Sphingosine 1-Phosphate Receptor 2 Signals Through Leukemia-Associated RhoGEF (LARG), to Promote Smooth Muscle Cell Differentiation

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Objective—The goals of this study were to identify the signaling pathway by which sphingosine 1-phosphate (S1P) activates RhoA in smooth muscle cells (SMC) and to evaluate the contribution of this pathway to the regulation of SMC phenotype.

Methods and Results—Using a combination of receptor-specific agonists and antagonists we identified S1P receptor 2 (S1PR2) as the major S1P receptor subtype that regulates SMC differentiation marker gene expression. Based on the known coupling properties of S1PR2 and our demonstration that overexpression of Go12 or Go13 increased SMC-specific promoter activity, we next tested whether the effects of S1P in SMC were mediated by the regulator of G protein-signaling-Rho guanine exchange factors (RGS-RhoGEFs) (leukemia-associated RhoGEF [LARG], PDZ-RhoGEF [PRG], RhoGEF [p115]). Although each of the RGS-RhoGEFs enhanced actin polymerization, myocardin-related transcription factor-A nuclear localization, and SMC-specific promoter activity when overexpressed in 10T1/2 cells, LARG exhibited the most robust effect and was the only RGS-RhoGEF activated by S1P in SMC. Importantly, siRNA-mediated depletion of LARG significantly inhibited the activation of RhoA and SMC differentiation marker gene expression by S1P. Knockdown of LARG had no effect on SMC proliferation but promoted SMC migration as measured by scratch wound and transwell assays.

Conclusion—These data indicate that S1PR2-dependent activation of RhoA in SMC is mediated by LARG and that this signaling mechanism promotes the differentiated SMC phenotype. (Arterioscler Thromb Vasc Biol. 2010;30:1779-1786.)

Key Words: G proteins ■ gene expression ■ lipids ■ S1P ■ S1PR2 ■ LARG
RhoA activity is tightly regulated by GTPase-activating proteins that facilitate RhoA’s intrinsic GTPase activity (inhibiting RhoA), guanine exchange factors (GEFs) that facilitate exchange of GDP for GTP (activating RhoA), and Rho GDP-dissociation inhibitors that sequester RhoA into an inactive fraction. However, the major regulators of RhoA activity in SMC are not completely clear, and even less is known about the signaling mechanisms by which these proteins are activated. We were the first to demonstrate that the lipid agonist, sphingosine 1-phosphate (S1P), upregulates SMC-specific gene expression by activating RhoA. The goal of the current study was to identify the signaling pathway that mediates the effects of S1P on RhoA in SMC and to evaluate the contribution of this pathway to the regulation of SMC phenotype.

Materials and Methods

Plasmids and Reagents

S1P, FTY-720, SEW2871, and JTE-013 were purchased from Cayman Chemical. Leukemia-associated RhoGEF (LARG), PDZ-RhoGEF (PRG), and p115 cDNAs were kind gifts from T. Kozasa (University of Tokyo, Tokyo, Japan). Goq12 and Goq13 cDNAs were kind gifts from P. Gierschik (Ulm University, Ulm, Germany). The Goq12 cDNA was a kind gift from G. Johnson (University of North Carolina, Chapel Hill). All cDNAs were subcloned into pcDNA 3.1. Antibodies to LARG and PRG were kind gifts from Keith Burridge (University of North Carolina, Chapel Hill). The p115RhoGFP antibody was purchased from Santa Cruz Biotechnology, Inc.

Transient Transfections and Reporter Gene Assays

The 10T1/2 and SMC cell cultures, transient transfections, and promoter-luciferase assays have been previously described. Statistical comparisons between groups were made using the Student’s t test with statistical significance accepted at P<0.05.

GST G17A Pull-Downs

GST-Rho(G17A) was a kind gift from K. Burridge, and pull-downs were performed as previously described. In brief, SMCs were plated and starved for 24 hours and then treated with S1P (10 μmol/L) or 10% serum for 3.5 or 9 minutes. Protein lysates were incubated with 20 μg GST-Rho(G17A) beads at 4°C for 3 hours. Complexes were washed 3 times in lysis buffer before analysis by Western blotting.

Immunofluorescence

Phalloidin staining and measurements of MRTF localization were described previously.

