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THE ROLE OF SPHINGOSINE KINASE 2 IN CELL GROWTH AND APOPTOSIS

A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy at Virginia Commonwealth University.

by

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Acknowledgements

I would like to dedicate this work to my mother, Janet Sankala who lost her battle with breast cancer in June 2005. I will always carry with me her amazing love, courage, strength, sweetness, and graceful nature.

I would like to express my gratitude to my advisor, Dr. Sarah Spiegel, for her dedication and contribution to my education and training. I gratefully acknowledge the contribution of my graduate committee members: Dr. Suzanne Barbour, Dr. Rik Van Antwerpen, Dr. Carmen Sato-Bigbee, Dr. Hisashi Harada and Dr. Paul Dent. I would like to extend my appreciation to Dr. Steven Grant, Dr. Christopher Pallister, and Dr. Leanne Cartee for sparking my interest in research. I would like to thank fellow members of the Spiegel Lab. (past and present), special thanks must be given to Dr. Nitai Hait and Steven Paugh for their help with the project, and to Dr. Shawn Payne for putting up with my endless questions. I would also like to thank my father; Paul Sankala and brothers; Ben and Hefin Sankala for their constant support and encouragement. I have been lucky to have incredible friends who always carry me through. I would particularly like to thank Kensey Geis, Stacey Markendorff, Emma Butt, James Sciubba and Lynessa Bray. Finally, I would like to thank my fiancé J. Michael Bauer. Words cannot describe how much I appreciate your love and support.

This work was supported by NCI Grant R01CA61774 to Dr. Sarah Spiegel, and Department of Defense predoctoral fellowship to Heidi Sankala. Confocal microscopy and flow cytometry were supported in part by NIH Grant P30 CA16059 to the Massey Cancer Center.

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List of Abbreviations

Apaf-1	Apoptosis-Activating Factor-1
Bcl-2	B-Cell Lymphoma-2
BH3	Bcl-2 Homology Domain
BSA	Bovine Serum Albumin
CaM	Calmodulin
Cdk	Cyclin Dependent Kinase
CerK	Ceramide Kinase
Cox-2	Cyclooxygenase 2
DGK	Diacylglycerol
DHS	Dihydrosphingosine
DMEM	Dulbecco's Modified Eagle's Medium
DMS	*N,N*- Dimethylsphingosine
EDG	Endothelial Differentiation Gene
EGF	Epidermal Growth Factor
ER	Endoplasmic Reticulum
ERK	Extracellular Signal-Regulated Kinase
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
GFP	Green Fluorescent Protein

GPCR	G Protein-Coupled Receptor
HEK	Human Embryonic Kidney
IL	Interleukin
IMEM	Improved Minimal Essential Medium
LCB	Long Chain Base
LPA	Lysophosphatidic Acid
MEF	Mouse Embryonic Fibroblasts
NES	Nuclear Export Sequence
NGF	Nerve Growth Factor
NLS	Nuclear Localization Sequence
PARP	Poly(ADP-ribose) Polymerase
PCR	Polymerase Chain Reaction
PDGF	Platelet-Derived Growth Factor
PDI	Protein-Disulfide Isomerase
PECAM-1	Platelet Endothelial Cell Adhesion Molecule-1
PGE_2	Prostaglandin E2
PI	Propidium Iodide
PKC	Protein Kinase C
PMA	Phorbol 12-Myristate 13-Acetate
PS	Phosphatidylserine

PTX	Pertussis Toxin
Rb	Retinoblastoma Protein
SD	Standard Deviation
siRNA	Small Interfering RNA
SH3	Src Homology 3
SKIP	Sphingosine Kinase 1-Interacting Protein
SphK	Sphingosine Kinase
SPT	Serine Palmitoyltransferase
S1P	Sphingosine-1-Phosphate
TNF-α	Tumor Necrosis Factor-alpha
TLC	Thin Layer Chromatography
TRAF2	Tumor Necrosis Factor Receptor-Assosciated Factor 2
VEGF	Vascular Endothelial Growth Factor
WT	Wild Type

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Abstract

THE ROLE OF SPHINGOSINE KINASE 2 IN CELL GROWTH AND APOPTOSIS

By Heidi Sankala, Ph.D.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2007

Major Director: Sarah Spiegel, Ph.D.,
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Two isoforms of sphingosine kinase (SphK) catalyze the formation of sphingosine-1-phosphate (S1P). Whereas, SphK1 stimulates cell growth and survival, it was found that when overexpressed in mouse NIH 3T3 fibroblasts SphK2 enhances caspase-dependent apoptosis in response to serum deprivation, independently of S1P receptors. Sequence analysis revealed that SphK2 contains a 9 amino acid motif similar to that present in BH3-only proteins. Studies showed that the BH3-only domain, catalytic activity, endoplasmic reticulum (ER) stress, and uptake of calcium by the mitochondria may all contribute to the apoptotic effects of overexpressed SphK2 in NIH 3T3 cells. Further studies in human carcinoma cells showed that overexpression of SphK2 increased the expression of the cyclin dependent kinase (cdk) inhibitor p21, but interestingly had no effect on p53 or its

phosphorylation. Correspondingly, downregulation of endogenous SphK2 with small interfering RNA (siRNA) targeted to unique mRNA sequences decreased basal and doxorubicin-induced expression of p21 without affecting p53. In addition, downregulation of SphK2 decreased G2/M arrest in response to doxorubicin. Surprisingly however, siSphK2 markedly enhanced apoptosis induced by doxorubicin in MCF7 and HCT-116 cells. This result raises the question of how overexpression of SphK2 decreases cell growth and enhances apoptosis while its downregulation sensitizes cells to apoptosis. A partial answer may come from the possibility that when SphK2 is overexpressed it does not always have the same subcellular distribution as the endogenous protein. It may also be possible that proteolysis of overexpressed SphK2 might induce apoptosis due to liberation of its BH3 peptide domain, which does not occur at the levels at which endogenous SphK2 is expressed. Collectively, these results demonstrate that endogenous SphK2 is important for p53-independent induction of p21 expression by doxorubicin and suggest that SphK2 expression may influence the balance between cytostasis and apoptosis.

CHAPTER 1

INTRODUCTION

Balancing Cell Growth and Cell Death

A continuous cycle of cell production and cell death occurs essentially in all tissues with self-renewal capacity. New cells are produced through cell division (cell cycle) and older or damaged cells die principally through programmed cell death (apoptosis). Cancer is a disease of the cell that develops because of failures in the mechanisms controlling the delicate balance between cell growth and cell death. According to the American Cancer Society, cancer is the second leading cause of death in the United States. It is characterized by uncontrolled proliferation and the inappropriate survival of damaged cells. Targeting the pathways involved in cell cycle control and apoptosis has emerged as an attractive approach for the treatment of cancer.

Apoptosis

The signaling cascades that result in apoptosis are as elaborate as those that promote cell survival and it is clear that coordination of both protein- and lipid-mediated signals is crucial for proper cell execution. Malfunctions in the regulation of this vital physiological process have been implicated as an initiating event or contributing factor in many human diseases. Apoptosis is often impaired in cancer and can limit conventional

therapies. Apoptosis is characterized by chromatin condensation, nuclear fragmentation, cell shrinkage, and plasma membrane "blebbing" (Arends and Wyllie, 1991; Kerr et al., 1972). Molecular changes induced during apoptosis include internucleosomal DNA cleavage (Wyllie et al., 1980), redistribution of the phospholipid phosphatidylserine (PS) between the inner and outer leaflets of the plasma membrane (Fadok et al., 1998), and cleavage of a number of intracellular substrates (Martin and Green, 1995). Caspases (cysteine aspartyl-specific proteases) are the main initiators and executioners of the apoptotic process, they cleave various substrate proteins that affect the cytoskeleton, chromatin structures and the nuclear envelope resulting in the apoptotic morphology (Alnemri et al., 1996). Caspases are synthesized as inactive cytosolic precursor molecules and in cells induced to undergo apoptosis are converted by proteolytic cleavage to active heterodimers. Caspases can be divided into two main classes; initiator (caspase 2, 8, 9, and 10) and effector (caspase 3, 6, and 7) caspases (Bokoch et al., 1998).

There are two main pathways that control caspase-dependent apoptosis, termed intrinsic and extrinsic, which converge into a common pathway causing the activation of effector caspases. The intrinsic pathway is also known as the mitochondrial-mediated pathway and is characterized by depolarization of the mitochondrial membrane and subsequent formation of the apoptosome (a protein complex comprising cytochrome *c*, apoptosis-activating factor-1 (Apaf-1) and caspase 9). The extrinsic pathway is characterized by the activation of death receptors, recruitment of adapter proteins and the resultant activation and cleavage of caspase 8. It is known that Bid is cleaved by active caspase 8 (Luo et al., 1998), providing a basis for crosstalk between the extrinsic and

intrinsic apoptotic pathways. Upon cleavage truncated Bid translocates to mitochondrial membranes, inducing cytochrome c release. Interestingly, caspase 3 has been shown to process both caspase 8 and 9, thus generating a positive amplification loop and increasing apoptotic responses (Slee et al., 1999).

B-cell lymphoma-2 (Bcl-2) protein family members are key regulators of the intrinsic pathway and appear to be dysregulated in some cancers (Baliga and Kumar, 2002). They are characterized by the presence of one or more Bcl-2 homology domains, denoted BH1-BH4 (Oltvai et al., 1993). Pro-survival members, such as Bcl-2 and Bcl-x_L, all contain three or four BH domains. Pro-apoptotic Bcl-2 family members can be divided into two sub-groups. Multi-BH domain pro-apoptotic members such as Bax and Bak are structurally similar to the pro-survival members (Suzuki et al., 2000), whereas the BH3-only protein members such as Bid (Wang et al., 1996) and Noxa (Oda et al., 2000) share only the 9-16 amino acid BH3 domain (Huang and Strasser, 2000). The BH3 region is short and loosely conserved. Thus, it is difficult to identify BH3-only proteins through database searches. Many members of the BH3 only protein family have been identified by a yeast two hybrid system using Bcl-2 or Bcl-x_L as bait.

A two-class model has been proposed for the characterization of BH3-only family members: BID-like domains that induce oligomerization of the pro-apoptotic Bcl-2 family members Bax and Bak, and BAD-like domains that “sensitize” by occupying the BH3 pocket of pro-survival Bcl-2 family members (Letai et al., 2002). Owing to the mitochondrial localization of many of the Bcl-2 family members, they are often assumed to have a direct effect on the mitochondria, the pro-survival Bcl-2 proteins functioning at

least in part, by protecting the mitochondria during apoptosis, and the pro-apoptotic family members appear to disrupt mitochondrial function. However, Bcl-2 family members are also localized to the ER and the nucleus (Akao et al., 1994; Bartholomeusz et al., 2006; Breckenridge et al., 2003; Krajewski et al., 1993). Less attention has been paid to the roles of Bcl-2 family members in these other intracellular sites. Recently, pro-survival Bcl-2 family members have become an attractive new anti-cancer target through the discovery of novel classes of small-molecule inhibitors targeted at the BH3 binding pocket (Degterev et al., 2001; Letai et al., 2002).

Cell Cycle

The cell cycle represents a series of tightly integrated events that allow the cell to grow and proliferate. The cell cycle is divided into four phases G1, S (DNA synthesis), G2, and M (mitosis). The G1 and G2 phases represent "gaps" in the cycle that occur between DNA synthesis and mitosis. G0 or quiescent cells are not actively dividing. Growth factors primarily act on cells in G0 and G1. The point in G1 after which cells no longer respond to withdrawal of growth factors has been termed the restriction point. Cell cycle checkpoints maintain genetic stability and ensure accurate chromosome replication and separation. If a cell fails to meet the requirements of a phase it will not be allowed to proceed to the next phase. For example, in response to a DNA damaging agent such as ionizing radiation, cell cycle checkpoints that arrest cell cycle progression can be activated, allowing the cell to repair and prevent transmission of damaged or incompletely replicated chromosomes. If the damage is severe or irreparable, these checkpoints will drive the cell into apoptosis or

other forms of cell death (Evan and Littlewood, 1998). Disruption of these checkpoints is a common feature in cancer since this increases the chance for the cell to replicate damaged DNA thereby inducing mutation. Aneuploidy (a change in the number of chromosomes) is also a hallmark of many cancers (Kops et al., 2005).

Progression of a cell through the cell cycle is regulated by two protein classes; the cyclins, and their kinase partners, a family of serine/threonine protein kinases, cdks. Leland Hartwell, Timothy Hunt and Paul Nurse won the 2001 Nobel Prize in Physiology and Medicine for their discovery of these central molecules in the regulation of the cell cycle. The main function of cdks is the phosphorylation of substrates required for cell cycle progression (Morgan, 1997). Cdks are regulated predominantly at the post-transcriptional level, since protein concentrations remain constant throughout the cell cycle. Binding to a cyclin is necessary for the activation of cdks. Cyclins are specific regulatory proteins that also target the cdks to the nucleus. The cyclins were so named because of their cyclic expression during the cell cycle. Some cdks bind more than one cyclin. Corresponding cell cycle inhibitory protein cdk inhibitors serve as negative regulators of the cell cycle.

The p53 tumor suppressor is a DNA damage response protein that transcriptionally activates numerous genes (Vogelstein et al., 2000). The protein products of these genes arrest the cell cycle or induce apoptosis (el-Deiry et al., 1994). Under normal conditions, the p53 protein which exists as a tetramer is maintained at relatively low levels. Following cellular stress, p53 is stabilized, resulting in an increase in p53 levels, which can induce the expression of the cdk inhibitor $p21^{WAF1/CIP1}$ (hereafter referred to as p21), among other target genes. Mutations in the p53 gene leading to the inability of p53 to regulate the cell

cycle and apoptosis have been reported to contribute to drug resistance (Aas et al., 1996; el Rouby et al., 1993). Additionally, apoptosis induced by many anti-cancer agents seems at least in part to be p53-dependent (Lowe, 1995)

p21 is the founding member of the CIP/KIP family of cdk inhibitors which also includes p27 (Polyak et al., 1994; Toyoshima and Hunter, 1994) and p57 (Lee et al., 1995; Matsuoka et al., 1995). These three cdk inhibitors contain a conserved region at the amino terminus that is both required and sufficient for the inhibition of cyclin/cdk complexes, whereas the C terminal regions are variable in length and function (Chen et al., 1995; Lee et al., 1995; Polyak et al., 1994). They can bind and inhibit a broad range of cyclin/Cdk complexes, with a preference for those containing Cdk2 (Harper et al., 1995; Zhang et al., 1993). p21 is a key mediator of arrest at the G1 phase of the cell cycle and also contributes to G2 arrest (Bunz et al., 1998; Dulic et al., 1998; Niculescu et al., 1998). p21 inhibits the retinoblastoma (Rb)/E2F pathway. Rb is a cdk substrate. In its active state Rb is hypophosphorylated, forming an inhibitory complex with E2F. Hyperphosphorylation of Rb releases E2F, resulting in activation of E2F transcription factors and cell cycle progression (Trimarchi and Lees, 2002).

p15 (Hannon and Beach, 1994), p16 (Serrano et al., 1993), p18 (Guan et al., 1994) and p19 (Chan et al., 1995; Hirai et al., 1995) comprise the second family of cdk inhibitors, known as the INK4 family. In contrast to the broad spectrum of inhibitory effects on cyclin/cdk complexes of the CIP/KIP family the INK4 family members inhibit cdk4 or cdk6 specifically. The four members are closely related having four ankyrin motif tandem repeats in common and have very similar functions (Pei and Xiong, 2005). Cdk inhibitors

have been postulated to play critical roles in growth arrest and cell differentiation in response to growth-inhibitory signals transduced from the extracellular environment. As negative cell cycle regulators, the cdk inhibitors may be targets for inactivation in oncogenesis and tumor progression.

Sphingolipid Metabolism and the Sphingolipid Rheostat

Although this lipid class shows a great structural diversity and complexity, the characteristic feature of all sphingolipids is the presence of a sphingoid backbone, derived from the aliphatic amino alcohol sphingosine. The sphingoid backbone can be O-linked to a head group such as ethanolamine, serine, or choline. The backbone can also be amide-linked to an acyl group, such as a fatty acid. Fatty acids consist of a hydrocarbon chain with a carboxylic acid at one end. In addition to being structural constituents of cell membranes, sphingolipids play key roles as signaling molecules. Sphingolipid metabolism is a dynamic and tightly regulated process resulting in the formation of a number of bioactive metabolites, including ceramide, sphingosine, and S1P, which have all been implicated as important components of cell fate decisions (Cuvillier et al., 1996; Spiegel et al., 1998). Ceramide and sphingosine are usually associated with apoptosis and negative effects on cell growth and survival, whereas, S1P opposes these effects (Cuvillier et al., 1996). Increased ceramide and sphingosine levels have been shown to induce apoptosis in many cell types; for example ceramide and sphingosine enhance apoptosis of radiation-resistant prostate and breast cancer cells (Nava et al., 2000). In contrast, it has been shown

that exogenous addition of S1P protects oocytes *in vivo* from radiation-induced apoptosis (Morita et al., 2000).

This has led to the proposal that the balance between the cellular concentrations of ceramide and sphingosine versus S1P, the "sphingolipid rheostat", is important in determining whether cells survive or die (Cuvillier et al., 1996) (Figure 1). In agreement, studies from our lab show that sphingosine is involved in mitochondria-mediated apoptotic signaling induced by doxorubicin in human breast cancer cells (Cuvillier et al., 2001). Whereas in sharp contrast, S1P, formed by phosphorylation of sphingosine, protects against ceramide-mediated apoptosis and promotes estrogen-dependent tumorigenesis of breast cancer MCF7 cells (Nava et al., 2002). Despite intensive research, however, the molecular mechanisms that mediate the actions of sphingolipids in apoptosis remain unclear.

Ceramide has a variable length fatty acid chain attached by an amide bond at the carbon 2 position. Ceramide can be synthesized either in response to the agonist-dependent activation of sphingomyelinases or *de novo*. *De novo* ceramide synthesis begins within the ER and is initiated by the condensation of serine and palmitoyl-CoA, catalyzed by the pyridoxal-dependent enzyme serine palmitoyltransferase (SPT) to form 3-ketodihydroshingosine, which in turn undergoes reduction to dihydrosphingosine. A fatty acyl group is added by an amide linkage to form dihydroceramide, which is converted directly to ceramide through the actions of dihydroceramide desaturase (Bielawska et al., 1993; Futerman et al., 1990) (Figure 2). This pathway can be stimulated by drugs and

ionizing radiation and usually results in prolonged ceramide activation (Bose et al., 1995; Liao et al., 1999).

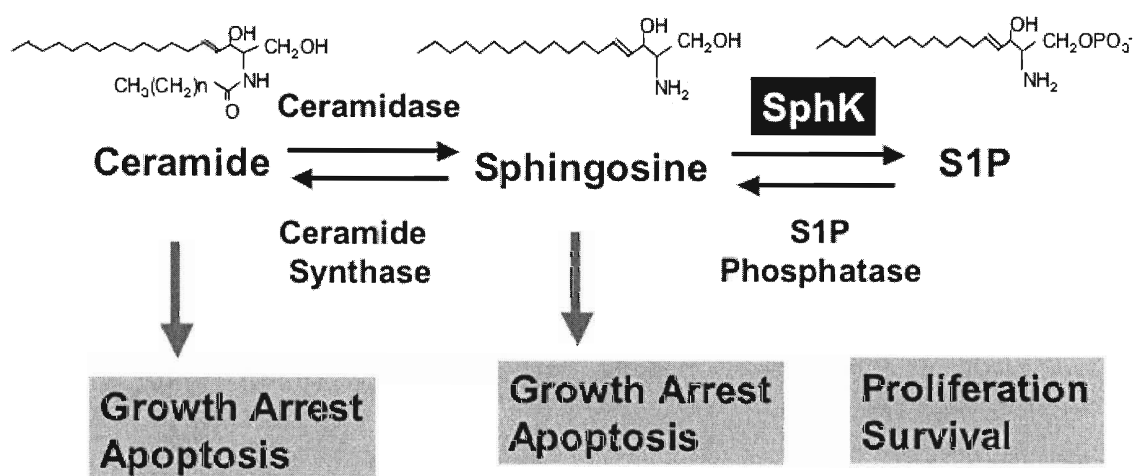

Figure 1. The Sphingolipid Rheostat: a balance between life and death

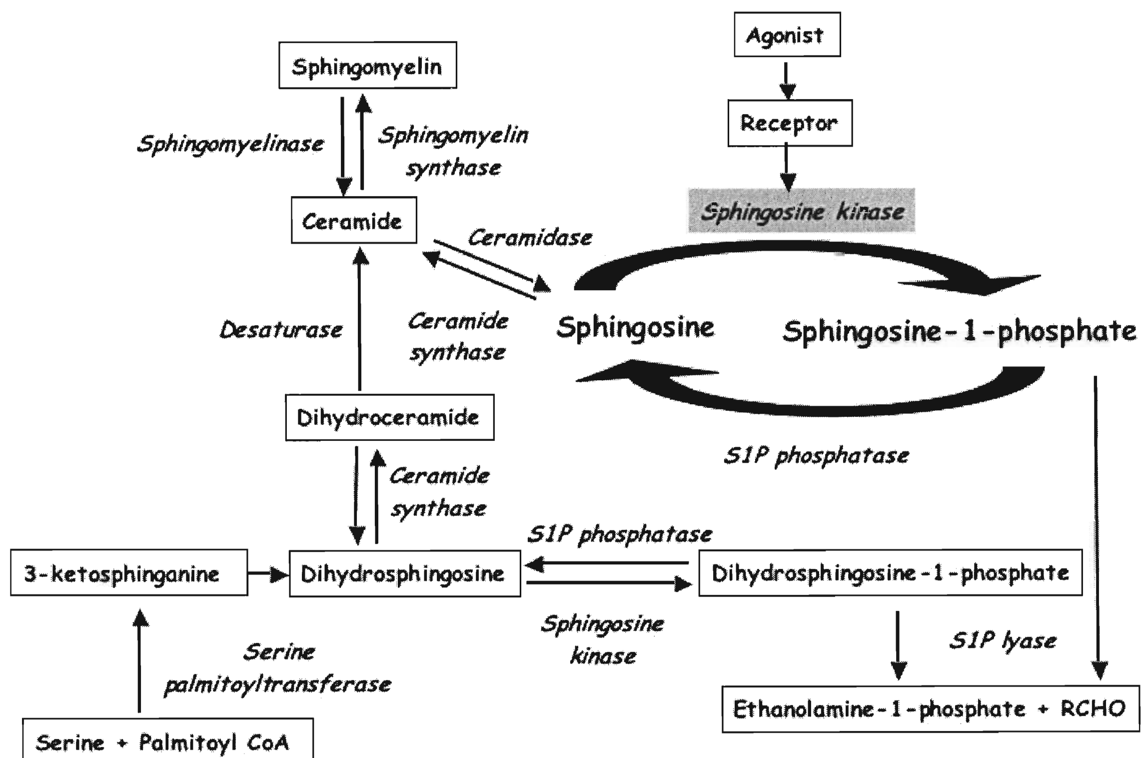

Figure 2. Overview of Sphingolipid Metabolism

Conversely, ceramide can be formed by the breakdown of sphingomyelin as a result of the action of sphingomyelinases (Figure 2). As much as two thirds of the total cellular sphingomyelin resides at the plasma membrane, where it plays important structural and functional roles. Sphingomyelinases are specialized enzymes with phospholipase C activity that hydrolyze the phosphodiester bond of sphingomyelin. Acidic sphingomyelinases are found in lysosomes, whereas neutral sphingomyelinases exhibit both cytosolic and plasma membrane forms (Bielawska et al., 1993; Futerman et al., 1990) (Hannun, 1994; Kolesnick and Kronke, 1998). Sphingomyelinases are rapidly stimulated by exogenous stimuli. *De novo* ceramide synthesis can be blocked at SPT by the fungal metabolite myriocin (Miyake et al., 1995), whereas the mycotoxin fumonisin B1 inhibits ceramide synthase (Wang et al., 1991).

