PTEN as an effector in the signaling of antimigratory G protein-coupled receptor

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PTEN, a tumor suppressor phosphatase, is important in the regulation of cell migration and invasion. Physiological regulation of PTEN (phosphatase and tensin homolog deleted on chromosome 10) by cell surface receptors has not been described. Here, we show that the bioactive lipid sphingosine 1-phosphate (S1P), which acts through the S1P2 receptor (S1P2R) G protein-coupled receptor (GPCR) to inhibit cell migration, utilizes PTEN as a signaling intermediate. S1P2R inhibition of cell migration is abrogated by dominant-negative PTEN expression. S1P was unable to efficiently inhibit the migration of *Pten*$^{\Delta loxP/\Delta loxP}$ mouse embryonic fibroblasts; however, the antimigratory effect was restored upon the expression of PTEN. S1P2R activation of Rho GTPase is not affected in *Pten*$^{\Delta loxP/\Delta loxP}$ cells, and dominant-negative Rho GTPase reversed S1P inhibition of cell migration in WT cells but not in *Pten*$^{\Delta loxP/\Delta loxP}$ cells, suggesting that PTEN acts downstream of the Rho GTPase. Ligand activation of the S1P2R receptor stimulated the coimmunoprecipitation of S1P2R and PTEN. Interestingly, S1P2R signaling increased PTEN phosphatase activity in membrane fractions. Furthermore, tyrosine phosphorylation of PTEN was stimulated by S1P2R signaling. These data suggest that the S1P2R receptor actively regulates the PTEN phosphatase by a Rho GTPase-dependent pathway to inhibit cell migration. GPCR regulation of PTEN maybe a general mechanism in signaling events of cell migration and invasion.

cell migration | signal transduction | sphingosine 1-phosphate | tumor suppressor

The tumor suppressor gene *PTEN* (phosphatase and tensin homolog deleted on chromosome 10) encodes a phosphatase with enzymatic activities toward 3′-phosphoinositides (1) and phosphorylated polypeptides (2). Inactivation of the *PTEN* gene is frequently observed in many human malignancies, including cancer of the breast, prostate, brain, and gastrointestinal tract (3–5). PTEN regulates the activity of critical signaling proteins, such as protein kinase B/Akt, which are activated by 3′-phosphoinositides. Thus, loss of PTEN function is associated with increased cell proliferation, resistance to apoptosis, and enhanced cell motility/invasion (6–9). Although the importance of the PTEN enzyme is appreciated, our knowledge of how this phosphatase is regulated under physiological conditions and how it is aberrantly regulated in pathology is limited. In *Dictyostelium discoideum*, PTEN enzyme localization is regulated by cAMP, a well known chemoattractant that signals by a specific G protein-coupled receptor (GPCR) (10, 11). Whether the cAMP receptor directly couples to the PTEN phosphatase is not understood. Moreover, in mammalian cells, little is known about the upstream regulatory pathways for PTEN.

Sphingosine 1-phosphate (S1P) is a multifunctional lipid mediator produced by sphingomyelin metabolism in eukaryotic cells. Extracellular S1P binds to and activates the endothelial differentiation gene family of GPCRs (12), which have been renamed S1P1–5 receptors (S1P1R–S1P5R) (reviewed in ref. 13). Although the standard nomenclature for these receptors does not include the "R" designation, it is indicated in this report to distinguish the receptor from the ligand. These receptors are coupled differentially to heterotrimeric G proteins such as G_i, $G_{12/13}$, and G_q (14). S1P1R couples exclusively to G_i, whereas S1P2R and S1P3R couple to G_i, G_q, and G_{13} (15, 16). Depending on the S1P receptor expression pattern in a given cell type, S1P mediates seemingly divergent effects. Thus, S1P1R is coupled to the G_i pathway and regulates the small GTPase Rac, cortical actin assembly (14), and cell migration (17) in a protein kinase B/Akt-dependent manner. In contrast, coupling of S1P2R to the heterotrimeric $G_{12/13}$ protein activates the small GTPase Rho and thereby inhibits Rac, cortical actin assembly, and cell migration (18–20). In endothelial cells, S1P1R and S1P2R receptors cooperatively function in embryonic blood vessel development. Thus, deletion of *S1P1R* and *S1P2R* genes results in a more severe bleeding phenotype and earlier embryonic lethality compared with *S1P1R* single null embryos (21, 22). In this report, we show that S1P2R actively regulates PTEN as a necessary downstream effector in the antimigratory response.

Materials and Methods

Reagents. Fatty acid-free BSA, gelatin, β-glycerophosphate, and guanosine 5′-[γ-thio]triphosphate were purchased from Sigma. Sphingosine and S1P were purchased from Biomol (Plymouth Meeting, PA). FTY720-phosphate (FTY720-P) was kindly provided by V. Brinkmann (Novartis, Basel). Pertussis toxin (PTx) and Y-27632 were purchased from Calbiochem.

