Cloning and Characterization of a Protein Kinase A Anchoring Protein (AKAP)-related Protein That Interacts with and Regulates Sphingosine Kinase 1 Activity*

Emanuela Lacaná‡‡, Michael Maceyka‡‡, Sheldon Milstien‡, and Sarah Spiegel†‡‡‡

From the †Department of Biochemistry and Molecular Biology, Georgetown University Medical School, Washington, D. C. 20007, the ‡Department of Biochemistry and Molecular Biophysics, Medical College of Virginia Campus, Virginia Commonwealth University, Richmond, Virginia 23298, and the §Laboratory of Cellular and Molecular Regulation, National Institute of Mental Health, Bethesda, Maryland 20892

Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid metabolite that has several dual actions. S1P is the ligand for a family of G protein-coupled receptors known as S1PRs that mediate various physiological functions. Growth factors rapidly activate sphingosine kinase type 1 (SPHK1) resulting in phosphorylation of sphingosine to form S1P, which plays important roles in cell growth regulation and protection from apoptosis. However, little is known of the mechanism(s) by which SPHK activity is regulated. Using a yeast two-hybrid screening approach, we cloned a 3-kb cDNA encoding a SPHK1-interacting protein (SKIP). BLAST analysis revealed that SKIP corresponded to the C-terminal region of a larger (~7 kb) CDNA that encoded a protein with a high degree of similarity to a family of protein kinase A anchor proteins (AKAP). In confirmation of the yeast two-hybrid assay, glutathione S-transferase (GST)-SPHK1 specifically pulled down SKIP, whereas GST did not. Moreover, immunoprecipitation of in vitro translated SPHK1 and SKIP revealed that SKIP and SPHK1 are tightly associated. Furthermore, SKIP overexpression in NIH 3T3 fibroblasts reduced SPHK1 activity and interfered with its biological functions. The apoptotic-sparing effect of SPHK1 against serum deprivation was reduced when co-transfected with SKIP. In addition, SPHK1-enhanced cell proliferation was also abolished by SKIP, with a corresponding decrease in activation of ERK. Taken together, these results indicate that SKIP is a novel protein likely to play a regulatory role in the modulation of SPHK1 activity.

Sphingolipid metabolites such as ceramide, sphingosine, and sphingosine 1-phosphate (S1P),† have been the subject of extensive studies in recent years and are implicated in the regulation of cell growth, survival, motility, and apoptosis (reviewed in Refs. 1–3). Ceramide (N-acylsphingosine) is generated from sphingomyelin hydrolysis by sphingomyelinase. This enzyme is activated by a variety of stress stimuli, including proinflammatory cytokines, growth factor withdrawal, radiation, and anticancer drugs (2, 4). Ceramide, in turn, regulates various events leading to cell growth arrest and apoptosis (2, 4, 5). Recent data also indicate that apoptosis can be mediated by de novo synthesis of ceramide (2, 4). Ceramide is further metabolized by ceramidase to sphingosine, which also exerts diverse biological effects, including inhibition of protein kinase C and induction of apoptosis (1, 4, 6).

In contrast to the growth inhibitory and pro-apoptotic effects of ceramide and sphingosine, a further metabolite, S1P, has been implicated in cell growth (7) and inhibition of ceramide-mediated apoptosis (8–13). S1P is produced by phosphorylation of sphingosine catalyzed by sphingosine kinase (SPHK). SPHK is activated by a variety of stimuli, including PDGF, EGF (epidermal growth factor), NGF (nerve growth factor), vitamin D3, TNF-α, and cross-linking of FceRI and FcyRI (reviewed in Refs. 3 and 14). The relative cellular levels of sphingosine and S1P determined by SPHK are decisive for mast cell activation after FceRI triggering (15). It has also been suggested that the intracellular balance of ceramide and sphingosine versus S1P acts as an internal sensor that can regulate the decision of a cell to either undergo apoptosis or survive (8–10, 12). This has important clinical implications, including prevention of sterility after chemo- or radiation therapy, as increased S1P or decreased ceramide can prevent anti-cancer drug and radiation-induced oocyte loss, the event that drives premature ovarian failure and infertility in female cancer patients (11, 13).

