Sphingosine Kinase Type 1 Induces $G_{12/13}$-mediated Stress Fiber Formation, yet Promotes Growth and Survival Independent of G Protein-coupled Receptors*

Ana Olivera‡, Hans M. Rosenfeldt§, Meryem Bektas§¶, Fang Wang§, Isao Ishii‖, Jerold Chun‡‡, Sheldon Milstien§§, and Sarah Spiegel§¶¶**

*From the ‡Molecular Immunology and Inflammation Branch, NIAMS, National Institutes of Health, and the §§Laboratory of Cellular and Molecular Regulation, National Institute of Mental Health, Bethesda, Maryland 20892, the §Department of Biochemistry, Virginia Commonwealth University School of Medicine, Richmond, Virginia 23298-0614, the ‖Department of Molecular Genetics, National Institute of Neuroscience, Tokyo 187-8502, Japan, and the **Department of Molecular Biology, Scripps Research Institute, La Jolla, California 92037*

Sphingosine 1-phosphate (S1P) is the ligand for a family of specific G protein-coupled receptors (GPCRs) that regulate a wide variety of important cellular functions, including growth, survival, cytoskeletal rearrangements, and cell motility. However, whether it also has an intracellular function is still a matter of great debate. Overexpression of sphingosine kinase type 1, which generated S1P, induced extensive stress fibers and impaired formation of the Src-focal adhesion kinase signaling complex, with consequent aberrant focal adhesion turnover, leading to inhibition of cell locomotion. We have dissected biological responses dependent on intracellular S1P from those that are receptor-mediated by specifically blocking signaling of $G\alpha_q$, $G\alpha_i$, $G\alpha_{12/13}$, and $G\beta\gamma$ subunits, the G proteins that S1P receptors (S1PRs) couple to and signal through. We found that intracellular S1P signaled "inside out" through its cell-surface receptors linked to $G_{12/13}$-mediated stress fiber formation, important for cell motility. Remarkably, cell growth stimulation and suppression of apoptosis by endogenous S1P were independent of GPCRs and inside-out signaling. Using fibroblasts from embryonic mice devoid of functional S1PRs, we also demonstrated that, in contrast to exogenous S1P, intracellular S1P formed by overexpression of sphingosine kinase type 1 promoted growth and survival independent of its GPCRs. Hence, exogenous and intracellularly generated S1Ps affect cell growth and survival by divergent pathways. Our results demonstrate a receptor-independent intracellular function of S1P, reminiscent of its action in yeast cells that lack S1PRs.

Sphingosine 1-phosphate (S1P),[1] a sphingolipid metabolite found in organisms as diverse as plants, yeast, worms, flies, and mammals, has been linked to a wide spectrum of biological processes, among which cell growth, survival, and motility are prominent (1, 2). S1P is formed by sphingosine kinase (SphK), a highly conserved enzyme that is activated by numerous stimuli (1, 3).

The most well known actions of S1P are mediated by binding to a family of specific G protein-coupled receptors (GPCRs). To date, five members, EDG-1/S1P$_1$, EDG-5/S1P$_2$, EDG-3/S1P$_3$, EDG-6/S1P$_4$, and EDG-8/S1P$_5$, have been identified (1, 2, 4, 5). S1P receptors (S1PRs) are differentially expressed; coupled to a variety of G proteins; and regulate angiogenesis, vascular maturation, cardiac development, neuronal survival, and immunity (1, 2, 4). In particular, S1PRs have been shown to play critical roles in cell migration (6–10). Activation of S1P$_1$ or S1P$_3$ by S1P in many cell types increases directional or chemotactic migration (6, 8, 10–12), whereas binding to S1P$_2$ abolishes chemotaxis and membrane ruffling (13).

Downstream of heterotrimeric G proteins, the S1PRs regulate tyrosine kinases such as focal adhesion kinase (FAK) and Src, which reside in focal adhesions, and the small GTPases of the Rho family that are important for cytoskeletal rearrangements (14). Whereas binding of S1P to S1P$_1$ mediates cortical actin assembly and Rac activation (8, 15), binding to S1P$_2$ and S1P$_3$ induces stress fiber formation and activation of Rho, and S1P$_2$ negatively regulates Rac activity (13), thereby inhibiting cell migration. In contrast, increasing intracellular levels of S1P in human breast cancer cells inhibits cell motility, leading us to suggest a possible role for intracellular S1P in inhibiting cell motility independent of its receptors (16). Moreover, other studies further support the notion that S1P also has second messenger functions important for calcium homeostasis (17, 18), cell growth (19–21), and suppression of apoptosis (22–24). In addition, expression of SphK1 in NIH 3T3 fibroblasts elevates intracellular levels of S1P, expedites the G$_1$/S transition, and protects against apoptosis (21) and enhances tumor formation in mice (25, 26). Because the involvement of S1PRs in these responses has not been conclusively ruled out and because intracellular targets of S1P have not yet been identified, whether S1P has direct intracellular effects remains controversial. Dissection of the intra- and extracellular actions of S1P is further complicated by the observation that binding of S1P to its receptors can stimulate SphK and generation of intracellular S1P (27). Conversely, binding of the platelet-derived growth factor (PDGF) to the PDGF receptor (PDGFR) activates and

[1] The abbreviations used are: S1P, sphingosine 1-phosphate; SphK, sphingosine kinase; GPCR, G protein-coupled receptor; EDG, endothelial differentiation gene; S1PR, sphingosine 1-phosphate receptor; FAK, focal adhesion kinase; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; MEFs, mouse embryonic fibroblasts; BSA, bovine serum albumin; PBS, phosphate-buffered saline; ERK, extracellular signal-regulated kinase; GFP, green fluorescent protein; BrdUrd, bromodeoxyuridine; MAPK, mitogen-activated protein kinase; SAPK1, stress-activated protein kinase-1; JNK, c-Jun N-terminal kinase; PDZ-RhoGEF, PDZ domain-containing Rho guanine exchange factor; GRK2, G protein-coupled receptor kinase-2; βARK, β-adrenergic receptor kinase; CDK2, cyclin-dependent kinase-2.

recruits SphK1 to the cell's leading edge (28), producing S1P, which spatially and temporally stimulates $S1P_1$ in an autocrine or paracrine manner (29) that results in activation and integration of downstream signals essential for cell locomotion (28, 29). Moreover, tethering of the PDGFR with $S1P_1$ may provide a platform for integrative signaling by these two types of receptors (30). On the other hand, S1P can specifically be transported into cells by the cystic fibrosis transmembrane regulator, a member of the ATP-binding cassette transporter family (31), which could serve to terminate signaling through the S1PRs (31) or initiate a second wave of signals acting inside the cells, making the S1P second messenger concept even more tenuous.

In this study, we examined the effects of overexpression of SphK1 and elevated intracellular S1P while simultaneously blocking signaling of the heterotrimeric G proteins that S1PRs couple to and signal through, to dissect biological functions dependent on intracellular S1P from those mediated by S1PRs. Whereas SphK1 overexpression stimulated "inside-out" signaling leading to cytoskeleton rearrangement and stress fiber formation mediated by a S1PR coupled to $G\alpha_{12/13}$, SphK1-promoted growth and survival were independent of S1PR signaling. Our results clearly demonstrate a receptor-independent intracellular function of S1P, reminiscent of its action in yeast and plants that lack S1PRs.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections—NIH 3T3 fibroblasts (American Type Culture Collection CRL-1658) stably transfected with pcDNA3-SphK1 or empty vector were cultured as described previously (32). For transient transfections, NIH 3T3 fibroblasts were plated on collagen-coated dishes or coverslips and, after 16 h, transfected using LipofectAMINE Plus (Invitrogen) according to the manufacturer's instructions. Transfection efficiencies were 30–40%.

Mouse embryonic fibroblasts (MEFs) were derived from embryonic day 14 embryos generated by wild-type or double knockout intercrosses of C57BL/6N mice as described previously (33). MEFs were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics. Only cells from passages 2 to 4 were used for experiments.

Chemotactic Motility—Boyden chamber chemotaxis assays were carried out exactly as described previously (28, 34).

Adhesion Assay—Six-well plates were coated with collagen I (0.1 mg/ml), fibronectin (0.5 mg/ml), polylysine (0.1 mg/ml), or Matrigel (1:10 dilution) and then incubated with 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 30 min to block nonspecific binding sites, followed by extensive PBS washes. NIH 3T3 fibroblasts, harvested by scraping in PBS and 10 mM EDTA, were resuspended in Dulbecco's modified Eagle's medium and 3% BSA at 1×10^5 cells/ml. 2×10^5 cells were added to each well and then incubated at 37 °C for the indicated times. Nonadherent cells were removed, and attached cells were fixed in 70% ethanol for 20 min and stained with crystal violet. Incorporated dye was dissolved in 0.1 M sodium citrate in 50% ethanol (pH 4.2), and the absorbance was measured at 540 nm (34).

