of SphK1 activity was found in the densest fractions (73.2%). Interestingly, we found that the specific activity of SphK1 present in

3.4.5 Sphingosine Kinase 1 Activity is Present in Membrane Rafts

Figure 12: Characterization of membrane raft associated SphK1. HEK293 cells were separated using the Triton X-100 detergent (D) or the high pH/carbonate detergent-free (DF) method of sucrose gradient centrifugation. The membrane raft markers caveolin-1 (a) and flotillin-1 (b) partitioned to membrane rafts (fractions 4 and 5). The membrane nonraft markers transferrin receptor (c) and calnexin (d) partitioned to the dense fractions 8 and 9. Overexpressed His6xSphK1 was identified in both membrane raft and dense fractions (e, g). No bands were observed in samples from HEK293 cells overexpressing empty vector (f). Endogenous SphK1 distributed predominately to the dense fractions, yet a significant amount was found in the membrane raft fractions (h).
the membrane raft fraction was disproportionately high relative to the specific activity of SphK1 in the dense fractions.

3.4.6 Sphingosine Kinase 1 is excluded from Membrane Rafts of Cholesterol Depleted Cells

We performed cholesterol depletion studies to further confirm the presence of SphK1 in membrane rafts. The characteristic detergent resistance of membrane rafts is due to the dense packing of lipids that are intercalated with cholesterol within these domains. Consequently, membrane rafts and their associated proteins are disrupted in the presence of the cholesterol binding agents methyl-β-cyclodextrin or filipin. Following methyl-β-cyclodextrin or filipin treatment, we found that caveolin-1 was depleted in the

Figure 13: NIH 3T3 cells were separated by sucrose gradient centrifugation. Each fraction was assayed for SphK1 activity (bar graph) as well as total protein concentration (line graph). * bar graph, p<0.05. # line graph, p<0.05.
membrane raft fraction and redistributed to the dense fraction (Figure 14A). Similarly, both overexpressed His$_{6\times}$-hSphK1 and endogenous SphK1 were depleted in the raft fractions following treatment with either methyl-$\beta$-cyclodextrin or filipin. A corresponding increase in His$_{6\times}$-hSphK1 in the dense fraction was not observed in the overexpression system. This is likely due to the fact that higher levels of SphK1 are present in cytosolic fractions. Thus, a small increase of SphK1 in these fractions is unlikely to be discernable. In agreement with the Western blot data, we observed a 39% decrease in SphK1 activity in the membrane raft fraction after methyl-$\beta$-cyclodextrin treatment (Figure 14B).

Figure 14: SphK1 is reduced in the membrane raft of cholesterol depleted cells. The presence of caveolin-1, His$_{6\times}$-SphK1, and endogenous SphK1 in membrane rafts is diminished in the presence methyl-$\beta$-cyclodextrin or filipin in HEK293 cells. B: Methyl-$\beta$-cyclodextrin decreases SphK1 activity found in membrane rafts. p<0.05.
3.4.7 Localization of GFP-Sphingosine Kinase 1 Within Membrane Rafts by Confocal Microscopy

We further characterized the membrane raft association of SphK1 using confocal microscopy. HEK293 cells stably overexpressing green fluorescent protein (GFP) labeled empty vector, wild type SphK1 (GFP-SphK1), dominant negative SphK1 (GFP-hSphK1Gly82Asp), or inactive truncated SphK1 (GFP-SphK1_1-220) were labeled with a red fluorescent dye that specifically binds to GM-1, a membrane raft marker. The raft label had a globular appearance along the cell membrane (Figure 15A, D, G, and J). The negative controls, empty vector and truncated SphK1, were distributed throughout the cytoplasm (Figure 15B and K). Both wild type SphK1 (Figure 15E) and the dominant negative SphK1 (Figure 15H) exhibited a cytoplasmic distribution with a concentration of GFP along the plasma membrane. Colocalization of GFP and membrane rafts was visualized by overlain confocal images. Colocalization was not observed in the cells transfected with empty vector or truncated SphK1 (Figure 15C and L). In contrast, we did observe colocalization of wild type SphK1 with membrane rafts (Figure 15F).

We next determined if catalytic activity was prerequisite for membrane raft localization of SphK1. Figure 15I shows a similar, though less pronounced, localization pattern for the dominant negative SphK1. We further examined the distribution of dominant negative and a truncated SphK1 by sucrose gradient (Figure 15M). We observed that wild type and dominant negative SphK1 exhibited similar membrane raft localization patterns, indicating that SphK1 activity is not necessary for membrane raft association. The SphK1 1-220 truncated mutant, which also lacked catalytic activity, did not translocate to the membrane raft, indicating that residues 221-384 contain a regulatory region necessary for membrane raft association.
Figure 15: Colocalization of sphingosine kinase 1 with membrane rafts. HEK293 cells stably overexpressing empty GFP vector (B), GFP-hSphK1 (E), dominant negative GFP-hSphK1Gly82Asp (H), and truncated GFP-hSphK1_1-220 (K) were labeled at membrane rafts (A, D, G, J) and imaged by confocal microscopy. Yellow fluorescence indicates a colocalization of GFP-hSphK1 (F) and GFP-hSphK1Gly82Asp (I) with membrane raft domains. No colocalization was observed in the vector control (C) or the truncated SphK1 (L). White arrows represent the presumed leading edge of the cell.

M: A dominant negative SphK1 also localizes to membrane rafts, but a truncated SphK1 does not. HEK293 cells overexpressing vector only, His6x-hSphK1, His6x-hSphK1Gly82Asp, and His6x-hSphK1_1-220 were separated by sucrose gradient centrifugation method and detected with an anti-His6x antibody.
3.4.8 Sphingosine is Highly Enriched in Membrane Rafts

Once we had confirmed that SphK1 is present in membrane rafts, we next examined whether its substrate, sphingosine, is also present there. We exogenously added $[^3]H$ sphingosine to the media of HEK293 cells and examined its distribution over a timecourse (Figure 16). After 5 min, we found that 86% of the $[^3]H$ sphingosine was retained in the media (gray bars), with 2% in the cytoplasmic fraction (white bars) and 12% having incorporated into the total membrane fraction (black bars). At 30 min, 57% was in the media, while 36% was in the total membrane fraction, and 7% was in the cytoplasm. By 60 min, 65% had been taken up into the total membrane fraction, with 16% in the cytoplasmic fraction, and only 19% remaining in the media. These data indicate that sphingosine preferentially partitions into membranes.

Figure 16: Sphingosine is present in membrane rafts. $[^3]H$ sphingosine was added to the culture media of HEK293 cells. The percentage of $[^3]H$ sphingosine that partitioned from the media (gray) to the cytoplasmic fraction (white) and the membrane fraction (black) over time was determined. All changes are significant (not indicated, $p<0.05$).
We next determined whether sphingosine specifically localizes to membrane rafts. HEK293 cells that had been preincubated for 30 min with $[^{3}\text{H}]$ sphingosine were separated by high pH/carbonate sucrose gradient centrifugation. An aliquot of each fraction was scintillation counted. We found that 56.9% of $[^{3}\text{H}]$ sphingosine was found in the membrane raft fraction (F4), with the remaining fractions containing less than 10% of $[^{3}\text{H}]$ sphingosine each (Figure 17).

We eliminated the possibility that the $[^{3}\text{H}]$ sphingosine tracer had been metabolized to other sphingolipid metabolites using thin layer chromatography (TLC). We preincubated HEK293 cells for 30 min with $[^{3}\text{H}]$ sphingosine and then performed high pH/carbonate sucrose gradient centrifugation. Each fraction was applied to a TLC plate and the spots corresponding to ceramide, sphinganine, or sphingomyelin were scraped and scintillation counted. The highest amount of radioactivity was consistently found in the membrane raft fraction (Figure 18). There was a small amount of radioactivity in the spots that corresponded to ceramide and sphinganine, however, the majority of radioactivity was found in the membrane raft fraction of the sphingosine spot.
3.4.9 Changes in Sphingosine and S-1-P Levels Following FBS Stimulation

In order to confirm that SphK1 is active at membrane rafts, we examined changes in the levels of sphingosine and S-1-P in different cellular compartments following stimulation of SphK1. HEK293 cells that had been stimulated with FBS over a timecourse were separated by high pH/carbonate sucrose gradient centrifugation into a raft fraction (pooled fractions 3-5) and a dense fraction (pooled fractions 7-9). The levels of sphingosine and S-1-P in each fraction were determined by liquid chromatography/mass spectrometry (LC/MS). We found that sphingosine levels did not change following FBS stimulation in the dense fraction (Figure 19A, gray bars). In contrast, the sphingosine levels in the membrane raft fraction decreased 73.4% after 5 min and remained decreased after 30 and 60 min (black bars). In contrast, the S-1-P
levels in the raft fraction did not change over time (Figure 19B, black bars). We instead observed an increase in the S-1-P levels in the dense fractions (gray bars) at 5 min (294%), 30 min (401%), and 60 min (345%). We observed similar results in the media (white bars) at 5 min (225%), 30 min (329%) and 60 min (344%).

To further confirm LC/MS/MS data, we used TLC analysis. HEK293 cells that had been preloaded with [3H] sphingosine were stimulated with 10% FBS over a timecourse of 60 min and separated by sucrose gradient centrifugation. The sphingolipid composition of the membrane raft fractions and the dense fractions was analyzed by LC/MS. A: Distribution of sphingosine following FBS stimulation. The sphingosine levels decreased in the raft fraction (black bars), but did not change in the dense fractions (gray bars). B: Distribution of S-1-P following FBS stimulation. The S-1-P levels in the raft fraction (black bars) did not change, while they increased in the dense fractions (gray bars) and in the media (white bars).

Figure 19: Changes in sphingosine and S-1-P levels following FBS stimulation. HEK293 cells that had been pretreated with [3H] sphingosine were stimulated with FBS over a timecourse of 60 min and separated by sucrose gradient centrifugation. The sphingolipid composition of the membrane raft fractions and the dense fractions was analyzed by LC/MS. A: Distribution of sphingosine following FBS stimulation. The sphingosine levels decreased in the raft fraction (black bars), but did not change in the dense fractions (gray bars). B: Distribution of S-1-P following FBS stimulation. The S-1-P levels in the raft fraction (black bars) did not change, while they increased in the dense fractions (gray bars) and in the media (white bars).
timecourse and were then fractioned by sucrose density gradient centrifugation. The lipids were extracted from each fraction and separated by TLC. The amount of $[^3]$H sphingosine found in the membrane raft fraction was determined (Figure 20). As expected, we observed a decrease in the amount of $[^3]$H sphingosine present in the membrane raft fraction over time following FBS stimulation. Specifically, there was a 16.2% decrease at 5 min, a 32.7% decrease at 30 min, and a 36.1% decrease at 60 min. By 120 min, the sphingosine levels returned to baseline, indicating that the sphingosine decrease at the membrane raft in response to FBS stimulation was a transient response.

3.4.10 SphK1 Levels at the Membrane Raft Following FBS Stimulation

We next examined the amount of SphK1 protein present in membrane rafts following a timecourse of FBS stimulation (Figure 21, upper panel). The SphK1 protein levels at the membrane raft increased from 15 min (196%) to 60 min (239%), and then returned to baseline by 120 min. This change was not observed in the SphK1 present in the nonraft dense fractions (Figure 21, lower panel). These data suggest that a subset of SphK1 transiently moves into membrane rafts following stimulation with FBS.
The data presented in this chapter provide evidence that a pool of SphK1 localizes to plasma membrane raft microdomains in HEK293 cells. This pool of SphK1 exhibits a high specific activity, yet catalytic activity is not prerequisite for the membrane raft association. Metabolic labeling and mass spectrometric analyses identify that sphingosine is highly enriched in membrane rafts, which is then an accessible substrate for membrane raft associated SphK1. We observed a transient increase in the amount of SphK1 protein in membrane raft domains following FBS stimulation, which paralleled a decrease in sphingosine from these domains and a concomitant increase in both intracellular and extracellular S-1-P levels. Collectively, these data provide evidence that membrane rafts are sites for localized production of S-1-P by SphK1.

Figure 21: The levels of SphK1 present in membrane raft fractions over a timecourse of FBS stimulation. Upper panel: Membrane raft fractions. Lower panel: Nonraft dense fractions.

