Research Article

The G Protein–Coupled Receptor $S1P_2$ Regulates Rho/Rho Kinase Pathway to Inhibit Tumor Cell Migration

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Abstract

Sphingosine 1-phosphate (S1P) is a lysophospholipid that exerts a variety of responses in cells such as proliferation, migration, and survival. These effects are mediated by G protein–coupled receptors on the cell surface ($S1P_{1-5}$), which activate downstream signaling intermediates such as Rac and Rho GTPases. Mechanisms of S1P action in human glioblastoma cells are not well defined. S1P receptors (1–5) and S1P-metabolizing enzymes were expressed in three human glioblastoma cell lines. S1P had a profound and differential effect on glioblastoma cell migration. U87 cells treated with S1P showed a significant increase in migration, whereas U118 and U138 cell lines were strongly inhibited. S1P-mediated inhibition correlated with $S1P_2$ receptor expression. FTY720-P, an S1P analogue that binds all S1P receptors except $S1P_2$, did not inhibit glioblastoma cell migration. Overexpression of $S1P_2$ further suppressed migration, and blockage of $S1P_2$ mRNA expression by small interfering RNA reversed the inhibitory effect. Contrary to previous reports showing bimodal regulation of Rac activity and migration by $S1P_2$ receptor stimulation, both Rac1 and RhoA GTPases were activated by S1P treatment in native cells and cells over-expressing $S1P_2$. Treatment of U118 cells with the Rho-associated protein kinase (ROCK) inhibitor Y-27632 restored migration suggesting that ROCK-dependent mechanisms are important. Actin staining of S1P stimulated U118 cells overexpressing β-galactosidase resulted in pronounced stress fiber formation that was exacerbated by $S1P_2$ overexpression, partially blocked by $S1P_1$, or totally abolished by pretreatment with Y-27632. These data provide evidence of a novel mechanism of S1P inhibition of tumor cell migration via Rho kinase–dependent pathway. (Cancer Res 2005; 65(9): 3788-95)

Introduction

Sphingosine 1-phosphate (S1P) is a bioactive lipid produced by the breakdown of the membrane phospholipid, sphingomyelin. S1P exerts a variety of responses in cells such as proliferation, differentiation, migration, and survival (1). These effects are mediated by the EDG family of extracellular G protein–coupled receptors, although some evidence suggests that unknown intracellular targets may also exist (2, 3). Five members of the EDG family bind with high affinity to S1P: $S1P_1$/EDG-1, $S1P_2$/EDG-5, $S1P_3$/EDG-3, $S1P_4$/EDG-6, and $S1P_5$/EDG-8 (4). Each of these receptors activates different intracellular signaling pathways depending on which G_α protein they couple to intracellularly (5). Unlike $S1P_1$ that only couples to Rac via G_i, $S1P_2$ and to a lesser extent $S1P_3$ also couple and stimulate Rho via $G_{12/13}$ (6). $S1P_4$, which is highly expressed in the lymphoid system, couples to G_i and even more effectively to $G_{12/13}$ but not to G_q (7). G protein–coupling properties of $S1P_5$, which is almost exclusively expressed in neuronal tissue, is not well characterized at present. Thus, the ability of S1P to bind to multiple S1P receptors, which in turn activate multiple Gα protein–coupled pathways, creates a complex signaling system downstream of S1P receptors.

Pathways controlling the activation of small GTPases Rac and Rho are of particular interest for their roles in cell migration during angiogenesis and cancer metastasis. The dichotomous effects of S1P on cell migration are cell type specific. S1P stimulates chemotaxis in vascular endothelial cells and embryonic fibroblasts that primarily express $S1P_1$ but inhibits cell migration in vascular smooth muscle cells, neutrophils, and B16 melanoma cells that predominantly express $S1P_2$ (8–12). Therefore, the balance of S1P receptor expression in any given cell seems to dictate its migration response to S1P. The model based on these observations ascribes Rac-dependent, chemoattractive responses primarily to G_i-coupled receptors $S1P_1$ and $S1P_3$ and chemorepellant activities via $S1P_2$/$G_{12/13}$-mediated activation of Rho. Recent evidence suggests an additional mechanism of Rac and Rho regulation, such that S1P-mediated activation of Rho by $S1P_2$ leads to the inhibition of Rac (13). Studies by Takuwa et al. have supported the notion that $S1P_2$-mediated inhibition of tumor cell migration and metastasis occurs as a consequence of down-regulation of cellular Rac. However, these results are largely based on work done in B16 mouse melanoma cells that are unique in that they constitutively express only $S1P_2$ (14). Data derived from human tumor cell lines and tissues show that multiple S1P receptors are typically present in a cell. Thus, the net result of tumor cell response to S1P stimulation represents the aggregate effect of the various S1P receptors and their downstream effectors.

Glioblastoma multiforme is a locally aggressive tumor associated with a poor prognosis despite aggressive therapy. Recently, Van Brocklyn et al. established that glioma cells express $S1P_1$, $S1P_2$, and $S1P_3$ and that S1P is a mitogen for glioblastoma multiforme cells (15). The authors also report that S1P enhances motility and invasion of glioblastoma cells independent of metalloproteinase secretion (16). Since these initial observations, we have more comprehensively quantitated the expression level of all of the S1P receptors and metabolizing enzymes in several human glioblastoma cell lines and studied the effects of S1P on glioblastoma multiforme proliferation and migration. Our data indicate that S1P has a profound and differential effect on glioblastoma cell migration dependent on S1P receptor, specifically

Note: Supplementary data for this article are available at Cancer Research Online (http://canres.aacrjournals.org).

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$S1P_2$, expression, and contrary to prior murine tumor studies, on Rho kinase activity without concomitant inhibition of Rac activation. These findings shed new light on our understanding of S1P regulation of tumor cell migration.

