Role of Sphingosine Kinase 2 in Cell Migration toward Epidermal Growth Factor*

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Sphingosine kinase (SphK) is a highly conserved enzyme found in organisms as diverse as mammals, flies, worms, slime mold, yeast, and plants that catalyzes the phosphorylation of sphingosine to generate sphingosine 1-phosphate (S1P), a potent lipid mediator (1). As a specific ligand for a family of five specific G protein-coupled receptors that regulate cytoskeletal rearrangements and cell motility. Whereas many growth factors stimulate SphK1, much less is known of the regulation of SphK2. Here we report that epidermal growth factor (EGF) stimulated SphK2 in HEK 293 cells. This is the first example of an agonist-dependent regulation of SphK2. Chemotaxis of HEK 293 cells toward EGF was inhibited by N,N-dimethylsphingosine, a competitive inhibitor of both SphKs, implicating S1P generation in this process. Down-regulating expression of SphK1 in HEK 293 cells with a specific siRNA abrogated migration toward EGF, whereas decreasing SphK2 expression had no effect. EGF contributes to the invasiveness of human breast cancer cells, and EGF receptor expression is associated with poor prognosis. EGF also stimulates SphK2 in MDA-MB-453 breast cancer cells. Surprisingly, however, down-regulation of SphK2 in these cells completely eliminated migration toward EGF without affecting fibronectin-induced haptotaxis. Our results suggest that SphK2 plays an important role in migration of MDA-MB-453 cells toward EGF.

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Materials—[γ-32P]ATP (3000 Ci/mmol) was purchased from Amer sham Biosciences. SIP and N,N-dimethylsphingosine (DMS) were from Biomol (Plymouth Meeting, PA). Serum and medium were from BioFl uids (Rockville, MD). EGF was obtained from Invitrogen. Collagen type I and fibronectin were purchased from BD Biosciences, collagen type IV was from Collagen Corp. (Palo Alto, CA). Polycarbonate filters were from Poretics (Livermore, CA). Rabbit polyclonal antibodies were raised against unique SphK1 and SphK2 peptide sequences (EPFPWSK-PQQMPPEEPL and QALHIQRLRPKPEARPR, respectively, Biosynthesis, Lewisville, TX). Antiserum was purified on a protein A column followed by affinity purification on a SulfoLink gel conjugated with the antigenic peptide according to the manufacturer’s instructions (Pierce).

EXPERIMENTAL PROCEDURES
was used to prepare catalytically inactive SphK1 (G82D mutation) as described (27) and catalytically inactive SphK2 (G213E mutation primers: forward, 5′-GTTCTGGAAGCCTGTTTACAGG-3′; and reverse, 5′-ATTTGGATAGCTTGGCAGAC-3′). All sequences were verified by DNA sequencing.

Cell Culture and Transfection—Human embryonic kidney cells (HEK 293, ATCC CRL-1753) and human MDA-MB-453 breast cancer cells (ATCC HB-8506) were cultured in high glucose Dulbecco’s modified Eagle’s medium containing 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine supplemented with 10% fetal bovine serum. Cells were transfected with Lipofectamine Plus (Invitrogen). In some experiments, SphK2 expression was also down-regulated by transfection with siRNA from Ambion (sense, 5′-GAGCUGCAAGGGCUCCUUTTCAAGG-3′; antisense, 5′-AGCCTTCTTAAAAAAGGCTGCTTCCACACTCCACCTTGATTGGAACAGGGCAGCCTTTTGGAAA-3′). Cells were transfected with OligofectAMINE (Invitrogen).

SphK2 expression was also down-regulated with sequence-specific siRNAs. siRNA for human SphK1 (GGGCAAGGCUCCUG-CAGGCUCdTT and GAGCGCAAGGGCUCCUUTTCAAGG-3′ and control siRNA were from Qiagen. Cells were transfected with OligofectAMINE (Invitrogen).