3.5 Discussion

The data presented in this chapter provide evidence that a pool of SphK1 localizes to plasma membrane raft microdomains in HEK293 cells. This pool of SphK1 exhibits a high specific activity, yet catalytic activity is not prerequisite for the membrane raft association. Metabolic labeling and mass spectrometric analyses identify that sphingosine is highly enriched in membrane rafts, which is then an accessible substrate for membrane raft associated SphK1. We observed a transient increase in the amount of SphK1 protein in membrane raft domains following FBS stimulation, which paralleled a decrease in sphingosine from these domains and a concomitant increase in both intracellular and extracellular S-1-P levels. Collectively, these data provide evidence that membrane rafts are sites for localized production of S-1-P by SphK1.
In all, we identified three distinct pools of SphK1 protein. The majority of SphK1 was found in the cytoplasmic fraction, as has been previously described [117, 129, 130]. This pool is likely basally active and may respond to mitogenic signals by translocating to the plasma membrane. We also identified a membrane associated pool of SphK1, which responded to FBS stimulation, while the cytoplasmic fraction did not. Within the plasma membrane, we identified a third SphK1 pool by confocal microscopy, which is present in membrane rafts. We confirmed the membrane raft association of SphK1 by pretreating cells with cholesterol binding agents, which disrupt membrane raft integrity. When we isolated membrane raft fractions following pretreatment, SphK1 protein and activity levels were depleted from those fractions.

Membrane rafts are believed to act as platforms for signaling molecules [112]. Although they contain only a small fraction of the total cellular protein, the majority of the protein present there is involved in cellular signaling events [131-133]. We found a disproportionately high amount of SphK1 enzymatic activity in membrane rafts. This enhancement of SphK1 activity in membrane rafts could indicate that these domains contain high levels of SphK1, which is unlikely based on the sucrose gradient data presented in Figure 12A. A second possibility is that the SphK1 protein present in membrane rafts could be in a highly activated state relative to the cytosolic or membrane associated pools, which would have been found in the dense fractions. A third possibility is that membrane rafts are highly enriched with substrate for SphK1, sphingosine.

Membrane rafts can distribute to specific locations on membranes, such as in polarized or migrating cells [134-136]. Similarly, activated SphK1 has been shown to translocate to the plasma membrane of extending neurons [57] and to the leading edge of migrating fibroblasts [129, 137]. We show a colocalization of SphK1 with the membrane raft marker GM-1 by confocal microscopy, particularly at the leading edge of HEK293 cells (Figure 15, white arrows). Recruitment of SphK1 to membrane rafts at the leading edge could result in localized formation of S-1-P, which could act extracellularly in the immediate microenvironment to stimulate S1PR1. This receptor has been shown to be associated with membrane rafts [138] and signals through molecules critical for
regulation of leading edge formation and migration, such as Rac, FAK [139], and Src [140]. These signaling molecules are also present at membrane rafts [141, 142], where they could locally and directionally amplify SphK1 signaling.

When we examined the cellular localization of sphingosine, we found that labeled sphingosine quickly accumulated in cell membranes. Specifically, we found that the membrane raft domain was highly enriched in sphingosine. This is in agreement with a previous report [143] that found the majority of endogenous sphingosine, but not phytosphingosine, in membrane rafts by HPLC analysis. The finding that sphingosine is predominantly a membrane raft associated sphingolipid is further supported by a study that demonstrated a specific interaction between sphingosine and cholesterol, resulting in the formation of condensed lipid complexes [144].

We observed that FBS stimulation results in a transient increase in SphK1 protein and depletion of sphingosine levels within membrane rafts. The decrease in sphingosine levels in the membrane raft could only be due to a conversion of sphingosine to ceramide or S-1-P. We did not observe a change in ceramide levels in any fraction (data not shown). We did observe an increase in S-1-P levels in the fractions that corresponded to the intracellular space (cytosolic) and the extracellular space (media). These results indicate that the pool of SphK1 that is found in membrane raft domains, though small, has preferential access to its substrate and is transiently inducible by FBS. Furthermore, the sphingosine that is depleted from membrane rafts is converted by SphK1 within the membrane raft microenvironment to the more soluble S-1-P, which is rapidly released to intracellular and extracellular spaces.

Further studies are necessary to determine how SphK1 biochemically associates with membrane rafts. Hydropathy-plot analysis of SphK1 does not predict any hydrophobic transmembrane regions [145]. Sphingosine has been described to be enriched in the outer leaflet of the plasma membrane. Preliminary studies using biotinylation or fluorescent labeling indicated that SphK1 is not cell surface exposed (data not shown), indicating that SphK1 could be partially inserted into the cytosolic face of the plasma membrane in order to access its substrate. Flippases may also cause sphingosine to translocate to the cytosolic leaflet of the plasma membrane. We had
previously observed that the catalytic activity of recombinant bacterial SphK1 protein responded positively to the reducing agents dithiothreitol (DTT) or β-mercaptoethanol in a dose dependent manner (shown in Appendix, Figure 41A). Interestingly, sucrose gradient samples prepared in lysis buffer containing β-mercaptoethanol have enhanced amounts of SphK1 in membrane raft fractions (Figure 41B), indicating that reducing a disulfide bond may be important in membrane raft association of SphK1. As there are no known disulfide bonds present within SphK1 itself, further study is necessary to explain these observations.

Myristoylation and palmitoylation can lead to raft localization. It has been reported that addition of a myristoylation sequence to SphK1 increased membrane associated SphK1 activity, resulting in a transformed phenotype [146]. The yeast sphingoid LCB (long chain base) enzymes do not show any membrane localization signals, yet significant SphK1 activity resides in the membrane fractions of these organisms [147]. Palmitylation at cysteine residues by the protein acyltransferase Akr1 was shown to be necessary for membrane localization of LCB4. Palmitylation by the human homologue (Hip14) at cysteine residues is a possible mechanism for human SphK1 membrane raft attachment [147]. Human SphK1 contains seven cysteine residues. However, preliminary studies using GFP-tagged site directed mutants at each of these residues did not result in a decrease of SphK1 present in membrane rafts as determined by confocal microscopy or sucrose gradient centrifugation (Appendix, Figure 42).

It is possible that SphK1 interacts with another membrane raft associated protein, such as lyn or a src family protein, which tethers SphK1 to the membrane raft. Studies performed by overexpressing SphK1 mutants show that truncating SphK1 after residue 220 abrogates membrane raft association of SphK1. This indicates that residues 221-384 must contain a regulatory region necessary for membrane raft association. This may include a phosphorylation event at Ser225 (Section 2.4.1). This interaction would be in addition to those involving Thr54 and Asp89, which are necessary for lipid selectivity and membrane targeting of SphK1 [59]. It is possible that a conformational change during enzymatic activation may target SphK1 to plasma membrane rafts and allow
SphK1 to be strongly associated there through multiple protein/protein and/or protein/lipid interactions.

The constitutively active SphK1 mutant truncated at residue 363 (Section 2.3.2) was observed to associate more strongly with the membrane raft fraction than wild type SphK1 (Appendix, Figure 43). This data suggests a link between SphK1 activity and membrane raft association. However, a dominant negative SphK1 was also identified in membrane rafts, in agreement with a report that SphK1 was able to translocate to membranes despite being catalytically inactive [148], indicating that catalytic activity is not prerequisite for SphK1 translocation to membrane rafts.

3.6 Model for Sphingosine Kinase 1 in Membrane Rafts

We propose a model for SphK1 signaling based on our findings as well as what is currently understood about the plasma membrane association of SphK1 (Figure 22). Upon stimulation, basally active cytosolic SphK1 is activated through Ca\(^{2+}\)/calmodulin-dependent signaling [149] and/or ERK-dependent phosphorylation of SphK1 [150], resulting in redistribution of SphK1 to the plasma membrane. Plasma membrane targeting of SphK1 has additionally been reported to derive from its selectivity for PS, which activates SphK1 by increasing substrate access and membrane affinity [151]. Calcium and other agonists have been demonstrated to cause PS to cluster together in membrane rafts, which could recruit SphK1 in membrane raft domains [119, 144].

Once at the membrane raft, the highly activated SphK1 catalyzes the phosphorylation of sphingosine to form S-1-P, which is released intracellularly, resulting in growth, differentiation, motility, and proliferative signaling. Alternatively, extracellularly released S-1-P can signal through S1PRs in an autocrine or paracrine fashion. We are not sure at this time how SphK1 encounters sphingosine found on the outer leaflet of the bilayer. It is possible that SphK1 is partially inserted in to the plasma membrane, or perhaps flippases are activated that make sphingosine more accessible. An alternative mechanism could be secretion of SphK1, where it would associate with the extracellular leaflet of membrane rafts.
Sphingomyelinases and ceramidases may be activated in concert with SphK1 activation, resulting in generation of additional sphingosine at membrane rafts [43, 152, 153]. SphK1 signaling at membrane rafts could be downregulated in a number of ways. SphK1 could be deactivated by dephosphorylation, it could be exported extracellularly [154], or S1PR receptors could be internalized [138]. Another possibility would be a negative feedback loop initiated from increased S-1-P levels, as described in mast cells [89].

Figure 22: Model of SphK1 localization and activation at the plasma membrane raft. Following stimulation (1), basally active cytosolic SphK1 is phosphorylated by ERK1/2 (2). Further interactions with Ca\(^2+/\)calmodulin are involved in the redistribution of activated SphK1 to the plasma membrane (3). Membrane rafts are highly enriched with sphingosine (sph), which is converted to S-1-P by SphK1 (4). Intracellular S-1-P is involved in multiple downstream signaling pathways (5). Alternately, S-1-P can be exported extracellularly (6). Compartmentalized signaling in the membrane raft microenvironment could be involved in downstream effects of S1PRs (7), such as migration (8).
3.7 Conclusions

Sphingolipid metabolites are now recognized as important messenger molecules in cell signaling pathways. SphK1 regulates cell growth by converting ceramide/sphingosine, proapoptotic signals, to S-1-P, a promitogenic signal. The studies presented in this thesis enable further understanding of SphK1 localization to the membrane raft microdomain of the plasma membrane, as well as transcriptional and post-translational activation of SphK1, in response to stimulation by growth factor.

An overall model of SphK1 activation based on the data generated in these studies is proposed in Figure 40. In response to a mitogenic stimulant, such as FBS or PMA, SphK1 has been observed to translocate from the cytosol, where it is basally active, to the plasma membrane, where it is in an activated state. Many events have been reported to be necessary for SphK1 plasma membrane association and enzymatic activation, including phosphorylation by ERK1/2 (gray rectangle) and calcium/calmodulin binding (dark gray moon). However, these events are not sufficient for stimuli-induced SphK1 membrane translocalization, indicating that additional factors or modifications are necessary. Growth factors also stimulate the activation of PKC (pathway shown in green). We show evidence that activated PKCα phosphorylates SphK1, resulting in a dually phosphorylated SphK1. This activated form of SphK1 is proposed to undergo a conformational change that allows key residues to bind favorably with PS at the plasma membrane, which further activates SphK1.
Chapter 4

Regulation of Sphingosine Kinase 1 Expression by PI3K/AKT

4.1 Introduction

SphK1 is implicated in signaling pathways that positively modulate vascular smooth muscle (VSM) cell growth [155, 156]. VSM cells are important structural components of blood vessels. While their activation at the site of a wound is beneficial, some pathological states occur as a result of VSM cell hyperproliferation, such as atherosclerosis [157, 158]. We believe that understanding how SphK1 is regulated at the expression level will identify novel therapeutic targets for treatment of hyperproliferative and inflammatory vascular diseases.

Activation of SphK1 is associated with migration, activation, differentiation, proliferation, and survival of activated VSM cells [159-162]. SphK1 is activated in response to factors that stimulate VSM cell growth, including PDGF [163], basic fibroblast growth factor [164], VEGF [165], oxLDL [166, 167], and hypoxic stress [168]. S-1-P present in serum has been shown to be a predictor of the occurrence and severity of coronary stenosis [69] and can be used as an early and accurate marker of myocardial infarction [169]. It has been proposed that the anti-atherogenic effects of HDL are due to inhibition of SphK1 activity in endothelial cells [170]. Therapeutic strategies using sphingolipid metabolites to target vascular lesions have been demonstrated by delivery of ceramide analogues, which prevented hyperproliferation of myocardium after injury [171, 172].

We believe that therapeutic strategies targeting the late phase (transcriptional/translational) activation of SphK1 in VSM cells would limit aberrant cellular proliferation. In this study, we use PDGF-stimulated human coronary artery smooth muscle (HCASM) cells. We examine which signaling pathways lie downstream of PDGF stimulation, yet upstream of SphK1 stimulation. There was some evidence that
the PI3K/AKT pathway might be involved. In the human breast cancer cell line MCF7, epidermal growth factor stimulated SphK1 late phase activation, accompanied by increased SphK1 mRNA and protein expression, which was determined to be dependent on PI3K [173]. OxLDL-induced VSM cell proliferation has been shown to require activation of both SphK1 and AKT [167, 174]. A study in monocytic cells also linked the activation of SphK1 to the PI3K/AKT pathway [175].