Recently, S1P was identified as the ligand for a family of G protein-coupled receptors known as the endothelial differentiation gene-1 (EDG-1) family, now collectively named S1PRs (14, 16–18). To date, five members, EDG-1/S1P1, EDG-5/S1P3, EDG-3/S1P2, EDG-6/S1P1, and EDG-8/S1P2, have been identified (14, 16–18). All bind both S1P and sphinganine 1-phosphate (dihydro-S1P) with high specificity. These receptors can couple to different G proteins to elicit a wide array of cellular responses including vascular maturation and angiogenesis (19–22), motility (23, 24), and heart development (25).

There is abundant evidence that S1P can also function as a **This work was supported by Research Grant RO1 CA61774 from the National Institutes of Health (to S. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This advertisement must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Present address: Laboratory of Immunology, Division of Therapeutic Proteins, Food and Drug Administration, Bldg. 29A, 9000 Rockville Pike, Bethesda, MD 20892.

** To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biophysics, Medical College of Virginia Campus, Virginia Commonwealth University, Richmond, VA 23298-0614. Tel.: 804-828-9330; Fax: 804-828-8999; E-mail: spiegel@vcu.edu.

† The abbreviations used are: S1P, sphingosine 1-phosphate; AKAP, protein kinase A anchoring protein; BSA, bovine serum albumin; BrdUrd, bromodeoxyuridine; EDG, endothelial differentiation gene; ERK, extracellular signal-regulated kinase; GFP, green fluorescent protein; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; SKIP, SPHK-interacting protein; SPHK, sphingosine kinase; TNF, tumor necrosis factor; TRAF2, TNF receptor-associated factor 2; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; GST, glutathione S-transferase; HA, hemagglutinin.
second messenger important for regulation of calcium homeostasis (26–29) and suppression of apoptosis (8, 13, 30–32). However, the intracellular targets of S1P have not yet been identified, making this a controversial issue (18). Understanding of the complex interplay between intra- and extracellular actions of S1P is further complicated because endogenously produced S1P activates SPHK1 in an autocrine or paracrine manner, and this transactivation plays a critical role in PDGF-directed cell migration (23, 32). Conversely, binding of S1P to its receptors can activate SPHK and increase S1P levels (33).

The importance of S1P as a lipid mediator prompted the purification and cloning of SPHK1 (34, 35) and of SPHK2 (36). Overexpression of SPHK1 promotes cell survival and protects cells from apoptotic insults, such as serum withdrawal (31). Recently it has been suggested that spk1 may be an oncogene as its overexpression induces colony formation in soft agar and formation of tumors in NOD/SCID mice (37). In agreement, it has been shown that γ-radiation sensitivity of prostate cancer cells correlates with SPHK1 activity (38). Despite its central roles, little is known of how SPHK1 activity is regulated. In this study, we used the yeast two-hybrid screening system to identify proteins that interact with SPHK1 and potentially regulate its function. We cloned a SPHK-interacting protein (SKIP) that interacts with SPHK1 in vitro and in vivo to regulate its activity and biological functions.

MATERIALS AND METHODS

Cell Lines, Cell Culture, and Transfection—HEK 293 cells were cultured in DMEM (Biofluids) supplemented with 10% fetal bovine serum (FBS) and NIH 3T3 fibroblasts in DMEM supplemented with 10% calf serum. HEK 293 cells were seeded at 2 × 10^6 cells/well 1 day prior to transfection. DNA (5 μg) was complexed with calcium phosphate and added to cells for 14–18 h as described (39). Cells were then washed three times with phosphate-buffered saline (PBS, Biofluids). NIH 3T3 cells were transfected using LipofectAMINE Plus (Invitrogen) according to the manufacturer's instructions. Transfection efficiencies were typically 80 and 30% for HEK 293 and NIH 3T3 cells, respectively.

Cloning and in Vitro Transcription/Translation—Human SPHK1 was cloned by PCR into the EcoRI and BamHI cloning sites of pGBKTT7. The forward primer sequence was 5'-ACCTGGAATTTCCCCG-3', and the reverse primer sequence was 5'-CTCTAGAGGCTC-3'. PCR was carried out with the following conditions: 3 min at 94°C, 30 s at 94°C, 1 min at 52°C, 1.5 min at 72°C (30 cycles), 7 min at 72°C. In vitro transcription/translation was performed using rabbit reticulocyte lysates (Promega) according to the manufacturer's instructions. Translation efficiencies were typically 80 and 30% for HEK 293 and NIH 3T3 cells, respectively.