Complex sphingolipids, such as cerebrosides and gangliosides, are synthesized by the insertion of different substituents at the 1-hydroxyl position of ceramide. Phosphorylation of ceramide by ceramide kinase (CerK) (Hannun et al., 2001; Mathias et al., 1998) generates ceramide-1-phophate (not shown). In addition, ceramide can also be converted back to sphingomyelin by transfer of phosphorylcholine from phosphatidylcholine through sphingomyelin synthase (Hannun et al., 2001; Mathias et al., 1998; Ullman and Sprecher, 1971; Ullman and Radin, 1974) (Figure 2). Alternatively, ceramidases catalyze the deacylation of ceramide to produce a free fatty acid and sphingosine. Sphingosine can be converted back to ceramide by ceramide synthase, through the salvage pathway.

SphKs catalyze the ATP-dependent phosphorylation of sphingosine to form S1P. S1P levels are regulated by S1P lyase and S1P phosphatase. S1P lyase cleaves S1P at the C2-C3 bond to produce palmitylaldehyde and ethanolamine-1-phosphate (Zhou and Saba, 1998).Whereas, S1P phosphatases dephosphorylate S1P (Mandala et al., 2000; Van Veldhoven et al., 1994) (Figure 2).

Sphingosine-1-Phosphate – An Important Signaling Molecule

S1P was originally considered to be formed merely as an intermediate in the detoxification of sphingosine by its phosphorylation and subsequent degradation (Stoffel and Assmann, 1970). However, it is now known that S1P is able to activate and regulate a diverse array of signaling pathways, resulting in a wide range of responses, including cell growth, survival, motility and invasion, as well as reorganization of the cytoskeleton and calcium mobilization (Hla, 2001; Pyne and Pyne, 2002; Spiegel and Milstien, 2002).

As a well-established extracellular regulator, S1P is a specific ligand for a family of specific G protein coupled receptors (GPCRs), which also bind dihydro-S1P. Cellular S1P, produced by SphKs, can be exported and act on S1P receptors, a process which has been termed 'inside-out signaling' (Spiegel and Milstien, 2003b). However, the mechanisms of S1P secretion are still unclear. Very recently, the involvement of the ABC transporter, ABCC1 in cellular S1P release was demonstrated in mast cells (Mitra et al., 2006). Inside-out signaling through $S1P_2$ has been shown to drive degranulation of mast cells, in response to SphK1 activation mediated by Fc receptor- cross-linking (Jolly et al., 2004).

A second messenger role for S1P was first proposed when a kinase-dependent sphingosine-stimulated release of calcium from intracellular stores was observed (Ghosh et al., 1990; Ghosh et al., 1994). In addition, several other lines of evidence support a role for intracellular actions of S1P. For example, activation of Ras and extracellular signal-regulated kinase (ERK1) signaling pathways by vascular endothelial growth factor (VEGF) requires SphK1 and is independent of S1P receptors (Shu et al., 2002; Wu et al., 2003). Also, intracellular S1P, released from caged S1P by photolysis, mobilized calcium from thapsigargin-sensitive stores even in cells that did not express S1P receptors (Meyer zu Heringdorf et al., 2003). Moreover, despite the ability to bind to and activate all the S1P receptors, dihydro-S1P does not mimic all of the biological effects of S1P, particularly those related to cell survival (Van Brocklyn et al., 1998). In addition, studies in *Dictostelium*, *Drosophila* and *Caenorhabiditis elegans* have revealed that these diverse lower organisms, which are devoid of S1P receptors (Herr et al., 2004; Herr et al., 2003; Min et al., 2005), still possess S1P metabolizing enzymes and are still responsive to S1P. Plants also lack S1P receptors but still respond to drought conditions by S1P dependent inhibition of stomatal opening and enhancement of stomatal closure (Coursol et al., 2003; Ng et al., 2001). However, the intracellular effects of S1P remain speculative since intracellular targets of S1P have not yet been identified.

The S1P Receptor Family

To date, five closely related GPCRs previously known as endothelial differentiation gene (EDG) family receptors have been identified as high-affinity receptors for S1P (Hla

and Maciag, 1990; MacLennan et al., 1994; Okazaki et al., 1993; Yamazaki et al., 2000), and are now called S1P receptors. The EDG family originally included three closely related yet distinct lysophosphatidic acid (LPA) receptors, LPA_{1-3} specifically bind LPA, a bioactive phospholipid with similar biological effects and structure to S1P (Contos and Chun, 1998; Fukushima et al., 2001). These receptors also share some homology with the cannabinoid receptor family (<30%), perhaps suggestive of a possible common ancestral gene (Lynch and Im, 1999). S1P receptors are integral membrane proteins that are probably glycosylated, they are predicted to have seven transmembrane domains and exhibit approximately 50% amino acid identity. The S1P receptor subfamily includes $S1P_1$ (EDG-1), $S1P_2$ (EDG-5/H218/AGR16), $S1P_3$ (EDG-3), $S1P_4$ (EDG-6), and $S1P_5$ (EDG-8/NRG-1) (Figure 3). The various S1P receptors are differentially expressed, and couple to different G proteins (G_q, $G_{i,}$, G_{12-13}) (Windh et al., 1999). This allows extracellular S1P the ability to regulate a diverse range of physiological processes (Figure 3).

The first of the S1P receptors to be identified, $S1P_1$ was cloned as an immediate early gene induced during endothelial differentiation (Hla and Maciag, 1990). It is expressed in most mammalian tissues with highest expression in skeletal structures undergoing ossification, endothelial cells, and the Purkinje cell layer of the cerebellum. $S1P_1$ signaling has been demonstrated to be coupled to G_i in a number of cell types and often results in ERK1/2 activation and inhibition of adenylyl cyclase (Okamoto et al., 1998; Zondag et al., 1998). $S1P_1$ receptor knockout mice show embryonic lethality due to severe hemorrhage as the result of deficient coverage of vessels by vascular smooth muscle

cells, a process that occurs during the last stages of angiogenesis and is necessary for stabilizing the vascular system (Allende et al., 2003; Liu et al., 2000b).

$S1P_2$ and $S1P_3$ are closely related and share 92% sequence identity. Thus, it is not surprising that their tissue distribution and the biological effects that they mediate are also very similar. These receptors are predominantly expressed in the lung, heart, kidney, liver, spleen, thymus, testis, and brain (Zhang et al., 1999). In contrast to $S1P_1$ null mice, $S1P_2$ and $S1P_3$ or $S1P_2$/$S1P_3$ receptor knockout mice are viable and show only modest phenotypic changes (Ishii et al., 2001; Ishii et al., 2002). $S1P_2$ and $S1P_3$ couple to G_1, G_q, G_{12} and G_{13} (An et al., 1999; Ancellin and Hla, 1999). The C-terminus of $S1P_3$ is unique amongst the S1P receptors in that it contains a putative class I Src homology 3 (SH3) interaction motif (RASPIQP), important in tyrosine kinase signaling.

$S1P_4$ and $S1P_5$ are the most recently identified and therefore the least well characterized S1P receptors. The expression of $S1P_4$ is confined to lymphoid and hematopoietic tissue and $S1P_5$ to the central nervous system. Currently, it is believed that $S1P_4$ can couple to the heterotrimeric G-protein, G_i, although this proposal is based only on the pertussis toxin (PTX) sensitivity of $S1P_4$-mediated ERK1/2 activation (Van Brocklyn et al., 2000). Reports indicate that $S1P_5$ can couple to the heterotrimeric G-proteins G_i and G_{12} but not to G_q or G_s (Malek et al., 2001).

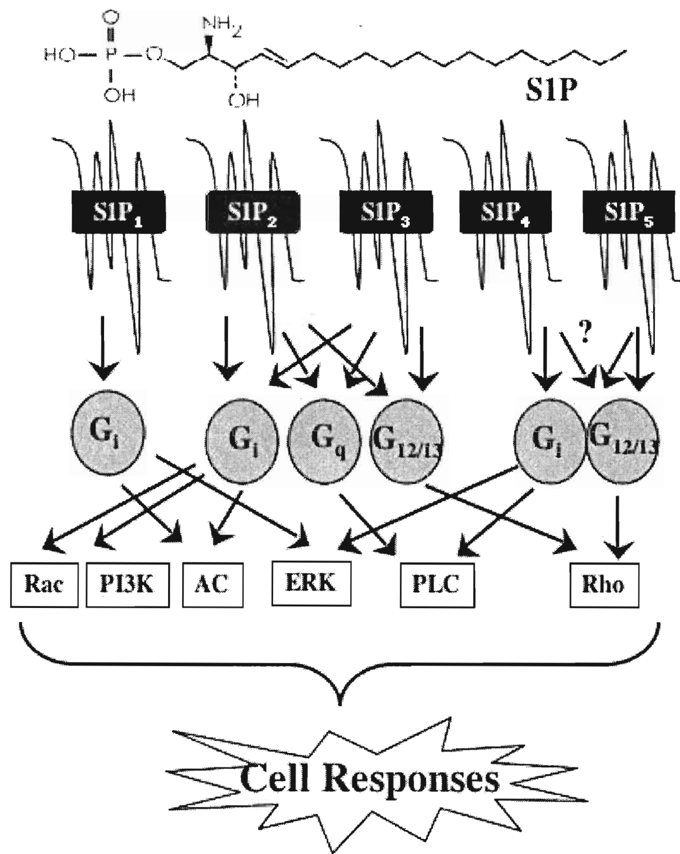

Figure 3. G Protein-Coupled Signaling by Sphingosine-1-Phosphate Receptors

Sphingosine Kinases – The Enzymes Responsible for S1P Formation

SphK proteins constitute a novel family of lipid kinases. These enzymes do not share homology with other lipid kinases and furthermore, do not show any homology with lipid binding domains found in other lipid-binding proteins. Five critical regions of highly conserved amino acids (C1-C5) have been identified in all SphK forms. The C1-C3 domains share homology with diacylglycerol kinase (DGK) and CerKs (Sugiura et al., 2002). The C4 domain is peculiar to SphKs and appears to be less conserved in CerK (Sugiura et al., 2002). The C5 domain is also conserved in ceramide kinase (Sugiura et al., 2002) as well as DGKs (Yokota et al., 2004). Recently, the ATP-binding site in SphK was shown to reside in the C2 domain within the consensus sequence $SGDGX_{17\text{-}21}K$ (Pitson et al., 2002).

SphK1, the first mammalian sphingosine kinase to be purified (Olivera et al., 1998) and cloned (Kohama et al., 1998) and thus the more studied isoform, has an apparent molecular mass of 49 kDa and is predominantly found in the cytosol although a small proportion is also associated with membrane fractions (Kohama et al., 1998). SphK1 specifically phosphorylates D-*erythro*-sphingosine. The sphingosine analog *N,N*-dimethylsphingosine (DMS), and D, L-*threo*-dihydrosphingosine (DHS) are competitive inhibitors of SphK1 (Edsall et al., 1998). DMS and DHS however, cannot be considered as specific inhibitors of SphK1 because they are also known to potently inhibit PKC and SphK2 (Igarashi and Yatomi, 1998; Jarvis et al., 1996). Sphingosine kinases have also been found in *Sacchoromyces cerevisiae*, and were identified as sphingolipid long chain base (LCB) kinases, hence the gene nomenclature *LCB4 and LCB5* (Nagiec et al., 1998).

Sphingosine kinases have also been identified in *Caenorhabditis elegans* (Kohama et al., 1998), *Drosophila melanogaster* (Herr et al., 2004), and *Arabidopsis thaliana* (Nishiura et al., 2000), as well as other organisms.

Many growth and survival factors have been reported to increase SphK1 activity, including platelet-derived growth factor (PDGF) (Olivera and Spiegel, 1993), epidermal growth factor (EGF) (Meyer zu Heringdorf et al., 1999), protein kinase C (PKC) activators (Johnson et al., 2002; Pitson et al., 2003), PS (Olivera et al., 1996), LPA (Young et al., 1999), and estrogen (Sukocheva et al., 2003). In many cases, increase of SphK1 activity is transient, reaching a peak within a few minutes after activation and declining typically within 30-60 minutes. The rapid onset of activation is suggestive of a post-translational modification; such as phosphorylation. Human SphK1 has been shown to be phosphorylated at Ser^{225} and this phosphorylation is mediated by ERK1/2 (Pitson et al., 2003). However, phosphorylation itself does not seem to enhance the intrinsic activity of SphK1, but rather facilitates the enzyme's association with membranes (Pitson et al., 2005; Xia et al., 2000). It is thought that SphK1 is mainly present in the cytosol and translocation of SphK1 to the plasma membrane appears to be an important and common feature of its activation (Spiegel and Milstien, 2003a). Phorbol 12-myristate 13-acetate (PMA) induction of PKC induces SphK1 phosphorylation and results in a decrease in cytosolic SphK and an increase in membrane associated SphK (Johnson et al., 2002). A recent study provided evidence that a phosphorylation-induced conformational change in SphK1 enhances the binding to PS and, thus, prolongs its retention at the plasma membrane (Stahelin et al., 2005). Although presently, the molecular mechanisms that mediate SphK1 translocation to

the plasma membrane remain unclear. Interestingly, SphK1 has a proline rich domain at its carboxyl terminus (C-terminus), which is reminiscent of SH3-binding domains (Albi et al., 1994; Ren et al., 1993). Two nuclear export sequences (NES1 and NES2) have also been identified in SphK1 and were shown to mediate shuttling of the protein from the nucleus to the cytosol (Inagaki et al., 2003).

Several SphK1-interacting proteins have been identified. These include TNF receptor-associated factor 2 (TRAF2) (Xia et al., 2002), calmodulin (CaM) (Young et al., 2003), platelet endothelial cell adhesion molecule-1 (PECAM-1) (Fukuda et al., 2004), δ-catenin (Fujita et al., 2004), aminoacylase 1 (Maceyka et al., 2004), an SphK1-interacting protein (SKIP) - a protein related to protein kinase A anchoring protein (Lacana et al., 2002), and RPK118 (Hayashi et al., 2002). There is accumulating evidence that the interaction of SphK1 with some of these proteins may influence the activity and subcellular localization of SphK1 but the physiological significance of their interaction remains unclear.

SphK2: A New Member of the SphK family

A second mammalian isoform of SphK (SphK2) was cloned and characterized in our laboratory (Liu et al., 2000a). Although highly similar in amino acid composition and sequence, SphK2 is much larger than SphK1 and diverges in its amino terminus and central region (Figure 4). Mouse SphK2 (mSphK2) and human SphK2 (hSphK2) encode proteins of 617 and 618 amino acids respectively. Human SphK1 localizes to chromosome 17, whereas, mouse SphK2 maps to chromosome 19 (Liu et al., 2000a). Although most

cells express both SphK1 and SphK2 (Kohama et al., 1998; Liu et al., 2000a), reverse transcription-polymerase chain reaction (RT-PCR) and Northern blot analysis revealed that SphK2 has a different tissue distribution compared to SphK1. SphK2 expression is highest in the liver, followed by kidney, brain, lung, spleen, and lymph nodes whereas, SphK1 expression is highest in lung and spleen, followed by brain kidney and lymph nodes, and low in liver (Billich et al., 2003; Kohama et al., 1998; Liu et al., 2000a). They also differ in the temporal patterns of their appearance during development (Liu et al., 2000a).

SphK2 appears to be more promiscuous in its substrates compared to SphK1. SphK2 demonstrates the surprising ability to phosphorylate DHS (Liu et al., 2000a), an inhibitor of SphK1 (Kohama et al., 1998; Pitson et al., 2000a). Also, unlike SphK1, SphK2 is able to phosphorylate phytosphingosine. In addition, while SphK1 phosphorylates the ether lipid 1-*o*-hexadecyl-2-desoxy-2-amino-*sn*-glycerol (Gijsbers et al., 2002), SphK2 catalyzes the phosphorylation about 10-fold more efficiently (Baumruker et al., 2005). Furthermore, recent studies have indicated that SphK2 is the main enzyme responsible for the phosphorylation and activation of the immunosuppressive drug FTY720 (2-amino-2[2-(4-octylphenyl) ethyl]propane-1,3-diol) (Billich et al., 2003; Paugh et al., 2003; Sanchez et al., 2003). FTY720 was developed by modifying myriocin and shares structural characteristics with sphingosine. Unlike myriocin, FTY720 does not inhibit SPT. (Kiuchi et al., 1998). FTY720 is gaining wide interest in the transplantation field due to its efficacy against autoimmune diseases and transplant rejection (Brinkmann et al., ; Brinkmann and Lynch, 2002). It is now in clinical trials for kidney transplantation and multiple sclerosis. Once phosphorylated, FTY720-P acts as an agonist on four of the five known S1P

receptors ($S1P_1$, $S1P_3$, $S1P_4$ and $S1P_5$) (Brinkmann et al., 2002). It has recently been shown that SphK2 is essential to lymphopenia induced with FTY720, mediated through agonism of the $S1P_1$ receptor by FTY720-P (Kharel et al., 2005; Zemann et al., 2006).

The activation of SphK2 has not been as well studied as that of SphK1, however, it has been reported to be activated by EGF (Hait et al., 2005), and in mast cells by Fc-receptor cross-linking (Olivera et al., 2006). Our laboratory recently reported that EGF and PMA can induce phosphorylation of human SphK2, which was markedly reduced by inhibition of the ERK1/2 pathway (Hait et al., 2007). Site-directed mutagenesis indicated that SphK2 is phosphorylated on Ser^{351} and Thr^{578} (Hait et al., 2007).

While DMS competitively inhibits SphK1, it non-competitively inhibits SphK2. Additionally, while SphK1 has high activity in the presence of Triton X-100 and low salt concentrations, SphK2 is inhibited by Triton X-100 and is activated in the presence of high salt concentrations (Liu et al., 2000a). Bovine serum albumin (BSA), which is often used as a vehicle for substrate presentation, has also been shown to inhibit SphK2 activity while having no effect on SphK1 (Liu et al., 2000a).

Similar to SphK1, SphK2 also has a proline rich domain, which is larger than that of SphK1 (Figure 4). SphK2 has been shown to associate with the murine interleukin-12 (IL-12) receptor, identified by a yeast two-hybrid approach and may play a role in modulating IL-12 signaling (Yoshimoto et al., 2003). However, it remains to be examined whether the interaction between SphK2 and the IL-12 receptor occurs in humans. A recent study also demonstrated that SphK2 binds CaM through a CaM binding region that is conserved with SphK1 (Sutherland et al., 2006). However, neither Ca^{2+}/CaM nor apoCaM

have any effect on the enzymatic activity of SphK2 *in vitro* (Sutherland et al., 2006). Thus, the physiological significance of the interaction of SphK2 with CaM remains unclear.

