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$G_{12/13}$ and G_q mediate $S1P_2$-induced inhibition of Rac and migration in vascular smooth muscle in a manner dependent on Rho but not Rho kinase

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1. Introduction

Migration of medial vascular smooth muscle cells (VSMCs) into the intima, together with the targeting mobilization and differentiation into VSMCs of bone marrow-derived vascular progenitor cells, is a crucial step that gives rise to the formation of vascular proliferative lesions including atheroma and post-angioplasty restenosis.[1,2] The control of vascular cell motility may confer a useful means to treat vascular proliferative lesions. Sphingosine-1-phosphate (S1P) is a recently established lipid mediator that exerts pleiotropic effects on diverse cell types including vascular smooth muscle cells (VSMCs) and vascular endothelial cells.[3-6] These include regulation of cell migration, cell shape and cell to cell adhesion, and stimulation of mitogenesis, most of which are mediated via S1P-specific G protein-coupled receptor (GPCR) family, including $S1P_1$ receptor ($S1P_1R$), $S1P_2R$, and $S1P_3R$.[3-6] S1P exhibits unique bimodal activities as an extracellular regulator on cell motility. It stimulates or inhibits cell migration, in a manner apparently dependent on cell types[7]; S1P stimulates migration of vascular endothelial cells[6] whereas S1P inhibits migration of VSMCs.[8] We previously demonstrtaed that this bimodal regulation by S1P is based upon a diversity of S1P receptor subtypes, which mediate receptor subtype-specific

stimulatory or inhibitory regulation for cell migration.[9] Thus, $S1P_1R$ and $S1P_3R$ act as attractant receptors to mediate migration directed toward S1P, whereas $S1P_2R$ acts as a repellant receptor to mediate inhibition of chemotaxis toward a chemoattractant.

Rho family small GTPases, primarily Rac, Cdc42, and Rho, are well-known regulators of actin reorganization and myosin motor function and thereby of cell motility.[10] We observed that platelet-derived growth factor (PDGF) stimulates Rac, but not Cdc42 or Rho in VSMCs and that Rac plays an essential role in PDGF-directed chemotaxis.[8] We found in heterologous expression of each S1P receptor subtype that the attractant receptors $S1P_1R$ and $S1P_3R$ mediate stimulation of Rac whereas the repellant receptor $S1P_2R$ inhibits chemoattractant-induced Rac activation.[9] Thus, S1PRs exert bimodal regulation on cell migration primarily through positively or negatively regulating cellular Rac activity. In VSMCs, S1P induced inhibition of PDGF-triggered Rac activation as well as migration.[8]

Pleiotropic actions of GPCRs are mediated primarily by four classes of heterotrimeric G proteins, G_s, G_i, G_q and $G_{12/13}$. Several GPCRs including prostaglandin E_2 (PGE_2) receptors, dopamine receptors and adrenomedullin receptors mediate inhibition of motility of VSMCs. These receptors are all canonical G_s-coupled type of receptors, and we recently showed that

upon PGE_2 receptor stimulation, cyclic AMP mediated Rac inhibition as a mechanism for inhibition of VSMC migration.[11] Different from these receptors, $S1P_2R$ is not a G_s-coupled type of receptor.[12,13] It is unknown which class of heterotrimeric G protein mediates S1P-induced inhibition of VSMC migration. Also, signaling mechanisms downstream of a heterotrimeric G protein for inhibition of Rac and migration in VSMCs are not fully defined. In the present study, we show in VSMCs that $S1P_2R$ induces Rho activation via both the G_q and $G_{12/13}$ classes, which in turn mediates inhibition of Rac and chemotaxis in a manner dependent on Rho but independent of Rho kinase.

2. Methods

2.1. Materials

S1P and human PDGF-B chain were purchased from BIOMOL (Plymouth Meeting, PA, U.S.A.) and Peprotech (London, U.K.), respectively. A mouse monoclonal anti-Rac1 antibody (23A8) and rabbit polyclonal anti-phospho (Thr^{850})-MYPT1 (36-003) were purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.).

Mouse monoclonal anti-RhoA antibody (26C4) was from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Rabbit polyclonal anti-MYPT1 antibody (PRB-457C) was bought from Covance (Berkley, CA, USA). Mouse monoclonal anti-myc antibody (9E10) was obtained from ATCC (Rockville, MA, U.S.A.). JTE-013 and HA-1077 were kind gifts from Japan Tabacco (Takatsuki, Japan) and Asahi Chemical Industry (Fuji, Japan), respectively. U73122 and BAPTA-AM were purchased from Merck-Calbiochem Biosciences (Darmstadt, Germany), and phorbol 12,13-dibutyrate (PDBu) and G418 were obtained from Sigma (St. Louis, MI, U.S.A.). Pertussis toxin was bought from List Biological Laboratories (Campbell, CA, U.S.A.). Botulinum C3 toxin, human PAK1 (amino acids 75 to 131(CRIB-domain)) and mouse Rhotekin (amino acids 7 to 89 (Rho-binding domain)) that were fused to glutathione-s-Sepharose (GST) (GST-C3, GST-PAK1 and GST-Rhotekin, respectively) were prepared as described previously.[28]

2.2.Plasmids and adenoviruses

The mammalian expression plasmid for $S1P_2$, pME18S-$S1P_2$, was described previously.[9] Mouse AT_1a receptor DNA encoding the entire coding region cloned from

mouse genomic DNA by PCR was ligated onto pME18S at B*stXI* site. The fusion receptor AT_1-$G\alpha_{12}$, in which the full length $G\alpha_{12}$ is fused to the C-terminus of AT_1a, was generated by the PCR-based method as described previously.[13] Replication-deficient adenoviruses carrying myc-tagged $G\alpha_{12}$-CT, $G\alpha_{13}$-CT, $G\alpha_q$-CT and $G\alpha_s$–CT were described previously.[13] The adenovirus encoding β-galactosidase (LacZ) were kindly donated by I. Saito (Institute of Medical Sciences, University of Tokyo). The cells were infected with adenoviruses at a multiplicity of 200 by incubating VSMCs with an adenovirus-containing medium for 1 h, which conferred successful gene transduction in nearly 100% of cells without significant cell damage.[8,14] After recovery in a fetal calf serum (FCS)-containing growth medium for 24 h, the cells were subjected to migration assay, or serum-deprived before each experiment.

2.3.Cells

Rat aortic VSMCs were isolated by the explant method from an 8-week old Wistar male rat, as described previously.[8] The present investigation using experimental animals conforms with the Guide for the Care and Use of Laboratory Animals published by the

US National Institutes of Health (NIH Publication No. 85-23, revised 1996). VSMCs were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FCS (Equitech-Bio, Ingram, TX, U.S.A.), 100U/ml of penicillin, and 100 μg/ml of streptomycin (Wako, Osaka, Japan). When indicated, VSMCs were pretreated with C3 toxin (10 μg/ml) in serum-free DMEM for 72 h.[13] The VSMCs stably overexpressing either AT_1 or AT_1-$G\alpha_{12}$ were established by co-transfecting VSMCs with one of the above expression plasmids and pKM3 vector that confers G418 resistance, and selecting in the presence of 0.2 mg/ml of G418 as described previously.[14] The stable VSMC transfectants were maintained in the presence of the same concentration of G418. VSMCs were treated with 10 ng/ml pertussis toxin for 24 h when indicated. Total RNA was isolated from VSMCs and analyzed for mRNA expression by Northern blotting using ^{32}P-labelld cDNA probe as described peviously.[8,14]

2.4.Transwell migration assay

Transwell migration of VSMCs was determined in a modified Boyden chamber (Neuroprobe, Gaithersburg, MD) using polycarbonate filters with 8-μm pores as

described in detail previously.[8,9] VSMCs (1×10^5) in 200 μl of serum-free DMEM containing 0.1% fatty acid-free bovine serum albumin were loaded into the upper wells, whereas the lower wells were filled with the same medium containing various concentrations of S1P, angiotensin II, and/or PDGF. The cells were allowed to migrate across the porous filter for 5 h at 37°C in a tissue culture incubator. After staining with Diff-Quick (Sysmex, Kobe, Japan) and scraping the upper surface of the filter, the number of cells that migrated to the lower side of the filter was determined by measuring optical densities at 595 nm using a 96-well microplate reader Model 3550 (Bio-Rad, Hercules, CA). There was a linear positive relationship between an optical density (OD) value and a counted number of migrated cells through pores in a range of less than 0.2 of OD value.[9] In the experiment to examine the effect of the expression of a dominant negative form of Rho kinase (DN-ROCK)[15] on transwell migration, VSMCs were co-transfected with the β-galactosidase expression vector (pCAGGS-LacZ) and either the DN-ROCK expression vector pME18S-DN-ROCK or the empty vector as described.[9] The filter was subjected to staining with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside as a substrate and the migrated cells

attached onto the lower side of the filters were counted under a microscope.[9]

