Sphingosine Kinase Type 2 Activation by ERK-mediated Phosphorylation*

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Sphingosine 1-phosphate (S1P), a potent lipid mediator, is a ligand for a family of five G protein-coupled receptors (S1P1–5) that have been shown to regulate a variety of biological responses important for cancer progression. The cellular level of S1P is low and tightly regulated in a spatio-temporal manner through its synthesis catalyzed by two sphingosine kinases, denoted SphK1 and SphK2. Many stimuli activate and translocate SphK1 to the plasma membrane by mechanisms that are dependent on its phosphorylation. Much less is known about activation of SphK2. Here we demonstrate that epidermal growth factor (EGF) as well as the protein kinase C activator, phorbol ester, induce rapid phosphorylation of hSphK2 which was markedly reduced by inhibition of MEK1/ERK pathway. Down-regulation of ERK1 blocked EGF-induced phosphorylation of SphK2. Recombinant ERK1 phosphorylated hSphK2 in vitro and increased its enzymatic activity. ERK1 also was found to be in a complex with hSphK2 in vivo. Site-directed mutagenesis indicated that hSphK2 is phosphorylated on Ser-351 and Thr-578 by ERK1 and that phosphorylation of these residues is important for EGF-stimulated migration of MDA-MB-453 cells. These studies provide the first clues to the mechanism of agonist-mediated SphK2 activation and enhance understanding of the regulation of SphK2 activity by phosphorylation and its role in movement of human breast cancer cells toward EGF.

Sphingosine kinase 1 (SphK1) and sphingosine kinase 2 (SphK2) are highly conserved lipid kinases found in such diverse organisms as mammals, flies, yeast, and plants (1). SphKs catalyze the ATP-dependent phosphorylation of the primary hydroxyl group of sphingosine to generate the potent lipid mediator, sphingosine 1-phosphate (S1P) (1). S1P exerts most of its actions as a ligand for five specific G protein-coupled receptors, S1P1–5, that regulate a wide variety of important cellular processes; such as growth, migration, and invasion; angiogenesis and vascular maturation; as well as lymphocyte trafficking, immunity, and allergy (reviewed in Refs. 2–6). Intracellular S1P has also been implicated in S1P receptor independent signaling leading to release of intracellular Ca$^{2+}$ stores, cellular proliferation, and protection from apoptosis (7–9).

Most cells express both SphK1 and SphK2 (10, 11). Many external stimuli, including growth factors such as EGF, ligands for G protein-coupled receptors, pro-inflammatory cytokines, and cross-linking of FcεRI receptors, stimulate SphK1 leading to its translocation to the plasma membrane where its substrate sphingosine resides, resulting in accumulation of intracellular S1P, and subsequently, increased cell proliferation and suppression of apoptosis (reviewed in Refs. 1, 4, and 12). Phorbol ester, phorbol 12-myristate 13-acetate (PMA), induces protein kinase C (PKC)-mediated phosphorylation of SphK1 and its localization to the plasma membrane, which leads to enhanced release of S1P to the media (13). It was later reported that both PMA and tumor necrosis factor-α induced phosphorylation of SphK2; the latter activated by activation of extracellular signal-regulated kinase (ERK) 1/2 (14). This phosphorylation induces its plasma membrane localization and activation (14) and drives its oncogenic signaling (15). Recently, it was suggested that phosphorylation of Ser-225 may induce a conformational change or electrostatic switch of SphK1 that promotes its interaction with phosphatidylserine in the plasma membrane (16). Whether SphK2 is also regulated by phosphorylation is still unknown. Moreover, although many agonists stimulate SphK1, to date, only EGF (17) and cross-linking of FcεRI (18) has been shown to activate SphK2. Interest in SphK2 has increased recently due to the discovery that the potent immunosuppressant FTY720 is a pro-drug that is phosphorylated by SphK2, but not by SphK1 (19–24), to a mimetic of S1P that regulates S1P1 receptor actions important for lymphocyte trafficking (2, 25). FTY720 has considerable therapeutic effects in the prevention of transplant rejection (26) and treatment of multiple sclerosis (27).