Cell Culture, cDNA Transfection, and Adenoviral Transduction. Human umbilical vein endothelial cells (HUVECs) (p3–10, Clonetics, San Diego) were cultured as described in ref. 23. WT, *Pten*$^{\Delta loxP/\Delta loxP}$, and S1P2R null mouse embryonic fibroblasts (MEFs) were prepared as described in ref. 7 and cultured in gelatin-coated dishes. HEK293T cells in 100-mm dishes were transfected with 6 μg of vectors containing V5-tagged S1P1R or S1P2R and epitope-tagged hemagglutinin (HA)-PTEN constructs by the calcium phosphate method (24). For adenoviral transduction, cells were infected with adenovirus containing β-gal (17), M2-tagged S1P2R, dominant-negative PTEN (dnPTEN), WT PTEN (25), and dominant-negative N19 Rho (26) for 16 h (20–100 multiplicity of infection). The supernatant was then removed, and cells were left to recover in complete medium for 12 h.

Migration Assays. HUVEC and MEF migration was assayed by using a 96-well chemotaxis microchamber (Neuroprobe, Cabin John, MD) as described in ref. 27. Quantification was done based

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Abbreviations: FTY720-P, FTY720-phosphate; GPCR, G protein-coupled receptor; HA, hemagglutinin; HUVEC, human umbilical vein endothelial cell; MEF, mouse embryonic fibroblast; PTEN, phosphatase and tensin homolog deleted on chromosome 10; S1P, sphingosine 1-phosphate; S1P*n*R: sphingosine 1-phosphate *n* receptor; PTx, pertussis toxin; dnPTEN, dominant-negative PTEN.

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on absorbance at 575 nm by a Spectramax 340 plate reader (Molecular Devices).

Western Blot Analysis and Immunoprecipitation. For determination of phospho-Akt levels, cells were homogenized in radioimmunoprecipitation assay buffer (0.1% SDS/0.5% sodium deoxycholate/1% Nonidet P-40/1 mM sodium orthovanadate/50 mM β-glycerophosphate/1$\times$ protease-inhibitor mixture) and centrifuged at 10,000 $\times$ g for 10 min. Equal amounts of proteins were separated on a 10% SDS/PAGE and blotted onto a nitrocellulose membrane. Immunoblot analysis was performed by using phospho-Akt and Akt antibodies (Cell Signaling Technology, Beverly, MA).

Anti-V5 (Invitrogen) and anti PTEN N19 (Santa Cruz Biotechnology) antibodies were covalently linked to protein A Trisacryl beads (Pierce) and protein G Sepharose beads (Amersham Biosciences), respectively, with dimethyl pimelimidate dihydrochloride (Pierce) as described in ref. 28. Twenty-four hours after transfection, HEK293T cells were serum-starved and stimulated with ligands. PTx treatment (200 ng/ml) was performed during serum starvation. After stimulation, cells were lysed for 45 min with radioimmunoprecipitation buffer and were immunoprecipitated as above. Immune complexes were released by incubating in sample buffer at room temperature for 45 min and were separated by SDS/PAGE.

To detect Ser/Thr-phosphorylated PTEN, MEFs and HUVECs were stimulated with S1P and lysed with radioimmunoprecipitation (RIPA) buffer containing protease-inhibitor mixture, 10 mM NaF, and 50 mM β-glycerolphosphate. To detect tyrosine-phosphorylated PTEN, MEFs and HUVECs were preincubated for 10 min with 30 μM pervanadate (29) and stimulated with different ligands. Cells were lysed with RIPA buffer containing protease-inhibitor mixture and 10 μM sodium orthovanadate (Sigma). PTEN was immunoprecipitated from 1 mg of cell lysates by using anti PTEN N19 antibody (Santa Cruz Biotechnology) covalently linked to protein G Sepharose beads (Amersham Biosciences). Ser/Thr-phosphorylated PTEN was detected by using anti-phosphoserine/threonine monoclonal antibody (BD Biosciences) and by using anti-phospho-PTEN Ser-380/Thr-382/383 antibody (Cell Signaling Technology). Tyrosine-phosphorylated PTEN was detected by using anti-phosphotyrosine antibody, clone 4G10 (Upstate Biotechnology, Lake Placid, NY).

Affinity Precipitation of Rho-GTP and Rac-GTP. After S1P stimulation, MEFs were lysed in radioimmunoprecipitation buffer, and 400 μg of cell lysates were incubated with GST-C21 [Rho binding domain of Rhotekin, kindly provided by Shuh Narumiya (Kyoto University, Kyoto)] or GST-PAK [Rac binding domain of p21 activated kinase, kindly provided by Martin Schwartz (The Scripps Research Institute, La Jolla, CA)] beads, as described in ref. 27.

PTEN Phosphatase Assay. HA-PTEN- and S1P2R- or HA-PTEN- and S1P1R-expressing HEK293T cells were serum-starved and stimulated with S1P for 5 min. Then, they were Dounce-homogenized in hypotonic buffer (20 mM Hepes-KOH, pH 7.5/10 mM KCl/1.5 mM $MgCl_2$/1 mM EGTA/1 mM EDTA/1 mM DTT). Nuclei were spun down (750 $\times$ g), and supernatant was centrifuged (100,000 $\times$ g) to obtain the membrane fraction, which in turn was solubilized and immunoprecipitated as described above.