Sphingosine Kinase Activity—Cells were lysed by freeze-thawing in SphK buffer (20 mM Tris (pH 7.4), 20% glycerol, 1 mM 2-mercaptoethanol, 1 mM EDTA, 5 mM sodium orthovanadate, 40 mM fluoride, and 0.5 mM 4-deoxyxylose). Lysates were centrifuged at 700 × g for 10 min to remove unbroken cells and then at 100,000 × g for 60 min to obtain cytosol and membrane fractions.

SphK1 activity was determined in the presence of 50 μM sphingosine and [γ-32P]ATP (10 μCi, 1 mM) containing MgCl2 (10 mM) in 0.25% Triton X-100, which inhibits SphK2, as described previously (23). SphK2 activity was determined with sphingosine added as a complex with 4 mg/ml BSA and [γ-32P]ATP in the presence of 1 μM KCl, conditions in which SphK2 activity is optimal and SphK1 strongly inhibited (23). SphK2 activity was also down-regulated by transfection with siRNA from Ambion (sense, 5′-GAAUUGCCGUCGGCUUCAUUA-3′; antisense, 5′-AGAAGAA-CCAGCGCAUCCGT-3′).

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Western Blotting—Equal amounts of proteins were separated by 10% SDS-PAGE and then transblotted to nitrocellulose. Blots were probed with rabbit polyclonal antibodies to SphK1 and SphK2 (1:1000), stripped, and re-probed with anti-calcxin, anti-tubulin, anti-PDI, or anti-EGFR antibodies (Santa Cruz Biotechnology) as loading controls. Immunoreactive bands were visualized by enhanced chemiluminescence and apparent horseradish peroxidase-conjugated secondary antibody (Jackson ImmuneResearch) and SuperSignal West Pico Chemiluminescent Substrate (Pierce).

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

Measurement of Intracellular S1P Levels—HEK 293 cells (8 × 106) were transfected with vector or SphK2 in poly-D-lysine-coated 10-cm plates. After 24 h, cells were serum-starved for 24 h and then stimulated with or without EGF (100 ng/ml) for 30 min. Cells were washed twice in ice-cold phosphate-buffered saline and scraped in 1 ml of methanol containing 2.5 μl of concentrated HCl. S1P was isolated and measured as previously described (28).

Subcellular Fractionation—Cells were suspended in buffer containing 20 mM Hepes (pH 7.4), 10 mM KCl, 2 mM MgCl2, 1 mM EDTA, 10 μg/ml each of aprotinin and leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.25% sodium deoxycholate. Subcellular fractionation was performed by sequential centrifugation, essentially as described (29). Briefly, cells were homogenized at 4 °C with a Dounce homogenizer and then centrifuged at 1,000 × g for 5 min at 4 °C to remove unbroken cells and nuclei. The postnuclear supernatants were further centrifuged for 10 min at 5,000 × g, and the pellets were resuspended in the same buffer. The 5,000 × g supernatants were centrifuged for 15 min at 17,000 × g, and the P2 pellets containing intracellular membrane fraction containing endoplasmic reticulum (endosome) and Golgi) were re-suspended in the same buffer. The remaining supernatants were centrifuged at 100,000 × g for 1 h to obtain cytosol (S) and pelleted plasma membrane fractions (PM).

Cell Migration Assays—Chemotaxis was measured in a modified Boyden chamber using polycarbonate filters (25 × 80 mm, 12-μm pore size) coated with collagen type I (50 μg/ml in 5% acetic acid) for HEK 293 cells and collagen type IV (10 μg/ml) for MDA-MB-453 cells (30). Briefly, chemoattractants were added to the lower chambers, and cells (5 × 104 per well) were added to the upper chambers. At the indicated times, nonmigratory cells on the upper membrane surfaces were mechanically removed and the cells that traversed and spread on the lower surfaces of the filters were fixed and stained with Diff-Quik. Migratory cells were counted using a microscope with a 10× objective.

Statistical Analysis—Experiments were repeated at least three times with consistent results. For each experiment, data from triplicate samples were calculated and expressed as means ± S.D. Statistics were performed using SigmaStat statistical software version 2.0.