4.2 Hypothesis

Although SphK1 is a critical modulator of VSM cell growth, the regulatory mechanisms of SphK1 expression during the late phase response in VSM cells are not known. The purpose of this study is to elucidate the mechanism(s) involved in the regulation of SphK1 expression in PDGF-stimulated HCASM cells. We expect that SphK1 is required for HCASM cell growth and that SphK1 mRNA and protein levels are elevated in response to PDGF stimulation. One promitogenic signaling pathway that is known to be activated in response to PDGF is the PI3K/AKT pathway. We hypothesize that regulation of SphK1 expression occurs through PI3K/AKT signaling.

4.3 Methods

4.3.1 Cell Culture

The primary HCASM cell line (CAMBREX, East Rutherford, NJ) was cultured in Medium 231 with 10% SMGS (smooth muscle growth supplement containing 5% fetal bovine serum, basic fibroblast growth factor, epidermal growth factor and insulin; Cascade Biologics, Portland, OR) in the presence of penicillin/streptomycin at 37°C in a humidified atmosphere of 5% carbon dioxide. All experiments were performed with cells that had been passaged fewer than ten times.
4.3.2 \[^3\text{H}\] Thymidine Incorporation

HCASM cells, plated at $5 \times 10^4$ cells/well into six-well culture dishes, were cultured in the absence of smooth muscle growth supplement (SMGS) for 18 h to induce growth arrest. Cells were subsequently treated with either vehicle (DMSO, dimethylsulphoxide) or a SphK1 inhibitor (2-(p-Hydroxyanilino)-4-(p-chlorophenyl) thiazole, Calbiochem, San Diego, CA)[3] at 1, 5, and 10 \(\mu\text{M}\) for 40 min. The cells were then stimulated with 10\% SMGS for 24 h. Cells were radiolabeled with 0.5 \(\mu\text{Ci/mL}\) \[^3\text{H}\] thymidine (ICN Radiochemicals, Costa Mesa, CA) during the last 4 h of stimulation with SMGS. The cells were then washed with cold PBS and genomic DNA was precipitated with cold 10\% TCA. Acid-precipitated DNA was solubilized in 0.1 N NaOH and the radioactivity was determined by scintillation counting. The amount of \[^3\text{H}\] thymidine incorporated into DNA was used as an indicator of DNA synthesis.

4.3.3 Cell Growth Studies

HCASM cells were seeded into six-well culture dishes ($5 \times 10^4$ cells/well). After 24 h, the media was changed to SMGS-free to induce growth arrest. After 18 h, the cells were treated with vehicle (DMSO) or 8 \(\mu\text{M}\) SphK1 inhibitor for 40 min and then stimulated with 10\% SMGS. Viable cells, as determined by trypan blue exclusion, were counted using a hemocytometer over a period of 5 days.

Growth studies in the presence of SphK1 siRNA were performed on HCASM cells that had been seeded into six-well culture dishes ($5 \times 10^4$ cells/well). After 24 h, cells were transfected with 200 nM AllStars Negative Control siRNA (Qiagen) or 200 nM SphK1 siRNA (Qiagen) using Lipofectamine 2000 (Invitrogen). The media was changed each day to provide optimal conditions for cell growth. Cells were counted using a hemocytometer as described above.
4.3.4 Real Time PCR

To quantitatively determine the SphK1 mRNA levels following PDGF treatment, HCASM cells were grown in the absence of SMGS for 18 h and then stimulated with 12.5 ng/mL PDGF (Cell Sciences, Canton, MA). Relative RT-PCR was used to determine the SphK1 mRNA levels following PI3K/AKT inhibitor treatment. Total RNA was extracted from HCASM cells using TRI reagent (Molecular Research Center, Cincinnati, OH) and treated with DNase (Ambion, Foster City, CA). cDNA was synthesized from equal quantities of RNA using the Superscript III Kit and Random Decamer Primers. Following the preliminary experiments to determine the linear range of DNA amplification, target primers were used to amplify PCR products for SphK1 (primers: FWD 5’-CCT TCC TCC TTC CCT AGG G-3’; REV 5’-TAG AAG GCC TTA CAT AGG CAG C-3’). As an internal control, 18S ribosomal RNA primers (QuantumRNA™ Classic 18S Internal Standard, Ambion) were also included in the PCR reaction mix with Promega’s GoTaq enzyme. Target and control products were quantified by normalizing to the 18S ribosomal RNA levels.

Quantitative real time PCR (QRT-PCR) was used to examine the effects of isoform-specific knockdown of AKT on SphK1 mRNA levels. Total RNA and protein were isolated using the Nucleospin RNA/Protein Kit (Machery & Nagel, Düren, Germany). Total RNA quality and quantity was assessed using the RNA 6000 Nano LabChip with an Agilent 2100 Expert Bioanalyzer (Agilent, Palo Alto, CA). cDNA synthesis was performed on total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). 500ng of total RNA from each sample was added to a 2X master mix (10X RT Buffer, 10X RT Random Primers, 25X dNTP Mix (100mM), Multiiscribe Reverse Transcriptase (50U/reaction) and RNase Inhibitor). The 20 µL reaction was then incubated at 25°C for 10 min, 37°C for 120 min and 85°C for 5 sec. QRT-PCR was performed on the ABI 7900HT Sequence Detection System using Assay on Demand primers and probes and TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA). Primer/probes used for analysis were: Sphk1 (Hs00184211_m1), Akt1 (Hs00178289_m1), Akt2 (Hs00609846_m1), Akt3
(Hs00178533_m1) and beta-actin (Hs99999903_m1). 10 µl final reaction volumes included: 2X TaqMan Universal Master Mix with UNG, 450nM unlabeled PCR primers, 125nM FAM dye-labeled TaqMan MGB probe, and 2 µL cDNA reaction product. PCR conditions were 2 min at 50°C, 10 min at 95°C and 40 cycles of 15 sec at 95°C and 1 min at 60°C. ABI SDS 2.2.2 software and the $2^{\Delta\Delta\text{Ct}}$ analysis method [176] were used to quantitate relative amounts of product using beta-actin as an endogenous control.

4.3.5 Western Blot Analysis

Refer to section 3.3.4 for detailed methods. Antibodies used in this chapter include our own anti-SphK1, anti-phospho-AKT (Cell Signaling Technology, Danvers, MA), anti-phospho-PKC (Cell Signaling Technology), anti-GAPDH, anti-AKT1, and anti-AKT2 (Santa Cruz Biotechnology), and β-actin (Chemicon International).

4.3.6 Sphingosine Kinase Activity Assay

Refer to section 3.3.

4.3.7 Treatment with PI3K, AKT, and mTOR Inhibitors

HCASM cells that had been grown in the absence of SMGS for 18 h were treated with 100 nM wortmannin (Biomol, Plymouth Meeting, PA), 5 µM SH-5 (Alexis Biochemicals), 100 nM rapamycin (Biomol), or vehicle (DMSO) for 30 min before stimulation with 12.5 ng/mL of PDGF. Analysis of SphK1 mRNA and protein expression levels were performed on cells harvested 3 h and 24 h post-stimulation, respectively. mRNA levels were determined by relative RT-PCR. Protein levels were determined by Western blot with β-actin or GAPDH as a loading control.
4.3.8 siRNA for AKT Isoforms

HCASM cells that were plated into six-well culture dishes (5 × 10^4 cells/well) were transfected with 100 nM siRNA for AKT1, AKT2 or AKT3 (Dharamcon, Lafayette, CO) using Lipofectamine 2000 (Invitrogen). Approximately a 50% transfection efficiency of siRNA was observed using 100 nM rhodamine labeled scrambled siRNA (Ambion). 24 h post-transfection, the media was changed to regular growth media. An additional 24 h later, the media was changed to SMGS free media for 20 h. The cells were then stimulated with 12.5 ng/mL PDGF. SphK1 mRNA (real time PCR) and protein (Western blot) analyses were performed 3 h and 10 h later, respectively.

4.4 Results

4.4.1 Growth Studies in the Presence of SphK1 Inhibitors

In order to determine if SphK1 is necessary for HCASM cell proliferation, [³H] thymidine incorporation was used to determine cell growth rates in the presence of a specific inhibitor of SphK1 (2-(p-Hydroxyanilino)-4-(p-chlorophenyl)). A dose response study was performed to establish the SphK1 inhibitor concentration required for growth inhibition of HCASM cells by measuring the rate of DNA synthesis. As shown in Figure 23A, HCASM cells pretreated with 1 µM or 5 µM SphK1 inhibitor showed an approximately 40% reduction in DNA synthesis compared to the vehicle treated cells. Greater inhibition of cell growth (74%) was observed with 10 µM SphK1 inhibitor, indicating that SphK1 activity is necessary for HCASM cell growth.

The necessity of SphK1 during cellular proliferation was confirmed by cell growth assays. The growth of HCASM cells treated with either vehicle (DMSO) alone or 10% smooth muscle growth supplement (SMGS) plus either vehicle or SphK1 inhibitor (8 µM) was determined over a period of 5 days. HCASM cells stimulated with SMGS exhibited a 4.8 fold increase in cell number by day 5 (Figure 23B, solid circles).
contrast, when HCASM cells were pretreated with SphK1 inhibitor, SMGS stimulated cell growth was completely abolished (open circles). The cells pretreated with SphK1 inhibitor appeared to growth arrest in a similar manner to cells that were grown in media without SMGS (closed triangles).

SphK1 siRNA was also used to further confirm that SphK1 is indispensable during HCASM cell growth (Figure 23E). Cells transfected with nonspecific siRNA grew normally (black circles), whereas cells transfected with SphK1 siRNA appeared to enter a state of growth arrest (white circles). Morphologically, the cells appeared healthy and free of cellular debris, indicating that the siRNA treatment did not induce cell death (not shown). The ability of the SphK1 siRNA to reduce SphK1 mRNA levels over a period of 4 days is shown in Figure 23C. Cells transfected with nonspecific siRNA are represented by gray bars. Cells transfected with SphK1 siRNA are represented by black bars. One day following the siRNA transfections, the SphK1 mRNA levels in SMGS-stimulated cells were decreased by 49% compared to cells that had been transfected with the nonspecific siRNA. By day 4, the SphK1 levels in the SphK1 siRNA treated cells were decreased to 77% compared to the nonspecific siRNA treated cells, indicating that the siRNA was effective up to at least 96 h following transfection. A corresponding decrease in SphK1 enzymatic activity was observed (Figure 23D). On day 1, the SphK1 activity was decreased by 43% in cells transfected with SphK1 siRNA compared to nonspecific siRNA. By day 3, this decrease reached 65%. These data indicate that SphK1 expression and activity are necessary for HCASM cell growth.
Figure 23: SphK1 is necessary for HCASM cell growth. A: $[3H]$ Thymidine incorporation following treatment with vehicle (DMSO) or increasing concentrations of SphK1 inhibitor. B: Growth studies in the presence of vehicle (DMSO, solid triangles), SMGS with vehicle (solid circles), or SMGS with 8 µM SphK1 inhibitor (open circles). C: SphK1 mRNA levels over a timecourse of transfection with nonspecific siRNA (gray bars) or SphK1 siRNA (black bars). D: SphK1 activity levels over a timecourse of transfection with 200 nM nonspecific siRNA (gray bars) or SphK1 siRNA (black bars). E: Growth studies in cells that had been transfected with 200 nM nonspecific siRNA (solid circles) or SphK1 siRNA (open circles). *p < 0.05, N=3.
4.4.2 PDGF Stimulates SphK1 mRNA, Protein Expression, and Catalytic Activity

Having established that SphK1 is required for HCASM cell proliferation, we next examined whether PDGF treatment would stimulate SphK1 mRNA expression in HCASM cells. In cells that were stimulated with PDGF (12.5 ng/mL), SphK1 mRNA levels significantly increased by 276% at 1 h and peaked at 385% at 3 h compared to the vehicle treated HCASM cells (Figure 24A). At 6 h post-stimulation, a sustained 295% elevation of SphK1 mRNA levels was observed. By 8 h, the SphK1 mRNA levels returned to the basal level.

