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Filamin A Links Sphingosine Kinase 1 and Sphingosine-1-Phosphate Receptor 1 at Lamellipodia To Orchestrate Cell Migration[▽]

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Sphingosine kinase 1 (SphK1) catalyzes the phosphorylation of sphingosine to produce the potent lipid mediator sphingosine-1-phosphate (S1P), which plays a critical role in cell motility via its cell surface receptors. Here, we have identified filamin A (FLNa), an actin-cross-linking protein involved in cell movement, as a bona fide SphK1-interacting protein. Heregulin stimulated SphK1 activity only in FLNa-expressing A7 melanoma cells but not in FLNa-deficient cells and induced its translocation and colocalization with FLNa at lamellipodia. SphK1 was required for heregulin-induced migration, lamellipodia formation, activation of PAK1, and subsequent FLNa phosphorylation. S1P directly stimulated PAK1 kinase, suggesting that it may be a target of intracellularly generated S1P. Heregulin also induced colocalization of $S1P_1$ (promotility S1P receptor) but not $S1P_2$, with SphK1 and FLNa at membrane ruffles. Moreover, an $S1P_1$ antagonist inhibited the lamellipodia formation induced by heregulin. Hence, FLNa links SphK1 and $S1P_1$ to locally influence the dynamics of actin cytoskeletal structures by orchestrating the concerted actions of the triumvirate of SphK1, FLNa, and PAK1, each of which requires and/or regulates the actions of the others, at lamellipodia to promote cell movement.

Sphingosine-1-phosphate (S1P) is a potent lipid mediator that has emerged as a key regulator of cytoskeletal rearrangements and cell movement, acting through five G protein-coupled S1P receptors, termed $S1P_{1-5}$. These differentially expressed receptors couple to a different subset of heterotrimeric G proteins, which in part, determines their distinct roles in cell migration (8, 38). Of the more widely expressed S1P receptors, $S1P_1$ and $S1P_3$ typically promote cell migration by activating the small GTPase Rac, leading to actin polymerization and lamellipodia formation (23, 26, 32). Conversely, $S1P_2$ has been called a repellent receptor as it stimulates Rho and antagonizes Rac, leading to stress fiber formation and cessation of migration (14, 37, 41).

S1P formation is catalyzed by sphingosine kinase 1 and 2 (SphK1 and SphK2). Many of the external stimuli and growth factors that enhance cell movement stimulate cytosolic SphK1, producing S1P that is secreted and that acts in an autocrine or paracrine manner to activate $S1P_1$ (17, 23). This "inside-out" signaling paradigm requires that SphK1 translocate to the plasma membrane, bringing it to the vicinity of its membrane-localized substrate sphingosine. The molecular mechanisms of SphK1 activation and/or translocation have not yet been completely elucidated. Several studies have suggested a role for calmodulin in SphK1 translocation (42, 44), while others have demonstrated the importance of extracellular signal-regulated kinase 1 and 2 (ERK1/2)-mediated phosphorylation of Ser225 of SphK1 in translocation (33, 34), which may cause changes in its conformation, to expose a phosphatidylserine-binding domain (39). In addition to posttranslational modifications, several proteins that interact with SphK1 have been identified that appear to localize cytosolic SphK1 to intracellular membranes, including RPK118 (16), PECAM-1 (13), aminoacylase 1 (24), and δ-catenin/NPRAP (12). However, none of these proteins has been shown to mediate the signal-induced translocation of SphK1 to the plasma membrane or to regulate the functions of SphK1 in motility. Here, we identify filamin A (FLNa), an actin binding protein that cross-links cortical actin filaments into dynamic three-dimensional actin webs at membrane ruffles (40) and acts as a scaffold protein that interacts with SphK1 and links it with $S1P_1$ at lamellipodia to orchestrate cell migration. Moreover, we have found that it is the concerted actions of the triumvirate of SphK1, FLNa, and p21-activated kinase 1 (PAK1), each of which requires and/or regulates the actions of the others, that are essential for lamellipodia formation and cell movement.

MATERIALS AND METHODS

Materials. All lipids were obtained from Biomol (Plymouth Meeting, PA). Labeled compounds [γ-^{32}P]ATP (3,000 Ci/mmol) and L-[4,5-^{3}H]leucine (150 Ci/mmol) were from Amersham Pharmacia Biotech (Piscataway, NJ). Recombinant human heregulin (Hrg) B1 was from Neomarkers (Fremont, CA). PAK, ERK2, and tubulin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). FLNa (Ser 2152), PAK1/2 (Thr423/Thr402), and ERK1/2 (Thr202/Tyr204) phospho-specific antibodies were from Cell Signaling Technology (Beverly, MA). Mouse anti-FLNa was from Chemicon (Temecula, CA), rat monoclonal anti-hemagglutinin (HA) (clone 3F10) was from Roche (Rockford, IL), mouse anti-V5 antibody was from Invitrogen (Carlsbad, CA), and rabbit anti-V5 was from Sigma-Aldrich (St. Louis, MO). Rabbit polyclonal SphK1 antibodies were described previously (15). Secondary antibodies were from Jackson ImmunoResearch (horseradish peroxidase [HRP] conjugated; West Grove, PA) or Molecular Probes (fluorescently labeled; Eugene, OR). Control and SphK1-specific short interfering RNAs (siRNAs) (15) were obtained from Qiagen (Valencia, CA). All other reagents were from Sigma-Aldrich.

Cell culture and transfection. Human embryonic kidney cells (HEK 293; ATCC CRL-1573) were cultured and transfected as previously described (2). M2 and A7 cells were maintained in minimal essential medium supplemented with 2% fetal and 8% newborn calf serum as described previously (29). A7 cell cultures were supplemented with 1 mg/ml G418. M2 and A7 cells were trans-

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fected with GeneJuice (EMD Biosciences, Gibbstown, NJ) for plasmids. For downregulation of SphK1, cells were plated in six-well plates at a density of 1.5×10^5 per well and were transfected with siRNA targeted to human SphK1 (5′-AAGGGCAAGGCCTTGCAGCTC-3′; Qiagen) and appropriate control siRNA, using Oligofectamine (Invitrogen) according to the manufacturer's instructions. In some experiments, to confirm the lack of off-target effects, cells were transfected with ON-TARGETplus SMARTpool siRNA targeting SphK1 (5′-CGACGAGGACUUUGUGCUA-3′; 5′-GAUGGGGAAUUGAUGGUUA-3′; 5′-GAAAUCUCCUUCACGCUGA-3′; and 5′-GGAAAGGUGUGUUUGCAGU-3′) or control siRNA (Dharmacon, Lafayette, CO).

Two-hybrid screening and cloning of C-terminal FLNa. A two-hybrid screen was carried out using a MatchMaker II kit from Clontech (Mountain View, CA) exactly as described previously (24). A clone was obtained that corresponded to a C-terminal fragment of FLNa (Ct-FLNa; amino acids [aa] 2380 to 2647) that passed all tests as a valid two-hybrid interactor with full-length SphK1. Ct-FLNa was cut from the library vector with EcoRI and BamH1 and cloned into pcDNA3-HA.