2.5.Determination of the activities of Rho and Rac

Pull-down assays to determine GTP-bound active forms of RhoA and Rac1 were performed as described in detail previously.[8,9,13] Briefly, cells that had been serum-deprived for 72 h were stimulated and cell lysates were prepared as described. Cell extracts were incubated with the GST-rhotekin (for determination of Rho activity) or the GST-PAK1 (for determination of Rac activity) that were immobilized onto glutathione-*s*-Sepharose 4B beads (GE Healthcare, Buckinghamshire, U.K.) at 4°C for 45 min, followed by three washes. Bound Rho and Rac proteins were detected by Western blotting using specific monoclonal antibodies against RhoA and Rac1 and quantitated by densitometry, as described. 1/100 of total RhoA and Rac1 present in the cell lysate of each sample were also shown in each figure. The amounts of GTP-bound active RhoA and Rac1 were normalized for the total amounts of RhoA and Rac1, respectively, and expressed as multiples of the value of non-treated control cells, which was expressed as 1.0.

2.6. Statistics

All data are shown as mean ± SEM and are representative of at least three independent experiments with similar results. ANOVA (analysis of variance) was followed by Bonferoni's test to determine the statistical significance of differences between mean values. For all statistical comparisons, $p<0.05$ was considered significant.

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3. Results

3.1. $S1P_2R$ mediates inhibition of PDGF-induced cell migration and Rac activation via $G_{12/13}$ and G_q

S1P inhibited migration directed toward PDGF-B chain of naive VSMCs and vector control VSMCs (data not shown) in a transwell migration assay in a dose-dependent manner with complete inhibition at 0.1 μM S1P (Fig. 1A). S1P alone slightly inhibited random migration of VSMC. The selective $S1P_2$ receptor antagonist JTE-013[16] abolished S1P inhibition of both random migration and PDGF–directed migration at up

to 0.1 μM S1P. On the other hand, overexpression of $S1P_2R$ in VSMCs shifted leftward the dose-response curve for S1P inhibition nearly by one order, compared to naive VSMCs. These observations indicate that S1P inhibition of cell migration is mediated via $S1P_2R$. PDGF stimulated Rac (Fig. 1B), which is essential for PDGF-directed VSMC migration.[8] S1P inhibited PDGF-induced Rac activation in a dose-dependent manner with complete inhibition at 0.1 μM S1P. JTE-013 totally abolished S1P inhibition of PDGF-induced Rac stimulation (Fig. 1C), indicating that Rac inhibition is also mediated via $S1P_2R$.

In order to identify which member of the heterotrimeric G proteins is responsible for $S1P_2R$-mediated migration inhibition, we determined the effects of specific inhibition of receptor-G protein coupling by adenovirus-mediated expression of the C-terminal peptides of heterotrimeric G protein α subunits (Gα-CTs) and pertussis toxin (PTX) pretreatment. The effectiveness and specificity of each Gα-CT peptide was previously demonstrated by us and other groups.[13,17] The expression of each myc-tagged Gα-CT peptide in adenovirus-infected VSMCs was confirmed by Western blot analysis (Fig. 2A). The expression of either of $G\alpha_{12}$-CT, $G\alpha_{13}$-CT and $G\alpha_q$-CT, but not that of

$G\alpha_s$-CT or LacZ (Fig. 2B) or PTX pretreatment (data not shown), abolished S1P inhibition of PDGF-directed migration. The expression of any of these Gα-CT peptides or PTX pretreatment in VSMCs did not affect PDGF-directed migration itself. Similarly, the expression of either of $G\alpha_{12}$-CT, $G\alpha_{13}$-CT and $G\alpha_q$-CT, but not that of $G\alpha_s$-CT or LacZ (Fig. 2C) or PTX pretreatment (data not shown), abolished S1P inhibition of PDGF-induced Rac activation. These observations indicate that the $G_{12/13}$ and G_q classes are both necessary for S1P-induced inhibition of cell migration and Rac.

3.2. $G_{12/13}$- and G_q -coupled Rho activation mediates S1P inhibition of cell migration and Rac in a Rho kinase-independent manner

S1P stimulated Rho activity in a dose-dependent manner with maximum activation at 0.1 μM S1P (Fig. 3A). JTE-013 abolished S1P-induced Rho activation (Fig. 3B), indicating that $S1P_2R$ mediated Rho activation. The expression of either of $G\alpha_{12}$-CT, $G\alpha_{13}$-CT and $G\alpha_q$-CT, but not that of $G\alpha_s$-CT or LacZ (Fig. 3C) or PTX pretreatment (data not shown), abolished S1P-induced Rho activation, indicating that Rho stimulation is mediated via both $G_{12/13}$ and G_q. Thus, Rac inhibition, migration

inhibition, and Rho stimulation induced by $S1P_2R$ activation are all mediated via both $G_{12/13}$ and G_q.

In order to investigate the role of Rho in inhibition of Rac and migration in VSMCs, we examined the effects of C3 toxin treatment on S1P inhibition of Rac and migration. Treatment of VSMCs with C3 toxin induced the mobility shift of RhoA which reflected ADP ribosylation, and abolished RhoA binding to the Rho-binding domain of the Rho effector Rhotekin, confirming effective inactivation of cellular Rho (Fig. 4A). C3 toxin strongly suppressed S1P inhibition of PDGF-directed migration but did not affect PDGF-directed migration itself (Fig. 4B). Similarly, C3 toxin treatment nearly abolished S1P suppression of PDGF-induced Rac activation (Fig. 4C). Thus, $S1P_2R$-mediated inhibition of PDGF-stimulated Rac activation and cell migration is dependent on Rho.

We examined the possible involvement of a Rho kinase, a downstream effector of Rho, in S1P inhibition of migration and Rac, because a Rho kinase inhibitor was previously reported to block S1P inhibition of cell migration in other cell types.[18] The Rho kinase inhibitor HA-1077 (fasudil) did not inhibit PDGF-directed cell migration itself or random migration (Fig. 5A). HA-1077 slightly (at most by 20%) suppressed

S1P-induced inhibition of PDGF-directed migration at the maximal concentration but did not block the most part of the S1P inhibition. Consistent with these observations, HA-1077 failed to reverse S1P inhibition of PDGF-induced membrane ruffling (Fig. 5B) and Rac activation (Fig. 5C). The effectiveness of HA-1077 in inhibiting Rho kinase in VSMCs was confirmed by the observations that this compound abolished S1P-induced stress fiber formatiomn (Fig. 5B) and S1P-induced stimulation of phosphorylation of the myosin-targeting subunit of myosin light chain phosphatase, MYPT1 (Fig. 5D). Similarly to the effect of HA-1077, the expression of a dominant negative form of Rho kinase, DN-ROCK, failed to suppress S1P-induced inhibition of PDGF-directed migration (Fig. 5E). The observations suggest that $S1P_2R$-mediated inhibition of PDGF-stimulated Rac activation and cell migration is independent of Rho kinase.

