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13. ABSTRACT (Maximum 200 Words) Sphingosine kinase 1 (SphK1) and its product sphingosine 1-phosphate have been shown to promote cell growth and inhibit apoptosis of tumor cells (reviewed in [1]). SphK1 has been shown to be responsible for radioresistance of certain prostate cancer cells [2]. To better understand SphK1 regulation, we undertook a two-hybrid screen for SphK1-interacting proteins. In the first report period, we focused on one of these interactors, aminoacylase 1. This work will not be discussed as it has been accepted for publication (appendix A). In this report period we studied a second interacting protein, filamin A. We show that SphK1 physically interacts with both the fragment of filamin found in the two-hybrid screen and full length. Though both C-terminal and full length proteins reduce SphK1 activity measured <i>in vitro</i> , the C-terminal fragment inhibits while the full length potentiates the effects of SphK1 on TNF- α signaling and motility. We further demonstrate that filamin is required for ligand-induced motility as well as activation of SphK1. Moreover, siRNA against SphK1 suggests the SphK1-filamin interaction is required for motility, indicating possible anti-metastasis drug targets.				
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Introduction

Sphingolipids are ubiquitous constituents of eukaryotic membranes characterized by the presence of an acylated sphingoid base, ceramide (Cer). Cer and its further metabolites sphingosine (Sph) and Sph-1-phosphate (S1P) are now recognized as potent bioactive molecules. In many cell types, increased Cer and Sph levels lead to cell growth arrest and apoptosis (reviewed in [1, 3, 4]). Conversely, S1P promotes cell growth and inhibits apoptosis (reviewed in [1, 5, 6]). Cells contain signal-regulated enzymes that can interconvert Cer, Sph, and S1P. Thus, conversion of Cer and Sph to S1P simultaneously removes pro-apoptotic signals and creates a survival signal, and vice versa. This led to the proposal of a “sphingolipid rheostat” as a factor determining cell fate [7]. According to this hypothesis, it is not the absolute levels but the relative amounts of these antagonistic metabolites that determines cell fate. In agreement, it has been shown that increased S1P protects against Cer-induced apoptosis, and depletion of S1P enhances Cer-induced apoptosis [7-10].

There are a number of agonists, especially growth and survival factors, that have been reported to increase SphK activity, including ligands for G-protein coupled receptors [11-13] and growth factor receptors [8, 14, 15]. Activation of SphK is required for at least some of the signaling effects observed. Requirement for SphK activation was typically based on the ability of inhibitors of SphK, including dominant negative SphK1 [16], to block agonist-induced effects and/or the ability of exogenously added S1P or a precursor to bypass the agonist. While many early studies suggested a role for S1P as an intracellular second messenger, it was later demonstrated that S1P is also a ligand for a family of G-protein coupled receptors (reviewed in [17]). Complicating matters, there is growing evidence that agonist-induced SphK activation leads to S1P secretion [18, 19] and autocrine and/or paracrine signaling to the cell surface S1P receptors [20, 21].

SphK1 and S1P have been linked to growth, metastasis, and radio- and chemotherapy resistance of tumors, including prostate tumors (reviewed in [1]). For example, it was shown that in radiation sensitive prostate cancer cells, γ -irradiation reduces SphK1 activity, leading to increased Cer and Sph levels and subsequent apoptosis. However, radiation-resistant prostate cancer cells showed no change in SphK activity or Cer levels. Furthermore, inhibitors of SphK sensitized these cells to γ -irradiation, demonstrating a role for SphK in prostate tumor radiation resistance [2].

In order to better understand the regulation and activation of SphK1, we had performed a two-hybrid screen for protein interactors of SphK1. In the initial proposal, we set out to characterize several of these interactors and their potential physiological influence on SphK1. In our first Annual Report, we discussed our results with one of these interactors, aminoacylase 1. That work was submitted and accepted for publication by FEBS Letters and is included as appendix A. Here we discuss the work with a second interacting protein, filamin A.

Updated Results

A C-terminal fragment of filamin A was pulled out of a kidney cDNA library with a two-hybrid screen as an interactor with mouse SphK1. Filamin, also known as filamin 1 and ABP280, is a 280 kDa protein that acts as a dimer. The N-terminus of the protein has an actin binding domain, while the central and C-terminal portions of the protein have coiled-coiled domains responsible for dimerization and protein-protein interactions (reviewed in [22]). While first thought of a structural protein of the cytoskeleton, filamin is emerging as an important scaffolding molecule involved in cell signaling and endocytosis, having been found to interact with TRAF2 [23], PAK [24], and integrins [25], among others. Intriguingly, filamin has also been shown to physically interact with and to be required for the proper localization of PSMA, a protein highly expressed in prostate cancer but not normal tissue [26]. As a first step in analyzing SphK1-filamin interactions, we confirmed the two-hybrid data by showing that SphK1 and the C-terminal fragment could interact when co-expressed in mammalian cells (**Task 1c**; figure 1, upper panel). It has been reported that SphK1 physically interacts with TRAF2 [27], and that this interaction is required for TRAF2-mediated signaling in response to TNF- α . Moreover, TRAF2 has been shown to interact with filamin [23]. Therefore, we re-probed our blot with antibodies to TRAF2. As expected, SphK1 co-purified TRAF2. Interestingly, the amount of TRAF2 that co-purifies with SphK1 is independent of filamin expression (figure 1, lower panel, lanes 1 and 4). This suggests

that SphK1 binds filamin and TRAF2 at independent sites, and that the three can be co-purified as a complex. There are good antibodies commercially available against endogenous filamin, and we have developed in our laboratory a polyclonal antibody which recognizes endogenous SphK1 (**Task 1a**). Using the antibody to SphK1, we were able to co-immunoprecipitate filamin from HEK 293 cells, (**Task 1d**; figure 2), demonstrating that the interaction between the two proteins is not an artifact of over-expression, and that the interaction occurs not just between mouse proteins (used in the original screen and in over-expression) but also the human proteins.