Materials and Methods

Cell culture and transfection. Glioblastoma cell lines U87 MG, U118 MG, and U138 MG (ATCC HTB-14, HTB-15, HTB-16) were cultured in DMEM supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and antibiotics (Life Technologies, Grand Island, NY). Transfection of small interfering RNA (siRNA) oligonucleotide duplexes to block $S1P_2$ expression was done using Oligofectamine reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Briefly, 19-mer oligonucleotide duplexes targeting the open reading frame of human $S1P_2$ were transfected at a concentration of 200 or 400 nmol/L in 200 μL volume containing 4 μL Oligofectamine diluted in OptiMEM (siRNA $S1P_2$ AAUACCUUGCUCUCUGGCUCU; siRNA HuD AACAACAGAAACUGUCCUUCU). Glioblastoma cells were plated the day before transfection at 400,000 cells per well in 6-well plates in complete growth medium. Cells were harvested 48 hours later for migration assays. S1P (BioMol, Inc., Plymouth Meeting, PA) was resuspended in PBS containing 0.4% (w/v) fatty acid–free bovine serum albumin (PBS/BSA). Adenovirus constructs of β-galactosidase (β-gal), human $S1P_1$, or rat- $S1P_2$ were added to U118 and U87 cells and cultured for 48 hours before harvest. Rho-associated protein kinase (ROCK) inhibitor Y-27632 (Calbiochem, San Diego, CA) was used at 10 μmol/L final concentration during the final 30 minutes of serum starvation.

Quantitative reverse transcription-PCR. Total RNA was prepared as previously described using the RNA STAT-60 single-step isolation procedure as previously described (17). Total RNA was treated with DNase I before reverse transcription to ensure removal of all contaminating DNA. PCR primers were designed with PrimerExpress software developed by Applied Biosystems (Foster City, CA) for optimal product length, GC content, and melting temperature for quantitative reverse transcription-PCR (RT-PCR) using SYBR Green I DNA binding dye technology (18). Primers span at least one intron whenever possible to ensure exclusive amplification of cDNA. Duplicate SYBR Green PCR reactions for each sample were done using an ABI 7900HT instrument. All reactions underwent a final dissociation curve determination to ensure a single PCR product at the correct melting temperature. Fluorescence data were exported and quantitated using a statistical model that corrects for PCR efficiency for each reaction (19). Results are expressed relative to the internal control gene glyceraldehyde-3-phosphate dehydrogenase. RT-PCR analysis of minus RT reactions for all genes were negative demonstrating that the observed products detected were indeed cDNA and not amplified from contaminating genomic DNA (data not shown).

Cell migration assay. Migration assays were done in 96-well transwell chambers with 8-μm polycarbonate membrane filters (Neuroprobe, Gaithersburg, MD) separating the lower and upper culture compartments. Cells were serum starved in DMEM containing 0.1% fatty acid–free BSA for 2 hours before trypsinizing and plated in triplicate into the top chamber at 5×10^4 per well in 0.39 mL serum-free medium containing 0.1% fatty acid–free BSA (Sigma, St. Louis, MO). The bottom chamber contained various concentrations of S1P in serum-free medium containing 0.1% fatty acid–free BSA or medium alone or complete growth medium. Cells were allowed to migrate for 5 hours in a humidified chamber at 37°C with 5% CO_2. After the incubation period, the filter was removed and nonmigrated cells on the upper side of the filter were removed with a cotton swab. The filters were fixed overnight with 4% formaldehyde in PBS at 4°C. Attached cells were stained with 0.1% crystal violet and quantitated by densitometric analysis using ImageQuant software (Molecular Dynamics). Absorbance in pixel values for equivalent areas of triplicate wells were averaged and used to calculate fold migration relative to control.

Rac and Rho activation assays. The GTPase-binding domain of p21-activated kinase (PAK; amino acids, 67-150) or rhotekin (C21; amino acids, 7-89; a kind gift of Dr. Shuh Narumiya, Kyoto University, Kyoto, Japan) were obtained in a bacterial expression vector and expressed in *Escherichia coli* as a fusion protein with glutathione *S*-transferase (GST; refs. 20, 21). All steps were done on ice or at 4°C. Bacteria from 500 mL of 2× LB culture containing 50 μg/mL Ampicillin and 0.1 mmol/L isopropyl-L-thio-B-D-galactopyranoside were pelleted and lysed with bacteria lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 1 mmol/L EDTA, 100 mmol/L NaCl, 5% glycerol, 0.1% Triton X-100, 1 mmol/L DTT, and 0.1 mmol/L phenylmethylsulfonyl fluoride]. GST-fusion proteins were purified from cleared bacterial cell lysates by adsorption to glutathione-Sepharose beads for 1 hour. Beads were washed with bacterial lysis buffer and aliquoted to 50 μL bead volume per reaction before adding 500 μg of 100 nmol/L S1P-treated cell extract in radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 7.5), 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.5 mol/L NaCl, 10 mmol/L $MgCl_2$, 1× protease inhibitors] and brought to a total reaction volume of 800 μL with PBS. Extracts loaded with GTP-γ-S were included as a positive control in all experiments. Affinity precipitation of GST-C21-$RhoA_{GTP}$ or GST-PAK-$Rac1_{GTP}$ was carried out for 1 hour at 4°C before washing thrice with Sander's bead wash [50 mmol/L Tris-HCl (pH 7.4), 0.1% Triton X-100, 150 mmol/L NaCl, 5 mmol/L $MgCl_2$, 10% glycerol, 1× protease inhibitors] and resuspending final bead pellet in 50 μL 4× Laemmli sample buffer. Proteins were separated on 12% SDS-PAGE gels, transferred to nitrocellulose membrane, and blotted with specific antibodies for RhoA (1:200, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or Rac1 (1:2,000, BD Biosciences, San Jose, CA). Equal loading was confirmed by blotting total extracts against RhoA or Rac1. Results were quantitated by densitometric analysis and expressed as fold above background.