RESULTS

EGF Activates SphK2—EGF contributes to the progression, invasion, and maintenance of the malignant phenotype in human breast cancers. Recently, it was suggested that intracellular metabolism of S1P may play an important role in EGF-directed cell migration (31). Because SphK1 and SphK2 are key enzymes in regulating S1P levels, it was important to determine their roles in motility of cells toward EGF. Although various growth factors, including PDGF, EGF, and VEGF, stimulate SphK1 and increase S1P levels (reviewed in Ref. 1), their effects on SphK2 have not yet been investigated. To first determine whether SphK2 can be activated by EGF, HEK 293 cells were transiently transfected with SphK2. These cells had significantly increased SphK2 activity compared with vector transfectants, and thus the ectopically expressed SphK2 contributed most of the measured activity in the cytosol and membrane fractions. Treatment with EGF markedly stimulated membrane-associated SphK2 (Fig. 1A). Because previous studies suggest that growth factors induce translocation of SphK1 to the plasma membrane (5, 32), we next examined whether EGF also induced changes in the distribution of ectopically expressed SphK2. However, localization of SphK2 was not affected by EGF (Fig. 1B).

It was important to determine whether EGF-stimulated SphK2 activity measured in vitro resulted in increased intracellular formation of S1P. Similar to previous reports demonstrating that overexpression of SphK1 results in only a modest increase in cellular S1P that does not correlate with the large-fold increase in SphK1 activity measured in vitro (33), overexpression of SphK2 caused a 2-fold increase in S1P levels (Fig. 1, C and D). Treatment with EGF significantly increased S1P levels in vector as well as in SphK2 expressing cells by 2- and 3.5-fold, respectively (Fig. 1C).

Because the localization of SphK2 appears to be cell type-specific (25), we examined its localization in HEK 293 cells after subcellular fractionation by differential centrifugation and Western blot analysis with anti-SphK2 antibodies. In lysates from cells expressing V5-tagged SphK2, the SphK2 antibody detected a single protein with the same molecular mass of ~70 kDa that was also detected by anti-V5 antibody (Fig. 1E). Although expression of SphK2 was enriched in the plasma membrane fraction, it was also present at significant levels in...
P2 (mitochondria), P3 (endoplasmic reticulum and Golgi), and cytosol fractions (Fig. 1E). In agreement with previous studies (25), there was little or no detectable expression of SphK2 in nuclear fractions of HEK 293 cells overexpressing SphK2 (data not shown).

Although overexpression of proteins is a useful approach, an associated caveat is that the ectopically expressed protein does not always exactly mimic the localization and functions of its endogenous counterpart particularly when overexpressed to a very high level. Although all experiments with overexpression of SphK2 were restricted to moderate increases in SphK2 expression (~10-fold compared with 100-fold in previous studies (23)), it was important to examine localization of endogenous SphK2. In parental HEK 293 cells, endogenous SphK2 was also expressed mainly in the plasma membrane fraction and was present at lower levels in the P2 and P3 fractions and at barely detectable levels in the cytosol (Fig. 1F).

**EGF Stimulates Endogenous SphK2 without Affecting Localization**—Next, we determined whether EGF activates endogenous SphK1 and/or more importantly, endogenous SphK2. Both SphK1 and SphK2 are expressed in HEK 293 cells and can be qualitatively distinguished by differences in enzymatic activities measured when the substrate sphingosine is added as a BSA complex in the presence of 1M KCl or when added in a micellar form with Triton X-100 (23). Whereas Triton X-100 strongly inhibits SphK2 but not SphK1, 1M KCl inhibits SphK1 but not SphK2.