In contrast to SphK1, overexpression of SphK2 suppresses cell growth and enhances apoptosis (28, 29), which could be due to its putative BH3 domain (28), or to localization to the nucleus (29) or the endoplasmic reticulum (30). The physiological role of endogenous SphK2 in cell growth and survival is still unclear as its down-regulation suppressed growth and enhanced apo-
ptosis of glioma cells (31), yet protected HEK 293 cells from apoptosis (29). Importantly, mice that are null for either SphK1 or SphK2 develop normally, yet the double knock-out is embryonically lethal due to severely abnormal neurogenesis and angiogenesis and neural tube closure defects (32). This suggests that SphK1 and SphK2 might also have overlapping and/or complementary functions. In agreement, both SphK1 and SphK2 were required for EGF-induced migration of MDA-MB-453 breast cancer cells (17). In this study, we show that EGF as well as PMA induce rapid phosphorylation of SphK2, which is mediated by ERK1. Furthermore, we found that phosphorylation of SphK2 not only results in an increase in its enzyme activity, but is also important for its ability to enhance EGF-induced chemotaxis of human breast cancer cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—[γ-³²P]ATP (3000 Ci/mmol) was purchased from Amersham Biosciences and [³²P]orthophosphate from PerkinElmer Life Sciences. SIP and GF109203X were from Biomol (Plymouth Meeting, PA). Serum and medium were from Biofluids (Rockville, MD). EGF and PMA were obtained from Invitrogen. U0126 was purchased from Calbiochem. NTA resin was from Qiagen Inc. (Valencia, CA). Protein A/G PLUS-agarose was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Collagen type IV was from Collagen Corp. (Palo Alto, CA). Polycarbonate filters were from Poretics (Livermore, CA), and rabbit polyclonal antibody against SphK2 was described previously (17).

**Cell Culture and Transfection**—Human embryonic kidney cells (HEK 293, ATCC CRL-1573) and human MDA-MB-453 breast cancer cells (ATCC HB-8506) were cultured in high glucose Dulbecco’s modified Eagle’s medium (DMEM) and transfected with wild-type or mutated SphK2 as described (17). siGenome SMARTpool ERK1 small interfering RNA (siRNA) and On-Target Plus negative control siRNA were purchased from Dharmacon (Lafayette, CO). Cells were transfected with 100 nM siRNA using Oligofectamine (Invitrogen, Gaithersburg, MD) according to the manufacturer’s protocol.

**SphK2 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 glycophosphate, 15 mM NaF, 10 μg/ml leupeptin, aprotinin, and soybean trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride, and 0.5 mM 4-deoxypyridoxine). Lysates were centrifuged at 700 × g for 10 min to remove unbroken cells. SphK2 activity was determined with 50 μM sphingosine, added as a complex with 4 mg/ml bovine serum albumin, and [γ-³²P]ATP in the presence of 1 mM KCl, conditions in which SphK2 activity is optimal and SphK1 strongly inhibited (17).

**Site-directed Mutagenesis**—Wild-type hSphK2 cDNA was cloned into V5/His pcDNA 3.1 expression vector (Invitrogen) and was used for site-directed mutagenesis with the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s protocol. The oligonucleotides used to generate the point mutant constructs were as follows: hSphK2S49A, 5’-GGGGTCGCTTCGGGGCGCCAG-3’ and 5’-GGTTGCTTCGGGGCGCCAG-3’; hSphK2S49A, 5’-GGGATGCTTGCGCCGCGGC-3’; hSphK2S49A, 5’-GGTTGCTTCGGGGCGCCAG-3’; hSphK2S49A, 5’-GGTTGCTTCGGGGCGCCAG-3’. All constructs were sequenced to verify the desired mutation.