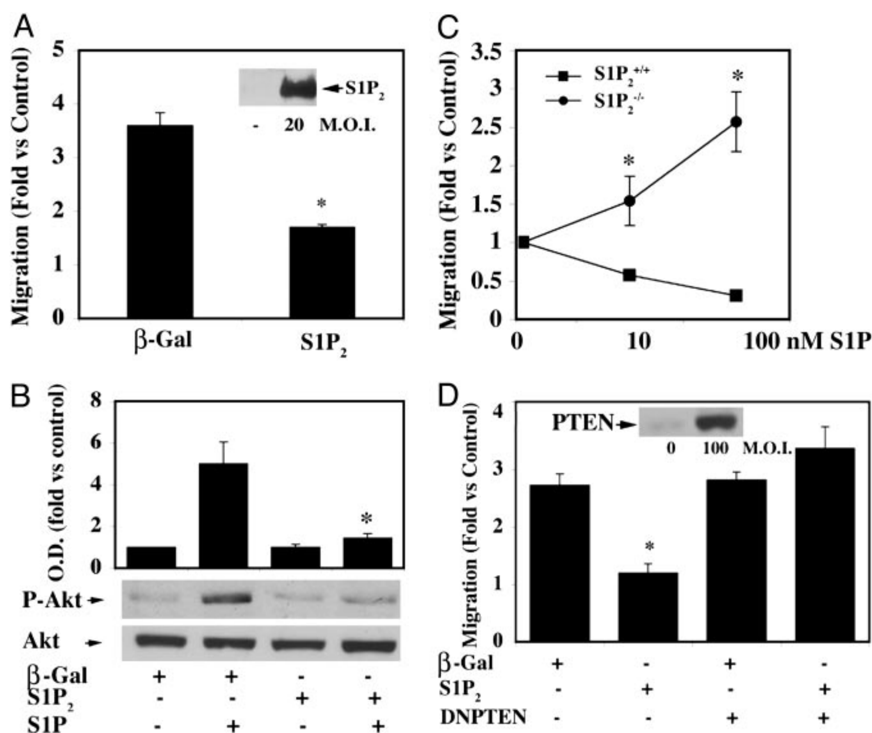

Fig. 1. S1P2R inhibits migration in HUVECs and MEFs. (*A*) S1P-induced migration in adenovirus control (β-gal) and S1P2R-transduced ($S1P_2$) endothelial cells. HUVECs were treated with 100 nM S1P or were left untreated, and cell migration was quantified as described. (*Inset*) S1P2R polypeptide expression in S1P2R virus-transduced HUVECs was determined by immunoprecipitation with anti-Flag antibody followed by immunoblotting with the same antibody, as described in *Materials and Methods*. *, $P < 0.01$ vs. β-gal-transduced HUVECs. (*B*) Phospho-Akt and total Akt levels in adenovirus control (β-gal) and S1P2R-transduced ($S1P_2$) endothelial cells stimulated with 100 nM S1P for 10 min. A representative blot of three is shown. Data are the mean ± SE; $n = 3$. *, $P < 0.01$ vs. β-gal-transduced HUVECs. (*C*) S1P-induced migration of MEFs derived from WT ($S1P_2^{+/+}$) or S1P2R null ($S1P_2^{-/-}$) mice. *, $P < 0.01$, WT vs. S1P2R null. (*D*) S1P-induced migration of endothelial cells infected with control (β-gal), S1P2R, or dnPTEN adenoviruses. Cells that were not infected with dnPTEN received the same dose of adenovirus control. Data are the mean ± SE of triplicates from a representative experiment; $n = 2$–4. *, $P < 0.01$ vs. control virus-infected cells. (*Inset*) PTEN levels after infection with 100 multiplicity of infection of dnPTEN virus.

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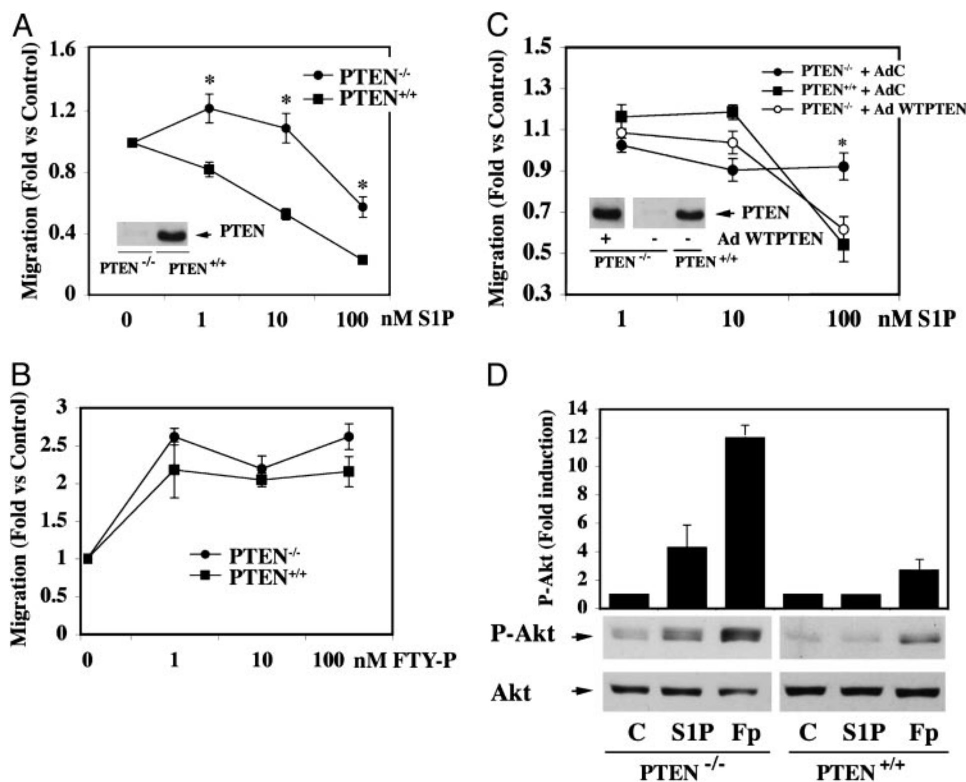