These results suggest that SphK1 and filamin physically interact *in vivo*. The next question was what are the physiological ramifications of this interaction. SphK1 assays were performed on TNF- α -stimulated HEK 293 cells expressing either vector or SphK1 and either vector or C-terminal filamin (**Task 1e**, figure 3). Intriguingly, the C-terminal fragment of filamin inhibited stimulated but not basal SphK1 activity, in both vector and SphK1 expressing cells, suggesting that it acts as a dominant negative inhibitor of SphK1 in response to TNF- α signaling. This is likely due at least in part to the fact that the C-terminal filamin construct lacks the actin binding domain, and thus would not be able to translocate SphK1 to the cytoskeleton. To determine the effect of inhibiting SphK1 activity, we examined a downstream effect of TNF- α stimulation by examining p38, a kinase phosphorylated in response to TNF- α . Again, cells expressing either vector or SphK1 and either vector or C-terminal filamin were stimulated with TNF- α and lysates blotted with phospho-p38 specific antibodies (figure 4, upper panel). Expression of SphK1 enhanced the phosphorylation of p38, and C-terminal filamin reduced this effect, again suggesting that it is acting as a dominant negative inhibitor of at least some aspects of SphK1 signaling. Similar results were obtained when phosphorylation of the related kinases, p44/p42-ERK (MAPK), were examined (figure 4, lower panel). Because TRAF2 shifts TNF- α to promote cell growth, we plan to extend these results by examining the effect of SphK1-filamin interactions in promoting cell growth and inhibiting apoptosis in response to TNF- α .

Because the two-hybrid screen yielded only a C-terminal portion of filamin, we obtained a full length clone from the lab of Dr. T.P. Stossel (**Task 1d**; data not shown). We also received two cell lines: M2, melanoma cells which express little or no filamin, and A7 cells, M2 cells engineered to stably express filamin [28]. These cells have been used to examine the role of filamin in cell motility, a major contributor to metastasis [24]. This is intriguing because much work has demonstrated that S1P, the product of SphK1, acts through cell surface G-protein coupled receptors to either promote or inhibit cell motility, depending on the receptor (reviewed in [29]). Moreover, we have demonstrated that SphK1 translocates from the cytosol to lamellapodia in response to chemoattractants [21], consistent with S1P being released and acting in an autocrine and/or paracrine manner [20, 30]. We hypothesize that SphK1 translocates to the leading edge of the cell by binding to filamin, which is also known to localize to the leading edge upon stimulation [24]. There, SphK1 makes S1P, which is secreted and activates pro-migratory S1P receptors. This "inside-out" signaling of ligand to SphK1 to S1Pr has been observed in several systems, including PDGF [20] and Fc ϵ receptor cross-linking [30].

As a first step, we determined by real time-PCR that M2 and A7 cells express SphK1 and receptors S1P1, 2, 3, and 5 but not S1P4 (data not shown). Heregulin (Hrg) stimulates migration in the filamin-containing A7 but not filamin-negative M2 cells [24]. When A7 cells were stimulated with (Hrg), SphK1 activity increased, while no change in activity was observed in M2 cells (figure 5, open bars). This is consistent with our hypothesis that filamin is required for activation of SphK1. Because we planned to use siRNA directed against SphK1 in these cells, as a control we tested this siRNA to ensure that it reduced SphK1 activity, which it does (figure 5, shaded bars). As a further control, we tested whether or not our antibody directed against SphK1 detected the protein in these cells (figure 5, lower panel). Indeed, our antibody recognizes a single band near the expected molecular weight. Additionally, this band is undetectable when cells are treated with siRNA directed against SphK1. Immunocytochemistry and cell fractionation experiments are ongoing to determine if SphK1 and filamin co-localize to the leading edge of migratory cells, and whether C-terminal filamin disrupts this localization.

We then performed modified Boyden chamber migration assays [20] to assess the role of SphK1 in motility in the M2 and A7 cells. In no case did we observe ligand-induced migration in the filamin-negative M2

cells (data not shown). As expected, Hrg induced migration in A7 cells (figure 6). Interestingly, when A7 cells were transfected with SphK1, no increase over vector stimulation was observed, suggesting that there is sufficient endogenous SphK1 to give maximal migration. However, when SphK1 levels were reduced with SphK1-specific siRNA or when C-terminal filamin was expressed, basal and Hrg-stimulated migration was reduced. Similar results were observed in HEK cells treated with EGF (data not shown). If the SphK1 recruitment is necessary for S1P production and secretion to activate S1P receptors, then A7 cells would be expected to migrate towards S1P. Indeed, this is exactly what was observed: S1P stimulated migration of A7 cells that was comparable to Hrg (figure 7). The decreasing response to higher concentrations of S1P has been observed many times (e.g. [20]), and may be due to stimulation of lower affinity S1P receptors which known inhibit cell motility (i.e. S1P2). To confirm the "inside-out" signaling, we plan to measure S1P secretion upon Hrg stimulation, and to use siRNA to determine which S1P receptor is involved, likely S1P1, 3 or both.

Key Research Results

- SphK1 physically interacts with both the C-terminus of filamin as well as full length.
- C-terminal fragment of filamin may act as a dominant negative inhibitor of SphK1 activity.
- C-terminal fragment of filamin may act as a dominant negative regulator of TNF- α .
- SphK1 likely forms a signaling complex with filamin and TRAF2 to mediate the pro-growth signaling of TNF- α .
- M2 and A7 cell data suggest SphK1-filamin interaction is required for cell migration in response to heregulin.
- Migration of A7 cells to S1P alone suggests "inside-out" signaling.

Reportable Outcomes

Published Paper: Michael Maceyka, Victor Nava, Sheldon Milstien, and Sarah Spiegel. *Sphingosine kinase 1 interacts with aminoacylase 1*. FEBS Lett, 2004 (In Press).

Conclusion

The data accumulated in the first reporting period strongly suggests that SphK1 physically and physiologically interacts with the C-terminal third of Acyl1, work which is in press (Appendix A). In the second reporting period, we have focused our efforts on a second SphK1 interacting protein, filamin a. We have found that the interaction of SphK1 with filamin is required for certain aspects of TNF- α signaling. This is important because TNF- α can promote cell growth or cell death, depending on the accessory molecules with interact with the activated receptor. The TRAF2-mediated signaling normally promotes cell growth and inhibits apoptosis, in part through its interaction with SphK1. Thus, the interaction between SphK1, filamin, and TRAF2 may provide useful targets for intervention in cancer therapy. Moreover, the interaction between filamin and SphK1 is involved in the regulation of motility, a necessity for metastasis. Intriguingly, a recent report demonstrates that PSMA, a protein up-regulated in prostate tumors but not normal tissue, interacts with filamin [26]. It may be that these three proteins, filamin, SphK1, and PSMA work in concert to promote prostate tumors. Thus, increased understanding of the interaction of these proteins may provide novel targets for disrupting the spread of prostatic tumors.