Fluorescence microscopy. Cells cultured in fibronectin-coated, glass bottom 35-mm dishes were fixed in 4% formaldehyde, permeabilized with 0.2% Triton X-100, and stained with tetramethyl rhodamine B isothiocyanate–labeled phalloidin (Sigma) to visualize filamentous actin and counter stained with 4′,6-diamidino-2-phenylindole to visualize nuclei. The cells were observed under on a Zeiss LSM 510 confocal microscope. The percentage of cells exhibiting stress fibers, cell rounding, or lamellipodia and membrane-ruffling structures were determined by counting the number of cells with each phenotype in five fields using standard fluorescence microscopy.

Statistical analysis. Data are presented as the mean ± SE and statistical differences between two groups were analyzed using Student's *t* test. For multiple comparisons one-way ANOVA with post hoc Dunnett's multiple comparisons correction where appropriate. $P < 0.05$ was considered significant.

Results

Expression of S1P receptors and sphingolipid metabolizing enzymes in human glioblastoma cell lines. We measured the mRNA expression of the five S1P receptors ($S1P_1$/EDG-1, $S1P_2$/EDG-5, $S1P_3$/EDG-3, $S1P_4$/EDG-6, and $S1P_5$/EDG-8) in three adult glioblastoma cell lines (U87, U118, and U138,) using quantitative RT-PCR. Transcripts for all receptors were detected at varying levels in all cell lines examined, except $S1P_4$/EDG-6 in U138 cells (Supplemental Fig. S1*A*). Overall, mRNA levels for $S1P_3$/EDG-3 were consistently high among the three cell lines, whereas $S1P_4$/EDG-6 and $S1P_5$/EDG-8 were barely detectable (Supplemental Fig. S1*A*).

Enzymes that metabolize S1P may alter signaling through this pathway. We next measured the mRNA expression level of SK1, SK2, SPP, and SGPL in the same cell lines. As shown in Supplemental Fig. 1*B*, SK1, SK2, SPP, and SGPL were expressed at different levels. Transcripts for SK1 or SK2 were highest of the four genes examined in U87, U118, and U138 (Supplemental Fig. S1*B*). These data indicate that the level of gene expression for the S1P receptors and metabolizing enzymes is highly variable, and suggests that sphingolipid metabolism and S1P signaling may contribute to biological responses.

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Differential effect of S1P on glioblastoma cell migration. S1P has been shown to either promote or inhibit cellular migration depending on the cell type examined (4). We therefore studied the role of S1P on glioblastoma migration *in vitro* and determined that S1P has a profound and differential effect on migration as shown in Fig. 1. U87 cells treated with 1 μmol/L S1P showed a significant increase in migration, whereas U118 and U138 cell lines were strongly inhibited (Fig. 1*A*). The effects of S1P on migration were dose-dependent and observed at 10 to 100 nmol/L concentrations, which is in the range of receptor activation (data not shown).

To elucidate the S1P receptor signaling pathways responsible for these differences, we tested the effects of 1 μmol/L S1P alone or in combination with pertussis toxin, a known inhibitor of G_i-linked pathways. Sphingosine had no effect on migration (Fig. 1*B*). Pertussis toxin blocked S1P-stimulated migration in U87 cells but not inhibition of migration in U118 cells. These data show that increased migration in response to S1P is G_i dependent, whereas S1P-mediated inhibition of migration is not.

$S1P_2$ mediates inhibition of migration in U118 cells. Because pertussis toxin was unable to block S1P-mediated inhibition of migration in U118 cells, a mechanism other than G_i is responsible. Interestingly, expression of $S1P_2$/EDG-5, which couples to $G_{12/13}$, correlated with the inhibitory effect on migration. For example, in

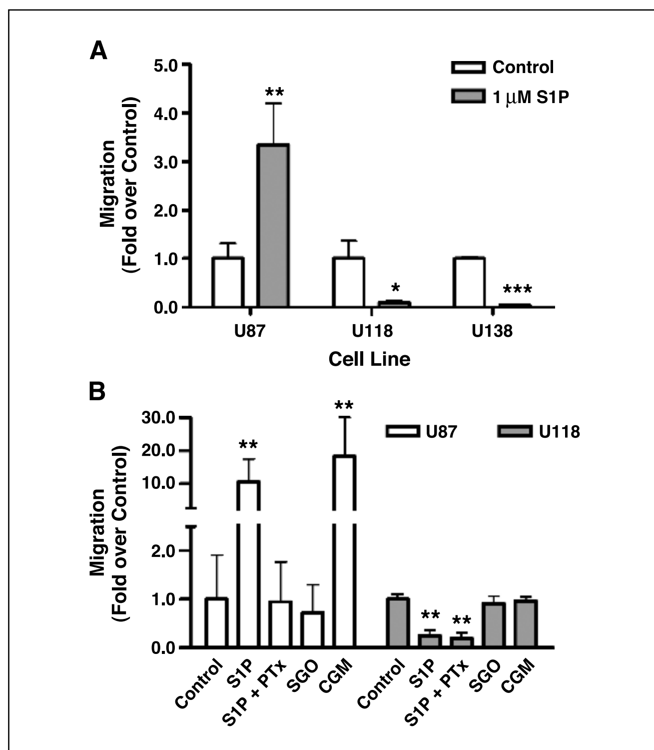

Figure 1. Differential effect of S1P on glioblastoma cell migration. Migration was assessed using a transwell chamber assay as described in Materials and Methods. Cells were plated in triplicate, and results densitometrically quantitated. Absorbances of each well were determined with ImageQuant software and represent the fold over control ± SE for triplicate wells. *A,* U87, U118, and U138 cells migrated through fibronectin-coated filters in the absence (*control*) or presence of 1 μmol/L S1P (*1 μM S1P*). Statistically significant differences from control were observed: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$ (Student's *t* test). *B,* pertussis toxin sensitivity of migration in response to S1P. Cells migrated in the presence or absence of 1 μmol/L S1P and pertussis toxin (*PTX*), or 1 μmol/L S1P or SGO alone. Complete growth medium (*CGM*) was included as a positive control for migration. **, $P < 0.01$ (ANOVA, Dunnett's test).