Fig. 2. PTEN mediates S1P2R-dependent inhibition of migration in MEFs. (*A*) Migration of *Pten*$^{\Delta loxP/\Delta loxP}$ (PTEN$^{-/-}$) and *Pten*$^{+/+}$ MEF cells toward S1P. (*Inset*) PTEN expression in *Pten*$^{\Delta loxP/\Delta loxP}$ and WT MEFs. *, $P < 0.01$ vs. *Pten*$^{+/+}$ MEFs. (*B*) Migration of *Pten*$^{\Delta loxP/\Delta loxP}$ (PTEN$^{-/-}$) and WT MEFs toward FTY720-P. (*C*) Migration of adenovirus control-transduced *Pten*$^{\Delta loxP/\Delta loxP}$ MEFs (PTEN$^{-/-}$ + AdC) and *Pten*$^{+/+}$ MEFs (*Pten*$^{+/+}$ + AdC) and WT PTEN adenovirus-transduced *Pten*$^{\Delta loxP/\Delta loxP}$ MEFs (PTEN$^{-/-}$ + Ad WT PTEN) toward different concentrations of S1P. (*Inset*) PTEN expression in *Pten*$^{\Delta loxP/\Delta loxP}$ and *Pten*$^{+/+}$ MEFs infected with the different adenoviruses. Fold induction vs. vehicle control is represented. Data represent the mean ± SE of triplicate values of a representative experiment; n = 2–4. (*D*) Phospho-Akt and total Akt levels in *Pten*$^{\Delta loxP/\Delta loxP}$ (PTEN$^{-/-}$) and *Pten*$^{+/+}$ MEFs stimulated for 10 min with vehicle control (C), 100 nM S1P (S1P), and 10 nM FTY720-P (Fp). A representative blot of three is shown. Values are the mean ± SE; n = 3.

PTEN immunoprecipitates from membrane fraction of HA-PTEN- and S1P2R- or HA-PTEN- and S1P1R-expressing HEK293T cells with HA.11 monoclonal antibody (Covance, Berkeley, CA) were assessed for phosphatase activity by using D-myo-phosphatidylinositol 3,4,5-triphosphate (Echelon Biosciences, Salt Lake City)-containing phospholipids vesicles. Phosphate released was determined by measuring the absorbance at 630 nm after addition of malachite green reagent (Upstate Biotechnology).

Statistical Analysis. In migration experiments, results represent mean ± SE of triplicate values. *P* values were calculated by Student's *t* test by using EXCEL (Microsoft). IC_{50} values were calculated by using PRISM 4 software and fitting the data to a sigmoidal dose-response (variable slope) equation.

Results and Discussion

Inhibition of PTEN Abrogates the Antimigratory Effect of the S1P2R Receptor. Ligand activation of HUVECs, which express the S1P1R receptor predominantly (14), results in strong stimulation of cell migration (Fig. 1*A*) concomitantly with the activation of protein kinase B/Akt (Fig. 1*B*). Expression of S1P2R in HUVECs by adenoviral transduction markedly inhibited S1P-induced migration and protein kinase B/Akt phosphorylation (Fig. 1 *A* and *B*). The inhibitory effect of S1P2R expression occurred even when low levels of receptor was expressed (20 multiplicity of infection of adenovirus) and was dose-dependent (data not shown). These data suggest that S1P2R antagonizes the promigratory S1P1R receptor signaling in endothelial cells by inhibiting protein kinase B/Akt phosphorylation.

To demonstrate the antimigratory effect of the endogenously expressed S1P2R receptor, we studied the migratory response of MEFs prepared from WT and *S1P2R* null mice (22). Consistent with previous results (30), WT MEFs expressed high levels of *S1P2R* and *S1P3R* and low but detectable levels of *S1P1R* receptor transcripts (data not shown). As shown in Fig. 1*C*, S1P inhibited the migration of WT MEFs in a dose-dependent manner. In sharp contrast, S1P stimulated migration in MEFs that lack the S1P2R receptor (2.57 ± 0.39-fold induction of basal motility). These results suggest that S1P2R inhibits cell migration in MEFs and HUVECs.

We next determined whether PTEN was involved in the S1P2R-mediated inhibition of protein kinase B/Akt phosphorylation and cell migration. Expression of enzymatically inactive dnPTEN, in which catalytic Cys-124 has been mutated to Ser (25), reversed S1P2R-mediated inhibition of migration in endothelial cells (Fig. 1*D*). In contrast, dnPTEN did not influence S1P-induced migration in β-gal virus-transduced HUVECs. These data suggest that PTEN activity is critical for S1P2R inhibition of migration.

Endogenous PTEN Is Required for S1P2R-Dependent Inhibition of Migration. To further study the role of PTEN in S1P2R antimigratory action, we used the *Pten*$^{\Delta loxP/\Delta loxP}$ MEFs, which were derived by Cre/lox recombination-based deletion of the *Pten* gene (9). Low levels of PTEN (5% of WT levels) were detected in *Pten*$^{\Delta loxP/\Delta loxP}$ MEFs, compared with their WT counterparts (Fig. 2*A Inset*). Real-time RT-PCR analysis showed that *S1P2R*, *S1P3R*, and, to a lesser extent, *S1P1R* transcripts were expressed in both MEF cell lines (data not shown). Interestingly, S1P did not inhibit migration in *Pten*$^{\Delta loxP/\Delta loxP}$ MEFs as potently as in their WT counterparts (Fig.