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Figure 1: SphK1 interacts with the C-terminus of filamin and with TRAF2. Vecotr (V) or V5-6xHis-tagged SphK1 (K1) was expressed in the absence and presence (K1H) of HA-tagged CT-filamin (HA fil) in HEK cells. The lysates were incubated with Ni-agarose to purify SphK1 and blotted for the presence of CT filamin. V, vector transfected cells. The same blot was also probed with antibodies to TRAF2. lower nanel.

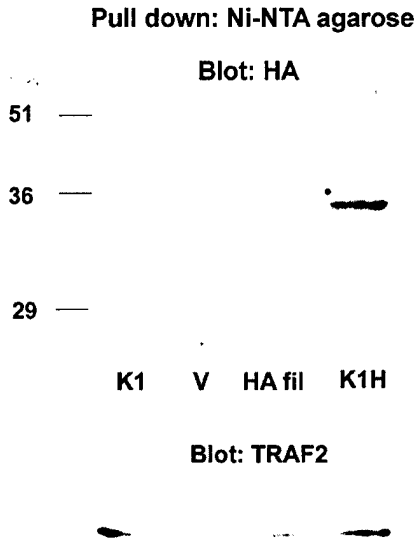


Figure 2: Endogenous SphK1 interacts with endogenous filamin. A lysate was prepared from naïve HEK cells, and equal portions were incubated with pre-immune (mock) or post-immune serum (hSK1) specific for SphK1. Immune complexes were precipitated, washed and blotted for endogenous filamin. Representative of 3 experiments

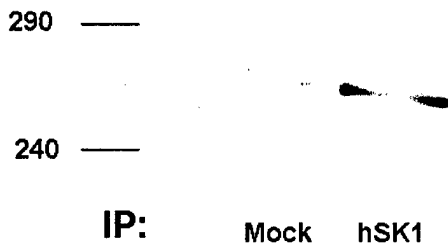


Figure 3: Expression of C-terminal filamin inhibits TNF- α stimulated SphK1 activity. HEK cells were transfected with the indicated constructs and stimulated without or with TNF for 10 min. Lysates were prepared and assayed for SphK1 activity. Representative of 3 experiments.

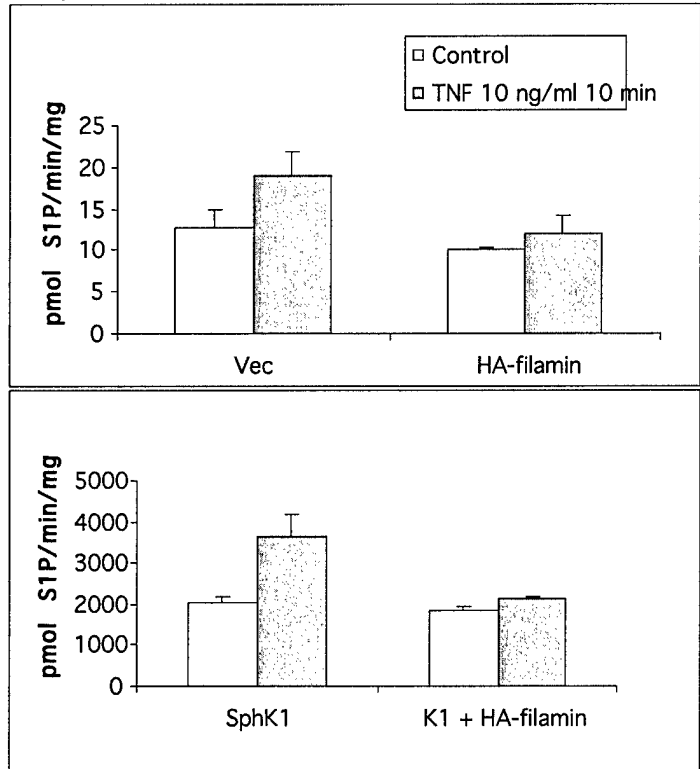


Figure 4: Expression of C-terminal filamin inhibits TNF- α stimulated signaling. HEK cells were transfected with the indicated constructs and stimulated without or with 10 ng/ml TNF for 10 min. Lysates were blotted for phospho-p38 (upper panel) and phospho-MAPK (lower panel). Loading control showed equal loading. Representative of 3 experiments.

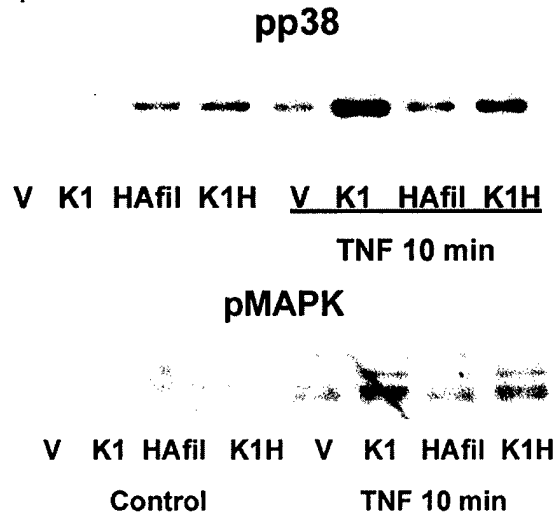


Figure 5: Hrg stimulates SphK1 in filamin expressing A7 but not filamin negative M2 cells, and siRNA directed against SphK1 reduces SphK1 activity. M2 and A7 were transfected with either control siRNA or siRNA directed against SphK1. Cells were then stimulated with Hrg for the indicated times, lysates prepared, and SphK1 activity measured. Representative of 3 experiments. Lower panel indicated western blot using anti-SphK1 antibodies. Left lane is molecular weight markers (in kDa), * indicates SphK1

