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Inhibitory and Stimulatory Regulation of Rac and Cell Motility by the $G_{12/13}$-Rho and G_i Pathways Integrated Downstream of a Single G Protein-Coupled Sphingosine-1-Phosphate Receptor Isoform

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The G protein-coupled receptors $S1P_2$/Edg5 and $S1P_3$/Edg3 both mediate sphingosine-1-phosphate (S1P) stimulation of Rho, yet $S1P_2$ but not $S1P_3$ mediates downregulation of Rac activation, membrane ruffling, and cell migration in response to chemoattractants. Specific inhibition of endogenous $G\alpha_{12}$ and $G\alpha_{13}$, but not of $G\alpha_q$, by expression of respective C-terminal peptides abolished $S1P_2$-mediated inhibition of Rac, membrane ruffling, and migration, as well as stimulation of Rho and stress fiber formation. Fusion receptors comprising $S1P_2$ and either $G\alpha_{12}$ or $G\alpha_{13}$, but not $G\alpha_q$, mediated S1P stimulation of Rho and also inhibition of Rac and migration. Overexpression of $G\alpha_i$, by contrast, specifically antagonized $S1P_2$-mediated inhibition of Rac and migration. The $S1P_2$ actions were mimicked by expression of V^{14}Rho and were abolished by C3 toxin and N^{19}Rho, but not Rho kinase inhibitors. In contrast to $S1P_2$, $S1P_3$ mediated S1P-directed, pertussis toxin-sensitive chemotaxis and Rac activation despite concurrent stimulation of Rho via $G_{12/13}$. Upon inactivation of G_i by pertussis toxin, $S1P_3$ mediated inhibition of Rac and migration just like $S1P_2$. These results indicate that integration of counteracting signals from the G_i- and the $G_{12/13}$-Rho pathways directs either positive or negative regulation of Rac, and thus cell migration, upon activation of a single S1P receptor isoform.

Regulation of cell migration is critical in such diverse biological processes as organogenesis, neuronal axon pathfinding, wound healing, inflammatory responses, vascular remodeling, and tumor cell dissemination (21). Extracellular cues called attractants and repellants, which are either soluble or membrane bound, instruct cells to advance and to retreat, respectively (36, 40). A number of chemokines, growth factors, cytokines, and other inflammatory mediators have been shown to stimulate directed cell migration, whereas a much more limited number of biological mediators have been shown to inhibit cell motility in a manner dependent on their concentration gradients. The latter include metastin (28), Slit, semaphorins, ephrins (44), and a lipid mediator, sphingosine 1-phosphate (S1P) (42). S1P is a bioactive lysophospholipid that exerts a wide variety of biological activities, most of which are mediated via Edg family G protein-coupled receptors (GPCRs), including $S1P_1$/Edg1, $S1P_2$/Edg5/AGR16/H218, and $S1P_3$/Edg3 (7, 16, 39, 43). S1P has been demonstrated to be quite unique as an extracellular regulator of motility in that it exerts either stimulatory or inhibitory actions on cell motility (42). These bimodal actions are apparently cell type specific; thus, S1P stimulates chemotaxis in vascular endothelial cells (22) and embryonic fibroblasts (24), whereas it inhibits cell migration in vascular smooth muscle cells (3, 33) and melanoma cells (34). We recently showed that this bimodal regulation by S1P is based upon a diversity of S1P receptor isotypes, which mediate either stimulatory or inhibitory regulation for cell migration (31, 42). Thus, we found that $S1P_2$ acts as a repellant receptor to mediate inhibition of chemotaxis toward attractants, whereas $S1P_1$ and $S1P_3$ act as attractant receptors to mediate migration directed toward S1P. Elimination of the S1P receptor gene in mice (24) and development of a drug to target S1P receptors (4, 25) have revealed that S1P is involved in regulation of cell migration in vivo, thus contributing to morphogenesis and regulation of lymphocyte homing.

Small GTPases of the Rho family, primarily Rac, Cdc42, and Rho, are well-known regulators of actin organization and myosin motor function and thereby of cell motility (10, 14, 47). These Rho GTPases show distinct activities on actin cytoskeletons: Rho mediates stress fiber formation and focal adhesion, while Rac and Cdc42 direct peripheral actin assembly that results in formation of lamellipodia and filopodia, respectively. Despite limitation of our understanding of intracellular signaling from the membrane to the cytoskeleton, a model has emerged from the observations in a variety of cell types that attractive extracellular cues activate Rac or Cdc42, while repulsive cues inhibit Rac or Cdc42 and stimulate Rho (9, 38, 42, 48). In fact, the repellant receptor $S1P_2$ negatively regulates cellular Rac activity through mechanisms involving stimulation of a GTPase-activating protein (GAP) for Rac (31). In contrast, the attractant receptors $S1P_1$ and $S1P_3$ mediate activation of Rac via G_i (22, 31, 32). Neither of these S1P receptors affects Cdc42 activity under our experimental conditions. Interestingly, the repellant receptor $S1P_2$ and the attractant receptor $S1P_3$ similarly mediate stimulation of cellular RhoA activity, most likely via $G_{12/13}$. Expression of N^{17}Rac, but not N^{19}RhoA or C3 toxin treatment, inhibited cell migration, indicating an essential role of Rac in cell migration (31, 33).

In the present study we explored the mechanisms by which $S1P_2$ receptor activation leads to Rac inhibition. The results of the present study demonstrate for the first time that inhibitory regulation of Rac by the GPCR is mediated via $G_{12/13}$ and Rho, through a downstream signaling mechanism not involving

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Rho kinase/ROCK/ROK. Our data also show that G_i exerts a stimulatory regulation for Rac which antagonizes and completely reverses $G_{12/13}$-mediated inhibitory regulation of Rac. Indeed, we found that the attractant receptor $S1P_3$ was converted to a repellant receptor upon pertussis toxin (PTX) treatment. Thus, these results indicate that integration of signals from G_i and $G_{12/13}$ determines cellular Rac activity, which directs migration toward or away from a GPCR agonist.

MATERIALS AND METHODS

Materials. S1P and 1-monooleoyl lysophosphatidic acid (LPA) were purchased from Biomol (Plymouth Meeting, Pa.) and Avanti (Birmingham, Ala.), respectively. They were dissolved, aliquoted, and stored as described previously (29). Recombinant human insulin-like growth factor I was purchased from R&D Systems. A rabbit polyclonal anti-$G\alpha_{q/11}$ and a mouse monoclonal anti-Rac antibody were purchased from Upstate Biotechnology. Rabbit polyclonal antibodies against $G\alpha_{s/olf}$ (C-18), $G\alpha_{i3}$ (C-10), anti-$G\alpha_{12}$ (S-20), $G\alpha_{13}$ (A-20), and GRK2 (H-222) and a mouse monoclonal anti-RhoA antibody were bought from Santa Cruz Biotechnology. A rabbit polyclonal anti-$G\alpha_{13}$ antibody (371778) was bought from Calbiochem. A mouse monoclonal anti-ERK1/2 antibody (clone 03-6600) was obtained from Zymed Laboratories Inc. An anti-FLAG M2 antibody and tetramethyl rhodamine isocyanate (TRITC)-labeled phalloidin were obtained from Sigma. PTX was bought from List Biological Laboratories. $AlCl_3$ and NaF were obtained from Wako Pure Chemicals (Osaka, Japan) and were added to media at a molar ratio of 1:4 ($AlCl_3$ to NaF) to generate AlF_4^-. Y-27632 and HA-1077 were supplied by Mitsubishi Pharma (Wako, Japan) and Asahi Chemical Industry (Fuji, Japan), respectively. Botulinum C3 toxin, the glutathione *S*-transferase (GST)–human PAK1 (amino acids 75 to 131) fusion protein, GST-mouse rhotekin (amino acids 7 to 89), and a mouse monoclonal anti-myc antibody (9E10) were prepared as described previously (41).

Plasmids, adenoviruses, and transfections. pME18S-myc-N^{19}RhoA, pME18S-myc-V^{14}RhoA, pGEX-2T-rhotekin, pGEX-2T-PAK, and an adenovirus encoding myc-N^{19}RhoA were described previously (31, 33, 35). cDNAs encoding full-length mouse $G\alpha_s$, $G\alpha_{i2}$, $G\alpha_{12}$, $G\alpha_{13}$, and $G\alpha_q$, and the C-terminal peptide of human β-adrenergic receptor kinase (βARK-CT; βARK residues 495 to 689), were obtained by reverse transcription-PCR (RT-PCR) from total mouse brain RNA and human brain RNA (Sawady Technology, Tokyo, Japan), respectively. PCR-based methods were used to generate the cDNAs encoding myc-tagged C-terminal regions of $G\alpha_s$ (residues 319 to 377), $G\alpha_{12}$ (residues 326 to 379), $G\alpha_{13}$ (residues 321 to 377), and $G\alpha_q$ (residues 306 to 359), which were designated $G\alpha_s$-CT, $G\alpha_{12}$-CT, $G\alpha_{13}$-CT, and $G\alpha_q$-CT; $S1P_2$ with a FLAG-epitope at its N terminus (FLAG-$S1P_2$); and fusion receptors $S1P_2$-$G\alpha_{12}$, $S1P_2$-$G\alpha_{13}$, and $S1P_2$-$G\alpha_q$, which have full-length $G\alpha_{12}$, $G\alpha_{13}$, and $G\alpha_q$, respectively, fused to the C terminus of $S1P_2$. The cDNAs of full-length Gα proteins, their C termini, and the FLAG-$S1P_2$ and $S1P_2$-Gα fusion receptors were ligated onto the mammalian expression vector pCAGGS (a gift from M. Miyazaki, Osaka University Medical School) to generate pCAGGS-$G\alpha_{i2}$, pCAGGS-$G\alpha_{12}$, pCAGGS-$G\alpha_{13}$, pCAGGS-$G\alpha_q$, pCAGGS-$G\alpha_s$-CT, pCAGGS-$G\alpha_{12}$-CT, pCAGGS-$G\alpha_{13}$-CT, pCAGGS-$G\alpha_q$-CT, pCAGGS-FLAG-$S1P_2$, pCAGGS-$S1P_2$-$G\alpha_{12}$, pCAGGS-$S1P_2$-$G\alpha_{13}$, and pCAGGS-$S1P_2$-$G\alpha_q$, respectively. βARK-CT cDNA was ligated onto the mammalian expression vector pME18S (a gift from K. Maruyama, Tokyo Medical and Dental University) to generate pME18S-βARK-CT. Replication-deficient adenoviruses encoding $G\alpha_{12}$-CT, $G\alpha_{13}$-CT, and $G\alpha_q$-CT were generated and amplified as described previously (8). pCAGGS-LacZ and an adenovirus encoding LacZ were kindly donated by I. Saito (Institute of Medical Sciences, University of Tokyo).