We next examined the relative expression levels of SphK1 protein following PDGF stimulation using Western blot analysis to determine if there is a correlation with increased mRNA levels (Figure 24B). A significant increase in SphK1 protein expression was observed at 8 h (156%), which was sustained for 24 h (161%). This increase in SphK1 protein levels correlated with an increase in SphK1 enzymatic activity levels (Figure 24C). Specifically, SphK1 activity levels were increased by 132% within 15 min of PDGF stimulation, indicative of an early phase response. A late phase, sustained upregulation of SphK1 activity, likely due to increased SphK1 protein expression levels, was also observed from 8 h (144%) up to 20 h (140%).
Figure 24: Effect of a timecourse of PDGF stimulation on SphK1 levels in smooth muscle growth supplement-deprived HCASM cells. A: SphK1 mRNA levels determined by relative RT-PCR. B: SphK1 protein expression levels determined by Western blot. C: SphK1 activity levels determined by the in vitro SphK1 activity assay. *p < 0.05, N=3.
4.4.3 PDGF Activates AKT

Once we had established that PDGF stimulation resulted in upregulation of SphK1 expression and its catalytic activity, we next examined the signaling molecules that modulate SphK1 expression and activation in HCASM cells. In response to mitogenic factors, two promitogenic signaling pathways that have been linked to activation of SphK1 signaling are the PI3K/AKT pathway [66, 101] and the PKC pathway [45, 94, 177]. To determine if either of these pathways are involved in upregulation of PDGF-stimulated SphK1 expression, we examined the levels of phospho-AKT and phospho-PKC levels during a timecourse of PDGF stimulation (Figure 25). Phospho-AKT levels showed at dramatic tenfold increase 15 min following stimulation, which decreased thereafter. In contrast, the phospho-PKC levels did not change in response to PDGF treatment in this cell type. These data indicate the PDGF activates the AKT signaling pathway rapidly and that signaling intermediates in the AKT pathway could lie upstream of transcriptional/translational regulation of SphK1 expression.
Figure 25: Levels of phospho-AKT and phospho-PKC during a timecourse of PDGF stimulation. A: Western blot analysis. B: Quantification of phospho-AKT levels. C: Quantification of phospho-PKC levels. *p < 0.05.
4.4.4 Pharmacological Inhibition of the PI3K/AKT Pathway

We demonstrated that PDGF induces SphK1 expression and catalytic activity. Additionally, we show that PDGF stimulates AKT activity in HCASM cells. To further determine whether the PI3K/AKT signaling pathway is involved in regulation of SphK1 expression, we used pharmacological inhibitors that target the PI3K, AKT, and mTOR pathways. HCASM cells grown in the absence of SMGS were pretreated for 30 min with a PI3K inhibitor (100 nM wortmannin), an AKT inhibitor (5 µM SH-5), an mTOR inhibitor (100 nM rapamycin), or vehicle (DMSO) for 30 min before stimulation with PDGF (12.5 ng/mL). The effect of these inhibitors on mRNA levels was examined at 3 h post-stimulation using RT-PCR (Figure 26A). There was a 244% increase in SphK1 mRNA expression in PDGF-stimulated cells compared to vehicle treated cells. In contrast, SphK1 mRNA levels in cells pretreated with rapamycin only increased 137% in response to PDGF. Similarly, increases in the levels of SphK1 mRNA were limited in PDGF-stimulated cells that had been pretreated with wortmannin (142%) or SH-5 (94%).

In agreement with SphK1 mRNA levels, SphK1 protein levels in HCASM cells stimulated with PDGF for 10 h increased 199% compared to vehicle treated cells, whereas PDGF stimulated SphK1 protein expression levels were inhibited in HCASM cells pretreated with PI3K/AKT pathway inhibitors (Figure 26B). Specifically, PDGF-stimulated SphK1 protein levels only increased 136% following pretreatment with rapamycin, only 137% after wortmannin pretreatment, and only 88% with SH-5 pretreatment. No significant change in SphK1 mRNA or protein expression was observed in cells pretreated with these inhibitors in the absence of PDGF (data not shown), indicating that inhibiting the PI3K/AKT pathway does not alter basal expression of SphK1.
Figure 26: PDGF-stimulated SphK1 expression is regulated through the PI3K/AKT pathway. A: SphK1 mRNA levels following pretreatment with vehicle (DMSO) or PI3K/AKT pathway inhibitors: 100 nM rapamycin, 100 nM wortmannin, or 5 µM SH-5, followed by stimulation with PDGF (12.5 ng/mL). Western blot analysis for SphK1 protein on PDGF-stimulated HCASM cell lysates that had been pretreated with PI3K/ATK signaling pathway inhibitors. Data in panel B was collected by Arpita Nag. *p < 0.05.
4.4.5 Isoform Specific Knockdown of AKT

Pharmacological inhibitor studies indicated that AKT is a key molecule in regulation of SphK1 expression. Hence, we next examined which AKT isoform specifically regulates SphK1 expression in HCASM cells using siRNAs (100nM) against AKT1, AKT2, and AKT3. Two days post-transfection with siRNA specific for each isoform, the media was changed to SMGS-free. The following day, the cells were stimulated with PDGF. RNA and protein samples were collected 3 h later. Isoform-specific knockdown of AKT mRNA levels was demonstrated using QRT-PCR (Figure 27A) normalized to β-actin levels. The siRNA were highly effective, knocking down AKT1 levels by 78%, AKT2 levels by 80%, and AKT3 levels by 91%. Knockdown of one AKT isoform did not significantly change the levels of other isoforms, demonstrating the isoform selectivity of the AKT siRNAs. Additionally, the PDGF stimulation did not increase levels of any of the AKT isoforms, indicating that AKT expression is not stimulated by PDGF treatment in HCASM cells (compare each white bar to the adjacent black bar).

We also examined the amount of protein knockdown resulting from AKT siRNA treatment (Figure 27B). We found the siRNA to be highly effective at reducing AKT1 levels by 83% and AKT2 levels to below detectable levels. Unfortunately we could not detect AKT3 protein levels due to limitations of the available anti-AKT3 antibodies. We do, however, expect that AKT3 levels would also be reduced based on the observation that AKT3 mRNA levels responded to siRNA treatment the most effectively.
Figure 27: Effect of isoform-specific AKT knockdown on PDGF-stimulated SphK1. A: AKT1, AKT2, and AKT3 mRNA levels. White and black bars indicate that cells were transfected with a nonspecific siRNA. Dark horizontal bars indicate AKT1 siRNA, light gray bars indicate AKT2 siRNA, and checkered bars indicate AKT3 siRNA (100 nM each). B: Evidence of knockdown of AKT1 and AKT2 protein levels.
4.4.6 SphK1 Expression is Regulated by AKT2

We next examined the effect of the isoform-specific AKT siRNAs on SphK1 mRNA levels. As we had previously observed, a 3 h PDGF stimulation of HCASM cells transfected with nonspecific control siRNA increased SphK1 mRNA levels almost twofold (Figure 28A). Reduction of AKT1 or AKT3 levels with siRNA did not have an effect on the PDGF-induced increase in SphK1 mRNA levels. In contrast, knockdown of AKT2 significantly blocked PDGF-stimulated increases in SphK1 mRNA levels, indicating that AKT2 plays a critical role in upregulating transcription of SphK1.

We also determined if knocking down the AKT isoforms would have an affect on SphK1 protein expression levels after 10 h of PDGF stimulation. We found that the protein levels correlated with the changes observed in mRNA levels (Figure 28B). We observed a 138% increase in SphK1 protein levels following PDGF stimulation in cells that had been transfected with the nonspecific siRNA. AKT2 knockdown suppressed the response to 112%. In contrast, suppressing AKT1 or AKT3 did not significantly alter the SphK1 response to PDGF at the protein level.

We observed similar changes in SphK1 enzymatic activity (Figure 28C). Stimulation with PDGF increased SphK1 catalytic activity 118%. This small but significant increase in SphK1 activity was not blocked by transfection with AKT1 or AKT3 siRNA. Knocking down AKT2, however, did reduce SphK1 activity levels to 79%, indicating that the downregulation of AKT2 modulates SphK1 activity. These results indicate that AKT2 is involved in regulation of SphK1 mRNA, protein, and enzymatic activity levels in response to PDGF stimulation in HCASM cells.
Figure 28: Knockdown of AKT2 reduces PDGF-stimulated increases in SphK1 levels. A: SphK1 mRNA levels determined by QRT-PCR. B: SphK1 protein levels by Western blot. C: SphK1 activity levels determined by the SphK1 activity assay. *p < 0.05, N=3.
4.5 Discussion

SphK1 is a critical signaling molecule that modulates VSM cell growth in response to growth factors. We report that SphK1 is necessary for proliferation of HCASM cells. As SphK1 is believed to be upregulated in hyperproliferative vascular diseases, we sought to determine regulatory mechanisms of SphK1 expression in VSM cells. It has been previously demonstrated that the PI3K/AKT signaling pathway also plays an integral role in proliferation of VSM cells [178, 179]. As SphK1 and AKT have been shown to both respond to promitogenic stimuli such as PDGF [178], NGF [180], and oxLDL [181], we hypothesized that the PI3K/AKT pathway could regulate SphK1 expression in HCASM cells.

PDGF has been demonstrated to stimulate SphK1 [182], possibly involving intracellular calcium release [183, 184]. We observed a biphasic activation of SphK1 activity following PDGF stimulation. The late phase activation of SphK1 occurred through transcriptional and translational upregulation of SphK1 expression, resulting in sustained heightened protein levels and catalytic activity. Our results are in agreement with reports using rat pancreatic islets [185], INS-1 cells [185], and EA.hy 926 human arterial endothelial cells [177], in which cytokines increased SphK1 mRNA levels at approximately 3 h post-stimulation and SphK1 protein and activity levels within 2-24 h.

We observed that PDGF rapidly increased phospho-AKT levels within 15 min, which decreased thereafter. The timeframe of AKT activation is realistic for AKT activation to be upstream of SphK1 transcriptional upregulation. SphK1 expression has also been shown to be regulated by PKC and ERK signaling pathways in other cell types [94, 177]. We did not observe that PDGF activated SphK1 though the PKC pathway, which is in agreement with a previous report [186]. Examination of ERK activation through PDGF signaling was inconclusive. It is likely that separate signaling pathways control the regulation of SphK1 expression in a stimulus- and cell type- specific manner.

Inhibiting PI3K or downstream targets, such as AKT and mTOR, significantly reduced PDGF-stimulated SphK1 mRNA and protein expression in HCASM cells, indicating that these signaling molecules are involved in regulating SphK1 translational
and transcriptional activation in response to PDGF stimulation. We further examined the regulatory role of AKT isoforms using siRNA against AKT1, AKT2, and AKT3. Silencing AKT2, but not AKT1 or AKT3, significantly inhibited the PDGF-stimulated increase in SphK1 mRNA expression that was observed 3 h post-stimulation. We observed a corresponding decrease in SphK1 protein and enzymatic activity levels following AKT2 siRNA treatment. Interestingly, AKT2 siRNA treatment reduced SphK1 activity levels, but not mRNA or protein levels, to below basal levels. We also observed that downregulating AKT3 enhanced the response of SphK1 protein and activity response to PDGF. We are not sure of the significance of AKT3 in regulation of SphK1 at this time.

It is logical to suggest that AKT2 could regulate SphK1 expression, as both SphK1 and AKT2 have been identified as possible oncogenes, propagate antiapoptotic signals, and are involved in migration. Among the members of AKT family, AKT2 is associated with the development of human cancers, including breast, ovarian [187], and pancreatic [188]. AKT2, but not AKT1, is a predictor for the development and progression of hepatocarcinogenesis [189]. There is evidence that AKT2 mediates PI3K-dependent effects on adhesion, motility, invasion, and metastasis in vivo [190]. AKT2 plays an important role in glioma cell motility and invasion [191]. A constitutively active AKT2 inhibited apoptosis in response to UV and TNFα-induced apoptosis [192]. AKT2 down-regulation suppressed morphological conversion and inhibited migration in EGF-stimulated cells [193]. During skeletal muscle differentiation, AKT2, but not AKT1, expression was significantly increased [194].

It has been reported that AKT2, but not AKT3, is upstream of mTOR activation [195], indicating that it is reasonable to suggest that PDGF signaling travels from the PDGF receptor, though AKT2 and mTOR, resulting in upregulation of SphK1 expression that stimulates HCASM cell proliferation. At the gene level, the transcription factor specificity protein 1 has been demonstrated to regulate NGF-induced SphK1 gene expression in PC12 cells [180]. It would be of interest to determine whether the same transcription factor was involved in PDGF-stimulated transcriptional activation of SphK1. Interestingly, one of the S-1-P receptors on the cell surface, S1PR3, has been
shown to cross-talk with the PDGF receptor, resulting in signals that activate AKT [105]. This suggests that S-1-P secreted in response to PDGF-stimulated upregulation of SphK1 could continue to activate the PDGF receptor, even in the absence of PDGF, thus perpetuating promitogenic signaling. A link to possible AKT2/SphK1 signaling in membrane rafts is possible, considering that membrane raft-associated AKT2 was found to be involved in enterocyte differentiation [196].