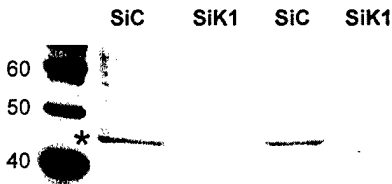
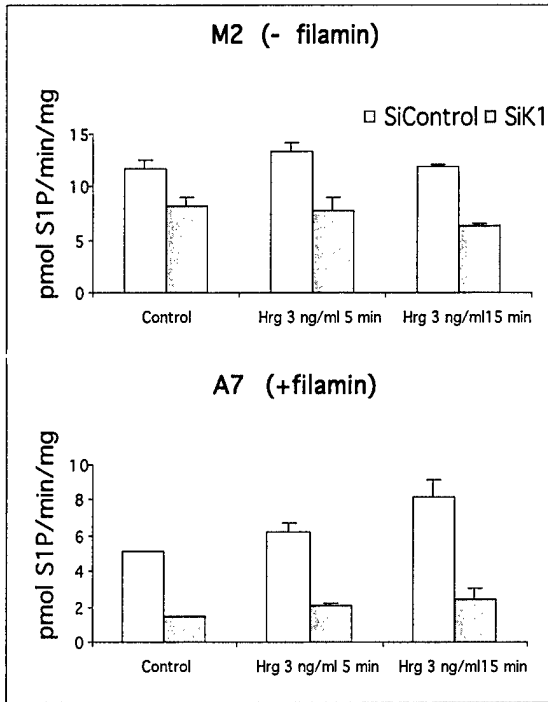


Figure 6: Hrg-stimulated migration in filamin expressing A7 cells is reduced by siRNA directed against SphK1 and by C-terminal filamin. A7 were transfected with the indicated constructs. Cells were then placed in a Boyden chamber and stimulated to migrate through a filter without (open bars) or with (shaded bars) Hrg for for 4 h. Representative of 2 experiments.

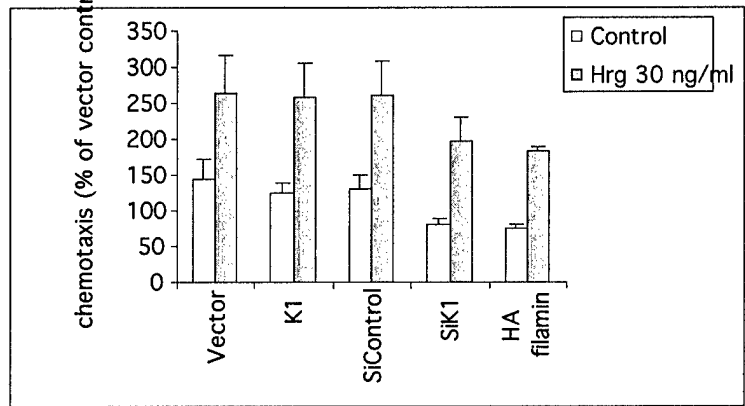
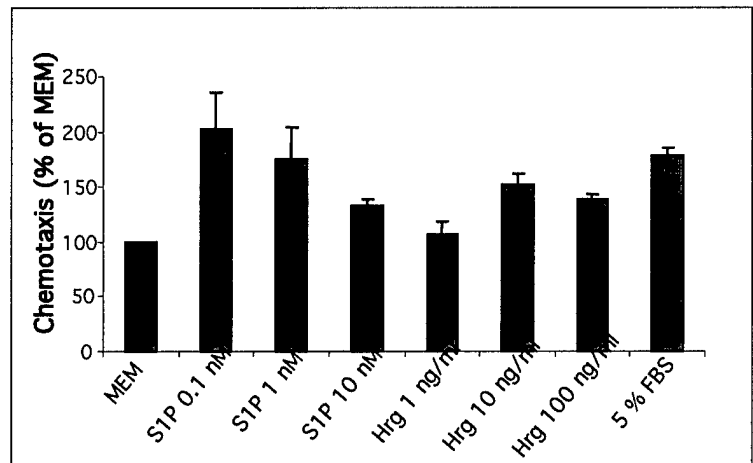


Figure 7: S1P-stimulated migration in filamin expressing A7 cells is reduced comparable to Hrg. A migration assay was performed with A7 cells. Cells were placed in a Boyden chamber and stimulated to migrate through a filter towards increasing concentrations of S1P or Hrg for for 4 h.



Aminoacylase 1 is a sphingosine kinase 1-interacting protein

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Abstract Sphingosine kinase type 1 (SphK1) and its product sphingosine-1-phosphate have been shown to promote cell growth and inhibit apoptosis of tumor cells. In an effort to further understand the regulation of SphK1, we used a yeast two-hybrid screen to find SphK1-interacting proteins. One of these was identified as aminoacylase 1 (Acy1), a metalloenzyme that removes amide-linked acyl groups from amino acids and may play a role in regulating responses to oxidative stress. Both the C-terminal fragment found in the two-hybrid screen and full-length Acy1 co-immunoprecipitate with SphK1. Though both C-terminal and full-length proteins slightly reduce SphK1 activity measured *in vitro*, the C-terminal fragment inhibits while full-length Acy1 potentiates the effects of SphK1 on proliferation and apoptosis. Interestingly, Acy1 induces redistribution of SphK1 as observed by immunocytochemistry and subcellular fractionation. Collectively, our data suggest that acyl physically interacts with SphK1 and may influence its physiological functions.
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Keywords: Aminoacylase 1; Sphingosine kinase; Sphingosine; Sphingosine-1-phosphate; Yeast two-hybrid

1. Introduction

Sphingolipids are ubiquitous constituents of eukaryotic membranes whose backbones consist of an acylated sphingoid base, ceramide. Ceramide and its further metabolites, sphingosine and sphingosine-1-phosphate (S1P), are now recognized as potent signaling molecules. In many cell types, increased ceramide and sphingosine levels lead to cell growth arrest and apoptosis [1,2]. Conversely, S1P promotes cell growth and inhibits apoptosis [3–5]. Cells contain enzymes that can rapidly interconvert ceramide, sphingosine, and S1P. Thus, conversion of ceramide and sphingosine to S1P simultaneously removes pro-apoptotic signals and creates a survival signal, and vice versa [6–9]. While many early studies suggested a role for S1P as an intracellular second messenger, it was later convincingly demonstrated that S1P is also a ligand for a family of G protein-coupled receptors [5,10]. Complicating matters, there

is growing evidence that agonist-induced sphingosine kinase type (SphK) activation leads to S1P secretion [11,12] and autocrine and/or paracrine signaling through cell surface S1P receptors [13–15].