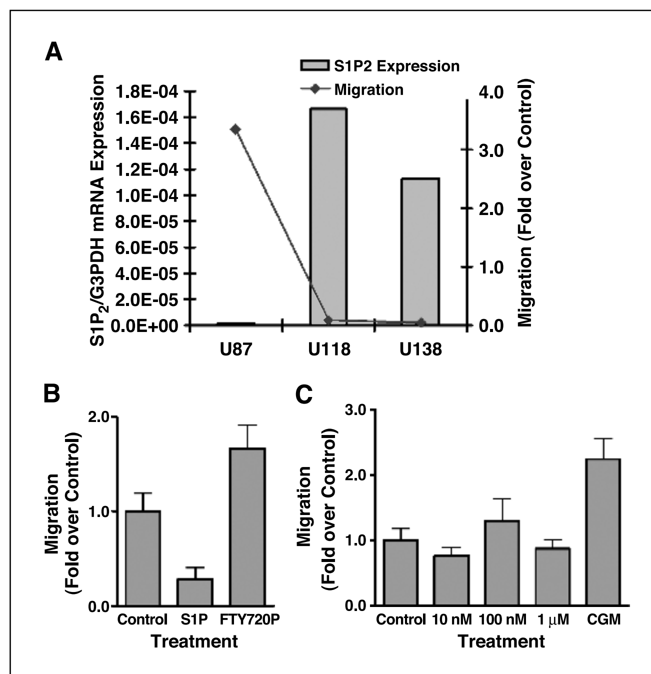

Figure 2. Migration in response to S1P correlates with $S1P_2$ gene expression. *A,* coanalysis of $S1P_2$ gene expression and migration in response to 1 μmol/L S1P in glioblastoma cell lines. *B,* effects of 100 nmol/L S1P or FTY720-P on migration of U118 cells. *C,* dose-response of FTY720-P on U118 cells. *Columns,* means for triplicate wells; *bars,* ±SE.

U87 cells that express low levels of $S1P_2$ mRNA, S1P is a potent inducer of migration (Fig. 2*A*). Conversely, in cell lines that express high levels of $S1P_2$, S1P strongly inhibits migration, particularly in U118 and U138 cell lines (Fig. 2*A*). Indeed, in previous findings from Takuwa's laboratory, $S1P_2$ was shown to inhibit migration of Chinese hamster ovary (CHO) cells and melanoma cells (22). To substantiate the notion that $S1P_2$ is involved in the inhibition of glioblastoma cell migration, we tested the effect of the S1P analogue FTY720-phosphate (FTY720-P), which is an agonist for all S1P receptors except $S1P_2$ (23). In U118 cells that express high levels of $S1P_2$, 100 nmol/L FTY720-P was unable to significantly inhibit migration in U118 cells, whereas the same concentration of S1P was inhibitory (Fig. 2*B*). Concentrations of FTY720-P ranging from 10 nmol/L to 1 μmol/L were unable to inhibit U118 cell migration, suggesting that S1P-mediated inhibition occurs via $S1P_2$ signaling pathways (Fig. 2*C*).

To further show that $S1P_2$ is indeed responsible for the inhibition of glioblastoma migration, we overexpressed $S1P_2$ and $S1P_1$ in U118 cells via adenoviral transduction. Cells overexpressing $S1P_2$ showed a more robust inhibition of migration at 10 to 100 nmol/L S1P compared with β-gal control virus transduced cells (Fig. 3*A*). However, as expected, overexpression of $S1P_1$ led to increased migration, thereby overcoming the endogenous $S1P_2$ inhibitory effects (Fig. 3*A*). Experiments were also done with U87 cells overexpressing $S1P_2$, which showed a pronounced inhibition of migration (Fig. 3*B*). Overexpression of $S1P_1$ increased basal levels of migration that were modestly increased upon S1P stimulation (Fig. 3*B*).

As a corollary, we hypothesized that down-regulation of endogenous $S1P_2$ by siRNA would antagonize the inhibitory effect of S1P on glioblastoma cell migration. To validate this approach we measured the level of $S1P_2$ mRNA by quantitative RT-PCR in cells

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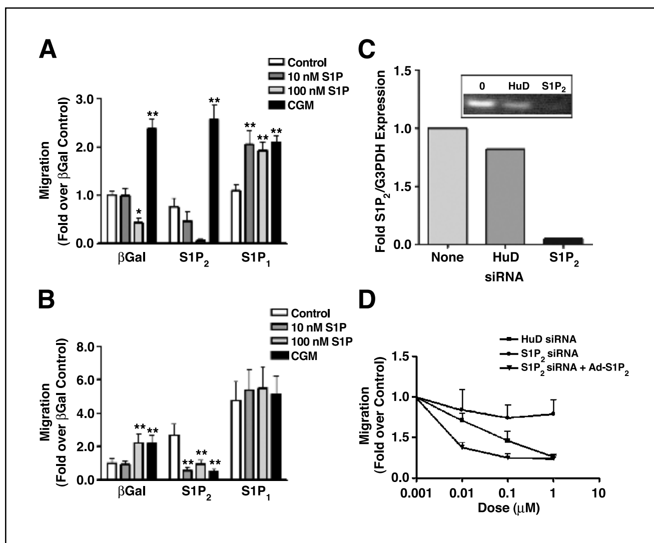