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2*A*). IC_{50} values were 5.32 nM for WT cells and 32 nM for $Pten^{\Delta loxP/\Delta loxP}$ MEFs. From 1–10 nM S1P, no inhibition was observed, whereas a more modest inhibition was seen at 100 nM when compared with WT cells. This effect could be due to residual PTEN levels in $Pten^{\Delta loxP/\Delta loxP}$ MEFs. Alternatively, S1P2R-mediated inhibition of migration may involve other signaling intermediates that are stimulated when higher doses of S1P are used. In contrast, FTY720-P, a S1P receptor agonist that activates S1P1R, S1P3R, S1P4R, and S1P5R but not S1P2R (31, 32), stimulated cell motility in $Pten^{\Delta loxP/\Delta loxP}$ cells (Fig. 2*B*). These results indicate that both cell types show a similar migratory response when S1P1R and S1P3R receptors are activated, whereas they clearly differ in the migratory response when S1P2R is activated, suggesting that S1P2R signaling efficiency is impaired in $Pten^{\Delta loxP/\Delta loxP}$ cells.

The lack of inhibition of migration by S1P in $Pten^{\Delta loxP/\Delta loxP}$ cells was reversible by adenoviral expression of enzymatically active WT PTEN (Fig. 2*C*). We also observed a modest increase in motility in both cell types after transduction with the control adenovirus, which may be due to the effect of adenoviral genes on MEF cell migration. Nevertheless, these results indicate that coupling to PTEN is critical for efficient inhibition of cell migration by the S1P2R receptor.

S1P stimulation did not trigger protein kinase B/Akt phosphorylation in WT MEFs, presumably because the activity of the S1P2R receptor predominates over that of S1P1R and S1P3R. Indeed, FTY720-P, which does not activate S1P2R but it is a strong agonist for S1P1R and S1P3R (31, 32), strongly stimulated the phosphorylation of protein kinase B/Akt (Fig. 2*D*), suggesting that S1P2R signaling in WT MEFs potently inhibited the S1P1R and S1P3R-mediated stimulation of protein kinase B/Akt phosphorylation. In $Pten^{\Delta loxP/\Delta loxP}$ MEFs, protein kinase B/Akt phosphorylation is stimulated by both S1P and FTY720-P, suggesting that PTEN levels regulate the ability of S1P2R to inhibit protein kinase B/Akt.

Rho, p160-ROCK, and PTEN Are Downstream of S1P2R in the Inhibition of Migration. The Rho family of GTPases plays a crucial role in regulating the actin cytoskeleton dynamics and cell migration. Active Rac is required at the leading edge of the cell to regulate actin polymerization and lamellipodia formation (33). Conversely, Rho regulates focal adhesion assembly and stress fiber formation (34). Previous studies on S1P2R-mediated inhibition of CHO cell migration suggest that signaling via the $G_{12/13}$/Rho pathway is essential for the inhibition of the small GTPase Rac, cortical actin assembly, and chemotaxis (18). However, recent work in neutrophil chemotaxis suggests that Rho and its effector, p160-ROCK (Rho-associated kinase), is important to inhibit Rac activation and contraction at the rear of the cell (uropod), thus providing polarity during chemotaxis (35).

S1P stimulation triggered a strong activation of Rho A in WT MEFs and, to a similar extent, $Pten^{\Delta loxP/\Delta loxP}$ MEFs (Fig. 3*A*), indicating that PTEN is not required for activation of Rho. In contrast, S1P did not increase Rac-GTP levels consistently in WT MEFs, although we observed some variability from experiment to experiment (fold induction was 1 ± 0.09 after 5 min and 1.2 ± 0.22 after 10 min). However, Rac was consistently activated in $Pten^{\Delta loxP/\Delta loxP}$ MEFs (with a fold induction after 5 and 10 min of S1P stimulation of 1.4 ± 0.18 and 1.6 ± 0.24, respectively). This result is in agreement with the fact that phosphatidylinositol-3,4,5-triphosphate (PIP_3) levels are higher in *Pten* null cells and that PIP_3 target the Rac guanine nucleotide exchange factor Tiam-1 to the plasma membrane (36). The small but consistent increase in Rac activation in $Pten^{\Delta loxP/\Delta loxP}$ MEFs by S1P together with the phosphorylation of Akt is in agreement with the notion that phosphorylation of S1P1R by Akt is necessary for Rac activation and S1P-induced migration (17).

Overexpression of N19 RhoA (dominant-negative construct) on WT MEFs by adenoviral transduction abrogated the inhibitory effect of S1P on migration. Indeed, S1P increased motility in WT MEFs expressing dominant-negative Rho (2.5 ± 0.25-fold; Fig. 3*B*).