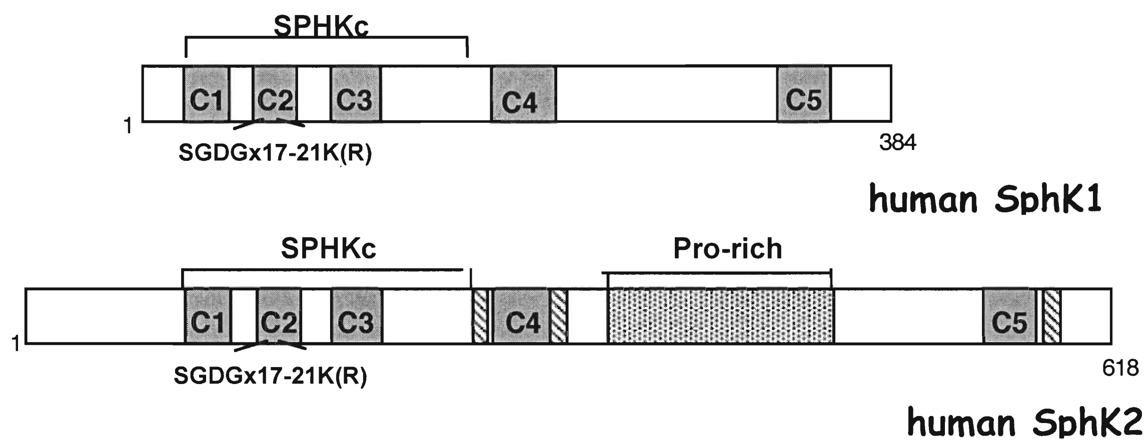

Figure 4. Human Sphingosine Kinase Isoforms

The Role of Sphingolipid Metabolites in Cancer

As previously mentioned, the balance between ceramide/sphingosine levels *vs* S1P provides a rheostat mechanism that determines whether a cell survives or undergoes apoptosis. One of the key regulatory enzymes is SphK1, and multiple evidence exists that SphK1 overexpression can abrogate apoptosis, notably by shifting the ceramide/S1P balance towards the cytoprotective S1P (Bektas et al., 2005; Olivera et al., 1999; Pchejetski et al., 2005). The first studies proposing SphK1 as an oncogene, observed that NIH 3T3 fibroblasts stably transfected with SphK1 exhibited enhanced cell proliferation, accompanied by increased S1P production and acquired the capability to form tumors in nude mice (Vadas and Gamble, 1996). Overexpression of SphK1 also enhances growth in soft agar, and protects cells from apoptosis - decreasing levels of pro-apoptotic ceramide and sphingosine (Edsall et al., 2001; Olivera et al., 1999). SphK1 expression has been found to be upregulated in many human solid tumors, such as those of the breast, lung, ovary, stomach, uterus, kidney and rectum (French et al., 2003; Johnson et al., 2005; Kawamori et al., 2006). The expression of SphK1 was also found to be upregulated in colon cancer tissues induced by azoxymethane, a well-characterized carcinogen that induces colon cancer in rodents (Kawamori et al., 2006). Enforced expression of SphK1 increased S1P levels and blocked MCF7 breast cancer cell death induced by sphingosine and tumor necrosis factor-alpha (TNF-α). When injected into mammary fat pads of ovariectomized nude mice implanted with estrogen pellets, MCF7/SphK1 cells formed more and larger tumors with higher microvessel density in their periphery than vector

tranfectants. Also, the sensitivity of MCF7 cells to the anti-cancer drug doxorubicin was potentiated by DMS (Nava et al., 2002). In addition, expression of SphK1 in human astrocytoma grade 4 (glioblastoma multiforme) tissue has been shown to correlate with a short patient survival (Van Brocklyn et al., 2005). SphK1 and S1P also mediate cycloxygenase-2 (Cox-2) and prostaglandin E_2 (PGE_2) induction, which are thought to play important roles in colon cancer (Kawamori et al., 2006). Furthermore, it has recently been suggested that SphK1 may also function to regulate autophagy, a cellular process that enables cells to degrade and recycle cytoplasmic contents during development, tissue homeostasis, and stress, to protect cells from apoptosis in response to nutrient deprivation (Lavieu et al., 2006). The use of SphK inhibitors, and expression of a dominant-negative SphK1 mutant have also been recently shown to sensitize many tumor cells to chemotherapeutics (Bektas et al., 2005; Pchejetski et al., 2005). SphK1 has therefore become an attractive target for the development of inhibitors in cancer therapy (Jendiroba et al., 2002).

Angiogenesis is the process by which new blood vessels are formed from pre-existing vasculature. The angiogenesis process associated with tumors is considered to be a crucial component of disease progression. A number of reports on the angiogenic role of S1P have been published. It has been reported that S1P can transactivate VEGF receptors in endothelial cells (Tanimoto et al., 2002). Work by Chae *et al.* has demonstrated that tumor cells upregulate the expression of $S1P_1$ during angiogenesis and that $S1P_1$ is necessary for the angiogenic response of the tumor *in vivo* (Chae et al., 2004). Cells that overexpress $S1P_1$ are resistant to the anti-angiogenic agents, thalidomide and neovastat

(Annabi et al., 2003). S1P may also aid tumor development by stimulating inflammatory responses since inflammation is known to participate in cancer promotion (Nakanishi and Toi, 2005; Philip et al., 2004). S1P has also been detected in the ascites fluid of ovarian cancer patients (Hong et al., 1999).

Recent data by Visentin *et* al., showed that an anti-S1P immunoneutralizing monoclonal antibody (sphingomab) reduced tumor progression by inhibiting cell migration, S1P-mediated proliferation, neovascularization, and the release of pro-angiogenic cytokines VEGF and IL-8; suggesting that S1P is a validated target (Visentin et al., 2006). Importantly, mice xenografted with orthotopically placed human cancer cells exhibited substantial reduction of tumor progression with sphingomab treatment (Visentin et al., 2006).

Conversely, consistent with the general idea that ceramide is a mediator of apoptosis, lack of ceramide generation has been linked to tumor cell resistance to death (Senchenkov et al., 2001). Furthermore, chemotherapy and radiotherapy elicit an increase in endogenous ceramide levels before the first biochemical signs of apoptosis and radio-resistant and chemo-resistant cell lines have been shown not to generate ceramide after irradiation of chemotherapy (Bruno et al., 1998; Yamagata et al., 2003). Also, attenuation of ceramide in clinical malignant astrocytoma patient samples correlates inversely with disease progression and severity of prognosis (Riboni et al., 2002). In addition, the total content of ceramide was decreased in ovarian tumors when compared to normal ovarian tissues (Rylova et al., 1998).

The Role of Sphingolipid Metabolites in Cell Cycle Progression

Although not definitive, there are indications that there is a mechanism for cell cycle control by the sphingolipid rheostat. Ceramide has been shown to induce cell cycle arrest at G0/G1 through the dephosphorylation of the Rb protein, activation of the cdk inhibitor p21, and inhibition of Cdk2 (Connor et al., 2001). Sphingosine has also been shown to induce dephosphorylation of the the Rb protein and induce cell cycle arrest (Chao et al., 1992). (Alesse et al., 1998; Pushkareva et al., 1995). Conversely, S1P has been shown to be required for entry of muscle satellite cells into the cell cycle due to its mitogenic properties (Nagata et al., 2006). Other studies have found that overexpression of SphK1 in NIH 3T3 cells reduced the fraction of cells in G0/G1 and, increased the proportion in the S phase (Olivera et al.). Also, decreasing endogenous SphK1 levels in MCF7 breast cancer cells using siRNA resulted in a shift of 16% of the cell population from the S to the G1 phase, suggesting that SphK1 may be required for normal progression through the cell cycle (Taha et al., 2006). Interestingly, it has been shown that the synthesis of sphingosine and S1P may be regulated by the cell cycle (Fatatis and Miller, 1999).

CHAPTER 2

STATEMENT OF WORK

It is hypothesized that the susceptibility of cancer cells to apoptosis can be altered by modulating levels of sphingolipids. As is typical of signaling molecules, intracellular levels of S1P are low and tightly regulated; SphK plays a major role in this regulation. In contrast to SphK1, much less is known about SphK2. Elucidating the mechanism of action of SphK2 in regulating growth and apoptosis of cells may increase our understanding of the roles of sphingolipids in cancer and chemotherapeutic drug resistance. The ability to alter susceptibility of cancer cells to apoptotic stimuli may provide the basis for enhancing existing therapeutic approaches.

Specific Aims

1. To investigate the role of overexpressed SphK2 in cell survival and apoptosis.

2. To investigate the functions of endogenous SphK2 (by downregulating SphK2) in human carcinoma cells and how it influences the balance between cytostasis and apoptosis in response to chemotherapeutics.

CHAPTER 3
SPHINGOSINE KINASE 2 IS A BH3-ONLY PROTEIN THAT INDUCES APOPTOSIS WHEN OVEREXPRESSED

The work presented in this chapter has been published in The Journal of Biological Chemistry 2003 Oct 10; 278 (41): 40330-6 and The Journal of Biological Chemistry 2005 Nov 5; 280 (44): 37118-29. Construction of SphK2 constructs was performed by Dr. H. Liu. Scoring of apoptotic cells was assisted by Dr. R. Toman.

Introduction

S1P is produced in cells by the phosphorylation of sphingosine, the backbone of sphingolipids, catalyzed by SphKs. Two isoforms of mammalian SphK (SphK1 and SphK2) have been cloned and characterized. [reviewed in ((Maceyka et al., 2005a)]. Northern blot analysis has shown that the two mammalian SphK isoenzymes differ in their temporal and spatial distribution, with SphK1 transcripts appearing before SphK2 transcripts in development, suggesting that they have distinct physiological functions (Liu et al., 2000a). It has been well established that SphK1 expression and formation of S1P are linked to cell growth and survival. In contrast to SphK1 much less is known about the functions of Sphk2. This study examined the biological effects of overexpressing SphK2 in cells. Overexpression of SphK2 in NIH 3T3 cells markedly enhanced apoptosis in response to serum withdrawal that was preceded by cytochrome *c* release and caspase activation, observations that are in sharp contrast to the effects of SphK1 overexression.

Materials and Methods

Reagents

Serum and medium were from Biofluids (Rockville, MD). BAPTA acetoxymethyl ester (BAPTA-AM; Molecular Probes, Eugene, OR) was resuspended in DMSO. The stock solution was diluted in the relevant medium for administration to cells. Control cultures received the equivalent amount of vehicle. Ru-360 (Calbiochem, San Diego, CA) and PTX (Sigma, St. Louis, MO) were resuspended in water. The stock solution was diluted in the relevant medium for administration to cells. Antibodies to cytochrome *c*, HA, ERK2, and tubulin were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-caspase 3 antibody was from Stressgen Biotechnologies (San Diego, CA). Caspase 12 antibody was kindly provided by Dr. J. Yuan (Nakagawa et al., 2000). Secondary antibodies were from Jackson Immunoresearch (West Grove, PA).

Cell Culture and Transfection

NIH 3T3 fibroblasts (ATCC CRL-1658) were grown in high glucose Dulbecco's modified Eagle's medium (DMEM) containing 2 mM L-glutamine supplemented with 10% calf serum. Wild type (WT), $S1P_2$, $S1P_3$, and $S1P_2/S1P_3$ knockout mouse embryonic fibroblasts (MEFs) (kindly provided by Dr. I Ishii) were derived from day 14 embryos generated from WT or knockout double intercrossed mice as described previously (Ishii et al., 2002). MEFs were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS). WT and Bax/Bak knockout MEFs (a kind gift from Dr. H. Harada) were grown in DMEM supplemented with 10% heat-inactivated FBS (Wei et al., 2001).

Cells were cultured at 37°C and 5% CO_2. Cells were transfected using LipofectAMINE/PLUS reagent (Invitrogen, Gaithersburg, MD) according to the manufacturer's instructions. Transfection efficiency was approximately 50%.

Western Blot Analysis

Unless otherwise indicated, cells were lysed in buffer containing 50 mM Hepes (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EDTA, 2 mM sodium orthovanadate, 4 mM sodium pyrophosphate, 100 mM NaF, and 1:500 dilution of protease inhibitor mixture (Sigma, St Louis, MO). For analysis of cytochrome *c* release from mitochondria, cytosolic fractions were prepared by resuspending cells in lysis buffer containing 75 mM NaCl, 1 mM NaH_2PO_4, 8 mM Na_2HPO_4, 1 mM EDTA, 250 mM sucrose and 700 μg/ml digitonin, lysates were then centrifuged at 14,000 x *g* for 15 min.

Equal amounts of proteins were separated by SDS-PAGE and transblotted to nitrocellulose, blocked with 5% non-fat dry milk for 2 h at room temperature, and then incubated with primary antibodies overnight. Appropriate horseradish peroxidase-conjugated secondary antibodies were added in Tris-buffered saline containing 5% non-fat milk. Immunoreactive signals were visualized by enhanced chemiluminescence (Pierce, Rockford, IL) and blots exposed to Kodak X-Omat film

Sphingosine Kinase Assay

Cells were lysed by freeze-thawing in SphK buffer (20 mM Tris (pH 7.4), 20% glycerol, 1 mM 2-mercaptoethanol, 1 mM EDTA, 5 mM sodium orthovanadate, 40 mM β-

glycerophosphate, 15 mM NaF, 10 μg/ml leupeptin, aprotinin, and soybean trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride, and 0.5 mM 4-deoxypyridoxine). SphK2 activity was determined with sphingosine added as a complex with 4 mg/ml BSA and [γ-^{32}P]ATP in the presence of 1 M KCl, conditions in which SphK2 activity is optimal and SphK1 strongly inhibited (Liu et al., 2000a). Labeled S1P was extracted and separated by thin layer chromatography (TLC) with chloroform/acetone/methanol/acetic acid/H_2O (10:4:3:2:1, v/v). Radioactive bands corresponding to S1P were quantified with a FX Molecular Imager (Bio-Rad, Oakland, CA). Specific activity is expressed as picomols of S1P formed/min/mg of protein.

Sphingosine Kinase Mutants

Mammalian expression constructs of cDNA for SphK2 were described previously (Liu et al., 2000a). Murine SphK2 was cloned into the Bg1II site of pSG5-HA. The QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) was used according to the manufacturers protocol to prepare HA-SphK2 mutants (G213E mutation primers: 5'-GTGTCTGGAGACGAGCTGCTTTACGAGG-3' and 5'-CCTCGTAAAGCAGCTCGTCTCCAGACAC-3'; L219A mutation primers: 5'-GCTTTACGAGGTGGCGAATGGGCTCCTTG-3' and 5'-CAAGGAGCCCATTCGCCACCTCGTAAAGC-3'). The G213E/L219A double mutant was prepared with the G213E primers using SphK2-L219A as a template. All mutations were confirmed by sequencing.

The following primers were used to construct expression vectors for amino and carboxyl terminal fragments of mSphK2: forward 5'-TGGAATTCTGGCCCCACCACCACTACTGCCAGT-3', reverse: 5'-CTCTCAGTCTGGCCGATCAAGGAG-3'; forward: 5'-AACATGGAGGATGCCGTGCGGATG-3', reverse: 5'-TGGTCCACCAACTCGCCATGCTT-3', respectively. PCR products were cloned into pcDNA3.1/V5-His Topo (Invitrogen, Gaithersburg, MD).

Cell Death Assays

Apoptotic cell death was measured by staining cell nuclei with 8 μg/ml Hoechst dye 33342 bisbenzimide (Sigma, St. Louis, MO) and apoptotic cells were identified by condensed, fragmented nuclear regions using a Nikon TE 300 fluorescent microscope. A minimum of 300 cells were scored. Alternatively, apoptosis was measured with the Cell Death Detection ELISA kit (Roche Applied Science, Indianapolis, IN) that determines cytoplasmic histone-associated DNA fragments. Apoptosis was confirmed by cytochrome *c* release and activation of caspase 3.

Statistical Analysis

For each experiment, data from triplicate samples were calculated and expressed as mean $\pm$ standard deviation (SD). Statistical analysis was performed using SigmaStat statistical software version 2.0.

Results

Overexpression of SphK2 Induces Apoptosis in NIH 3T3 Cells, which Proceeds through Cytochrome c Release and Caspase 3 Cleavage Independently of S1P Receptors

In general, the stimulation of SphK1 and subsequent formation of S1P has been associated with cellular growth and survival (Edsall et al., 2001; Olivera et al., 1999). In sharp contrast, when SphK2 was overexpressed in NIH 3T3 fibroblasts a higher percentage of apoptotic cells was observed in response to serum deprivation for 24 h compared to vector expressing cells, where shrinkage and condensation of nuclei were clearly evident (Figure 5). Mitochondria are a key regulatory element of the intrinsic pathway of apoptosis. Cytochrome *c* resides loosely attached in the mitochondrial intermembrane space where it functions as an electron carrier in the respiratory chain by interacting with redox partners of complex III and complex IV (Hatefi, 1985). Mitochondrial cytochrome *c* translocates to the cytosol in cells undergoing apoptosis, where it participates in the activation of caspases. Pro-survival Bcl-2 and Bcl-x_L can prevent the efflux of cytochrome *c* from mitochondria (Reed and Green, 2002). Overexpressed SphK2 increased release of cytochrome *c* from the mitochondria into the cytosol and activated caspase 3, as determined by processing to the p17 form, which preceded the appearance of fragmented nuclei after 24 h in response to serum withdrawal (Figure 6). Collectively, this suggests that apoptosis induced by SphK2 overexpression proceeds via the intrinsic mitochondrial death pathway.

Due to the fact that most of the well-known functions of S1P, the product of SphK2, are elicited by binding to S1P receptors, it was of interest to examine their

involvement in SphK2-enhanced apoptosis. MEF cells from single $S1P_2$ and $S1P_3$, or $S1P_2$ /$S1P_3$ double knockout mice (Ishii et al., 2002) were utilized. Overexpression of SphK2 induced apoptosis in these MEFs even in the presence of 10% serum and deletion of $S1P_2$, $S1P_3$, or both did not have a significant effect. In contrast to WT MEFs, which express $S1P_{1-3}$, $S1P_2$/$S1P_3$ knock-out cells only express $S1P_1$ (Ishii et al., 2002), which is solely coupled to G_i (Hla, 2001). PTX is a two component A+B bacterial exotoxin. The A subunit (S1) is an ADP ribosyl transferase. The B component, composed of five polypeptide subunits (S2 through S5), binds to specific carbohydrates on cell surfaces. Following binding of the B component to host cells, the A subunit is inserted through the membrane and released into the cytoplasm directly. The A subunit gains enzymatic activity and transfers the ADP ribosyl moiety of NAD to the membrane-bound regulatory protein G_i. The G_i protein is inactivated and cannot perform its normal functions. Treatment with PTX to inactivate G_i, did not abrogate the ability of SphK2 to induce apoptosis even in these cells devoid of functional S1P receptors. These observations are in contrast to inhibition by PTX of numerous other biological responses of S1P (Hla, 2001; Spiegel et al., 2002).

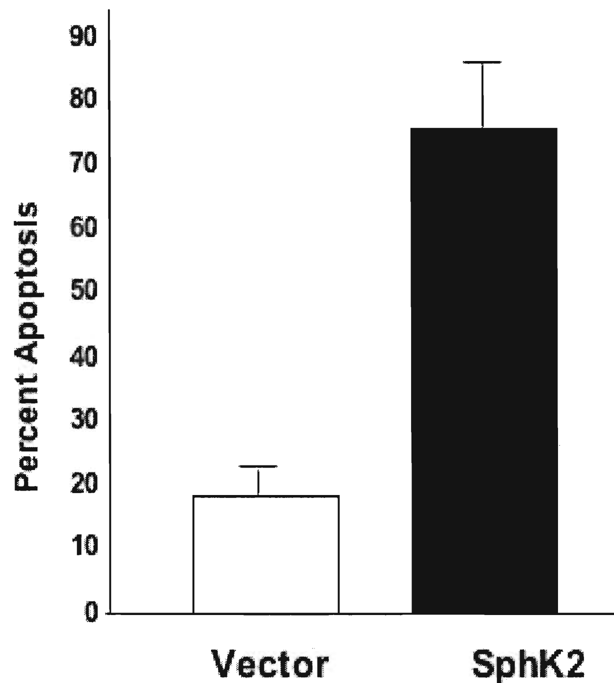

Figure 5. Overexpression of SphK2 Induces Apoptosis in NIH 3T3 Cells in Response to Serum Withdrawal. NIH 3T3 cells were transiently co-transfected with vector or mSphK2 and green fluorescent protein (GFP) in a 5 to 1 ratio. 24 h after transfection, cells were washed and cultured in serum free medium. 24 h later, cells were fixed and stained with Hoechst. The percentage of intact and apoptotic nuclei in cells expressing GFP fluorescence was determined. Each point is the mean ± SD of 3 samples. A total of at least 300 transfected cells were scored in a double-blinded manner. The data are representative of at least 3 separate experiments. $p<0.01$ for SphK2 induced apoptosis compared to vector transfected cells.

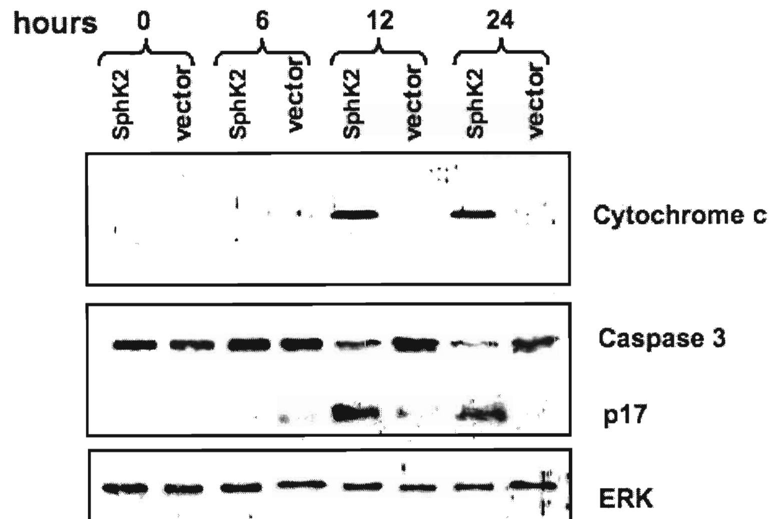

Figure 6. Overexpression of SphK2 Induces Cytochrome *c* Release and Caspase 3 Cleavage in NIH 3T3 Cells in Response to Serum Withdrawal. NIH 3T3 vector or SphK2 transient transfectants were deprived of serum for the indicated times. Cytosolic proteins were resolved by SDS-PAGE and analyzed by immunoblotting antibodies against cytochrome *c* and caspase 3, p17 is the active cleaved fragment of caspase 3. Blots were subsequently stripped and re-probed with anti-ERK2 antibody to demonstrate equal loading.