**Role of SphK in EGF-induced Chemotaxis**

![Figure 1](image1.png) **Fig. 1. EGF activates SphK2 and increases intracellular S1P levels.** A, HEK 293 cells transiently transfected with V5-SphK2 were stimulated with vehicle or EGF (100 ng/ml) for 5 min and lysed, and membrane and cytosol fractions were prepared by centrifugation at 100,000 x g. SphK2 activity was measured with sphingosine added as a BSA complex in the presence of 1M KCl. Data are means ± S.E. of three independent experiments, each performed in duplicate. *p < 0.01 by Students t test. B, HEK 293 cells expressing V5-SphK2 were stimulated with EGF (100 ng/ml) for the indicated times and equal amounts of proteins from membrane fractions (M) and supernatants (S) separated by SDS-PAGE and immunoblotted with anti-SphK2 antibody. Blots were stripped and reprobed with anti-calnexin and anti-tubulin antibodies as loading controls for the membrane and cytosolic fractions, respectively. C, vector and SphK2 transfectants were treated with EGF (100 ng/ml) and cellular S1P levels measured. Data are means ± S.E. from three independent experiments, each performed in duplicate. A representative TLC showing formation of S1P is shown in D, E and F, subcellular localization of SphK2. Lysates from HEK 293 cells transfected with SphK2-V5 (E) or from parental cells (F) were subcellularly fractionated into P2 (mitochondria), P3 (endoplasmic reticulum and Golgi), PM (plasma membrane), and cytosol (S), as described under “Experimental Procedures.” Proteins (5 μg in E and 40 μg in F) were resolved by SDS-PAGE and immunoblotted with anti-SphK2 antibodies. Blots were stripped and reprobed with anti-calnexin and anti-tubulin antibodies as loading controls for membrane and cytosol fractions, respectively.

![Figure 2](image2.png) **Fig. 2. EGF activates endogenous SphK1 and SphK2.** A, naive HEK 293 cells were treated with EGF (100 ng/ml) for the indicated times, and SphK activity was measured in cell lysates with 50 μM sphingosine added in 0.5% Triton X-100 (dashed line) or as a complex with BSA (dotted line). Data are means ± S.E. of three independent experiments, each performed in duplicate. B, homogenates of HEK 293 cells expressing SphK2 treated with EGF (100 ng/ml) for the indicated times were centrifuged at 100,000 x g and SphK2 activity measured in membrane and cytosol fractions with 50 μM sphingosine added as a BSA complex in the presence of 1M KCl. C, equal amounts of proteins (40 μg) from the membrane (M) and cytosol fractions (S) in B were separated by SDS-PAGE and immunoblotted with anti-SphK2 antibodies. Blots were stripped and reprobed with anti-calnexin and anti-tubulin antibodies as loading controls for the membrane and cytosol fractions, respectively.
SphK2 (23). SphK1 activity in lysates from naive HEK 293 cells measured in the presence of Triton X-100 was rapidly increased by EGF, reaching a maximum within 1 min (Fig. 2A). EGF stimulation of sphingosine phosphorylating activity, as measured with sphingosine complexed with BSA (a measure of SphK1 plus SphK2 activities), appeared somewhat slower, with a maximum increase at 5–10 min (Fig. 2A), and was higher than when measured with sphingosine in Triton X-100 micelles. To examine whether EGF activates and translocates endogenous SphK2, cytosolic and membrane fractions were prepared from naive and EGF-stimulated HEK 293 cells. Similar results were obtained in at least three independent experiments. B, HEK 293 cells transiently transfected with vector, SphK1, SphK2, catalytically inactive SphK1 (SphK1G82D) or SphK2 (SphK2G213E) and chemotaxis was measured in the absence (open bars) or presence (filled bars) of EGF (100 ng/ml). The data are the means ± S.D. of three individual wells.

Fig. 3. SphK1, not SphK2, is important for EGF-induced motility of HEK 293 cells. A, EGF-induced chemotaxis is inhibited by DMS. HEK 293 cells were pretreated for 20 min without or with DMS (10 μM), allowed to migrate toward vehicle (open bars) or 100 ng/ml EGF (filled bars) for 4 h and chemotaxis was measured. The average number of migrating cells in four random fields was determined and data are means ± S.D. of triplicate determinations. B, HEK 293 cells transiently transfected with vector, SphK1, SphK2, catalytically inactive SphK1 (SphK1G82D) or SphK2 (SphK2G213E) and chemotaxis was measured in the absence (open bars) or presence (filled bars) of EGF (100 ng/ml). The data are the means ± S.D. of three individual wells.