3.3. G_q is necessary but not sufficient for activation of Rho or inhibition of Rac and migration

The GPCR agonist angiotensin II (Ang II) effectively couples to G_q to induce robust

phospholipase C stimulation and Ca^{2+} mobilization via AT_1R in VSMCs.[19] Ang II did not stimulate Rho or inhibit PDGF-induced Rac activation or migration in vector-transfected (control) VSMCs (Figs. 6B-6D), suggesting that activation of G_q alone is not sufficient for Rho stimulation or resultant inhibition of Rac and migration. In order to explore the role of $G_{12/13}$ in AT_1R signaling, we established VSMCs stably overexpressing either wild type AT_1R or the AT_1-$G\alpha_{12}$ fusion receptor (AT_1-$G\alpha_{12}R$) in which $G\alpha_{12}$ is fused to AT_1 at its C-terminus and, thereby, the coupling to G_{12} is facilitated (Fig. 6A), and compared their abilities to stimulate Rho and to inhibit Rac and migration. In AT_1R-overexpressing VSMCs, Ang II tended to only slightly stimulate Rho, whereas Ang II induced a 1.8-fold increase in GTP-Rho in AT_1-$G\alpha_{12}R$-overexpressing cells (Fig. 6B). Ang II did not at all affect PDGF-induced Rac activation in either vector-transfected or AT_1R-overexpressing VSMCs, but inhibited PDGF-induced Rac activation in AT1-$G\alpha_{12}R$-overexpressing VSMCs approximately by 50% (Fig. 6C). Consistent with this observation, Ang II inhibited PDGF-directed migration in AT1-$G\alpha_{12}R$-overexpressing VSMCs, but not in vector-transfected or AT_1R-overexpressing VSMCs (Fig. 6D). Ang II alone slightly

stimulated cell migration in vector control VSMCs. Thus, the G_q signaling pathway by itself seems to be insufficient for stimulating Rho, but additional activation of the $G_{12/13}$ pathway results in inhibition of Rac and cell migration with Rho stimulation.

3.4. Gα_q mediates Rho activation through a Ca^{2+}- and protein kinase C-independent mechanism

G_q mediates stimulation of phospholipase C (PLC), resulting in Ca^{2+} mobilization and protein kinase C activation. Previous studies[20,21] suggested the involvement of Ca^{2+} and protein kinase C in Rho-induced cellular responses. We examined the involvement of PLC, Ca^{2+}, and protein kinase C in S1P-induced Rho activation. The PLC inhibitor U73122, which totally inhibited S1P-induced increase in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in VSMCs, did not affect S1P-induced Rho activation (supplementary Figure). The cell permeable Ca^{2+}-chelator BAPTA-AM did not inhibit S1P stimulation of Rho. The protein kinase C activator phorbol-12,13-dibutyrate (PDBu) did not stimulate Rho (Fig. 8C). Thus, the mechanism of the G_q involvement in $S1P_2R$-mediated Rho stimulation does not appears to involve PLC, Ca^{2+} or protein

kinase C.

3. Discussion

Migration of VSMCs into the intima is a critical step that gives rise to the formation of atheroma and vascular stenotic lesions.[1] Migration of VSMCs is positively and negatively regulated by chemoattractants and inhibitory mediators, respectively.[22] Upon ligand binding, chemoattractant receptors, which include receptor tyrosine kinases, GPCRs and cytokine type receptors, activate complex signaling cascades involving protein tyrosine kinases, PI (phosphoinositide) 3-kinase, and the small G proteins, particularly Rho family GTPases.[22] Among Rho family GTPases, Rac is activated by numerous chemoattractants and serves essential roles for cell migration, as well as Cdc42.[10] Compared with chemoattractant receptors, much less is known about the signaling mechanisms of inhibitory or repellant receptors. Among these, we identified $S1P_2R$ as the first example of a GPCR that mediates negative regulation of Rac and, thereby, cell migration in Chinese hamster ovary (CHO) cells overexpressing $S1P_2R$ and

other cell types.[7,9]

We previously demonstrated in the same type of VSMCs as employed in the present study that the repellant receptor $S1P_2R$ is a predominantly expressed isoform whereas the expression levels of the attractant receptors $S1P_1R$ and $S1P_3R$ are moderate or very low.[14] Consistent with these observations, S1P inhibited both random migration and PDGF-directed migration of VSMCs in a manner sensitive to the selective $S1P_2R$ antagonist JTE013 (Fig. 1A). We previously showed in CHO cells that $S1P_2R$-induced inhibition of Rac, a molecular switch for actin reorganization and cell migration, is a major mechanism for $S1P_2R$-mediated inhibition of cell migration.[9] S1P inhibited PDGF-induced Rac activation via $S1P_2R$ in VSMCs (Fig. 1B and 2C) with a similar dose-response relationship as that for inhibition of cell migration (Fig. 1A), suggesting that $S1P_2R$-mediated Rac inhibition is at least in part responsible for S1P inhibition of migration in VSMCs.

$S1P_2R$ couples to multiple heterotrimeric G proteins.[9] We took advantage of adenovirus-mediated expression of specific inhibitor peptides and pertussis toxin to identify a heterotrimeric G protein that couples $S1P_2R$ to inhibition of Rac and cell

migration. The results showed that $S1P_2R$-mediated inhibition of Rac and cell migration required both G_q and $G_{12/13}$ in VSMCs (Figs. 2B and 2C). $G_{12/13}$ is well known to mediate coupling of GPCR activation to Rho stimulation.[20] $S1P_2R$-mediated Rho activation in VSMCs was dependent on not only $G_{12/13}$ but also on G_q, similarly to inhibition of Rac and migration (Fig. 3). We previously observed in CHO cells overexpressing $S1P_2R$ that $S1P_2R$-mediated inhibition of Rac and cell migration was dependent on Rho.[13] This was also the case in VSMCs (Fig. 4). Our previous study[9] showed that $G\alpha_{13}$-CT is a selective inhibitor for G_{13} whereas $G\alpha_{12}$-CT inhibits both G_{12} and G_{13}. In addition, either $G\alpha_{12}$-CT or $G\alpha_{13}$-CT does not affect the G_q coupling, and vice versa. Therefore, our observations suggest that all of G_{12}, G_{13} and G_q are required, or both G_{13} and G_q are required for S1P-induced Rac inhibition in VSMCs. Previous studies[20, 23] on non-muscle cells demonstrated that stimulation of GPCRs with various receptor agonists including lysophosphatidic acid, endothelin-1 and thrombin induced Rho activation through receptor coupling to the $G_{12/13}$ family of the heterotrimeric G proteins. Direct physical and functional interaction of $G\alpha_{12/13}$ with a group of Rho-GEFs with the conserved structural motif known as the regulator of G protein signaling

domain (the RGS box), which include p115RhoGEF, PDZ-RhoGEF and LARG, was demonstrated.[23] More recent studies[20] showed that G_q also had the ability to mediate Rho stimulation through the similar mechanisms in certain non-muscle cell types.

In the present study, G_q activation by Ang II did not induce either Rho stimulation or inhibition of Rac or cell migration (Fig. 6) although $G\alpha_q$ was reported to directly associate with RhoGEFs in non-muscle cells.[20] The observations suggest that G_q activation by itself is not sufficient for effective Rho activation and the following inhibition of Rac and migration in VSMCs. Forced coupling of AT_1R to G_{12} by fusing $G\alpha_{12}$ at the C-terminus of AT_1R enabled Ang II-induced Rho activation and inhibition of Rac and migration. These observations, together with the finding that $S1P_2R$-mediated Rho activation is dependent on both $G_{12/13}$ and G_q, suggest that G_q may exert a permissive effect on Rho activation in cooperation with $G_{12/13}$. A previous study[24] showed that the expression of activated forms of $G\alpha_{12}$ and $G\alpha_{13}$, but not $G\alpha_q$, in VSMCs induced contraction that was inhibited by C_3 toxin, which is also consistent with the notion that G_q plays a permissive role in Rho activation in VSMCs. Previous investigations[20,21] in non-muscle cells showed the involvement of Ca^{2+} and protein

kinase C in Rho-dependent responses induced by expression of a constitutively active mutant of $G\alpha_q$. However, it was unclear in these studies whether Ca^{2+} and protein kinase C affected the process of Rho activation itself or at a site(s) downstream of Rho activation. Our results suggest that PLC or its downstream second messengers, Ca^{2+} or protein kinase C, are likely not involved in $S1P_2R$-mediated Rho activation (supplementary Fig. 1).

A number of downstream effectors of Rho have been identified, including Rho kinase, Dia, protein kinase N (PKN), rhotekin, rhophilin, citron and citron kinase.[25] Among them, Rho kinase has been well-investigated and found to be essential for Rho-induced formation of stress fibers and focal adhesions and Rho-induced negative regulation of myosin phosphatase.[25] In the present study, either the Rho kinase inhibitor HA-1077 or the expression of a dominant negative form of Rho kinase did not suppress $S1P_2R$-mediated inhibition of Rac, cell migration, or membrane ruffling although HA-1077 effectively inhibited $S1P_2R$-mediated Rho-dependent stress fiber formation (Fig. 5). These our results indicate that a Rho effector other than Rho kinase participates in $S1P_2R$-mediated Rac inhibition in VSMCs. The responsible Rho effector molecule

remains to be identified.