Recently, progress has been made in elucidating the molecular mechanisms of activation of SphK type 1 (SphK1). It has been shown that PKC can phosphorylate SphK1, both activating SphK1 and inducing its translocation to the plasma membrane [12]. More recently, it has been demonstrated that activation and translocation of SphK1 from the cytosol to the plasma membrane results directly from phosphorylation at Ser225 by ERK1/2 [16]. SphK1 interacts with TRAF2, an interaction that is required for suppression of apoptosis by TNF- α [17]. Several other SphK1-interacting proteins have also recently been identified, including PECAM-1 [18], RPK118 [19], and AKAP-related protein SKIP1 [20], which are involved in the translocation of SphK1 to the plasma membrane, endosomes, and signaling complexes, respectively.

In a yeast two-hybrid search for additional SphK1-interacting proteins, we cloned aminoacylase 1 (Acy1) and showed that it interacted with SphK1 and affected its activity and biological functions.

2. Materials and methods

2.1. Cell culture and transfection

Cos7, HEK 293, and NIH 3T3 cells were obtained from ATCC. Cells were cultured in DMEM supplemented with 10% fetal bovine serum (Cos7, HEK) or 10% calf serum (NIH) and maintained at 37 °C in a humidified environment in 5% CO₂. All culture reagents were from BioFluids. HEK 293 cells, plated on poly-D-lysine, and NIH 3T3 cells were transfected using Lipofectamine Plus and Cos7 cells with Lipofectamine 2000 (Invitrogen).

2.2. Two-hybrid screen and cloning

The two-hybrid screen was carried out using the MatchMaker II Kit from Clontech as described [20] with mouse SphK1a as bait against a mouse kidney cDNA library (Clontech). A clone of the C-terminal portion of Acy1 (CT-Acy1) was obtained from this screen that passed all tests as a valid two-hybrid interactor. The CT-Acy1 was removed from the library vector using *EcoRI* and *BamHI* and cloned into pcDNA3-HA (N-terminal tag). Full-length Acy1 was cloned by PCR from a mouse kidney library using the V5-His-Topo Cloning Kit (Invitrogen).

2.3. Sphingosine kinase assay

SphK1 activity was measured essentially as described [21] with sphingosine solubilized in Triton X-100 (0.25% final concentration).

2.4. GST pulldown and immunoprecipitation

The CT-Acy1 was transcribed and translated *in vitro* with the TnT Kit (Promega) in the presence of [³H]leucine. The translation mix was

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Abbreviations: Acy1, aminoacylase 1; S1P, sphingosine-1-phosphate; SphK1, sphingosine kinase type 1

95 incubated with either GST or GST-SphK1 as described [20], then af-
 96 finity-purified using glutathione-Sepharose beads (Pierce), and washed
 97 three times with SphK assay buffer containing 1% Triton X-100. The
 98 pellet was resuspended in sample buffer and proteins resolved by SDS-
 99 PAGE. Gels were dried and exposed to film. For immunoprecipitation,
 100 HEK 293 transfectants were lysed and 800 µg lysate incubated with
 101 anti-myc antibodies for 24 h at 4 °C. Anti-myc immunocomplexes were
 102 precipitated with protein A/G Sepharose (Santa Cruz) and washed
 103 three times with SphK assay buffer containing 1% Triton X-100. The
 104 pellets were resuspended in sample buffer, proteins resolved by SDS-
 105 PAGE, and immunoblotted with anti-HA (CT-Acy1) or anti-V5
 106 (Acy1).

107 2.5. Apoptosis and MTT assays

108 48 h after transfection, NIH 3T3 cells were serum-starved for 24 h to
 109 induce apoptosis. Cells were fixed with 4% paraformaldehyde in 4%
 110 sucrose-PBS and stained with 8 µg/ml Hoechst. Apoptotic nuclei were
 111 scored essentially as described [20]. Cell viability was assessed by the
 112 MTT dye reduction assay (Roche).

113 2.6. Fractionation and immunofluorescence

114 Cells were plated on 10-cm dishes. 48 h after transfection, cells were
 115 washed and harvested in SphK buffer. Cells were lysed by freeze-thaw
 116 and then centrifuged at 100 000 × g. Supernatants were removed (cy-
 117 tosol) and pellets washed with SphK buffer. Pellets were then resus-
 118 pended in SphK buffer containing 1% Triton X-100 and solubilized on
 119 ice for 1 h. Solubilized pelletS were centrifuged at 100 000 × g for 30
 120 min and supernatants (Triton soluble, TS) and pellets (Triton insol-
 121 uble, TI) were then separated. TI pellets were resuspended in SphK
 122 buffer plus 1% Triton X-100. Western blotting was used to determine
 123 protein expression with either anti-myc (9E10; Santa Cruz), anti-HA
 124 (3F10; Roche), or anti-V5 (monoclonal from Invitrogen or rabbit
 125 polyclonal from Sigma-Aldrich) as primary antibodies followed by
 126 HRP-conjugated secondary antibodies (1:10 000, Jackson Immuno-
 127 Research Laboratories). Immunocomplexes were visualized by en-
 128 hanced chemiluminescence (Pierce) as described previously [22].

129 For immunofluorescence, cells were plated on #1 coverslips, trans-
 130 fected, and after 48 h, fixed in 3.7% formalin and stained essentially as
 131 described [20]. Briefly, after washing with PBS containing 10 mM
 132 glycine, cells were permeabilized for 3 min with 0.5% Triton X-100 in
 133 PBS-glycine, washed again, and incubated for 20 min at room tem-
 134 perature with mouse monoclonal anti-myc (2 µg/ml) for detection of
 135 SphK1 and rabbit anti-V5 (4 µg/ml) for Acy1. After washing, cells
 136 were incubated for 20 min with Texas Red-conjugated anti-mouse and
 137 FITC-conjugated anti-rabbit secondary antibodies (1 µg/ml each;
 138 Jackson ImmunoResearch). Coverslips were then mounted with glycer-
 139 ol containing 10 mM *n*-propyl gallate and images collected with a
 140 Nikon TE-200 fluorescence microscope.