Fig. 4. Down-regulating SphK1 suppresses migration of HEK 293 cells toward EGF. A, HEK 293 cells were transfected with control siRNA (siControl) or siRNA targeted to SphK1 (siSphK1). RNA was isolated and mRNA levels of SphK1 and SphK2, and 18 S RNA were determined by quantitative real-time PCR. B, HEK 293 cells transfected with siControl or siSphK1 were lysed, and membrane and cytosol fractions were prepared by centrifugation at 100,000 × g. SphK1 activity was measured in the presence of 0.25% Triton X-100. C, equal amounts of proteins (40 μg) from 100,000 × g supernatants (Cytosol) and pellets (Membrane) were immunoblotted with anti-SphK1. Lysates from untransfected MCF7 cells (40 μg) and MCF7 cells overexpressing AU1-tagged SphK1 (2 μg) were included as controls. Blots were stripped and reprobed with anti-calnexin and anti-tubulin to confirm equal loading. Duplicate samples were immunoblotted with anti-AU1 antibody. The asterisk indicates nonspecific immunostained bands. Membrane and cytosol fractions (5 μg) from HEK 293 cells transiently transfected with untagged SphK1 were also immunoblotted with anti-SphK1 antibody to indicate the mobility of SphK1. D, HEK 293 cells were transfected with siControl or siSphK1 and proteins from 100,000 × g supernatants (S) and pellets (Membrane) immunoblotted with anti-SphK2. E, HEK 293 cells were transfected with siControl or siSphK1, serum-starved overnight, then allowed to migrate for 6 h toward medium (None), EGF (10 ng/ml), or serum (20%) in a modified Boyden chamber assay. Data are expressed as mean number of cells per field ± S.D. of triplicate determinations.

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with a SphK2-specific antibody, was present in the membrane fraction and was not cytosolic (Fig. 2C). Similar to ectopically expressed SphK2, EGF did not induce redistribution of endogenous SphK2 protein (Fig. 2C).

SphK1 but Not SphK2 Is Involved in EGF-induced Migration of HEK 293 Cells—As a first approach to examining the involvement of SphKs in EGF-induced migration, we utilized DMS, a potent and specific inhibitor of SphK1 and SphK2 (22, 23, 34). DMS markedly decreased migration of HEK 293 cells toward EGF (Fig. 3A). Overexpression of SphK1 increased EGF-induced migration by nearly 2-fold (Fig. 3B). In contrast, SphK2 expression had no effect on migration toward EGF (Fig. 3B), even though its activity was significantly stimulated by EGF (Figs. 1 and 2).

Cells were transfected with catalytically inactive SphK mutants to further examine the involvement of specific SphK isoforms. Both SphK1 and SphK2 have an ATP binding sequence (SGDGX7-21K(R)) present within their conserved C2 domains (23, 35), and a single point mutation of the second conserved glycine residue in this motif to aspartate, SphK1G82D, has been shown to block SphK1 activation by various agonists and some of its functions (36, 37). Similarly, site-directed mutagenesis of the equivalent residue in SphK2 (G213E) resulted in a complete loss of sphingosine-phosphorylating activity (data not shown). Although there were no significant differences in expression of these mutants, transient expression of SphK1G82D in HEK 293 cells decreased cell migration toward EGF (Fig. 3B). In contrast, expression of SphK2G213E had no effect on EGF-induced chemotaxis (Fig. 3B). Together, these results suggest that, although EGF stimulates both SphK1 and SphK2 in HEK 293 cells, only SphK1 seems to have a role in their migration toward EGF.

A molecular approach was then used to substantiate the involvement of specific SphK isoforms in EGF-induced chemotaxis. To this end, we utilized small interfering RNA (siRNA) targeted to a specific sequence of SphK1 that has been successfully used to down-regulate SphK1 protein and activity (38–43). In agreement, down-regulation of SphK1 with siRNA not only significantly reduced SphK1 mRNA (Fig. 4A), but also SphK1 activity (Fig. 4B) and protein levels (Fig. 4C). Moreover, siSphK1 had no effect on expression of SphK2 (Fig. 4A) or protein, which was mainly membrane-associated (Fig. 4D). Importantly, down-regulation of endogenous SphK1 also significantly decreased migration toward EGF.