Figure 3. $S1P_2$ inhibits migration in glioblastoma cells. Migration of U118 cells (*A*) or U87 cells (*B*) overexpressing β-gal (*βGal*), $S1P_2$, or $S1P_1$ in response to S1P. *Columns,* fold over *β*-gal control for triplicate wells; *bars,* ±SE. Statistically significant differences from control were observed: *, $P < 0.05$; **, $P < 0.01$ (ANOVA, Dunnett's test). *C,* selective blockade of $S1P_2$ expression by siRNA in U118 cells transfected with 200 nmol/L siRNA to the open reading frame of human $S1P_2$ or *HuD* as a control and were harvested 48 hours later and assayed for the level of $S1P_2$ mRNA by quantitative RT-PCR. *Columns,* fold over untransfected (*control*). *Inset,* gel electrophoresis of $S1P_2$ RT-PCR products after 30 cycles: *lane 1,* 0 nmol/L siRNA; *lane 2,* 200 nmol/L HuD siRNA; *lane 3,* 200 nmol/L $S1P_2$ siRNA. *D,* migration of U118 cells transfected with 200 nmol/L control siRNA to HuD (■), 200 nmol/L siRNA $S1P_2$ (●), or a combination of 200 nmol/L siRNA $S1P_2$ and adenovirus rat $S1P_2$ (▼). *Points,* fold over control for triplicate wells; *bars,* ±SE. Experiments were performed twice with similar results.

treated with siRNA against human $S1P_2$ at 24- and 48-hour time points. siRNA against $S1P_2$ was extremely effective at reducing the level of $S1P_2$ mRNA (Fig. 3*C*). The expression of $S1P_2$ was nearly abolished after 48 hours with 200 nmol/L $S1P_2$ siRNA, whereas directed against an irrelevant neuronal protein, HuD, had no effect on $S1P_2$ mRNA levels (Fig. 3*C*).

$S1P_2$ siRNA treatment of U118 cells completely abrogated S1P inhibition of cell migration, whereas a control siRNA was without an effect (Fig. 3*D*). The specificity of $S1P_2$ siRNA was further confirmed in a recovery experiment using $S1P_2$ siRNA with the rat $S1P_2$ adenovirus expression vector, which is not targeted by the human $S1P_2$ siRNA. These cells were inhibited by S1P similarly to cells in Fig. 3*A* that overexpressed $S1P_2$ (Fig. 3*D*). These data corroborate overexpression studies establishing that $S1P_2$ mediates inhibition of migration of glioblastoma cells.

Lack of inhibition of the Rac GTPase in S1P-mediated inhibition of glioblastoma cell migration. Having established the requirement for $S1P_2$ on S1P-mediated inhibition of migration in U118 cells, we investigated whether small GTPases such as Rho and Rac are involved. In particular, we checked for Rho-dependent inhibition of Rac, which was shown recently in melanoma and CHO cells (24). As shown in Supplemental Fig. S2, treatment of U118 cells with 100 nmol/L S1P led to a robust activation of Rho by 1 minute that was returned to baseline after for 30 minutes. Interestingly, Rac was also activated nearly 2-fold and did not return to baseline within 30 minutes of stimulation, despite the fact that S1P inhibits migration in these cells (Supplemental Fig. S2). To confirm these findings we did a similar time course in U118 cells overexpressing $S1P_2$ or β-galactosidase as a control (Fig. 4*A*). As shown in Fig. 4*A*, stimulation of U118 cells overexpressing $S1P_2$ with 100 nmol/L S1P resulted in a robust activation of Rho and Rac. In fact, overexpression of $S1P_2$ led to a more potent activation of Rac compared with β-galactosidase transduced cells. To rule out cell type–specific effects on Rac and Rho, we also did overexpression experiments with the U87 cell line. As shown in Fig. 4*B*, a 1-minute stimulation of U87 cells overexpressing $S1P_2$ led to a dramatic induction of activated Rho with a concomitant modest increase in Rac activity. Taken together, these results do not reveal any evidence for inhibition of Rac GTPase during S1P-mediated inhibition of migration in glioblastoma cells, further confirming that modulation of $S1P_2$ receptor expression alone inhibits migratory responses.

Rho/Rho-associated protein kinase pathway mediates $S1P_2$ inhibition of glioblastoma cell migration. Activated GTP-bound Rho activates several downstream signaling pathways, among which ROCK is a prominent player. ROCK is a serine/threonine kinase and phosphorylates a number of substrates involved in actin-filament assembly and contractility (25). We tested the effects of a ROCK inhibitor, Y-27632, on glioblastoma cell migration. Cells were pretreated with Y-27632 for the last 30 minutes of a 2-hour serum starvation before harvest for migration assays. As shown in Fig. 5*A*, U118 control cells were inhibited by S1P in a dose-dependent manner as expected. However, cells pretreated with Y-27632 were considerably less inhibited at 10 and 100 nmol/L S1P compared with control cells. Experiments were also carried out on the U87 cell line and showed that Y-27632 does not effect S1P-induced migration (Fig. 5*B*). These data suggest that S1P activates Rho via $S1P_2$, and that downstream ROCK activity is required for the inhibition of migration.