**Labeling of Cellular hSphK2**—Naïve or transfected HEK 293 cells were serum-starved overnight in phosphate-free DMEM, metabolically labeled in the same medium with [³²P]orthophosphate (70 μCi/ml) for 2.5 h at 37 °C, then treated as indicated in figure legends. Cells were harvested in cold PBS and hSphK2 was immunoprecipitated from lysates with anti-SphK2 antibody or pulled down with Ni-NTA-agarose as described below. Proteins were separated by SDS-PAGE and transferred to nitrocellulose, and incorporation of ³²P in hSphK2 was determined with a phosphorimager or by densitometry after exposing to x-ray film. Identification of the ³²P-labeled SphK2 band was confirmed by Western blotting.

**In Vitro Phosphorylation of hSphK2**—Recombinant V5-Flag tagged SphK2s expressed in HEK 293 cells were purified with Ni-NTA-agarose (33). In brief, cells were lysed in buffer A (50 mM Tris, pH 7.5, 10% glycerol, 150 mM NaCl, 0.1% Triton X-100, 1 mM 2-mercaptoethanol, 1 mM sodium orthovanadate, 40 mM β-glycerophosphate, 15 mM NaF, 1:500 mammalian protease inhibitor mixture (Sigma), and 0.5 mM 4-deoxypyridoxine and 10 mM imidazole) by freeze-thawing. After centrifugation at 500 × g for 10 min at 4 °C, equal amount of cell-free lysates were incubated with buffer A-equilibrated Ni-NTA resin for 30 min at 4 °C with constant agitation. Following centrifugation at 1000 × g, the Ni-NTA-agarose was washed with Buffer A, then Buffer A containing 50 mM imidazole and 1 mM NaCl and bound proteins eluted with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol containing 150 mM imidazole. The buffer was exchanged and imidazole removed with Microcon YM-10 centrifugal filter (10 kDa cutoff, Millipore Corp., Bedford, MA) with buffer containing 50 mM Tris-HCl, pH 7.5, 10% glycerol, 150 mM NaCl, 0.25 mM dithiothreitol, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride. The eluted recombinant SphK2 proteins were examined by silver staining SDS-PAGE gels and by immunoblotting with anti-V5 or anti-SphK2 antibodies. In vitro phosphorylation of these proteins was performed by incubating with 0.4 ng/μl of purified recombinant active ERK1 (Stressgen, Victoria, British Columbia, Canada) or 0.05 unit/μl of purified recombinant ERK2 (New England Biolabs), 0.1 mM [γ-³²P]ATP, and 4 mM MgCl₂ at 25 °C for 30 min. Reactions were stopped with 2X SDS sample buffer for SDS-PAGE and autoradiography or placed on ice for SphK2 enzyme activity assays. Incorporation of ³²P into hSphK2 was determined as described above and normalized to SphK2 protein determined by immunoblotting.

**Immunoprecipitation, Pull Down, and Western Blotting**—Cell lysates were preclarified by incubation with protein A/G-agarose and then incubated with SphK2 antibody for 18 h at
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**RESULTS**

EGF and PMA Stimulate Phosphorylation of hSphK2—It has been shown that agonist-induced activation of hSphK1 results from its phosphorylation at Ser-225 by ERK1/2 (14). This phosphorylation was not only involved in translocation of SphK1 to the plasma membrane, it was essential for its oncogenic properties (15). In contrast, little is known of the mechanisms of SphK2 activation. Since SphK1 and SphK2 are highly homologous, it was of interest to examine whether activation of hSphK2 also proceeds via its phosphorylation. To this end, HEK 293 cells ectopically expressing hSphK2 were metabolically labeled with $^{32}$P for 2.5 h prior to treatment without or with 100 ng/ml EGF for 20 min. Cell lysates were immunoprecipitated with anti-hSphK2 antibody, proteins separated by SDS-PAGE, and $^{32}$P incorporation determined by autoradiography. Duplicate unlabeled samples were immunoprecipitated and analyzed by immunoblotting with anti-SphK2 antibodies (A) and SphK2 activity in the immunoprecipitates was determined (B). Data are expressed as pmol of S1P produced per min and are means ± S.D. C–E, HEK 293 cells transfected with V5-tagged hSphK2-S or hSphK2-L were labeled with $^{32}$P as described in A and treated with 100 ng/ml EGF or 100 nM PMA for the indicated times (C, D) or for 20 min (E). $^{32}$P incorporation into hSphK2 was determined by autoradiography. As a loading control, hSphK2 in the immunoprecipitates was analyzed by immunoblotting with anti-SphK2 antibody.