FIG. 5. Effect of down-regulating SphK2 expression on motility. A, HEK 293 cells were transfected with pSilencer-Vector or pSilencer-SphK2. After 24 h, mRNA levels of SphK1 and SphK2, and 18 S RNA were determined by quantitative real-time PCR. B, duplicate cultures were lysed and centrifuged at 100,000 × g to isolate membrane (M) and cytosol (S) fractions. Equal amounts of proteins were analyzed by immunoblotting with anti-SphK2 or anti-SphK1 antibodies. Blots were stripped and re-probed with anti-calnexin and anti-tubulin antibodies as loading controls for membrane and cytosolic fractions, respectively. C, SphK2 activity was measured in fractions from B with sphingosine added as a BSA complex in the presence of 1 M KCl and SphK1 activity was measured with sphingosine added as Triton X-100 mixed micelles. *, p < 0.01. D, HEK 293 cells were transfected with pSilencer-vector (open bars) or pSilencer-SphK2 (filled bars), serum-starved overnight, and then treated with EGF (10 ng/ml) for the indicated time periods. Cells were lysed and SphK2 activity measured in the presence of BSA and 1 M KCl. E, HEK 293 cells were transfected with pSilencer-vector (open bars) or pSilencer-SphK2 (filled bars), serum-starved overnight, then allowed to migrate for 4 h toward medium (None), EGF (10 ng/ml), or serum (20%) in a modified Boyden chamber assay. Data are expressed as mean number of cells per field ± S.D. of triplicate determinations.

FIG. 6. Down-regulation of SphK1 reduces migration of MDA-MB-453 cells toward EGF. A, MDA-MB-453 cells were transfected with control siRNA or siRNA targeted to SphK1, serum-starved overnight, then allowed to migrate toward vehicle (None), EGF (100 ng/ml), serum (20%), or S1P (1 μM). Data are expressed as mean number of cells per field ± S.D. of triplicate determinations. B, after 48 h, mRNA levels of SphK1 and SphK2, and 18 S RNA from duplicate cultures were determined by quantitative real-time PCR.
Fig. 7. Down-regulation of SphK2 reduces migration of MDA-MB-453 cells toward EGF. A, MDA-MB-453 cells were transfected with pSilencer-Vector or pSilencer-SphK2. After 24 h, cultures were lysed and centrifuged at 100,000 \( \times g \) to isolate membrane (M) and cytosol (S) fractions. Equal amounts of proteins were analyzed by immunoblotting with anti-SphK2 antibodies. Blots were stripped and re-probed with anti-calnexin as loading control. B, SphK2 activity was measured in fractions from A with sphingosine added as a BSA complex in the presence of 1 M KCl. C, MDA-MB-453 cells were transfected with pSilencer-Vector (open bars) or pSilencer-SphK2 (filled bars), allowed to migrate for 24 h toward vehicle (None), serum (20%), EGF (100 ng/ml), S1P (1 \( \mu \)M), or fibronectin (20 \( \mu \)g/ml), and chemotaxis was determined. The data are the means \( \pm \) S.D. of three individual wells. D, MDA-MB-453 cells were homogenized and subcellular fractions prepared: internal membranes (IM), endoplasmic reticulum, and Golgi) and plasma membranes (PM) and cytosol (S) (100,000 \( \times g \) for 1 h, pellet and supernatant fractions, respectively). Equal amounts of proteins (40 \( \mu \)g) were resolved by SDS-PAGE and immunoblotted with anti-SphK2 or anti-SphK1, as indicated. Blots were stripped and re-probed with anti-calnexin, anti-EGFR, or anti-tubulin as specific organelle markers. Similar results were obtained in two additional experiments. E and F, naive MDA-MB-453 cells were stimulated with EGF (10 ng/ml) for the indicated times, lysed and endogenous SphK2 (E) and SphK1 (F) activity measured. Data are means \( \pm \) S.D. of triplicate determinations. 