Two-hybrid Screening—Two-hybrid screening was performed using the Matchmaker II two-hybrid system (CLONTECH). The bait construct was transformed in yeast and plated on synthetic medium lacking tryptophan. One positive clone was amplified, mated with a pretransformed Matchmaker library generated from human brain in the yeast strain Y187, and plated on synthetic medium lacking tryptophan and leucine. Transformants were then scored for the ability to grow on medium lacking histidine and adenine and activation of the lacZ promoter. A total of 12 × 10^6 independent clones were screened. 181 clones scored positive for growth capability, and of these, 96 also scored positive for LacZ activation. The 96 positive clones were analyzed by frequent cutter restriction analysis, and the group was further reduced to 40.

The 40 independent clones were re-transformed and tested for transcriptional activation either with SPHK1 or with laminin, an unrelated protein. Five clones were then confirmed to be true positives.

Lysate Preparation and Sphingosine Kinase Assay—Cells were scraped and lysed by seven cycles of freeze-thawing in buffer A (0.2 M Tris, pH 7.4, 1 mM EDTA, 0.5 mM deoxyribonuclease, 15 mM NaF, 0.1 mM 2-mercaptoethanol, 10 μg/ml each leupeptin, aprotinin, and trypsin inhibitor, 40 μg/ml glycerol phosphate, 1 mM phenylmethylsulfonyl fluoride, 16% glycerol). Lysates were centrifuged at 100,000 × g for 40 min at 4°C and the pellet was resuspended in buffer A with Dounce homogenizer. Protein concentrations were measured using the Bradford microassay (Bio-Rad). Cytosolic and membrane-associated SPHK activity was determined in the presence of 50 μM sphingosine, dissolved in 5% Triton X-100 (final concentration 0.25%), and [32P]ATP (10 μCi, 1 mM) containing MgCl2 (10 mM) as described previously (35) and expressed as pmol of S1P formed per min per mg of protein.

Western Blotting and Immunoprecipitation—For Western blot analysis, cytosolic fractions (10–25 μg) were separated on 12% SDS-PAGE, blotted onto nitrocellulose membranes (Bio-Rad), probed with antibodies, and immunocomplexes detected by enhanced chemiluminescence (ECL). The antibodies used were: anti-phosphotyrosine clone 4G10 (Upstate Biotechnology), anti-ERK1/2 (New England Biolabs), anti-ERK1 (Santa Cruz Biotechnology), anti-c-Myc (Zymed Laboratories Inc.), anti-HA clone 3F10 (Roche Molecular Biochemicals), anti-β-tubulin (Roche Molecular Biochemicals), and anti-c-Src (Upstate Biotechnology, Lake Placid, NY).

Immunoprecipitation of in vitro translated proteins was performed by diluting reactions in 500 μl of PBS and incubating overnight at 4°C. Transformants were then scored for the ability to grow on medium lacking histidine and adenine and activation of the lacZ promoter either with SPHK1 or with laminin, an unrelated protein. We cloned a SPHK-interacting protein (SKIP) that interacts with SPHK1 in vitro and in vivo to regulate its activity and biological functions.

RESULTS

Cloning and Characterization of SKIP—SPHK activity and formation of S1P have been shown to be rapidly and transiently increased by many stimuli (3, 14), yet little evidence has surfaced to suggest this is due to posttranslational modifications. Thus, the yeast two-hybrid approach was employed to identify potential SPHK1-interacting proteins that might regulate its activity in vivo. Human SPHK1 fused to the DNA-binding domain of GAL4 was used as bait, and the prey consisted of a human brain DNA library fused to the transcriptional activation domain of GAL4. The yeast two-hybrid system that we used mitigates against false positives by having three different promoter-reporter gene constructs, each with differing affinities for the GAL4 DNA-binding domain. Prior to screening, we verified that the prey construct did not act on its own by binding regions around the GAL4 DNA binding site or to spec-
specific TATA boxes. After putative SPHK1 interactors were pulled out, they were tested for duplicate clones and then transformed back into yeast and re-screened, to ensure that the activation was due to the prey plasmid and not mutations in the yeast. These plasmids were also transformed into yeast containing a control protein-DNA binding domain fusion, to remove prey that did not require interaction with the SPHK1 bait to activate transcription. Using the most stringent interaction tests, five cDNA clones were considered to be SPKH1-specific two-hybrid interactors. Three of these clones contained 3-kb cDNAs with identical sequences, and the corresponding encoded protein (Fig. 1A) was named SKIP (sphingosine kinase-interacting protein).