Western Blotting and Immunoprecipitation—Cells were plated in 100-mm dishes coated with 50 μg/ml collagen at 1.4×10^6 cells/dish. After the indicated treatments, cells were lysed for 10 min in buffer A (50 mM HEPES (pH 7.4), 1% Triton X-100, 150 mM NaCl, 1.5 mM MgCl$_2$, 1 mM EDTA, 2 mM orthovanadate, 4 mM sodium pyrophosphate, 100 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) and scraped off the plates. After centrifugation of the cell lysates for 15 min at $10,000 \times g$, equal amounts of the Triton X-100-insoluble and -soluble fractions were separated by 7 and 10% SDS-PAGE, respectively, and then transblotted onto nitrocellulose. Antibodies to paxillin and FAK (BD Transduction Laboratories, Lexington, KY); pan-c-Src (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); phospho-Try[418] Src, phospho-Tyr[577] FAK, and phospho-Tyr[397] FAK (BIOSOURCE); phospho-p38, p38, and phospho-ERK1/2 (New England Biolabs Inc); and PDGFR, vinculin, and phosphotyrosine (monoclonal antibody 4G10, Upstate Biotechnology, Inc., Lake Placid, NY) were used as primary antibodies. Immunocomplexes were visualized by enhanced chemiluminescence as described (28). The blots shown are representative of at least three independent experiments. Where indicated, quantification of immunocomplexes was performed by den-

sitometric scanning of the bands and integration with NIH Image software. Blots were further edited with Adobe PhotoShop Version 5.5 and/or Microsoft PowerPoint 2001 for Macintosh.

For immunoprecipitation studies, cells were lysed in buffer A containing 0.5% deoxycholate and 0.1% SDS. 400 μg of the clarified lysates were incubated with 1–2 μg of anti-paxillin or anti-pan-Src antibodies at 4 °C overnight and then with protein A/G-Sepharose beads (Santa Cruz Biotechnology, Inc.) for an additional 1 h to capture immunocomplexes. After pelleting and washing by brief spins at $10,000 \times g$, the beads were resuspended in 2× sample buffer, and proteins were resolved by SDS-PAGE.

Immunostaining—Cells grown on glass coverslips coated with collagen I (50 μg/ml) were incubated overnight in Dulbecco's modified Eagle's medium supplemented with 2 μg/ml transferrin and 20 μg/ml BSA. After treatment, cells were washed with PBS, fixed in 1.8% formalin and 0.1% Triton X-100 for 30 min, and then permeabilized with 0.5% Triton X-100 for 10 min. Actin filaments were visualized with Alexa 488-conjugated phalloidin (Molecular Probes, Inc., Eugene, OR), and focal complexes were visualized with antibody to paxillin, followed by staining with Texas Red-conjugated secondary antibody. After washing three times with PBS, coverslips were mounted on slides using an anti-fade kit (Molecular Probes, Inc.), and >80 cells were examined with a confocal laser scanning microscope (Olympus Fluoview). All experiments were repeated at least three times.

Rho Activation—Cytosolic extracts were incubated with freshly prepared glutathione *S*-transferase-rhotekin fusion protein bound to glutathione-agarose beads for 30 min at 4 °C. The bound proteins were separated by 15% SDS-PAGE, transferred to nitrocellulose, and blotted with anti-Rho antibody (Santa Cruz Biotechnology, Inc.). GTP-Rho was quantified using NIH Image software and normalized with total cellular Rho.

Incorporation of Bromodeoxyuridine—Cells were plated on collagen-coated 5-cm^2 coverslips at 2×10^5 cells and transfected the next day with the various constructs at a 5:1 ratio with green fluorescent protein (GFP). 24 h after transfection, NIH 3T3 cells were serum-starved in Dulbecco's modified Eagle's medium supplemented with 2 μg/ml transferrin and 20 μg/ml BSA for 8 h and then stimulated with various agents. Cells were incubated for 3 h with bromodeoxyuridine (BrdUrd; 10 μM) and fixed in 4% paraformaldehyde containing 5% sucrose (pH 7.0) for 20 min at room temperature. Nuclei incorporating BrdUrd were stained exactly as described (21). Coverslips were mounted on slides, and cells expressing GFP and cells with positive BrdUrd staining were counted using a Zeiss fluorescence microscope. At least 400 cells were scored per point, which included at least four different randomly chosen fields.

Staining of Apoptotic Nuclei—Apoptosis was assessed by staining cells with 8 μg/ml Hoechst dye in 30% glycerol and PBS for 10 min at room temperature as described previously (21). Cells expressing GFP were examined with an inverted fluorescence microscope, and apoptotic cells were distinguished by condensed fragmented nuclear regions. The percentage of intact and apoptotic nuclei in cells expressing GFP fluorescence was determined. A minimum of 500 cells were scored in a double-blind manner.

DNA Synthesis—[³H]Thymidine incorporation into DNA was measured as described (21). Values are the means of triplicate determinations, and S.D. values were routinely <10% of the mean.

RESULTS

SphK Inhibits Cell Motility toward PDGF and Serum without Affecting Cell Adhesion or PDGFR Signaling through the MAPK Family—We have recently shown that PDGF-induced chemotaxis requires SphK stimulation, S1P formation, and consequent transactivation of $S1P_1$ (28, 29). In contrast, SphK1 overexpression and concomitant increased S1P levels inhibit the motility of MCF-7 and MDA-MB-231 human breast cancer cells, which do not express $S1P_1$ (16). The inside-out signaling by S1P, whereby its intracellular generation can lead to activation of S1PRs (29, 35), prompted us to reinvestigate the roles of S1PRs in PDGF-induced chemotaxis. Similar to human breast cancer cells (16), overexpression of SphK1 in NIH 3T3 fibroblasts, which do express $S1P_1$, markedly reduced chemotactic responses toward PDGF and serum (Fig. 1*A*). It is possible that this could result from altered adhesion to the collagen I matrix, which coated the filters utilized in the Boyden chamber assay. However, overexpression of SphK1 had no sig-
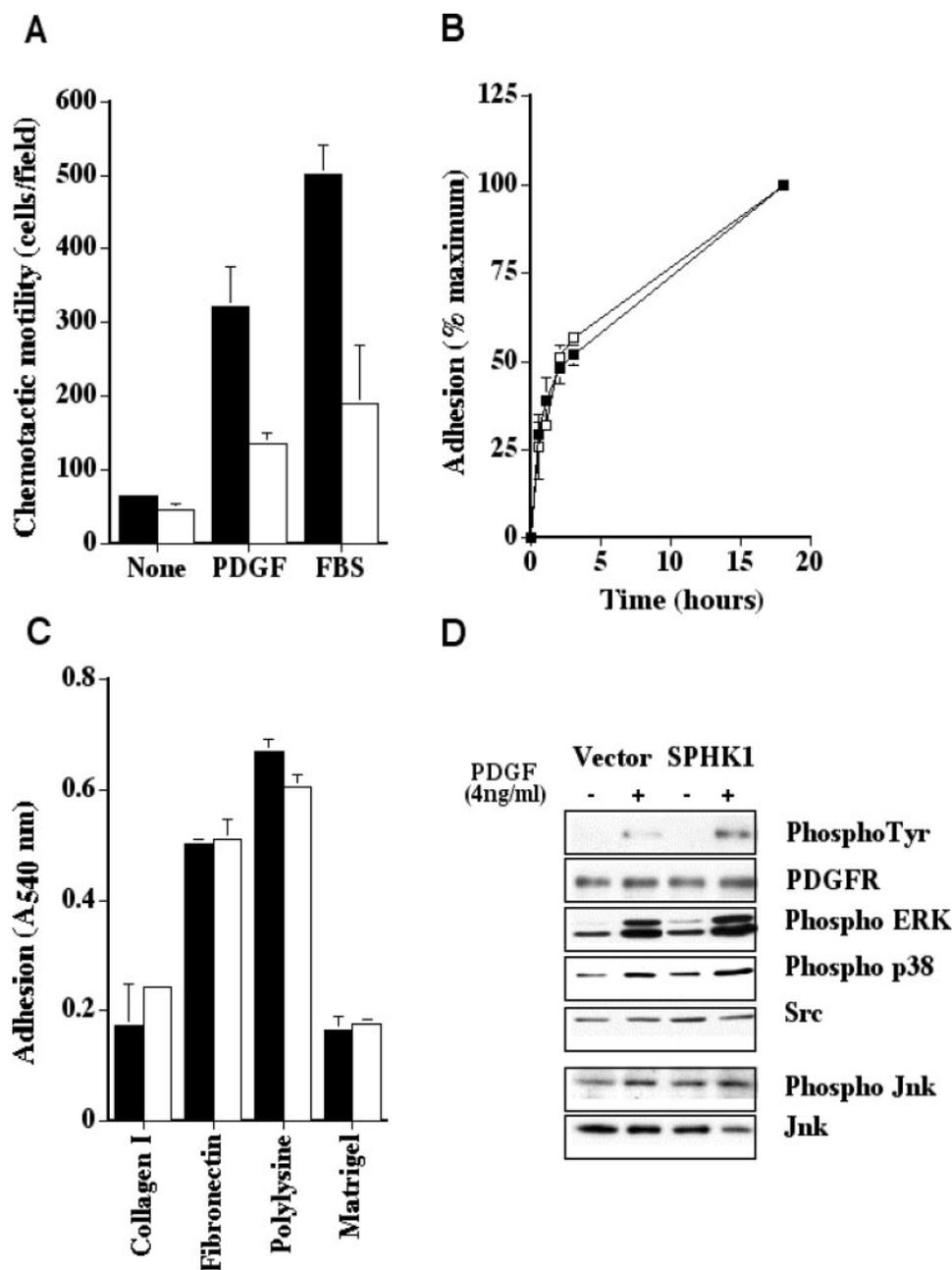
FIG. 1. **Expression of SphK1 inhibits chemotactic motility of NIH 3T3 fibroblasts without affecting adhesion or PDGF-induced MAPK signaling.** *A*, NIH 3T3 cells stably transfected with empty vector (*black bars*) or SphK1 (*white bars*) were allowed to migrate toward PDGF-BB (20 ng/ml) or 10% fetal bovine serum (*FBS*), and chemokinesis (*None*) and chemotaxis were measured after 24 h. Data are means ± S.D. Each determination is the average of three random microscope fields. *B* and *C*, attachment of cells to collagen I-coated plates or to fibronectin-, polylysine-, and Matrigel-coated plates, respectively, was determined as described under "Experimental Procedures." *D*, shown is the effect of SphK1 on PDGF-induced tyrosine phosphorylation of the PDGFR and activation of ERK, p38, and JNK. Cells stably transfected with vector or SphK1 were plated on collagen-coated dishes and treated without or with PDGF-BB (4 ng/ml) for 5 min. Cell lysates were immunoprecipitated with anti-PDGFR antibody, and the immunoprecipitates were analyzed by Western blotting using anti-phosphotyrosine or anti-PDGFR antibody. Cell lysates (20 µg) were also analyzed by Western blotting using phospho-specific anti-ERK1/2, anti-p38, and anti-JNK antibodies. Blots were stripped and reprobed with anti-Src or anti-JNK antibody to show equal loading.