Statistical Analysis—Experiments were repeated at least three times with consistent results. Statistics were performed using SigmaStat statistical software version 2.0.

**Chemotaxis Assays**—Chemotaxis was measured in a modified Boyden chamber using polycarbonate filters (25 × 80 mm, 12 μm pore size) coated with collagen type IV (10 μg/ml) as described (17). For each experiment, data from triplicate samples were calculated and expressed as means ± S.D.

**FIGURE 1. Agonist-induced phosphorylation of hSphK2.** A and B, HEK 293 cells transfected with hSphK2-S or empty vector were serum-starved overnight in phosphate-free DMEM and metabolically labeled with $^{32}$P for 2.5 h prior to treatment without or with 100 ng/ml EGF for 20 min. Cell lysates were immunoprecipitated with anti-hSphK2 antibody, proteins separated by SDSPAGE, and $^{32}$P incorporation determined by autoradiography. Duplicate unlabeled samples were immunoprecipitated and analyzed by immunoblotting with anti-SphK2 antibodies (A) and SphK2 activity in the immunoprecipitates was determined (B). Data are expressed as pmol of S1P produced per min and are means ± S.D. C–E, HEK 293 cells transfected with V5-tagged hSphK2-S or hSphK2-L were labeled with $^{32}$P as described in A and treated with 100 ng/ml EGF or 100 nM PMA for the indicated times (C, D) or for 20 min (E). $^{32}$P incorporation into hSphK2 was determined by autoradiography. As a loading control, hSphK2 in the immunoprecipitates was analyzed by immunoblotting with anti-SphK2 antibody.

**FIGURE 2.** MEK1/2 and EGFR inhibitors block EGF-mediated hSphK2 phosphorylation. HEK 293 cells transfected with either hSphK2 or empty vector were serum-starved overnight in phosphate-free DMEM and metabolically labeled with $^{32}$P for 2.5 h. Cells were washed and pretreated with vehicle, 10 μM U0126 (A, B), 10 μM PD98059 (C), or 250 nM AG1478 (D) for 1 h as indicated and then stimulated without or with 100 ng/ml EGF for 20 min. Cell lysates were immunoprecipitated with anti-hSphK2 antibody, proteins separated by SDS-PAGE, and $^{32}$P incorporation determined by autoradiography. Duplicate unlabeled samples were immunoprecipitated and analyzed by immunoblotting with anti-V5 antibody (A–D), anti-SphK2, or anti-pERK1/2 (B). The blot was stripped and re-probed with anti-ERK2 antibody as a loading control (B).
interest to examine whether PMA can also stimulate phosphorylation of hSphK2. Indeed, PMA stimulated phosphorylation of hSphK2 in a time-dependent manner, increasing \( ^{32}P \) incorporation by more than 8-fold within 20 min (Fig. 1C).

Recently, a 36-amino acid N-terminally extended SphK2 splice variant, SphK2-L (21), which is expressed in humans but not in mice, has been identified by sequence analysis (29). hSphK2-L appears to be the major isoform expressed in some tissues and cells as shown by PCR (29). EGF and PMA also markedly enhanced phosphorylation of ectopically expressed hSphK2-L (Fig. 1E).