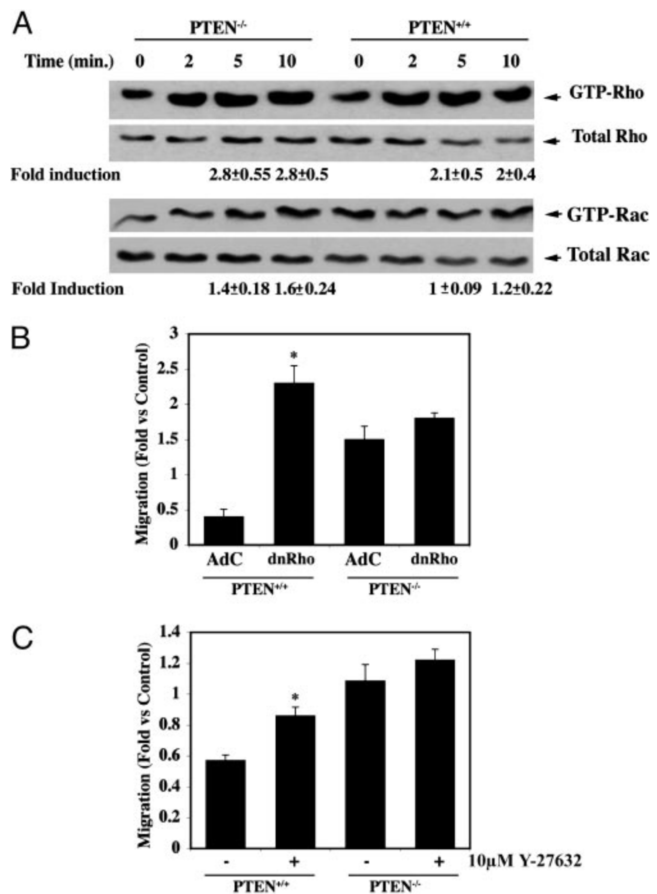

Fig. 3. Rho, p160-ROCK, and PTEN mediate S1P2R antimigratory action. (*A*) Levels of active Rho (GTP-Rho), total Rho, active Rac (GTP-Rac), and total Rac in $Pten^{\Delta loxP/\Delta loxP}$ ($PTEN^{-/-}$) and WT ($PTEN^{+/+}$) MEFs after 2-, 5-, and 10-min S1P stimulation. A representative blot of four is shown. Values are the mean ± SE of the fold induction; $n = 4$. (*B*) Migration of control adenovirus (AdC) and dominant-negative Rho adenovirus (dnRho)-transduced $Pten^{+/+}$ ($PTEN^{+/+}$) and $Pten^{\Delta loxP/\Delta loxP}$ ($PTEN^{-/-}$) MEF cells toward 10 nM S1P. *, $P < 0.01$ vs. adenovirus control-transduced cells. (*C*) $Pten^{+/+}$ ($PTEN^{+/+}$) and $Pten^{\Delta loxP/\Delta loxP}$ ($PTEN^{-/-}$) MEFs were pretreated with vehicle control or 10 μM Y-27632 for 30 min. Then, a migration experiment toward 10 nM S1P was performed as described. These treatments were also present at the upper and lower chamber during the migration experiment. *, $P < 0.01$ vs. nontreated cells. Fold induction vs. vehicle control (basal motility in the absence of S1P) is plotted. Data are the mean ± SE of triplicates from one representative experiment; $n = 2$–3.

These data indicate that the GTPase Rho is a major regulator of S1P2R- mediated inhibition of migration in MEFs, in agreement with the findings by Sugimoto *et al.* (18) in S1P2R-overexpressing CHO cells. Similar results were found in S1P2R-transduced HUVECs (data not shown). Interestingly, 10 nM S1P stimulated migration in $Pten^{\Delta loxP/\Delta loxP}$ cells, but inhibition of Rho did not further increase motility, indicating that the ability of Rho to modulate cell migration depends on PTEN levels. Together with the fact that PTEN is not required for Rho activation, these data suggest that PTEN is downstream of Rho in the S1P2R antimigratory pathway.

To determine the involvement of p160-ROCK in S1P2R-dependent inhibition of migration in MEFs, we tested the effect of Y-27632, a specific inhibitor of this protein kinase. As shown in Fig. 3*C*, in WT MEF cells, Y-27632 treatment enhanced basal motility and significantly abrogated the antimigratory effect of S1P. Although p160-ROCK is involved in S1P2R chemorepellant activity, a stronger migratory response was observed by inhibiting Rho (compare 2.3 ± 0.25-fold induction by 10 nM S1P in N19 Rho-

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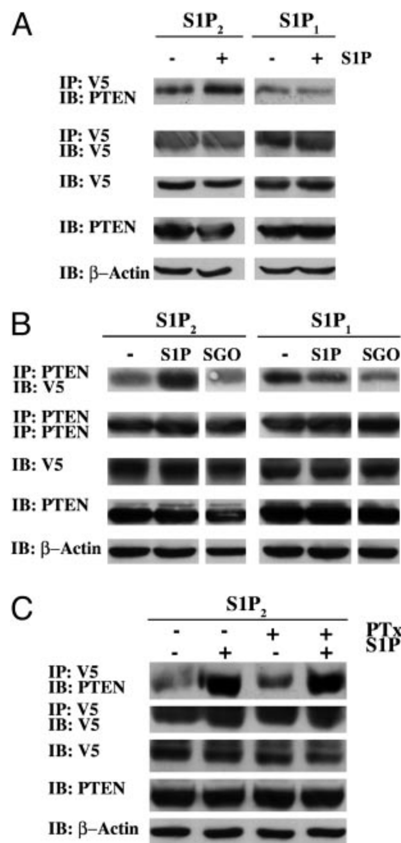