nificant effects on the adhesiveness of cells to collagen (Fig. 1, *B* and *C*) or even to fibronectin, Matrigel, or polylysine (Fig. 1*C*). Cellular responses induced by PDGF, including chemotaxis, are initiated by activation and tyrosine phosphorylation of PDGFR, and this also was not affected by SphK1 overexpression (Fig. 1*D*). Moreover, activation of MAPK family members, in particular ERK1/2 and p38, which have been implicated in PDGF-mediated chemotaxis (36), was not reduced by overexpression of SphK1, whereas SAPK1/JNK was not stimulated by PDGF (Fig. 1*D*). These results suggest that the decreased motility is not due to a general impairment of PDGFR signaling.

Expression of SphK1 Enhances Formation of Stress Fibers and Focal Adhesions—We next examined by histochemistry whether SphK1 influences the architecture of the actin cytoskeleton by phalloidin staining of actin filaments and using antibodies to focal adhesion components. In agreement with other studies (14, 37, 38), in the absence of PDGF, vector-transfected cells had few thin actin filaments in the cell body, and only sparse small focal points at the cell periphery were detected with antibody against paxillin (Fig. 2*C*) or vinculin (data not shown). In contrast, even in the absence of PDGF, 3T3 cells expressing SphK1 had numerous stress fibers and

focal adhesions (Fig. 2, *E* and *G*) that resembled those formed after PDGF stimulation (Fig. 2, *B* and *D*). Addition of PDGF to SphK1 transfectants did not further enhance stress fiber or

FIG. 2. **SphK1 overexpression induces stress fiber and focal adhesion formation.** NIH 3T3 fibroblasts stably transfected with empty vector (*A–D*) or SphK1 (*E–H*) were plated on collagen-coated glass coverslips, serum-starved overnight, and stimulated for 30 min without (*A, C, E,* and *G*) or with (*B, D, F,* and *H*) 4 ng/ml PDGF-BB. Cells were fixed and permeabilized, and actin filaments were stained with fluorescein isothiocyanate-labeled phalloidin (*A, B, E,* and *F*). Focal adhesions were stained with anti-paxillin antibody and Texas Red-conjugated second antibody (*C, D, G,* and *H*). Cells were visualized with a confocal fluorescence microscope.

focal adhesion formation (Fig. 2, *F* and *H*). These cytoskeletal rearrangements were mediated by increased formation of S1P, as the stress fibers in SphK1-expressing cells were completely eliminated by the SphK inhibitor *N,N*-dimethylsphingosine.

It is well established that Rho triggers formation of contractile stress fibers and focal adhesion complexes (39). Expression of SphK1 increased the cellular amount of the GTP-bound active form of Rho (Fig. 3*A*). In agreement with the established link between PDGF-mediated receptor stimulation and the Rho family of GTPases (40, 41), PDGF induced transient activation of Rac (data not shown) and rapid inactivation of Rho (Fig. 3*A*), which was blunted by SphK1.

Hyperphosphorylation of Src and FAK and Translocation of Paxillin and Vinculin to Focal Adhesions in SphK1 Transfectants—FAK and the Src family protein-tyrosine kinases (Src, Yes, and Fyn; hereafter referred to as Src) have been implicated in the organization and turnover of focal adhesions (42, 43). In agreement, PDGF rapidly increased phosphorylation of cytoskeleton-associated FAK at Tyr[577], which is located in the kinase catalytic domain and is required for maximal activity, whereas in SphK1 transfectants, PDGF had no effect, and Tyr[577] appeared to be constitutively hyperphosphorylated (Fig. 3*B*). PDGF also induced activation of cytoskeleton-associated Src, as determined with an antibody specific for Src phosphorylated at Tyr[418], an autophosphorylation site located in its catalytic domain required for full activity. By contrast, basal Src activation was higher, and PDGF did not further increase its phosphorylation in SphK1 transfectants (Fig. 3*B*). SphK1 also increased phosphorylation of FAK at its autophosphoryl-
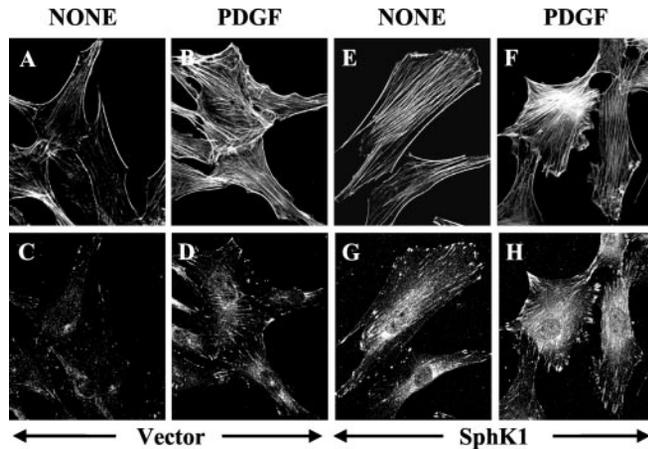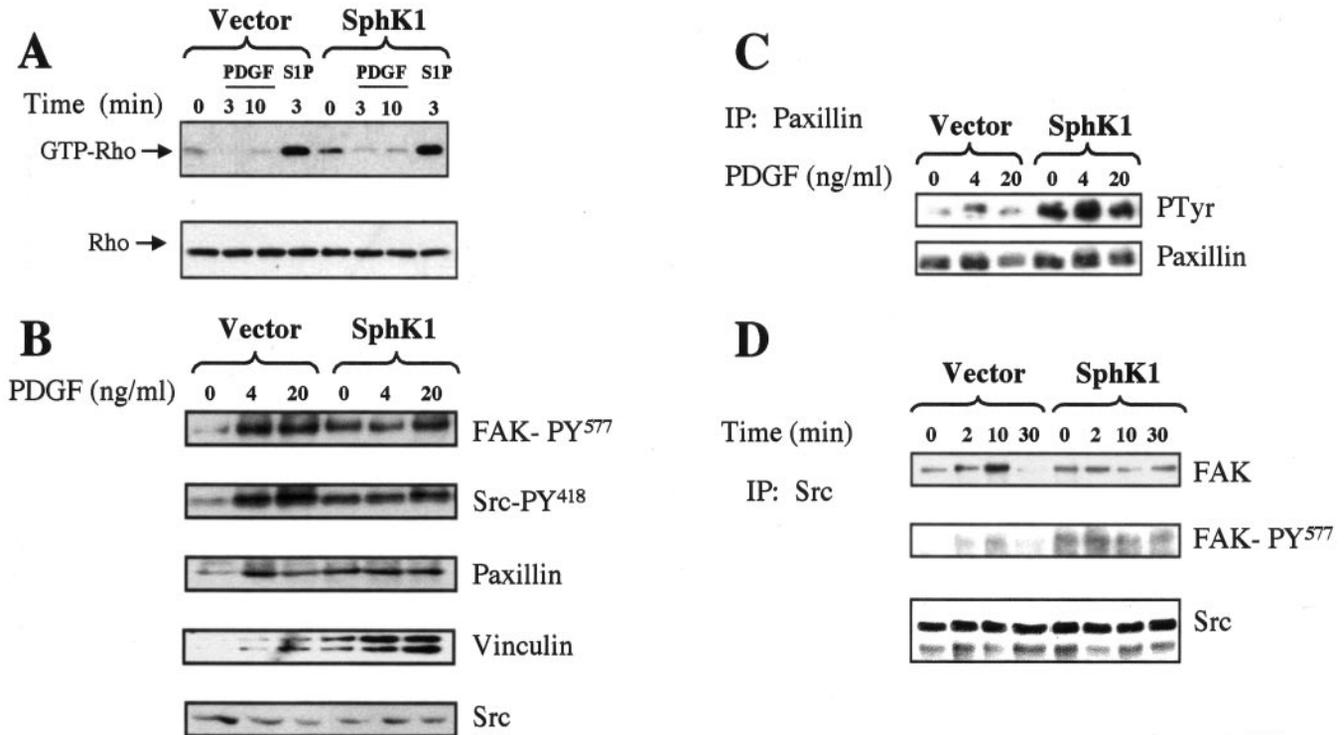