**ERK1/2 Phosphorylates hSphK2 in Vivo**—The NetPhos phosphorylation web site (34) predicts that hSphK2 possesses 33 potential serine and threonine (but no tyrosine) phosphorylation sites, including putative sites for PKC, PKA, ERK, and casein kinase. ERK1/2 are closely related members of the mitogen-activated protein kinase family that have been implicated in phosphorylation and activation of SphK1 (14). Therefore, we examined their possible involvement in EGF-induced phosphorylation of SphK2. Pretreatment of cells with U0126, a specific MEK1/2 inhibitor (35), inhibited EGF-induced phosphorylation of both SphK2 (Fig. 2A) and ERK1/2 (Fig. 2B). Similar results were obtained with PD98059, another MEK1/2 inhibitor (Fig. 2C). Moreover, the EGFR tyrosine kinase inhibitor AG1478, which also inhibited EGF-induced activation of ERK1/2 (data not shown), likewise abolished EGF-induced phosphorylation of hSphK2 (Fig. 2D). Collectively, these data suggest that ERK1/2 might be the kinase(s) that phosphorylates SphK2 downstream of EGFR.

**PKC Activates hSphK2 via ERK1/2**—Activation of PKC by PMA is known to induce phosphorylation of hSphK1 (36), although there is no evidence that purified PKC can directly phosphorylate hSphK1 (14). Treatment of HEK 293 cells ectopically expressing hSphK2 with PMA dramatically increased phosphorylation of hSphK2 (Fig. 3A). Moreover, this PMA-induced phosphorylation was inhibited by GF109203X (Fig. 3A), a specific PKC inhibitor (37). Of note, PMA also activated MEK1/2 and ERK1/2, as determined with phospho-specific antibodies, which was completely abolished by GF109203X (Fig. 3B), suggesting that PMA activates MEK1/2 that in turn regulates ERK1/2. Interestingly, these results also imply that ERK1/2 may be responsible for PMA-induced phosphorylation of SphK2 (Fig. 3C).

**EGF and PMA Induce Phosphorylation of hSphK2 at Ser-351 and Thr-578**—We next focused on the five putative ERK1/2 substrate recognition motifs found in SphK2 (PX[S/T], where X represents a neutral or basic amino acid) (Fig. 4) for several reasons. First, our results suggest that ERK1/2 might be involved in EGF- and PMA-induced phosphorylation of SphK2. Second, the sequence of amino acids from 181 to 199 in hSphK2 is similar to the consensus ERK1/2 docking site motif (Fig. 4A), which has been shown to confer specificity and efficiency of phosphorylation in other ERK1/2 substrates (38). Third, the SphK2 Pro-Ala-Ser-351 sequence is identical to the Pro-Ala-Ser-225 ERK1/2 phosphorylation site of hSphK1 (14) (Fig. 4B). To examine
the five putative ERK1/2 phosphorylation sites, we mutated the Ser or Thr residues to alanine (S351A, S401A, S430A, S441A, T578A) (Fig. 4A). All five mutants were enzymatically active, although the SphK activities of T578A and S441A mutants were lower in comparison with the wild-type hSphK2 (Fig. 5A). Three of the five mutations had no effect on EGF- or PMA-mediated hSphK2 phosphorylation, as demonstrated by comparable $^{32}$P incorporation into mutants and wild-type SphK2 (Fig. 5B). In contrast, EGF- and PMA-induced incorporation of $^{32}$P into the Ser-351 $\rightarrow$ Ala and Thr-578 $\rightarrow$ Ala mutants was completely abolished (Fig. 5B). Interestingly, the Ser-225 $\rightarrow$ Ala SphK1 mutation, which is equivalent to Ser-351 $\rightarrow$ Ala SphK2 mutation, also completely ablated basal phosphorylation (14).

It should be pointed out that the Thr-578 putative ERK1/2 phosphorylation site of hSphK2 is conserved in mammalian SphK2s and it is not present in SphK1 sequences (Fig. 4C), suggesting that this phosphorylation site may allow specific regulation that is unique to SphK2.