Immunoblotting. Unless otherwise indicated, after various treatments, cells were washed with ice-cold phosphate-buffered saline (PBS) and scraped into buffer containing 20 mM Tris (pH 7.4), 1 mM EDTA, 2 mM sodium orthovanadate, 4 mM sodium pyrophosphate, 100 mM NaF, and 1:500 protease inhibitor cocktail (Sigma-Aldrich). Lysates were prepared by subjecting cells to five to seven freeze-thaw cycles (HEK 293 cells) or by adding 1% Triton X-100 and incubating the cells for 30 min on ice (M2 and A7 cells), followed by centrifugation at $1,000 \times g$ for 5 min to remove unbroken cells. Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transblotted to nitrocellulose. Blots were incubated with the indicated primary antibodies overnight, washed, and incubated with the appropriate HRP-conjugated secondary antibodies (at a 1:10,000 dilution; Jackson ImmunoResearch Laboratories). Immunocomplexes were visualized by enhanced chemiluminescence (Pierce Chemical Co., Rockford, IL) according to the manufacturer's instructions, with X-ray film or an Alpha Innotech FluorChem SP (San Leandro, CA) chemiluminescence imager.

Cell migration assay. Cell migration was measured in a modified Boyden chamber using polycarbonate filters (12-μm pores) from Poretics (Livermore, CA) essentially as described previously (15). Briefly, serum-starved cells were trypsinized and loaded into the upper well of the Boyden chamber (50,000 cells/well). The lower side of the separating filter was coated with 20 μg/ml fibronectin, and the lower chamber contained either serum-free medium or chemoattractants as indicated. Cells were permitted to migrate for 3 h, and nonmigrating cells were removed mechanically. Migrated cells were fixed, stained with DiffQuick, and counted using an 10× magnification objective. Each data point is the average number of cells in three random fields and is the mean ± standard deviation (SD) value of three individual wells.

Sphingosine kinase assay. Lysates were prepared, and SphK1 activity was measured as described previously (31), in the presence of 5 μM sphingosine and 0.25% Triton X-100.

Pulldown and immunoprecipitation assays. Ct-FLNa was transcribed and translated in vitro using a TnT kit (Promega, San Louis Obispo, CA) in the presence of L-[4,5-^{3}H]leucine. The translation mixture was incubated with either glutathione *S*-transferase (GST) or GST-SphK1 (20) and then affinity purified using glutathione-Sepharose beads (Pierce). After they were washed extensively with SphK assay buffer containing 1% Triton X-100, proteins in the pellet were resolved by SDS-PAGE. Gels were then dried and exposed to X-ray film. For the pulldown assay of V5-His-SphK1, equal amounts of lysates were incubated with Ni-nitrilotriacetic acid (NTA)-agarose (Pierce) in lysis buffer containing 10 mM imidazole for 30 min at 4°C with constant agitation. Protein-bound Ni-NTA-agarose was recovered by centrifugation at $1,000 \times g$ for 5 min and then washed with buffer containing 10 mM imidazole and 1 M NaCl.

Immunofluorescence. Cells cultured on coverslips were transfected, treated as indicated, and then fixed in 3% paraformaldehyde and stained (25). Briefly, fixation was quenched with 10 mM glycine in PBS and was followed by permeabilization with 0.5% Triton X-100. Coverslips were washed and incubated with Alexa 488 phalloidin or with primary antibodies for 30 min, followed by washing and a 30-min incubation with secondary antibodies. Coverslips were then washed and mounted on slides with 10 mM *n*-propylgallate in 100% glycerol. Images were collected with a Zeiss LSM 510 Meta confocal microscope with a 63× 1.4 numerical aperture Plan-Apochromat objective, with the optical thickness set to 1 μm for all channels. Single-fluorophore secondary controls were routinely negative for the other channels, and sequential scanning was used to ensure that there was no cross-talk between channels. All images were exported directly using Zeiss LSM Image Examiner (v. 3.2.0.70) software to 8-bit tagged image file format (TIFF) files without compression, contrast, or gamma adjustments.

In vitro PAK1 kinase assays. PAK1 activity was measured with 10-ng recombinant PAK1, 1 μg of myelin basic protein (MBP) substrate, and ATP (20 μM, 5 μCi) in buffer containing 50 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 2 mM $MnCl_2$, and 0.2 mM dithiothreitol (9) in the presence of fatty acid-free bovine serum albumin vehicle, sphingosine, S1P, or 1 ng of Cdc42-GTPγS prepared as described previously (18). Reactions were carried out at 30°C and stopped at the indicated times by the addition of SDS sample buffer. Proteins were resolved by SDS-PAGE and blotted to nitrocellulose, and the levels of radioactivity incorporated into the MBP and PAK1 were determined with a Bio-Rad FX phosphorimager (Hercules, CA) and Quantity One software. Parallel reactions were performed in the presence of unlabeled ATP, and proteins were immunoblotted with anti-phospho-threonine antibodies (Cell Signaling).

Statistical analysis. Statistical significance was assessed by one-way analysis of variance with a Tukey post hoc test.

RESULTS

Identification of FLNa as a SphK1-binding protein. To identify proteins that interacted with SphK1 and regulated its functions, a yeast two-hybrid screen of a kidney cDNA library was performed using full-length SphK1 as the bait, as described previously (24). Several of the SphK1-interacting clones contained a cDNA insert that encoded a C-terminal fragment of the actin binding protein FLNa (aa 2380 to 2647), hereafter referred to as Ct-FLNa (Fig. 1A). Cotransfection of the Ct-FLNa and SphK1 constructs into yeast cells conferred the ability of transformed colonies to grow in medium lacking adenosine, histidine, tryptophan, and leucine. Ct-FLNa also specifically activated three different promoter-reporter gene constructs with differing affinities for the GAL4 DNA-binding domain in the presence SphK1, suggesting that SphK1 and Ct-FLNa are bona fide two-hybrid interactors.

To confirm the physical interaction between these two proteins, Ct-FLNa was cloned into a mammalian expression vector and transcribed and translated in vitro in the presence of L-[4,5-^{3}H]leucine. Only GST-SphK1, but not GST alone, specifically pulled down ^{3}H-labeled Ct-FLNa (Fig. 1B). Next, it was important to confirm that Ct-FLNa and SphK1 interact in vivo in mammalian cells. To this end, SphK1 was specifically pulled down from lysates of cells expressing both HA-tagged Ct-FLNa and V5-His-tagged SphK1 with Ni-NTA-agarose. Ct-FLNa was detected only in the pulldown assay when SphK1 was coexpressed, indicating that both proteins are capable of association in cells (Fig. 1C). For the SphK1-FLNa interaction to have biological significance, it is important to demonstrate that endogenous full-length FLNa is also able to bind to endogenous SphK1. First, a 280-kDa band corresponding to endogenous FLNa was specifically pulled down with ectopic V5-His-tagged SphK1 (Fig. 1D). Second, endogenous full-length FLNa was coimmunoprecipitated with endogenous SphK1 by a specific anti-SphK1 antibody but not by control antiserum (Fig. 1E). Together, these results provided evidence that SphK1 physically interacts with FLNa in mammalian cells.