FIG. 3. **SphK1 overexpression induces hyperphosphorylation and recruitment of focal adhesion proteins to cytoskeleton adhesion complexes.** *A*, shown is the effect of SphK1 on Rho. Cells were serum-starved overnight and treated without or with 4 ng/ml PDGF or 200 nM S1P (positive control) for the indicated times. Active GTP-Rho was specifically pulled down from the cell lysates and analyzed by Western blotting using anti-Rho antibody. Total cell lysate Rho is shown below. *B*, vector- or SphK1-overexpressing cells were treated with the indicated concentrations of PDGF for 5 min, and the 1% Triton X-100-insoluble proteins were analyzed by Western blotting using anti-phospho-Tyr[577] FAK, anti-phospho-Tyr[418] Src, anti-paxillin, or anti-vinculin antibody and subsequently with anti-Src antibody to show equal loading. *C*, shown is the hyperphosphorylation of paxillin in SphK1-overexpressing cells. Paxillin was immunoprecipitated (*IP*) from 400 μg of cell lysates treated as described for *B*. The immunoprecipitated proteins were analyzed by Western blotting using anti-phosphotyrosine antibody, and the blots were stripped and reprobed with anti-paxillin antibody. *D*, shown is the impaired formation of the Src-FAK signaling complex. Empty vector- or SphK1-overexpressing cells were treated with 4 ng/ml PDGF for the indicated times, lysed, and immunoprecipitated as described under "Experimental Procedures." Src immunoprecipitates were analyzed by Western blotting using anti-FAK or anti-phospho-Tyr[577] FAK antibody and subsequently with anti-Src antibody to show equal loading.

ation site (Tyr[397]), which may be the first of several signaling events necessary for focal adhesion turnover to promote PDGF-stimulated cell migration (43), and PDGF had no further effect (data not shown).

FAK and Src function as part of a large cytoskeleton-associated network of signaling proteins, including paxillin and vinculin, a structural protein of focal adhesions that links talin and actin (44). In vector transfectants, PDGF rapidly induced translocation of vinculin and paxillin to the cytoskeleton-associated, Triton X-100-insoluble fraction containing the focal adhesion complexes (Fig. 3*B*). Their levels appeared to be enhanced in SphK1-overexpressing cells and were not further altered by PDGF (Fig. 3*B*). Tyrosine phosphorylation of paxillin by FAK is important for focal adhesion formation (44); and in agreement, paxillin was constitutively hyperphosphorylated in SphK1-expressing cells (Fig. 3*C*).

Upon recruitment of Src to FAK Tyr[397], active Src phosphorylates FAK at Tyr[577], enhancing its activity. The Src-FAK-linked activities subsequently promote focal adhesion dissociation and degradation of FAK, leading to turnover of focal adhesions, required for cell motility (45, 46). In control cells, PDGF induced a transient increase in Src-FAK complex formation with a corresponding increase in FAK phospho-Tyr[577] at 10 min, which rapidly decreased thereafter (Fig. 3*D*). By contrast, in cells expressing SphK1, Src was associated with FAK even in the absence of PDGF. This complex and the association of Src with hyperphosphorylated FAK remained intact even 30 min after treatment with PDGF (Fig. 3*D*).

$G_{12/13}$ *Is Necessary for SphK1-induced Stress Fiber Formation*—Because activation of S1PRs coupled to $G_{12/13}$ induces Rho activation and stress fiber formation (11, 13), we examined the role of G proteins in stress fiber formation induced by SphK1. To this end, cells were transfected with the GFP-tagged PDZ-RGS (regulator of G protein signaling) domain of the PDZ domain-containing Rho guanine exchange factor (PDZ-Rho-GEF), which acts as a dominant-negative for $G_{12/13}$-induced activation of Rho (47). The PDZ-RGS domain has the RGS domain responsible for the binding of PDZ-RhoGEF to $G\alpha_{12}$ or $G\alpha_{13}$, but lacks the Dbl homology/pleckstrin homology domain of PDZ-RhoGEF required for the conversion of GDP-bound Rho into GTP-bound Rho, the active form of Rho. This construct inhibits the activation of the serum response element by lysophosphatidic acid and thrombin receptors (48). In agreement, the GFP-tagged PDZ-RGS domain completely blocked the formation of stress fibers induced by S1P or lysophosphatidic acid (Fig. 4, *g–j*). Notably, it also totally eliminated stress fibers in SphK1-overexpressing cells (Fig. 4, *c–f*), whereas transfection of these cells with GFP alone did not have any effect on the architecture of stress fibers induced by SphK1 (Fig. 4, *a* and *b*). Nevertheless, transient expression of RGS3CT, with a truncated N terminus that stimulates the GTPase activity of G_i and G_q proteins, resulting in their inactivation (49), had no effect on stress fibers induced by SphK1 (Fig. 5, *a* and *b*). Similarly, pertussis toxin, which inactivates G_i, also did not reduce stress fiber formation in SphK1 transfectants (data not shown). These results suggest that $G_{12/13}$ (but not G_i or G_q) is involved in SphK1-induced stress fiber formation.

To further substantiate the involvement of S1PRs in cytoskeletal rearrangements, cells were transfected with G protein-coupled receptor kinase-2 (GRK2), a GRK family member that phosphorylates agonist-activated GPCRs, including S1PRs (50), thereby leading to a desensitization of G protein-linked signaling. FLAG-tagged GRK2 blocked formation of stress fibers induced by S1P (Fig. 5, *g* and *h*) without affecting those induced by lysophosphatidic acid (Fig. 5, *i* and *j*). This also indicates that the effects of GRK2 are specific and unlikely
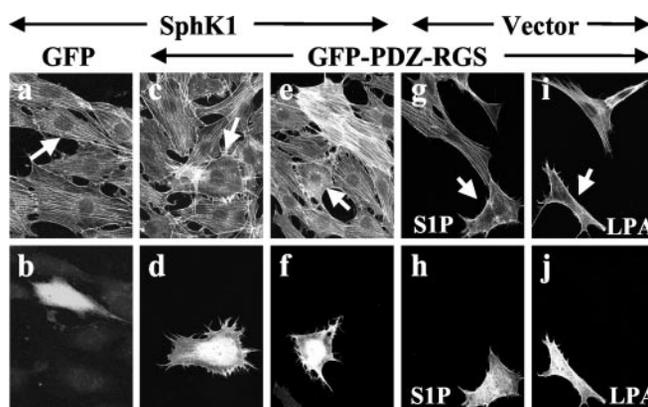

FIG. 4. **The PDZ-RGS domain blocks stress fiber formation induced by SphK1 and S1P.** NIH 3T3 cells stably transfected with SphK1 (*a–f*) or with empty vector (*g–j*) were plated on collagen-coated coverslips and transiently transfected with GFP (*a* and *b*) or GFP-PDZ-RGS (*c–j*). After 24 h, cells were serum-starved for 16 h and treated with vehicle (*a–f*), 200 nM S1P (*g* and *h*), or 100 nM lysophosphatidic acid (*LPA*; *i* and *j*). Cells were fixed and permeabilized, and actin filaments were stained using Texas Red-labeled phalloidin (*a*, *c*, *e*, *g*, and *i*). Cells expressing GFP (*b*) or GFP-PDZ-RGS (*d*, *f*, *h*, and *j*) are shown. GFP-PDZ-RGS totally eliminated stress fibers in 95% of the SphK1-overexpressing cells, whereas transfection with GFP had no noticeable effect.
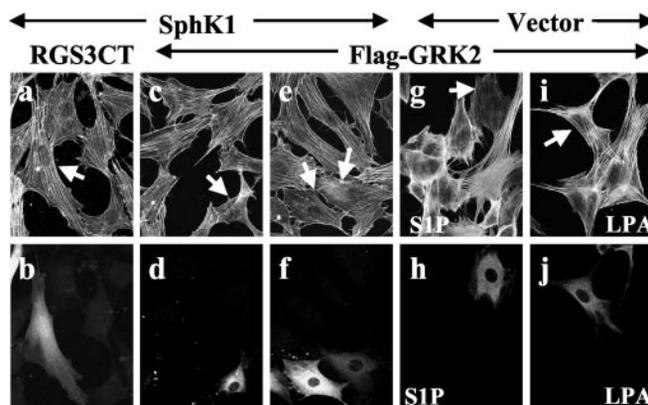

FIG. 5. **Expression of GRK2 (but not RGS3CT) prevents stress fiber formation induced by SphK1 and S1P.** NIH 3T3 cells stably transfected with SphK1 (*a–f*) or empty vector (*g–j*) were plated on collagen-coated coverslips and transiently transfected with GFP and RGS3CT at a ratio of 1:5 (*a* and *b*) or with FLAG-GRK2 (*c–j*). After 24 h, cells were serum-starved for 16 h and treated with vehicle (*a–f*), 200 nM S1P (*g* and *h*), or 100 nM lysophosphatidic acid (*i* and *j*). Actin filaments were stained with Texas Red-labeled phalloidin (*a*) or fluorescein isothiocyanate-labeled phalloidin (*c*, *e*, *g*, and *i*). Cells expressing GFP-RGS3CT (*b*) or FLAG-GRK2 (*d*, *f*, *h*, and *j*) were visualized with anti-FLAG antibody followed by Texas Red-conjugated secondary antibody. In 43 and 50% of the SphK1-overexpressing cells examined, FLAG-GRK2 totally and markedly, respectively, reduced stress fibers. In contrast, neither empty FLAG vector nor GFP-RGS3CT had discernable effects. All images were obtained with a confocal fluorescence microscope.

due to a direct effect on the cytoskeleton (51). GRK2 likewise abolished SphK1-induced stress fiber formation (Fig. 5, *c–f*). Collectively, these data suggest that enforced expression of SphK1, which increases intracellular S1P, activates a S1PR coupled to heterotrimeric $G\alpha_{12/13}$, which in turn leads to activation of Rho and stress fiber formation.