Based on our findings and other studies, we propose a model of SphK1 expression and activation in HCASM cells (Figure 29). In this model, binding of PDGF to its receptor activates PI3K and its downstream effectors, including AKT2 and mTOR. Subsequently, SphK1 transcription levels are enhanced, resulting in increased SphK1 protein synthesis. The accumulated SphK1 enzyme phosphorylates sphingosine to elevate S-1-P levels, resulting from increased SphK1 enzymatic activity. S-1-P transmits mitogenic signals via binding to unidentified intracellular targets or via secretion extracellularly, where it can bind to S-1-P receptors on the cell surface of the same or neighboring cells. Aberrant activation of SphK1 could alter sphingolipid metabolism in favor of S-1-P, resulting in pro-inflammatory and/or hyperproliferative cellular responses, making SphK1 activation through AKT2 signaling an attractive target for vascular diseases such as atherosclerosis and cancer.
4.6 Conclusion

We provide evidence that SphK1 is an indispensable signaling molecule in the growth of HCASM cells. We further demonstrate that PDGF-stimulated upregulation of SphK1 transcription and translation takes place though PI3K/AKT signaling. Using a panel of pharmacological inhibitors, we show that the PI3K/AKT/mTOR pathway modulates SphK1 expression. Specifically, we have identified that this regulation takes place in an AKT isoform-specific manner. PDGF-stimulated increases in SphK1 mRNA, protein, and enzymatic activity levels were abrogated in cells that had been transfected with AKT2 siRNA, but not AKT1 or AKT3 siRNA. Our results indicate that targeting AKT2 activation of SphK1 may result in decreased proliferation of VSM cells.
Chapter 5

Regulation of Sphingosine Kinase 1 Activation by Protein Kinase C

5.1 Introduction

PKC comprises a large family of promitogenic serine/threonine kinases (Section 2.4.2). Several lines of evidence suggest that SphK1 activation is downstream of PKC signaling. Firstly, inhibitors of PKC block SphK1 activation in HEK293 cells [45], human leukemia HL-60 cells [48], HUVECs [197], human erythroleukemia (HEL) cells [56], and T24 bladder tumor cells [197]. Secondly, inhibition of apoptosis has been reported to result from activation of PKC signaling through SphK1 [28]. Thirdly, ischemic preconditioning has been reported to activate SphK1 in a PKC-dependent manner [93]. Interestingly, sphingosine, which opposes the effects resulting from SphK1 activation, is a potent competitive inhibitor of PKC [198].

Activators of PKC have been shown to regulate both phases of SphK1 activation. PMA transiently stimulated SphK1 activity in T24 cells, peaking at 20 min [197]. This is indicative of an early phase activation, involving translocation of SphK1 to the plasma membrane where it may be brought in close proximity with activating anionic lipids [79, 199]. In HEK293 cells, PMA has been reported to stimulate a rapid increase in membrane-associated SphK1 activity, which is accompanied by translocation of SphK1 to the plasma membrane [45]. PKC inhibitors block SphK1 translocation, indicating that plasma membrane translocation of activated SphK1 is mediated by PKC. Late phase activation of SphK1, involving transcription/translation, was observed to occur in a PMA- and DAG-specific manner in human erythroleukemia cells [56]. PMA treatment also led to sustained upregulation of the SphK1 protein expression and activity in endothelial cells [177].

PKC-mediated early phase activation of SphK1 is presumed to involve a post-translational modification, such as a phosphorylation event. PMA has been shown to
induce phosphorylation of SphK1, but not basal SphK1 activity, indicating that PKC-mediated phosphorylation of SphK1 may be required for translocation of SphK1 [197]. In vitro phosphorylation and activation of recombinant SphK1 by purified PKC has been shown to occur in an ATP-dependent manner, which is blocked by inhibitors of PKC [197]. In vivo studies have demonstrated that SphK1 is phosphorylated in response to PMA [45] and VEGF [197], further suggesting that SphK1 may be a direct substrate of PKC.

5.2 Hypothesis

Although the exact molecular mechanisms of SphK1 activation are unknown to date, there is evidence that PKC is involved in translocation and activation of SphK1. We hypothesize that PKC directly phosphorylates SphK1 in an isoform-specific manner, resulting in heightened SphK1 activity. The goal of this study is to identify an isoform of PKC that directly phosphorylates SphK1 and also to determine which residue of SphK1 is phosphorylated by PKC.

5.3 Methods

5.3.1 Cloning of Wild Type and Mutant Sphingosine Kinase 1

Human SphK1 was cloned from HEK293 cells as described in Section 3.3.2. The SphK1 cDNA was directionally cloned into the EcoRI (5’) and Not I (3’) sites of pGEX4t-3 (Amersham Biosciences) for bacterial expression as an NH$_2$-terminally tagged GST-fusion protein. For mammalian expression, an amino-terminally tagged vector (HISB) and a carboxyl-terminally tagged vector (myc HIS) were created in the same way as the pGEX4t-3 vector. The resulting wild-type SK1 clone was sequence verified and used for subsequent mutagenesis experiments.
SphK1 mutants were generated using the Quikchange Site-Directed Mutagenesis system (Stratagene, La Jolla, CA) according to the manufacturer's recommendations. The primer sets used are:

- **Thr54Glu**
  - FWD: 5’-CACGCTGATGCTTGAGGAGCGGCGGAACC-3’
  - REV: 5’-GGTTCCGCGCTCTGAGCATCAGCGTG-3’

- **Ser371Ala**
  - FWD: 5’-AGCCCCCGCCCCGCTGGAAAGCCCC-3’
  - REV: 5’-GGGGCTTCCAGGCAGGCGGGGGGCT-3’

- **Ser371Asp**
  - FWD: 5’-GAGCCCCCGCCCCGACTGGAAGCCCCA-3’
  - REV: 5’-TGCCCCGCTTCCAGGCGGGGGGCTC-3’

- **Ser181Ala**
  - FWD: 5’-CTGATGTTGACCTAGAGGCTGAGAAGTCG-3’
  - REV: 5’-CGCCGATACCTTCTCAGCTCTAGGCTACCATCA-3’

- **Ser181Asp**
  - FWD: 5’-GCTGATGTTGACCTAGAGGATGAGAAGTCG-5’
  - REV: 5’-ACGCCGATACTTCTCATCTCTAGGCTACATCAG-3’

- **Thr205Ala**
  - FWD: 5’-CAGCCCTGCGCGCATCCGCGGGCC-3’
  - REV: 5’-GGCCGCGGTAGGCGCGCAGGGCTG-3’

- **Thr205Glu**
  - FWD: 5’-CGTCTGGCAGCCCTGCGCGAGTCGCGCGCG-3’
  - REV: 5’-GGCCACGGCGGTACTCGCGCGAGGGGCTCAG-3’

- **Ser225Ala**
  - FWD: 5’-AAGACACCTGCGCCCGCCCTTGTGTC-3’
  - REV: 5’-GACCACAACGGGCGGCGAGGTG-3’

5.3.2 Cell Culture

HEK293 cells and NIH-3T3 fibroblasts were cultured as described in Section 3.3.1.
5.3.3 Western Blot Analysis

Please refer to section 3.3.4 for detailed methods. Antibodies used in this section include anti-HIS (BD Biosciences), anti-GST and anti-GFP (Santa Cruz Biotechnology). We also used anti-phosphoserine and anti-phosphothreonine (Invitrogen).

5.3.4 Sphingosine Kinase Activity Assay

Refer to Section 3.3.7. Membrane and cytoplasmic fraction isolation is described in Section 3.3.3.

5.3.5 Purification of Recombinant Sphingosine Kinase 1

For purification of GST-tagged recombinant SphK1 from bacteria, the pGEX-SphK1 vectors were transformed into BL21-AI E. coli (Invitrogen). Inclusion of the GST-fusion partner aided in solubilization of overexpressed proteins and allowed for protein purification. Overnight cultures (4 mL) of transformed isolates were grown with shaking (250 rpm) at 37°C in 2xYT (16 g/L bacto tryptone, 10g/L bacto yeast extract, and 5 g/L NaCl, pH 7.0) containing ampicillin (100mg/L). The cultures were diluted 1:10 into fresh medium and grown at 30°C until reaching an OD₆₀₀ of 0.8. The culture was then induced with arabinose (0.2%) followed 30 min later with IPTG (40 uM) to activate the promoter and transcribe the transgene, respectively. The culture was then further incubated at 30°C for 3 h. The cells were then harvested by centrifugation at 700 x g for 10 min at 4°C. The resulting pellet was resuspended in 10 mL of Resuspension Solution (1x PBS containing 15 mM NaF, 1 mM sodium orthovanadate, 5 mg lysozyme, protease inhibitors). Resuspended samples were rocked at 4°C for 1 h, followed by sonication 3x each for 30 s on ice. The lysate was then clarified by centrifugation at 22,000 x g for 10 min at 4°C. The supernatant was centrifuged a second time at 100,000 x g for 30 min. The lysate was filtered through 0.45 µm filters and applied to a pre-equilibrated GST
HiTrap Affinity Column (Amersham). The column was washed with Binding Buffer (1x PBS containing 140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.3). GST-SphK1 fusion proteins were eluted with Elution Buffer (50 mM Tris HCl pH 8, 10 mM reduced glutathione, 5 mM DTT, and protease inhibitors). The fractions containing purified protein were concentrated (Amicon Ultra, Millipore).

For purification of HIS-tagged SphK1 from mammalian cells, HEK293 cells were transfected with wild type or mutant HIS-SphK1 and stable lines were generated. Just before reaching confluency, the cells were harvested. To limit non-specific binding to the HIS column, mammalian lysates were preincubated for 30 min at 37°C with 50 mM Tris HCl (pH 7.4), 2 mM ATP, and 10 mM MgSO₄. After 10 min, the mixture was diluted to 6 mL in Bind Solution (1 mM EDTA, 1 mM EGTA, 1x TBS, 5 mM DTT) before filtration through a 0.45 micron filter and application to a pre-equilibrated HIS affinity column (BD Biosciences). The column was washed with 20 mL Bind Solution. Purified protein was eluted with 50 mM imidizole as four 1 mL fractions. The fractions were concentrated and then analyzed by Western Blotting or buffer exchanged (Biorad) into SphK Activity Assay Buffer (Section 3.3.7).

5.3.6 Silver Staining

The purity of column purified SphK1 proteins was determined using a silver stain protocol modified from the method of Blum [200]. Gels were incubated at least 1 h in Fix Solution (50% MeOH, 12% AcOH, 0.5 mL/L formalin). The gel was then rinsed twice in boiling water and sensitized for 1 min in 1.5 mL/L 10% Na₂S₂O₃. The gel was then rinsed three times with water and soaked for 20 min in 0.012 M AgNO₂ with 0.75 mL/L formalin. After three brief rinses in water, the gel was soaked in Developing Solution (30 g/L Na₂CO₃, 0.5 mL/L formalin, 75 µL/L 10% Na₂S₂O₃) until desired band intensity was obtained. Development was halted with 1% acetic acid.
5.3.7 Calmodulin Sepharose Binding

Calmodulin sepharose binding was used as an indicator that the recombinant SphK1 proteins were correctly folded. This assay was performed as described previously [86, 199] with slight modifications. Aliquots of purified recombinant wild type and mutant SphK1 were added to pre-equilibrated calmodulin/Sepharose 4B beads (Amersham). Cleared SphK1 lysate (20 µL) in 180 µL Binding Buffer (50 mM Tris–HCl, pH 7.4, 200 mM NaCl, 10% glycerol, 0.2% Triton X-100, 1 mM DTT, 2 mM Na₃VO₄, 10 mM NaF and protease inhibitors) was added to 20 µL pre-equilibrated resin and incubated with rotation at 4°C for 2 h. The resin was then pelleted at 1,000 x g for 2 min at 4°C and washed in 1 mL Binding Buffer. The pelleting and wash steps were repeated twice. The proteins were eluted in 50 µL 1x NuPage LDS loading buffer (Invitrogen) and incubated at 70°C for 10 min. The resin was pelleted by 2 min centrifugation at 1,000 x g. The supernatant containing proteins of interest was loaded onto a SDS-PAGE gel.