The cells were infected with adenoviruses at a multiplicity of infection of 200 by incubating cells with an adenovirus-containing medium for 1 h, which conferred successful gene transduction in nearly 100% of cells. After recovery in growth medium for 24 h, the cells were serum deprived for 24 h before experiments.

Transient transfection with expression plasmid vectors was carried out by using LipofectAMINE (Invitrogen) 48 h before each experiment. To study the migration of transiently transfected cells, the cells were cotransfected with either one of the Gα-CT expression plasmids, the βARK-CT expression plasmid, or the empty vector and pCAGGS-LacZ as a transfection marker (31) for 3 h. In some experiments in which the actin cytoskeleton was evaluated (Fig. 2C), the green fluorescent protein (GFP) expression vector pEGFP-C1 (Clontech) was employed as a transfection marker. After recovery in growth medium for 21 h, the cells were serum deprived for 24 h.

To establish CHO cells that stably express $S1P_2$-Gα fusion receptors, cells were cotransfected with pCAGGS-$S1P_2$-Gα and the neomycin resistance gene expression vector pKM3 (27) and were selected in the presence of 0.7 mg of G418 (Nacalai, Kyoto, Japan)/ml. To establish CHO-$S1P_2$ cells that stably express full-length Gα protein and $G\alpha_q$-CT, CHO-$S1P_2$ cells were cotransfected with either pCAGGS-Gα, pCAGGS-$G\alpha_q$-CT, or the Zeocin resistance gene expression vector pCMV/Zeo (Invitrogen) and were selected in the presence of 50 μg of Zeocin (Invitrogen)/ml and 0.7 mg of G418/ml. Cloned cells were isolated and tested for expression of transduced genes.

In the experiments using CHO-$S1P_2$ cells that express N^{19}Rho or V^{14}Rho, CHO-$S1P_2$ cells were cotransfected with either pME18S-myc-N^{19}RhoA or pME18S-myc-V^{14}RhoA and pCMV/Zeo and were selected in the presence of 50 μg of Zeocin/ml. The Zeocin-resistant cell populations were employed in these experiments.

Cells. CHO-K1 (CHO) cells, Swiss 3T3 cells, and COS7 cells were grown in Ham's F-12 (CHO) or Dulbecco's modified Eagle medium (3T3 and COS7) supplemented with 10% fetal bovine serum (Equitech-Bio, Ingram, Tex.), 100 U of penicillin/ml, and 100 μg of streptomycin/ml (Wako Pure Chemicals). CHO cells that stably overexpress either $S1P_1$, $S1P_2$, or $S1P_3$, i.e., CHO-$S1P_1$, CHO-$S1P_2$, and CHO-$S1P_3$ cells, respectively, have been described previously (13, 29, 30) and were maintained in the presence of 0.7 mg of G418/ml. Cells were treated with C3 toxin (10 μg/ml) in F-12 medium containing 10% fetal bovine serum for 48 h and then in serum-free F-12 medium for a further 24 h. PTX (200 ng/ml) treatment was carried out by incubating cells in serum-free F-12 medium containing PTX for 24 h before experiments.

Transwell migration assay. Chemotactic migration of cells was measured in a modified Boyden chamber (Neuroprobe) using polycarbonate filters with 8-μm pores as described in detail previously (31, 33). In migration assays using transiently transfected cell populations, the migratory cells attached to the lower side of the membrane were subjected to staining with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside as a substrate. The number of migratory cells staining positive with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside was determined by using a microplate reader as described above.

Determination of the activities of Rho and Rac. Pulldown assay methods to determine GTP-bound active forms of Rho and Rac have been described in detail previously (31, 33, 35). Briefly, cell extracts were incubated with the GST-rhotekin Rho-binding domain (for determination of Rho activity) or the GST-PAK CRIB domain (for determination of Rac activity) immobilized to glutathione-Sepharose 4B beads (Pharmacia Amersham Biotech) at 4°C for 45 min, followed by three washes. Bound Rho and Rac proteins were quantitatively detected by Western blotting using specific, monoclonal antibodies against RhoA and Rac.

Western blotting, $[Ca^{2+}]_i$ measurement, and fluorescence microscopy. Western blotting was performed as described previously (29). The band shift of activated p42 and p44 extracellular signal-regulated kinase (ERK) was detected by Western blot analysis of total cell lysates with a mouse monoclonal anti-ERK antibody (8). Intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured as described previously (29) in Fura-2-loaded cells with a CAF-110 spectrofluorimeter (Japan Spectroscopy, Inc., Tokyo, Japan) with excitation at 340 and 380 nm and emission at 500 nm.

To evaluate actin cytoskeletons, cells were transfected as indicated 48 h before experiments and were serum starved for 24 h. After treatment with receptor agonists and/or Rho kinase inhibitors for indicated times, the cells were fixed in 3.7% formaldehyde in phosphate-buffered saline and processed as described previously (41). F-actin was visualized with TRITC-labeled phalloidin under an inverted fluorescence microscope IX70 (Olympus, Tokyo, Japan).

Statistics. Values are presented as means ± standard errors of three or more determinations and are representative of at least two independent experiments with similar results.

RESULTS

AlF_4^- mimics $S1P_2$ actions in inhibiting Rac and migration in IGF I-stimulated cells. Most of the actions of GPCRs are mediated through heterotrimeric G protein coupling. However, recent studies provide evidence that mechanisms independent of heterotrimeric G protein coupling mediate certain actions of GPCRs, which include regulation of a Na^+-H^+ exchanger and a Ca^{2+} channel, as well as activation of STAT (5). To understand in more depth the molecular mechanisms

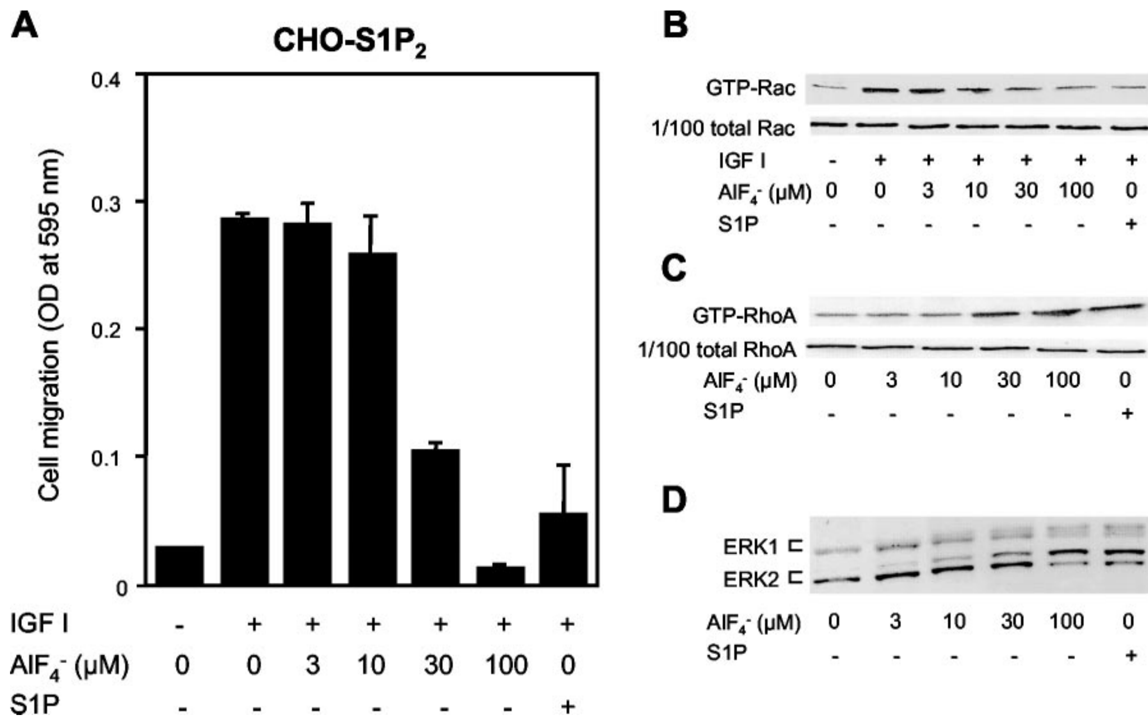

FIG. 1. AlF_4^- mimicks S1P actions in inhibiting Rac and migration and stimulating Rho and ERK in $S1P_2$ receptor-expressing cells. (A) AlF_4^- and S1P inhibit IGF I-directed chemotaxis. Transwell migration of CHO-$S1P_2$ cells toward IGF I (100 ng/ml) was determined in the presence or absence of various concentrations of AlF_4^- and 10^{-7} M S1P in the lower chamber. (B) AlF_4^- and S1P inhibit IGF I-induced Rac stimulation. CHO-$S1P_2$ cells were treated with various concentrations of AlF_4^- or 10^{-7} M S1P for 10 min and then stimulated with IGF I (100 ng/ml) for 1 min. Cells were then subjected to a pulldown assay for GTP-Rac as described in Materials and Methods. GTP-Rac bound to the GST-PAK1 CRIB domain immobilized onto Sepharose beads was analyzed by Western blotting using an anti-Rac antibody (top), and 1/100 of total Rac present in the cell lysate is also shown to confirm loading of equal amounts of proteins (bottom). (C) AlF_4^- and S1P stimulate Rho. CHO-$S1P_2$ cells were stimulated with various concentrations of AlF_4^- or 10^{-7} M S1P for 3 min. Cells were then subjected to a pulldown assay for GTP-RhoA as described in Materials and Methods. (D) AlF_4^- and S1P stimulate ERK1 and -2. CHO-$S1P_2$ cells were stimulated with various concentrations of AlF_4^- or 10^{-7} M S1P for 5 min. ERK activation was determined by band shift analysis using Western blotting.