EGF, but did not affect migration toward serum (Fig. 4E). A similar approach was used to examine the functions of endogenous SphK2. Endogenous SphK2 expression was significantly down-regulated by transfection with pSilencer siSphK2, as determined by real-time PCR analysis, without affecting SphK1 mRNA (Fig. 5A). SphK2 protein (Fig. 5B) and its enzymatic activity were also markedly reduced by siSphK2 (Fig. 5C), but it had no effects on SphK1 protein expression (Fig. 5B) or activity (Fig. 5C). Importantly, in agreement with the observation that EGF activated SphK2 in HEK 293 cells (Figs. 2B) this stimulation was prevented by knockdown of SphK2 expression (Fig. 5D). However, down-regulation of SphK2 had no effect on either EGF- or serum-induced chemotaxis (Fig. 5E).

Both SphK1 and SphK2 Are Required for EGF-directed Motility of MDA-MB-453 Cells—EGF is an important growth factor associated with the development and spread of breast cancer. In addition to its mitogenic effects, EGF has been shown to be motogenic for breast cancer cells and to induce chemotaxis (46, 47). In agreement with previous studies (48), we found that chemotaxis of MDA-MB-453 human breast cancer cells was stimulated by EGF and S1P (Fig. 6A). Similar to HEK 293 cells, down-regulation of SphK1, which specifically decreased its expression in MDA-MB-453 cells (Fig. 6B), markedly reduced migration of these cells toward EGF (Fig. 6A) but did not inhibit migration toward exogenous S1P (Fig. 6A). As in HEK 293 cells, transfection of MDA-MB-453 cells with pSilencer-SphK2 but not pSilencer-vector significantly decreased SphK2 expression (Fig. 7A) and enzymatic activity (Fig. 7B). Surprisingly, however, in striking contrast to the effect on HEK 293 cells, knocking down expression of SphK2 in MDA-MB-453 cells also drastically reduced their migration toward EGF (Fig. 7C). This was not a general inhibitory effect on migratory responses, because migration toward S1P, serum, and fibronectin was not significantly affected by down-regulation of SphK2.

Because of the different effects of down-regulation of SphK2 on EGF-induced migration of HEK 293 and MDA-MB-453 cells, it was of interest to compare its localization in these cells. However, similar to HEK 293 cells, SphK2 is highly expressed in the plasma membrane of MDA-MB-453 cells and present to a lesser extent in internal membranes (Fig. 7D). Moreover, in both types of cells, there was little expression of SphK2 in the cytosol, whereas SphK1 was mainly cytosolic. In addition, like in HEK 293 cells, EGF markedly stimulated the activity of endogenous SphK2 (Fig. 7E) as well as SphK1 (Fig. 7F) in MDA-MB-453 cells.

To exclude nonspecific off-target effects, SphK2 expression was also down-regulated with siRNA targeted to another region of the SphK2 sequence (Fig. 8). This siSphK2 also markedly reduced expression of SphK2 mRNA and protein, but not SphK1, in both MDA-MB-453 cells (Fig. 8, A and B) and HEK 293 cells (data not shown). As with down-regulation of SphK2 with pSilencer (Fig. 7C), this siSphK2 almost completely abolished EGF-induced migration of MDA-MB-453 cells, but did not alter migration toward fibronectin or serum (Fig. 8C). Importantly, siSphK2 had no effect on migration of HEK 293 cells toward EGF (Fig. 8D).

DISCUSSION

Abnormalities in EGFR expression and downstream signaling pathways contribute to the progression, invasion, and
pressed SphK2 was mainly cytosolic; in HeLa cells, it was
idly stimulated endogenous SphK2 activity. This is the first
Both in HEK 293 cells and MDA-MB-453 cells, EGF also rap-
expressed SphK2 and SphK2-dependent formation of S1P.