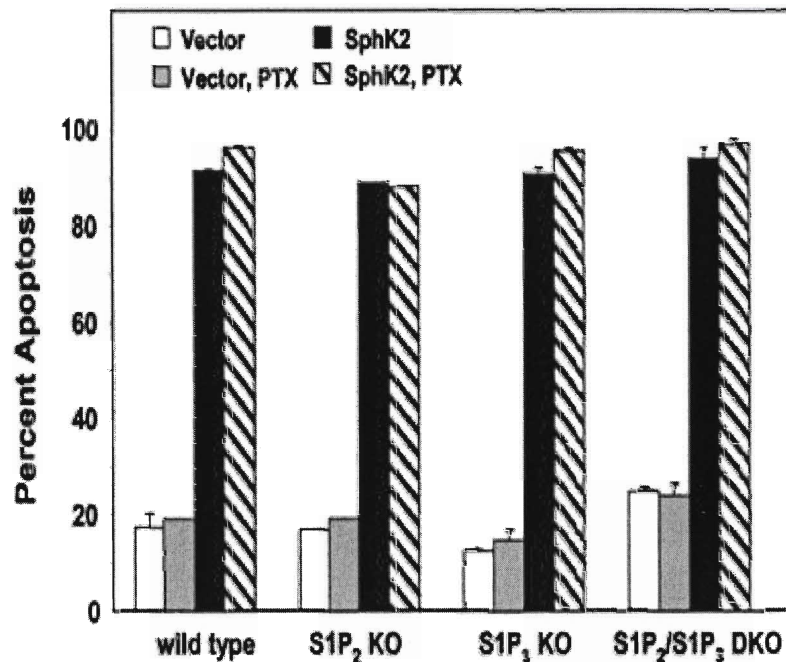

Figure 7. Overexpressed SphK2-Induced Apoptosis is Independent of S1P Receptor Activation. WT, $S1P_2$ or $S1P_3$ single or $S1_2/S1_3$ double knockout MEFs were transiently transfected with GFP vector or GFP-SphK2 and cultured in 10% serum with or without PTX (20 ng/ml), as indicated. The percentage of apoptotic nuclei in cells expressing GFP fluorescence was determined. Each point is the mean ± SD of 3 samples. A total of at least 300 transfected cells were scored in a double-blinded manner.

SphK2 Contains a Putative BH3-Only Domain: Induction of Apoptosis by SphK2 Overexpression Requires Both the Catalytic Activity and the BH3-Only Domain of SphK2

Sequence analysis revealed that both human and mouse SphK2, but not their close SphK1 relatives, contain a nine amino acid sequence reminiscent of the BH3-only domain (Figure 8). These pro-apoptotic members of the Bcl-2 family have sequence homology only within this amphiphathic α-helical segment that allows their interaction with pro-survival Bcl-2 family members to trigger apoptosis (Cheng et al., 2001; Huang and Strasser, 2000). Similar to the association of other BH3-only proteins, results from this laboratory indicated that overexpressed SphK2 immunoprecipitated with co-expressed Bcl-x_L (data not shown). The BH3-only domain of SphK2 is unusual in that most BH3-only domains have a glycine residue before the highly conserved aspartic acid, at least three, Bnip3, Egl-1, and Bak, do not and SphK2 is the only one with leucine in this position. Substitution of the highly conserved leucine residue in the BH3 domain of these proteins has been shown to interfere with their function (Huang and Strasser, 2000). Thus, a similar substitution was introduced in SphK2. This L219A mutation diminished the apoptotic effect of overexpressed SphK2 in NIH 3T3 cells, but not completely (Figure 9). In contrast to similar mutations in other BH3-only proteins, including Bmf (Puthalakath et al., 2001) which render them totally inactive, although less than WT SphK2-L219A still retained significant kinase activity (Figure 10).

SphK activity requires the ATP binding sequence $SGDGX_{17\text{-}21}K$, which is present within the conserved C2 domain of all the members of the SphK family (Pitson et al.,

2002). It has been demonstrated that a single point mutation of the second conserved glycine residue to aspartate produces a catalytically inactive SphK1 (Pitson et al., 2000b). In a similar manner, site-directed mutagenesis of the equivalent residue in SphK2 (G213E) resulted in a complete loss of sphingosine kinase activity (Figure 10). This catalytically inactive mutant was much less effective in promoting apoptosis than WT overexpressed SphK2 in NIH 3T3 cells, but it retained some pro-apoptotic activity, albeit much less than WT SphK2 (Figure 9). Notably, the double G213E/L219A mutation not only eliminated the enzymatic activity of Sphk2 (Figure 10), it also totally abrogated its apoptotic ability (Figure 9). These effects did not result from differential expression, as the levels of the mutant SphK2 proteins were essentially the same as the WT protein (Figure 9).

In order to further characterize the role of the BH3 domain in the apoptosis-inducing ability of SphK2, further studies in the lab in which SphK2 was split after its BH3 domain, forming a 227 amino acid N-terminal fragment and a 391 amino acid C-terminal fragment, both fragments lacked catalytic activity due to fragmentation of the catalytic domain. Only the N-terminal fragment, which contained the BH3-only domain, was capable of inducing apoptosis (although less than full length overexpressed SphK2) in NIH 3T3 cells, in response to serum deprivation (data not shown). Collectively, these results indicate that induction of apoptosis by SphK2 overexpression requires both the catalytic activity and the BH3-only domain of SphK2.

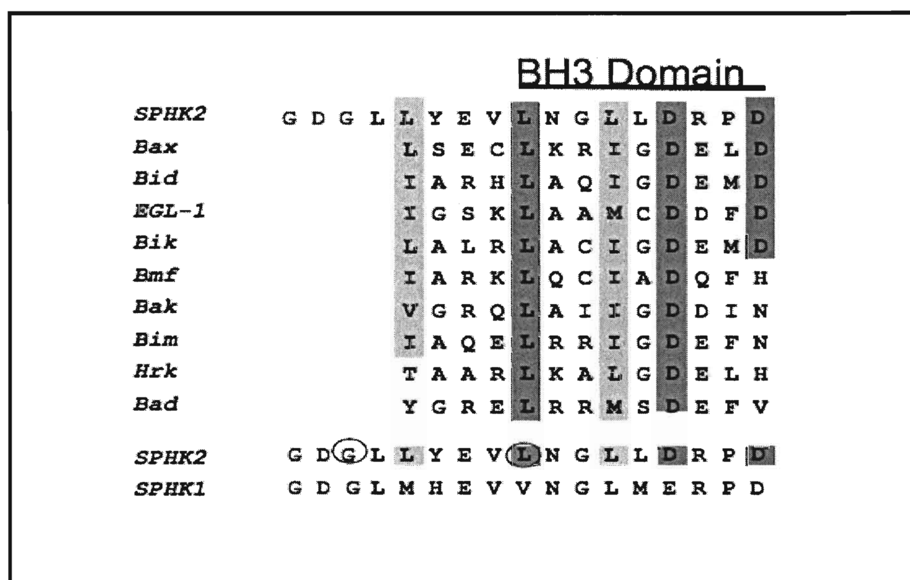

Figure 8. BH3-Only Domain of SphK2. The alignment of the putative BH3-only domain of SphK2 with other BH3 domains from pro-apoptotic Bcl-2 family members. Dark shaded boxes indicate identical amino acids and grey shaded boxes indicate very similar residues. The alignment of SphK1 and SphK2 are also shown below, the first three amino acids residues shown, GDG, are residues that form part of the SphK nucleotide binding motif (SGDGX$_{17\text{-}21}$K). The circled residues were mutated.

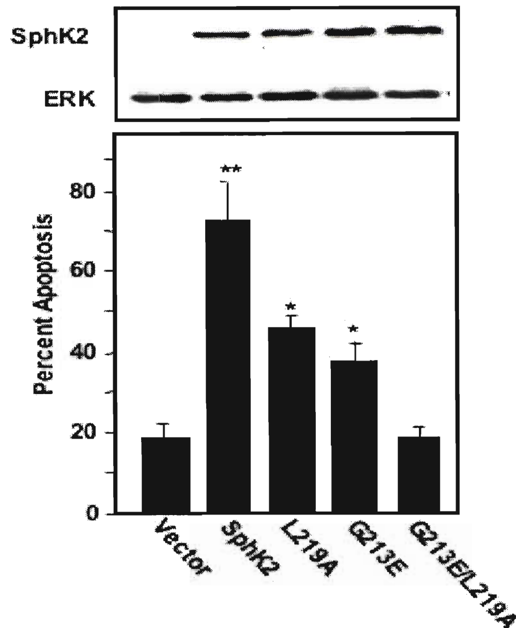

Figure 9. Overexpressed SphK2-Induced Apoptosis Requires the BH3-Only Domain and Catalytic Activity. NIH 3T3 cells were transiently co-transfected with vector, SphK2, SphK2-L219A, SphK2-G213E or SphK2-L219A/G213E with GFP at a 5 to 1 ratio. Cells were cultured in serum-free medium for 24 h, fixed, and stained with Hoechst. Total GFP-expressing cells and GFP-expressing cells displaying condensed nuclei indicative of apoptosis were scored. Each point is the mean ± SD of 3 samples. A total of at least 300 transfected cells were scored. The data are representative of at least 3 separate experiments. Equal expression of constructs was confirmed by western blotting. Lysates from duplicate cultures of NIH 3T3 fibroblasts were separated by SDS-PAGE and transferred to nitrocellulose. After blotting with anti-HA antibody to detect HA-tagged SphK2 proteins, the blot was stripped and re-probed with an anti-ERK2 antibody as a loading control. **, $p<0.001$; *, $p<0.05$.

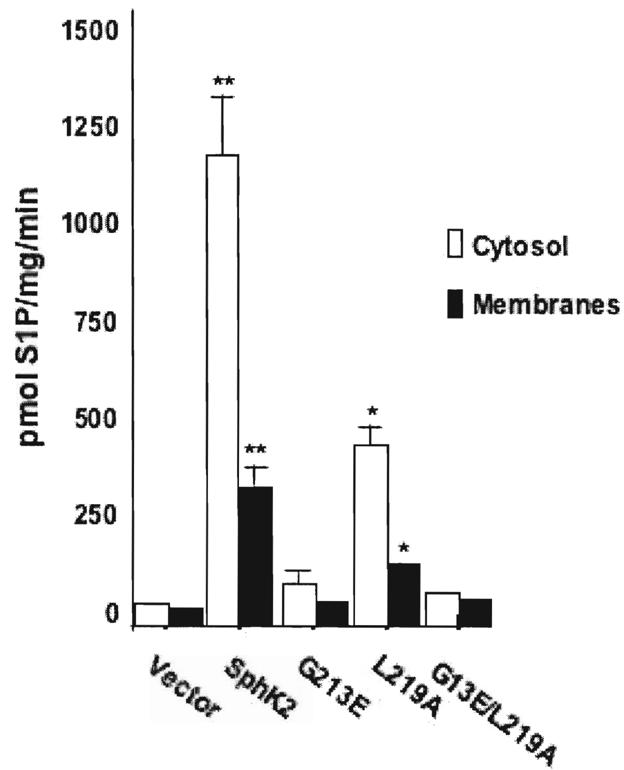

Figure 10. Sphingosine Kinase Activity of SphK2 Mutant Constructs. NIH 3T3 cells were transiently transfected with vector, SphK2, SphK2-L219A, SphK2-G213E or SphK2-L219A/G213E as indicated and SphK activity was measured in cytosolic (open bars) and membrane fractions (filled bars). **, $p<0.001$; *, $p<0.05$.

Endoplasmic Reticulum Stress and Uptake of Calcium by the Mitochondria also Contribute to the Apoptotic Effects of Overexpressed SphK2.

Calcium is necessary for cell growth and survival, but it has been suggested that an inappropriate increase in levels of cytosolic free calcium may lead to cell death (Berridge et al., 2000). Release of calcium from the ER has been observed in response to many apoptotic-inducing agents. Importantly, BH3-only proteins have been implicated in ER calcium release (Scorrano et al., 2003; Smith and Deshmukh, 2007). Calcium release from the ER can be rapidly taken up by closely juxtaposed mitochondria (Rizzuto et al., 1998). In addition, calcium uptake by the mitochondria is essential for cytochrome *c* release during apoptosis, particularly that induced by the pro-apoptotic Bcl-2 protein Bax (Nutt et al., 2002).

My colleagues have reported that serum starvation in SphK2 overexpressing cells increases intracellular calcium (data not shown). Furthermore, treatment of cells with BAPTA-AM, a cell-permeant calcium chelator, slightly enhanced apoptosis of vector transfectants, but substantially reduced apoptosis induced by SphK2 overexpression in serum starved NIH 3T3 cells (Figure 11). These findings were further substantiated with the use of Ru-360, an inhibitor of the mitochondrial calcium antiporter (Matlib et al., 1998). Ru-360 significantly reduced overexpressed SphK2-induced cell killing of serum-starved NIH 3T3 cells (Figure 11). As previously shown, SphK2 overexpression caused the release of cytochrome *c* from the mitochondria of NIH 3T3 cells cultured in the absence of serum (Figure 6 and 12). To confirm that the protective effect of Ru-360 resulted from inhibition of the intrinsic pathway of apoptosis, treatment with Ru-360

markedly reduced this cytochrome *c* release (Figure 12). Taken together, these results suggest that overexpressed SphK2-induced apoptosis in response to serum withdrawal is calcium dependent and that mitochondrial calcium uptake is critical.

Previous studies have suggested the existence of a novel apoptotic pathway in which caspase 12 is specifically activated by ER stresses, particularly due to perturbation of calcium homeostasis (Nakagawa and Yuan, 2000). Accordingly, caspase 12-null mice and cells are partially resistant to apoptosis induced by ER stress but not by other apoptotic stimuli (Nakagawa et al., 2000). Following its activation at the ER, caspase 12 may directly process downstream caspases in the cytosol or target other as yet unidentified substrates that influence the progression of apoptosis (Morishima et al., 2002). Alignment of the murine caspase 12 cDNA with the human genome sequence localized the human caspase 12 gene at a single locus within the caspase 1 gene cluster on chromosome 11q22.3. Reverse transcrition-PCR and molecular cloning revealed that nine alternatively spliced transcripts of this gene are expressed. However, a frame shift mutation and a premature stop codon which is present in all splice variants preclude the expression of a full length protein. An additional loss-of-function mutation, prohibits any proteins, if they are produced, from acting catalytically. This data indicates that functional caspase 12 is lost in humans (Fischer et al., 2002). However, it has been reported that caspase 4 can function as an ER stress-specific caspase in humans (Hitomi et al., 2004). Consistent with its effects on calcium, SphK2 activated pro-caspase 12 as shown by its cleavage (Figure 13), which preceded the appearance of fragmented nuclei. This result suggests that overexpressed SphK2-induced apoptosis is also partially mediated through ER stress.

The ER stress response occurs when the ability of the ER to properly fold proteins is overwhelmed or compromised, resulting in the activation of the unfolded protein response (Wu and Kaufman, 2006). The mechanism by which apoptosis is induced in response to ER stress has been examined in multiple cell types. In addition to the established roles of pro-apoptotic Bax and Bak at the mitochondria, both are also found localized to the ER membrane and are known to be involved in ER stress-induced calcium release, caspase 12 activation, and apoptosis (Scorrano et al., 2003; Zong et al., 2003). Bax and Bak are important in this pathway, since MEFs doubly deficient in Bax and Bak are resistant to ER stress-induced apoptosis (Scorrano et al., 2003; Zong et al., 2003).

To examine their involvement, embryonic fibroblasts from Bax/Bak double knockout mice were utilized. As shown previously (Figure 7), SphK2 expression markedly induced apoptosis of WT MEFs in the absence of serum (Figure 14). However, the Bax/Bak double knockout MEF cells transiently transfected with SphK2 were almost completely resistant to apoptosis induced by serum withdrawal (Figure 14). Collectively, these results suggest that ER stress and uptake of calcium by the mitochondria also contribute to the apoptotic effects of overexpressed SphK2 in response to serum withdrawal.

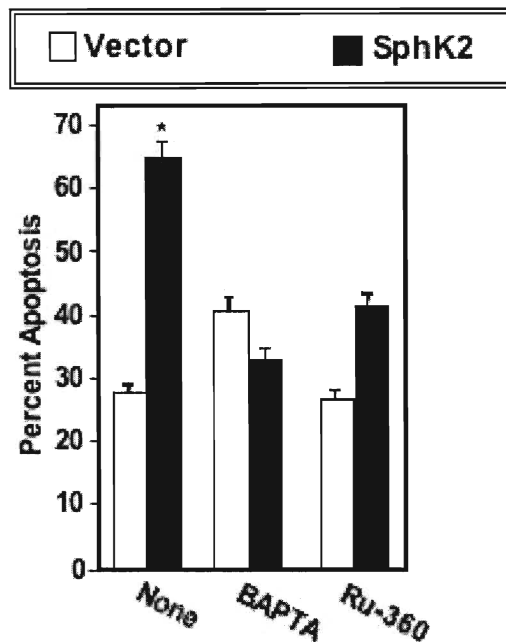

Figure 11. Overexpressed SphK2-Induced Apoptosis Requires Calcium Uptake by Mitochondria. NIH 3T3 cells were transiently co-transfected vector (open bars) or SphK2 (filled bars), together with GFP at a 5 to 1 ratio. Cells were then cultured in serum-free medium in the presence or absence of BAPTA-AM (10 μM) or Ru-360 (10 μM). 24 h later cells, the percentage of apoptotic nuclei in cells expressing GFP was determined with Hoechst staining. Similar results were obtained in at least three independent experiments and statistical difference is indicated by the asterisk ($p<0.001$). Each point is the mean ± SD of 3 samples. A total of at least 300 transfected cells were scored in a double-blinded manner. *, $p<0.001$.

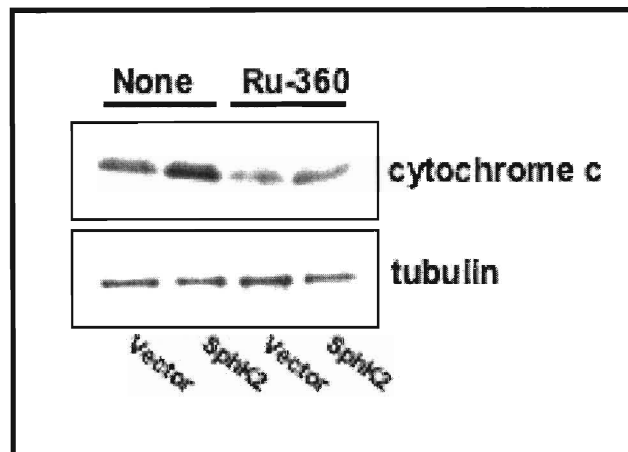

Figure 12. Ru-360 Reduces Overexpressed SphK2-Induced Cytochrome *c* Release. NIH 3T3 cells transiently expressing either vector or SphK2 were serum-starved for 24 h in the presence or absence of Ru-360 (10 μM). Cytochrome *c* release to the cytosol was assessed by immunoblotting. Equal loading was verified by blotting with anti-tubulin antibody.

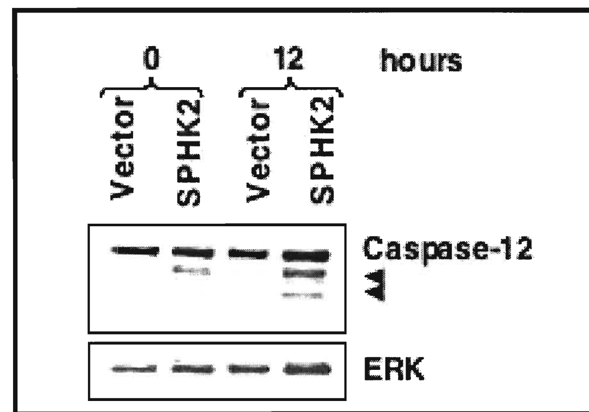

Figure 13. Overexpressed SphK2-Induced Apoptosis Proceeds through Activation of Caspase 12. NIH 3T3 vector or SphK2 transient transfectants were deprived of serum for the indicated times. Cytosolic proteins were resolved by SDS-PAGE and analyzed by immunoblotting with anti-caspase 12 antibody and subsequently with anti-ERK2 antibody to show equal loading. Arrowheads indicate cleaved fragments.

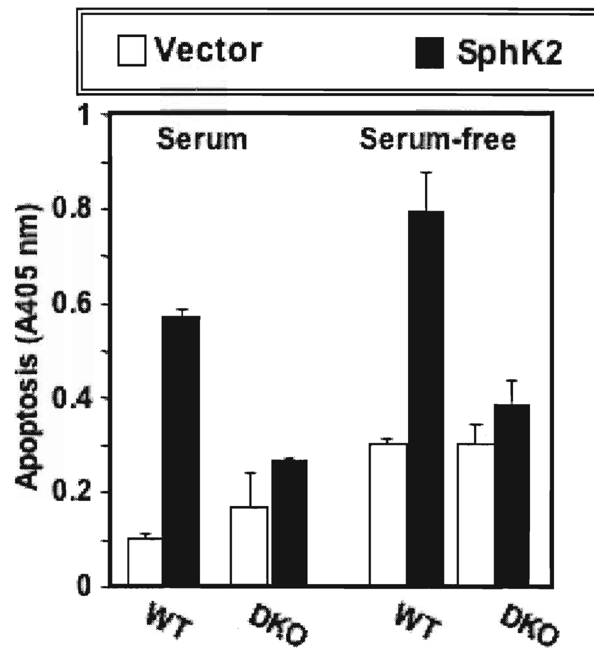

Figure 14. Induction of Apoptosis by Overexpressed SphK2 Depends on Bax and Bak. MEF cells from WT and Bax/Bak double knockout mice were transfected with vector (open bars) or SphK2 (filled bars), cultured without or with 10% serum for 24 h and apoptosis determined by ELISA for histone-associated DNA fragments.