Northern blot analysis using labeled SKIP cDNA as a probe revealed a single cDNA species of about 7 kb, whose expression was restricted to spleen, ovary, brain, and heart, with highest expression in the heart (Fig. 1B). BLAST analysis revealed that SKIP was 99% identical to the C-terminal region of a larger cDNA (KIAA1678) previously identified from a brain cDNA library (42), encoding for a protein of about 140 kDa. Surprisingly, as shown in the ClustalW alignment (Fig. 2), there was strong sequence homology with the C-terminal region of protein kinase A anchoring proteins (AKAPs), with 75–80% amino acid similarity and 35–40% identity between SKIP and AKAP110 and human and rat AKAP220. AKAP110 is a testis-specific protein that may participate in PKA-mediated sperm functions (43). Rat AKAP220 is a peroxisomal anchor protein expressed in the cytosol (Fig. 4A) and Ref. 31), it was of interest to examine the effect of their co-expression on cellular localization. To this end, NIH 3T3 fibroblasts were transiently transfected with either HA-tagged SKIP, AU1-tagged SPHK1, or both and analyzed by confocal microscopy after staining with the appropriate antibodies. SPHK1 is mainly cytoplasmic, in agreement with previous reports (31, 45), serum stimulated SPHK1 activity. SKIP transfection markedly reduced SPHK1 activity in untreated as well as in FBS stimulated cells (Fig. 4A). The same cell lysates were also analyzed by Western blotting with anti-Myc antibody, to identify SPHK1, or with anti-HA antibody, to identify SKIP. Both proteins were readily detected in the cell lysates, and their levels were not affected by the co-transfections (Fig. 4B). Furthermore, when HEK 293 cells were transfected with SPHK1, in agreement with previous studies (31, 35), 80% of the SPHK1 activity was cytosolic and 20% was membrane associated. Co-transfection with SKIP significantly decreased SPHK1 activity in both fractions. The activity in the cytosol was reduced 2-fold, while the membrane-associated activity was reduced by almost 5-fold, suggesting that SKIP also affected SPHK1 distribution between the cytosolic and particulate fractions (Fig. 4C).

**Localization of SKIP and SPHK1**—As SKIP contains several putative nuclear localization motifs, and SPHK1 is mainly expressed in the cytosol (Fig. 4C and Ref. 31), it was of interest to examine the effect of their co-expression on cellular localization. To this end, NIH 3T3 fibroblasts were transiently transfected with either HA-tagged SKIP, AU1-tagged SPHK1, or both and analyzed by confocal microscopy after staining with the appropriate antibodies. SPHK1 is mainly cytoplasmic, in agreement with previous results (31). SKIP appears to have a cytoplasmic expression pattern (Fig. 5) as well despite the presence of a putative nuclear targeting sequence and signal peptide determined by PSORTII (psort.nibb.ac.jp), although occasionally, nuclear envelope localization could be observed (Fig. 5A). When the proteins were co-expressed, there was clear co-localization, and no change was observed in the localization of SPHK1 (Fig. 5, B and C). Overexpression of SKIP, particularly in the presence of SPHK1, appeared to cause the cells to be more rounded than cells expressing only one of the constructs (Fig. 5C).