underlying GPCR-mediated inhibition of cell migration, we first investigated whether $S1P_2$-mediated Rac inhibition occurs through heterotrimeric G protein coupling, and, if so, which heterotrimeric G protein mediates Rac inhibition. In CHO cells expressing the $S1P_2$ receptor (CHO-$S1P_2$), but not in naive CHO cells or CHO cells expressing $S1P_1$ or $S1P_3$, S1P dose-dependently and potently inhibited IGF I-directed chemotaxis, which is a Rac-dependent process (31). Direct stimulation of the heterotrimeric G proteins with AlF_4^- (17) dose-dependently inhibited chemotaxis of CHO-$S1P_2$ cells toward IGF I, like S1P stimulation of the $S1P_2$ receptor (Fig. 1A). AlF_4^- also mimicked the action of $S1P_2$ in inhibiting IGF I-induced increases in cellular amounts of a GTP-bound, active form of Rac (GTP-Rac) (Fig. 1B) and in stimulating RhoA (Fig. 1C), ERK (Fig. 1D), and Ca^{2+} mobilization (data not shown). These observations strongly suggest that the heterotrimeric G protein(s) mediated inhibition of Rac and cell migration as well as the other actions of $S1P_2$.

Endogenous G_{12} and G_{13} couple $S1P_2$ to inhibition of cell migration. To examine which member of the heterotrimeric G proteins is responsible for mediating suppression of cell migration, we determined the effects of specific inhibition of receptor-G protein coupling by either transient expression of C-terminal peptides (1, 11) of heterotrimeric G protein α subunits (Gα-CTs) or pretreatment with PTX. Shown in Fig. 2A are Western blot analyses of the expression of $G\alpha_s$-CT, $G\alpha_q$-CT, $G\alpha_{12}$-CT, and $G\alpha_{13}$-CT. CHO-$S1P_2$ cells were cotransfected with one of these peptides and β-galactosidase (LacZ) and subjected to a migration assay (31). As in naive CHO-$S1P_2$ cells (31), S1P inhibited IGF I-directed chemotaxis in vector-transfected CHO-$S1P_2$ cells with a bell-shaped dose-response curve and maximal inhibition at 10^{-7} M (Fig. 2B).Neither expression of any of these C-terminal peptides nor pretreatment with PTX affected chemotaxis toward IGF I in the absence of S1P (Fig. 2B). Expression of $G\alpha_{12}$-CT or $G\alpha_{13}$-CT, but not $G\alpha_s$-CT or $G\alpha_q$-CT, specifically abolished the S1P inhibition of IGF I-directed chemotaxis. We confirmed that expression of $G\alpha_q$-CT effectively inhibited the S1P-induced increase in $[Ca^{2+}]_i$, a G_q-mediated response, compared to that with the vector control in S1P-expressing cells (Fig. 2C). Inhibition of $G_{i/o}$ by PTX pretreatment, and expression of βARK-CT, which acts as a scavenger for βγ subunits (20), to a lesser extent, rather potentiated S1P inhibition of IGF I-directed chemotaxis at lower S1P concentrations. PTX pretreatment or the expression of βARK-CT substantially attenuated S1P-induced ERK activation (data not shown), confirming the effectiveness of PTX and βARK at inhibiting G_i. These observations are consistent with the notion that endogenously expressed G_{12} and G_{13} are responsible for mediating a signal

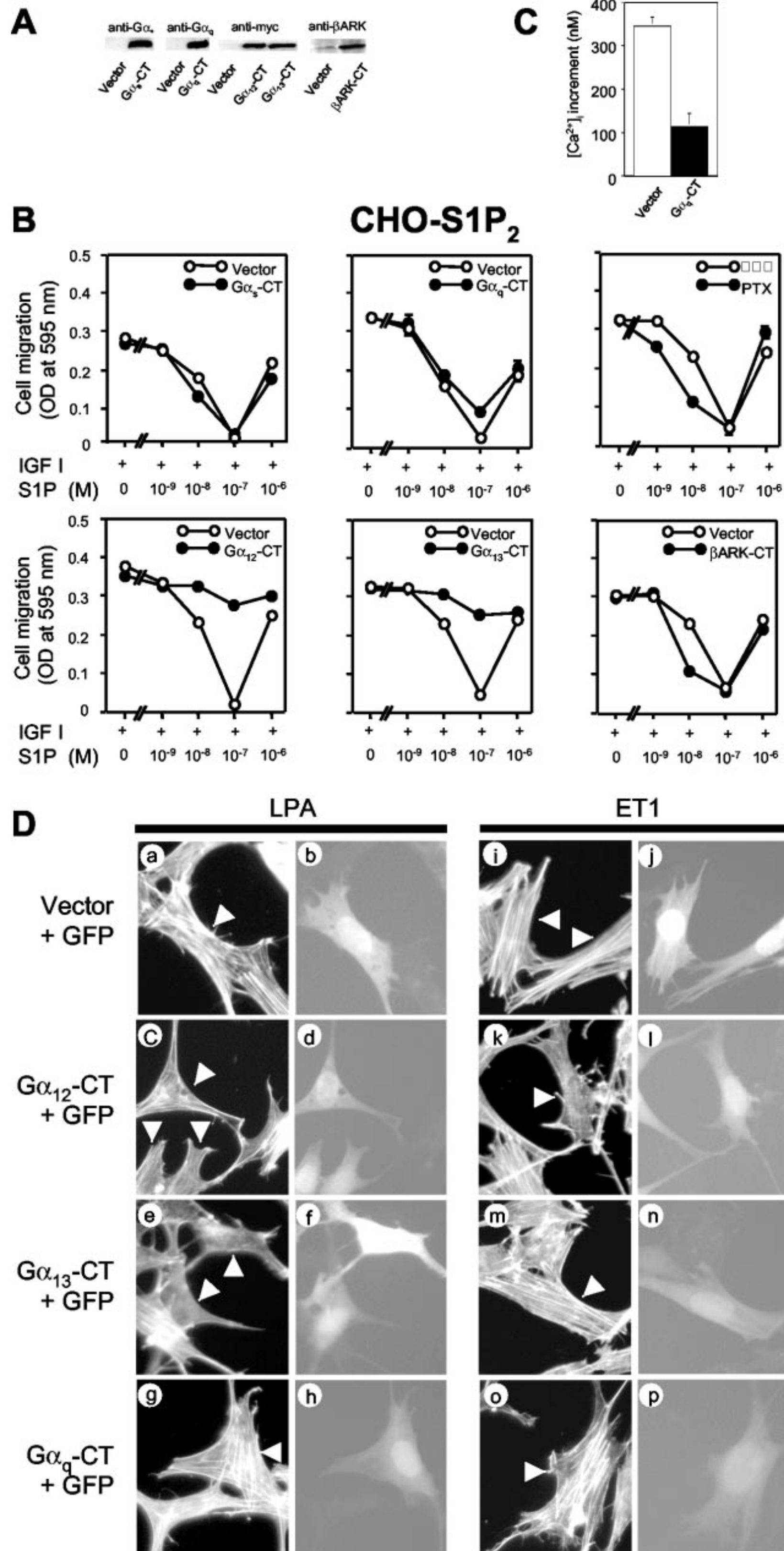

FIG. 2. Selective blockade of G_{12} and G_{13}, but not G_s, G_i, or G_q, relieves S1P inhibition of migration in $S1P_2$ receptor-expressing cells. (A) Western blot analysis showing expression of the Gα C-terminal peptides and βARK-CT. CHO-$S1P_2$ cells were transfected with either expression vectors for the Gα-CTs and βARK-CT or an empty vector and were subjected to Western blot analysis using respective, specific antibodies and an anti-myc tag antibody. (B) Expression of G_{12}-CT and G_{13}-CT treatment relieves S1P inhibition of IGF I-directed chemotaxis.

that leads to inhibition of migration upon S1P stimulation of the $S1P_2$ receptor.