Heregulin induces FLNa-dependent colocalization of SphK1 at lamellipodia. To gain insight into the physiological role of the FLNa and SphK1 interaction, we used a matched pair of melanoma cell lines, M2 cells that do not express detectable levels of FLNa and the M2-derived A7 cells, stably expressing FLNa at near normal levels and that rescues the motility defect of M2 cells (7). In agreement with previous studies, Hrg, an epider-

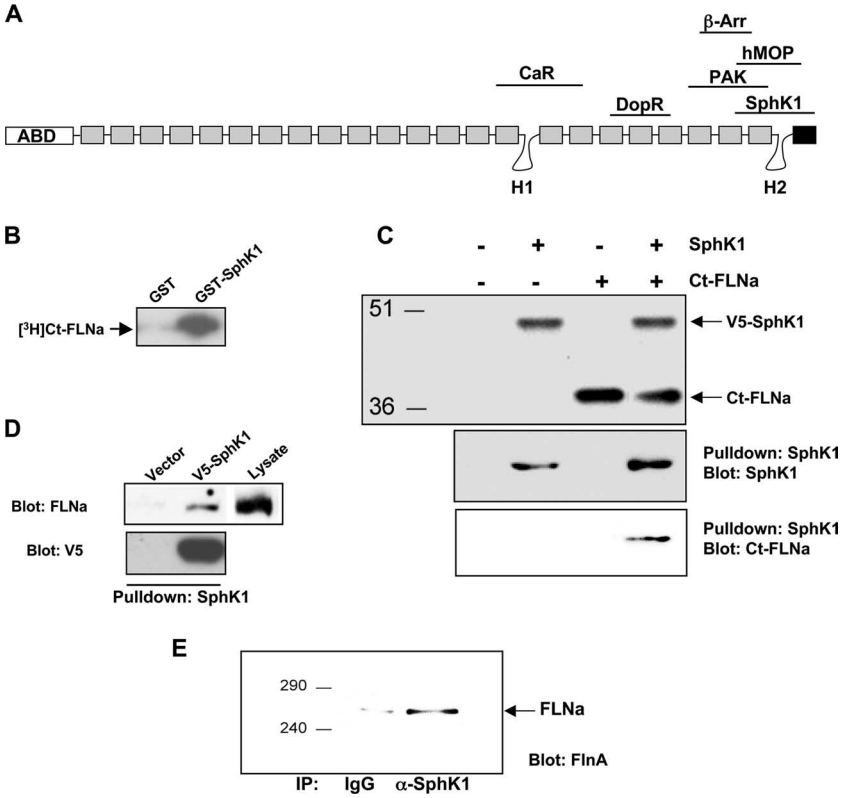


FIG. 1. The C terminus of FLNa interacts with SphK1. (A) Diagram of full-length FLNa, with gray boxes representing immunoglobulin-like repeats. Filled box, FLNa dimerization domain; ABD, actin binding domain; H1 and H2, hinge regions 1 and 2. Bars indicate regions of FLNa that have been found to bind SphK1, calcium sensing receptor (CaR), β-arrestin (β-Arr), dopamine receptor 2 and 3 (DopR), human μ opioid receptor (hMOP), and PAK1. (B) The Ct-FLNa (aa 2380 to 2647), identified as a SphK1 interactor in a yeast two-hybrid screen, was cloned into pcDNA3.1-HA and transcribed and translated in vitro in the presence of L-[4,5-^{3}H]leucine. Equal portions of labeled proteins were then incubated with GST or GST-SphK1, pulled down with glutathione-Sepharose beads, washed, resolved by SDS-PAGE, and autoradiographed. (C) HEK 293 cells were transfected with vector, V5-His-SphK1, or with Ct-FLNa or with both, as indicated. After 48 h, cells were lysed, and proteins were resolved by SDS-PAGE, transblotted, and probed with anti-V5 and anti-HA for SphK1 and Ct-FLNa, respectively (upper panel). Equal amounts of cell lysates were also incubated with Ni-NTA-agarose to pull down SphK1, and bound proteins were examined by immunoblotting with V5 (middle panel) or HA antibodies (lower panel). Note that Ct-FLNa was pulled down only in the presence of V5-His-SphK1. (D) Equal amounts of lysates from HEK 293 cells transfected with vector or V5-SphK1 were incubated with Ni-NTA-agarose to pull down SphK1, and bound proteins were resolved by SDS-PAGE and blotted with anti-FLNa for endogenous FLNa (upper panel) or anti-V5 for SphK1 (lower panel). (E) Lysates from naïve HEK 293 cells were incubated with preimmune serum or anti-SphK1 antibody. Immune complexes were isolated with protein A/G agarose, washed, resolved by SDS-PAGE, and immunoblotted with anti-FLNa antibodies.

mal growth factor (EGF)-like ligand, induced lamellipodia formation and promoted localization of FLNa only in A7 cells (Fig. 2A and B), although both A7 and M2 cells express similar levels of the Hrg receptors ErbB-2 and ErbB-3 (28, 43). Although SphK1 had a similar subcellular distribution in both M2 and A7 cells, Hrg-induced translocation of SphK1 to the lamellipodia only in A7 cells (Fig. 2A and B), where SphK1 colocalized with FLNa. Ectopic expression of FLNa in M2 cells rescued the Hrg-induced formation of membrane ruffles (Fig. 2B and C) and restored colocalization of FLNa and SphK1 at actin-rich lamellipodia (Fig. 2B and D). It should be noted that, although Hrg induced the formation of lamellipodia in only about 40% of A7 cells (Fig. 2C), FLNa and SphK1 were colocalized in >95% of the lamellipodia observed (compare Fig. 2D and C). This colocalization is consistent with the physical interaction between SphK1 and FLNa and the requirement of FLNa for lamellipodia formation.

It is well established that EGF stimulates SphK1, which is required for EGF-induced motility (10, 15, 27). Likewise, Hrg induced a twofold increase in SphK1 activity in FLNa-expressing A7 cells but did not activate SphK1 in M2 cells (Fig. 2E), suggesting that FLNa is required for both the translocation of SphK1 to lamellipodia and its activation. It has been shown that activated ERK1/2 can phosphorylate SphK1 and that this phosphorylation is responsible for SphK1 translocation to the membrane in response to some stimuli (33). However, Hrg stimulated ERK1/2 in both M2 and A7 cells (Fig. 2F), suggesting that the lack of translocation of SphK1 in M2 cells is not due to the inability to activate ERK1/2.

SphK1 is required for FLNa-dependent cell migration. We next determined whether SphK1 was required for FLNa-dependent motility by downregulating its expression. In both M2 and A7 cells, siRNA targeted to SphK1 reduced its mRNA level by more than 70%, without affecting SphK2 expression (data not shown), and greatly reduced the level of SphK1 protein expression and activity (Fig. 3A). In agreement with previous observations that M2 cells have a defective migratory ability (7), these cells did not migrate toward Hrg in the mod-