Fig. 4. Ligand-dependent association of S1P2R and PTEN. (*A*) HEK293T cells were transfected with HA-PTEN and nV5-S1P2R ($S1P_2$) or HA-PTEN and V5-S1P1R ($S1P_1$). Twenty-four hours after transfection, cells were serum-starved and stimulated with vehicle control (−) or 100 nM S1P (+) for 10 min. Cell lysis and immunoprecipitation were performed. (*B*) Cells were treated with vehicle control (−), 100 nM S1P (S1P), or 100 nM sphingosine (SGO) for 10 min. (*C*) PTEN-S1P2R association is not inhibited by PTx treatment. HEK293T cells were transfected with HA-PTEN and V5-S1P2R. During serum starvation, cells were treated with 200 ng/ml PTx where indicated. They were then stimulated with 100 nM S1P (S1P) for 10 min. Cell lysis and immunoprecipitation were performed as indicated above. A representative experiment of three is shown.

overexpressing WT MEFs with 0.87 ± 0.039-fold induction in Y-27632-treated WT MEFs). This observation is consistent with the fact that Rho is upstream of p160-ROCK and that multiple Rho-regulated targets are likely involved in the migration inhibitory pathway. In $Pten^{\Delta loxP/\Delta loxP}$ MEF cells, Y-27632 treatment did not enhance further motility, suggesting that p160-ROCK and PTEN cooperate in the S1P2R-mediated antimigratory action.

Ligand Stimulation Triggers S1P2R and PTEN Complex Formation in the Membrane Compartment. We next determined whether PTEN and S1P2R are found in a protein complex by performing a coimmunoprecipitation experiment. HEK293T cells expressing S1P2R and PTEN were treated with S1P, and the GPCR was immunoprecipitated. S1P treatment induced PTEN association with S1P2R (Fig. 4*A*). In contrast, although a weaker basal interaction was observed between PTEN and the S1P1R receptor, this interaction was not increased by ligand stimulation. PTEN and S1P2R association was ligand-dependent because sphingosine was not able to induce this association. Immunoprecipitation of PTEN also resulted in S1P2R association, which was further stimulated by the ligand (Fig. 4*B*). The recruitment of PTEN was independent of G_i signaling because pretreatment with PTx did not block this association (Fig. 4*C*). These data suggest that S1P2R regulates PTEN function by recruiting it to the same subcellular compartment as a protein complex. Previous studies show that PTEN can interact with the multi-PDZ-containing scaffold protein membrane-associated guanylate kinase inverted-2 through its C terminus type 1 PDZ binding motif, (S/T)XV (37). Interestingly, among S1P receptors, only the S1P2R C terminus contains this highly conserved motif (TVV) (38). Among the other GPCRs that contain this motif are the β_1 adenoreceptor and serotonin receptors 2A and 2C (39). The ability of GPCR to interact with scaffold proteins through the PDZ binding motif may facilitate the efficient coupling of the receptor to specific signaling intermediates and/or intracellular effectors.

S1P2R Stimulation Triggers an Increase in PTEN-Specific Activity and Tyr Phosphorylation. Because PTEN is cytosolic and its main substrate is the membrane lipid phosphatidylinositol-3,4,5-triphosphate, the activation of PTEN must involve its recruitment to the membrane. Ligand stimulation increased the specific activity of membrane-associated PTEN in S1P2R-expressing HEK293T cells (Fig. 5*A*), indicating an active regulation of PTEN by S1P