Chapter Summary

Surprisingly, in contrast to the known anti-apoptotic effects of SphK1, overexpression of SphK2 was found to enhance caspase-dependent apoptosis in response to serum withdrawal in NIH 3T3 fibroblasts. These effects are not due to transactivation of S1P receptors because treatment of $S1P_2/S1P_3$ double knockout cells with PTX did not interfere with the ability of overexpressed SphK2 to induce apoptosis. Additionally, the apoptotic effects of overexpressed SphK2 have also observed in other cell types that have completely different patterns of S1P receptor expression. Sequence analysis revealed that SphK2 contains a 9 amino acid motif which is not found in SphK1 but is similar to that present in BH3-only proteins and associates with Bcl-x_L *in vivo*. Single mutations of critical residues in; (1) the BH3-only domain and (2) the catalytic domain of SphK2 only partially reduced apoptosis. However, a double mutation of both the BH3-only domain and the catalytic activity totally abrogated the apoptotic activity of SphK2, suggesting that both the catalytic activity and the BH3-only domain of SphK2 are important for the induction of apoptosis. Additionally, further results suggest that ER stress and uptake of calcium by the mitochondria also may contribute to the apoptotic effects of overexpressed SphK2. Using Ru-360 it was found that overexpressed SphK2-induced apoptosis in response to serum withdrawal is calcium dependent and that mitochondrial calcium uptake is critical, since Ru-360 prevented cytochrome *c* release by overexpression of SphK2 and serum withdrawal. Overexpressed SphK2-induced apoptosis was found to proceed through activation of caspase 12 and to require Bax and Bak, since Bax/Bak double knockout MEF

cells transiently transfected with SphK2 were almost completely resistant to apoptosis induced by serum withdrawal.

Similarly, Igarashi *et al.,* have also reported that SphK2 inhibits DNA synthesis when overexpressed in mammalian cells (Igarashi et al., 2003). Also, deletion of the yeast homologue LCB4 but not LCB5 prevented growth inhibition and cell death suggesting that similar to this study demonstrating opposite functions of overexpressed SphK1 and SphK2, the yeast homologues LCB4 and LCB5, also appear to have different functions in yeast (Kim et al., 2000).

An explanation for the opposing effects of overexpressed SphK1 and SphK2 may come from the results of further experiments from the laboratory. My colleagues found that overexpression of SphK2 increased production of ceramide in the sphingolipid salvage pathway, whereas SphK1 decreased it (data not shown). These results suggest that SphK2 may act in concert with S1P phosphatase to convert S1P back to sphingosine and then to ceramide (Maceyka et al., 2005b). Moreover, ceramide generated at the ER has been linked to increased calcium release, leading to apoptosis. (Scorrano et al., 2003). Additionally, in the studies by my colleagues, it was found that overexpressed SphK2 was localized to the ER in NIH 3T3 cells in response to serum starvation, which was partly dependent on its BH3-only domain (Maceyka et al., 2005b). These results imply that the cellular location of where S1P is produced may determine how two closely related enzymes that use the same substrate and generate the same product, have opposite effects on cell survival. Consistent with this notion my colleagues have also found that targeting

cytosolic SphK1 to the ER in NIH 3T3 cells also resulted in increased apoptosis (Maceyka et al., 2005b).

In conclusion, these studies revealed that the BH3-only domain, catalytic activity, ER stress, and uptake of calcium by the mitochondria all contribute to the apoptotic effects of overexpressed SphK2. These results indicate that SphK2 may be an important component in apoptotic cell death.

CHAPTER 4

THE INVOLVEMENT OF SPHINGOSINE KINASE 2 IN THE INDUCTION OF p21 BY THE CHEMOTHERAPEUTIC DRUG DOXORUBICIN

The manuscript for the work presented in this chapter is currently in preparation. A portion of this chapter has been published in FEBS Letters 2005 Oct 10; 579 (24): 5313-7. Construction of the SphK2-L construct, SphK2 antibody purification, quantitative PCR, and localization studies were performed by Dr. N. Hait. Cell cycle analysis was assisted by S. Paugh.

Introduction

As described, previous studies revealed that overexpression of SphK2 suppressed cell growth and also markedly enhanced apoptosis that was preceded by cytochrome *c* release and caspase activation in response to serum withdrawal (Igarashi et al., 2003; Liu et al., 2003). Induction of apoptosis by overexpressed SphK2 was independent of activation of S1P receptors but required its putative BH3-only domain, catalytic activity, ER stress, and uptake of calcium by the mitochondria (Liu et al., 2003; Maceyka et al., 2005b).

Subsequently, an N-terminal extended hSphK2 (SphK2-L) has been revealed by BLAST searches of the human genome sequence database. The SphK2-L gene is ~10 kb long and consists of 6 exons separated by 5 introns. In contrast, the originally reported hSphK2-S lacks exon 2 of SphK2-L, and the first initiation codon for translation is within exon 3. The variant form is composed of 654 amino acids that encode a protein of 72 kDa

(SphK2-L), while SphK2-S is composed of 618 amino acids that encode a protein of 68 kDa. However, the physiological functions of these Sphk2 isoforms are currently not understood.

It has been reported that SphK1 is mainly cytosolic and several growth factors induce its translocation to the plasma membrane (Johnson et al., 2003; Pitson et al., 2003; Rosenfeldt et al., 2001), which is important for its oncogenic functions (Pitson et al., 2005). In contrast, the localization of endogenous SphK2 is still unclear as it may be different in different cell types (Igarashi et al., 2003; Maceyka et al., 2005b; Okada et al., 2005; Olivera and Rivera, 2005). Intriguingly though, it has been suggested that SphK2 contains a putative nuclear localization signal (NLS) that resides within the amino terminus of SphK2 (Igarashi et al., 2003) (Figure 15). Indeed, SphK activity has been detected in nuclear fractions (Kleuser et al., 2001). However, overexpressed SphK2 was found to be mainly cytosolic in COS7 cells, yet was localized to the nucleus of HeLa cells (Igarashi et al., 2003). In addition, in HEK 293 cells the Sphk2-S isoform was associated with the nucleus as well as being present in the cytoplasm. This is in contrast with the SphK2-L isoform, which showed minimal nuclear localization in HEK 293 cells (Sanchez et al., 2003). However, results by Okada *et al.*, showed that serum deprivation resulted in the translocation of SPHK2-L into the nuclei of HEK 293 cells (Okada et al., 2005).

Currently, the few SphK2 studies reported have mainly investigated the functions of SphK2 by overexpression, and the functions of endogenous SphK2 are not yet known. Additionally, little information exists regarding the effect of SphK2 expression in cancer cell lines in response to anti-cancer agents. The anthracyclin doxorubicin, a major anti-

tumor agent used for the treatment of a variety of human cancers (Culine et al., 1998), is known to cause cellular damage through a number of mechanisms including free radical formation and inhibition of topoisomerase II by maintaining the enzyme-dependent state of DNA double-strand breaks (Liu, 1989). Like ionizing radiation, doxorubicin also induces oxidative DNA damage via hydoxyl radicals (Breimer, 1990), DNA double-strand breaks and induction of DNA repair and apoptosis (Liu, 1989).

To further investigate the role of SphK2 in cell growth and apoptosis this study investigated the functions of endogenous SphK2 in human carcinoma cells and how it influences the balance between cytostasis and apoptosis in response to the chemotherapeutic anthracycline doxorubicin.

Materials and Methods

Reagents

Doxorubicin was purchased from Sigma (St. Louis, MO), reconstituted in molecular biology grade water, and stored protected from light. The stock solution was diluted in the relevant medium for administration to cells. Serum and medium were from Biofluids (Rockville, MD). Antibodies to p21, lamin A/C, caspase 7, phospho-p53 (Ser^{15}), and Poly(ADP-Ribose) Polymerase (PARP) were purchased from Cell Signaling (Beverly, MA). Anti-p53 antibody was from Oncogene (San Diego, CA). Anti-under-phosphorylated Rb antibody was from Transduction Laboratories (San Diego, CA). Anti-PDI antibody was from Stressgen Biotechnologies (San Diego, CA). Anti-tubulin antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-V5 antibody was purchased from Invitrogen

(Gaithersburg, MD). SphK2 rabbit polyclonal antiserum raised against a unique SphK2 peptide sequence (QALHIQRLRPKPEARPR) (Biosynthesis, Lewisville, TX) was purified on a protein A column followed by affinity purification on a Sulfolink gel conjugated with the antigenic peptide according to the manufacturer's instructions (Pierce, Rockford, IL). Secondary antibodies were from Jackson Immunoresearch (West Grove, PA).

cDNA Cloning and Expression Vectors

Construction of human SphK2 and SphK1 (UniProt: Q9NRA0-2) expression vectors have been described previously (Hait et al., 2005; Liu et al., 2000a; Olivera et al., 1999). SphK2 with an N-terminal extension of 36 amino acids (SphK2-L, UniProt: Q9NRA0-1) was amplified from cDNA isolated from HEK 293 cells by PCR using Platinum taq polymerase High Fidelity (Invitrogen, Gaithersburg, MD) with 5'-CACCATGAATGGACACCTTGAA-3' and 5'-TCCGGGCTCCCGCCCCGG-3' sense and antisense primers, respectively. The PCR product was cloned into pcDNA 3.1 V5-His vector (Invitrogen, Gaithersburg, MD). All sequences were verified by DNA sequencing and protein expression after transfection of HEK 293 cells was confirmed by western blotting with anti-SphK2 or anti-V5 antibody (Hait et al., 2005).

Cell Culture and Transfection

MCF7 human breast carcinoma cells were grown in phenol red-free improved minimal essential media (IMEM) supplemented with 0.25% glucose and 10% heat-inactivated FBS. HEK 293 and HCT-116 human colon carcinoma (WT, $p21^{-/-}$, and $p53^{-/-}$)

cells were grown in DMEM supplemented with 10% heat-inactivated FBS. Cells were cultured at 37°C and 5% CO_2. MCF7 cells were transiently transfected using LipofectAMINE/PLUS reagent (Invitrogen, Gaithersburg, MD) as described by the manufacturer and cultured overnight. Transfection efficiency was approximately 50%.

SphK2 expression in MCF7 and HCT-116 cells was downregulated by transfection with sequence specific siRNA for human SphK2 (sense, 5'-GGAUUGCGCUCGUCGCUUUCAU-3'; antisense, 5'-AUGAAAGCGAGCGCAAUCCTG-3', Ambion) and control siRNA (Ambion) using Oligofectamine (Invitrogen, Gaithersburg, MD), according to the manufacturer's instructions. In some experiments, siRNA targeted to another human SphK2 sequence (5'-GCTGGGCTGTCCTTCAACCT-3', Qiagen, Valencia, CA) and control siRNA (Qiagen, Valencia, CA) was utilized. In addition ON-TARGETplus SMARTpool siRNA against SphK2 and control siRNA from Dharmacon (Lafayette, CO) were used to confirm lack of off-target effects.

SphK1 expression in MCF7 cells was downregulated by transfection using Oligofectamine (Invitrogen, Gaithersburg, MD) with sequence specific siRNA for human SphK1 (sequence targeted: GGGCAAGGCCTTGCAGCTC) from Qiagen (Valencia, CA).

Western Blot Analysis

Unless otherwise indicated, cells were lysed in buffer containing 50 mM Hepes (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EDTA, 2 mM sodium orthovanadate, 4 mM sodium pyrophosphate, 100 mM NaF, and 1:500 dilution of protease

inhibitor mixture (Sigma, St Louis, MO). For analysis of cytochrome *c* release from mitochondria, cytosolic fractions were prepared by resuspending cells in lysis buffer containing 75 mM NaCl, 1 mM NaH_2PO_4, 8 mM Na_2HPO_4, 1 mM EDTA, 250 mM sucrose and 700 μg/ml digitonin, lysates were then centrifuged at 14,000 x *g* for 15 min.

Equal amounts of proteins were separated by SDS-PAGE and transblotted to nitrocellulose, blocked with 5% non-fat dry milk for 2 h at room temperature, and then incubated with primary antibodies overnight. Appropriate horseradish peroxidase-conjugated secondary antibodies were added in Tris-buffered saline containing 5% non-fat milk. Immunoreactive signals were visualized by enhanced chemiluminescence (Pierce, Rockford, IL) and blots exposed to Kodak X-Omat film.

Immunofluorescence and Confocal Microscopy

MCF7 cells were grown on four-chambered slides (Nalge/Nunc) and transfected with V5-tagged SphK2-S or SphK2-L. Cells were washed with PBS, fixed for 20 min at room temperature with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100 in PBS for 5 min. After washing, cells were incubated for 45 min with anti-V5 antibody in PBS containing 1% BSA (Sigma, St Louis, MO), and then for 45 min with secondary antibody conjugated with Fluorescein isothiocyanate (FITC). Coverslips were mounted on glass slides using an Anti-Fade kit (Molecular Probes, Eugene, OR) and examined with a Zeiss LSM 510 laser confocal microscope.

Nuclear Extraction

Cytoplasmic and nuclear fractions were isolated from MCF7 cells using the NE-PER nuclear and cytoplasmic isolation kit (Pierce, Rockford, IL) according to the manufacturer's instructions.

Quantitative PCR

Total RNA was isolated with TRIzol Reagent (Invitrogen, Gaithersburg, MD). At least 1 μg of RNA was reverse transcribed with Superscript II (Invitrogen, Gaithersburg, MD). For real-time PCR, pre-mixed primer-probe sets were purchased from Applied Biosystems (Foster City, CA) and cDNA amplified with ABI 7900HT.

Sphingosine Kinase Assay

Cells were lysed by freeze-thawing in SphK buffer (20 mM Tris (pH 7.4), 20% glycerol, 1 mM 2-mercaptoethanol, 1 mM EDTA, 5 mM sodium orthovanadate, 40 mM β-glycerophosphate, 15 mM NaF, 10 μg/ml leupeptin, aprotinin, and soybean trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride, and 0.5 mM 4-deoxypyridoxine). SphK2 activity was determined with sphingosine added as a complex with 4 mg/ml BSA and [γ-^{32}P]ATP in the presence of 1 M KCl, conditions in which SphK2 activity is optimal and SphK1 strongly inhibited (Liu et al., 2000a). Labeled S1P was extracted and separated by TLC with chloroform/acetone/methanol/acetic acid/H_2O (10:4:3:2:1, v/v). Radioactive bands corresponding to S1P were quantified with a FX Molecular Imager (Bio-Rad,

Oakland, CA). Specific activity is expressed as picomols of S1P formed/min/mg of protein.

Cell Cycle Analysis

After treatment, equal numbers of cells were trypsinized, washed, fixed, and stained with propidium iodide (PI) (0.05 mg/ml in 3.8 μM sodium citrate, 0.1% Triton X-100, and 7 kU/ml RNase B) for 2 h and analyzed on a Beckman-Coulter XL-MCL flow cytometer (Hialeah, FL). Data were analyzed using Mod Fit LT 3.0 (Verity Software House; Topsham, ME).

Cell Death and Proliferation Assays

Apoptotic cell death was measured by staining cell nuclei with 8 μg/ml Hoechst dye 33342 bisbenzimide (Sigma, St. Louis, MO) and apoptotic cells were identified by condensed, fragmented nuclear regions using a Nikon TE 300 fluorescent microscope. A minimum of 300 cells were scored. Apoptosis was confirmed by determining PARP cleavage and activation of caspase 7. Cell proliferation was determined with the WST-1 reagent (Roche Applied Science, Indianapolis, IN) and incubating at 37 °C for 1 h. Absorbance was measured at 450 nm with background subtraction at 630 nm.

Statistical Analysis

For each experiment, data from triplicate samples were calculated and expressed as mean $\pm$ SD. Statistical analysis was performed using Microsoft Excel statistical software.

Densitometry was performed using Alpha Ease Version 3.24 on an Alpha Imager 2000 (Alpha Innotech Co., San Leandro, CA).

Results

SphK2 is Predominantly Localized to the Nucleus in MCF7 Cells

To examine the localization of SphK2 in MCF7 cells, cells were transiently transfected with V5-tagged SphK2-S or SphK2-L. Western blot analysis of lysates from these cells detected major protein bands with the predicted molecular masses (Figure 16a). As expected, transient transfection with SphK2-S and SphK2-L also resulted in significantly increased sphingosine kinase activity (Figure 16b). Both SphK2-S and SphK2-L were found to be expressed mainly in the nucleus of MCF7 cells when examined by confocal fluorescence microscopy (Figure 17). Moreover, the localization of either splice variant was not affected by serum deprivation in MCF7 cells (Figure 17).

mSphK2	87	R G **R R** G G **R R** R	95
hSphK2	86	R G **R R** G A **R R** R	94
Rev protein	36	Q A **R R** N R **R R** R	44
Rex protein	7	R P **R R** S Q **R K** R	15
Tat protein	50	K K **R R** Q R **R R** A	57
PKCλ	144	Q A **K R** F N **R R** A	152
Conesensus NLS		R / **K R** x x **R R** / E	

Figure 15. Comparison of NLS Sequences of SphK2 and Other Proteins. The position and sequences of putative NLS regions from SphK2, Rev protein, Rex protein, Tat protein, and PKCλ are shown. The basic amino acid residues (arginine and lysine) known to be important for NLS function are shown in bold. A consensus sequence of the NLS is also shown.

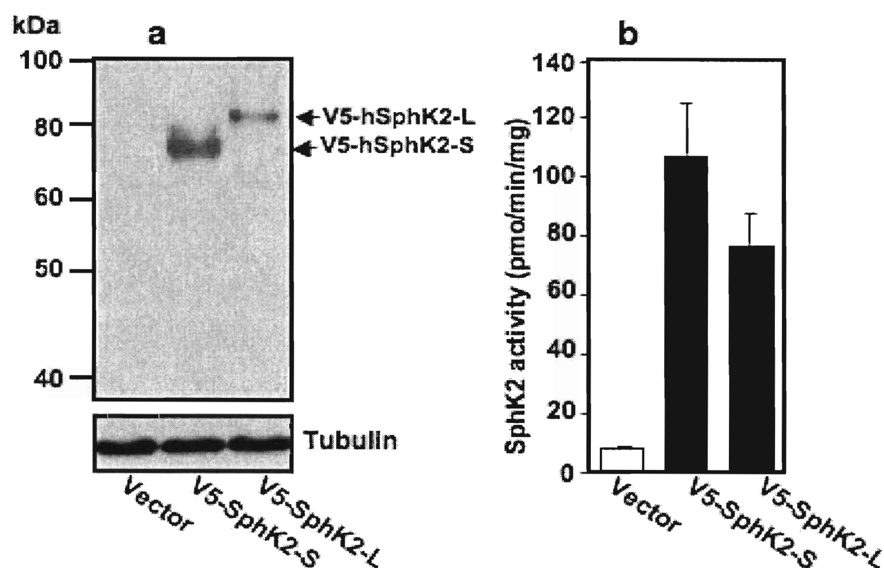

Figure 16. Expression of SphK2-S and SphK2-L in MCF7 Cells. (a) MCF7 cells were transiently transfected with vector, V5-SphK2-S, or V5-SphK2-L. Cell lysate proteins were separated by SDS-PAGE and then immunoblotted with anti-V5 antibody. Blots were stripped and re-probed with anti-tubulin antibody to demonstrate equal loading. (b) SphK2 activity was measured in cell lysates prepared from duplicate cultures with sphingosine added as a BSA complex in the presence of 1 M KCl. Data are means ± SD of triplicate determinations.

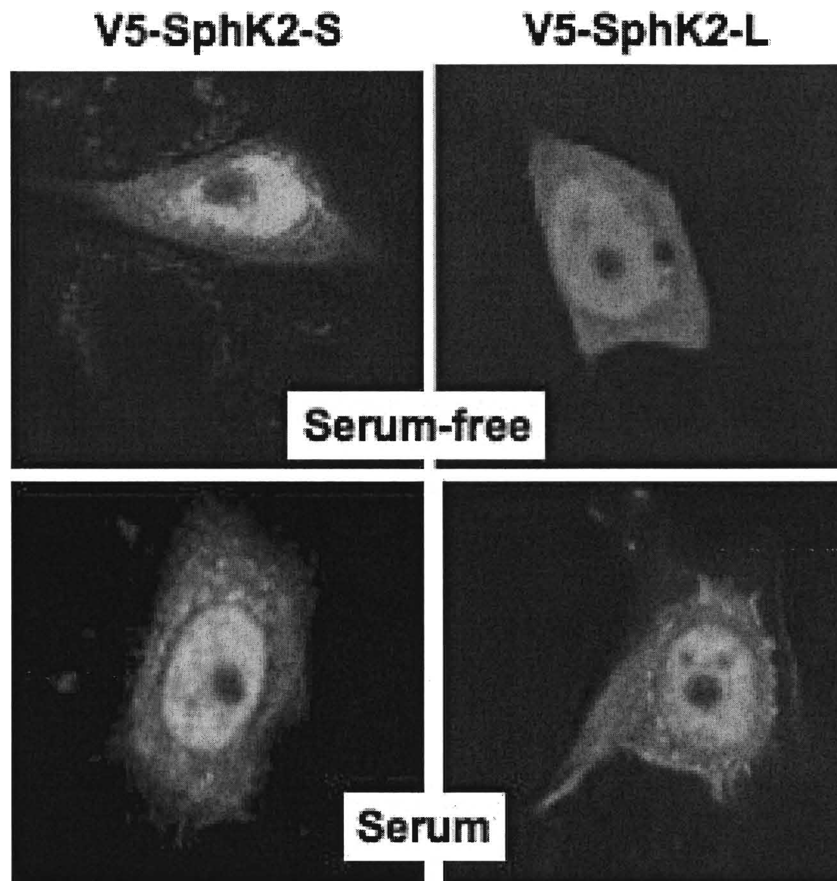

Figure 17. Localization of SphK2-S and SphK2-L in MCF7 Cells. MCF7 cells grown on coverslips were transiently transfected with vector, V5-SphK2-S or V5-SphK2-L and subsequently cultured for 24 h in the absence or presence of serum, fixed with paraformaldehyde, and immunostained with anti-V5 antibody followed by FITC-conjugated secondary antibody. Cells were visualized by confocal fluorescence microscopy. Representative cells of approximately 100 cells examined are shown.