Previous investigations suggested that S1P exerts both atherogenic and anti-atherogenic effects. $S1P_1R$ but not $S1P_3R$ was shown to suppresses adhesion of leukocytes onto endothelial cells in the isolated aorta,[26] whereas in cultured endothelial cells both $S1P_1R$ and $S1P_3R$ were shown to stimulate expression of adhesion molecules and monocytic cell adhesion.[27] In the latter investigations $S1P_1R$ also exerted inhibitory effects on adhesion molecule expression through PI 3-kinase and eNOS in cultured endothelial cells. Thus, there is still some controversy concerning the roles of S1P receptors in the regulation of adhesion molecule expression in endothelial cells. In addition, $S1P_1R$ suppresses vascular permeability[28] whereas $S1P_2R$ induces vascular hyperpermeability.[29] *In vivo*, the synthetic S1P analogue FTY720 inhibited development of atherosclerosis in murine models of hypercholesterolemia probably through modulating functions of lymphocytes and macrophages via $S1P_1R$ and $S1P_4R$, both of which are major targets of FTY720.[30] $S1P_1R$, which is upregulated in neointimal VSMCs, stimulates migration of neointimal VSMCs, suggesting its stimulatory role in neointima formation.[31] In contrast to $S1P_1R$, $S1P_2R$ inhibits migration of VSMCs. A

very recent report[32] showed that neointima formation *in vivo* in the carotid artery ligation model is increased in $S1P_2R$-null mice compared with wild type mice, indicating that $S1P_2R$ mediates an inhibitory effect on neointima formation. Thus, in atherosclerotic lesions and other vasoocclusive lesions, S1P appears to exert complicated effects on lesion formation through multiple mechanisms in receptor subtype- and cell type-specific manners. Selective activation of $S1P_2R$ in VSMCs by local drug delivery methods including a drug-eluting stent at stenotic sites could inhibit accumulation of VSMCs into the luminal surface by uniquely activating a chemorepellant activity.

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Conflict of interest

All authors have no potential conflict of interest.

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Figure legends

Figure 1. S1P inhibits PDGF-triggered chemotaxis and Rac activation via $S1P_2R$ in VSMCs. A, Dose-dependent inhibition of PDGF-directed migration. Transwell migration across the porous filter of non-transfected, VCMCs (naive) and VSMCs stably overexpressing $S1P_2R$ ($S1P_2$) toward PDGF (0.41 nM) was determined in the presence and absence of various concentrations of S1P and JTE-013 (1 μM) in the lower well of the Boyden chamber. OD indicates optical density. ∗ and ∗∗ denote $p<0.05$ and $p<0.01$, respectively, compared with the values in the absence of S1P. B, Dose-dependent inhibition of PDGF-induced Rac activation by S1P. VSMCs were pretreated with various concentrations of S1P for 5 minutes and then stimulated with PDGF (0.41 nM) for 2 minutes. The cells were then subjected to a pulldown assay for GTP-Rac as described in Material and Methods. 1/100 of total Rac present in each sample is also shown to confirm loading of equal amounts of proteins (bottom). ∗ and ∗∗ denote $p<0.05$ and $p<0.01$, respectively, compared with PDGF alone. C, Blockade of S1P inhibition of PDGF-induced Rac activation by the $S1P_2R$-selective inhibitor JTE-013. VSMCs were pretreated or not with JTE-013 (1 μM) for 10 minutes and

treated with 0.1 μM S1P for 5 min, followed by stimulation with PDGF (0.41 nM) for 2 minutes.

Figure 2. Effects of selective blockade of G_{12}, G_{13}, G_q, and G_s on S1P inhibition of PDGF-induced chemotaxis and Rac activation in VSMCs. A, Expression of myc-tagged $G\alpha_{12}$-CT, $G\alpha_{13}$-CT, $G\alpha_q$-CT, and $G\alpha_s$-CT. VSMCs were infected with indicated adenoviruses and analyzed for expression of each Gα-CT peptide by Western blotting using anti-myc antibody. B and C, Effects of Gα-CT peptides on S1P inhibition of PDGF-directed chemotaxis (B) and PDGF-induced Rac activation (C). VSMCs were infected with adenovirus encoding $G\alpha_{12}$-CT, $G\alpha_{13}$-CT, $G\alpha_q$-CT, $G\alpha_s$-CT or LacZ as described in Material and Methods, and then subjected to transwell migration assay (B). VSMCs were stimulated with PDGF (0.41nM). In (C), VSMCs were pretreated with 0.1 μM S1P for 5 minutes and then stimulated with PDGF (0.41 nM) for 2 minutes, followed by pull-down assay for GTP-Rac. ** denotes $p<0.01$ compared with PDGF alone.

Figure 3. Effects of selective blockade of G_{12}, G_{13}, G_q, and G_s on S1P-induced Rho activation in VSMCs. A, Dose-dependent stimulation of Rho by S1P. VSMCs were stimulated with various concentrations of S1P for 3 minutes and were subjected to a pull-down assay for GTP-Rho as described in Material and Methods. 1/100 of total Rho present in each sample is also shown to confirm loading of equal amounts of proteins (bottom). ** denotes $p<0.01$ compared with no stimulation. B, Blockade of S1P-induced Rho activation by the $S1P_2R$-selective inhibitor JTE-013. VSMCs were pretreated or not with JTE-013 (1 μM) for 10 minutes and stimulated with 0.1 μM S1P for 3 min. C, Effects of Gα-CT peptides on S1P-induced Rho activation. VSMCs were infected with adenovirus encoding $G\alpha_{12}$-CT, $G\alpha_{13}$-CT, $G\alpha_q$-CT, $G\alpha_s$-CT or LacZ as described in Material and Methods. VSMCs were stimulated with 0.1 μM S1P for 3 min. In C, ** denotes $p<0.01$ compared with S1P stimulation in LacZ virus-infected cells.

Figure 4. S1P inhibition of PDGF-induced chemotaxis and Rac activation is dependent on Rho. A, ADP-ribosylation of RhoA and inhibition of binding to rhotekin by C3 treatment. Note the mobility shift of RhoA from C3-pretreated cells and the inability to

bind to GST-rhotekin beads. In B and C, VSMCs that had been pretreated or not with C3 toxin were subjected to transwell migration (B) and pull-down assay for Rac (C). VSMCs were treated with S1P and stimulated with PDGF as described in Fig. 1A and Fig. 2B. In B, ** denotes $p<0.01$ compared with PDGF stimulation in the absence of S1P.

Figure 5. Neither a Rho kinase inhibitor nor a dominant negative form of Rho kinase suppresses S1P inhibition of PDGF-induced chemotaxis, lamellipodium formation, Rac activation or MYPT1 phosphorylation. A, VSMCs were pretreated or not with HA-1077 (HA) (10, 30 or 50 μM) for 15 minutes before transwell migration assay. Transwell migration was determined in the presence or absence of PDGF (0.41 nM) and S1P (0.1 μM) in the lower chamber. HA-1077 was present in both the upper and lower chambers, where indicated. B, VSMCs were pretreated or not with HA-1077 (20 μM) for 15 minutes and then treated with S1P (0.1 μM) for 10 min when indicated. The cells were then stimulated with PDGF (0.41 nM) for 30 min. F-actin was visualized by staining with TRITC-labeled phalloidin as described in Materials and Methods. C, VSMCs were

pretreated or not with S1P (0.1 μM) for 5 min and stimulated with PDGF (0.41 nM) for 2 minutes, followed by pull-down assay for GTP-Rac. HA-1077 (20 μM) was added 15 min before the addition of S1P when indicated. D, VSMCs were pretreated with HA-1077 or left untreated as in C and stimulated with S1P (0.1 μM) for 3 min. The cell lysate was analyzed for phosphorylation at Thr^{850} of MYPT1 and total amount of MYPT1. E, VSMCs were co-transfected with LacZ-expression vector and either expression vector of a DN-ROCK or empty vector, and subjected to transwell migration assay followed by quantification of migrated cells as described in "Materials and Methods". The numbers of migrated cells in the presence of PDGF alone were 72±8 and 152±7 cells/five low power fields in vector- and DN-ROCK-transfection, respectively. * and ** denote $p<0.05$ and $p<0.01$, respectively, compared with the values in the presence of PDGF alone.