SphK1-induced Cell Proliferation and Survival Are Independent of GPCR Activation—Although a few studies have suggested that proliferation and suppression of apoptosis by S1P are mediated via intracellular actions, many others have argued for the involvement of S1PRs, making this a controversial area (reviewed in Ref. 4). Although the GFP-tagged PDZ-RGS domain totally blocked stress fiber formation induced by SphK1 expression (Fig. 5, *c–f*), this dominant-negative RhoGEF
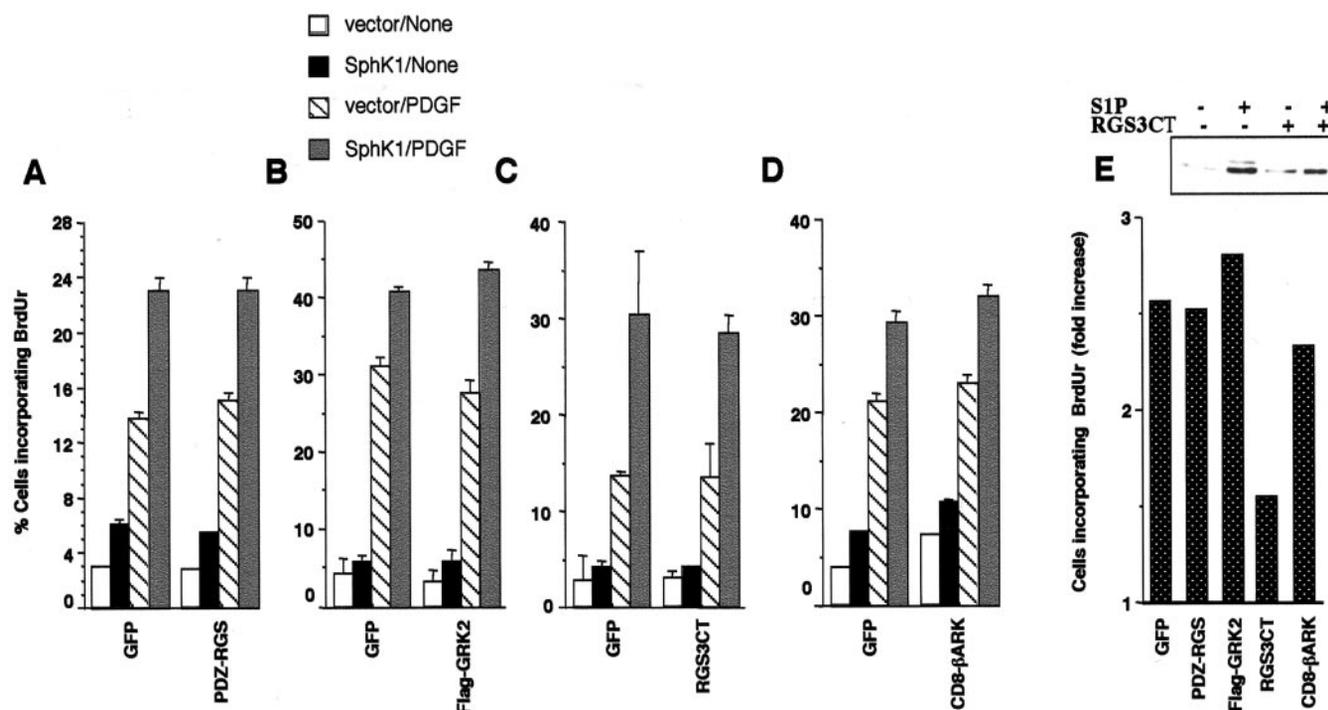
FIG. 6. **Inhibition of heterotrimeric G protein signaling pathways does not abrogate the growth advantage induced by SphK1.** NIH 3T3 fibroblasts stably transfected with vector (*white* and *hatched bars*) or SphK1 (*black* and *gray bars*) were transiently transfected with GFP or GFP-PDZ-RGS (*A*), GFP or FLAG-GRK2 at a ratio of 1:5 (*B*), GFP or RGS3CT at a ratio of 1:5 (*C*), or GFP or CD8-βARK at a ratio of 1:5 (*D*). After 24 h, cells were cultured in serum-free medium containing 2 μg/ml transferrin and 20 μg/ml BSA without (*white* and *black bars*) or with (*hatched* and *gray bars*) 10 ng/ml PDGF. After 16 h, BrdUrd was added for an additional 3 h. Double immunofluorescence was used to visualize transfected cells and BrdUrd incorporation into nascent DNA. The proportion of cells incorporating BrdUrd among total transfected cells (expressing GFP) was determined. Data are means ± S.D. of triplicate cultures from a representative experiment. At least three different fields with a minimum of 130 cells/field were scored. Expression of RGS3CT inhibited proliferation induced by exogenous S1P (*E*). NIH 3T3 vector cells were transfected as described above. After 24 h, cells were cultured in serum-free medium containing 2 μg/ml transferrin and 20 μg/ml BSA in the absence or presence of 4 μM S1P, and BrdUrd incorporation was measured. Data are expressed as -fold stimulation of BrdUrd incorporation compared with cells treated with vehicle only and are means of three independent experiments. *Inset*, Western blot showing activation of ERK1/2 by 200 nM S1P in cells transfected with vector (−) or RGS3CT (+).

did not have a significant effect on SphK1-induced proliferation either in the absence of serum or after stimulation with PDGF as measured by BrdUrd incorporation into nascent DNA in the transfected cells (Fig. 6*A*). It should be noted that the growth advantage of SphK1 is more pronounced in the presence of low concentrations of serum or when insulin is added to serum-free medium (21).

In agreement with previous studies (21, 24), overexpression of SphK1 markedly reduced serum deprivation-induced apoptosis by 50%, as determined by shrinkage and condensation of nuclei (Fig. 7). GFP-PDZ-RGS did not abrogate this cytoprotective effect. Thus, although $G_{12/13}$ is important for cytoskeletal reorganization and stress fiber formation induced by SphK1, this G protein does not seem to be involved in its proliferative and survival responses. However, this does not exclude other α subunits of heterotrimeric G proteins downstream of S1PRs that have been shown to be important transducers of mitogenic signals (52, 53). Nevertheless, transfection with RGS3CT to inhibit signaling through $G\alpha_q$ and $G\alpha_i$ or with GRK2, which phosphorylated and desensitized the GPCR involved in S1P-induced stress fiber formation (Fig. 5, *c–f*), did not abrogate the growth advantage of SphK1 overexpression (Fig. 6, *B* and *C*). Moreover, they did not decrease the cytoprotective effect of SphK1 (Fig. 7). In agreement, the increase in cell numbers induced by overexpression of SphK1 (1.7-fold) or even after PDGF stimulation (2.5-fold) was also not affected. However, it should be emphasized that RGS3CT, a potent G_i and G_q subfamily effector antagonist, markedly reduced proliferation induced by exogenous S1P (Fig. 6*E*) and also suppressed S1P-induced activation of ERK1/2 (Fig. 6*E*, *inset*), in contrast to its

inability to regulate the effects induced by intracellular generated S1P (Fig. 6*C*).

In many cases, it is the Gβγ dimers rather than the Gα subunits that transmit the signals for proliferation and survival (54). Thus, we examined whether Gβγ signaling downstream of S1PRs might be involved in the proliferative and survival effects of SphK1. However, a chimeric construct containing the extracellular and transmembrane domains of CD8 and the C-terminal domain of the β-adrenergic receptor kinase (CD8-βARK), which includes the βγ-binding domain and is able to bind free βγ dimers, inhibiting their signaling thereafter (55), did not prevent the proliferative effect of SphK1 (Fig. 6*D*). Moreover, although overexpression of CD8-βARK by itself somewhat enhanced apoptosis, even in the presence of serum, it did not prevent the apoptosis-sparing effect of SphK1 (Fig. 7).