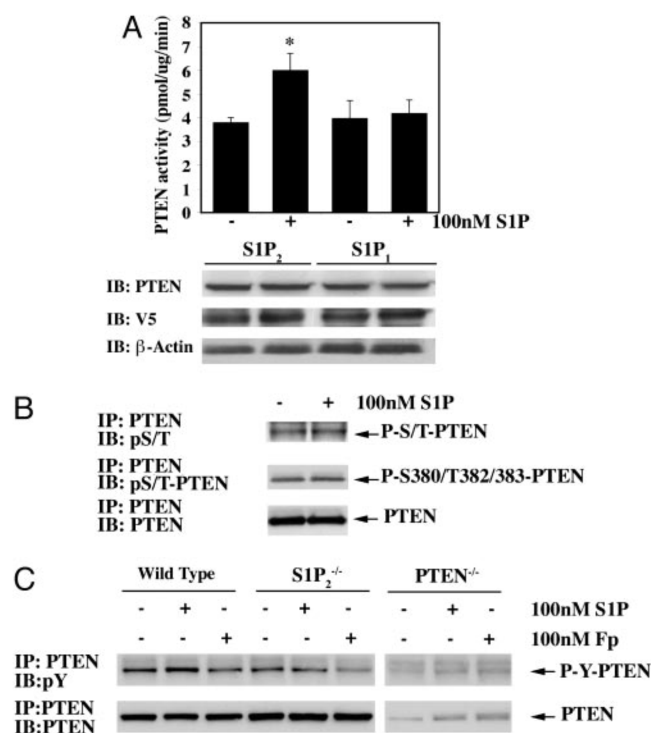

Fig. 5. S1P stimulation triggers an increase in PTEN-specific activity and tyrosine phosphorylation. (*A*) S1P stimulation triggers an increase in PTEN-specific activity in HA-PTEN and S1P2R HEK293T cells. Cells were transfected with HA-PTEN and nV5-S1P2R ($S1P_2$) or HA-PTEN and nV5-S1P1R ($S1P_1$). After serum starvation, cells were stimulated for 5 min with vehicle control (−) or 100 nM S1P (+). Membrane fraction was isolated, and phosphatase activity toward phosphatidylinositol-3,4,5-triphosphate present in lipid vesicles was measured from immunoprecipitated PTEN from membrane fraction as described in *Materials and Methods*. Data represent the mean ± SE of quadruplicates from a representative experiment; $n = 2$. *, $P < 0.05$ vs. vehicle control. (*B*) S1P stimulation did not induce changes in Thr/Ser-phosphorylated PTEN. MEFs were serum-starved and stimulated with S1P or vehicle control for 15 min. Cell extract (1 mg) was immunoprecipitated with PTEN antibody, followed by immunoblotting with anti-phospho-serine and -threonine general antibody (P-S/T-PTEN), anti-phospho-Ser-380/Thr-382 and 383-PTEN antibody (P-S380/T382/383-PTEN), or PTEN antibody. (*C*) S1P stimulation induced tyrosine phosphorylation of PTEN. MEFs from WT, *S1P2R* null ($S1P_2^{-/-}$), or *Pten* null ($PTEN^{-/-}$) mice were serum-starved, preincubated with 30 μM pervanadate for 15 min, and stimulated with S1P, FTY720-P (Fp), or vehicle control for another 15 min. Cells were lysed, and 1 mg of cell extract was immunoprecipitated with PTEN antibody, followed by immunoblotting with anti-phospho-tyrosine antibody (pY) or PTEN antibody. A representative blot is shown; $n = 2–4$.

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through the S1P2R receptor. No changes in PTEN activity were detected in S1P1R-expressing HEK293T cells.

Little is known about the signaling pathways that regulate PTEN activity. PTEN function may be regulated by subcellular localization and phosphorylation. Several structural motifs in PTEN have been shown to contribute to its function. These include the phosphatase domain, a C2 domain, the PDZ binding motif at the C terminus, TKV, and several putative Tyr and Ser/Thr phosphorylation sites (40). PTEN function, stability, and ability to interact with other proteins have been shown to depend on the phosphorylation of Ser and Thr residues in its C-terminal tail (41–44). In addition, mutations in two putative Tyr phosphorylation sites at the C2 domain of PTEN show their critical role in the phosphatase and tumor suppressor activities (45). These posttranslational modifications of PTEN may control its localization and interaction with the substrates. Because PTEN is required for the S1P2R-mediated S1P-chemorepellant activity in MEFs, we aimed to study whether S1P stimulation could affect the phosphorylation status of PTEN. We immunoprecipitated PTEN from MEFs and blotted with an anti-phospho Ser and Thr antibody or with a specific anti-phospho-Ser-380/Thr-382/383-PTEN antibody. As shown in Fig. *5B*, no changes in Ser and Thr-phosphorylated PTEN after S1P stimulation were detected in MEFs (Fig. *5B*). Nor did we detect any changes in Ser- or Thr-phosphorylated PTEN by S1P in HUVECs (data not shown).

Previous studies showed that to detect Tyr-phosphorylated PTEN, it was necessary to inhibit tyrosine phosphatases by pervanadate preincubation or to overexpress Src kinases (29). We were not able to detect Tyr-phosphorylated PTEN unless we preincubated with pervanadate. Under these conditions, S1P stimulation induced a 1.7 ± 0.2-fold increase in Tyr phosphorylation of PTEN in MEFs (Fig. *5C*). This increase was S1P2R-dependent, because it was not triggered either by S1P stimulation in S1P2R null MEFs or by FTY720-P in WT MEFs. The fact that preincubation with pervanadate was necessary to detect Tyr-phosphorylated PTEN is in agreement with studies that indicate that other phosphatases, such as SHP-1, can interact with and dephosphorylate PTEN (29). Moreover, Tyr-phosphorylation mutants of PTEN underscore the physiological relevance of these sites (45).

The broader implication of this work is that PTEN activity can be modulated acutely by GPCR signaling. Many chemorepellant factors regulate animal development and are implicated in tumorigenesis. For example, ephrin signaling, which is clearly shown to be critical in development (46, 47), was recently shown to be important in intestinal tumorigenesis (48). S1P2R is also important in vascular development and cooperates with S1P1R to achieve the formation of a functional vasculature (22). While this manuscript was in preparation, White *et al.* (49) reported the involvement of PTEN in the antimigratory activity of prostaglandin E_2 in fibroblasts. Thus, GPCR regulation of PTEN maybe a general mechanism in cell signaling.

Sphingolipid signaling is now recognized as an important signaling system that regulates cell metabolism, growth, death, and fate and cell–cell communication in higher organisms (38, 50). Recently, it has begun to be appreciated that S1P metabolism and function may be important in cancer development and progression. For example, dietary sphingomyelin can potently inhibit intestinal tumorigenesis (51). The precise mechanism of action is not clear, but it is assumed that metabolic conversion of sphingomyelin to sphingolipid mediators, such as sphingosine, ceramide, and S1P, may be involved. Data in this report prompt the speculation that inhibition of tumorigenesis by sphingolipid mediators, in particular the S1P2R, may involve the function of the tumor suppressor PTEN.

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