Figure 6. Facilitation of AT_1 receptor coupling to G_{12} enables stimulation of Rho and inhibition of Rac and migration. A, Expression of AT_1R and AT_1-$G\alpha_{12}$ fusion receptor (AT_1-$G\alpha_{12}R$) in VSMCs. VSMCs were transfected with either of receptor expression

vectors and empty vector and selected in the presence of G418 as described in Materials and Methods. The mRNA levels of AT_1R and AT_1-$G\alpha_{12}R$ were analyzed by Northern analysis using AT_1R cDNA. B-D, The effects of angiotensin II (Ang II) on the amounts of GTP-Rho (B), PDGF-induced increases in GTP-Rac (C), and PDGF-directed cell migration (D) were determined. VSMCs were pretreated or not with 0.1 μM Ang II for 5 min, and stimulated with PDGF (0.41 nM) for 2 min (GTP-Rac assay) or for 3 min (GTP-Rho assay). The cells were then subjected to pull-down assays for GTP-Rac and GTP-Rho. Transwell migration of VSMCs toward PDGF (0.41 nM) was determined in the presence and absence of various concentrations of Ang II (0.1 μM) in the lower well of the Boyden chamber. In B, ∗ denotes $p<0.05$ compared with no stimulation. In C and D, ∗ denotes $p<0.05$ compared with PDGF alone.

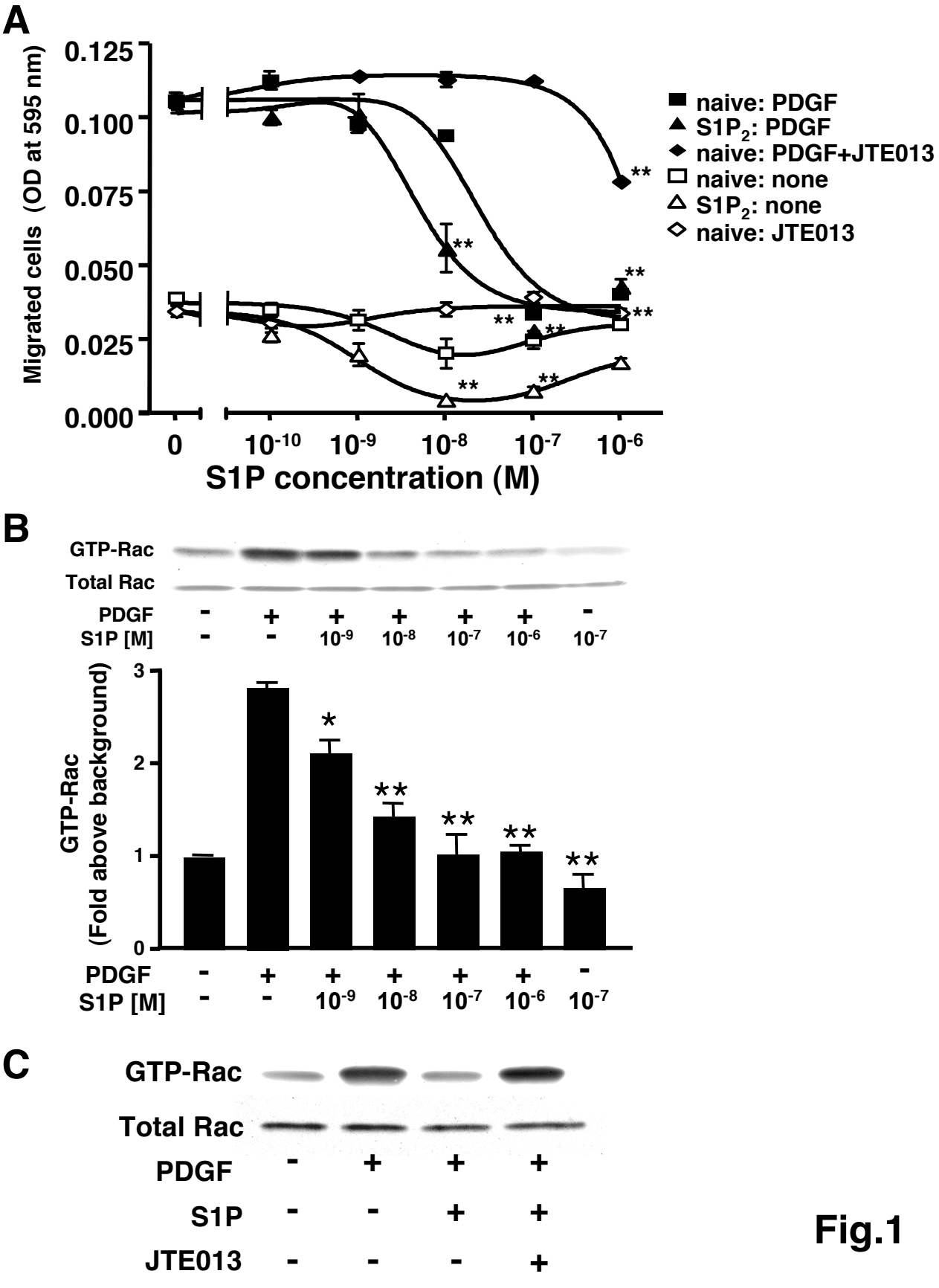

A
Migrated cells (OD at 595 nm)
0.125
0.100
0.075
0.050
0.025
0.000
0
10-10
10-9
10-8
10-7
10-6
S1P concentration (M)
naive: PDGF
S1P2: PDGF
naive: PDGF+JTE013
naive: none
S1P2: none
naive: JTE013
**
B
GTP-Rac
Total Rac
PDGF
S1P [M]
GTP-Rac (Fold above background)
C
GTP-Rac
Total Rac
PDGF
S1P
JTE013