Overexpression of SphK2 Upregulates p21 in MCF7 Cells Independently of p53

As discussed, overexpression of SphK2 in contrast to SphK1, reduces cell growth and enhances apoptosis in response to apoptotic stimuli (Igarashi et al., 2003; Liu et al., 2003; Maceyka et al., 2005b; Okada et al., 2005). However, it is not known how SphK2 inhibits cell proliferation. As previously mentioned, p21 is a member of the family of cdk inhibitors, and is an important negative regulator of cell cycle progression and prevents aberrant mitosis leading to cell death (Weiss, 2003). Both p53-dependent and –independent pathways of p21 induction in cells exposed to DNA damaging and maturation/differentiation inducing agents have been described (Jiang et al., 1994; Macleod et al., 1995; Waldman et al., 1995). Therefore, it was of interest to examine the effects of SphK2 overexpression on p21 and p53. Overexpressed SphK2-S increased expression levels of p21 compared to vector transfected cells (Figure 18). Cdks, the main cell cycle relevant targets of p21 keep pRb in an inactive, hyperphosphorylated state during cell cycle progression. In agreement with the increase in p21 induced by overexpression of SphK2-S, it also concomitantly increased hypophosphorylated/underphosphorylated pRb (Figure 18). Similarly, overexpression of SphK2-L also increased expression levels p21 (Figure 19).

In agreement with numerous reports (Mahyar-Roemer and Roemer, 2001; Mukherjee and Conrad, 2005; Seoane et al., 2002), treatment with the DNA damaging agent doxorubicin markedly increased levels of p21 and its transcriptional activator p53 (Figure 18 and 19). However, neither SphK2-S nor SphK2-L expression altered p53 levels. Moreover, neither SphK2 splice form influenced p53 levels induced by doxorubicin

(Figure 18 and 19). Amongst the multiple modification sites on p53, phosphorylation at Ser^{15} in response to DNA damage correlates with both the accumulation of total p53 protein and its functional activation (Canman et al., 1998; Shieh et al., 1997). As expected, doxorubicin induced phosphorylation of p53 at Ser^{15}. However, SphK2 expression had no significant effects on this phosphorylation (Figure 18). Interestingly, reminiscent of the effects of genotoxic stress on SphK1 (Taha et al., 2004), it was also noticed that treatment with doxorubicin for 24 h decreased levels of endogenous SphK2 (Figure 18). The significance of this effect is not known. However, in contrast to SphK2, expression of SphK1 had no effect on p21 expression (Figure 20).

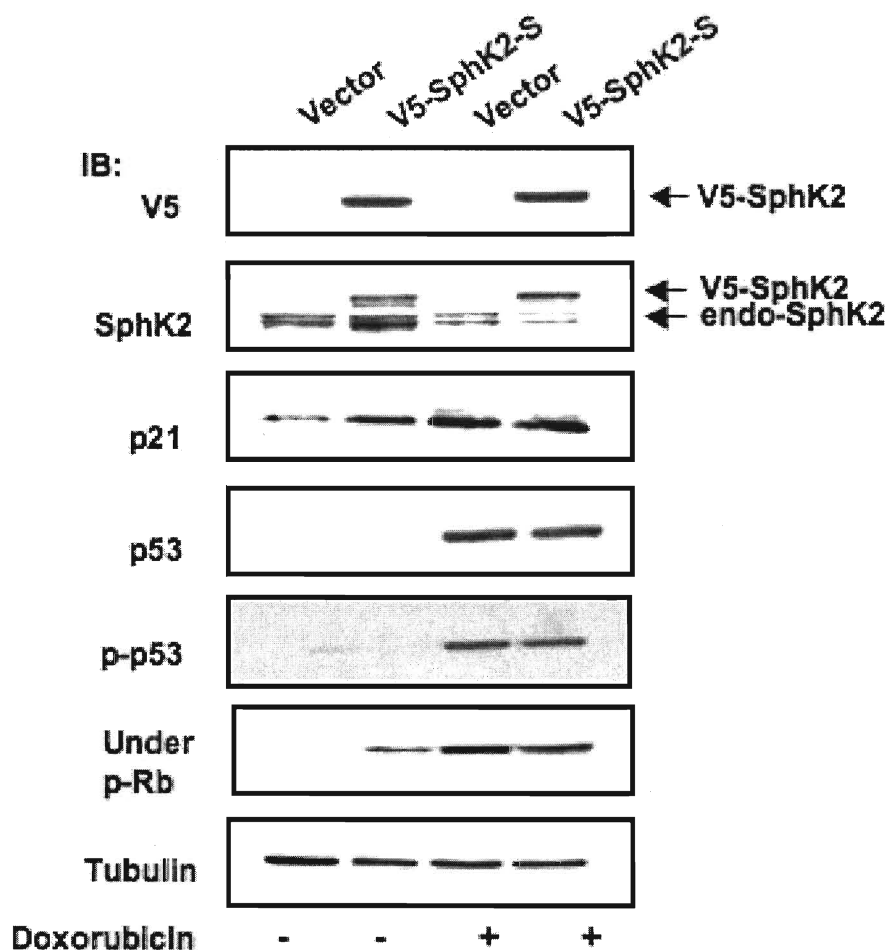

Figure 18. SphK2-S Increases p21 Expression in MCF7 Cells Without Affecting p53. MCF7 cells transiently transfected with vector or V5-SphK2-S were cultured in the absence or presence of doxorubicin (1.7 μM) for 24 h. Equal amounts of cell lysate proteins were separated by SDS-PAGE and then immunoblotted with antibodies directed toward SphK2, V5, p21, p53, phospho-p53 (Ser^{15}), and underphosphorylated Rb, as indicated. Membranes were subsequently probed with anti-tubulin antibody to show equal loading. Similar results were obtained in two additional experiments.

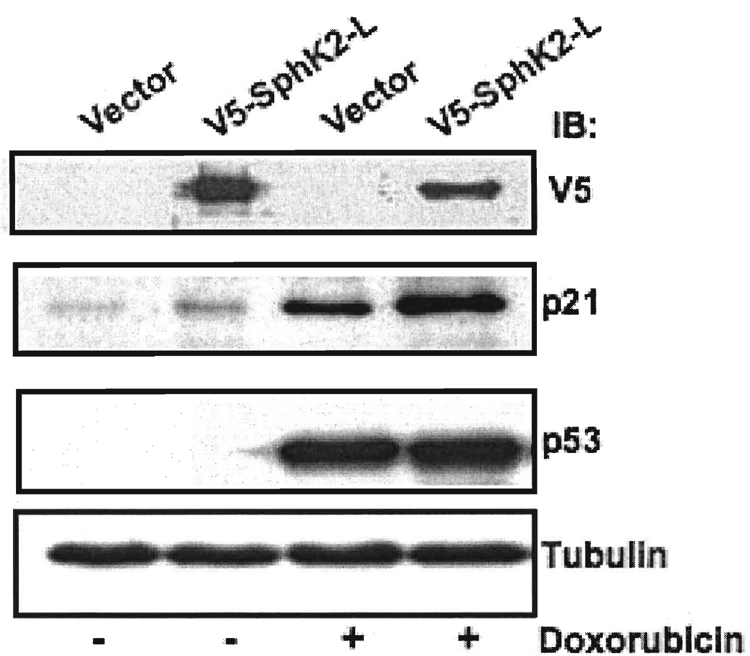

Figure 19. SphK2-L Increases p21 Expression in MCF7 Cells Without Affecting p53. MCF7 cells transiently transfected with vector or V5-SphK2-L were cultured in the absence or presence of doxorubicin (1.7 μM) for 24 h. Equal amounts of cell lysate proteins were separated by SDS-PAGE and then immunoblotted with antibodies directed toward SphK2, V5, p21, and p53, as indicated. Membranes were subsequently probed with anti-tubulin antibody to show equal loading. Similar results were obtained in two additional experiments.

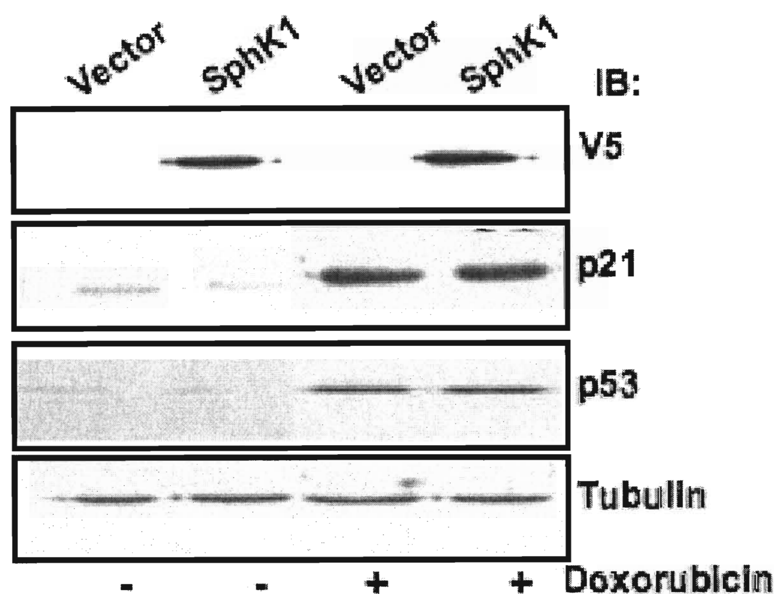

Figure 20. SphK1 Expression has no Effect on p21 and p53 Expression in MCF7 Cells. MCF7 cells transiently transfected with vector or V5-SphK1 were cultured in the absence or presence of doxorubicin (1.7 μM) for 24 h. Equal amounts of cell lysate proteins were separated by SDS-PAGE and then immunoblotted with antibodies directed toward V5, p21, and p53, as indicated. Membranes were subsequently probed with anti-tubulin antibody to show equal loading. Similar results were obtained in two additional experiments.

Localization of Endogenous SphK2 in MCF7 Cells

Although experiments with overexpression of SphK2 were restricted to moderate increases in SphK2 expression (Figure 16), it was important to examine localization and functions of endogenous SphK2. To this end, rabbit polyclonal anti-SphK2 antibodies were utilized, which were previously used to identify endogenous SphK2 in HEK 293 cells (Hait et al., 2005), in western blotting analyses. In MCF7 cells, an immunoreactive band with the same apparent molecular mass as the endogenous SphK2 previously detected in HEK 293 cells was present in the cellular membrane fraction but undetectable in the cytosol (Figure 21). Based on initial studies in the laboratory with subcellular fractions prepared by differential centrifugation, it was observed that in contrast to previous results in HEK 293 and MDA-MB-453 cells where the majority of endogenous SphK2 was in the plasma membrane fraction (Hait et al., 2005), endogenous SphK2 in MCF7 cells was readily detected in the nuclear fraction and barely detectable in other subcellular fractions (data not shown). Because the nuclear proteins are very dilute after subcellular fractionation, more concentrated nuclear and cytoplasmic fractions were prepared with NE-PER reagents. In agreement with confocal microscopy (Figure 17), endogenous SphK2 was clearly localized in the nuclei of MCF7 cells (Figure 22). It should be noted that overexpressed SphK2-L is also detected in the cytosol fraction (Figure 22).

It has recently been suggested that SphK2-L is the predominant splice variant in several human cell lines and tissues (Okada et al., 2005). However, although mRNA for SphK2-L could be detected by quantitative PCR, endogenous nuclear SphK2 in MCF7 cells had similar electrophoretic mobility as untagged SphK2-S rather than untagged

SphK2-L (Figure 22). As expected, SphK2-S migrated faster than the SphK2-L species (Figure 22).

To further examine the localization and function of endogenous SphK2 in MCF7 cells, its expression was downregulated by siRNA targeted to a specific SphK2 sequence (Figure 22 and 23). siRNA is a recent and powerful technology. First discovered as an endogenous property of plants (Napoli et al., 1990), RNA interference is a multistep process involving the generation of siRNAs from double-stranded RNA through the actions of an RNase III-like enzyme, Dicer. The resulting siRNAs mediate the degradation of their complementary RNA [reviewed in (McManus and Sharp, 2002)]. siRNA constructs directed toward SPHK2 were developed by first designing an appropriate target sequence. To ensure that the target sequence is not homologous to any other genes, a BLAST search of the target sequence was performed to prevent unwanted silencing of other genes with a similar sequence. Ideally, it is suggested that the GC ratio should be as close to 50% as possible. It was also ensured that the sequences chosen were present in both SphK2-S and SphK2-L forms.

siRNA directed toward SphK2 decreased SphK2 protein and mRNA by more than 80% (Figure 23a and b), without changing the level of $S1P_2$, $S1P_3$, or $S1P_5$ mRNA (data not shown). It should be noted that reduction of SphK2 protein levels in the nucleus with siSphK2 was also accompanied by a decrease in nuclear SphK2 activity (Figure 23c). Collectively, this data suggests that the predominant form of SphK2 in MCF7 cells, which is expressed in the nucleus, is the short form of SphK2. Notably, SphK2-L is not thought to be present in mice (Okada et al., 2005).

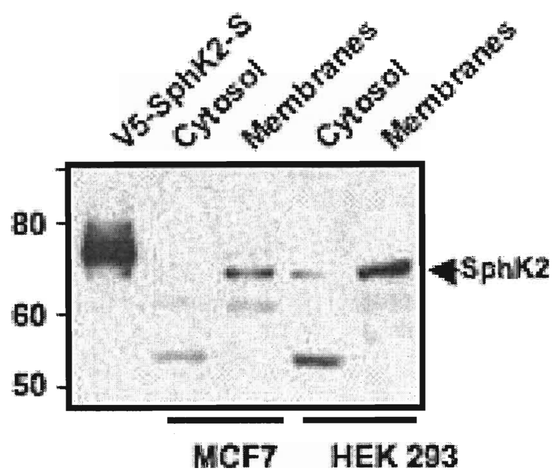

Figure 21. Comparison of Endogenous SphK2 Expression in MCF7 and HEK 293 Cells. Cytosol and membrane fractions from naive MCF7 and HEK 293 cells were prepared by centrifugation at 100,000 x g. Equal amounts of proteins were separated by SDS-PAGE and immunoblotted with anti-SphK2 antibody. Cell lysate from MCF7 cells transiently transfected with V5-tagged SphK2-S was used as a positive control.

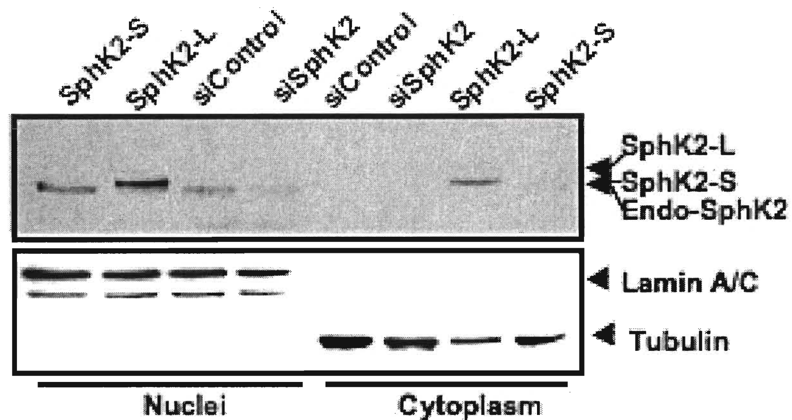

Figure 22. SphK2 is Mainly a Nuclear Protein in MCF7 Cells. MCF7 cells were transfected with control siRNA or siRNA targeted to SphK2. After 48 h, Nuclear and cytosolic fractions were prepared using NE-PER cytoplasm and nuclear extraction reagents. Equal amounts of proteins were separated by SDS-PAGE and immunoblotted with anti-SphK2. Antibodies against lamin A/C and tubulin were used as nuclei and cytoplasm markers, respectively. Cell lysates from MCF7 cells transiently transfected with untagged SphK2-S or untagged SphK2-L were included to indicate the molecular weight of these proteins. Similar results were obtained in two additional experiments.

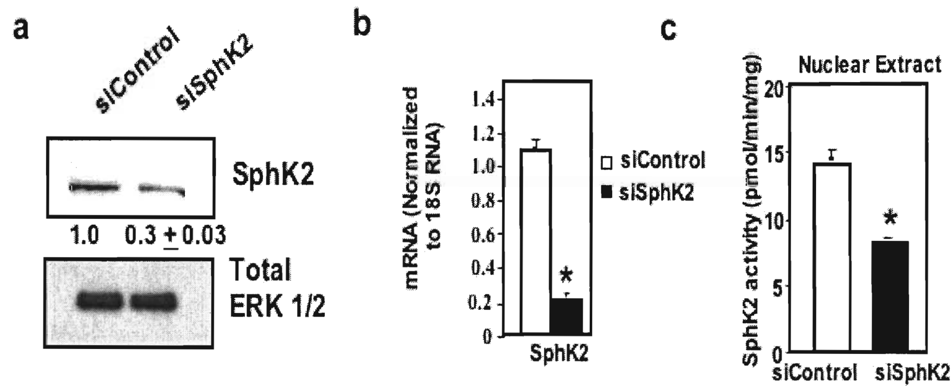

Figure 23. Downregulation of SphK2 with siRNA. (a) MCF7 cells were transfected with control siRNA or siRNA targeted to SphK2. Equal amounts of cell lysate proteins were separated by SDS-PAGE and immunoblotted with anti-SphK2 antibody. Equal loading was verified with anti-ERK1/2 antibody. (b) MCF7 cells were transfected with control siRNA (open bars) or siRNA targeted to SphK2 (filled bars). After 48 h, RNA was isolated and mRNA levels of SphK2, and 18s RNA (for normalization), were determined by quantitative real-time PCR. (c) MCF7 cells were transfected with control siRNA or siRNA targeted to SphK2. After 48 h nuclear fractions were isolated and SphK2 activity was measured. *, $p<0.05$.

Involvement of Endogenous SphK2 in Doxorubicin-Induced Upregulation of p21, and Cell Cycle Arrest in MCF7 Cells

To explore the role of endogenous SphK2 in the responses of MCF7 cells to doxorubicin, the effect of SphK2 knockdown on doxorubicin-induced p21 and p53 expression was examined. Decreasing endogenous levels of SphK2 using siSphK2 decreased basal levels of p21 and induction of p21 upon exposure to doxorubicin but had no effect on doxorubicin induced p53 expression in MCF7 cells (Figure 24). To exclude nonspecific off-target effects, SphK2 expression was also downregulated with siRNA targeted to two other regions of the SphK2 sequence. These siRNAs directed toward SphK2, but not scrambled siRNA controls, also markedly reduced expression of SphK2 mRNA and protein (data not shown). They also almost completely abolished p21 expression induced by doxorubicin, without significantly altering expression of p53 (data not shown).

To characterize the involvement of endogenous SphK2 in doxorubicin-induced MCF7 cell cycle checkpoints, the effect of downregulating its expression on the cell cycle profile was examined by flow cytometry analysis (Figure 25). In agreement with several previous reports (Fornari et al., 1996; Mahyar-Roemer and Roemer, 2001; Seoane et al., 2002), doxorubicin treatment resulted in a marked increase in the proportion of cells in G2/M (Figure 25). Downregulation of SphK2 almost completely prevented these effects of doxorubicin (Figure 25).

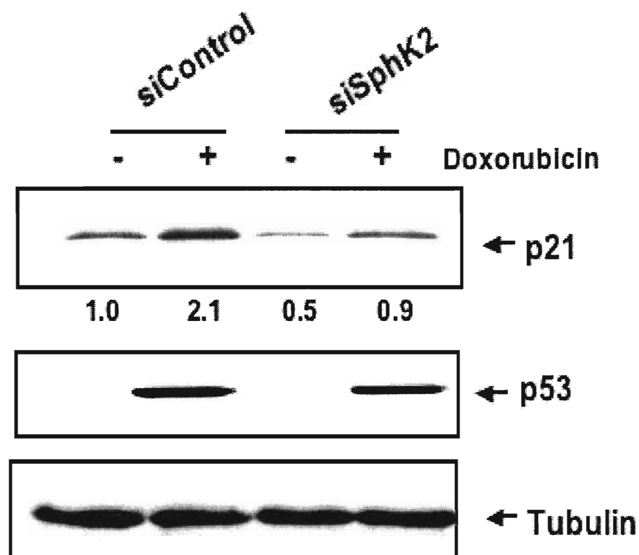

Figure 24. Effect of Downregulation of SphK2 on Doxorubicin-Induced Increases of p21 in MCF7 Cells. MCF7 cells transfected with siRNA directed to SphK2 or control siRNA were cultured in the absence or presence of doxorubicin (1.7 μM) for 24 h. Equal amounts of cell lysate proteins were separated by SDS-PAGE and immunoblotted with antibodies directed towards p21 or p53. Equal loading was verified with anti-tubulin antibody. Similar results were obtained in five additional experiments.