It has been reported for Swiss 3T3 fibroblasts (12) that endothelin-1 (ET1) and LPA induce stress fiber formation through G_{12} and G_{13}, respectively. By employing Swiss 3T3 cells and these GPCR agonists, we determined the specificity of the inhibitory actions of $G\alpha_{12}$-CT and $G\alpha_{13}$-CT. We observed that expression of $G\alpha_{13}$-CT abolished LPA-induced stress fiber formation (Fig. 2Da, b, e, and f) but not ET1-induced stress fiber formation (Fig. 2Di, j, m, and n), while expression of $G\alpha_{12}$-CT abolished both ET1- and LPA-induced stress fiber formation (Fig. 2Dc, d, k, and l). The results indicate that the $G\alpha_{13}$-CT peptide acts as a selective inhibitor for G_{13} whereas the $G\alpha_{12}$-CT peptide acts as an inhibitor for both G_{12} and G_{13}. Together with the observation using βARK-CT (Fig. 2B), we conclude that the α subunits, but not the βγ dimer, of G_{13} or both G_{12} and G_{13} mediate inhibition of migration.

Endogenous G_{12} and G_{13} couple $S1P_2$ to inhibition of Rac and stimulation of Rho. We next expressed myc-tagged $G\alpha_{12}$-CT, $G\alpha_{13}$-CT, and $G\alpha_q$-CT, and LacZ as a control, by adenovirus-mediated gene transduction in CHO-$S1P_2$ cells, and we determined the effects of their expression on the activities of RhoA and Rac (Fig. 3) and also on the actin cytoskeleton (Fig. 4). Expression of the inhibitor proteins was confirmed by Western blot analysis (Fig. 3A). Expression of any of these C-terminal peptides did not affect IGF I-induced Rac activation (Fig. 3B) or membrane ruffling (Fig. 4) in the absence of S1P, nor did it affect S1P stimulation of ERK (Fig. 3D), indicating that their expression did not compromise cellular activity in a nonspecific manner. Interestingly, however, expression of either $G\alpha_{12}$-CT or $G\alpha_{13}$-CT, but not $G\alpha_q$-CT or LacZ, abolished S1P inhibition of Rac activation in response to IGF I (Fig. 3B). Expression of either $G\alpha_{12}$-CT or $G\alpha_{13}$-CT, but not $G\alpha_q$-CT, also greatly inhibited S1P-induced RhoA stimulation compared to LacZ transfection (Fig. 3C). Consistent with the effects on Rho and Rac, expression of $G\alpha_{12}$-CT or $G\alpha_{13}$-CT, but not of $G\alpha_q$-CT or LacZ, abolished S1P suppression of peripheral actin filament assembly in response to IGF I, as well as S1P stimulation of stress fiber formation (Fig. 4). These results are consistent with the observations on cell migration and indicate that G_{12} and G_{13} are responsible for mediating suppressive effects of S1P on cellular Rac activity, membrane ruffling, and cell migration in CHO-$S1P_2$ cells.

$S1P_2$-$G\alpha_{12}$ and $S1P_2$-$G\alpha_{13}$ fusion receptors, but not $S1P_2$-$G\alpha_q$, mediate inhibition of Rac and migration. Consistent with the observations showing functional coupling of $S1P_2$ to G_{12} and G_{13} (Fig. 2 to 4), we observed coimmunoprecipitation of $S1P_2$ and either G_{12} or G_{13} from the cells coexpressing these molecules (Fig. 5A). We further studied and compared the

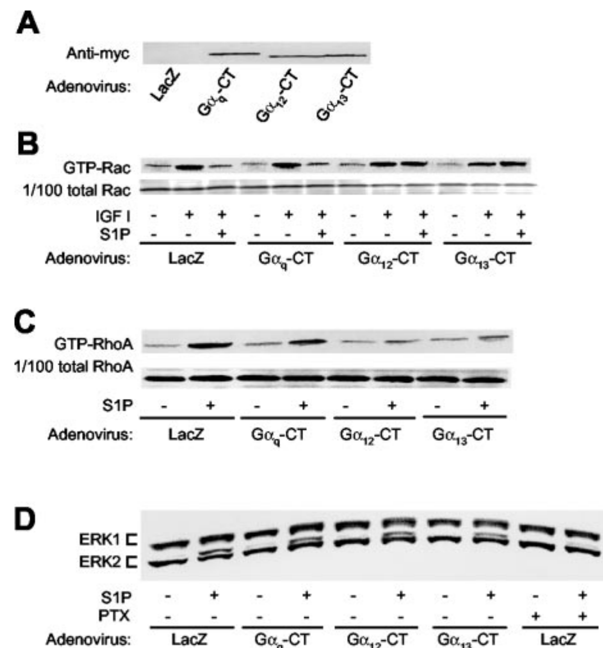

FIG. 3. Adenovirus-mediated expression of $G\alpha_{12}$-CT and $G\alpha_{13}$-CT abolishes S1P inhibition of Rac and stimulation of Rho in $S1P_2$ receptor-expressing cells. (A) Western blot analysis of expression of the Gα C-terminal peptides. CHO-$S1P_2$ cells were infected with adenoviruses encoding myc-tagged $G\alpha_q$-CT, $G\alpha_{12}$-CT, $G\alpha_{13}$-CT, and LacZ 48 h before experiments and were subjected to Western blot analysis using an anti-myc tag antibody. (B and C) Expression of $G\alpha_{12}$-CT and $G\alpha_{13}$-CT, but not $G\alpha_q$-CT abolishes S1P inhibition of IGF I-induced Rac stimulation and S1P stimulation of Rho. CHO-$S1P_2$ cells that had been were infected with the adenoviruses were stimulated with IGF I (100 ng/ml) for 1 min in the presence of S1P (10^{-7} M) (for the Rac assay) or with S1P (10^{-7} M) for 3 min (for the Rho assay). For the Rac assay S1P was added 10 min before addition of IGF I. Cells were then subjected to a pulldown assay for GTP-Rac or GTP-RhoA. (D) Inhibition of S1P-induced ERK stimulation by PTX, but not by expression of G_q-CT, G_{12}-CT, or G_{13}-CT. CHO-$S1P_2$ cells were either infected with the adenoviruses as described above or pretreated with PTX as for Fig. 2B. Cells were then stimulated with S1P (10^{-7} M) for 5 min and subjected to band shift analysis.

effects of S1P on IGF I-directed migration in CHO cells that stably expressed fusion receptors designated $S1P_2$-$G\alpha_{12}$, $S1P_2$-$G\alpha_{13}$, and $S1P_2$-$G\alpha_q$, which have either of the full-length Gα subunit sequences fused to the C terminus of $S1P_2$ (37). In CHO cells expressing either the $S1P_2$-$G\alpha_{12}$ or the $S1P_2$-$G\alpha_{13}$ fusion receptor, S1P potently inhibited chemotaxis toward IGF I (Fig. 5B). Adenovirus-mediated expression of $G\alpha_{12}$-CT or $G\alpha_{13}$-CT completely abolished S1P inhibition of IGF I-directed chemotaxis in cells expressing the respective fusion re-

CHO-$S1P_2$ cells were either cotransfected with LacZ and one of the expression vectors for $G\alpha_s$-CT, $G\alpha_q$-CT, $G\alpha_{12}$-CT, $G\alpha_{13}$-CT, and βARK-CT at a weight ratio of 1:2.5 or pretreated with PTX (200 ng/ml) as described in Materials and Methods. Transwell migration of transfected CHO-$S1P_2$ cells was determined in the presence of IGF I (100 ng/ml) and various concentrations of S1P in the lower chamber. (C) Expression of $G\alpha_q$-CT inhibits the S1P-induced $[Ca^{2+}]_i$ increase in $S1P_2$-overexpressing CHO cells. CHO-$S1P_2$ cells stably expressing $G\alpha_q$-CT and CHO-$S1P_2$ cells expressing the vector control were stimulated by S1P at 10^{-8} M, and the peak increment in the $[Ca^{2+}]_i$ was determined. (D) Inhibition of LPA- and ET1-induced stress fiber formation by expression of $G\alpha_{12}$-CT or $G\alpha_{13}$-CT. Swiss 3T3 cells were cotransfected with pEGFP-C1 and either an expression vector for $G\alpha_{12}$-CT or $G\alpha_{13}$-CT or an empty vector at a weight ratio of 1:10. Cells were stimulated with LPA or ET1 at 10^{-7} M for 10 min. F-actin was visualized with TRITC-labeled phalloidin. Arrowheads indicate transfected cells identified with GFP fluorescence.