5.3.8 In Vitro Phosphorylation of Sphingosine Kinase 1 by Protein Kinase C

Purified SphK1 (30 µL) was added to 70 µL of Reaction Mix (10 mM MgCl₂, 100 µM CaCl₂, 100 µM ATP, 2 µCi [γ³²P] ATP [ICN Radiochemicals], 100 µg/mL PS, 20 µg/mL DAG, 20 mM HEPES, pH 7.4, 0.03% Triton X-100). Recombinant PKC alpha or epsilon (Invitrogen) were diluted to 50 ng/µL in Dilution Buffer (10 mM HEPES, pH 7.4, 5 mM DTT, and 0.01% Triton X-100). Diluted PKC (2 µL) was added to initiate the reaction, which was agitated at 37°C for 30 min. The reaction mix was then added directly to the SphK Activity Assay or loaded onto a SDS-PAGE gel for Western Blot analysis. Autoradiography was used to determine if SphK1 had been phosphorylated by PKC isoforms at the expense of [γ³²P] ATP.
5.3.9 Cell Growth in Soft Agar Assay

NIH-3T3 cells were selected by flow cytometry (Flow Cytometry Core Facility, Penn State College of Medicine) to be stably overexpressing GFP-SphK1 wild type and mutant proteins. 1.2% SeaKem GTG agarose (Fisher) was autoclaved. 2x DMEM (6.7 g DMEM powder, 1.85 g sodium bicarbonate, bring volume to 250 mL, sterile filter and add 0.5 mL penicillin/streptomycin) was made with 10% FCS. The agarose was microwaved and kept in a 50°C water bath. 50 mL DMEM was mixed with 16.7 mL water and warmed in a 37°C water bath. 33.3 mL warm agarose was added and swirled. 2.5 mL was dispensed into each well of a 6 well plate (up to 40 wells) and put in 37°C incubator (bottom layer is 0.4% agarose). For every 4 wells, 2 mL DMEM was warmed in 37°C water bath. Cells in the log growth phase were trypsonized, counted and diluted (enough cells for 4 wells into 1.2 mL media was ~50,000 cells). Warm media was added (with growth factor if desired) and then 0.8 mL warm agarose was added. 1 mL was quickly dispensed into each well and placed in 37°C incubator (middle layer is 0.2% agarose). The wells were then sealed with 500 µL of 0.4% agarose. After 2 weeks, the plates were stained with MTS and photographed.

5.3.10 Mass Spectrometry

Lysates from BL21-AI bacteria overexpressing GST-SphK1 or HEK293 cells that had been stably transfected with an HIS-SphK1 or SphK1-HIS were purified over GSTtrap HP or HITrap HP columns (Amersham Biosciences), respectively. The partially purified eluate was resolved by SDS-PAGE and stained overnight with SyproRuby Protein Stain (Molecular Probes) according to the manufacturer’s recommendations. Bands corresponding to the protein of interest were excised under UV light. The gel slices were diced up and incubated in 15 µL 5 mM DTT in 20 mM ammonium bicarbonate for 30 min at 60°C. The liquid was removed and 15 µL 55 mM iodoacetomide in 20 mM ammonium bicarbonate was added, followed by a 30 min
incubation at 25°C in the dark. The liquid was removed and 100 µL 50 mM ammonium bicarbonate/50% acetonitrile was added, followed by 20 min incubation at 25°C. This step was repeated, and then 100 µL 75% acetonitrile was added, followed by 20 min incubation. The liquid was removed and the gel slices were dried by speedvacing for 30 min with heat.

An in-gel enzyme digestion was performed on SphK1 protein using trypsin (Pierce Biotechnology) for bacterially produced SphK1, or using chymotrypsin (Roche Applied Science) for SphK1 purified from HEK293 cells. For the trypsin digest, gel slices were incubated in 15 µL of 20 µg/mL trypsin in 20 mM ammonium bicarbonate at 37°C overnight. For the chymotrypsin digest, chymotrypsin was dissolved in 100 µL 1 mM HCl and added to 900 µL 100 mM Tris-HCl, 10 mM CaCl₂, pH 7.8. 15 µL was to each well and the plate was incubated at 25°C overnight. The next day, 50 µL of 50% acetonitrile/0.1% TFA was added, followed by 20 min incubation. The solution was then transferred to a new 96 well plate. This step was repeated. The gel slices were then dried in a speedvac for 30 min with heat. The dried slices were then resuspended in 10 µL 0.5% TFA.

Enzyme digested samples were cleaned up using ZipTipC18 (Millipore, Billerica, MA), spotted onto a stainless-steel MALDI target plate, and overlaid with matrix (cyanohydroxycinnamic acid). MALDI-TOF-MS (matrix-assisted laser-desorption for ionization-time-of-flight mass spectrometry) analysis was performed on samples using an Applied Biosystems 4700 Proteomics Analyzer (Mass Spectrometry/Proteomics Core Facility of the Pennsylvania State College of Medicine). All data with spectra in the range of m/z 800-4000 were acquired using 4000 Series software to acquire data and the GPSExplorer 3.0 is used for database searching (Applied Biosystems). The obtained data was searched against the SwissProt database limited to human proteins.
5.4 Results

5.4.1 PMA is an activator of Protein Kinase C and Sphingosine Kinase 1

PMA, a direct activator of PKC, has been shown to activate SphK1 in many cell types. In order to confirm these observations in our HEK293 cells, we stimulated serum-starved cells with 300 nM PMA over a timecourse of 30 min. We determined that the PKC signaling pathway had been activated by monitoring phospho-PKC levels by Western blot. We observed a rapid increase in phospho-PKC levels compared to β-actin levels (Figure 30A). Phospho-PKC levels had increased 500% by 15 minutes, and remained elevated beyond 30 min.

We next examined whether PMA could activate SphK1 activity. We stimulated serum-starved HEK293 cells with 300 nM PMA over a timecourse of 30 min. Despite multiple optimization attempts, we were unable to show that PMA stimulated SphK1 activity, which had been widely described in the literature. It was not until we separated the membrane fraction from the cytoplasmic fraction that we identified activation in the membrane fraction (Figure 30B). The cytoplasmic fraction did not respond to PMA stimulation (solid line), while we observed a 162% increase in SphK1 activity 5 min following PMA stimulation in the membrane fraction (dotted line). Increased SphK1 activity was maintained at 148% at 15 min following PMA stimulation, and was approaching baseline levels by 30 min. Our findings are consistent with recent reports in which treatment of cells with PMA stimulates SphK1 activity and translocation to the plasma membrane [28, 41, 45, 51, 56].
Figure 30: Effects of PMA stimulation in HEK293 cells. A: Phospho-PKC levels following a timecourse of 300 nM PMA treatment. B: 300 nM PMA stimulation of SphK1 activity in the cytoplasmic (solid line) vs. the membrane (dotted line) fraction.
5.4.2 Characterization of Recombinant Sphingosine Kinase 1 Produced in E. coli

Once we had confirmed that PMA activated SphK1, we wanted to determine if PKC could phosphorylate recombinant SphK1 in vitro. We first optimized the expression of GST-tagged bacterially expressed protein. Using the BL-21AI strain of E. coli, we were able to express enough GST-SphK1 to purify over a GST column (Figure 31A). The silver stain shows that the majority of bacterial proteins that were present in the starting material (lane 1) passed through the column without binding to it (lane 2, flow-through). The wash step (lane 3) was devoid of discernable protein. There were three primary bands present in the eluate (lane 4). Western Blot with an anti-GST antibody indicated that the middle band was purified GST-SphK1 (lane 5), which was later confirmed by mass spectrometric analyses (section 5.3.).

The SphK1 enzymatic activity of the purified recombinant SphK1 was also determined (Figure 31B). The bacterial lysates that were overexpressing GST-SphK1 exhibited a greater than 1000-fold increase in SphK1 activity compared to those that were overexpressing empty vector, indicating that overexpressed SphK1 was enzymatically active. Of the 3 mLs that were eluted from the GST column, E2 exhibited the highest enzymatic activity. This fraction was concentrated and used in subsequent in vitro experiments.
Figure 31: A: Purification of recombinant SphK1. A: Bacterially expressed proteins were purified over a GST column. Silver stain: 1. Total lysate. 2. Column flow through. 3. Wash. 4. Elution. 5. Detection of GST-SphK1 by Western blot. B: Activity of GST purified fractions compared to whole cell lysates from vector- and SphK1- transformed BL21-AI bacteria. The activity of the first three fractions (E1-E3) of GST-SphK1 eluted from a GST column are also shown.
5.4.3 In Vitro Phosphorylation of Sphingosine Kinase 1 by Protein Kinase C

Many reports have presented circumstantial evidence that SphK1 is a substrate of PKC [45, 197]. We tested the ability of a classical isoform of PKC (alpha) and a novel PKC isoform (epsilon), to directly phosphorylate our purified recombinant SphK1 by performing an in vitro PKC phosphorylation assay using GST-SphK1 as a substrate. As a positive control, we observed that PKC was able to phosphorylate its known substrate, histone H3 (data not shown). Figure 32 shows that both PKCα (70 kD) and PKCε (80kD) are able to autophosphorylate, as seen by the upper bands. The presence of a lower band in the PKCα + SphK1 sample (and its absence in the PKCε + SphK1 sample) at the expected molecular weight of GST-SphK1 (63 kDa) is evidence that PKCα, and not PKCε, is able to phosphorylate SphK1.

Figure 32: PKCα (70 kD), but not PKCε (80 kD), is able to directly phosphorylate GST-SphK1 (63 kD). The upper bands represent autophosphorylated PKC.
5.4.4 Protein Kinase C alpha Activates Sphingosine Kinase 1

We next examined if preincubation with PKCα was sufficient to increase SphK1 activity. Increasing amounts of PKCα caused a corresponding increase in the activity of SphK1 (Figure 33). Lipid or PKCα alone did not exhibit any SphK1 activity. Recombinant PKCα activated SphK1 activity in a dose-dependent manner, with the highest dose (5 ng) increasing SphK1 activity by 180%. These results indicate that PKCα is sufficient to activate SphK1 in vitro.

Figure 33: Preincubation with PKCα directly activates SphK1 in a dose-dependant manner.
5.4.5 Characterization Sphingosine Kinase Mutants

Our in vitro data indicate that PKCα is able to both directly activate and directly phosphorylate SphK1 in the absence of any accessory proteins. In order to confirm if phosphorylation of SphK1 by PKCα is involved in the activation of SphK1, we mutated each of the four putative PKC phosphorylation sites on SphK1 (Thr54, Ser181, Thr205, Ser371) to a non-phosphorylatable residue (alanine) or to a phosphomimetic (aspartate or glutamate). The Netphos 2.0 phosphoprotein prediction server [201] predicted that Ser181 or Ser 371 were the most likely of these sites to be phosphorylated (Figure 34).

Point mutants at each of the four putative phosphorylation sites were purified from BL-21AI E. coli. Western blot analysis was used to confirm that the GST-tagged SphK1 proteins expressed properly (Figure 35A). As SphK1 is known to interact with calmodulin, proper folding of SphK1 is indicated by an ability to bind to calmodulin sepharose [202]. We found that each of our mutants were able to bind to calmodulin, suggesting that they were not misfolded (Figure 35B). Subsequent experiments demonstrated that the difference in band intensity between the mutants was a variable of

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**Figure 34**: Netphos Prediction of residues on SphK1 that are likely to be phosphorylated. Putative PKC phosphorylation site are indicated by arrows. Residues that have been mutated in previous studies are indicated by asterisks.
the experiment, not of the binding efficiency of individual mutants to the calmodulin (not shown).

The SphK1 enzymatic activities of each of the purified putative PKC phosphorylation site mutants of GST-SphK1 were examined (Figure 35C). The Thr54 and Ser181 mutants were essentially catalytically inactive. The Thr205 mutants had about 30% of wild type activity. The Ser371 mutants (Ser371Ala and Ser371Asp) exhibited greater than wild type activity. Using this data, we eliminated Thr54, Ser181, and Thr205 as candidate phosphorylation site on SphK1 for PKC. We next examined the Ser371 site further, as it was the most likely site of PKCα phosphorylation of SphK1.