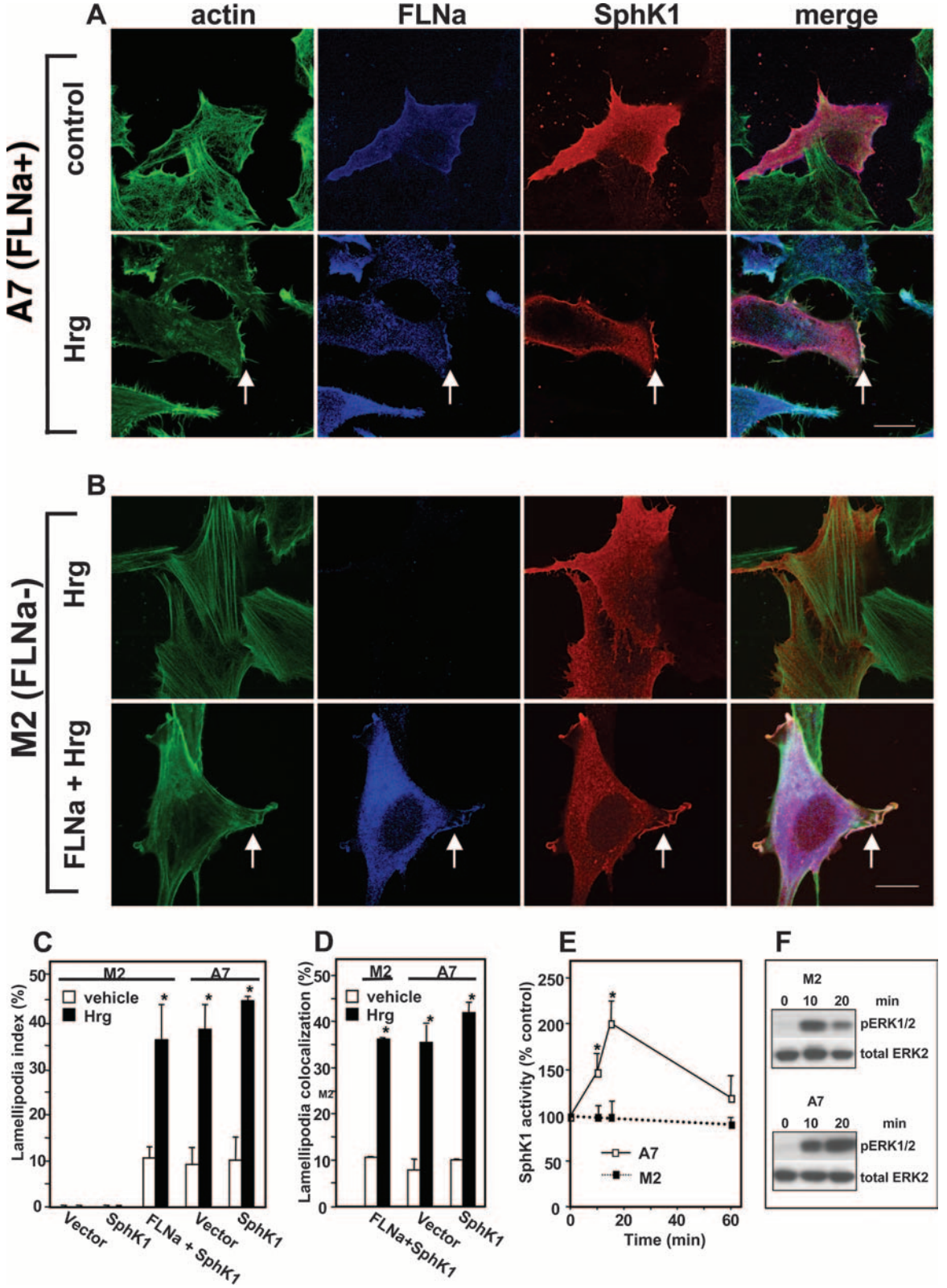


FIG. 2. Hrg induces colocalization of SphK1 and FLNa to lamellipodia. FLNa-expressing A7 (A) and FLNa-deficient M2 (B) cells were transfected with vector, V5-SphK1, or FLNa, as indicated. After 48 h, cells were serum starved overnight and then treated with vehicle or Hrg (30 ng/ml) for 30 min; fixed; stained with Alexa 488-labeled phalloidin for F-actin (green), polyclonal antibody against V5 to visualize SphK1 (red), or monoclonal antibody against FLNa (blue); and visualized by confocal microscopy. White areas indicate colocalization of SphK1, FLNa, and actin. Arrows indicate colocalization of actin, FLNa, and SphK1 in lamellipodia, defined as membrane ruffles at the edge of the cell, visualized by DIC that also stain strongly for actin. Bar, 10 μm. Results are representative of at least four independent experiments. (C) The percentage of

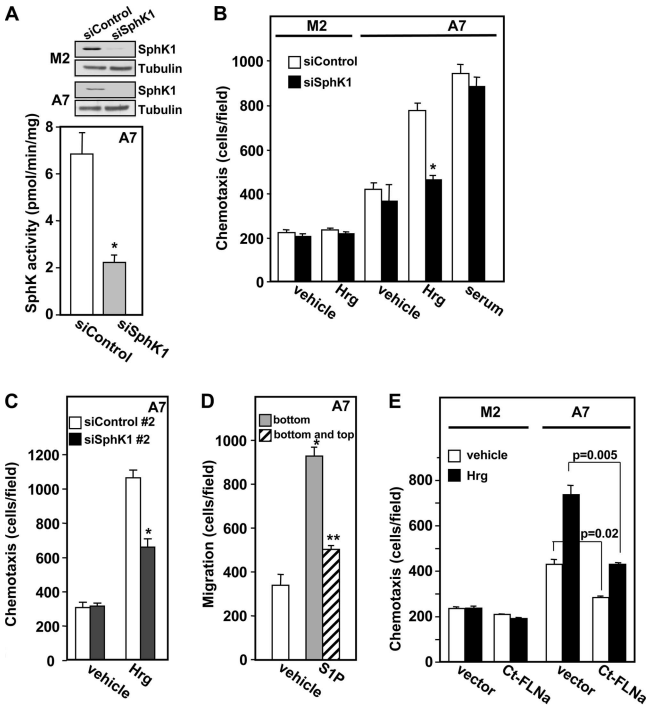


FIG. 3. SphK1 is required for Hrg-induced cell migration. (A and B) M2 and A7 cells were transfected with control siRNA (siControl) or siRNA targeted to SphK1 (siSphK1) as indicated. (A) Equal amounts of cell lysates were separated by SDS-PAGE and immunoblotted with anti-SphK1 antibody. Blots were reprobed for tubulin to ensure equal loading and transfer. SphK1 activity was determined in lysates from A7 cells (bottom panel). (B) Cells were placed into a modified Boyden chamber and allowed to migrate toward serum-free medium (vehicle), Hrg (30 ng/ml), or 10% serum for 3 h as indicated. Data are expressed as the number of migrating cells per field. Similar results were obtained for three independent experiments. *, $P < 0.001$. (C) A7 cells were transfected with ON-TARGETplus SMARTpool siRNA targeted to SphK1 (filled bars) or control siRNA 2 (open bars), and cells were allowed to migrate toward Hrg (10 ng/ml) for 3 h. Data are expressed as the number of migrating cells per field. *, $P < 0.001$. (D) S1P (1 nM) was added to either the bottom chamber or to both the top and bottom chambers, and the migration of A7 cells was determined. *, $P < 0.001$; **, $P < 0.05$. (E) M2 and A7 cells were transfected with vector or with Ct-FLNa, and chemotaxis toward serum-free medium (vehicle) or Hrg (30 ng/ml) was measured in a modified Boyden chamber. Data are expressed as the number of migrating cells per field and are representative of three independent experiments.

ified Boyden chamber assays (Fig. 3B). In contrast, the chemotaxis of A7 cells was markedly stimulated by Hrg, S1P, or serum (Fig. 3B and D). Downregulation of SphK1 expression completely blocked the chemotaxis of A7 cells toward Hrg yet did not alter the ability of these cells to migrate toward serum (Fig. 3B). Serum, which contains many other chemoattractants in addition to S1P, was used as a positive control, indicating that the inhibition of migration due to a reduction of SphK1 expression is specific and not a result of a general reduction in cell viability or global impairment of motility. To exclude nonspecific off-target effects, SphK1 expression was also downregulated with siRNA targeted to other regions of the SphK1 sequence. ON-TARGETplus SMARTpool siRNA targeted for SphK1 but not control siRNA, which markedly reduced the expression of SphK1 mRNA by more than 85%, also significantly reduced Hrg-stimulated migration (Fig. 3C). A7 cells also migrated toward S1P (Fig. 3D), and it was of interest to determine whether this migration was mediated by enhanced directed migration in response to a gradient of chemoattractant (chemotaxis) or by increased random motility due to the presence of the chemoattractant itself (chemokinesis). The greatest numbers of cells were found to migrate toward S1P when it was added only to the bottom chamber, whereas migratory responses were greatly reduced when concentrations of S1P were the same in the top and bottom chambers (Fig. 3D), indicating that S1P stimulates chemotactic rather than chemokinetic responses in these cells.