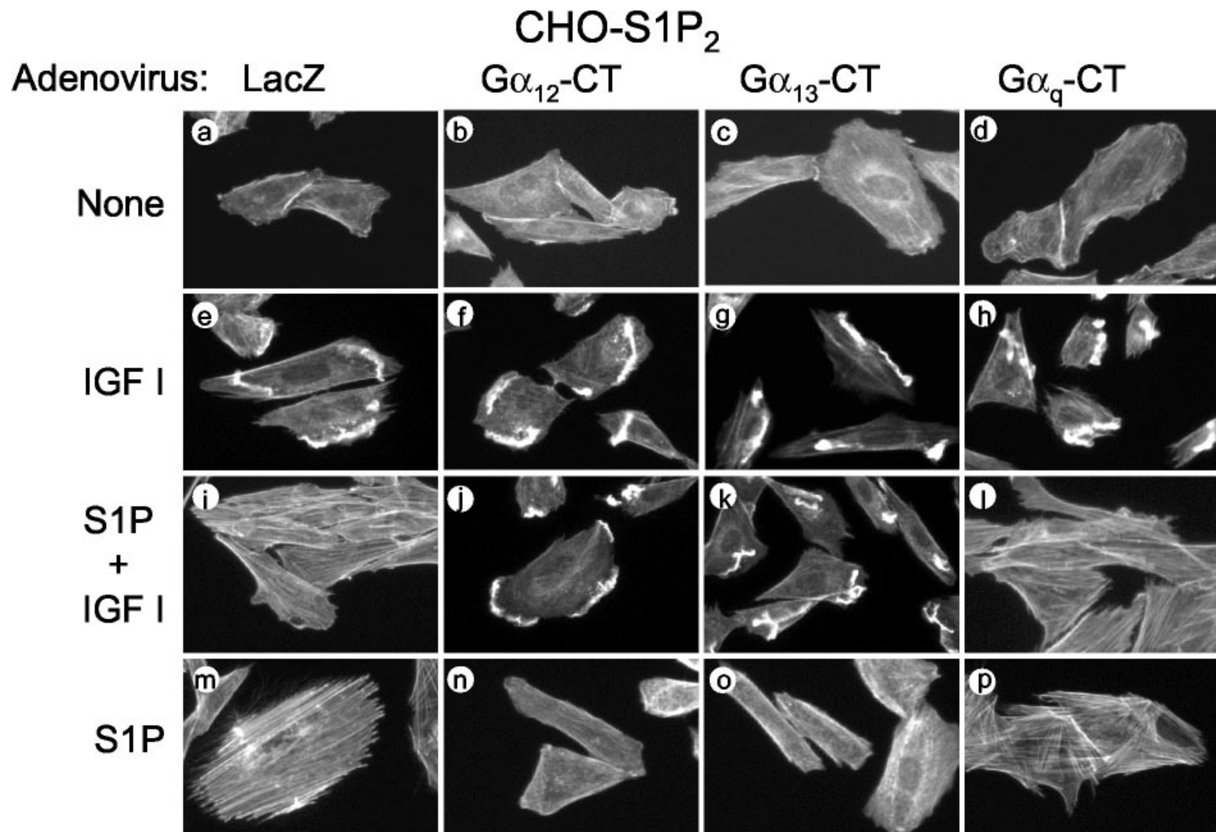

FIG. 4. Expression of $G\alpha_{12}$-CT and $G\alpha_{13}$-CT, but not $G\alpha_q$-CT, abolishes S1P inhibition of IGF I-induced membrane ruffling and also S1P stimulation of stress fiber formation in $S1P_2$ receptor-expressing cells. CHO-$S1P_2$ cells were infected with adenoviruses encoding $G\alpha_{12}$-CT, $G\alpha_{13}$-CT, $G\alpha_q$-CT, or LacZ as for Fig. 3A. Cells were stimulated with IGF I (100 ng/ml) and/or S1P (10^{-7} M) for 30 min. Cells were fixed, permeabilized, and stained with TRITC-labeled phalloidin for F-actin.

ceptor $S1P_2$-$G\alpha_{12}$ or $S1P_2$-$G\alpha_{13}$. Cells expressing $S1P_2$-$G\alpha_q$ showed a prominent increase in the $[Ca^{2+}]_i$ in response to S1P (Fig. 5C); however, they responded to S1P with only a marginal inhibition of cell migration (Fig. 5B). In vector-transfected cells and cells expressing either of the three fusion receptors, IGF I similarly stimulated Rac activity (Fig. 5D). In cells expressing either $S1P_2$-$G\alpha_{12}$ or $S1P_2$-$G\alpha_{13}$, but not vector-transfected cells or cells expressing $S1P_2$-$G\alpha_q$, S1P induced a nearly complete inhibition of Rac activation in response to IGF I, as in CHO-$S1P_2$ cells (Fig. 5D). As expected, cells expressing $S1P_2$-$G\alpha_{12}$ or $S1P_2$-$G\alpha_{13}$, but not vector-transfected cells or cells expressing $S1P_2$-$G\alpha_q$, showed strong activation of RhoA in response to S1P (Fig. 5D). These observations provided further evidence that $G_{12/13}$ coupled with $S1P_2$ for inhibition of Rac and migration.

Rho mediates inhibition of cellular Rac activity and migration through a mechanism not involving Rho kinase. We tested the possibility that Rho, which is an effector of $G_{12/13}$, was involved in the signaling pathway leading to suppression of Rac activity. Pretreatment of $S1P_2$-expressing cells with botulinum C3 toxin, which inactivates Rho, did not affect migration in response to IGF I; however, it completely abolished the $S1P_2$-mediated inhibition of IGF I-directed migration (Fig. 6A). Similarly, adenovirus-mediated expression of N^{19}RhoA did not affect chemotaxis toward IGF I but totally abolished S1P inhibition of IGF I-directed migration (Fig. 6A). Consistent with these observations, either C3 treatment or N^{19}RhoA expression abolished S1P inhibition of IGF I-induced Rac stimulation, while these treatments did not affect Rac activation in response to IGF I alone (Fig. 6B). On the other hand, we observed that cells that stably expressed a myc-tagged constitutively active RhoA mutant, myc-V^{14}RhoA, showed reductions in both chemotaxis and Rac activation in response to IGF I, compared to vector-control cells (Fig. 6C and 6D).

We next studied whether Rho kinase, which is one of the direct effectors of Rho (23, 46), was involved in $S1P_2$-mediated suppression of Rac activity and migration. It was previously reported that a Rho kinase inhibitor abolished V^{14}Rho-induced inhibition of Rac activity (50). HA-1077 and Y-27638, which are two structurally unrelated Rho kinase inhibitors (26, 49), effectively inhibited S1P-induced stress fiber formation in CHO-$S1P_2$ cells (Fig. 7); however, they were totally ineffective in preventing the $S1P_2$-mediated inhibition of IGF I-directed cell migration (Fig. 6E), Rac activation (Fig. 6F), or membrane ruffling (Fig. 7). In contrast, C3 toxin abolished the $S1P_2$-mediated inhibition of IGF I-induced membrane ruffling (Fig. 7f) as well as induction of stress fiber formation (Fig. 7j). $S1P_2$ mediates actin cytoskeletal changes in two ways through Rho: it inhibits membrane ruffling in IGF I-stimulated cells, and it stimulates stress fiber formation. These observations clearly indicate that inhibition of membrane ruffle formation requires a Rho-dependent signal other than the Rho kinase pathway,

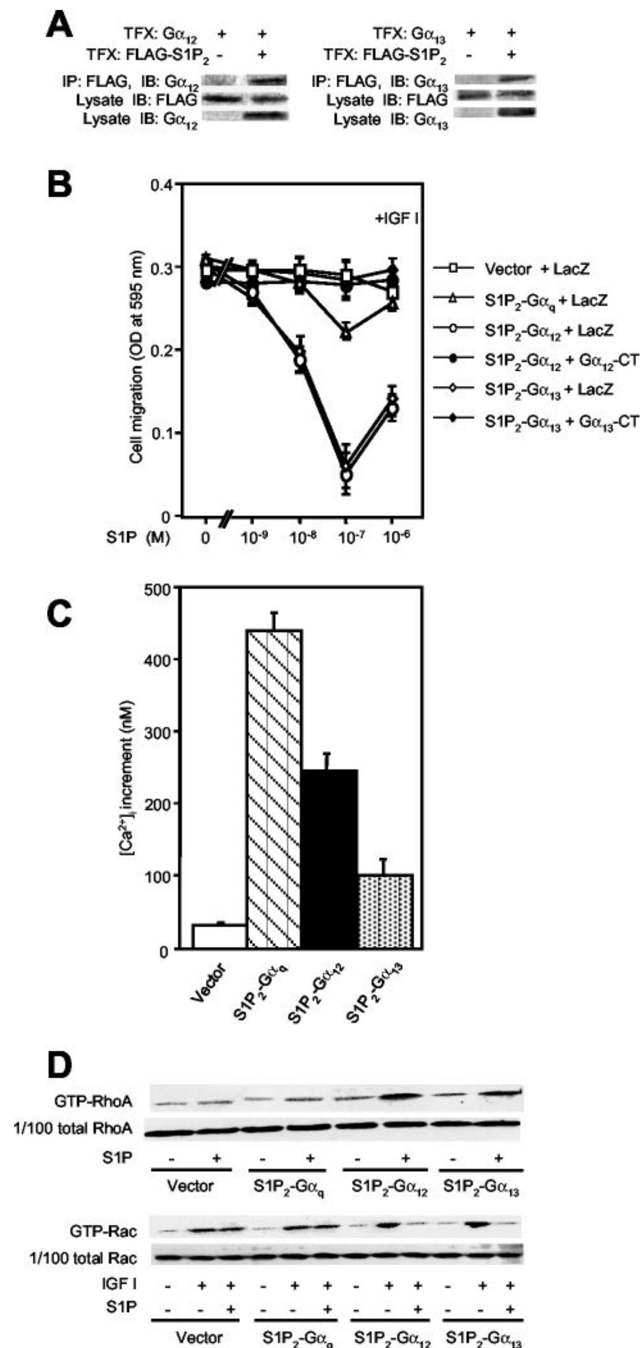

FIG. 5. The fusion receptors $S1P_2$-$G\alpha_{12}$ and $S1P_2$-$G\alpha_{13}$, but not $S1P_2$-$G\alpha_q$, mediate inhibition of Rac and migration. (A) Coimmunoprecipitation of $S1P_2$ and either G_{12} or G_{13}. COS7 cells were transiently cotransfected with pCAGGS-FLAG-$S1P_2$ and either pCAGGS-$G\alpha_{12}$ or pCAGGS-$G\alpha_{13}$, and FLAG-tagged $S1P_2$ was immunoprecipitated with an anti-FLAG (M2) antibody. The anti-FLAG immunoprecipitates were analyzed by Western blotting using anti-G_{12} or anti-G_{13} antibodies. Portions of cell lysates were analyzed by Western blotting using anti-FLAG, anti-G_{12}, and anti-G_{13} antibodies. TFX, transfection; IP, immunoprecipitation; IB, immunoblotting. (B) S1P inhibits IGF I-directed chemotaxis in CHO cells expressing $S1P_2$-$G\alpha_{12}$ and $S1P_2$-$G\alpha_{13}$, but not $S1P_2$-$G\alpha_q$ or vector, which is sensitive to expression of $G\alpha_{12}$-CT and $G\alpha_{13}$-CT inhibitor peptides. CHO cells stably expressing $S1P_2$-$G\alpha_{12}$, $S1P_2$-$G\alpha_{13}$, or $S1P_2$-$G\alpha_q$ were infected with adenoviruses encoding $G\alpha_{12}$-CT, $G\alpha_{13}$-CT, or LacZ and then subjected to transwell migration as for Fig. 2B. (C) S1P-induced $[Ca^{2+}]_i$ response in CHO cells expressing the fusion receptors. Cells were stimulated with S1P (10^{-7} M), and the peak increments of the $[Ca^{2+}]_i$ responses were determined. (D) S1P inhibits IGF I-induced Rac stimulation and stimulates Rho in CHO cells expressing $S1P_2$-$G\alpha_{12}$ and $S1P_2$-$G\alpha_{13}$, but not $S1P_2$-$G\alpha_q$ or vector. Cells were stimulated as described in the legend for Fig. 3B and C and were subjected to a pulldown assay for GTP-Rac and GTP-Rho.

whereas induction of stress fiber formation requires Rho kinase.