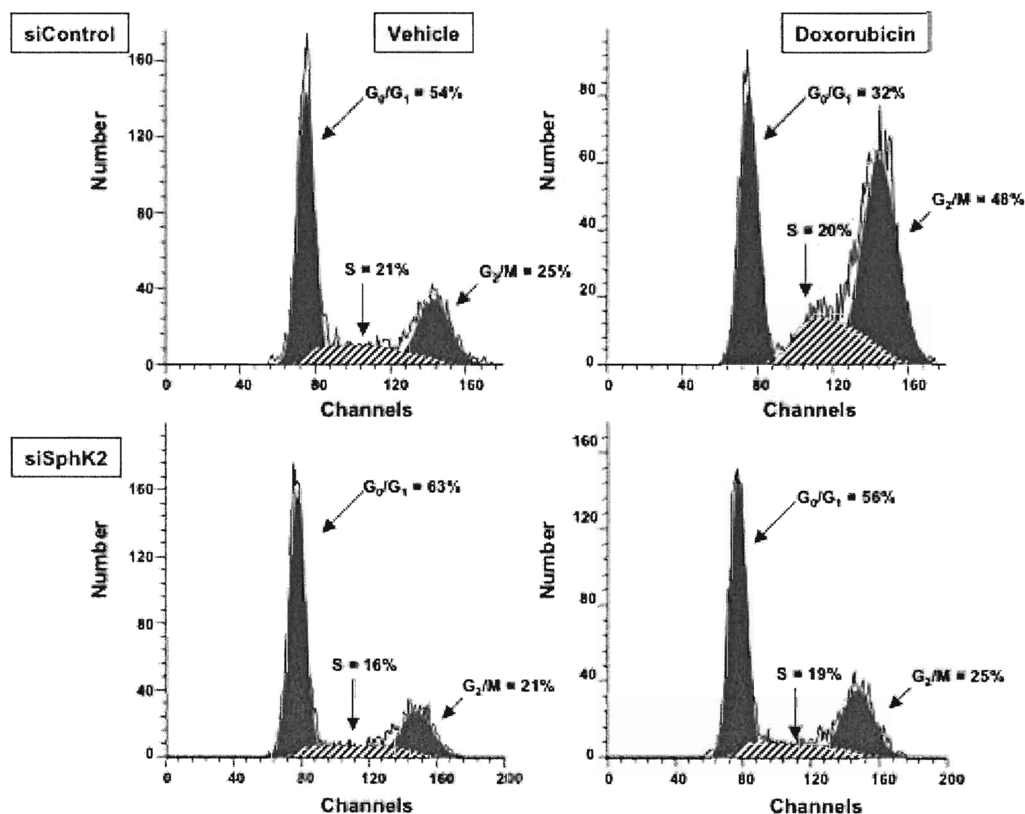

Figure 25. Effect of Downregulation of SphK2 on Doxorubicin-Induced Cell Cycle Arrest in MCF7 Cells. Cellular DNA content of MCF7 cells transfected with siControl or siSphK2 and cultured in the absence or presence of doxorubicin (1.7 μM) for 72 h were analyzed by flow cytometry after staining with PI. Each plot represents the analysis of 5,000 events. It should be noted that floating dead and fragmented cells were removed prior to staining with PI. $p<0.05$ for G2/M for siSphK2 cells treated with doxorubicin compared to G2/M for siContol cells treated with doxorubicin.

Involvement of Endogenous SphK2 in Doxorubicin-Induced Apoptosis in MCF7 Cells

Studies have demonstrated that in contrast to SphK1, overexpression of SphK2 suppresses growth and enhances apoptosis (Igarashi et al., 2003; Liu et al., 2003; Maceyka et al., 2005b). Surprisingly, RNA interference to knockdown SphK2 expression inhibited glioblastoma cell proliferation more potently than SphK1 knockdown did (Van Brocklyn et al., 2005). Similarly, downregulation of SphK2 in MCF7 cells with siSphK2 reduced their growth even when cultured in the presence of serum (Figure 26). Due to the fact that downregulating SphK2 also reduced p21 (Figure 24), and inhibiting p21 expression influences the outcome of a p53 response to doxorubicin in favor of cell death (Seoane et al., 2002), the effect of siSphK2 on doxorubicin-induced apoptosis was next examined.

It should be noted that MCF7 cells have lost caspase 3 expression as a consequence of a 47-base pair deletion within exon 3 of the *CASP-3* gene and show some defects in activating the apoptosis execution machinery (Wendt et al., 2006). Exposure of MCF7 cells to doxorubicin for 24 h only induced minimal apoptosis (Figure 27 and 28), in agreement with previous studies (Fornari et al., 1996; Mahyar-Roemer and Roemer, 2001; Seoane et al., 2002). Notably, although downregulation of SphK1 itself did not induce apoptotic cell death of MCF7 cells but sensitized MCF7 cells to doxorubicin-induced apoptosis (Figure 27) (Sarkar et al., 2005), siSphK2, but not siControl, significantly induced apoptosis (Figure 28). Furthermore, siSphK2 also sensitized MCF7 cells to apoptosis induced by 24 h treatment with doxorubicin (Figure 28). In agreement, downregulation of SphK2 induced apoptotic traits, including activation of caspase 7, the main effector caspase in MCF7 cells, and cleavage of PARP, a marker of caspase-mediated

proteolysis during the apoptotic response (Figure 29). Cleavage of the 116 kDa intact form generates an 89 kDa fragment that is used as a marker for apoptosis. Moreover, downregulation of SphK2 further enhanced doxorubicin-induced PARP cleavage, caspase 7 activation, and increased cytochrome c release to the cytosol (Figure 29), suggesting that loss of SphK2 enhances sensitivity to chemotherapy.

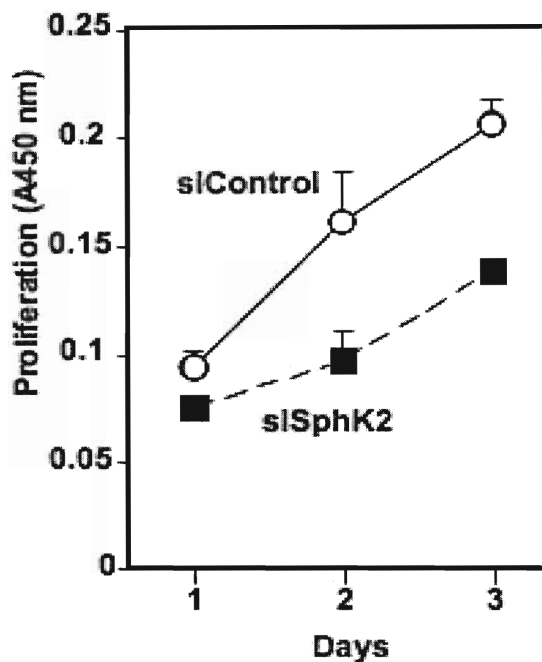

Figure 26. Downregulation of SphK2 Decreases Proliferation in MCF7 Cells. MCF7 cells were transfected with siControl or siSphK2 and after 48 h, plated in 24-well tissue culture dishes. Cells were cultured in media containing 5% serum and at the indicated times, proliferation was determined with the WST-1 reagent. Data are means ± SD of triplicate determinations. The data are representative of at least 3 separate experiments.

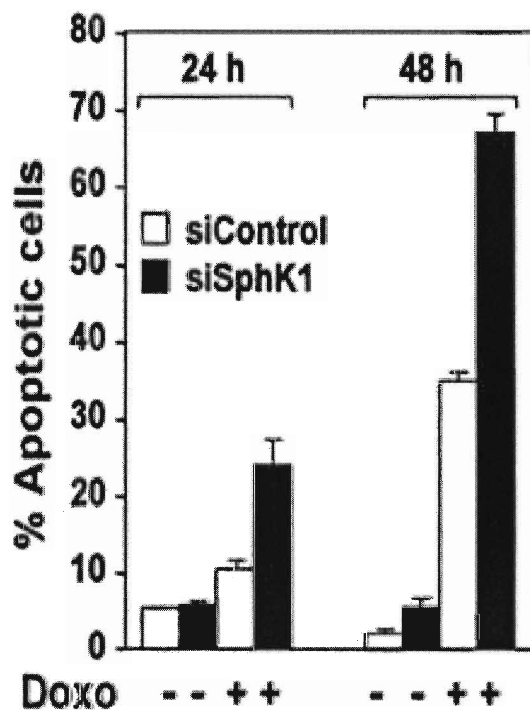

Figure 27. Downregulation of SphK1 Sensitizes MCF7 Cells to Doxorubicin-Induced Apoptosis. MCF7 cells transfected with siControl or siSphK1 were cultured in the absence or presence of doxorubicin (1.7 μM) for 48 h, fixed, and nuclei stained with Hoechst. Apoptosis was determined by scoring the percentage of cells displaying fragmented, condensed nuclei indicative of apoptosis. A minimum of three different fields were analyzed, scoring a minimum of 300 cells.

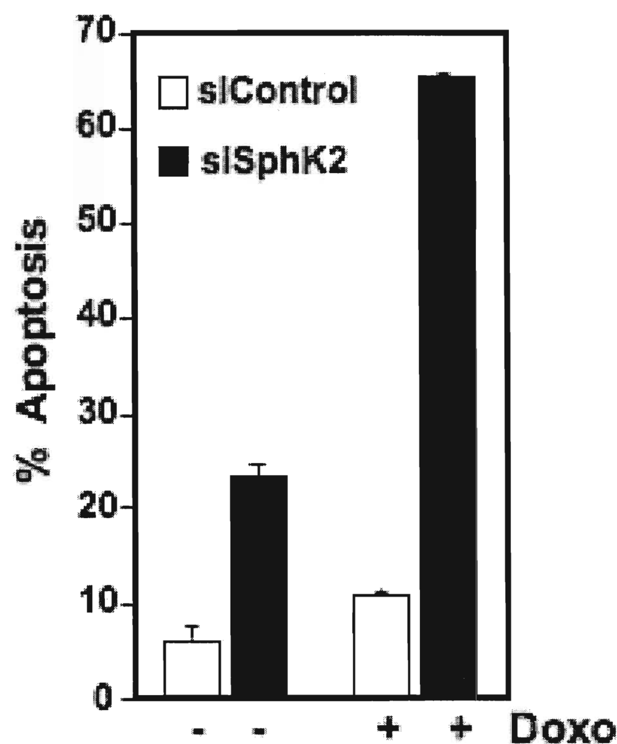

Figure 28. Downregulation of SphK2 Sensitizes MCF7 Cells to Doxorubicin-Induced Apoptosis. MCF7 cells transfected with siControl or siSphK2 were cultured in the absence or presence of doxorubicin (1.7 μM) for 48 h, fixed, and nuclei stained with Hoechst. Apoptosis was determined by scoring the percentage of cells displaying fragmented, condensed nuclei indicative of apoptosis. A minimum of three different fields were analyzed, scoring a minimum of 300 cells. Each point is the mean ± SD of 3 triplicate determinations. The data are representative of at least 3 separate experiments.

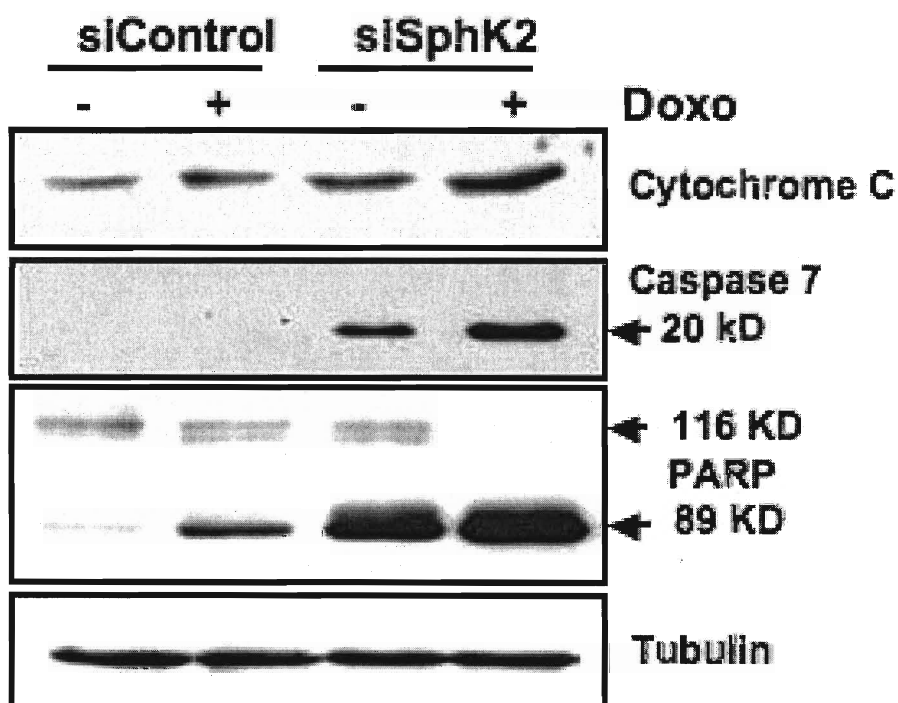

Figure 29. Downregulation of SphK2 Sensitizes MCF7 Cells to Doxorubicin-Induced Cytochrome *c* Release, PARP and Caspase 7 Cleavage. MCF7 cells transfected with siControl or siSphK2 were cultured in the absence or presence of doxorubicin (1.7 μM) for 48 h. Cytosolic proteins were resolved by SDS-PAGE and analyzed by immunoblotting with antibodies against cytochrome *c*, cleaved caspase 7, and cleaved PARP. Blots were stripped and re-probed with anti-tubulin antibody to demonstrate equal loading. Arrows indicate active p20 subunit of caspase 7 and p116 full length PARP and its p89 fragment.

Involvement of Endogenous SphK2 in Doxorubicin-Induced Upregulation of p21, and Apoptosis in MCF7 Cells Lacking p53

To further substantiate the notion that endogenous SphK2 is important for p53-independent induction of p21 by doxorubicin, MCF7 cells infected with HPV-16 E6 were utilized. Expression of HPV-16 E6 protein targets p53 protein for ubiquitination and degradation, thereby inactivating p53 function (Scheffner et al., 1990). In agreement with previous studies (Elmore et al., 2002), there was no induction of p53 by doxorubicin in these cells (Figure 30). Nevertheless, doxorubicin induced a small increase in p21 in MCF7/E6 cells, although as expected, this response was greatly attenuated compared to MCF7 cells infected with pLXNS vector. Downregulation of SphK2 in MCF7/E6 cells also abolished the increase in p21 induced by doxorubicin and sensitized these cells to apoptosis, as determined by cleavage of PARP (Figure 31). These results further substantiate that the effect of SphK2 on p21 is independent of p53. It should also be noted here that downregulation of SphK2 also results in a decrease in basal p21 expression and induction of p21 in response to doxorubicin in vector cells, as shown previously (Figure 24).

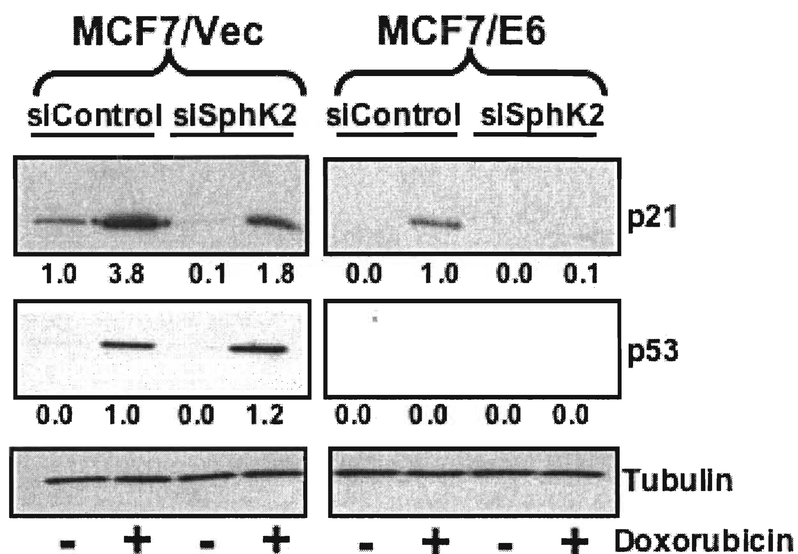

Figure 30. Effect of Downregulation of SphK2 on Doxorubicin-Induced Upregulation of p21 in MCF7 Cells Lacking p53. MCF7 infected with vector pLXSN only (MCF7/Vec) or HPV-16 E6 (MCF7/E6) were transfected with siControl or siSphK2 and cultured in the absence or presence of doxorubicin (1.7 μM) for 24 h. Equal amounts of cell lysate proteins were separated by SDS-PAGE and immunoblotted with the indicated antibodies.

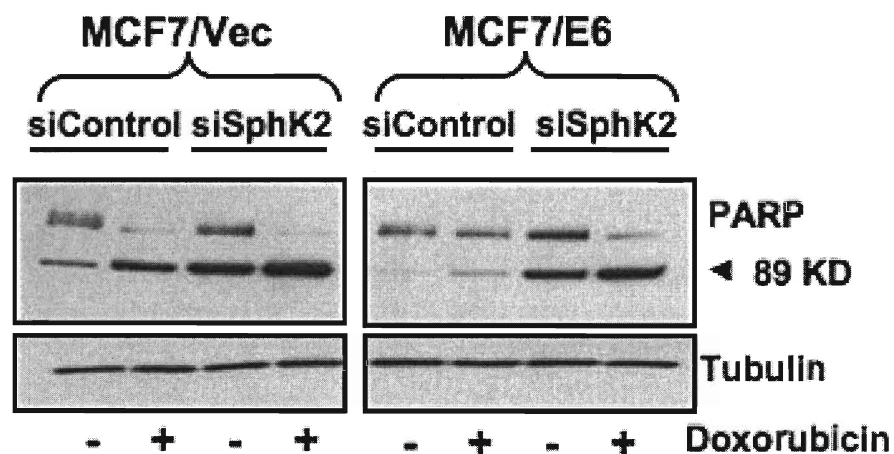

Figure 31. Effect of Downregulation of SphK2 in Doxorubicin-Induced Apoptosis in MCF7 Cells Lacking p53. MCF7 infected with vector pLXSN only (MCF7/Vec) or HPV-16 E6 (MCF7/E6) were transfected with siControl or siSphK2 and cultured in the absence or presence of doxorubicin (1.7 μM) for 24 h. Equal amounts of cell lysate proteins were separated by SDS-PAGE and immunoblotted with the indicated antibodies.

Involvement of Endogenous SphK2 in Doxorubicin-Induced Upregulation of p21 in a p53 Independent Manner in HCT-116 Human Colon Carcinoma Cells

To determine whether the effect of SphK2 on doxorubicin-induced upregulation of p21 independently of p53 was specific to MCF7 cells or generalizable to other cancer cell lines isogenic cell lines derived from human HCT-116 colon carcinoma cells in which p53 and p21 were inactivated by targeted homologous recombination (Bunz et al., 1998; Waldman et al., 1996) were used. It should be noted here that endogenous SphK2 is also enriched in the nucleus in these cells (data not shown). Since HCT-116 cells express caspase 3 and are therefore more sensitive to chemotherapeutics than MCF7 cells, a lower dose of doxorubicin was used. In agreement with the results in MCF7 cells, decreasing endogenous levels of SphK2 using siSphK2 decreased basal expression of p21 and moreover, decreased induction of p21 upon exposure to doxorubicin but had no effect on doxorubicin induced p53 expression in HCT-116 WT cells (Figure 32). In addition, doxorubicin induced a small increase in p21 in HCT-116 $p53^{-/-}$ cells and as expected this response was greatly attenuated compared to HCT-116 WT cells (Figure 32). Downregulation of SphK2 in HCT-116 $p53^{-/-}$ cells also abolished the increase in p21 induced by doxorubicin (Figure 32). Treatment of HCT-116 WT, $p21^{-/-}$, and $p53^{-/-}$ cells with siSphK2 also sensitized these cells to apoptosis, as determined by cleavage of PARP and caspase 7 (Figure 33).

A lower dose of doxorubicin (0.2 μM) arrested the parental HCT-116 in the G2/M stage of the cell cycle, with little evidence of DNA fragmentation indicative of cell death (sub G1 DNA content) (Figure 34). In agreement with several previous reports (Bilim et

al., 2000; Lee et al., 2005), doxorubicin produced a marked increase in the proportion of cells in G2/M (Figure 35). Downregulation of SphK2 almost completely prevented these effects of doxorubicin (Figure 34).

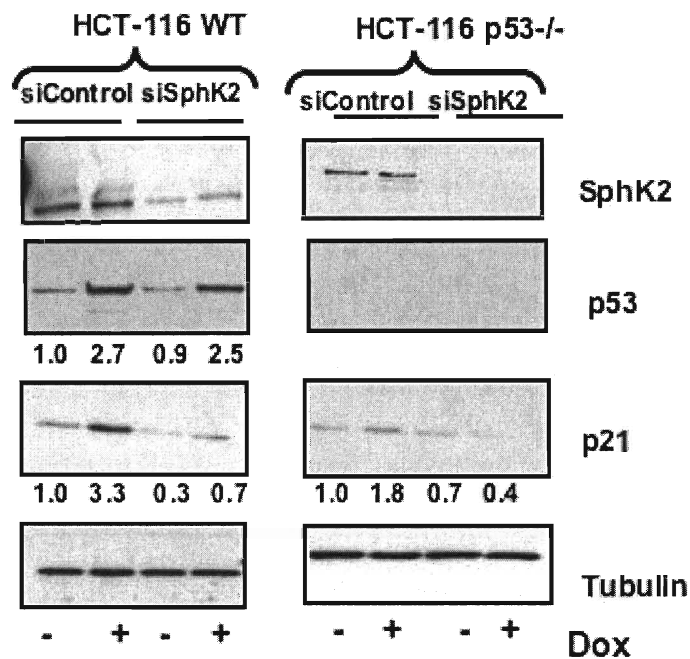

Figure 32. Effect of Downregulation of SphK2 in Doxorubicin-Induced Upregulation of p21 in HCT-116 WT and $p53^{-/-}$ Cells. HCT-116 WT and $p53^{-/-}$ were transfected with siControl or siSphK2 and cultured in the absence or presence of doxorubicin (1 μM) for 24 h. Equal amounts of cell lysate proteins were separated by SDS-PAGE and immunoblotted with antibodies against SphK2, p53, and p21. Blots were stripped and re-probed with anti-tubulin antibody to demonstrate equal loading.