Having shown that both Rac and Rho are activated in glioblastoma cells in response to S1P and that Rho/ROCK pathway is involved in the chemorepellant activity of $S1P_2$, we used confocal microscopy to delineate the changes in actin cytoskeleton in response to S1P in U118 and U87 cells overexpressing $S1P_1$ or $S1P_2$ treated or not with Y-27632. As shown in Fig. 6*A*, activation of endogenous $S1P_2$ receptors in β-gal overexpressing U118 cells increased the percentage of cells containing stress fibers from 3% in control to 48% in S1P treated cells (*e*). This Rho signaling pathway was exacerbated in U118 cells overexpressing $S1P_2$ as evidence by significant cell rounding (Fig. 6*A*, *c* and *g*). Approximately 2% of U118 cells overexpressing $S1P_2$ exhibited the cell rounding phenotype compared with 32% of cells treated with S1P. The Rho kinase inhibitor Y-27632 substantially reduced the percentage of $S1P_2$ overexpressing cells exhibiting either stress fibers or cell rounding to 13% and 3%, respectively (Fig. 6*A*, *d* and *h*). Stimulation of U118 cells overexpressing $S1P_1$ resulted in the redistribution of actin to both stress fibers and lamellipodia (Fig. 6*A*, *b* and *f*). S1P stimulation of U87 cells that endogenously expresses low levels of $S1P_2$ resulted in 66% of the cells forming lamellipodia and membrane-ruffling structures compared with 32% of control cells indicative of pronounced Rac activation (Fig. 6*B*, *e*). These cells were flatter than β-gal control cells, a property indicative of increased adhesion and focal contact site formation around the cell periphery by $S1P_1$-mediated Rac signaling (26). Overexpression of $S1P_2$ in U87 cells led to 55% of cells rounding compared with <1% in control (Fig. 6*B*, *c* and *g*), which was reduced to 6% by the Rho kinase inhibitor Y-27632 (Fig. 6*B*, *d* and *h*). Confocal microscopy data correlates with migration data showing that S1P-$S1P_2$ receptor signaling results in ROCK-dependent inhibition of migration and stress fiber formation and, conversely, activation

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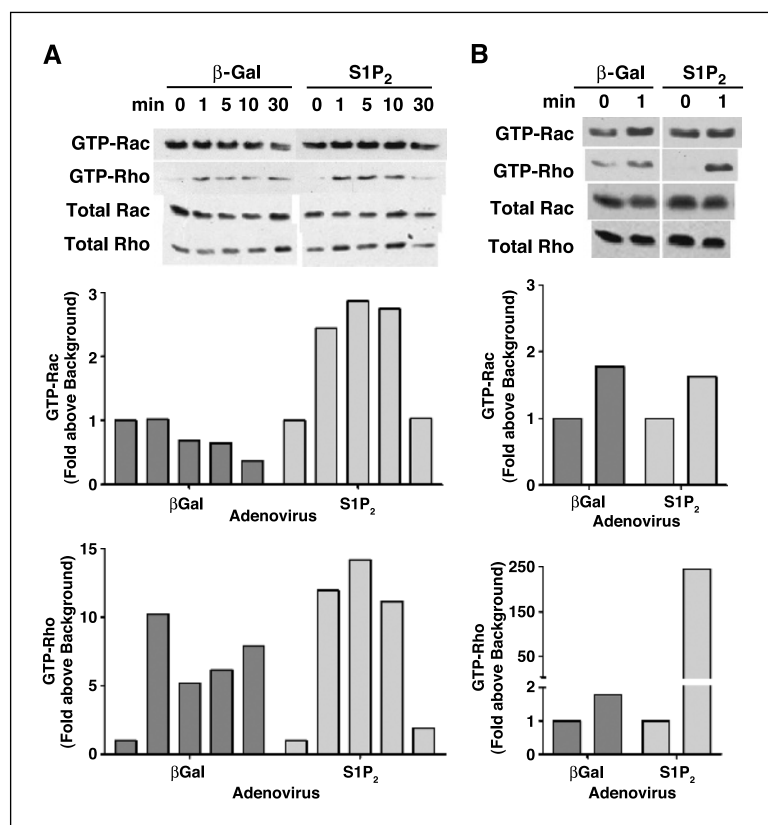

Figure 4. Rac and Rho are activated in cells overexpressing $S1P_2$. *A,* time course activation of Rac and Rho in U118 cells overexpressing β-gal or $S1P_2$ in response to 100 nmol/L S1P. Activated Rac and Rho were isolated in pull-down assays as described in Materials and Methods. Representative experiment that was repeated thrice. *B,* Rac and Rho activation in U87 cells overexpressing β-galactosidase or $S1P_2$ following a 1-minute stimulation with 100 nmol/L S1P. Western blot analysis of activated Rac or Rho and total Rac and Rho protein levels (*top*) and quantitated results (*bottom*). Experiments were performed twice with similar results.

of S1P-$S1P_1$ signaling pathways leads to stimulation of migration through Rac activation and lamellipodia formation.

Proliferation of glioblastoma cells in response to S1P or dimethyl-sphingosine. Having established that the S1P receptor profile in glioblastoma cells dictates its migratory response to S1P, such that high $S1P_2$ expression inhibits migration, we were interested in knowing whether receptor expression also conferred a higher proliferative response to S1P. Cells were cultured in the presence of increasing concentrations of S1P for 48 or 96 hours at which time they were assayed for growth by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction assay. Our results indicate that S1P is weakly mitogenic in some cell lines, such as U118, and, as in the case for U87 cells, may even inhibit growth at higher concentrations (0.1-1 μmol/L) after 96 hours (4 days) of culture (Supplemental Fig. S3*A*). Similar results were obtained for the U138 cell line (data not shown).

Because U87 and U138 cell lines expressed relatively high levels of SK1 mRNA (Supplemental Fig. S1*B*), we investigated whether the SK inhibitor dimethyl-sphingosine (DMS) could influence the growth of these cells *in vitro*. DMS potently inhibited proliferation of U87 (high SK1) and U138 (lower SK1) at concentrations >1 μM (Supplemental Fig. S3*B*). DMS-treated cells rapidly died as evidenced by the cells rounding up and detaching from the dish. U138 cells behaved similarly (data not shown).