Figure 35: Characterization of putative PKC phosphorylation site mutants of GST-SphK1. A: Western Blot using anti-GST. B: Mutants are properly folded enough to bind to calmodulin sepharose. C: Activity of putative PKC phosphorylation site SphK1 mutants.
5.4.6 In Vivo Characterization of Ser371Ala and Ser371Asp Sphingosine Kinase 1

We next overexpressed HIS-SphK1_371Ala and HIS-SphK1_371Asp in HEK293 cells in order to determine if phosphorylation of SphK1 at Ser371 was critical for SphK1 activity in vivo. We performed kinetic analysis to test the ability of the Ser371Ala and the Ser371Asp mutants to phosphorylate sphingosine compared to wild type SphK1 (Figure 36). The apparent specificity constants were 4.5 cpm/OD/µM for the wild type, 0.7 cpm/OD/µM for Ser371Ala, and 2.3 cpm/OD/µM for S371Asp. Samples from cells transfected with empty vector did not exhibit SphK1 activity over background levels (data not shown). These values indicate that mutating at Ser371 affects sphingosine binding, with an alanine mutation having a greater affect on catalytic efficiency. It is interesting that the Vmax values were similar for all three fusion proteins. One possible explanation for this outcome would be if the Ser371Ala clone contained a higher copy number of the SphK1-containing plasmid or if it had inserted downstream of a more active promoter than the Ser371Asp did.
Figure 36: Kinetic analysis of HIS-SphK1 (open circles), HIS-SphK1_Ser371Ala (closed squares), and HIS-SphK1_371Asp (closed triangles). The inset shows the relative expression level of SphK1 protein partially purified from HEK293 cells in duplicate.
5.4.7 Cell Growth Studies

We further analyzed the effect of mutating Ser371 in HEK293 cells by performing cell growth studies in soft agar. The ability of a cell to form colonies in soft agar is an indication of cellular transformation. GFP vector, Ras, GFP-SphK1, GFP-SphK1_371Ala, GFP-SphK1_371Asp, GFP-SphK1_Ser225Ala, and GFP-SphK1_371A/Ser225Ala were stably transfected into NIH-3T3 cells. We chose to use the GFP tag so that we could check for expression of the GFP-SphK1 fusion proteins by fluorescence microscopy (data not shown). The inset in Figure 37 shows that we observed consistent expression of the fusion proteins in all of the stable lines as determined by Western blot of total cell lysates using the anti-GFP antibody. Panel A shows that nontransfected NIH-3T3 cells did not spontaneously grow in soft agar. Panel B shows that some colonies did form when the empty GFP vector was stably expressed. We cannot explain why there were colonies in this negative control, however, the levels were low compared to the size of colonies observed in the positive control, ras-transfected cells (Panel C). It is unfortunate that there was difficulty obtaining quantitative data from this experimental design, which would have allowed a more direct comparison between the growth of individual mutants.

We observed that cells overexpressing wild type SphK1 also grew colonies (panel D), as has been previously described [5]. These colonies were more numerous than the ras-transfected cells, though not as large. Cells overexpressing Ser371Ala SphK1 (panel E) grew similar to vector transfected cells (panel B), indicating that blocking this phosphorylation site prevented transformation. Cells overexpressing Ser371Asp SphK1 (panel F) grew more like the ras transfected cells (panel C), indicating that a phosphorylation event at this site (Ser371) causes cells to grow rapidly and overcome cell contact inhibition. Cells overexpressing Ser225Ala SphK1, which has been shown to be active but not translocate to the plasma membrane (section 2.4.1) did not grow colonies at all (panel H). Cells overexpressing the double mutant, Ser371Ala/Ser225Ala (panel H) grew in a similar fashion to the Ser371Ala mutant (panel E).
5.4.8 Phosphoprotein Analyses

We next wanted to identify if SphK1 was phosphorylated using phosphoantibodies and a phosphoprotein stain. Bacterially produced purified GST-SphK1 was incubated with PCKα and ATP to allow PKC to phosphorylate SphK1. The reaction mix was then resolved by SDS-PAGE and either used for Western blotting or directly stained by the ProQ-Diamond Phosphoprotein Stain. Immunodetection for phosphoserine residues indicated that PKC autophosphorylated in the presence of ATP.
(Figure 38, lane 3). This is in agreement with data presented in Figure 32. Unexpectedly, GST was also detected by the anti-phosphoserine antibody (lanes 1-3). GST-SphK1 appeared to be phosphorylated at a serine residue in the absence of phosphorylation by PKC (lanes 1-2), which should not have been possible as bacteria should not have phosphorylated the recombinant protein. There was an increase in the intensity of the phosphoserine antibody detection of SphK1 after preincubation with PKC and ATP (lane 3). This increase was only slight, indicating that if PKC does indeed phosphorylate SphK1, that it was not very efficient in our in vitro system. The phosphothreonine antibody detected PKC (lanes 4-6) but not SphK1. This is in agreement with our hypothesis that SphK1 is not phosphorylated by PKC at a threonine site.

Figure 38: Analysis of phosphoproteins using antibodies (anti-phosphoserine, lanes 1-3; anti-phosphothreonine, lanes 4-6) and a phosphoprotein stain (lanes 7-12).

The ProQDiamond Phosphoprotein Stain recognized autophosphorylated PKCα (Figure 38, upper band, lanes 8, 10, 12) as well as faintly recognizing all purified proteins (compare to silver stain, Figure 31A), with the exception of the Ser371Ala SphK1 mutant (lanes 9 and 10). The GST-SphK1 band did not appear to intensify in the presence of PKCα and ATP (lane 8) as we had observed in lane 3 with the anti-phosphoserine. The
Ser371Ala mutant appeared to be entirely nonphosphorylated (lanes 9-10), while the Ser371Asp mutant was detected with the same faint intensity as wild type (lanes 7-8, 11-12).

5.4.9 Mass Spectrometry to Identify Post Translational Modification of Sphingosine Kinase 1

In order to definitively determine if Ser371 was a phosphorylation site on SphK1, we used MALDI-TOF-MS analysis. We began by examining bacterially expressed GST-SphK1 than had been phosphorylated by PKC in vitro. Figure 39A shows a SyproRuby protein stain of GST-SphK1 and its copurifying proteins from the GST column (lane 2). Lane 3 shows the purified SphK1, PKCα, and ATP reaction mix. Figure 39B shows the purification scheme for GST-SphK1 by a silver stain. Lane 5 shows all of the proteins present in the whole bacterial lysate (flow through off of GST column). Lane 6 shows that the wash step is pure of protein. Lane 7 shows that GST-SphK1 co-purifies with other proteins, similar to lane 2. In order to be sure which band was SphK1, we separated purified GST-SphK1 by a 2D gel (Figure 39C). Each of the spots that were excised for protein identification by MALDI-TOF-MS analysis are shown in a red box. We were able to identify which band was SphK1 and to also identify the co-purifying proteins as DNA K, chaperonin, a heat shock protein, and an elongation factor (indicated by arrows).

Once we had identified which protein was SphK1, we then examined if Ser371 was phosphorylated in the sample that had been preincubated with PKC and ATP. This was challenging, as the fragment containing Ser371 was not the correct size for analysis when digested with trypsin. In order to get the correctly sized peptide, we digested with chymotrypsin. At this time we switched over to analyzing partially purified SphK1 from HEK293 cells. Due to concerns that a HIS tag could interfere with phosphorylation by PKC, we tried both amino tagged (HIS-SphK1) and carboxyl tagged (SphK1-HIS) constructs. The white boxes in Figure 39D represent which bands were excised and used for MALDI-TOF-MS analysis. Lanes 3 and 4 contained partially purified SphK1-HIS. Lanes 6 and 7 contained partially purified HIS-SphK1.
The results of the MALDI-TOF-MS analysis are shown in Table 1. We confirmed that the bands that we excised were indeed SphK1. Unfortunately, the equipment was not sensitive enough to determine if any of the peptides of interest had been phosphorylated. We were not even able to detect the known phosphorylation event at Ser225. Subsequent analysis at the University of Victoria Genome British Columbia
Table 1: SphK1 peptides identified by MALDI-TOF-MS. Bolded fragments were detected in both bacterial and mammalian preparations.

<table>
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<th>Expression</th>
<th>Fragment</th>
<th>Mass</th>
<th>Position</th>
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<td>GVLPPPRCR</td>
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<td></td>
<td>VLVNNRPRL</td>
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<td>VLVNNRPGK</td>
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<td>RLEIPKDGKGVF</td>
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<td>FWQGSGCVEPPPSWV</td>
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Proteomics Centre concluded that the Thr54 site is not phosphorylated (data not shown). This eliminates one of the putative PKC phosphorylation sites, but is not sufficient to determine if one of the remaining three was phosphorylated.

5.5 Discussion

It has been widely reported in the literature that activators of PKC also activate SphK1 activity and membrane translocation, which can be blocked by preincubation with PKC inhibitors [45]. We have demonstrated that PMA, an activator of conventional and novel PKCs, stimulates a rapid activation of PKC, which takes place concurrent with the early phase activation of SphK1 activity. We observed a membrane-specific activation of SphK1 activity following PMA stimulation, which is in agreement with stimulation of SphK1 by FBS presented in Section 3.4.

We performed in vitro studies to determine if PKC directly activates SphK1 by phosphorylation. We show that we were able to purify bacterially produced active SphK1. This recombinant SphK1 was used in a PKC phosphorylation assay, from which we determined that PKCα (a conventional PKC isoform), and not PKCε (a novel PKC isoform), could directly phosphorylate SphK1. This is in agreement with a report that tested the effect of isoform-specific knockdown of PKCs using siRNA in endothelial cells. In this study, only depletion of PKCα blocked PMA-induced SphK1 induction [177]. Similar to SphK1, PKCα has been shown to translocate to the plasma membrane from the cytosol due to selectivity for PS, which induces a conformational change that increases membrane penetration and opens the PKC active site [203]. Based on these results and our own, we tested the ability of PKCα to stimulate SphK1 activity in vitro. We found that PKCα preincubation increased the activity of SphK1 in a dose-dependent manner.

In order to determine the site of phosphorylation of SphK1 by PKCα, we identified four conserved putative PKC phosphorylation sites within SphK1: Thr54, Ser181, Thr205, and Ser371. To determine which of these conserved PKC sites is
necessary for stimulation of SK1 activity, these sites were mutated to alanine to eliminate
the PKC phosphorylation site(s) or to a negatively charged residue (glutamic acid or
aspartic acid) to act as a phosphomimetic. These mutants were overexpressed in bacteria
and determined to possess activity.

Predictions generated using the Netphos 2.0 Phosphoprotein Prediction Server
[201] predicted that Ser181 or Ser371 were the most likely phosphorylation sites.
However, the threonine residues were also candidates, as the phosphorylation potential
for these residues was higher than that predicted for the only previously known
phosphorylation site on SphK1, Ser225 (Figure 34, third asterisk from the left). We
found that the Thr54 and Ser181 mutants were essentially catalytically inactive. These
results are supported by a previous report that Ser181 is unable to be phosphorylated [1].
The Thr205 mutants had about 30% of wild type activity. It is not surprising that the
Ser181 and Thr205 mutants exhibited a low activity, as they are predicted to be close to
the sphingosine binding pocket (residues 165-198). Most interesting were the Ser371Ala
and Ser371Asp mutants, which both exhibited greater than wild type activity. We are not
certain why the alanine mutant also exhibited elevated activity, as this mutant did not
possess a pseudophosphorylation site. However, these data enabled us to eliminate
Thr54, Ser181, and Thr205 as candidate phosphorylation site on SphK1 for PKC, leaving
Ser371 as the most likely candidate.

We performed kinetic studies and found that HEK 293 cells expressed much
higher levels of Ser371Ala in order to achieve a comparable Vmax to wild type SphK1.
Unexpectedly, the wild type SphK1 protein exhibited a higher catalytic efficiency than
the Ser371Asp mutant. This indicates that if PKC phosphorylates SphK1 at Ser371, that
this one phosphorylation event is not enough to fully activate SphK1.

Overexpressing SphK1 has been shown to be sufficient to cause cells to become
transformed [5]. We tested the ability of mutant SphK1 to grow in soft agar and
confirmed previous reports that Ser225 is a critical site for SphK1 activity leading to cell
transformation, as the Ser225Ala mutant did not spontaneously transform. Our data also
indicate that Ser371 is also a critical site, as the Ser371Asp mutant grew larger colonies
than wild type SphK1 did. However, it is not clear why the Ser371Ala/Ser225Ala double
mutant was able to form colonies. It appears that modification at Ser225 may occur upstream of a modification at Ser371, as the double mutant exhibited the phenotype of the Ser371 mutant.