There were no obvious changes in cell shape or cytoskeletal arrangement after transfection with siSphK1 (Fig. 4A). Nevertheless, the downregulation of SphK1 markedly reduced the number of actin-rich lamellipodia produced in response to Hrg (Fig. 4A and B). Sphingosine, the substrate for SphK1, has previously been shown to promote membrane ruffle formation in a FLNa-dependent manner (43). In agreement, a rapid and robust increase in lamellipodia formation was observed after cells were exposed to sphingosine. However, knocking down SphK1 expression significantly reduced sphingosine-induced lamellipodia formation and also the translocation of FLNa (Fig. 4A and B), suggesting that the conversion of sphingosine to S1P is the critical event. Moreover, the Ct-FLNa fragment, which binds SphK1 but not actin (Fig. 1A), acted as a dominant-negative construct by inhibiting Hrg-induced chemotaxis of A7 cells (Fig. 3E).

Hrg- and sphingosine-induced activation of PAK1 and FLNa phosphorylation are dependent on S1P formation. The serine/threonine kinase PAK1 is a downstream effector of the small GTPases Cdc42 and Rac and serves as an important regulator of cytoskeletal dynamics and cell motility (3). Intriguingly, both sphingosine and Hrg activate PAK1, which in turn phosphorylates FLNa on serine 2152, an event that is required for FLNa-mediated actin cytoskeleton assembly, including ruffle formation (43). In agreement, in A7 cells, Hrg and sphingosine increased phosphorylation of PAK1 at Thr423 (Fig. 5A and B), a site essential for full activity (46), and also enhanced phosphorylation at Ser2152 on FLNa (Fig. 5D and E). Treatment of M2 cells with Hrg or sphingosine did not alter their levels of PAK phosphorylation (data not shown). This result is consistent with previous results demonstrating that Hrg did not

lamellipodia-positive cells was calculated, and the lamellipodia index was expressed as the mean ± standard error of the mean (SEM). *, $P < 0.005$. A minimum of 300 cells was scored in a double-blind manner. (D) The percent of cells in which SphK1 and FLNa were colocalized to lamellipodia is expressed as the mean ± SEM. *, $P < 0.005$. (E) FLNa is essential for the activation of SphK1 by Hrg. M2 and A7 cells were serum starved overnight and then treated with Hrg (30 ng/ml). At the indicated times, cells were washed, lysed, and assayed for SphK1 activity. (F) Hrg stimulates ERK1/2 in both M2 and A7 cells. M2 and A7 cells were treated with 30 ng/ml Hrg for the indicated times. Equal amounts of cell lysates were immunoblotted with anti-phospho-ERK1/2 antibodies. Anti-ERK2 was used as a loading control.

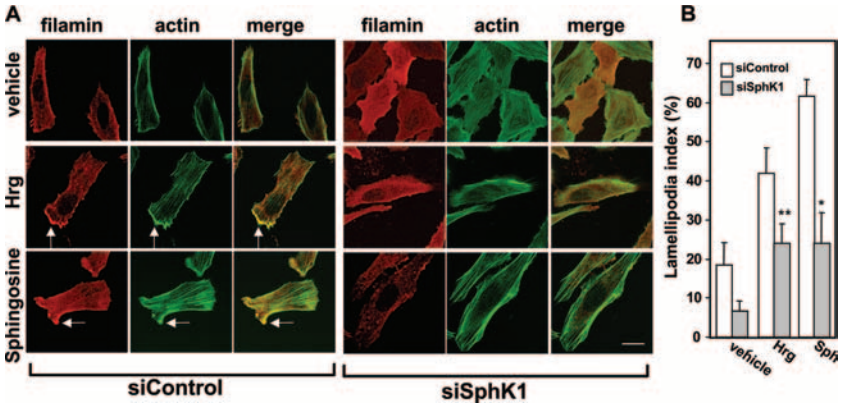


FIG. 4. SphK1 is required for Hrg- and sphingosine-induced lamellipodia formation. A7 cells were transfected with control siRNA (siControl) or siRNA targeted to SphK1 (siSphK1) as indicated, serum-starved overnight, and then stimulated with vehicle, Hrg (30 ng/ml), or sphingosine (Sph; 5 μM) for 30 min. Cells were then fixed, stained for actin (green) and FLNa (red), and visualized by confocal microscopy. (A) Yellow areas in merged images indicate colocalization of FLNa and actin. Arrows indicate regions of colocalization of FLNa and actin in lamellipodia. Bar, 10 μm. (B) The percentage of lamellipodia-positive cells was calculated, and the lamellipodia index is expressed as the mean ± standard error of the mean. *, $P < 0.05$; **, $P < 0.01$. Data are representative of at least three independent experiments.

stimulate the phosphorylation of the known PAK substrate cofillin in M2 cells (43).

Downregulation of SphK1 markedly attenuated the activation of PAK1 and FLNa phosphorylation in A7 cells, suggesting that SphK1 and the formation of S1P are required for activation of PAK1 and concomitant FLNa phosphorylation induced by Hrg or sphingosine. If so, then the exogenous addition of S1P should bypass the requirement for SphK1.