**ERK1 Activates hSphK2 by Direct Phosphorylation**—Next, we determined whether ERK1 or ERK2 can directly phosphorylate SphK2 in vitro. Purified ERK1 and ERK2 catalyzed the phosphorylation of recombinant hSphK2-S and hSphK2-L (Fig. 6A). ERK1 was more potent than ERK2 in phosphorylating both isoforms of SphK2. However, hSphK2-S was phosphorylated to a greater extent than SphK2-L (Fig. 6A). Furthermore, phosphorylation of both hSphK2-S and hSphK2-L isoforms by ERK1 was accompanied by a significant increase in their enzymatic activity (Fig. 6B). The enhanced enzymatic activity resulted from both an increase in catalytic activity and also a change in SphK2 activation by phosphorylation.
substrate affinity as the stimulation was greater when activity was measured with 5 μM sphingosine, a subsaturating substrate concentration, than at 50 μM (Fig. 6B).

To establish whether there is a direct interaction between ERK1/2 and hSphK2, the ability of ERK1 or ERK2 to bind SphK2 was assessed. Specific pull down of His-tagged V5-SphK2 also brought down ERK1, but not ERK2, suggesting that ERK1 does form a complex with hSphK2 (Fig. 7). In agreement with previous results (14), we also confirmed that hSphK1 physically interacts with ERK1 (Fig. 7).

To confirm that ERK1 is involved in EGF-induced phosphorylation of SphK2, its expression was down-regulated with siRNA targeted to a specific sequence of ERK1. siERK1, but not siControl, drastically reduced ERK1 but not ERK2 in both HEK 293 and MDA-MB-453 cells (Fig. 8, B and D). Reduction of ERK1 expression markedly reduced EGF-induced SphK2 phosphorylation in these two cell lines (Fig. 8, A and C). Similar to the results in HEK 293 cells (Fig. 5), EGF induced phosphorylation of hSphK2 at Ser-351 and Thr-578 in MDA-MB-453 cells, as incorporation of 32P into the Ser-351 and Thr-578 into Ala mutants was completely abolished compared with wild-type SphK2 (Fig. 8E).

To further substantiate that ERK1 can phosphorylate specific sites on SphK2, in vitro kinase assays were carried out with recombinant wild-type SphK2 and the various mutants. In agreement with the in vivo 32P incorporation data (Fig. 5B), mutations of Ser-351 and Thr-578 to Ala markedly reduced ERK1-dependent phosphorylation in vitro, while the S401A, Ser-430 → Ala, and Ser-441 → Ala mutations only slightly decreased 32P incorporation compared with wild-type SphK2 (Fig. 9). These results further indicate that Ser-351 and Thr-578 sites are bona fide sites of phosphorylation of hSphK2 by ERK1.

S351A and T578A Mutations Prevent SphK2 Enhancement of EGF-mediated Chemotaxis of MDA-MB-453 Breast Cancer Cells—Although the biological functions of SphK2 are not completely clear, in MDA-MB-435 human breast cancer cells, SphK2, similar to SphK1, is critical for cell migration toward EGF (17). Transient expression of wild-type hSphK2 in MDA-MB-453 cells was transfected with siControl or siERK1, as indicated. 24 h later, cells were transfected with empty vector or hSphK2 and after an additional 24 h were metabolically labeled with 32P for 2.5 h. Cells were washed and treated with vehicle or with 100 ng/ml EGF for 20 min. SphK2 was pulled down from lysates with Ni-NTA-agarose, bound proteins separated by SDS-PAGE, and 32P incorporation determined by autoradiography and protein levels by Western blot with anti-V5 antibody. B and D, duplicate lysates from unlabeled samples were analyzed by immunoblotting with anti-V5 antibody or anti-ERK1. Blots were stripped and re-probed with ERK2 antibody as a loading control. E, EGF induces phosphorylation of SphK2 at Ser-351 and Thr-578 in MDA-MB-453 cells. MDA-MB-453 cells transiently transfected with wt-hSphK2, hSphK2S351A, hSphK2T578A, or empty vector were labeled with 32P, for 2.5 h prior to treatment with vehicle or 100 ng/ml EGF for 20 min. SphK2 was pulled down from lysates with Ni-NTA-agarose, bound proteins separated by SDS-PAGE, visualized on Western blots with anti-V5 antibody, and 32P incorporation determined by autoradiography.
MB-435 cells enhanced chemotaxis toward EGF (Fig. 10A). Consistent with our previous studies (17), overexpression of SphK2 had no effect on migration of MDA-MB-453 cells toward serum or S1P. Importantly, in accordance with their ability to be phosphorylated in vivo and in vitro by ERK1, expression of the SphK2 phosphorylatable mutants also enhanced chemotaxis toward EGF (Fig. 10B and data not shown). In contrast, the S351A and T578A mutants, which were not phosphorylated by ERK1, did not increase the migration of cells toward EGF (Fig. 10B).