Overexpression of $G\alpha_i$ counteracts AlF_4^-- and S1P-induced inhibition of Rac and migration. $S1P_2$ couples not only to $G_{12/13}$ but also to G_i (13, 30, 49). PTX pretreatment of CHO-$S1P_2$cells potentiated S1P inhibition of chemotaxis (Fig. 1B), suggesting that G_i conveyed a signal which counteracted inhibition of migration. To study in more depth the role of $G\alpha_i$ in the regulation of Rac and cell migration by the S1P receptor, we evaluated the effects of overexpression of $G\alpha_{i2}$, which is an endogenously expressed $G\alpha_i$ isoform in CHO cells, on AlF_4^-- and S1P-induced inhibition of Rac and migration in CHO-$S1P_2$ cells (Fig. 8). In cells overexpressing $G\alpha_{i2}$, S1P by itself stimulated chemotaxis and Rac moderately, unlike the situation in the vector control cells (data not shown). Overexpression of $G\alpha_{i2}$ nearly completely abolished AlF_4^-- and S1P-induced inhibition of chemotaxis (Fig. 8B and C). The results contrast sharply with those obtained with overexpression of either $G\alpha_{12}$or $G\alpha_{13}$, which potentiated AlF_4^-- and S1P-induced inhibition of IGF I-directed chemotaxis (Fig. 8B and C). Overexpression of $G\alpha_q$ did not affect inhibition by AlF_4^- or S1P. In agreement with these observations on migration, overexpression of $G\alpha_{i2}$ nearly abolished S1P inhibition of IGF I-induced stimulation of Rac, whereas overexpression of $G\alpha_{12}$ and $G\alpha_{13}$ potentiated S1P inhibition of IGF I stimulation of Rac compared to that in vector control cells (Fig. 8D). Expression of $G\alpha_q$ did not affect S1P inhibition of Rac activity. Overexpression of these Gα subunits did not affect chemotaxis or Rac activation in response to IGF I alone (Fig. 8C and D). Thus, G_i generates a stimulatory signal for Rac and consequently migration to antagonize $G_{12/13}$-mediated inhibition of Rac and migration in S1P receptor signaling.

$S1P_3$ mediates S1P inhibition, rather than stimulation, of Rac and cell migration upon G_i inactivation. As we reported previously (31), S1P by itself directed chemotaxis in both $S1P_1$- and $S1P_3$-expressing CHO cells (CHO-$S1P_1$ and CHO-$S1P_3$ cells, respectively) (Fig. 9A and B). This contrasts with CHO-$S1P_2$ cells, in which S1P by itself does not stimulate chemotaxis (31). PTX pretreatment of both CHO-$S1P_1$ and CHO-$S1P_3$ cells totally inhibited migration toward S1P, indicating that S1P-directed chemotaxis is $G_{i/o}$ dependent (Fig. 9A and B). When CHO-$S1P_1$ cells were stimulated with a combination of S1P and IGF I, they showed a chemotactic response slightly greater than that to stimulation with IGF I alone (Fig. 9C). PTX pretreatment did not affect CHO-$S1P_1$ cell migration stimulated by IGF I alone, and it slightly reduced cell migration stimulated by the combination of S1P and IGF I. In CHO-$S1P_3$ cells, the combination of S1P and IGF I induced a stimulation of chemotaxis slightly larger than that with IGF I alone, as in CHO-$S1P_1$ cells (Fig. 9D). PTX did not affect the response to IGF I alone in this cell type, either. In contrast to

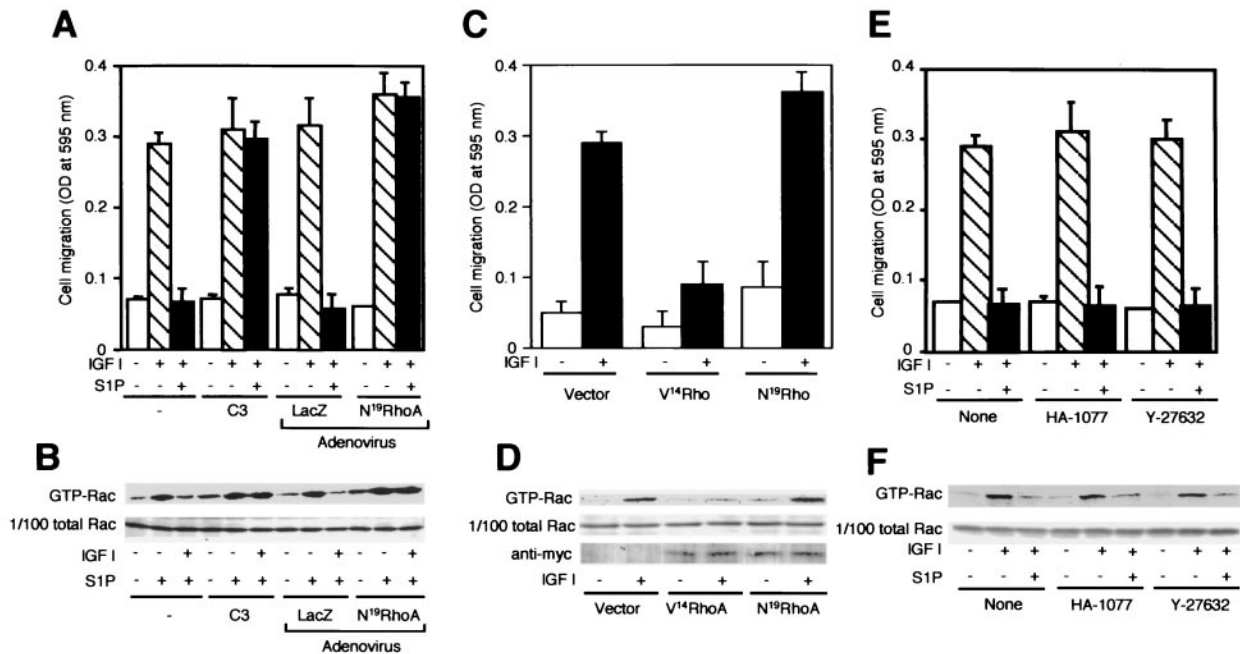

FIG. 6. Rho, but not Rho kinase, mediates S1P inhibition of Rac activity and migration in $S1P_2$-expressing cells. (A and B) C3 pretreatment and N^{19}Rho expression abolish S1P inhibition of IGF I-stimulated chemotaxis and Rac activity. CHO-$S1P_2$ cells were either infected with adenoviruses encoding N^{19}Rho or LacZ as for Fig. 3A, pretreated with C3 toxin (10 μg/ml) as described in Materials and Methods, or not pretreated. Transwell migration was determined in Fig. 2B. For the GTP-Rac pulldown assay, the cells were stimulated as for Fig. 3B. (C and D) Expression of V^{14}Rho inhibits IGF I-stimulated chemotaxis and Rac activation. CHO-$S1P_2$ cells were cotransfected with either myc-tagged N^{19}Rho, myc-tagged V^{14}Rho, or an empty vector and pCMV/Zeo, and they were selected in the presence of Zeocin. Zeocin-resistant cell populations, which expressed either of the myc-tagged Rho mutants, were employed in these experiments. Transwell migration was determined in the presence or absence of IGF I (100 ng/ml) in the lower chamber. For the GTP-Rac pulldown assay, cells were stimulated with IGF I (100 ng/ml) for 1 min. Expression of myc-tagged N^{19}Rho and V^{14}Rho proteins in the cell lysate are shown in the bottom gel of panel D. (E and F) Rho kinase inhibitors fail to prevent S1P inhibition of IGF I-stimulated chemotaxis and Rac activation. CHO-$S1P_2$ cells were pretreated or not with HA-1077 (20 μM) or Y-27632 (10 μM) for 30 min before migration and Rac assays. Transwell migration was determined in the presence or absence of IGF I (100 ng/ml) and S1P (10^{-7} M) in the lower chamber. HA-1077 (20 μM) or Y-27632 (10 μM) was present in both the upper and lower chambers, where indicated. For the Rac assay, cells were stimulated with IGF I (100 ng/ml) for 1 min, with or without a 10-min pretreatment with S1P (10^{-7} M) and/or HA-1077 (20 μM) or Y-27632 (10 μM).