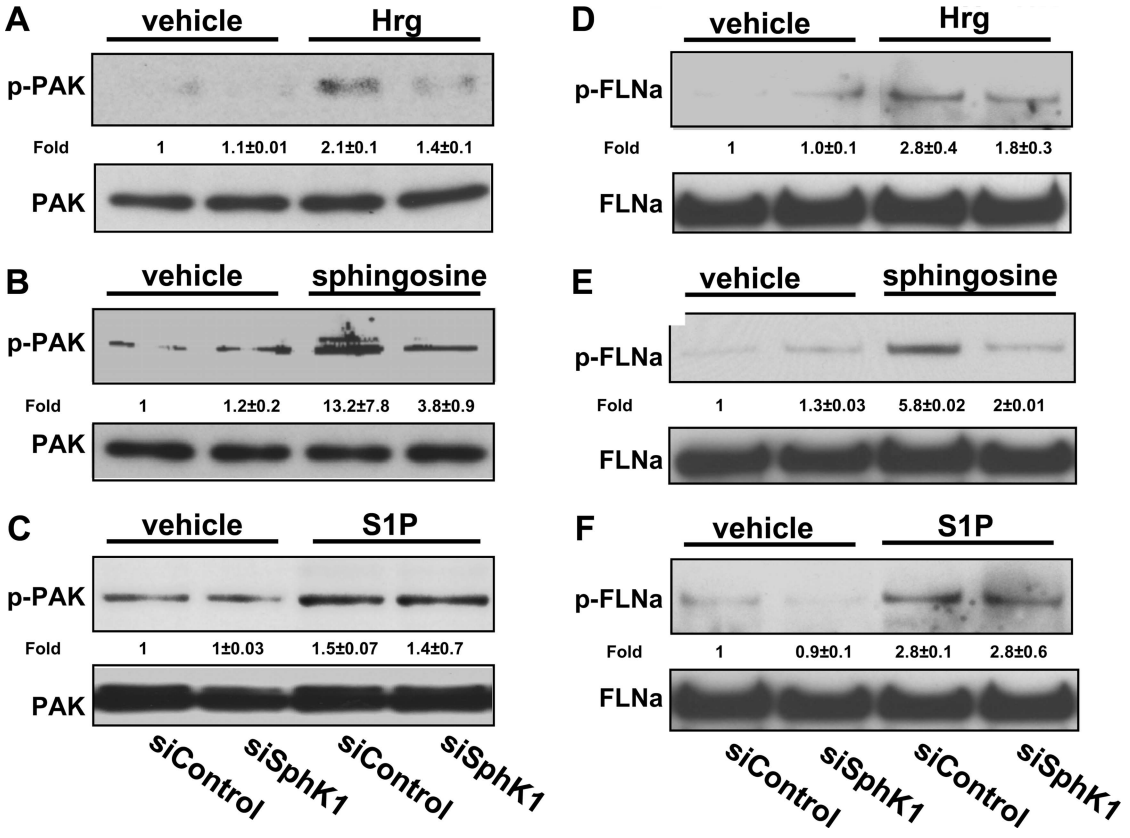


FIG. 5. SphK1 is required for Hrg- and sphingosine-induced phosphorylation of FLNa and PAK. A7 cells were transfected with control siRNA (siControl) or siRNA targeted to SphK1 (siSphK1), starved overnight, and then stimulated with Hrg (30 ng/ml), sphingosine (5 μM), or S1P (100 nM) for the indicated times. Equal amounts of lysates were resolved by SDS-PAGE and immunoblotted with the phospho-specific antibodies p-PAK1/2 (Thr432/Thr402) (A to C) or p-Ser2152 FLNa (D to F). Blots were reprobed for total PAK or FLNa to ensure equal loading and transfer. The upper band in panel B is PAK2 phosphorylated at Thr402. Densitometry was used to determine the change in stimulation of the samples compared to that of the untreated siControl, and values are expressed as the means ± standard errors of the means from three independent experiments.

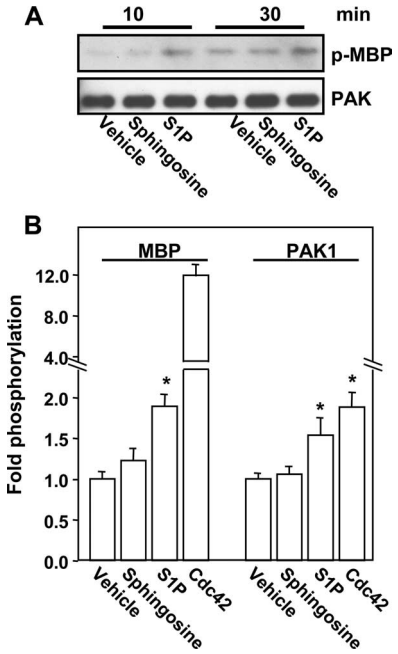


FIG. 6. S1P stimulates in vitro PAK1 activity. (A) Recombinant PAK1 was incubated with vehicle, sphingosine (2 μM), or S1P (2 μM) in the presence of ATP and MBP as substrates. Reactions were stopped at the indicated times, and phosphorylation of MBP was determined by immunoblotting with anti-phospho-threonine. Blots were stripped and reprobed with anti-PAK1 as a loading control. (B) Recombinant PAK1 activity was measured in duplicate samples with [γ-^{32}P]ATP in the absence or presence of vehicle, sphingosine, S1P, or 1 μg of GTPγS-loaded recombinant Cdc42 for 30 min. ^{32}P-labeled MBP and autophosphorylated PAK1 were determined with a phosphorimager after the proteins were resolved by SDS-PAGE and transblotted to nitrocellulose. Data are expressed as the change in stimulation of the vehicle compared to that of the untreated controls and are expressed as the means ± standard deviations from four independent experiments. *, $P < 0.005$.

Indeed, treatment of A7 cells with 100 nM S1P stimulated phosphorylation of both PAK1 and FLNa, which was unaltered by SphK1 downregulation (Fig. 5C and F).

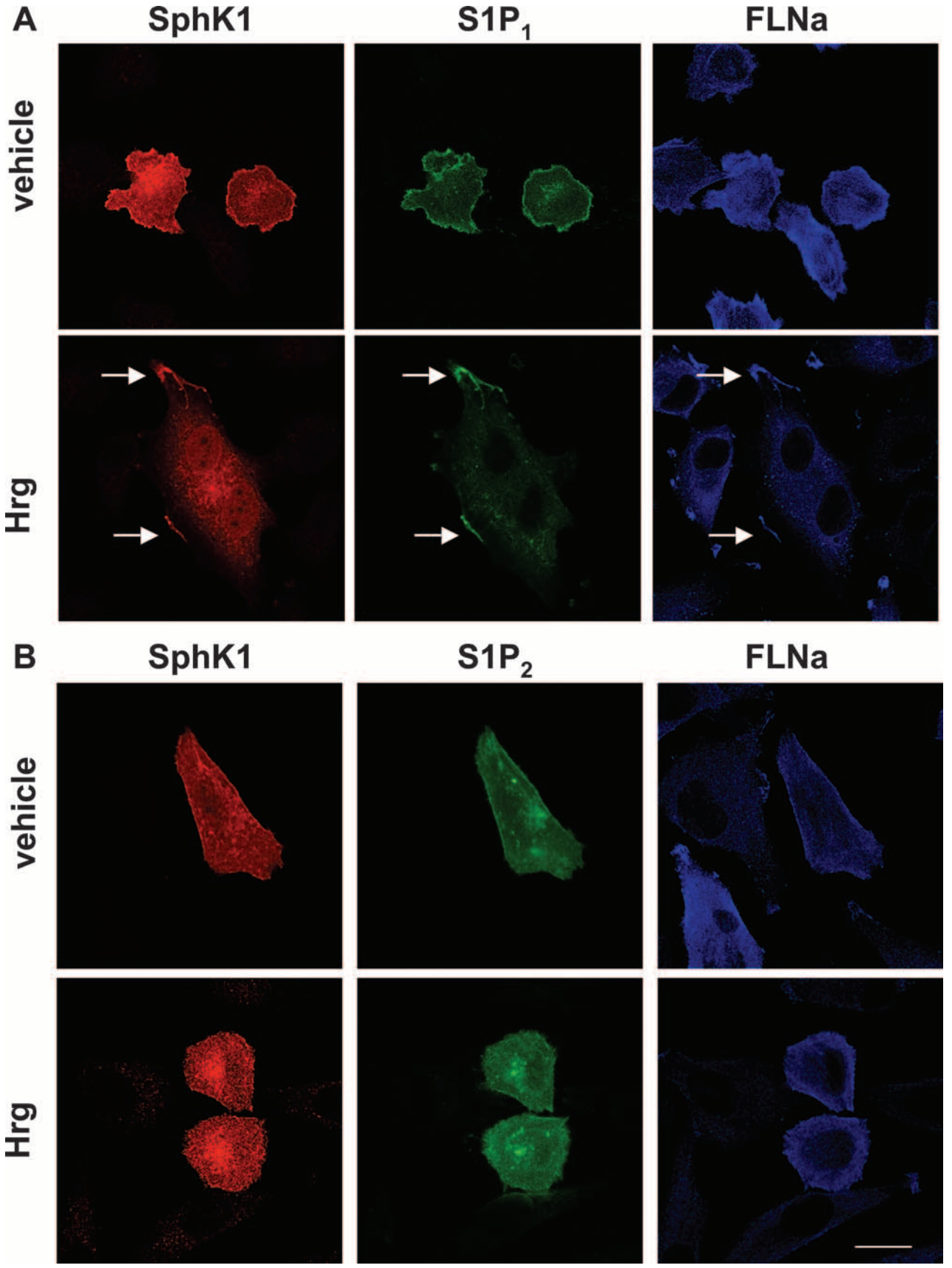
Previously, we found that high concentrations of sphingosine stimulated immunoprecipitated PAK1 activity and that *N*,*N*-dimethylsphingosine, a pan-SphK inhibitor, blocked its stimulation by Cdc42 (4). Given that PAK coimmunoprecipitated with FLNa (43), and we found that FLNa coimmunoprecipitated with SphK1, we next addressed the possibility that SphK1-produced S1P directly stimulated PAK1. To test this possibility, in vitro kinase assays using recombinant PAK1 produced in bacteria were performed. We found that low concentrations of S1P (2 μM), but not sphingosine, stimulated PAK1-mediated phosphorylation of MBP in a time-dependent manner (Fig. 6A), which was determined by immunoblotting with anti-phospho-threonine antibodies. Moreover, in vitro PAK1 kinase assays with [γ-^{32}P]ATP confirmed that a low concentration of S1P, but not sphingosine, stimulated PAK1-mediated incorporation of ^{32}P into MBP, albeit to a lesser extent than its known activator, Cdc42 (Fig. 6B). However, this concentration of S1P stimulated autophosphorylation of PAK1 to almost the same extent as that of Cdc42 (Fig. 6B). Similarly, we previously noted that when PAK1 was activated by sphingosine, it did not effectively phosphorylate MBP, which serves as an excellent substrate for Cdc42-activated PAK1 (4). This could be due to differences in substrate utilization by differentially phosphorylated PAK1 species (4) or, alternatively, due to differences in the ability of S1P-activated PAK1 to utilize only certain substrates.