Discussion

The role of S1P receptor signaling in human tumors is not well characterized. As a basis for our studies, we began by comprehensively characterizing S1P receptor ($S1P_{1\text{-}5}$) expression and S1P metabolizing enzymes in three different adult human glioblastoma cell lines using SYBR green quantitative RT-PCR. All S1P receptors were present at varying levels in each cell line, except for lack of expression of $S1P_4$ in U138 cells (Supplemental Fig. S1*A*). SK1 and SK2, as well as SPP and SGPL were expressed at 3 to 4 orders of magnitude higher than S1P receptors (Supplemental Fig. S1*B*). In

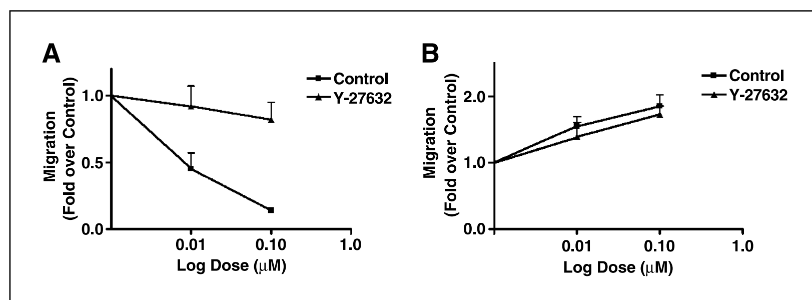

Figure 5. Rho kinase activity is required for $S1P_2$-mediated inhibition of migration in U118 cells. U118 (*A*) or U87 (*B*) and control cells (■) or cells pretreated with Y-27632 (▲) were assayed for their ability to migrate in response to increasing doses of S1P. *Points,* fold over control for triplicate wells from an experiment that was repeated twice; *bars,* ±SE.

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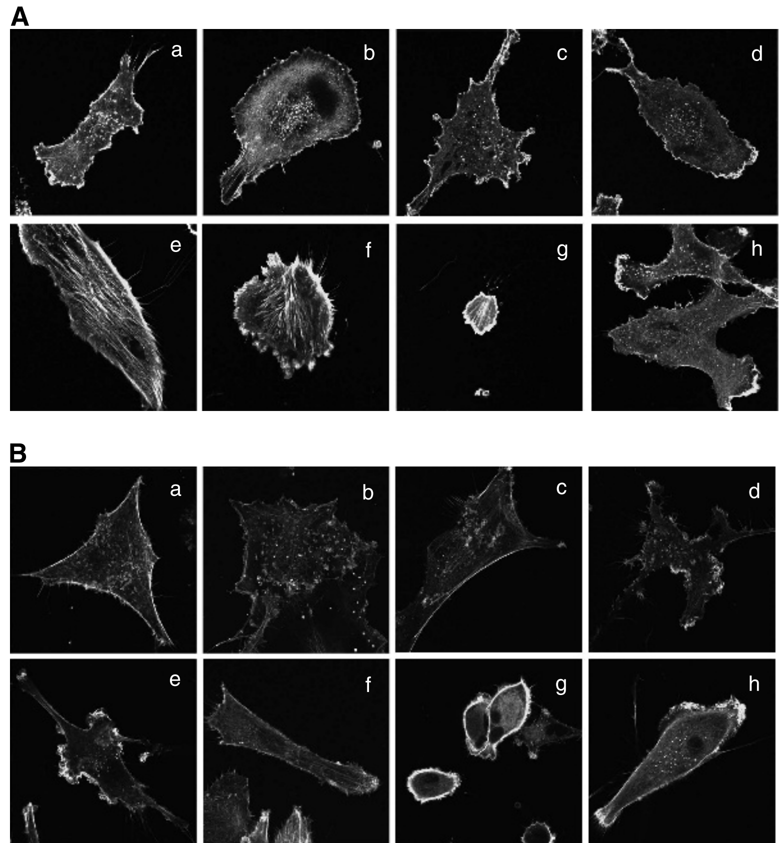

Figure 6. Effect of Rho kinase inhibition on $S1P_2$ induced F-actin in glioblastoma cells. U118 cells (*A*) and U87 cells (*B*) overexpressing β-gal (*a* and *e*), $S1P_1$ (*b* and *f*), $S1P_2$ (*c* and *g*), or $S1P_2$ in combination Y-27632 (*d* and *h*) were either nonstimulated (*a-d*) or stimulated (*e-h*) with 100 nmol/L S1P for 1 hour before staining with TRITC-phalloidin to visualize filamentous actin. Experiments done twice with similar results.

addition, we confirmed expression of these genes in primary pediatric glioblastoma tumor specimens obtained from the children's oncology group biopathology center (data not shown).

We found that S1P has a profound and differential effect on glioblastoma motility. Only one cell line, U87, of three migrated in a pertussis toxin sensitive manner in response to S1P (Fig. 1). U118 and U138 cell lines were strongly inhibited by S1P (Fig. 1*A*). Inhibition of migration by S1P was insensitive to pertussis toxin, thereby implicating non-G_i linked signaling pathways (Fig. 1*B*). Our data support several reports in the literature that define G_i-linked $S1P_1$ and $S1P_3$ as chemoattractant receptors and $G_{12/13}$-linked $S1P_2$ as a chemorepellant receptor (24).

A major finding of the present study is the correlation between the level of $S1P_2$ mRNA expression and degree of S1P-mediated inhibition of migration (Fig. 2). This correlation was confirmed in migration experiments using FTY720-P, an S1P analogue that binds to all S1P receptors except $S1P_2$ (23), which was ineffective at inhibiting migration (Fig. 2*B*). FTY720-P does not significantly induce migration of U118 cells despite their expression of $S1P_3$. This may be due to the fact that the affinity of FTY720-P for S1P receptors varies and is reported to be greatest for $S1P_1$ (23). Consequently, a different effect between responses mediated via $S1P_1$ versus $S1P_3$ could be expected. Furthermore, unlike $S1P_1$, $S1P_3$ can also signal through $G_{12/13}$ thereby inhibiting migration. Finally, despite having similar receptor profiles, tumor cells may exhibit varied responses to FTY720-P due to other signal transduction abnormalities. Our data agree with that obtained by Osada et al., who used a recently developed $S1P_2$ antagonist, JTE-013, to reverse the inhibitory actions of S1P on migration in vascular endothelial cells and smooth muscles cells (27).