Fig.1

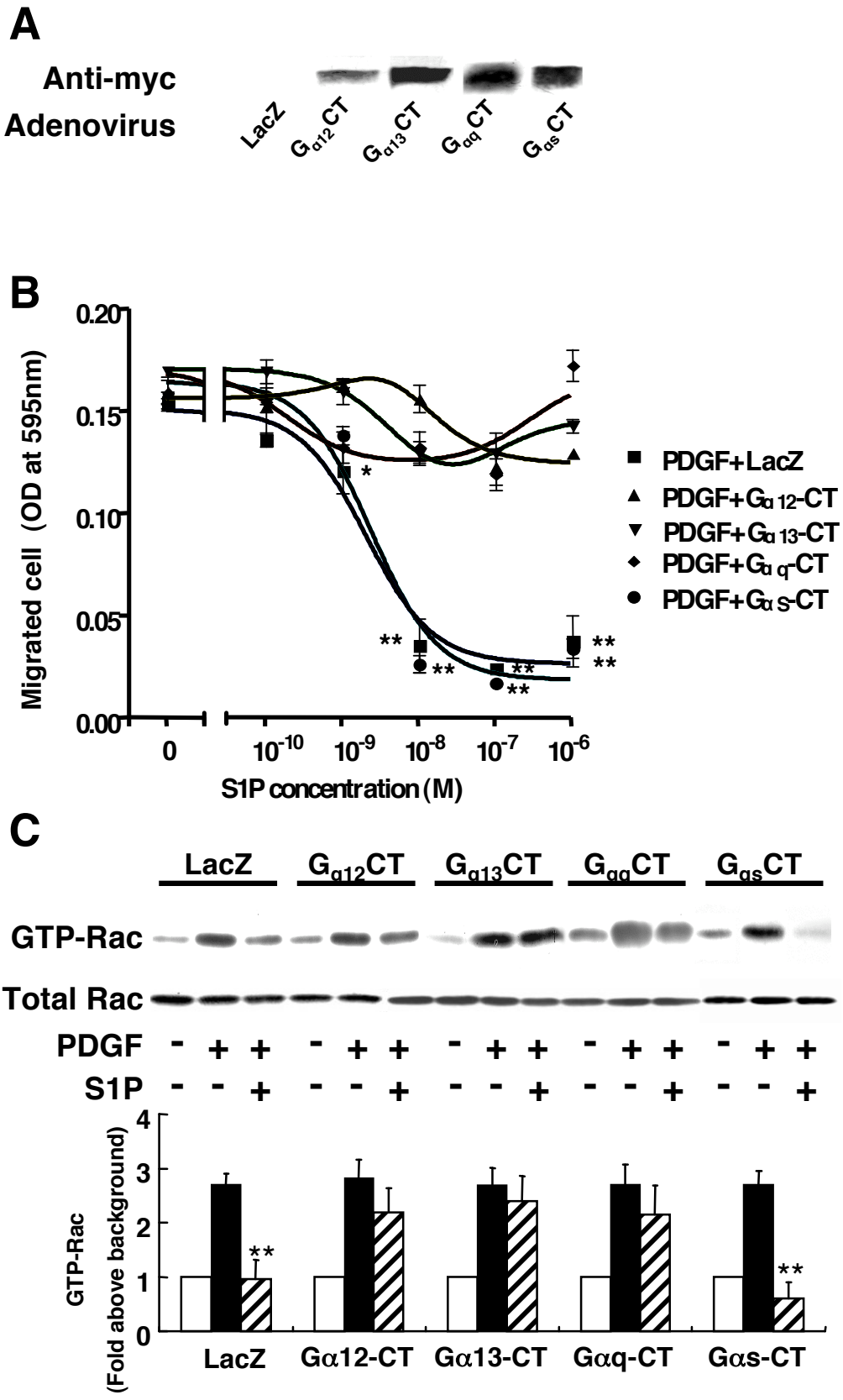

A
Anti-myc
Adenovirus
LacZ
Gα12CT
Gα13CT
GαqCT
GαsCT
B
Migrated cell (OD at 595nm)
0.20
0.15
0.10
0.05
0.00
0
10-10
10-9
10-8
10-7
10-6
S1P concentration (M)
PDGF+LacZ
PDGF+Gα12-CT
PDGF+Gα13-CT
PDGF+Gαq-CT
PDGF+GαS-CT
C
LacZ
Gα12CT
Gα13CT
GαqCT
GαsCT
GTP-Rac
Total Rac
PDGF
S1P
GTP-Rac
(Fold above background)
LacZ
Gα12-CT
Gα13-CT
Gαq-CT
Gαs-CT

Fig.2

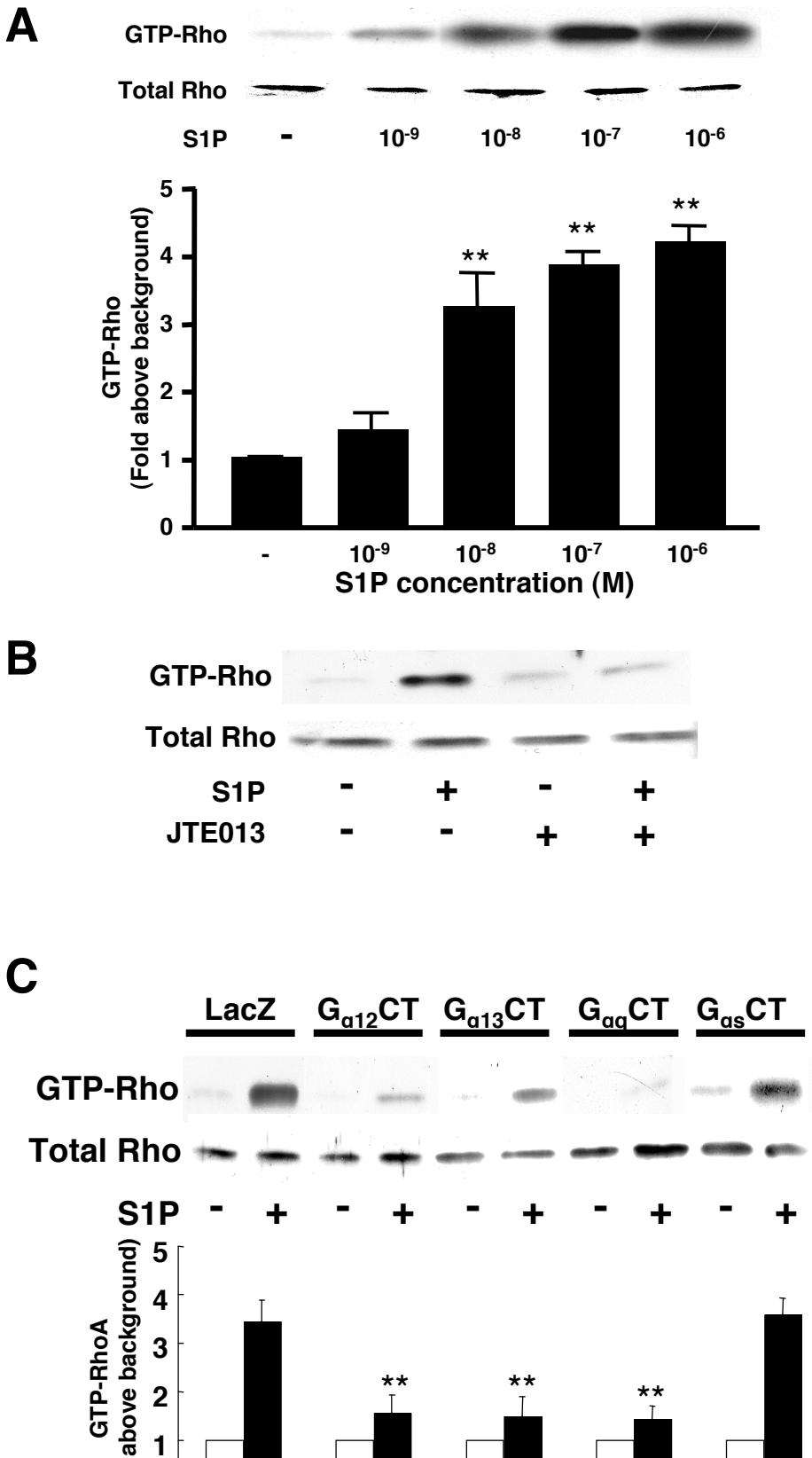

A
GTP-Rho
Total Rho
S1P
-
10-9
10-8
10-7
10-6
GTP-Rho
(Fold above background)
S1P concentration (M)
**
B
GTP-Rho
Total Rho
S1P
JTE013
C
LacZ
Gα12CT
Gα13CT
Gαq CT
Gαs CT
GTP-Rho
Total Rho
S1P
GTP-RhoA
(Fold above background)
LacZ
Gα12-CT
Gα13-CT
Gαq-CT
Gαs-CT