CHO-$S1P_1$ cells, however, inactivation of G_i with PTX in CHO-$S1P_3$ cells dramatically changed the responses to S1P: the cells showed inhibition, rather than stimulation, of IGF I-directed chemotaxis in response to S1P, with a nearly complete inhibition at 10^{-6} M (Fig. 9D). Moreover, adenovirus-mediated expression of either $G\alpha_{12}$-CT or $G\alpha_{13}$-CT abolished S1P inhibition of IGF I-directed chemotaxis in PTX-treated CHO-$S1P_3$ cells (Fig. 9D).

In CHO-$S1P_1$ cells S1P by itself stimulated Rac, which was totally inhibited by PTX (Fig. 10A). IGF I also stimulated Rac, but in a PTX-insensitive manner. The combination of S1P and IGF I induced a slightly but consistently greater response in Rac activation compared to that with stimulation by either alone. In CHO-$S1P_3$ cells S1P by itself stimulated Rac in a PTX-sensitive manner (Fig. 10C), which was similar to the findings for CHO-$S1P_1$ cells. In contrast to the situation in PTX-pretreated CHO-$S1P_1$ cells, however, in PTX-pretreated CHO-$S1P_3$ cells S1P markedly inhibited IGF I-stimulation of Rac (Fig. 10C), which was consistent with the S1P inhibition of IGF I-directed chemotaxis. Expression of either $G\alpha_{12}$-CT or $G\alpha_{13}$-CT abolished the S1P inhibition of Rac in PTX-pretreated, IGF I-stimulated CHO-$S1P_3$ cells (Fig. 10C), just as with cell migration (Fig. 9D). In CHO-$S1P_3$ cells, S1P induced

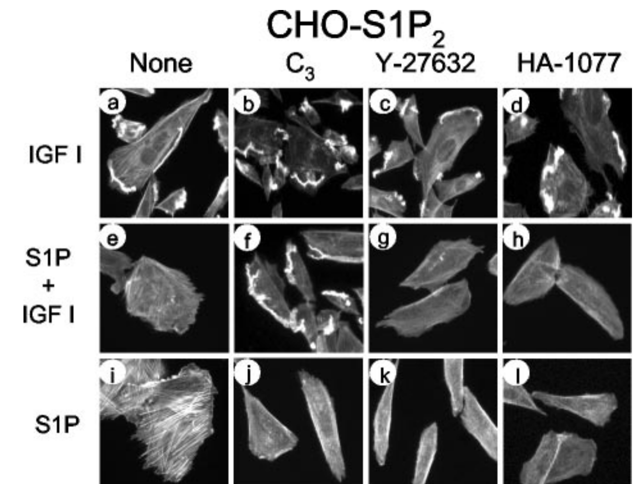

FIG. 7. Rho kinase inhibitors do not affect S1P inhibition of IGF I-induced membrane ruffling but do abolish S1P-induced stress fiber formation. CHO-$S1P_2$ cells were pretreated either with C3 toxin, as described in Materials and Methods, or with HA-1077 (20 μM) or Y-27632 (10 μM) for 30 min. Cells were then stimulated with IGF I (100 ng/ml) and/or S1P (10^{-7} M) for 30 min. Cells were stained with TRITC-labeled phalloidin for F-actin. Note that C3, but not HA-1077 or Y-27632, abolishes S1P inhibition of membrane ruffling in response to IGF I, although the Rho kinase inhibitors effectively suppress S1P-induced stress fiber formation.

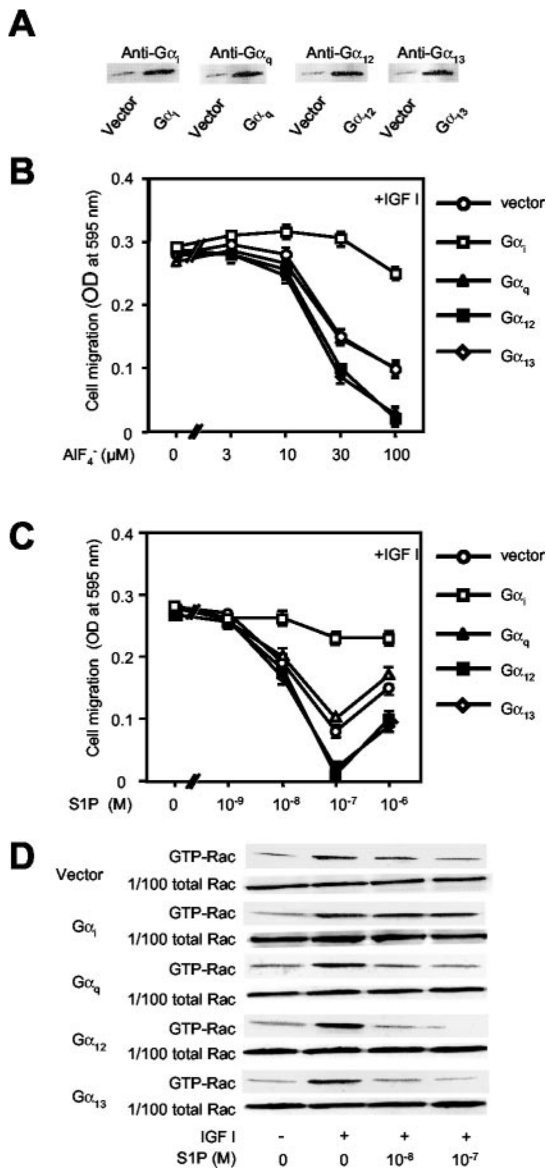

FIG. 8. Overexpression of $G\alpha_i$ counteracts AlF_4^-- and S1P-induced inhibition of Rac and migration in $S1P_2$ receptor-expressing cells. (A) Western blot analysis of expression of full-length Gα proteins. CHO-$S1P_2$ cells stably expressing either $G\alpha_{i2}$, $G\alpha_q$, $G\alpha_{12}$, $G\alpha_{13}$, or an empty vector were subjected to Western blot analysis using respective, specific anti-Gα antibodies described in Materials and Methods. (B and C) Overexpression of $G\alpha_i$ markedly attenuates AlF_4^-- and S1P-induced inhibition of IGF I-directed chemotaxis, but overexpression of $G\alpha_{12}$ or $G\alpha_{13}$ enhances such inhibition. Transwell migration of the CHO-$S1P_2$ cells that stably express one of these Gα subunits or the empty vector was determined as for Fig. 1A and 2B. (D) Overexpression of $G\alpha_i$ markedly attenuates S1P inhibition of IGF I-induced Rac stimulation. CHO-$S1P_2$ cells that stably express one of the Gα subunits or an empty vector were stimulated as for Fig. 3B and then subjected to a pulldown assay for GTP-Rac.

stimulation of RhoA via $G_{12/13}$ irrespective of PTX pretreatment (Fig. 10D). S1P did not change the level of GTP-RhoA in CHO-$S1P_1$ cells (Fig. 10B). Thus, inactivation of G_i in CHO-$S1P_3$ cells converts S1P-induced stimulation of Rac and migration to inhibition of Rac and migration. These observa-

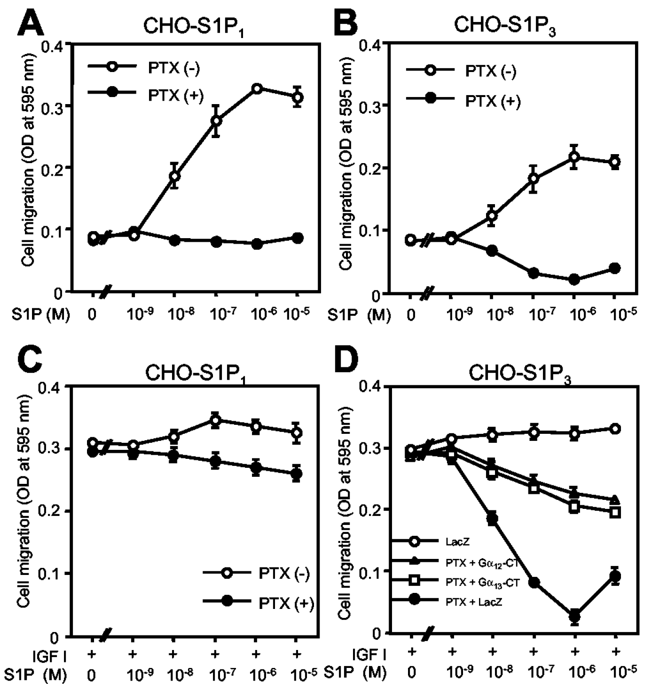

FIG. 9. PTX pretreatment abolishes S1P stimulation of migration in $S1P_1$- and $S1P_3$-expressing cells and uncovers $S1P_3$-mediated, $G_{12/13}$-dependent inhibition of IGF I-directed chemotaxis. (A and B) PTX pretreatment abolishes chemotaxis toward S1P in CHO-$S1P_1$ and CHO-$S1P_3$ cells. Cells were pretreated or not with PTX as described in Materials and Methods and were then subjected to a transwell migration assay in the presence of various concentrations of S1P in the lower chamber. (C and D) PTX pretreatment unveils S1P-induced, $G_{12/13}$-dependent inhibition of IGF I-directed chemotaxis in CHO-$S1P_3$ cells, but not in CHO-$S1P_1$ cells. CHO-$S1P_3$ cells were infected with adenoviruses encoding $G\alpha_{12}$-CT, $G\alpha_{13}$-CT, and LacZ and were then pretreated or not with PTX, as for Fig. 3D. Cells were then subjected to a transwell migration assay as for Fig. 2B.

tions indicate that the S1P receptor isoforms $S1P_1$, $S1P_2$, and $S1P_3$ exert distinct regulatory actions on Rac and cell migration through differential coupling to $G_{12/13}$ and G_i, which convey signals to inhibit and stimulate Rac and migration, respectively.