**Effect of SKIP Overexpression on Sphingosine Kinase Activity**—To assess whether SKIP overexpression has a functional effect on SPHK1 activity, Myc-SPHK1 stably transfected HEK 293 cells were transfected either with HA-tagged SKIP or with empty vector. 12 h after transfection, cells were serum starved for 24 h and then stimulated with 10% FBS for 10 min. In agreement with previous reports (31, 45), serum stimulated SPHK1 activity. SKIP transfection markedly reduced SPHK1 activity in untreated as well as in FBS stimulated cells (Fig. 4A). The same cell lysates were also analyzed by Western blotting with anti-Myc antibody, to identify SPHK1, or with anti-HA antibody, to identify SKIP. Both proteins were readily detected in the cell lysates, and their levels were not affected by the co-transfections (Fig. 4B). Furthermore, when HEK 293 cells were transfected with SPHK1, in agreement with previous studies (31, 35), 80% of the SPHK1 activity was cytosolic and 20% was membrane associated. Co-transfection with SKIP significantly decreased SPHK1 activity in both fractions. The activity in the cytosol was reduced 2-fold, while the membrane-associated activity was reduced by almost 5-fold, suggesting that SKIP also affected SPHK1 distribution between the cytosolic and particulate fractions (Fig. 4C).
SKIP Overexpression Blocks the Biological Effects of SPHK1—Previously, it was found that SPHK1-overexpressing cells proliferate faster than vector-transfected cells and have an increased proportion of cells in the S phase of the cell cycle (31). Because SKIP expression markedly reduced SPHK1 activity, we examined whether this also affects the growth-promoting ability of SPHK1. To this end, NIH 3T3 fibroblasts stably expressing SPHK1 or empty vector were transfected with SKIP together with GFP to visualize transfected cells. After 8 h of serum starvation, cells were stimulated overnight with serum and analyzed for their capability to incorporate BrdUrd into nascent DNA. In agreement with previous studies (31, 37, 46), after stimulation with serum, NIH 3T3 cells overexpressing SPHK1 had a higher proportion of cells in the S phase of the cell cycle than the vector transfectants (Fig. 6). However, overexpression of SKIP reduced the incorporation of BrdUrd in NIH 3T3-SPHK1 cells to levels comparable with NIH 3T3-vector cells, indicating that SKIP abolished the ability of SPHK1 to promote proliferation. Moreover, SKIP transfection also inhibited serum-induced proliferation of vector cells, albeit to a lesser extent.

Overexpression of SPHK1 has been shown to specifically protect cells from apoptosis induced by serum deprivation, but not from cell death induced by staurosporin, a protein kinase inhibitor (30, 31). Thus it was relevant to also assess whether SKIP inhibits the cytoprotective effect of SPHK1. As expected (31), NIH 3T3 cells overexpressing SPHK1 had a higher proportion of cells in the S phase of the cell cycle than the vector transfectants (Fig. 6A). However, overexpression of SKIP reduced the incorporation of BrdUrd in NIH 3T3-SPHK1 cells to levels comparable with NIH 3T3-vector cells, indicating that SKIP abolished the ability of SPHK1 to promote proliferation. Moreover, SKIP transfection also inhibited serum-induced proliferation of vector cells, albeit to a lesser extent.

Overexpression of SPHK1 has been shown to specifically protect cells from apoptosis induced by serum deprivation, but not from cell death induced by staurosporin, a protein kinase inhibitor (30, 31). Thus it was relevant to also assess whether SKIP inhibits the cytoprotective effect of SPHK1. As expected (31), NIH 3T3 cells overexpressing SPHK1 had a higher proportion of cells in the S phase of the cell cycle than the vector transfectants (Fig. 6A). However, overexpression of SKIP reduced the incorporation of BrdUrd in NIH 3T3-SPHK1 cells to levels comparable with NIH 3T3-vector cells, indicating that SKIP abolished the ability of SPHK1 to promote proliferation. Moreover, SKIP transfection also inhibited serum-induced proliferation of vector cells, albeit to a lesser extent.

FIG. 2. SKIP has a high degree of similarity to AKAPs. Sequence alignments of SKIP and the C terminus of the indicated AKAP family members were made with ClustalW. Dark shaded boxes represent identical residues, while light shaded boxes represent similar residues. There is 35–40% identity and 70–75% similarity between SKIP and the C-terminal region of AKAPs.

FIG. 3. In vitro interaction between SKIP and SPHK1. A, 20 µl of in vitro translated [3H]leucine-labeled SKIP was incubated either with GST-agarose beads or GST-SPHK1 bound to glutathione-conjugated agarose beads. After overnight incubation at 4 °C, beads were washed and bound proteins separated by SDS-PAGE. GST-SPHK1 precipitated 21-kDa radiolabeled SKIP. In lane 1, 5 µl of total in vitro reaction were loaded for control. B, in vitro translated [3H]leucine-labeled SKIP was incubated in the absence (lane 1) or presence of in vitro translated SPHK1 with an N-terminal AU1 tag (lane 2). The samples were immunoprecipitated with anti-AU1 antibodies (Covance) overnight at 4 °C, washed, and resolved by SDS-PAGE.