Fig.3

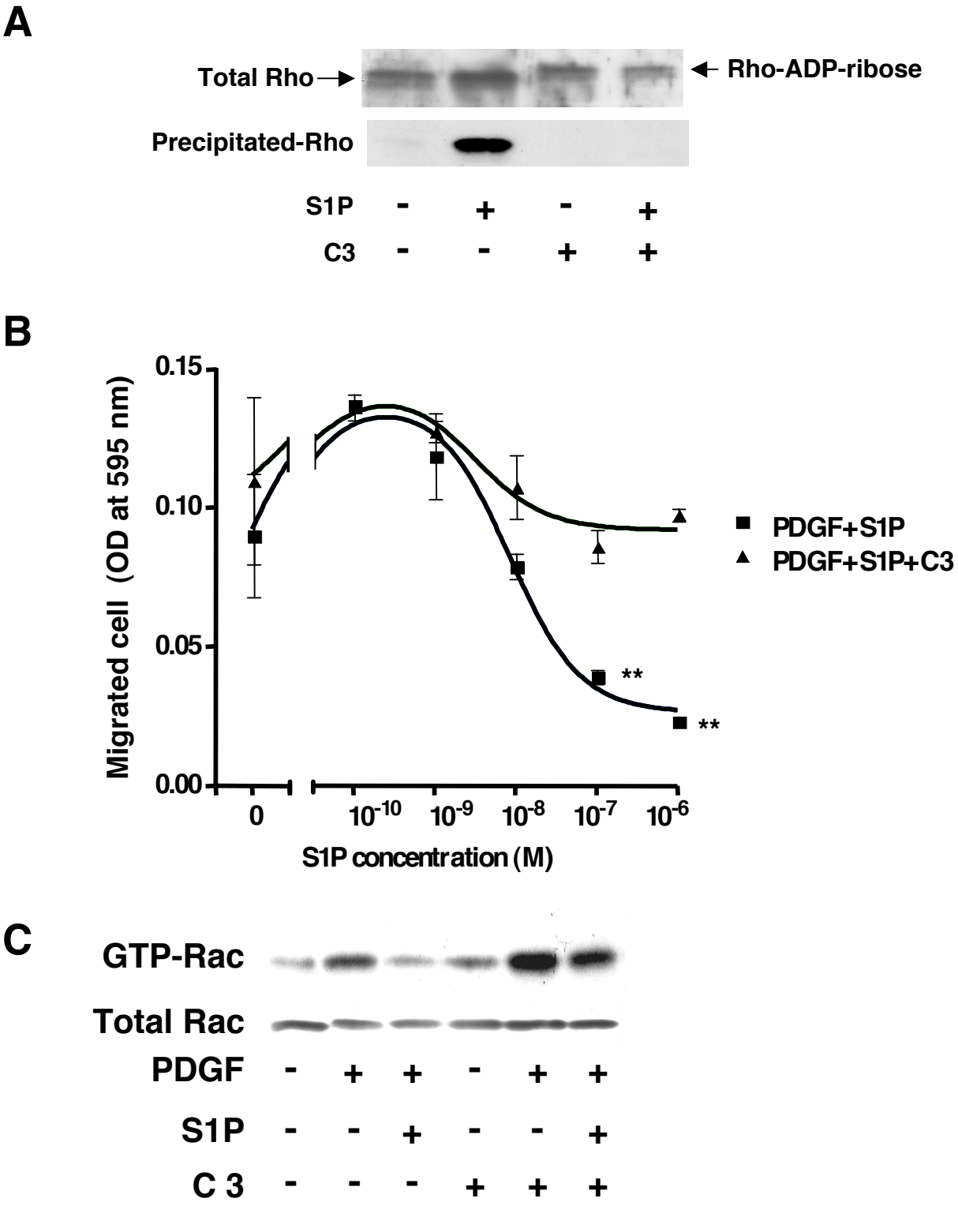

A
Total Rho
Rho-ADP-ribose
Precipitated-Rho
S1P - + - +
C3 - - + +
B
Migrated cell (OD at 595 nm)
0.15
0.10
0.05
0.00
0
10-10
10-9
10-8
10-7
10-6
S1P concentration (M)
PDGF+S1P
PDGF+S1P+C3
**
**
C
GTP-Rac
Total Rac
PDGF - + + - + +
S1P - - + - - +
C 3 - - - + + +

Fig.4

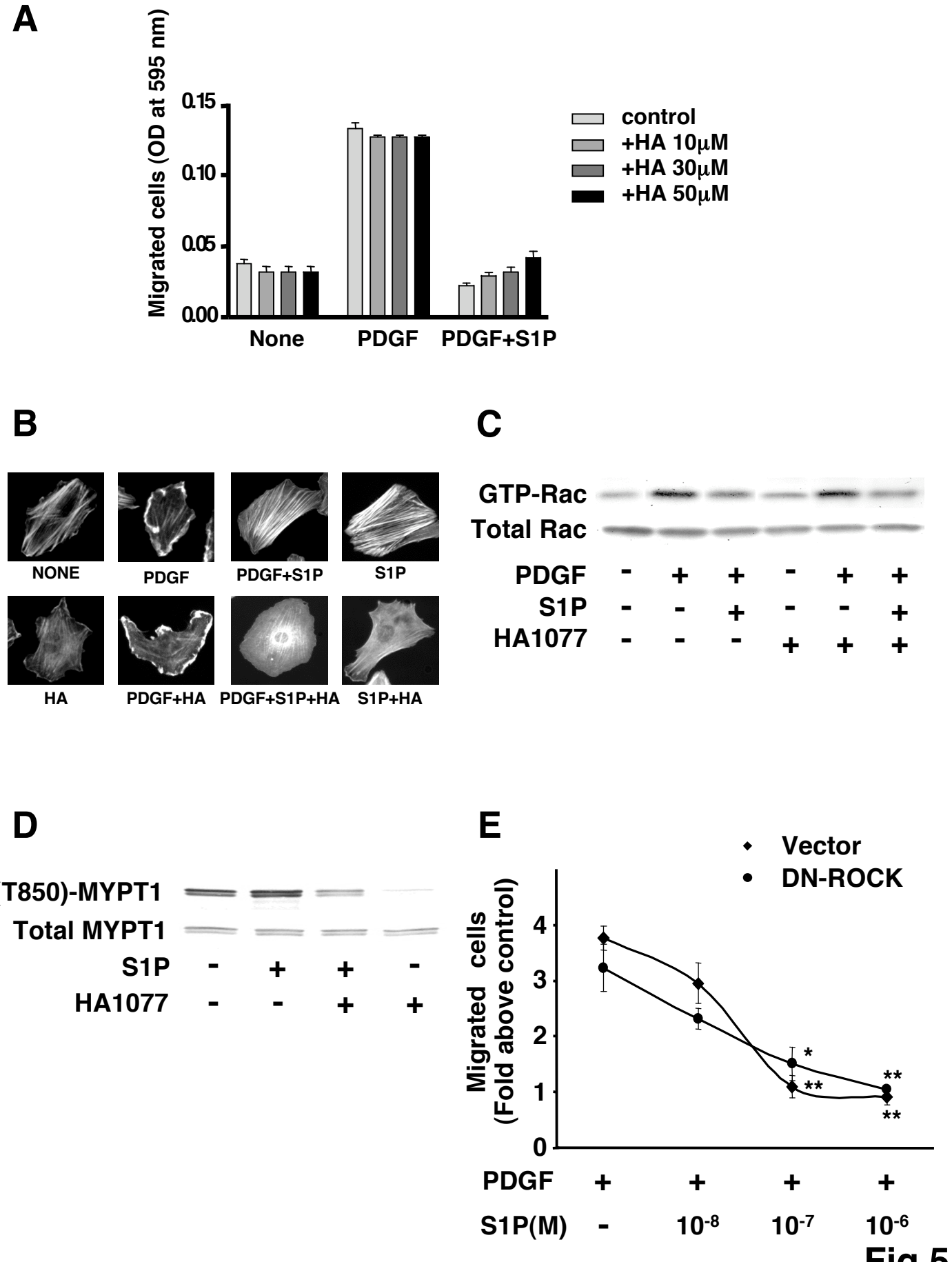

A
Migrated cells (OD at 595 nm)
0.15
0.10
0.05
0.00
None
PDGF
PDGF+S1P
control
+HA 10μM
+HA 30μM
+HA 50μM
B
NONE
PDGF
PDGF+S1P
S1P
HA
PDGF+HA
PDGF+S1P+HA
S1P+HA
C
GTP-Rac
Total Rac
PDGF - + + - + +
S1P - - + - - +
HA1077 - - - + + +
D
P(T850)-MYPT1
Total MYPT1
S1P - + + -
HA1077 - - + +
E
Vector
DN-ROCK
Migrated cells (Fold above control)
0
1
2
3
4
*
**
**
**
PDGF + + + +
S1P(M) - 10-8 10-7 10-6