Role of S1PRs—Collectively, our data suggest that, in contrast to exogenously added S1P, expression of SphK1 and generation of endogenous S1P stimulate cell proliferation and inhibit apoptosis independent of S1PRs coupled to G proteins. This is a remarkable finding because inside-out signaling by S1P can induce $G_{12/13}$-mediated stress fiber formation important for the regulation of cell motility. We utilized MEFS isolated from $S1P_2/S1P_3$ double knockout mice (33) to definitively determine whether S1PRs are the mediators of the growth and survival effects of SphK1. Expression of SphK1 in the double knockout MEFs stimulated BrdUrd incorporation into nascent DNA to a greater extent than in wild-type MEFs (Fig. 8*A*). In contrast to wild-type MEFs, which express $S1P_{1-3}$, these MEFs express only $S1P_1$ (33), which is coupled solely to G_i (20, 56). Pertussis toxin, which ADP-ribosylates and inactivates G_i, did
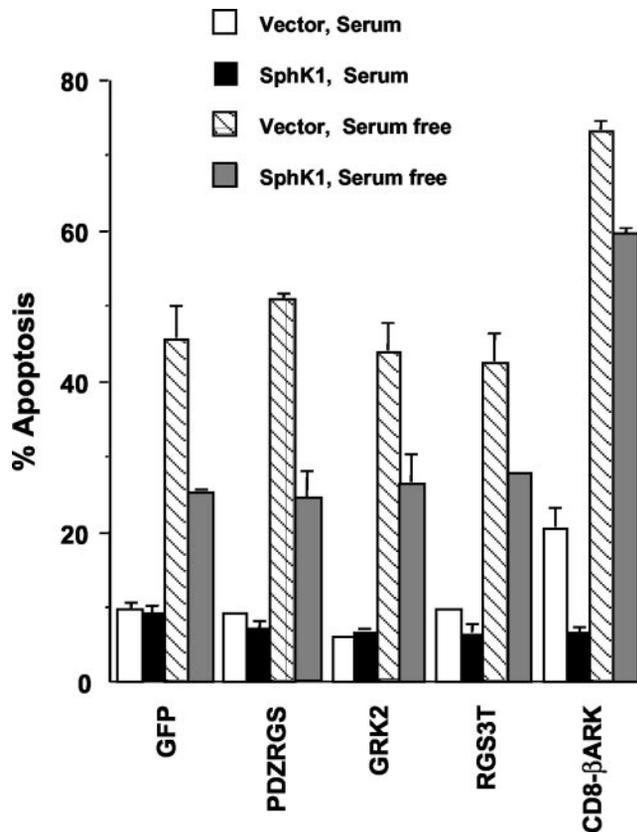
FIG. 7. **Inhibition of $G_{12/13}$, $G\alpha_q$, $G\alpha_i$, and $G\beta\gamma$ signaling or down-regulation of GPCRs does not influence the cytoprotective effect of SphK1 overexpression.** NIH 3T3 fibroblasts stably transfected with empty vector (*white* and *hatched bars*) or SphK1 (*black* and *gray bars*) were transfected as described in the legend to Fig. 6. After 24 h, cells were serum-starved for 48 h or grown in 5% serum. Cells were fixed, and nuclei were stained with Hoechst dye. Total GFP-expressing cells and GFP-expressing cells displaying condensed nuclei indicative of apoptosis were scored. The results are from a representative experiment performed in duplicate, and data are means ± S.D. At least three different fields with a minimum of 500 cells/field were scored.

not abrogate the ability of SphK1 to induce cell proliferation in wild-type or null MEFs. Unexpectedly, SphK1 stimulated proliferation even more potently than PDGF in the absence or presence of pertussis toxin (Fig. 8*A*). In contrast, exogenous S1P was a less potent mitogen than PDGF in wild-type MEFs, and its effect was markedly reduced in the S1PR-deficient MEFs (Fig. 8*B*). In contrast to the strong mitogenic effect of SphK1 in pertussis toxin-treated knockout MEFs (Fig. 8*A*), no significant responses were observed with exogenous S1P (Fig. 8*B*). These results suggest that S1PRs are dispensable for the mitogenic effect of SphK1, but contribute to that of exogenous S1P.

Survival Effects of SphK1 Are Not Compromised by Lack of S1PRs—There are several reports showing that activation of S1PRs by S1P protects cells from apoptosis (57, 58), whereas others suggest that suppression of apoptosis is mediated via intracellular actions (21–23, 59). Therefore, examination of the cytoprotective effects of SphK1 and exogenous S1P in the null MEFs should aid in settling this controversy. In agreement with previous studies (28), serum deprivation induced apoptosis of MEFs in a time-dependent manner. Apoptosis was clearly evident after 48 h, and deletion of $S1P_2$ and $S1P_3$ did not have a significant effect. Addition of micromolar concentrations of exogenous S1P, but not nanomolar concentrations (data not shown), suppressed apoptosis in a dose-dependent manner in these MEFs (Fig. 9). Pertussis toxin slightly reduced the pro-

tective effects of serum and high concentrations of S1P. In contrast, pertussis toxin did not decrease the strong cytoprotective effect of SphK1 overexpression in either wild-type or null MEFs (Fig. 9). Thus, even in the absence of all S1PR signaling, SphK1 still markedly induced growth and survival.

DISCUSSION

How Does Intracellularly Generated S1P Inhibit Cell Movement?—Cell movement is orchestrated by the complex interplay of actin polymerization at the leading edge under the control of members of the Rho family of small GTPases (Rac, Cdc42, and Rho) (14) and the formation of nascent focal adhesion complexes that tether the cell to the extracellular matrix, modulated by tyrosine kinases that reside within these complexes, such as FAK (60) and Src (42, 45, 61). Stress fiber and focal adhesion formation and especially their dynamic turnover play a crucial role in S1P-directed cell migration (11, 16, 38, 62). Indeed, SphK1-overexpressing cells had numerous and prominent focal adhesions that localized at the tips or along the length of omnipresent stress fibers and showed a defect in focal adhesion turnover.

Consistent with the increase in focal adhesions, SphK1 also caused tyrosine hyperphosphorylation of cytoskeleton-associated paxillin, Src, and FAK. The latter was phosphorylated at Tyr[577], important for its activation, and at Tyr[397], the major autophosphorylation site (42, 43, 64). Whereas FAK activity is dispensable for PDGF-induced cell chemotaxis, phosphorylation of Tyr[397] seems to be essential, as it is the Src recruitment signal (43). This association not only results in Src-mediated maximal activation of FAK and enhanced recruitment of other focal adhesion components and signaling proteins (42, 64), but also in Src-mediated dissociation of FAK from the complex, which is necessary for the proper turnover of focal adhesions (45). Notably, the delicate dynamics of association and dissociation of Src-FAK complexes following PDGF treatment were dysregulated in SphK1 transfectants. In these cells, continuous interaction between Src and FAK was PDGF-independent. Interestingly, the autophosphorylation site of FAK is required for the inhibitory effects of S1P on cell motility (34). Formation of S1P by overexpression of SphK1 impairs focal adhesion remodeling and normal coordination of focal adhesion turnover, leading to aberrant cell migration. Furthermore, the constitutive extensive stress fiber formation in SphK1 transfectants is consistent with the observation that Rho stimulates contractile actin-myosin filaments, resulting in the formation of stress fibers and focal adhesions that likely inhibit cell locomotion (65, 66).

Involvement of $G_{12/13}$ Downstream of S1PRs—Here we have shown that inhibition of $G\alpha_{12/13}$ (but not $G\alpha_i$ or $G\alpha_q$) signaling or uncoupling of S1PRs from G protein signaling by overexpression of GRK2 completely inhibited stress fiber formation induced by exogenous S1P or SphK1 expression. These results further support the notion of inside-out signaling by S1P, whereby intracellularly generated S1P activates a S1PR (10, 29) coupled to $G\alpha_{12/13}$, which in turn leads to activation of Rho and stress fiber formation.

Does S1P Exert Its Action Solely through S1PRs?—Heterotrimeric G proteins couple cell-surface receptors to signals that regulate proliferation and survival, and asynchronous activation of $G\alpha$ subunits can lead to oncogenic transformation (52). Moreover, in some cases, it is the $G\beta\gamma$ dimers that transmit signals for proliferation via ERK1/2 and promote cell survival by activation of phosphatidylinositol 3-kinase (54). The preponderance of evidence implicating G protein-coupled S1PRs in the biological activities of S1P has overshadowed its intracellular roles, mainly due to the difficulty of dissociating pathways that originate at the receptor-ligand boundary from those poten-
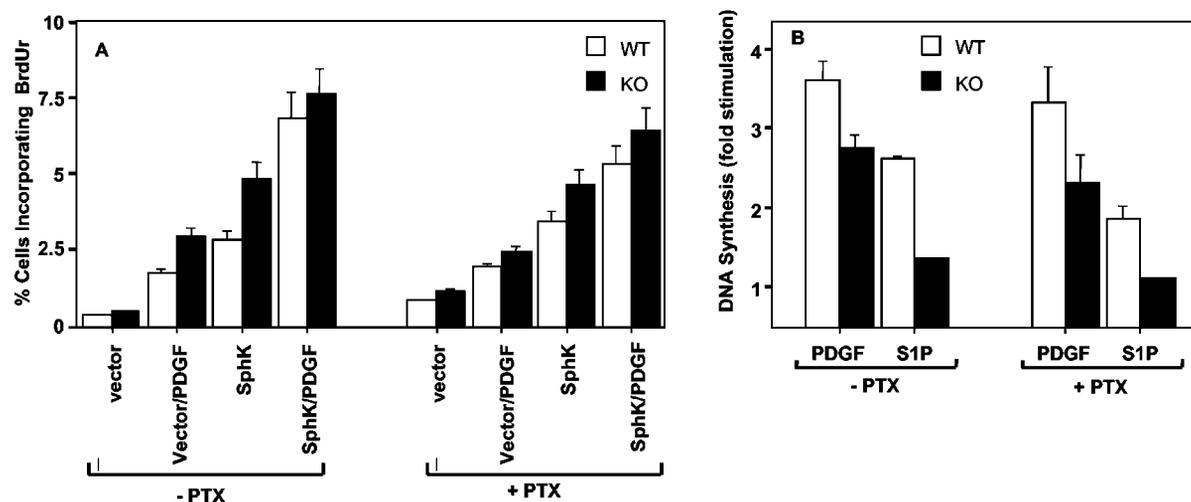
FIG. 8. **S1PRs are dispensable for DNA synthesis induced by SphK1.** *A*, wild-type (*WT*; *white bars*) and S1P$_2$/S1P$_3$ double knockout (*KO*; *black bars*) MEFs were transiently transfected with GFP vector or GFP-SphK1 and, after 24 h, cultured in serum-free medium containing 2 μg/ml transferrin and 20 μg/ml BSA without or with 10 ng/ml PDGF in the absence or presence of pertussis toxin (*PTX*; 100 ng/ml). After 16 h, BrdUrd was added for an additional 3 h. The proportion of cells incorporating BrdUrd among total transfected cells was determined as described in the legend to Fig. 6. *B*, shown is the effect of exogenous S1P on proliferation. Wild-type (*white bars*) and S1P$_2$/S1P$_3$ double knockout (*black bars*) MEFs were treated with PDGF-BB (10 ng/ml) or S1P (10 μM) in the absence or presence of pretreatment with 20 ng/ml pertussis toxin, and DNA synthesis was measured. Data are expressed as -fold stimulation and are means $\pm$ S.D. [^{3}H]Thymidine incorporation in unstimulated wild-type and double knockout MEFs was 3500 $\pm$ 400 and 4700 $\pm$ 500 cpm/well, respectively.
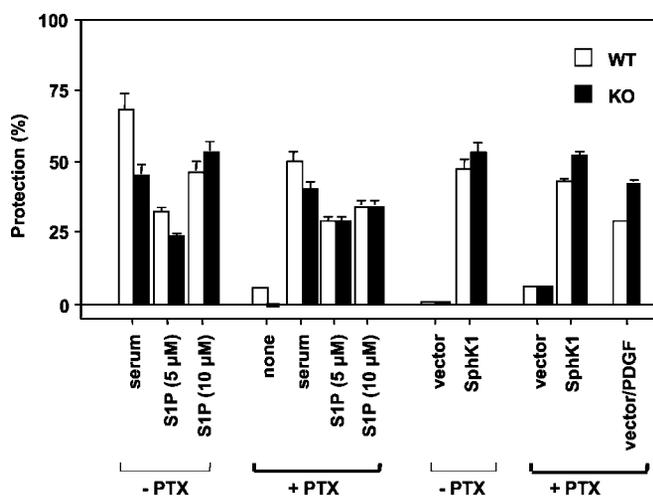