SKIP Overexpression Blocks the Biological Effects of SPHK1—Previously, it was found that SPHK1-overexpressing cells proliferate faster than vector-transfected cells and have an increased proportion of cells in the S phase of the cell cycle (31). Because SKIP expression markedly reduced SPHK1 activity, we examined whether this also affects the growth-promoting ability of SPHK1. To this end, NIH 3T3 fibroblasts stably expressing SPHK1 or empty vector were transfected with SKIP together with GFP to visualize transfected cells. After 8 h of serum starvation, cells were stimulated overnight with serum and analyzed for their capability to incorporate BrdUrd into nascent DNA. In agreement with previous studies (31, 37, 46), after stimulation with serum, NIH 3T3 cells overexpressing SPHK1 had a higher proportion of cells in the S phase of the cell cycle than the vector transfectants (Fig. 6A). However, overexpression of SKIP reduced the incorporation of BrdUrd in NIH 3T3-SPHK1 cells to levels comparable with NIH 3T3-vector cells, indicating that SKIP abolished the ability of SPHK1 to promote proliferation. Moreover, SKIP transfection also inhibited serum-induced proliferation of vector cells, albeit to a lesser extent.
data suggest that SKIP blocked the ability of SPHK1 to protect from cell death.

**SKIP Overexpression Reduces Prolonged Activation of ERK**—The mitogen-activated protein kinase ERK is known to play an important role in cell growth and survival (47). In agreement with many previous studies (reviewed in Ref. 47), serum transiently stimulated ERK1/2, reaching a maximum within 10 min. In the same lysates were resolved by SDS-PAGE and analyzed by Western blot with anti-Myc, anti-HA and anti-β-tubulin as a loading control. C, HEK 293 cells were transiently transfected with SPHK1 plus empty vector or SKIP by the calcium phosphate method. 24 h after transfection, cells were lysed, and SPHK1 activity was assessed in both cytosolic (open bars) and crude membrane fractions (filled bars). Despite its importance in regulating sphingolipid metabolite levels, and its potential roles in cell growth and survival, there are only a few studies on its regulation. Previously, it was shown that acidic phospholipids activate SPHK1 in vitro (49). In agreement, aggregation of the high-affinity receptor FcγRI in monocytes resulted in activation of phospholipase D by tyrosine kinases, leading to activation of SPHK, which in turn resulted in transient release of stored calcium (50). However, no phospholipid binding motifs are present in SPHK1 or SPHK2. Initially, protein kinase C (PKC) was thought to be involved in SPHK activation (51). However, more recently, using tyrosine phosphorylation site mutants of PDGFR, it was shown that although the tyrosine residue responsible for binding of phospholipase Cγ is required for PDGF-induced activation of SPHK1, calcium mobilization downstream of phospholipase Cγ, but not PKC activation, seems to be required for activation of SPHK by PDGF (52). SPHK1 is rapidly activated in many cell types by a variety of stimuli (reviewed in Refs. 14 and 53), but almost nothing is known of the molecular mechanisms involved. Using the yeast two-hybrid system, we have now identified SKIP as a SPHK1-interacting protein that regulates its activity both in vitro and in situ. Several lines of evidence indicate that SKIP is a bona fide SPHK1 interactor, including specific SPHK1-GST pull-down assays and co-immunoprecipitation experiments. Moreover, transfection of SKIP reduced SPHK1 activity without affecting its expression and abolished its biological effects, namely the ability to protect cells from apoptosis induced by serum withdrawal and enhanced cell proliferation. Interestingly, SKIP also suppressed ERK activation induced by serum or by SPHK1 overexpression, implicating ERK activation in signaling pathways downstream of S1P.