141 3. Results and discussion

142 3.1. Acy1 is a SphK1-interacting protein

143 To search for proteins that interact with SphK1 and regulate
 144 its activity or translocation to the plasma membrane, a two-
 145 hybrid screen was carried out using mouse SphK1 fused to
 146 the DNA binding domain of GAL4 as bait. The prey consisted
 147 of a mouse kidney cDNA library (Clontech) fused to the
 148 transcriptional activation domain of GAL4. Interaction be-
 149 tween SphK1 and a library protein brings together the two
 150 domains necessary for transcription of reporter genes. The
 151 MatchMaker II system mitigates against false positives by
 152 having three different promoter-reporter gene constructs, with
 153 differing affinities for the GAL4 DNA-binding domain. This
 154 reduces the chances that the prey construct activates on its own
 155 by binding regions around the GAL4 DNA binding site or to
 156 specific TATA boxes and allows for control of stringency.
 157 Using the most stringent interaction test, a clone of the CT-
 158 Acy1, starting at amino acid 232 of the full-length protein
 159 (Fig. 1A), was obtained. Acy1 has been characterized as a

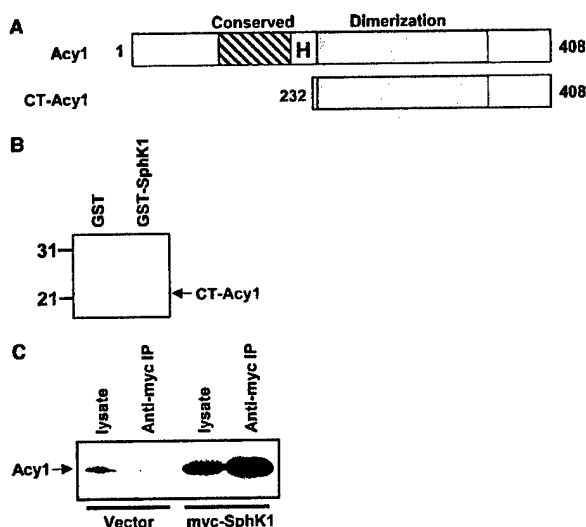


Fig. 1. SphK1 physically interacts with Acy1. (A) Schematic representation of full-length Acy1 (top) and CT-Acy1, the C-terminal fragment pulled out of the two-hybrid screen. Hatched box indicates conserved regions (aa 78–148) amongst Acy1 family members across kingdoms, H indicates the conserved catalytic histidine, and shaded boxes indicate putative dimerization domains. (B) [³H]-labeled CT-Acy1 prepared by in vitro transcription-translation was incubated with either GST or GST-SphK1. Glutathione-Sepharose beads were then added. After overnight incubation at 4 °C, beads were washed and bound proteins resolved by SDS-PAGE and autoradiographed. GST-SphK1 precipitated 22 kDa radiolabeled CT-Acy1. The data are representatives of two independent experiments. (C) HEK 293 cells were co-transfected with V5-Acy1 and either vector or myc-SphK1. Cells were then lysed and immunoprecipitated with anti-myc antibodies followed by protein A/G-Sepharose. The pellets were resolved by SDS-PAGE and immunoblotted with anti-V5. Lysate indicates 1/100 of the total protein immunoprecipitated. Similar results were obtained in two additional experiments.

160 cytosolic homodimeric metalloenzyme of amino acid salvage
 161 [23], catalyzing the hydrolysis of amide-linked Acy1 chains of
 162 amino acids. It is the major acylase that degrades *N*-acetyl-
 163 cysteine [24], and thus may play a role in the regulation of
 164 cellular redox status. Acy1 is abundant in the kidney and brain
 165 [24], two tissues with high SphK1 levels [25]. CT-Acy1 is not
 166 expected to be active because it lacks conserved residues necessary
 167 for binding essential Zn ions and it has a truncated
 168 catalytic domain [26] (Fig. 1A).

169 To examine whether CT-Acy1 interacts physically with
 170 SphK1, [³H]-labeled CT-Acy1 was synthesized by in vitro
 171 transcription-translation, incubated with either GST or GST-
 172 SphK1 [20] and binding was determined using glutathione-
 173 Sepharose beads. Sepharose-bound proteins were then re-
 174 solved by SDS-PAGE and ³H-labeled proteins visualized by
 175 autoradiography. CT-Acy1 specifically interacted with GST-
 176 SphK1, but not with GST alone (Fig. 1B).

177 3.2. Acy1 interacts with SphK1 in vivo

178 To determine if Acy1 interacts with SphK1 when expressed
 179 in mammalian cells, HEK 293 cells were co-transfected with
 180 Acy1 and SphK1. Lysates were immunoprecipitated with an-
 181 tibodies to SphK1 and the blots probed with antibodies to
 182 either CT-Acy1 or Acy1. Both CT-Acy1 (data not shown) and
 183 full-length Acy1 co-immunoprecipitated with SphK1
 184 (Fig. 1C). This result, coupled with the GST pull-down results