FLNa links SphK1 to S1P$_1$ at membrane ruffles. In many cell systems, S1P has been shown to promote motility through the activation of cell surface S1P receptors. Intriguingly, S1P$_1$ and S1P$_2$ receptors have opposing actions on cytoskeletal rearrangements and cell movement (reviewed in references 38 and 41). Thus, it was of interest to examine the localization and involvement of these receptors in Hrg-induced lamellipodia formation. As expected, in unstimulated A7 cells, S1P$_1$ was evenly distributed at the plasma membrane (Fig. 7A), and its expression did not alter the localization of SphK1 or FLNa (compare Fig. 7A with Fig. 2A). However, when A7 cells were stimulated with Hrg, there was a striking concentration of S1P$_1$ in lamellipodia that colocalized with both SphK1 and FLNa (Fig. 7A). S1P$_1$ expression did not alter the number of lamellipodia in untreated or Hrg-stimulated cells (Fig. 7C). In contrast, Hrg did not induce colocalization of S1P$_2$ with either SphK1 or FLNa (Fig. 7B). Moreover, the expression of S1P$_2$ completely eliminated lamellipodia formation in untreated cells or in response to Hrg (Fig. 7C).

Colocalization of S1P$_1$ and SphK1 requires FLNa, as this was undetectable in FLNa-deficient M2 cells treated with Hrg (data not shown). That S1P$_1$, but not S1P$_2$, colocalizes in lamellipodia with SphK1 and FLNa suggests that these proteins may cooperate to promote lamellipodia formation in response to Hrg. Indeed, pretreatment of naïve A7 cells with VPC23019, an antagonist of S1P$_1$ and of S1P$_3$, significantly reduced sphingosine- and Hrg-induced lamellipodia formation (Fig. 7D). Conversely, the S1P$_2$-specific antagonist JTE-013 had no effect (Fig. 7D). Together, these data confirm the importance of localized S1P produced by SphK1 at lamellipodia and signaling thereafter through S1P$_1$ to locally influence the dynamics of actin polymerization and to potentiate lamellipodia formation and cell movement.

DISCUSSION

SphK1 and its product S1P play significant roles in regulating actin cytoskeleton dynamics in a variety of cell types. Many chemoattractants and growth factors stimulate motility in a SphK1-dependent manner and transactivate S1P receptors (11, 38). Here we show that SphK1 physically associates with the actin binding protein FLNa in a physiologically relevant manner. SphK1 interacted with a C-terminal portion of FLNa consisting of the anti-parallel β-sheet repeats 22 to 24 (Fig. 1A) in a yeast two-hybrid screen and in vitro and in vivo affinity precipitation assays. Moreover, we found that endogenous SphK1 physically associated with endogenous FLNa in mammalian cells and that the two proteins colocalized at lamellipodia. A recent study demonstrated that SphK1 interacts with filamentous actin in stimulated macrophages and that its localization to membrane ruffles is coordinately regulated with actin cytoskeletal dynamics (19). However, it is unlikely that actin is involved in the interaction between SphK1 and Ct-FLNa as the latter lacks the FLNa actin binding domain (Fig.


A
SphK1
S1P1
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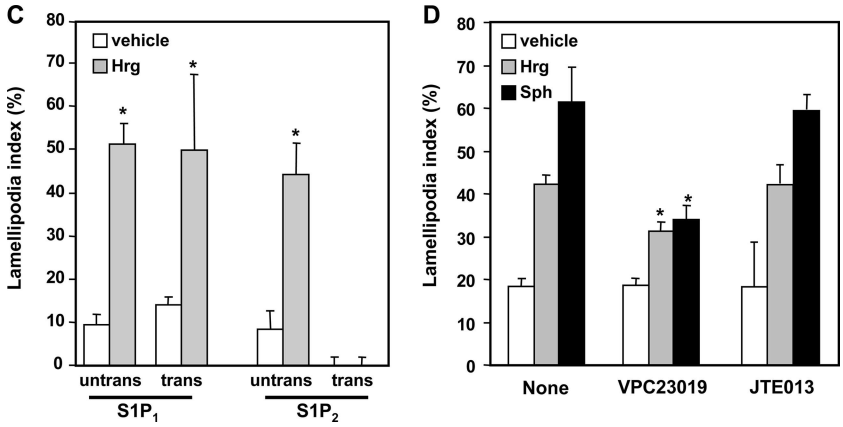

FIG. 7. Colocalization of SphK1 and S1P$_1$, but not S1P$_2$, induced by Hrg at lamellipodia. A7 cells grown on glass coverslips were transfected with V5-SphK1 and green fluorescent protein (GFP)-S1P$_1$ (A) or GFP-S1P$_2$ (B). After 48 h, the cells were serum starved overnight and then treated with vehicle or Hrg (30 ng/ml) for 30 min. Cells were then fixed; stained for SphK1 (red), FLNa (blue), and either S1P$_1$ or S1P$_2$ (green); and visualized by confocal microscopy. Bar, 10 μm. (C) Fields in panels A and B were scored for the number of nontransfected (untrans) and neighboring transfected cells (trans) exhibiting actin-positive lamellipodia, and the lamellipodia index was expressed as the mean ± standard error of the mean (SEM). *, $P < 0.01$. Data are representative of at least three independent experiments. (D) A7 cells were serum starved; pretreated with vehicle, VPC 23019 (10 μM), or JTE013 (1 μM) for 30 min; and then exposed to vehicle, Hrg (30 ng/ml), or sphingosine (5 μM) for an additional 30 min. Cells were fixed, stained for actin, and visualized by fluorescent microscopy. The percentage of lamellipodia-positive cells was calculated, and the lamellipodia index was expressed as the mean ± SEM. *, $P < 0.01$. Data are representative of at least three independent experiments.