**DISCUSSION**

SPHK1 belongs to a family of enzymes expressed in a wide variety of organisms from plants through higher mammals (48). Despite its high sequence homology to several
members of the AKAP family. AKAPs were identified on the basis of their interaction with PKA, and their major role is to function as scaffolds and anchors to a variety of subcellular structures for its regulatory subunit (54, 55). Since PKA has pleiotropic functions in many aspects of signaling, specificity is achieved through its targeting to the precise site of action. However, recent structural and functional analysis of AKAPs indicate a more complex role for these proteins in signal transduction. AKAPs also bind to a variety of other important signaling kinases and phosphatases, including PKC, PP1, PP2A, and Abl (56–58). For example, the AKAP Yotiao directly binds to the N-methyl-D-aspartic acid receptor and targets both PKA and PP1 to the receptor, bringing together signaling enzymes with a potential substrate (58). The similarity between the AKAP family and SKIP suggests that it might also act as a SPHK1 scaffolding or anchoring protein. For example, SPHK1 distribution within cells is mainly cytoplasmic (31), although its substrate sphingosine is membrane-associated. SKIP might participate in tethering SPHK1 to membranes in the proximity of sphingosine and thereby promoting its activity. Recent studies have shown that following PDGF stimulation, SPHK1 shows a late phase of activation and nuclear localization, concomitantly with the initiation of DNA synthesis (41), and SKIP might be involved in recruiting SPHK1 to the nucleus.

A novel mechanism of SPHK activation by TNF-α was recently described (59). This study revealed an association of SPHK with TNF receptor-associated factor 2 (TRAF2) one of the major mediators of TNF actions. This interaction between SPHK and TRAF2 was important for TNF-induced activation of NF-kB and prevention of apoptosis (59). However, there is little sequence homology or similarity between TRAF2 and SKIP, raising the intriguing possibility that there are different families of SPHK-interacting proteins that play different roles in its regulation.

Acknowledgments—We thank Debyani Chakravarty and Samantha Poulton for technical assistance and Francis Flomerfelt for kindly providing the Matchmaker III yeast two-hybrid system and brain library.

REFERENCES
1. Merrill, A. H., Jr., Schmelz, E.-M., Dillehay, D. L., Spiegel, S., Shayman, J. A., Schroeder, J. D., Riley, R. T., Voss, K. A., and Wang, E. (1997) Tissue Cell.

Fig. 6. The effect of SKIP on SPHK1-enhanced cell growth. NIH 3T3 cells stably expressing Myc-SPHK1 or Myc vector were transiently transfected with either SKIP or empty vector, together with pCEFl-GFP. Cells were then serum-starved and cultured without (open bars) or with 10% calf serum (filled bars). After 16 h, BrdUrd was added for an additional 3 h. Double immunofluorescence was used to visualize transfected cells and BrdUrd incorporation, and the proportion of cells incorporating BrdUrd among total transfected cells (expressing GFP) was determined. Data are means ± S.D. of duplicate cultures from a representative experiment. At least three different fields were scored with a minimum of 100 cells scored per field. Similar results were obtained in two independent experiments.

Fig. 7. SKIP abolishes the cytoprotective effect of SPHK1 on serum withdrawal. NIH 3T3 cells stably transfected with Myc-SPHK1 or vector were transiently transfected with either SKIP or empty vector with pCEFl-GFP and serum-starved (empty bars) or cultured in the presence of 10% serum (solid bars) for 24 h. Total GFP-expressing cells and GFP-expressing cells displaying fragmented nuclei indicative of apoptosis were counted as described under “Materials and Methods.” Data are means ± S.D. Three independent wells were counted for each treatment, with a minimum of 100 cells scored per well. Data are representative of three independent experiments.

Fig. 8. Effects of SKIP expression on ERK activation. A, prolonged ERK activation is abolished when SKIP is overexpressed. HEK 293 cells stably transfected with Myc-SPHK1 or empty vector were transiently transfected with SKIP or vector. After serum starvation, cells were treated without or with 10% FBS for the indicated minutes. Cell lysates were separated by SDS-PAGE and immunoblotted with an antibody that recognizes the phospho-tyrosine, active form of pp42/44 ERK. Blots were then stripped and re-probed with an antibody against total ERK to show loading. B, SKIP overexpression does not affect tyrosine phosphorylation. Lysates from cells treated as in A were immunoblotted with anti-phosphotyrosine antibody. The arrows on the left indicate proteins whose phosphorylation level increased following serum addition. Blots were then stripped and re-probed with anti-Src antibodies to demonstrate equal loading.