FIGURE 9. In vitro phosphorylation of hSphK2 mutants by ERK1 and ERK2. HEK 293 cells were transfected with wt-hSphK2, hSphK2S351A, hSphK2T578A, hSphK2S430A, or hSphK2S441A. Lysates were prepared and recombinant SphK2s purified with Ni-NTA-agarose as described under “Experimental Procedures” and incubated without or with recombinant ERK1 in the presence of [γ-32P]ATP. Labeled proteins were separated by SDS-PAGE. 32P incorporation into proteins was determined by autoradiography and SphK2 protein was analyzed by immunoblotting using anti-V5 antibody as loading controls. X-ray films and blots were scanned and quantified by densitometry. Data are normalized with the appropriate loading controls. *p < 0.01. **p < 0.001.

FIGURE 10. Wild-type SphK2 but not Ser-351 → Ala and Thr-578 → Ala hSphK2 mutations enhance EGF-mediated migration of MDA-MB-453 breast cancer cells. MDA-MB-453 cells were transfected with empty vector (open bars), wt-hSphK2 (black bars), hSphK2S430A (gray striped bars), hSphK2S441A (gray bars), or hSphK2T578A (stippled bars) as indicated. Cells were serum-starved overnight and allowed to migrate for 24 h toward vehicle (None), serum (20%), EGF (100 ng/ml), or S1P (1 μM), as indicated, and chemotaxis was determined in a modified Boyden chamber. Data are expressed as mean number of cells per field ± S.D. of triplicate determinations. *p < 0.01.

DISCUSSION

Although SphK2 has recently been implicated in regulation of important physiological processes including lymphocyte trafficking (24), and neural and vascular development (32), nothing is yet known about its regulation. A recent study demonstrated that hSphK2 binds calmodulin (CaM) through a CaM binding region that is conserved with hSphK1 (39). However, neither Ca2+/CaM nor apoCaM has any effect on the enzymatic activity of hSphK2 in vitro (39). Thus, the physiological significance of the interaction of CaM with hSphK2 remains unclear. Previously, we showed that EGF activates hSphK2 and this was critical for EGF-directed cell motility of MDA-MB-453 breast cancer cells (17). Now we have demonstrated that activation of hSphK2 by EGF is mediated by ERK1-dependent phosphorylation at Ser-351 and Thr-578. Moreover, recombinant ERK1 also catalyzed the phosphorylation of wild-type hSphK2 but not Ser-351 → Ala or Thr-578 → Ala hSphK2 mutants in vitro.