DISCUSSION

Multiple classes of cell surface receptors including receptor tyrosine kinases and GPCRs trigger chemotactic behavior of cells (21, 36). In GPCR-activated chemotactic signaling, liberation of βγ subunits from the heterotrimeric G_i, activation of phosphatidylinositol 3-kinases, and stimulation of the small GTPase Rac are considered to constitute the important signaling cascade for stimulating chemotaxis (15). However, much less is known about signaling mechanisms of chemorepellant receptors. We recently identified $S1P_2$ as the first example of a GPCR that mediates negative regulation of Rac, which serves as a signal for inhibiting cell migration directed toward a chemoattractant (31, 33, 42). In the present study, first, we observed that direct activation of heterotrimeric G proteins with AlF_4^- (17) mimicked all of the known S1P actions in $S1P_2$ receptor-expressing cells (Fig. 1A to D), strongly

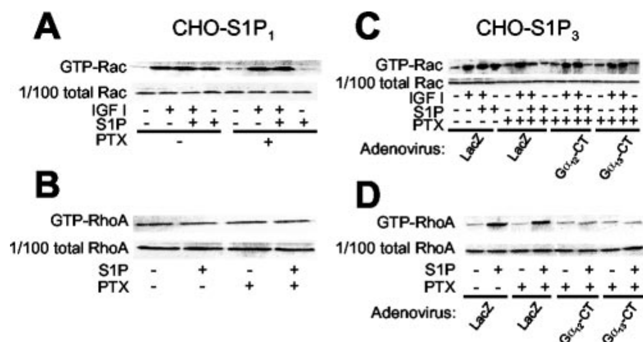

FIG. 10. PTX pretreatment abolishes S1P stimulation of Rac in $S1P_1$- and $S1P_3$-expressing cells and uncovers $S1P_3$-mediated, $G_{12/13}$-dependent inhibition of IGF I-induced Rac stimulation. (A and B) Effects of PTX pretreatment on Rac and Rho activities in CHO-$S1P_1$ cells. Cells were pretreated or not pretreated with PTX, stimulated with IGF I and/or S1P, and subjected to a pulldown assay for GTP-Rac and GTP-Rho, as described in the legends for Fig. 3B, C, and D. (C and D) Effects of PTX treatment and expression of $G\alpha_{12}$-CT and $G\alpha_{13}$-CT on Rac and Rho activities in CHO-$S1P_3$ cells. Cells were infected with adenoviruses encoding $G\alpha_{12}$-CT, $G\alpha_{13}$-CT, and LacZ and then pretreated or not with PTX, as for Fig. 3D. Cells were stimulated in the same way as for panels A and B and were subjected to a pulldown assay for GTP-Rac and GTP-Rho.

suggesting that heterotrimeric G protein coupling mediates inhibition of Rac and migration downstream of $S1P_2$ stimulation. By utilizing specific G protein inhibitors (1, 11), we demonstrated that it is the $G_{12/13}$ family protein that couples $S1P_2$ to inhibition of Rac, cell migration, and membrane ruffling (Fig. 2B, 3, and 4). Consistent with the critical roles of $G_{12/13}$, the fusion receptors $S1P_2$-$G\alpha_{12}$ and $S1P_2$-$G\alpha_{13}$, but not $S1P_2$-$G\alpha_q$, mediated S1P inhibition of Rac and cell migration (Fig. 5), and overexpression of either $G\alpha_{12}$ or $G\alpha_{13}$, but not $G\alpha_q$, potentiated the inhibitory effects of S1P (Fig. 8). In addition, the observation that expression of βARK-CT, which sequesters βγ subunits (20), did not abolish S1P inhibition of migration but rather slightly augmented it suggests that $G\alpha_{12}$ and $G\alpha_{13}$, but not βγ subunits of $G_{12/13}$, mediate S1P inhibition of migration (Fig. 2B). It is of note that the metastin receptor hTOT7T175 and the thrombin receptor PAR1, which are GPCRs capable of inhibiting cell migration, are also $G_{12/13}$ coupled (18, 28), although these receptors are not known to be able to inhibit Rac. However, the present study also indicates that GPCRs that couple to $G_{12/13}$ do not always inhibit Rac or cell migration (see below).

Since Rho is a well-established effector of the $G_{12/13}$ class of heterotrimeric G proteins (12), we next examined its involvement in the $S1P_2$ signaling leading to Rac inhibition. Inhibition of endogenous Rho activity by C3 toxin treatment or N^{19}RhoA expression abolished $G_{12/13}$-mediated inhibition of Rac, cell migration, and membrane ruffling (Fig. 6A, 6B, and 7). Conversely, expression of V^{14}Rho mimicked S1P inhibition of Rac and also of migration, like N^{17}Rac expression (Fig. 6C and D). These observations, together with the observation that $S1P_2$ mediates Rho stimulation via $G_{12/13}$ (Fig. 1 and 3) (31), clearly indicate that Rho mediates Rac inhibition. Although it was previously demonstrated that expression of V^{14}Rho resulted in inhibition of cellular Rac activity (50), this is the first demonstration that receptor activation of the $G_{12/13}$-Rho signaling pathway mediates Rac inhibition. In certain cell types (6, 19), G_q as well as $G_{12/13}$ was demonstrated to mediate Rho stimulation. However, in CHO-$S1P_2$ cells, the G_q inhibitor peptide did not affect S1P-induced Rho stimulation (Fig. 3B). Besides the inhibitory action on cellular Rac activity, Rho may also act to inhibit cell motility through its stimulating effects on contractile actin-myosin filaments, which result in the formation of stress fibers and focal adhesions (2, 27), although precise mechanisms relating these structures to inhibition of migration remain to be elucidated.

Rho kinase is a candidate Rho effector that mediates Rac inhibition. Indeed, a previous study showed that expression of V^{14}Rho in PC12 cells inhibited nerve growth factor-induced activation of Rac and that this inhibition was reversed by the Rho kinase inhibitor Y-27632 (50). It was also reported recently that Y-27632 inhibition of Rho kinase unveiled previously unrecognized Rho-dependent activation of Rac via mDia, which is another direct effector of Rho (45). In contrast to these previous reports, however, we did not observe any prevention of $S1P_2$-mediated inhibition of Rac, cell migration, or membrane ruffling by two structurally different Rho kinase inhibitors, Y-27632 and HA-1077, although they effectively inhibited $S1P_2$-mediated, Rho-dependent stress fiber formation (Fig. 6E, 6F, and 7). Our results indicate that a Rho effector other than Rho kinase plays a critical role in $S1P_2$ receptor-mediated Rac inhibition. It is possible that the effects of activation of endogenous Rho by receptor stimulation and V^{14}RhoA expression could be distinct both temporally and spatially, resulting in different effector stimulation. Although the exact explanation for the discrepancy is not known at present, these disparate observations suggest that there exist both Rho kinase-dependent and -independent mechanisms for Rho regulation of Rac activity. The differential effects of C3 toxin and the Rho kinase inhibitors on the actin cytoskeletal changes (Fig. 7) also indicate that the $S1P_2$ receptor stimulates Rho to mediate dissolution of membrane ruffles and induction of stress fiber formation, which are uncoupled processes, through Rho kinase-independent and -dependent pathways, respectively, in CHO cells.

Another point that should be noted in the present results is the counteraction between $G_{12/13}$ and G_i with regard to Rac regulation. Thus, overexpression of $G\alpha_i$ reversed $S1P_2$-mediated, $G_{12/13}$-dependent suppression of Rac and migration (Fig. 8C and D), like that of the $G_{12/13}$ inhibitor peptides. Moreover, we demonstrated in the present study that $S1P_3$, an isoform of $S1P_2$, exerts dual regulation for cellular Rac activity via $G_{12/13}$ and G_i (Fig. 10C). As we and others (31, 32) reported previously, $S1P_3$ as well as $S1P_1$ mediate G_i-dependent stimulation of Rac activity and migration (Fig. 9 and 10). $S1P_1$ couples exclusively to G_i, whereas $S1P_3$, like $S1P_2$, couples to G_q and $G_{12/13}$ in addition to G_i (43, 49). Inactivation of G_i by PTX treatment uncovered $S1P_3$-mediated, $G_{12/13}$-dependent inhibitory regulation for Rac and migration (Fig. 9D and 10C). The $S1P_2$ receptor also couples to G_i to activate ERK (Fig. 3D) (13); however, this coupling appears to be less efficient than those of $S1P_1$ and $S1P_3$, as evidenced by the fact that the dose-response curve for S1P-induced, G_i-dependent ERK activation in CHO-$S1P_2$ cells is shifted at least 1 order rightward from that in $S1P_1$- and $S1P_3$-expressing cells (30). Robust activation by $S1P_2$ of the $G_{12/13}$-Rho pathway likely masks the

G_i-mediated, modest stimulatory effect on Rac, resulting in evident inhibition of cellular Rac activity and migration, even in naive CHO-$S1P_2$ cells (Fig. 1 to 3).

Besides the $S1P_2$ and $S1P_3$ receptors, a number of GPCRs have been shown to couple to G_i, $G_{12/13}$, and also G_q (18, 28, 39). In certain cell types, G_q as well as $G_{12/13}$ may transmit a stimulatory signal to Rho (6, 19). It is likely that integration of G_i- and $G_{12/13}$- or G_q-dependent, positive and negative regulatory signals for cellular Rac activity determines the eventual regulatory activities of the GPCRs with regard to cell migration.

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