We proved that $S1P_2$ mediates inhibition of migration in response to S1P by over expression of $S1P_2$, as well as $S1P_2$ gene silencing via siRNA (Fig. 3). Importantly, U118 cells are capable of S1P-stimulated migration as shown by adenoviral overexpression of $S1P_1$ (Fig. 3*A*), so the effects of S1P are not due to the lack of downstream signaling machinery. Recent findings by Yamaguchi et al. show that S1P treatment of mouse B16 melanoma cells that express only $S1P_2$ significantly reduced lung metastasis after tail vein injection, and these effects could be potentiated or aggravated by overexpression of $S1P_2$ or $S1P_1$, respectively (28). Because $S1P_2$ expression varied in the three glioblastoma lines we tested, more comprehensive studies on primary tumors are warranted to determine whether $S1P_2$ expression changes during tumor progression, and whether changes have value in predicting subsequent invasion, tumor sequelae development after surgery, and poor prognosis.

In the present study, we explored the mechanism of $S1P_2$-mediated inhibition of migration in glioblastoma cells by examining downstream effectors of $S1P_2$. Functional data for $S1P_2$ indicates that this receptor couples primarily to G_{12} or G_{13}, which activate the small GTPase Rho (5). Whereas it has been shown that Rac and Rho are both required for establishing cell polarity in terms of "frontness" and "backness," respectively, excessive Rho activity seems to inhibit polarization and motility (29). Rho mediates stress fiber formation and focal adhesion, processes associated with stationary cells, through a phosphorylation

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cascade beginning with p160 Rho kinase (ROCK), and ultimately impeding activity of cofilin and myosin light-chain phosphatase thereby stabilizing actin filaments (26). Not surprisingly, U118 cells stimulated with 100 nmol/L S1P resulted in a 5-fold induction of RhoA activity and 2-fold activation of Rac after 1 minute (Supplemental Fig. S2). Contrary to the model proposed by Takuwa et al., Rac was also activated in both cell lines even when $S1P_2$ was overexpressed (Fig. 4). These data clearly indicate that $S1P_2$-specific inhibition of Rac activity is not involved in S1P-mediated inhibition of migration in the human glioblastoma cell lines examined.

To gain further insight into events following Rho activation, we examined the effect of the specific ROCK inhibitor Y-27632 on S1P-mediated migration (Fig. 5). ROCK activity was found required for S1P-mediated inhibition of U118 cells but independent of S1P-induced migration of U87 (Fig. 5). Our data contradicts a recent report by Sugimoto et al. who did not observe any prevention of S1P-$S1P_2$–mediated inhibition of migration by Y-27632 or a structurally unrelated Rho kinase inhibitor HA-1077 in CHO cells stably transfected with $S1P_2$ (13). These disparate observations suggest that there exist both Rho-kinase–dependent and –independent mechanisms for Rho regulation of migration in different cell types.

The ability of cells to coordinately reorganize the actin cytoskeleton is a hallmark of motile cells. Confocal microscopy of actin-stained S1P-stimulated U118 cells that are inhibited to migrate in response to S1P resulted in dramatic stress fiber formation, as expected (Fig. 6*A*, *e*). U87 cells, on the other hand, are potently stimulated to migrate in response to S1P and showed lamellipodia and membrane ruffling structures in response to S1P, as anticipated (Fig. 6*B*, *e*). Our findings support results by Okamoto et al. who used CHO cells expressing $S1P_1$ or $S1P_2$ mediated lamellipodia and membrane ruffling or stress fibers, respectively, in response to S1P (22). Stress fiber formation was blocked in U118 cells pretreated with the Rho kinase inhibitor Y-27632, which agrees with previous findings by Sugimoto et al. using CHO cells overexpressing $S1P_2$ (13). Confocal microscopy data correlates with migration assays with U118 and U87 cells showing that S1P-$S1P_2$ receptor signaling results in ROCK-dependent inhibition of migration and stress fiber formation and, conversely, activation of S1P-$S1P_1$ signaling pathways leads to stimulation of migration through Rac activation and lamellipodia formation.

Having established the requirement for $S1P_2$ in S1P-mediated inhibition of migration, we were interested in knowing whether $S1P_2$ also influenced the proliferative capacity of glioblastoma cells *in vitro*. Interestingly, a recent finding showed that S1P inhibits proliferation of rat hepatocytes by activating Rho via $S1P_2$ (30). In proliferation experiments we consistently found S1P to be a weak mitogen for glioblastoma cell lines tested at doses ranging from 10 nmol/L to 1 μmol/L (Supplemental Fig. S3*A*). Our data on the proliferative capacity of U87 and U118 glioblastoma cells in response to S1P agrees with that observed by Van Brocklyn et al. who showed that U87 and U118 cells treated with 100 nmol/L S1P resulted in a 1.0- and 1.02-fold increase, respectively (15). We conclude that whereas $S1P_2$ has an important role in glioblastoma cell migration, it does not correlate with proliferation.

Enzymes involved in S1P metabolism can perturb the levels of S1P. Previous reports in the literature have shown that overexpression of sphingosine kinase in NIH 3T3 cells leads to increased proliferation and acquisition of a transformed phenotype (31). We used a competitive inhibitor of sphingosine kinase, DMS, to assess whether high sphingosine kinase mRNA expression identified in U87 cells conferred a growth advantage (Supplemental Fig. S3*B*). Our results did not support a mechanism of enhanced growth potential in high SK expressing cells, because both U87 and U118 cells were equally inhibited by DMS (Supplemental Fig. S3*B*).

In conclusion, our results show that $S1P_2$ mediates inhibition of migration *in vitro* in response to S1P, and this inhibition is dependent on Rho kinase activity but not concomitant inhibition of Rac activity. Manipulating $S1P_2$ expression, receptor activity, or downstream effectors such as Rho-kinase may be a point for intervention in human cancers.

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The G Protein–Coupled Receptor $S1P_2$ Regulates Rho/Rho Kinase Pathway to Inhibit Tumor Cell Migration

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