FIG. 9. **The cytoprotective effect of SphK1 is independent of functional S1PRs.** Wild-type (*WT*; *white bars*) and S1P$_2$/S1P$_3$ double knockout (*KO*; *black bars*) MEFs were cultured in serum-free medium for 48 h without or with S1P (5 and 10 μM) or 10% serum in the absence or presence of pertussis toxin (*PTX*; 20 ng/ml), and apoptosis was determined. Where indicated, cells were transiently transfected with GFP vector or GFP-SphK1. Total GFP-expressing cells and GFP-expressing cells displaying condensed nuclei indicative of apoptosis were scored as described in the legend to Fig. 6. Data are expressed as percent protection compared with untreated cells. 45 $\pm$ 5 and 43 $\pm$ 2% of untreated serum-starved wild-type and knockout MEFs, respectively, were apoptotic.

tially originating inside cells. Previously, pertussis toxin was used to implicate a Gα_i-mediated pathway in the proliferative and survival effects induced by exogenous S1P (20, 67, 68). However, a SphK inhibitor (but not pertussis toxin) inhibits the proliferation and cytoprotective effects induced by SphK1 overexpression (21). In agreement, we found that inhibiting Gα_i and Gα_q (but not G$\alpha_{12/13}$) drastically reduced proliferation and ERK1/2 activation induced by exogenous S1P. In sharp contrast, blocking signaling of the various Gα subunits and G$\beta\gamma$ dimers, the G proteins that S1PRs couple to and signal through, did not influence growth and survival promoted by SphK1 and intracellularly generated S1P. Hence, although the mitogenic effect of exogenous S1P is mediated by ligation of cell-surface receptors, S1P formed by overexpression of SphK1 nonetheless promoted growth and survival independent of its GPCRs. In further support of this conclusion, SphK1 markedly stimulated growth and survival of S1P$_2$/S1P$_3$ double knockout MEFs treated with pertussis toxin, which then have no functional S1PRs. Thus, surprisingly, exogenous and intracellularly generated S1Ps affect cell growth and survival by divergent pathways.

The paramount importance of our results is that, although intracellularly generated S1P can signal inside-out to regulate cytoskeletal rearrangements and cell movement, this is not the case for the regulation of cell growth and suppression of apoptosis, which is independent of S1PRs. Several other lines of evidence further support the notion of such intracellular actions of S1P. First, sphinganine 1-phosphate (dihydro-S1P), which is identical to S1P and lacks only the 4,5-*trans*-double bond, binds to all of the S1PRs and activates them, yet does not mimic the effects of S1P on cell survival (20, 23, 28, 69). Second, microinjection of S1P, which elevates intracellular S1P, has been shown to mobilize calcium (18) and to enhance proliferation and survival (20, 23, 70). Third, SphK1 and conversion of sphingosine to S1P mediate vascular endothelial growth factor-induced activation of Ras and, as a consequence, the ERK pathway and cell division by regulating Ras GTPase-activating protein activity without involving S1PRs (71). Finally, yeast cells also do not possess GPCRs, yet levels of phosphorylated long chain sphingoid bases regulate environmental stress responses and survival (72–74), in a manner reminiscent of the function of S1P in mammalian cells. A yeast strain with deletion of both Lcb4 and Lcb5, the homologs of mammalian SphK, was delayed in entering S phase (74). As the accumulated sphingoid bases in yeast induce G$_0$/G$_1$ arrest, these data suggest that SphK removes the sphingoid block, allowing progression to S phase (74). Likewise, overexpression of SphK1 in NIH 3T3 fibroblasts expedites the G$_1$/S transition and increases the percentage of cells in S phase (21). Additionally, PDGF-induced activation of CDK2, a cyclin-dependent kinase that promotes progression through the G$_1$/S transition, is blocked by inhibiting SphK (75). Intriguingly, the time course for CDK2 activation correlates with increased nucleoplasmic SphK activity and translocation to the nuclear envelope (63). Finally, our study

suggests an intracellular action of S1P independent of its GPCRs and signaling inside-out, which not only is an important concept in signaling, but also has important clinical implications for SphK inhibitors in cancer (25) and also for prevention of radiation-induced premature ovarian failure and infertility (23, 70).