1A). Rather, our data indicate that FLNa acts as a linker between SphK1 and filamentous actin.

FLNa-dependent translocation of SphK1 to lamellipodia allows this cytosolic enzyme access to its lipid substrate, sphingosine, resulting in the spatially restricted formation of S1P and polarized secretion. Spatially restricted secretion of S1P is particularly important for oriented cell movement, as extracellular S1P can potentially simultaneously stimulate cell migration through S1P$_1$ and S1P$_3$ (23, 26, 32) and inhibit it through its repellant receptor, S1P$_2$ (14, 37, 41). However, we found that S1P$_1$, but not S1P$_2$, colocalized with SphK1 and FLNa at lamellipodia in response to cell activation, and thus, FLNa appears to link both SphK1 and S1P$_1$ together at the leading edge, ensuring that S1P$_1$ but not S1P$_2$ is activated by the ligand S1P. This could serve to amplify autocrine signals to control gradient sensing and to direct cell orientation along chemotactic gradients (Fig. 8). A similar autocrine amplification system was recently reported in neutrophils, where ATP was released at the leading edge of the cells in response to chemoattractants to initiate and accelerate directional movement through P2Y2 and A3 receptors (5). Intriguingly, it was recently reported that P2Y2 receptors also bind FLNa, an interaction required for aortic smooth muscle cell migration toward nucleotides (45), suggesting a general role for FLNa in promoting chemotaxis through the localization of G protein-coupled receptors.

How do SphK1, FLNa, and PAK1 enhance each others' actions and cooperate to promote lamellipodia formation and forward movement? FLNa, which binds SphK1 and is essential for its activation by Hrg, is a scaffold for many other signaling proteins, including Rho family GTPases, GEFs, GAPs, and downstream effectors, including PAK1, whose switching on and off coordinate with FLNa actions to assemble and/or disassemble actin filaments (28). PAK1, a downstream effector of Rac and Cdc42, binds to FLNa within repeat 23 (43), which is within the C-terminal fragment of FLNa that binds SphK1 (Fig. 1A), suggesting that these proteins bind to FLNa in close proximity or at overlapping sites. PAK1 phosphorylates FLNa, which in turn, contributes to the local stimulation of PAK1 activity and its targets in the cytoskeleton (43). We observed a similar role for SphK1, which like PAK1 (43) is colocalized with FLNa to lamellipodia and is required for both PAK1 activation and the consequent FLNa phosphorylation. Hence, SphK1 is part of a feed-forward mechanism that is important for polarized lamellipodia formation and directed cell movement. Several potential pathways can be envisioned by which S1P formed by SphK1 could be involved in this activation/amplification loop, which are not mutually exclusive (Fig. 8). First, it is well known that the activation of S1P$_1$ by S1P stimulates Rac in a $G_{\alpha i}$-dependent and phosphatidylinositol 3-kinase-dependent manner (30, 36), and it has been suggested that the phosphorylation of S1P$_1$ at threonine 236 by Akt is critical in transducing S1P signals to Rac activation and lamellipodia formation (21, 22). Alternatively, Akt can also directly phosphorylate PAK1 and activate it through a GTPase-independent mechanism (47). The requirement of SphK1 for the activation of PAK1 might also explain the original observation that high concentrations of sphingosine, the substrate of SphK1, stimulated PAK1 by a GTPase-independent pathway (4, 6, 35). Moreover, stimulation of PAK1 by sphingosine was effectively suppressed by the SphK1 inhibitor *N*,*N*-dimethylsphingosine (4). Coupled with our observation that the knockdown of SphK1 inhibits sphingosine-induced activation of PAK1 in intact cells, our data suggest that SphK1-catalyzed phosphorylation of sphingosine to S1P is responsible for the effects observed for sphingosine on PAK1 activation (4). Indeed, we found that low concentrations of S1P, but not sphingosine, directly stimulated recombinant PAK1 in vitro. This is the first example of a potential direct intracellular target for S1P and suggests that S1P acts in a dual manner, by activating PAK1 intracellularly and by "inside-out signaling" through

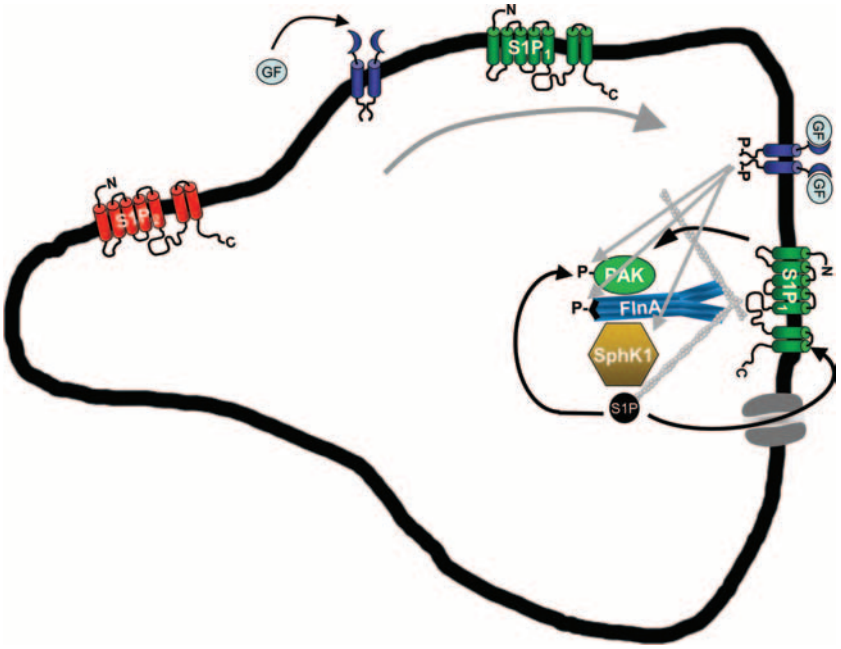

FIG. 8. Proposed roles for the concerted actions of SphK1, S1P, and $S1P_1$ with FLNa and PAK1 at lamellipodia to promote motility. We propose that the activation of growth factor receptors induces the FLNa-dependent recruitment of SphK1 and PAK1 to nascent lamellipodia and also initiates the signaling pathways leading to their phosphorylation. Activated SphK1 produces S1P that can further enhance PAK1 phosphorylation by direct stimulation. In addition, S1P can also act in an autocrine manner to activate $S1P_1$, and this autocrine loop potentiates motility as G_i-coupled $S1P_1$ can activate Rac, which in turn activates PAK1. The FLNa-dependent localization of $S1P_1$, but not $S1P_2$, to lamellipodia further amplifies motility signaling and averts the locally released S1P from inhibiting cell migration through $S1P_2$ (see Discussion for more details). Note that other components associated with FLNa that are relevant to actin filament remodeling are not shown.

$S1P_1$ and its downstream target Rac to promote cell migration. Taken together, our data indicate that PAK1 is an important mediator of the effects of S1P on cytoskeletal rearrangements and motility. Once PAK is activated, it can turn off inhibitory Rho signaling, thus enhancing its own phosphorylation and activity (1). In addition to FLNa, PAK1 can also regulate actin reorganization through other substrates, including LIM kinases and P41-ARC, a subunit of the Arp2/3 complex (3). Taking these findings together, this study suggests that tridirectional communication between PAK1-FLNa-SphK1 in a feed-forward mechanism is critical for the regulation of the dynamics of the actin cytoskeleton at the leading edge of the cell.

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