Fig.5

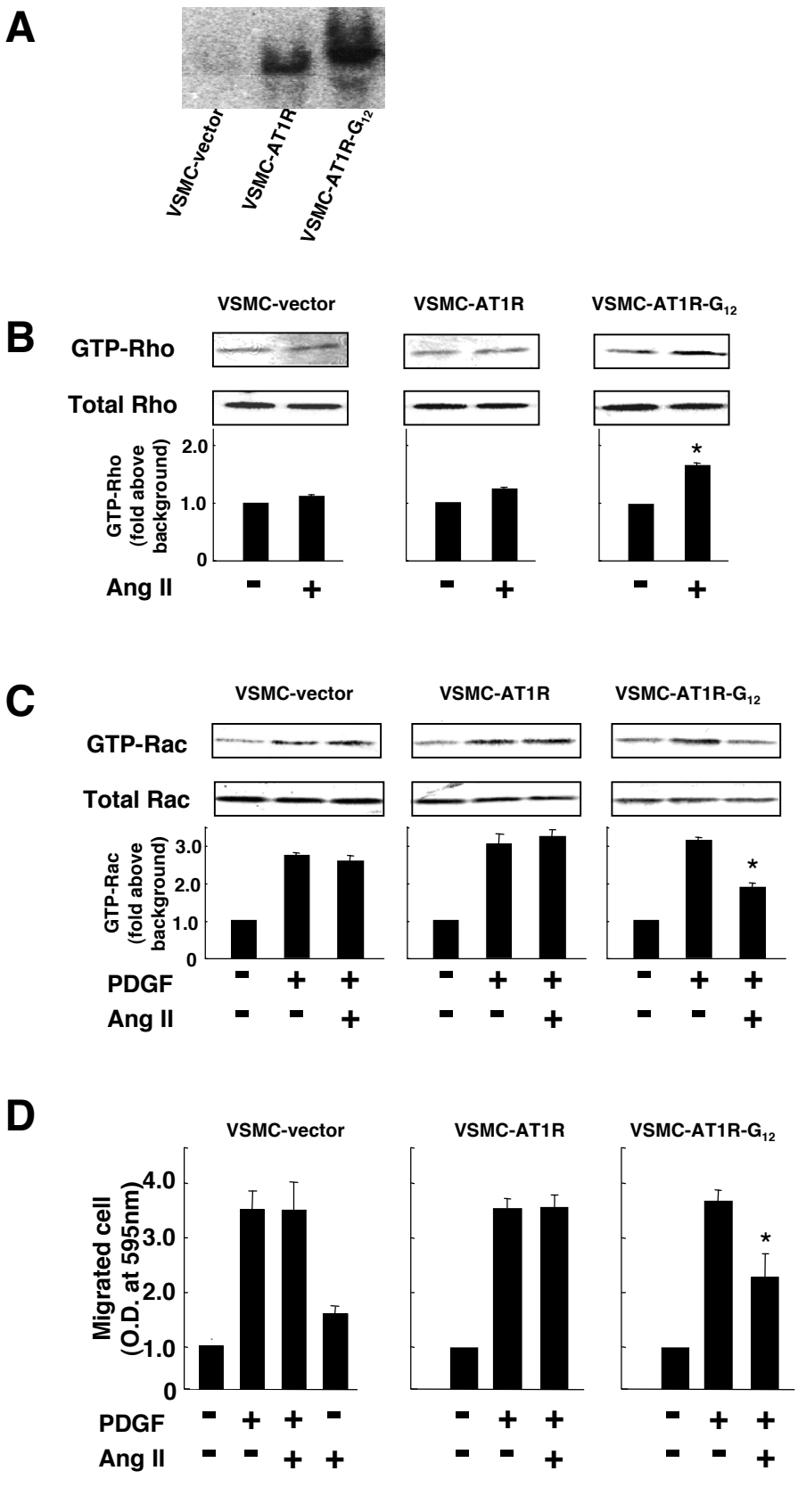
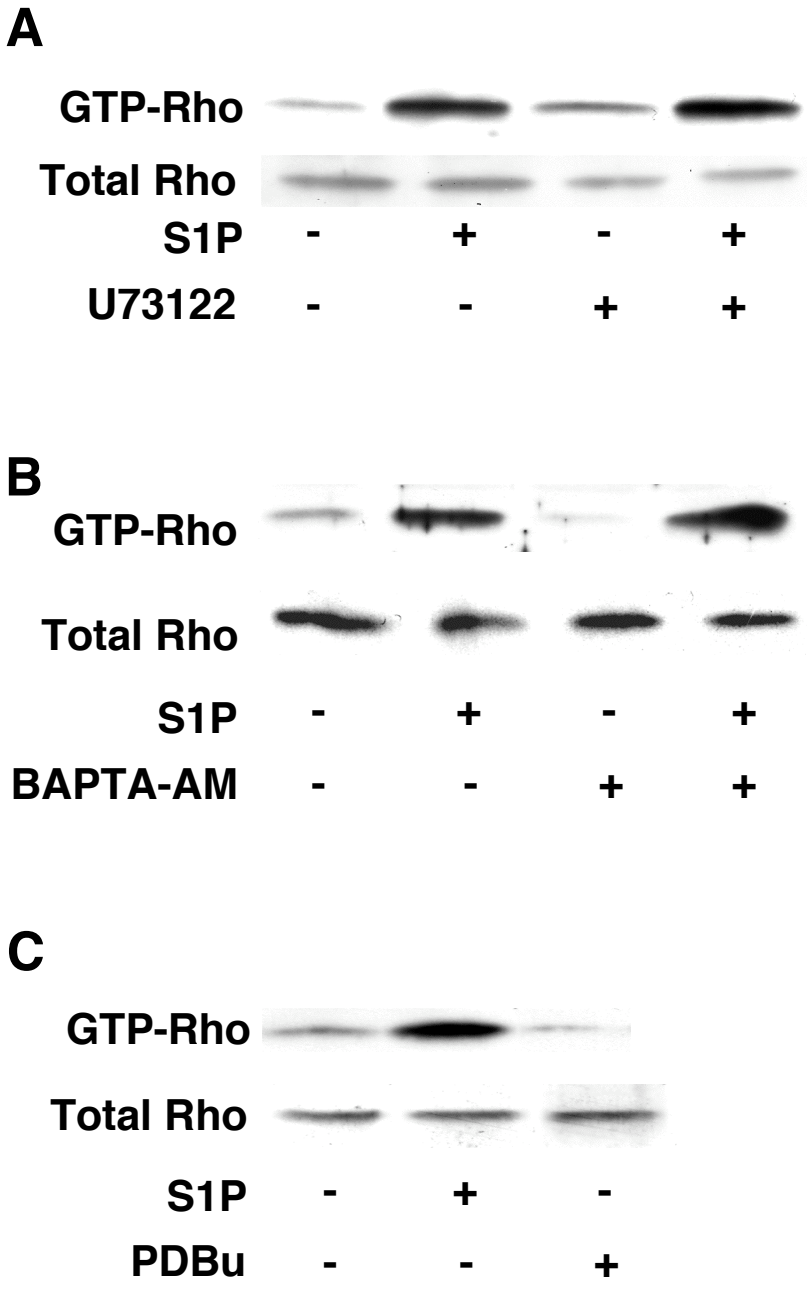

A
VSMC-vector
VSMC-AT1R
VSMC-AT1R-G12
B
VSMC-vector
VSMC-AT1R
VSMC-AT1R-G12
GTP-Rho
Total Rho
GTP-Rho (fold above background)
2.0
1.0
0
*
Ang II
- + - + - +
C
VSMC-vector
VSMC-AT1R
VSMC-AT1R-G12
GTP-Rac
Total Rac
GTP-Rac (fold above background)
3.0
2.0
1.0
0
*
PDGF
Ang II
D
VSMC-vector
VSMC-AT1R
VSMC-AT1R-G12
Migrated cell (O.D. at 595nm)
4.0
3.0
2.0
1.0
0
*
PDGF
Ang II

Fig.6

Supplementary Figure.

$S1P_2R$-mediated Rho activation is not dependent on phospholipase C stimulation, calcium mobilization or protein kinase C activation. A, Effects of the PLCβ inhibitor on S1P-induced Rho activation. VSMCs were pretreated or not with the PLCβ inhibitor U73122 (10 μM) for 10 minutes followed by stimulation with S1P (0.1 μM) for 3 minutes and then subjected to a pulldown assay for GTP-Rho. B, Effects of the Ca^{2+}-chelator BAPTA-AM on S1P-induced Rho activation. VSMC were incubated with BAPTA-AM (50 μM) for 30 minutes and stimulated with S1P (0.1 μM) for 3 minutes, followed by GTP-RhoA pulldown assay. C, Effects of a PKC activator on Rho activity. VSMCs were stimulated with PDBu (0.1 μM) for 8 min or with S1P (0.1 μM) for 3 min, followed by GTP-RhoA pulldown assay.


A
GTP-Rho
Total Rho
S1P - + - +
U73122 - - + +
B
GTP-Rho
Total Rho
S1P - + - +
BAPTA-AM - - + +
C
GTP-Rho
Total Rho
S1P - + -
PDBu - - +

Supple. Fig.