REFERENCES

1. Spiegel, S., and Milstien, S. (2002) *J. Biol. Chem.* **277,** 25851–25854
2. Fukushima, N., Ishii, I., Contos, J. J., Weiner, J. A., and Chun, J. (2001) *Annu. Rev. Pharmacol. Toxicol.* **41,** 507–534
3. Olivera, A., and Spiegel, S. (2001) *Prostaglandins* **64,** 123–134
4. Hla, T., Lee, M. J., Ancellin, N., Paik, J. H., and Kluk, M. J. (2001) *Science* **294,** 1875–1878
5. Chun, J., Goetzl, E. J., Hla, T., Igarashi, Y., Lynch, K. R., Moolenaar, W., Pyne, S., and Tigyi, G. (2002) *Pharmacol. Rev.* **54,** 265–269
6. Wang, F., Van Brocklyn, J. R., Hobson, J. P., Movafagh, S., Zukowska-Grojec, Z., Milstien, S., and Spiegel, S. (1999) *J. Biol. Chem.* **274,** 35343–35350
7. English, D., Kovala, A. T., Welch, Z., Harvey, K. A., Siddiqui, R. A., Brindley, D. N., and Garcia, J. G. (1999) *J. Hematother. Stem Cell Res.* **8,** 627–634
8. Lee, O. H., Kim, Y. M., Lee, Y. M., Moon, E. J., Lee, D. J., Kim, J. H., Kim, K. W., and Kwon, Y. G. (1999) *Biochem. Biophys. Res. Commun.* **264,** 743–750
9. Kupperman, E., An, S., Osborne, N., Waldron, S., and Stainier, D. Y. (2000) *Nature* **406,** 192–195
10. Spiegel, S., English, D., and Milstien, S. (2002) *Trends Cell Biol.* **12,** 236–242
11. Ohmori, T., Yatomi, Y., Okamoto, H., Miura, Y., Rile, G., Satoh, K., and Ozaki, Y. (2001) *J. Biol. Chem.* **276,** 5274–5280
12. English, D., Welch, Z., Kovala, A. T., Harvey, K., Volpert, O. V., Brindley, D. N., and Garcia, J. G. (2000) *FASEB J.* **14,** 2255–2265
13. Okamoto, H., Takuwa, N., Yokomizo, T., Sugimoto, N., Sakurada, S., Shigematsu, H., and Takuwa, Y. (2000) *Mol. Cell. Biol.* **20,** 9247–9261
14. Hall, A. (1998) *Science* **280,** 2074–2075
15. Liu, Y., Wada, R., Yamashita, T., Mi, Y., Deng, C.-X., Hobson, J. P., Rosenfeldt, H. M., Nava, V. E., Chae, S. S., Lee, M. J., Liu, C. H., Hla, T., Spiegel, S., and Proia, R. L. (2000) *J. Clin. Invest.* **106,** 951–961
16. Wang, F., Van Brocklyn, J. R., Edsall, L., Nava, V. E., and Spiegel, S. (1999) *Cancer Res.* **59,** 6185–6191
17. Meyer zu Heringdorf, D., Lass, H., Alemany, R., Laser, K. T., Neumann, E., Zhang, C., Schmidt, M., Rauen, U., Jakobs, K. H., and van Koppen, C. J. (1998) *EMBO J.* **17,** 2830–2837
18. van Koppen, C. J., Meyer zu Heringdorf, D., Alemany, R., and Jakobs, K. H. (2001) *Life Sci.* **68,** 2535–2540
19. Olivera, A., and Spiegel, S. (1993) *Nature* **365,** 557–560
20. Van Brocklyn, J. R., Lee, M. J., Menzeleev, R., Olivera, A., Edsall, L., Cuvillier, O., Thomas, D. M., Coopman, P. J. P., Thangada, S., Hla, T., and Spiegel, S. (1998) *J. Cell Biol.* **142,** 229–240
21. Olivera, A., Kohama, T., Edsall, L. C., Nava, V., Cuvillier, O., Poulton, S., and Spiegel, S. (1999) *J. Cell Biol.* **147,** 545–558
22. Cuvillier, O., Pirianov, G., Kleuser, B., Vanek, P. G., Coso, O. A., Gutkind, S., and Spiegel, S. (1996) *Nature* **381,** 800–803
23. Morita, Y., Perez, G. I., Paris, F., Miranda, S. R., Ehleiter, D., Haimovitz-Friedman, A., Fuks, Z., Xie, Z., Reed, J. C., Schuchman, E. H., Kolesnick, R. N., and Tilly, J. L. (2000) *Nat. Med.* **6,** 1109–1114
24. Edsall, L. C., Cuvillier, O., Twitty, S., Spiegel, S., and Milstien, S. (2001) *J. Neurochem.* **76,** 1573–1584
25. Xia, P., Gamble, J. R., Wang, L., Pitson, S. M., Moretti, P. A., Wattenberg, B. W., D'Andrea, R. J., and Vadas, M. A. (2000) *Curr. Biol.* **10,** 1527–1530
26. Nava, V. E., Hobson, J. P., Murthy, S., Milstien, S., and Spiegel, S. (2002) *Exp. Cell Res.* **281,** 115–127
27. Meyer zu Heringdorf, D., Lass, H., Kuchar, I., Lipinski, M., Alemany, R., Rumenapp, U., and Jakobs, K. H. (2001) *Eur. J. Pharmacol.* **414,** 145–154
28. Rosenfeldt, H. M., Hobson, J. P., Maceyka, M., Olivera, A., Nava, V. E., Milstien, S., and Spiegel, S. (2001) *FASEB J.* **15,** 2649–2659
29. Hobson, J. P., Rosenfeldt, H. M., Barak, L. S., Olivera, A., Poulton, S., Caron, M. G., Milstien, S., and Spiegel, S. (2001) *Science* **291,** 1800–1803
30. Alderton, F., Rakhit, S., Choi, K. K., Palmer, T., Sambi, B., Pyne, S., and Pyne, N. J. (2001) *J. Biol. Chem.* **276,** 28578–28585
31. Boujaoude, L. C., Bradshaw-Wilder, C., Mao, C., Cohn, J., Ogretmen, B., Hannun, Y. A., and Obeid, L. M. (2001) *J. Biol. Chem.* **276,** 35258–35264
32. Kohama, T., Olivera, A., Edsall, L., Nagiec, M. M., Dickson, R., and Spiegel, S. (1998) *J. Biol. Chem.* **273,** 23722–23728
33. Ishii, I., Ye, X., Friedman, B., Kawamura, S., Contos, J. J., Kingsbury, M. A., Yang, A. H., Zhang, G., Brown, J. H., and Chun, J. (2002) *J. Biol. Chem.* **277,** 25152–25159
34. Wang, F., Nohara, K., Olivera, A., Thompson, E. W., and Spiegel, S. (1999) *Exp. Cell Res.* **247,** 17–28
35. Johnson, K. R., Becker, K. P., Facchinetti, M. M., Hannun, Y. A., and Obeid, L. M. (2002) *J. Biol. Chem.* **277,** 35257–35262
36. Ronnstrand, L., and Heldin, C. H. (2001) *Int. J. Cancer* **91,** 757–762
37. Wang, F., Nobes, C. D., Hall, A., and Spiegel, S. (1997) *Biochem. J.* **324,** 481–488
38. Machesky, L. M., and Hall, A. (1997) *J. Cell Biol.* **138,** 913–926
39. Ridley, A. J., and Hall, A. (1992) *Cell* **70,** 389–399
40. Sander, E. E., ten Klooster, J. P., van Delft, S., van der Kammen, R. A., and Collard, J. G. (1999) *J. Cell Biol.* **147,** 1009–1022
41. Ryu, Y., Takuwa, N., Sugimoto, N., Sakurada, S., Usui, S., Okamoto, H., Matsui, O., and Takuwa, Y. (2002) *Circ. Res.* **90,** 325–332
42. Salazar, E. P., and Rozengurt, E. (1999) *J. Biol. Chem.* **274,** 28371–28378
43. Sieg, D. J., Hauck, C. R., Ilic, D., Klingbeil, C. K., Schaefer, E., Damsky, C. H., and Schlaepfer, D. D. (2000) *Nat. Cell Biol.* **2,** 249–256
44. Turner, C. E. (2000) *Nat. Cell Biol.* **2,** E231–E236
45. Fincham, V. J., and Frame, M. C. (1998) *EMBO J.* **17,** 81–92
46. Geiger, B., Bershadsky, A., Pankov, R., and Yamada, K. M. (2001) *Nat. Rev. Mol. Cell. Biol.* **2,** 793–805
47. Fukuhara, S., Murga, C., Zohar, M., Igishi, T., and Gutkind, J. S. (1999) *J. Biol. Chem.* **274,** 5868–5879
48. Fukuhara, S., Chikumi, H., and Gutkind, J. S. (2000) *FEBS Lett.* **485,** 183–188
49. Scheschonka, A., Dessauer, C. W., Sinnarajah, S., Chidiac, P., Shi, C. S., and Kehrl, J. H. (2000) *Mol. Pharmacol.* **58,** 719–728
50. Watterson, K. R., Johnston, E., Chalmers, C., Pronin, A., Cook, S. J., Benovic, J. L., and Palmer, T. M. (2002) *J. Biol. Chem.* **277,** 5767–5777
51. Pitcher, J. A., Hall, R. A., Daaka, Y., Zhang, J., Ferguson, S. S., Hester, S., Miller, S., Caron, M. G., Lefkowitz, R. J., and Barak, L. S. (1998) *J. Biol. Chem.* **273,** 12316–12324
52. Radhika, V., and Dhanasekaran, N. (2001) *Oncogene* **20,** 1607–1614
53. Gutkind, J. S. (1998) *J. Biol. Chem.* **273,** 1839–1842
54. Schwindinger, W. F., and Robishaw, J. D. (2001) *Oncogene* **20,** 1653–1660
55. Crespo, P., Cachero, T. G., Xu, N., and Gutkind, J. S. (1995) *J. Biol. Chem.* **270,** 25259–25265
56. Ancellin, N., and Hla, T. (1999) *J. Biol. Chem.* **274,** 18997–19002
57. Lee, M. J., Thangada, S., Claffey, K. P., Ancellin, N., Liu, C. H., Kluk, M., Volpi, M., Sha'afi, R. I., and Hla, T. (1999) *Cell* **99,** 301–312
58. Hisano, N., Yatomi, Y., Satoh, K., Akimoto, S., Mitsumata, M., Fujino, M. A., and Ozaki, Y. (1999) *Blood* **93,** 4293–4299
59. Xia, P., Wang, L., Gamble, J. R., and Vadas, M. A. (1999) *J. Biol. Chem.* **274,** 34499–34505
60. Ilic, D., Furuta, Y., Kanazawa, S., Takeda, N., Sobue, K., Nakatsuji, N., Nomura, S., Fujimoto, J., Okada, M., and Alzawa, S. (1995) *Nature* **377,** 539–544
61. Thomas, S. M., and Brugge, J. S. (1997) *Annu. Rev. Cell Dev. Biol.* **13,** 513–609
62. Paik, J. H., Chae, S., Lee, M. J., Thangada, S., and Hla, T. (2001) *J. Biol. Chem.* **276,** 11830–11837
63. Kleuser, B., Maceyka, M., Milstien, S., and Spiegel, S. (2001) *FEBS Lett.* **503,** 85–90
64. Schaller, M. D. (2001) *Biochim. Biophys. Acta* **1540,** 1–21
65. Hall, A. (1998) *Science* **279,** 509–514
66. Nobes, C. D., and Hall, A. (1999) *J. Cell Biol.* **144,** 1235–1244
67. Goodemote, K. A., Mattie, M. E., Berger, A., and Spiegel, S. (1995) *J. Biol. Chem.* **270,** 10272–10277
68. An, S., Zheng, Y., and Bleu, T. (2000) *J. Biol. Chem.* **275,** 288–296
69. Xia, P., Gamble, J. R., Rye, K. A., Wang, L., Hii, C. S. T., Cockerill, P., Khew-Goodall, Y., Bert, A. G., Barter, P. J., and Vadas, M. A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 14196–14201
70. Paris, F., Perez, G. I., Fuks, Z., Haimovitz-Friedman, A., Nguyen, H., Bose, M., Ilagan, A., Hunt, P. A., Morgan, W. F., Tilly, J. L., and Kolesnick, R. (2002) *Nat. Med.* **8,** 901–902
71. Shu, X., Wu, W., Mosteller, R. D., and Broek, D. (2002) *Mol. Cell. Biol.* **22,** 7758–7768
72. Mandala, S. M., Thornton, R., Tu, Z., Kurtz, M. B., Nickels, J., Broach, J., Menzeleev, R., and Spiegel, S. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 150–155
73. Mao, C., Saba, J. D., and Obeid, L. M. (1999) *Biochem. J.* **342,** 667–675
74. Jenkins, G. M., and Hannun, Y. A. (2001) *J. Biol. Chem.* **276,** 8574–8581
75. Rani, C. S., Berger, A., Wu, J., Sturgill, T. W., Beitner-Johnson, D., LeRoith, D., Varticovski, L., and Spiegel, S. (1997) *J. Biol. Chem.* **272,** 10777–10783