In addition to EGF, PMA also induced rapid phosphorylation of hSphK2 that was dependent on ERK activation. Although previous studies have shown that PMA indirectly stimulated phosphorylation and translocation of SphK1 (14), unlike hSphK1, exposure of cells to PMA did not result in detectable redistribution of hSphK2 (39). Similarly, EGF also did not alter the cellular localization of hSphK2 (17). These results suggest that in contrast to hSphK1, hSphK2 may not change cellular localization in an agonist-dependent manner as it is mostly present in the membrane fraction (17). On the other hand, a recent study showed that cross-linking of the IgE receptor on murine and human mast cells by antigen induced translocation of cytosolic SphK1 and SphK2 to the membrane fraction (18). SphK1 interacts with the members of the Src protein tyrosine kinase family, Lyn and Fyn, FcεRI proximal kinases that initiate the signaling events following cross-linking of this receptor on mast cells but not with Src or other tyrosine kinases such as Syk. Following interaction with Lyn, SphK1 is recruited to membrane rafts where conformational changes may occur in both kinases that favor their respective activities (40). Fyn kinase was also required for FcεRI coupling to SphK2 and for subsequent S1P production. However, Fyn kinase increased SphK1 but not SphK2 activity in vitro and, together with Lyn kinase, was important for membrane translocation of SphK2 (18). Since SphK2 does not contain a tyrosine phosphorylation site, it is reasonable to assume that SphK2 is not a direct target of Fyn. It is also possible that ERK phosphorylates SphK2 during mast cell activation as Fyn is upstream of ERK in FcεRI signaling in these cells.

It is likely that phosphorylation of hSphK2 at Ser-351 or Thr-578 by ERK1 induces a conformational change to facilitate the observed increase in its catalytic activity and increased affinity for its substrate sphingosine. Nevertheless, the 7-fold increase in catalytic activity that results from phosphorylation of hSphK2 by ERK1 in vitro is similar to the 6-fold increase in SphK2 activity observed in cells following

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FIGURE 10. Wild-type SphK2 but not Ser-351 → Ala and Thr-578 → Ala hSphK2 mutations enhance EGF-mediated migration of MDA-MB-453 breast cancer cells. MDA-MB-453 cells were transfected with empty vector (open bars), wt-hSphK2 (black bars), hSphK2S430A (gray striped bars), hSphK2S441A (gray bars), or hSphK2T578A (stippled bars) as indicated. Cells were serum-starved overnight and allowed to migrate for 24 h toward vehicle (None), serum (20%), EGF (100 ng/ml), or S1P (1 μM), as indicated, and chemotaxis was determined in a modified Boyden chamber. Data are expressed as mean number of cells per field ± S.D. of triplicate determinations. *p < 0.01.
treatment with EGF (17), supporting the importance of phosphorylation in the regulation of the activity of SphK2. Several lines of evidence also support an in vivo role for ERK1 in the activation of hSphK2. First, blocking activation of ERK with specific inhibitors markedly reduced EGF- and PMA-induced phosphorylation of SphK2. Second, mutation of consensus ERK1 phosphorylation sites drastically reduced EGF- and PMA-induced phosphorylation of SphK2 in vivo. Down-regulating ERK1 expression with siRNA also nearly abolished EGF-mediated phosphorylation of SphK2. Finally, ERK1 directly binds to SphK2 in cells.

EGF stimulates SphK2 activity in MDA-MB-435 human breast cancer cells and is required for EGF-directed cell movement, as shown by down-regulating its expression with specific siRNA (17). However, decreasing expression of SphK2 did not alter migration toward serum, S1P, or fibronectin (17). In agreement, we found that overexpression of SphK2 significantly enhanced migratory responses toward EGF in MDA-MB-435 cells. These results suggest that endogenous SphK2 is important but not saturating for EGF-induced motility. Similarly, previous studies have shown that SphK1 is important for migration of HEK 293 cells toward EGF by down-regulation or overexpression which decreased or increased, respectively, migration of these cells toward EGF (17). Importantly, in this study, we found that phosphorylation/activation of SphK2 by ERK1 is physiologically relevant as ectopic expression of wild-type hSphK2, but not the non-phosphorylatable SphK2 mutants, also enhanced EGF-driven chemotaxis.

In sum, our studies provide the first evidence for regulation of SphK2 by agonist-mediated phosphorylation. Better understanding of the regulation of SphK2 activity by phosphorylation will lay the groundwork for more systematic and functional studies of this important lipid kinase.

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