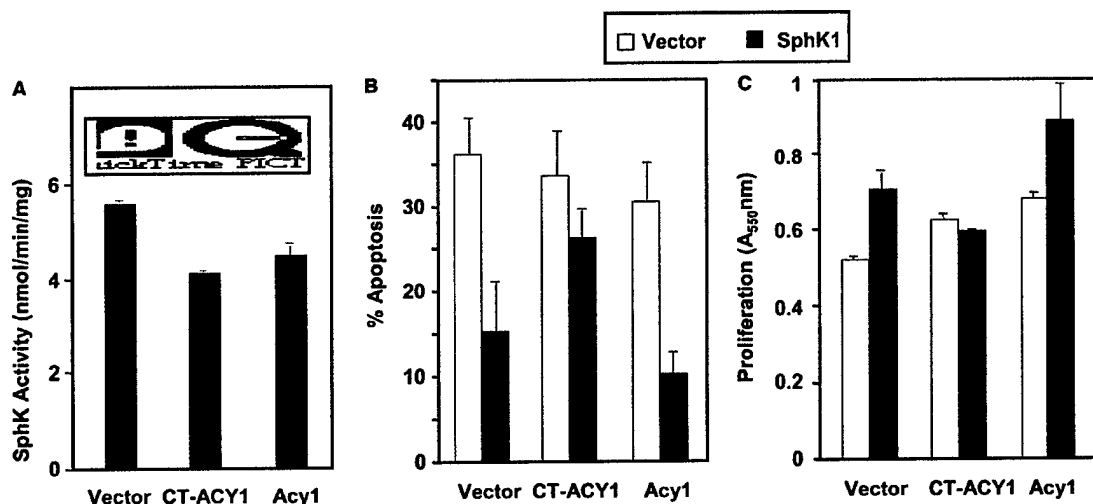


Fig. 2. Effect of Acyl1 on activity and function of SphK1. (A) SphK1 activity. HEK 293 cells were co-transfected with myc-SphK1 and vector, CT-Acy1, or Acyl1. After 48 h, cells were lysed and SphK1 activity measured. Inset shows equal expression of SphK1 as determined by Western blotting with anti-myc. The data are representatives of three independent experiments. (B) Cytoprotective effects of SphK1. NIH 3T3 cells stably transfected with vector (open bars) or SphK1 (filled bars) were transiently transfected with either Acyl1, CT-Acy1, or empty vector, together with GFP at a 5:1 ratio, and then serum-starved. After 24 h, cells were fixed and stained with Hoechst. Total GFP-expressing cells and GFP-expressing cells displaying fragmented nuclei indicative of apoptosis were enumerated. Data are means \pm S.D. Three independent wells were counted for each treatment, with a minimum of 100 cells scored per well. Data are representatives of two independent experiments. (C) Proliferative effects of SphK1. Cells transfected with the indicated constructs were plated at equal density and allowed to grow for 24 h. Cell proliferation was determined by MTT dye reduction.

185 and the original two-hybrid data, indicates that SphK1 and
186 Acyl1 physically interact in vivo.

187 3.3. Effects of Acyl1 on SphK1 activity and biological functions

188 We next examined whether the physical interaction with
189 Acyl1 affects SphK1 biological functions. Co-transfection of
190 SphK1 with either CT-Acy1 or Acyl1 slightly decreased SphK1
191 activity measured in vitro, without affecting its expression level
192 (Fig. 2A). The best characterized biological responses of
193 SphK1 are suppression of apoptosis and stimulation of cell
194 proliferation and entry into S phase [4,21]. NIH 3T3 cells
195 expressing either vector or SphK1 were co-transfected with
196 CT-Acy1 or Acyl1 and effects on apoptosis induced by serum-
197 withdrawal determined by examining chromosomal condensa-
198 tion and fragmentation. Interestingly, in contrast to their
199 inhibitory effects on SphK1 activity, CT-Acy1 reduced while
200 Acyl1 potentiated the anti-apoptotic effect of SphK1 (Fig. 2B).

201 To address the possibility that interaction of Acyl1 with
202 SphK1 regulates its mitogenic effect, we also examined the
203 effect of CT-Acy1 or Acyl1 on proliferation. In agreement with
204 other studies [27–30], expression of SphK1 increased cell
205 growth as determined by MTT dye reduction assay. Once
206 again, CT-Acy1 had a different effect than full-length Acyl1.
207 Whereas CT-Acy1 reduced the growth-promoting effect of
208 SphK1, Acyl1 enhanced it (Fig. 2C).

209 3.4. Acyl1 induces redistribution of SphK1

210 SphK1 is a cytosolic enzyme, while its substrate sphin-
211 gosine is a lipid found in membranes. Therefore, it is likely
212 that SphK1 activity is regulated in part by its translocation
213 from the cytosol to membranes. Indeed, several previous
214 studies have shown that SphK1 translocates to membranes
215 upon activation [12,15,16,31]. It was therefore of interest to
216 determine whether Acyl1 alters the localization of SphK1.
217 First, we examined the localization of both proteins by

immunocytochemistry. In agreement with its cytoplasmic
218 expression [32], Acyl1 had a diffuse cytosolic localization
219 when expressed in Cos7 cells (Fig. 3A and B). When SphK1
220 was expressed alone, it also showed a diffuse cytosolic ex-
221 pression pattern (Fig. 3C and D), with dispersed punctate
222 staining as reported previously [27]. However, when Acyl1
223 and SphK1 were co-expressed, although both were still
224 predominantly cytosolic, there was also co-localization in
225 tubular structures (Fig. 3E–G, arrows) and at or near the
226 plasma membrane as indicated by the yellow color in the
227 merged pictures.
228

229 To further substantiate that expression of Acyl1 induces
230 redistribution of SphK1 to the plasma membrane, we ex-
231 amined their localization by subcellular fractionation.
232 Transfected cells were lysed by freeze-thawing and centri-
233 fuge at 100 000 \times g. Pellets were then extracted with 1%
234 Triton X-100, generating a soluble fraction and a TI fraction
235 that contains cytoskeleton proteins, focal adhesions, and li-
236 pid rafts. As expected from the immunofluorescence data,
237 when expressed alone, both proteins were predominantly
238 localized to the cytosolic fraction (Fig. 4). Interestingly,
239 when co-expressed with Acyl1, a portion of SphK1 shifted
240 from the cytosol to the TS fraction (Fig. 4).

241 4. Conclusions

242 Our results suggest that Acyl1 is a bona fide SphK1-inter-
243 acting protein that can influence not only its activity but also
244 its cellular localization. Acyl1 also potentiated the mitogenic
245 and cytoprotective effects of SphK1 effects. Surprisingly, the
246 CT-Acy1, which also binds SphK1, reduced these effects. Al-
247 though the physiological significance of these observations is
248 not yet clear, our data suggest that CT-Acy1 may act as a