Because intracellular S1P levels are regulated not only by its
degradation but also by its synthesis catalyzed by SphK1 and
SphK2, it was important to examine whether they are acti-
dated to the plasma membrane, present at lower levels in inter-
nal membranes, and nearly undetectable in the cytosol. These
results are consistent with another report that SphK2 associ-
ates with the interleukin-12 receptor β1 at the plasma mem-
brane (51). It has been suggested that phosphorylation-induced
translocation of SphK1 to the plasma membrane, rather than
enhanced catalytic activity of this enzyme, is important for its
function (32, 52). However in this study, activation of SphK2 by
EGF, in contrast to SphK1, did not influence its membrane
localization, suggesting a different mechanism of regulation of
this isozyme by EGF.

Although it has been suggested that SphK1 and SphK2 have
opposing functions on cell growth and survival, to date, only
SphK1 has been linked to regulation of cell motility (20, 21, 27,
53–56). Moreover, only SphK1 and not SphK2 was required for
migration of mast cells toward increasing concentrations of
antigen (21) and for C5a receptor-dependent chemotaxis of
macrophages (55). Similarly, we found that down-regulation or
overexpression of SphK2 had no effect on migration of HEK 293
cells toward EGF. In sharp contrast, down-regulation or over-
expression of SphK1 reduced or enhanced migratory responses
of these cells toward EGF, respectively.

Because EGF is also known to play a critical role in induction
of breast cancer cell motility, a key step in invasion and me-
tastasis, we also examined the roles of SphK1 and SphK2 in
EGF-induced motility of MDA-MB-453 human breast cancer
cells. These cells were selected because they express both
SphK1 and SphK2 and, in agreement with previous studies
(48), we found that these cells migrate toward EGF and S1P. As
with HEK 293 cells, down-regulating SphK1 expression re-
duced migration of MDA-MB-453 cells toward EGF. However,
down-regulation of SphK2 in these cells, which had no signifi-
cant effect on motility of HEK 293 cells, markedly reduced
EGF-directed migration. Using another siRNA targeted to a
different SphK2 sequence, once more, EGF-induced motility of
HEK 293 cells was unaffected while motility of MDA-MB-453
cells was inhibited. Importantly, knockdown of SphK2 had no
effect on migration of MDA-MB-453 cells toward S1P (the pro-
duct of this kinase) or toward fibronectin. These results suggest
that inhibition of cell migration by knockdown of SphK2, as
with SphK1, is due to inhibition of specific pathways and not a
generalized defect in migratory responses. Hence, in MDA-MB-
453 cells, the two SphK isoenzymes have overlapping and/or
complementary functions in EGF-induced migration.

It is not clear why SphK2 is involved in migration toward
EGF in one cell type but not in another. One possibility is that
SphK2 is present in a unique cell compartment important for
motility in certain cell types. This seems unlikely, however, as
the majority of endogenous SphK2 was present in the plasma
membranes of both HEK 293 and MDA-MB-453 cells, even
before EGF treatment, and EGF did not induce any obvious
changes in its localization. However, it is still possible that
SphK2 might be brought to a specialized plasma membrane
compartment and produce S1P in the vicinity of its receptors.
Alternatively, a threshold level of S1P may be required for
EGF-induced motility that is only reached in some cell types
when both SphK1 and SphK2 are activated.

Because SphK1 stimulates growth and survival of cancer
cells, whereas SphK2 has been implicated in growth inhibition
and induction of apoptosis, anticancer therapeutics targeting

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2 S. Sarkar, M. Maceyka, and S. Spiegel, unpublished data.
SphK1 might be useful adjuncts for management of many types of cancer. Moreover, SphK1 is overexpressed in a variety of solid tumors, including breast, stomach, ovary, kidney, and lung, with compared with normal tissues from the same patients (57). Non-isozyme specific inhibitors of SphK, such as DMS and 1-threo-dihydrosphingosine (known as safingol), induce apoptosis regardless of multidrug resistance expression (58). Moreover, SphK inhibitors reduce gastric tumor growth (59) and sis regardless of multidrug resistance expression (58). More-ther studies are still needed to clarify the biological functions of SphK1.

Our demonstration that SphK2 has a role for motility in certain cells has implications for the targeting of SphKs for inhibition of metastasis and angiogenesis. Specifically, inhibiting SphK1 may not be sufficient to decrease metastasis if SphK2 can compensate. In this case, a broad specificity inhib-

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