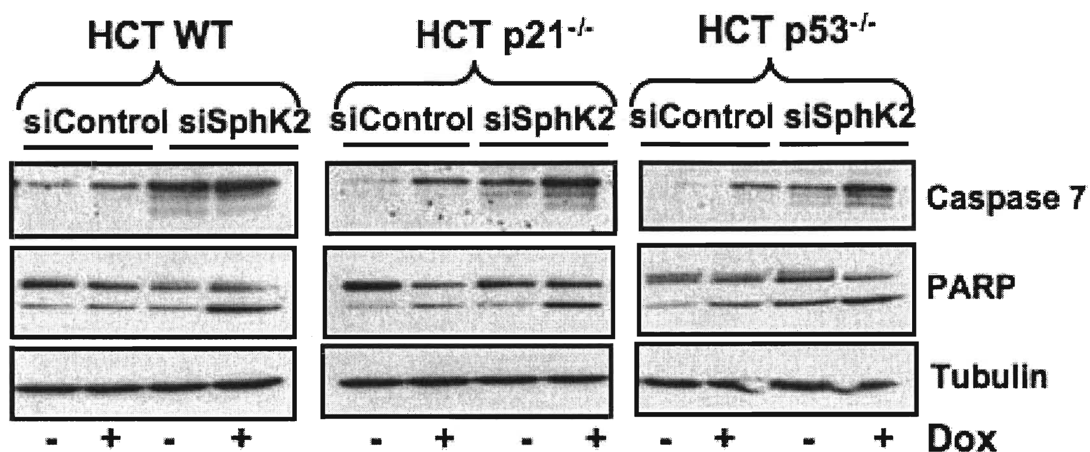

Figure 33. Downregulation of SphK2 Sensitizes HCT-116 WT, p21$^{-/-}$, and p53$^{-/-}$ Cells to Doxorubicin-Induced Apoptosis. HCT-116 WT, p21$^{-/-}$, and p53$^{-/-}$ cells were transfected with siControl or siSphK2 and cultured in the absence or presence of doxorubicin (1 μM) for 18 h. Equal amounts of cell lysate proteins were separated by SDS-PAGE and immunoblotted with antibodies against caspase 7 and PARP. Blots were stripped and re-probed with anti-tubulin antibody to demonstrate equal loading.

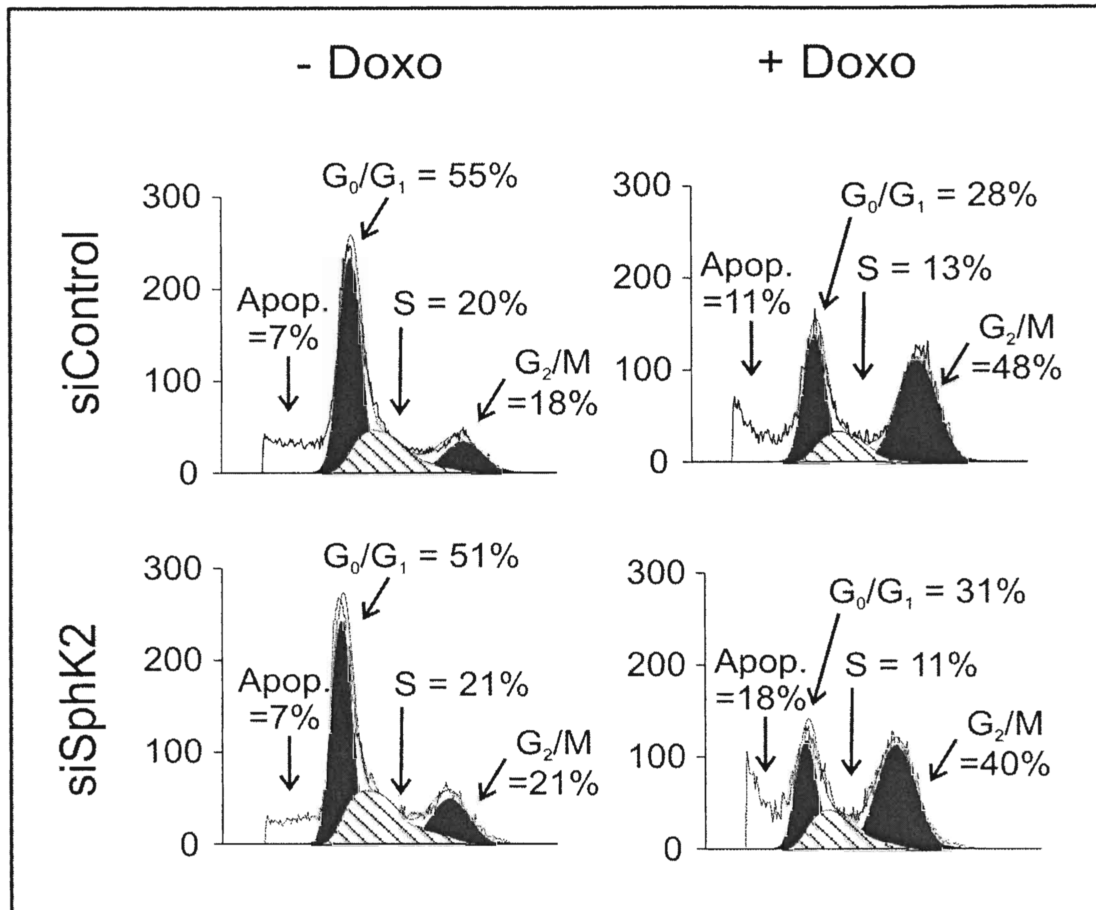

Figure 34. Effect of Downregulation of SphK2 on Doxorubicin-Induced Cell Cycle Arrest in HCT-116 Cells. Cellular DNA content of HCT-116 WT cells transfected with siControl or siSphK2 and cultured in the absence or presence of doxorubicin (0.2 μM) for 72 h. was analyzed by flow cytometry after staining with PI. Each plot represents the analysis of 5,000 events. It should be noted that in contrast to Figure 23 floating dead and fragmented cells were also collected and stained with PI. $p<0.05$ for G2/M for siSphK2 cells treated with doxorubicin compared to G2/M for siContol cells treated with doxorubicin.

Chapter Summary

Two SphK2 splice variants have been described, the originally reported 68 kDa form, designated SphK2-S (Liu et al., 2000a), and the N-terminal extended 72 kDa SphK2-L (Billich et al., 2003; Okada et al., 2005). Based mainly on real-time quantitative PCR of mRNA using primer sets that differentiate between SphK2-S and SphK2-L, it has been suggested that SphK2-L is the predominant form in human cell lines and tissues (Okada et al., 2005). However, the presence of endogenous SphK2 protein forms was not directly examined in these studies (Okada et al., 2005). Using immunoblotting with an antibody directed against a peptide sequence present in both SphK2-S and SphK2-L, an immunopositive band that was downregulated by siSphK2 that had a similar electrophoretic mobility as SphK2-S rather than SphK2-L was detected. These results clearly indicate that the endogenous form of SphK2 expressed in MCF7 cells, which is particularly enriched in the nucleus, is not SphK2-L.

Overexpression of SphK2 increased p53-independent expression of p21 and hypophosphorylation of Rb in MCF7 cells. In contrast, overexpression of SphK1 had no effect on either p21 or p53. Importantly, downregulation of endogenous SphK2 in both MCF7 and HCT-116 cells markedly reduced doxorubicin-induced p21, without affecting p53 expression. Although the function of SphK2 in the nucleus is not clear, we found that endogenous SphK2 is involved in doxorubicin-induced expression of p21 in a p53-independent manner.

The mechanisms that regulate p21 expression fall into two general categories: those that are either dependent or independent of the tumor suppressor, p53. Induction of the p21

gene by p53 is mediated by transcriptional activation via cis-elements located 1.95 and 2.85 kb upstream from the transcriptional start site in the mouse and 2.4 kb in the human p21 gene (el-Deiry et al., 1993; Macleod et al., 1995). p53-independent mechanisms of p21 induction and growth arrest have been less extensively studied. Emerging data has identified a variety of other transcription factors and signaling molecules that can regulate levels of p21 independently of p53 (Gartel and Radhakrishnan, 2005). A number of transcription factors, including STATs, E2Fs, AP2, C/EBPα, C/EBPβ, and the homeobox transcription factor gax, can regulate p21 trancription through *cis*-acting elements in the p21 promoter (Gartel and Tyner, 1999). p21 expression may also be regulated posttranscriptionally by both ubiquitin-dependent and -independent proteasome-mediated degradation (Sheaff et al., 2000; Uozumi et al., 1997).

p21 appears to be a major determinant of cell fate in response to anticancer therapy (Figure 35) as it plays an essential role in growth arrest after DNA damage (Gartel and Tyner, 2002; Weiss, 2003), and can function to inhibit p53-dependent and -independent apoptosis (Mahyar-Roemer and Roemer, 2001). Thus, repression of p21 by gene targeting, c-Myc or chemical p21 inhibitors, all sensitize tumor cells to apoptosis by anticancer drugs (Seoane et al., 2002; Yu et al., 2003). Indeed, downregulation of SphK2 inhibited doxorubicin-induced G2 checkpoint arrest in MCF7 and HCT-116 cells, consistent with the failure of these cells to up-regulate p21 in response to doxorubicin. Importantly, it also sensitized them to apoptosis induced by doxorubicin with a concomitant activation of caspase 7 and PARP cleavage, suggesting that endogenous SphK2 may regulate sensitivity

to chemotherapy. Thus, similar to SphK1 (Sarkar et al., 2005), SphK2 may also be important for growth and survival of MCF7 cells.

According to this idea, doxorubicin-induced expression of p53, which in turn can induce p21, leads to cell cycle arrest, or induction of pro-apoptotic factors, including Puma and Noxa, leading to cell death (Seoane et al., 2002). SphK2 is required for maximal increases in p21 (independently of p53), enabling cell cycle arrest, DNA repair, and preventing execution of the cell death program. Downregulation of SphK2 expression, represses p21 and switches the response from cell cycle arrest to apoptosis (Figure 35 and 36), suggesting that SphK2 may influence the balance between cytostasis and apoptosis of human breast cancer cells. Thus, targeting SphK2 to decrease p21 expression may have the potential to improve the action of anticancer drugs.

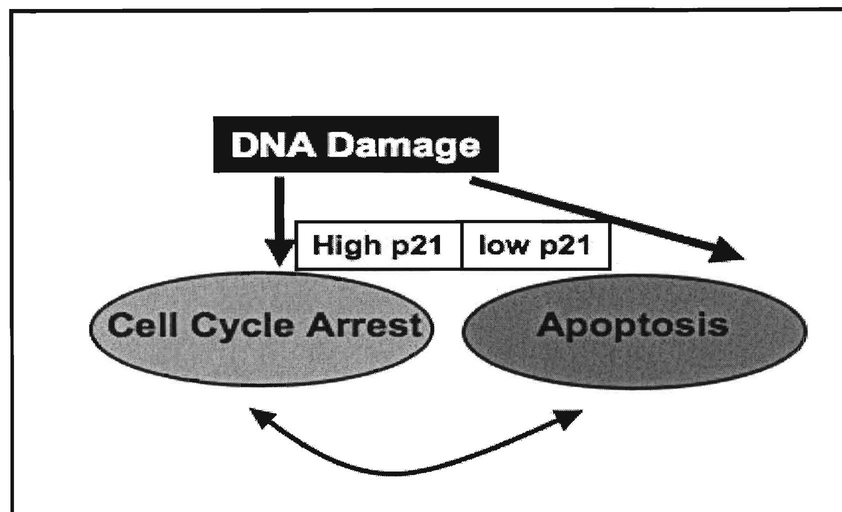

Figure 35. The Effects of p21 Expression on Cell Fate After DNA Damage. The molecular balance between apoptosis and cell cycle arrest in response to DNA damaging agents can be directed by the level of p21. When p21 expression is high, cells are arrested whereas blockage of p21 directly leads to apoptosis. Thus, if p21 expression does not reach a critical level, cells are directly removed by apoptosis. Alternatively, the cells are arrested and then either persist in a state of terminal arrest, or escape this state to die by apoptosis, or recover completely.

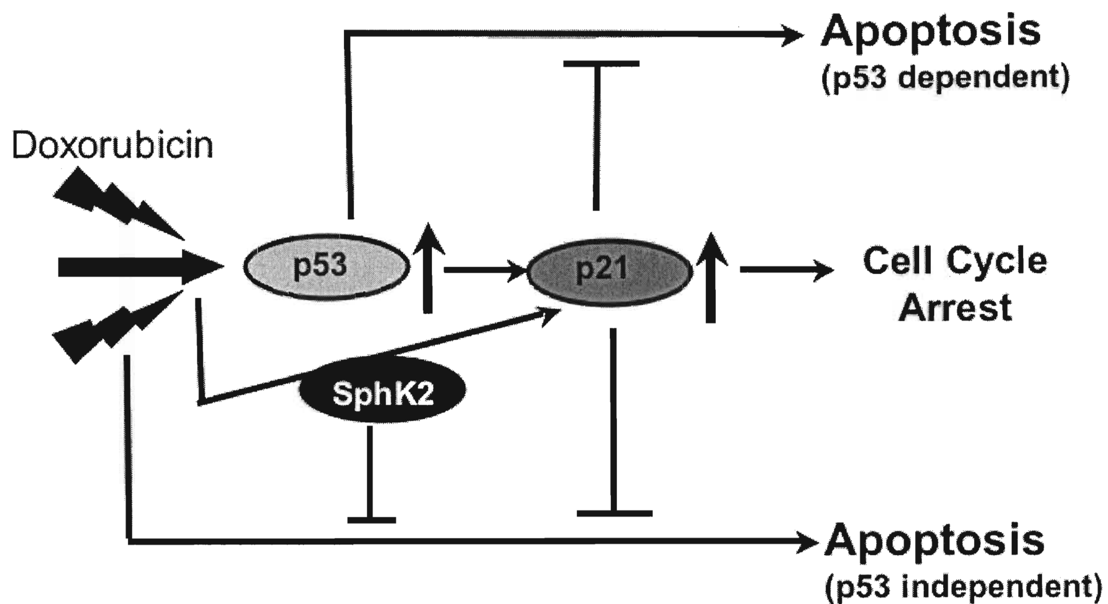

Figure 36. Proposed Model Depicting how Endogenous SphK2 may Protect Cells from DNA Damage-Induced Apoptosis. Doxorubicin induces expression of p53 leading to induction to pro-apoptotic factors, such as Bax, Noxa, and Puma, and cell death, and also induces p21 leading to cell cycle arrest. Doxorubicin can also enhance expression of p21 in a p53-independent manner. SphK2 contributes to induction of p21 independently of p53. Downregulation of SphK2 prevents induction of p21 and removes p21-mediated protection against apoptosis, enabling cell death. In addition, downregulation of SphK2 increases apoptosis in response to doxorubicin independently of p21.

CHAPTER 5

GENRAL DISCUSSION AND FUTURE EXPERIMENTS

Collectively, these results raise the conundrum of how overexpression of SphK2 decreases cell growth and enhances apoptosis, as described in Chapter 3 (Igarashi et al., 2003; Liu et al., 2003; Maceyka et al., 2005b; Okada et al., 2005), while its downregulation sensitizes cells to apoptosis, as described in Chapter 4. However, other reports of this phenomenon have been shown in the literature, Daxx protein (an essential protein found in mice) for example. Overexpressed Daxx was shown to induce apoptosis. However, increased apoptosis was observed in Daxx knockout embryos. siRNA studies confirmed that endogenous Daxx functioned as a pro-survival protein rather than a pro-apoptotic protein, as initially thought (Michaelson and Leder, 2003). Similarly, our studies suggest that endogenous SphK2 functions as a pro-survival protein.

Additionally, similar to our results with siSphK2, a study by Van Brocklyn *et al.,* found that decreasing either SphK2 or SphK1 expression by RNA interference decreased growth of U-1242 MG and U-87 MG glioblastoma cells. Surprisingly, RNA interference to knockdown SphK2 expression inhibited glioblastoma cell proliferation more potently than SphK1 knockdown did (Van Brocklyn et al., 2005). Together these results suggest that similar to SphK1 (Sarkar et al., 2005), SphK2 may also be important for growth and survival of cancer cells.

Importantly, although neither SphK1 nor SphK2 null mice have an obvious phenotype or marked deficiency of S1P, the double knockouts completely lack S1P and have severely disturbed neurogenesis, including neural tube closure, and angiogenesis defects, resulting in early embryonic lethality before day 13.5 because of severe bleeding (Mizugishi et al., 2005). Thus, it seems that SphK1 and SphK2 can complement for the loss of one of the isozymes, and during development S1P formation is absolutely critical.

A partial answer may also be provided by results showing that when SphK2 is overexpressed it does not always have the same subcellular distribution as the endogenous protein. As described in Chapter 3 overexpressed SphK2 was found to be localized to the ER, in addition targeting pro-survival SphK1 to the ER, also resulted induced apoptosis in response to serum withdrawal (data not shown). Moreover, similar to the demonstration that SphK1 is degraded during apoptosis (Taha et al., 2004), we noticed that overexpressed SphK2 is also degraded. The physiological significance of SphK2 cleavage however is not yet known, and may be a crucial focus of future studies. Alternatively, as described in Chapter 3 SphK2 contains an α-helical BH3 domain analogous that present in other BH3-only pro-apoptotic proteins (Liu et al., 2003), such as Bim and Bid. It has been shown that short BH3 domain peptides can induce oligomerization of Bak and Bax releasing cytochrome *c* to sensitize mitochondrial apoptosis (Letai et al., 2002) and thus, it is possible that proteolysis of overexpressed SphK2 might induce apoptosis due to liberation of its BH3 peptide domain.

An important topic of future studies will be to determine how SphK2 influences p21 levels and whether it affects p21 transcription, mRNA stability, or protein turnover

and whether its nuclear localization contributes to this function. Initial data from the laboratory suggests that SphK2 regulates p21 protein stability rather than transcription (data not shown). Another potential clue to this is a finding that specific downregulation of SphK2 reduced formation of ceramide in the sphingolipid salvage pathway (Maceyka et al., 2005b). In this regard, exogenous ceramide has been reported to increase p21 and its association with cdk2 leading to activation of Rb and subsequent cell cycle arrest (Lee et al., 2000). Conversely, more recently it was shown that downregulation of neutral sphingomyelinase 2, which decreases endogenous ceramide levels, downregulates p21 in growth-arrested MCF7 cells, substantiating a link between endogenous ceramide levels and p21 (Marchesini et al., 2004).

Clarifying the functions of SphK1 and SphK2 has important implications in terms of anticancer therapeutics targeting. A broad specificity inhibitor of SphKs may be counterproductive if SphK2 has negative growth-regulatory effects. However, specific inhibitors of SphK1 may not be sufficient to decrease metastasis if SphK2 can compensate. With this in mind interestingly, non-isospecific inhibitors of SphK, such as DMS and DHS induce apoptosis (Jendiroba et al., 2002), which may be a further indication that endogenous SphK2 has pro-survival effects.

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Abstracts and Presentations

Sankala H, Hait NC, Paugh SW, Milstien S, Spiegel S. p53 independent regulation of p21 expression by sphingosine kinase 2. The American Society for Cell Biology 46th Annual Meeting, San Diego, CA, December 9-13, 2006.

Sankala H. Sphingosine kinase 2 regulates p21 independently of p53 in MCF7 cells. 34th Annual John C. Forbes Graduate Student Honors Colloquium, VCU, May 2006.

Sankala H, Hait NC, Paugh SW, Milstien S, Spiegel S. Sphingosine kinase 2 regulates doxorubicin-induced cell cycle arrest and p21 independently of p53 in MCF7 cells. 22nd Annual Daniel T. Watts Research Poster Symposium, VCU, October 25-26, 2005.

Sankala H, Hait NC, Paugh S, Milstien S, Spiegel S. The role of sphingosine kinase 2 in doxorubicin-induced cell cycle arrest in MCF7 cells. Integrative Cellular and Molecular Signaling Symposium, VCU, October 28-29, 2005.

Abstracts and Presentations (continued)

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Sankala H, Liu H, Toman R, Goparaju S, Maceyka M, Bektas M, Payne S, Milstien S, Spiegel S. Dual roles of sphingosine kinase 2 in apoptosis. Massey Cancer Center Research Retreat. VCU, November 2004

Sankala H, Hait N, Milstien S, Spiegel S. The role of sphingosine kinase 2 in apoptosis of human breast cancer cells. 21st Annual Daniel T. Watts Research Poster Symposium, VCU, October 2004.

Awards

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The John C. Forbes Award for Excellence in Biochemistry
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Publications

Sankala H, Hait N, Paugh SW, Milstien S, Spiegel S. Sphingosine kinase 2 mediates induction of p21 by doxorubicin independently of p53 in MCF-7 breast cancer cells. In preparation.

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Honors

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