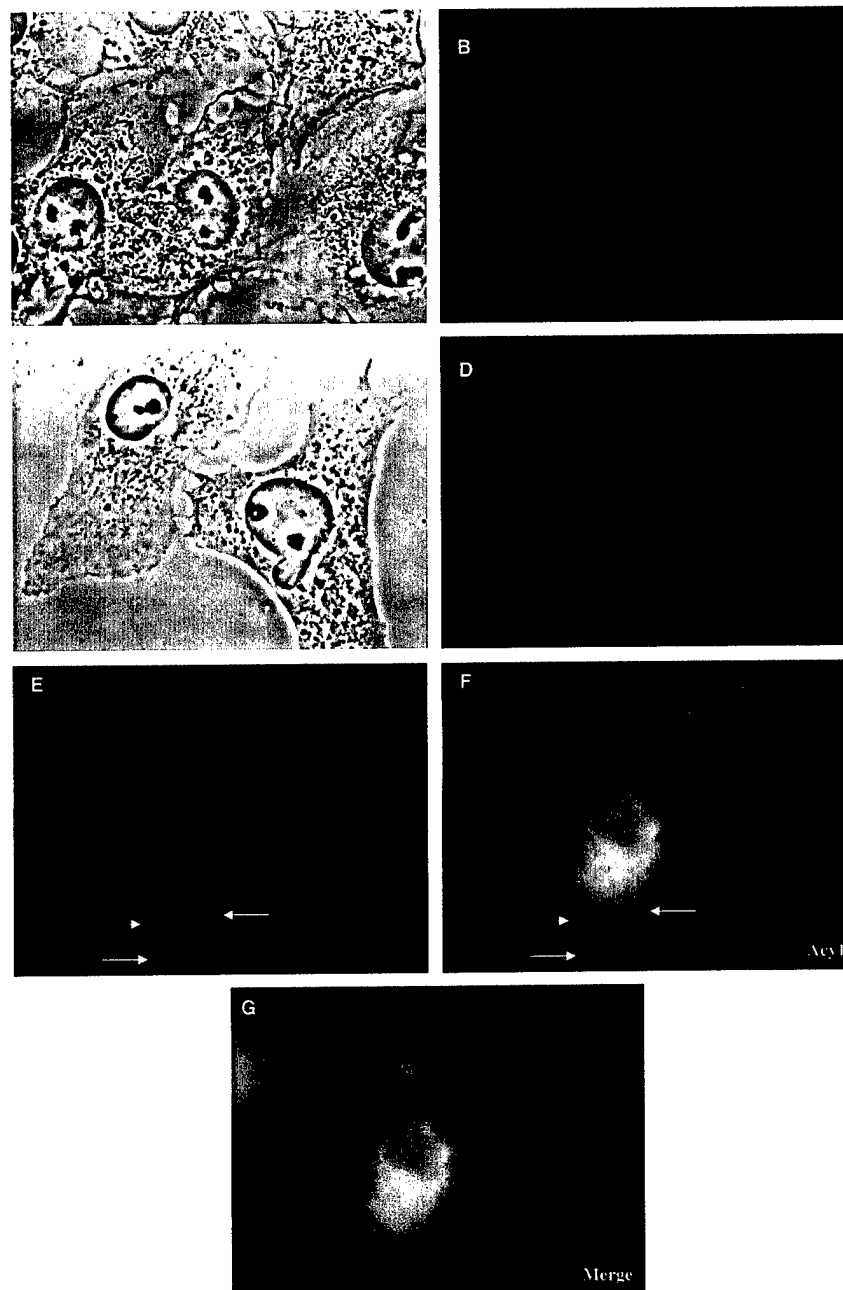


Fig. 3. Acyl1 alters the intracellular distribution of SphK1. Cos7 cells were transfected with V5-Acy1 (A,B) or myc-SphK1 (C,D) or both (E-G) and fixed 48 h later. Cells were then incubated with anti-myc and anti-V5 antibodies and stained with Texas red anti-mouse IgG and FITC anti-rabbit IgG. Phase (A,C) and fluorescent (B,D,E-G) images were obtained with a Nikon TE-200 using a CoolSnap camera driven by MetaMorph software. Panel G shows the superimposed merged pictures, yellow color represents co-localization of the two proteins. Arrows indicate long tubular structures observed only when proteins were co-transfected.

249 dominant-negative inhibitor of SphK1. We suspect that over-
 250 expression of CT-Acy1 blocks the ability of SphK1 to interact
 251 with endogenous, active Acyl1. This would block the pro-
 252 growth and anti-apoptotic effects of SphK1 if the aminoacyl-
 253 lase activity of Acyl1 is required for its SphK1 regulatory
 254 effects, because CT-Acy1 is enzymatically inactive. It is also
 255 possible that the N-terminus of Acyl1, missing from CT-Acy1,
 256 may have binding sites for other proteins required for the

SphK1-Acy1 complex to inhibit apoptosis and promote cell
 growth or for its translocation to its site of action.

Because cellular levels of the bioactive sphingolipid mediator
 S1P are low and tightly regulated, it is not surprising that cells
 have evolved many mechanisms to control the activity of
 SphK1, the critical enzyme responsible for formation of S1P,
 as suggested by the discovery of a plethora of SphK1-inter-
 acting proteins [17-20]. Most of them, including Acyl1, have in

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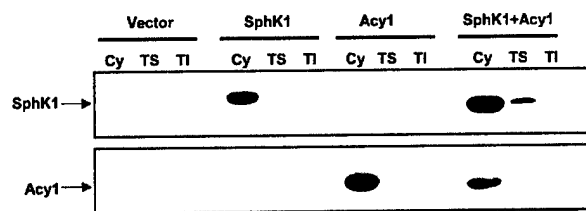


Fig. 4. Acy1 translocates SphK1 from the cytosol to the Triton-soluble (TS) membrane fraction. HEK 293 cells were transfected with SphK1, Acy1, or both. After 48 h, cells were harvested and lysed by freeze-thawing. The lysates were centrifuged at 100 000 × g to generate cytosol (Cy) and pellet fractions. 1% Triton X-100 was added to the pellet fractions and after centrifugation at 100 000 × g, equal amounts of the Triton X-100-insoluble (TI) and TS fractions were separated on 10% SDS-PAGE, transblotted to nitrocellulose, and probed with antibodies to myc (SphK1) and V5 (Acy1) epitopes.

265 common the ability to reduce SphK1 enzymatic activity and
 266 affect its cellular localization, directing it from a diffuse cyto-
 267 plasmic expression to specific membranes where S1P produc-
 268 tion can then be spatially and temporally regulated to influence
 269 both intracellular and extracellular signaling pathways.

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 271 Health Grant R01CA61774 (S.S.) and US Department of Defense
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