Phosphoprotein analyses indicated that PKC\(\alpha\) phosphorylates SphK1 at a serine residue, but not at a threonine residue. The Ser371Ala mutant was absent by phosphoprotein stain, however the wild type and the Ser371Asp mutant were equally stained. In an attempt to definitively determine if Ser371 was a phosphorylation site on SphK1, we used MALDI-TOF-MS analysis. These analyses positively identified HIS-SphK1, SphK1-HIS, and GST-SphK1. We also identified co-purifying proteins that are involved in correct folding of bacterial proteins. Unfortunately, the available equipment was not sensitive enough to identify if the fragment that contained Ser371 was a phosphopeptide. We were able to eliminate Thr54 as a possible phosphorylation site, which is in agreement with our observation that GST-SphK1-Thr54Ala and GST-SphK1-Thr54Asp lacked enzymatic activity.

The evidence that PKC phosphorylates near the carboxyl-terminus of SphK1 is not surprising. We have shown that recombinant SphK1 tagged at the amino-terminus is more active than the same construct tagged at the carboxyl-terminus, even though SphK1 tagged at the carboxyl-terminus overexpresses much higher levels of recombinant protein (Appendix Figure 44), similar to how Ser371Ala was expressed at much higher levels than wild type SphK1 in the kinetic studies. This indicates that blocking a possible activating modification site with a bulky tag impedes catalytic efficiency. We had previously hypothesized that the carboxyl-tail of SphK1 is modified during activation, resulting in increased accessibility of sphingosine to the active site. This is supported by the fact that Ser371 is not conserved in SphK2. SphK2 does contain Thr184 (analogous to SphK1 Thr54), Ser311 (analogous to SphK1 Ser181), and Thr335 (analogous to SphK1 Thr205). As SphK2 is not inducible, the absence of a PKC phosphorylation site near the carboxyl terminus could be the key difference between the regulation of the two SphK isoforms. One alternate conclusion is that PKC activation of SphK1 activity is not via direct phosphorylation, but that autophosphorylated PKC binds to SphK1 and enhances its active conformation.
5.6 Conclusion

We propose a model for activation of SphK1 by PMA whereby PMA activates PCKα, which phosphorylates SphK1 at Ser371. PMA has also been shown to induce phosphorylation of SphK1 at Ser225 involving ERK1/2 [1]. We believe that dually phosphorylated SphK1 (P-Ser371 and P-Ser225) results in a fully activated SphK1 that then translocates to the plasma membrane, perhaps involving an interaction with calcium/calmodulin [58]. We are not certain at this time if one phosphorylation causes a posttranscriptional change that then makes the second phosphorylation event more favorable. Fully activated SphK1 may undergo a conformational change, resulting in exposure of residues, perhaps Thr54 and Asn89, which bind to the plasma membrane and further activate SphK1 [59]. Further studies are necessary to determine if phosphorylation at Ser371 is definitively involved in translocation of SphK1 to the plasma membrane.
Chapter 6
Overall Conclusions

Sphingolipid metabolites are now recognized as important messenger molecules in cell signaling pathways. SphK1 regulates cell growth by converting ceramide/sphingosine, proapoptotic signals, to S-1-P, a promitogenic signal. The studies presented in this thesis enable further understanding of SphK1 localization to the membrane raft microdomain of the plasma membrane, as well as transcriptional and post-translational activation of SphK1, in response to stimulation by growth factor.

An overall model of SphK1 activation based on the data generated in these studies is proposed in Figure 40. In response to a mitogenic stimulant, such as FBS or PMA, SphK1 has been observed to translocate from the cytosol, where it is basally active, to the plasma membrane, where it is in an activated state. Many events have been reported to be necessary for SphK1 plasma membrane association and enzymatic activation, including phosphorylation by ERK1/2 (gray rectangle) and calcium/calmodulin binding (dark gray moon). However, these events are not sufficient for stimuli-induced SphK1 membrane translocalization, indicating that additional factors or modifications are necessary. Growth factors also stimulate the activation of PKC (pathway shown in green). We show evidence that activated PKCα phosphorylates SphK1, resulting in a dually phosphorylated SphK1. This activated form of SphK1 is proposed to undergo a conformational change that allows key residues to bind favorably with PS at the plasma membrane, which further activates SphK1.
Figure 40: Model of SphK1 activation as a result of stimulation with PMA (green), FBS (red), or PDGF (blue). Dotted lines indicate putative signaling.
Once at the plasma membrane, we believe that SphK1 diffuses laterally to the membrane raft microdomain (colored red), where PS has been reported to be concentrated. We show that it is here that the substrate of SphK1, sphingosine, is enriched. It has been reported that sphingomyelinases and ceramidases, also concentrated in membrane rafts, are also activated in response to growth factor stimulation, resulting in more sphingosine available for conversion by SphK1 to S-1-P. We demonstrate that the S-1-P generated is quickly released intracellularly and secreted extracellularly, where it is involved in prosurvival signaling including differentiation, migration, and mitogenesis. We believe that distribution of SphK1 to membrane rafts is critical in activation of downstream signaling pathways, as membrane rafts are believed to serve as platforms for amplification of cell signaling events. Compartmentalization of SphK1 signaling in membrane rafts could spatially and temporally activate S1PRs that localize to membrane rafts in stimulated cells, such as S1PR1, resulting in directional cellular migration and providing positive feedback for sustained and localized activation of SphK1.

Concurrent with plasma membrane raft translocation of SphK1, growth factor stimulation of SphK1 is further regulated at the transcriptional and translational levels. We have characterized a mechanism of SphK1 activation initiated by PDGF binding to its receptor, leading to activation of PI3K at the plasma membrane and downstream activation of AKT (pathway shown in blue). We have identified that it is specifically the AKT2 isoform that results in transcriptional activation of the SphK1 gene. Increased SphK1 mRNA levels are translated into increased SphK1 protein levels. We have observed both a transient and a sustained increase in SphK1 enzymatic activity levels following PDGF stimulation. The transient response resulted from translocation of existing SphK1 to the membrane raft microdomain. The sustained response resulted from newly synthesized SphK1 being produced over a longer period of time and subsequently being targeted to membrane rafts.

We believe that abundant cross-talk is involved between signaling pathways required for activation of SphK1. For example, PKC has been shown to activate AKT [204]. AKT2 has been reported to colocalize in membrane rafts with PI3K [196].
Another example is signaling through calcium mobilization from intracellular stores, which is a well characterized mitogenic signal. Increases in intracellular calcium levels have been shown to activate SphK1 [205], which amplifies calcium signaling through certain GPCRs [44]. PDGF, which results in upregulation of SphK1 expression, has been demonstrated to induce release of intracellular calcium [206]. Increased intracellular calcium levels also result in activation of PKC at the plasma membrane [207], which activates SphK1. Calcium flux within a cell modulates the binding of calmodulin to SphK1, an event that is associated with SphK1 translocation to the plasma membrane. Cholesterol depleting agents that disrupt membrane rafts have been reported to shorten the duration of secondary calcium spikes, suggesting that the opening of calcium channels is associated with the integrity of plasma membrane raft microdomains [208].

Increases in cellular calcium are partially attenuated by SphK inhibition, suggesting that SphK1 is a downstream effector for activation by calcium as well as an upstream mediator of calcium waves in the cell. Although a mechanism for downregulation of SphK1 activation has not yet been identified, it is possible that fluxes in intracellular calcium levels could serve as an on/off switch for SphK1 activation.

The overall significance of the studies presented in this thesis is that the information generated can be applied to the development of rationally designed drugs that target the activation of SphK1. As was mentioned in the introduction, there is evidence that SphK1 is abnormally overactive in hyperproliferative diseases, making it an attractive therapeutic target. The clinical implication of our work is that deregulation of SphK1 phosphorylation and localization may be key elements in the acquisition of hyperproliferative phenotypes. Blocking promitogenic signaling at the level of SphK1 activation offers a new pharmacological approach to fighting diseases such as cancer.

One interesting avenue of future study worth pursuing would be to examine a proposed link between elevated levels of cell signaling occurring in the membrane rafts of cancer patients. It has been reported that cancer cells contain increased levels of membrane rafts and suggest a potential use of membrane raft-modulating agents as anti-cancer drugs [209]. It would be worthwhile to examine if the membrane raft pool of SphK1 is proportionally larger or more catalytically active in cancer vs. normal cell lines.
As a myristoylated SphK1 mutant was reported to be oncogenic [121], and myristoylated and palmitoylated proteins have been shown to be enriched at membrane raft domains [210], myristoylation of SphK1 could be a mechanism for membrane raft targeting of SphK1, which could be dysregulated in cancer cells. As S1PRs associated with migration are known to be present in membrane rafts, SphK1 activation and signaling though membrane rafts could be important in migration and metastasis of tumor cell precursors. This is supported by the observation of increased SphK1 mRNA stability in cancer cells, which could result in high levels of SphK1 protein, which could be in turn be targeted to membrane rafts and result in aberrant promitogenic signaling. [3].

There is ample evidence that SphK1 is a validated druggable target. SphK1 is upregulated in many different types of cancer, including colon [4], lung [3], ovary [3], and glioblastoma [75]. Several small molecule inhibitors specific for SphK have been designed and have been demonstrated to exhibit antitumor activity in mice with limited toxicity [3]. Based on the results shown in Figure 23 using siRNA specific for SphK1, we believe that a drug targeting SphK1 activation would be cytostatic, causing cells to growth arrest, rather than to enter apoptosis. A drug that would inhibit SphK1 activity would function by disrupting the sphingolipid rheostat (Figure 3), causing an accumulation of ceramide and sphingosine, both anti-proliferative signals. Without a mechanism of forming S-1-P, promitogenic signals would be suppressed, resulting in decreased cell growth. The approach of limiting cell proliferation by blocking SphK1 activity could be used to treat other hyperproliferative diseases in addition to cancer, such as atherosclerosis, asthma, and inflammatory diseases (Section 2.2.4).
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Appendix

Supplemental Figures

This chapter contains data that is tangential to points being made in preceding chapters. Most of these experiments are very preliminary and should be repeated in order to confirm their findings. As discussed on page 52, Figure 41 demonstrates the effect of reducing agents on SphK1 activity (panel A) and membrane raft localization (panel B). Black bars represent DTT and gray bars represent β–mercaptoethanol.

![Figure 41: Reducing agents increase the in vitro enzymatic activity and membrane raft localization of SphK1. A: Effects of DTT and β–mercaptoethanol on SphK1 activity and localization. A: DTT (black) and β–mercaptoethanol (gray) increase recombinant GST-SphK1 enzymatic activity in a dose dependent manner. N=3. B: Sucrose gradient centrifugation of HIS-SphK1 overexpressing HEK293 cell lysates in the absence (top) and presence (bottom) of β–mercaptoethanol. Detected with anti-HIS antibody. F4 represents the membrane raft fraction. N=1.](image-url)
Also discussed on page 52 is Figure 42. This experiment was performed to determine if mutating SphK1 at a cysteine residue would interfere with its ability to localize to membrane rafts. Across several experiments, each SphK1 mutant was able to localize equally well with the membrane raft. Although the wild type condition is absent, this indicates that palmitoylation at one particular cysteine residue is probably not the means of SphK1 membrane raft association.

Figure 42: Localization of His-SphK1 Cys site directed mutants after sucrose gradient centrifugation. Red dots correspond to mutants that had low expression. N=2.
Figure 43 examines the ability of a constitutively active SphK1 mutant truncated after residue 363 (introduced on page 19). Truncating after residue 270 abrogates SphK1 activity. The 1-315 mutant exhibits approximately 50% wild type activity. The 1-363 truncation acts as a constitutively active mutant, exhibiting greater than threefold higher activity than wild type SphK1 activity. As discussed on page 53, the 1-363 truncated SphK1 mutant localizes much more strongly to membrane rafts than wild type SphK1 does (inset).

![Figure 43: Sphingosine kinase 1 enzymatic activities of recombinant GST-tagged truncated mutants. N=4. Inset: A greater fraction of the HIS-SphK1_1-363 truncation mutant localizes to membrane rafts compared to wild type as determined by Western blot following the detergent method of sucrose density gradient centrifugation. N=2.](image)
Figure 44 is referred to on page 104 in a discussion about the importance of the carboxyl region of SphK1 in regulation of its activation. A HIS tag at the carboxyl terminus of SphK1 results in higher protein expression levels, but lower enzymatic activity levels as compared to an amino HIS tag. Experiments performed on recombinant GST-SphK1 and SphK1-GST gave similar results (data not shown), indicating that amino-tagged SphK1 has greater relative SphK1 enzymatic activity, implying that tagging at the carboxyl terminus could block a regulatory region necessary for full activation and/or optimal catalytic efficiency.

Figure 44: Properties of tagged recombinant sphingosine kinase 1 indicate that the carboxyl terminus contains a regulatory region. Top panel shows SphK1 activity assay data. Bottom panel shows Western blotting using anti-HIS. N=3.
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