Small Interfering RNA (siRNA) Knockdowns

siRNA oligos were purchased from Invitrogen, and transfections were performed using the Dharmafect transfection reagent as per protocol. RGS-RhoGEF knockdown was confirmed in all experiments by Western blot analysis. See supplemental methods for additional details.

RhoA Activity Assays

The G-LISA RhoA activation assay kit (Cytoskeleton, Inc.) was used as per protocol.

Quantitative RT-PCR

The TRIzol reagent was used to extract RNA from control and LARG K/D cells. SM22, SM α-actin, and 18S mRNAs were measured as previously described. See supplemental methods for additional details.

Migration Assays

Transwell assays were performed as previously described. For wound healing assays, confluent cultures of control and LARG knockdown SMCs were scraped with a P200 tip and then placed on an inverted microscope equipped with a heated, humidified, and O2/CO2-perfused stage. Pictures taken every 3.5 minutes for 9 hours were assembled into movies using Quicktime. At the end of each experiment, cell lysates were subjected to Western blot analysis to ensure LARG knockdown. See supplemental methods for additional details.

5-Bromodeoxyuridine Incorporation

Assays in control and LARG knockdown SMC were conducted as previously described.

Results

SIP Signals Through S1PR2 to Activate SMC Differentiation Marker Gene Expression

As shown in Figure 1, treatment of serum-starved mouse SMC with S1P strongly upregulated the expression of multiple SMC differentiation marker genes, including smooth muscle myosin heavy chain (SM MHC), SM22, SM α-actin, and calponin. These results are in excellent agreement with previous studies from our laboratory and others. S1P signals through a family of G protein-coupled receptors (S1PR1 through S1PR5). Because S1P receptor expression levels and coupling properties can vary significantly, it is often difficult to identify the receptor subtype that mediates a specific S1P-dependent response. RT-PCR analysis revealed that our aortic SMC cultures express S1PR1, S1PR2, S1PR3, and low levels of S1PR5 (data not shown). To help determine which of these receptor subtypes was responsible for activating SMC-specific transcription, we used several receptor subtype-specific agonists and antagonists. SMC differentiation marker gene expression in SMC was not enhanced by S1PR2-specific agonist, SEW2871, an S1PR1-specific agonist, or FTY720, an S1PR2 agonist that activates all 5 S1P receptors (data not shown). As shown in Figure 1D, JTE-013 dose dependently inhibited SM22 and SM MHC protein expression in SMC (Figure 1C). To determine whether these effects were mediated transcriptionally, we also tested JTE-013 on S1P-dependent SMC-specific promoter activity in multipotent 10T1/2 cells. This SMC precursor line is frequently used to study SMC-specific gene activation and our RT-PCR analyses indicated that 10T1/2 cells express all 5 S1P receptors (data not shown). As shown in Figure 1D, JTE-013 dose dependently inhibited SM22 and SM α-actin promoter activity. JTE-013 did not affect the expression of housekeeping genes or the activity of a minimal thymidine kinase promoter in these studies.

Goq12/13 and the RGS-RhoGEFs Stimulate SMC-Specific Transcription

The G protein-dependent signaling pathways activated by S1PR1, S1PR2, and S1PR3 have been fairly well characterized in a number of heterologous cell culture systems. S1PR1 couples almost exclusively to Goi and regulates
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MAPK activity and cell growth, whereas S1PR2 and S1PR3 have been shown to couple somewhat promiscuously to Gα12/13 and Goq. Because both Gα12/13 and Goq have been associated with RhoA activation,29 we tested whether overexpression of these G proteins was sufficient to stimulate SMC-specific transcription. As shown in Figure 2, overexpression of these G proteins was sufficient to stimulate transcription by 3-fold, whereas expression of Gα12 or Goq has been shown to couple somewhat promiscuously to Gα12/13-coupled receptors and RhoA activation. All 3 RGS-RhoGEFs are expressed in rat aortic SMC,30,31 and interestingly, Becknell et al.31 demonstrated that LARG expression in the lung and gastrointestinal tract of the mouse was specific for the SMC layers in those organs. Of additional importance to our studies, the RGS-RhoGEFs, unlike most other GEFs, specifically activate RhoA but not the closely related small GTPases, Rac and Cdc42. Based on these observations and our data implicating S1PR2 and Gα12/13 in the activation of SMC-specific gene expression, we hypothesized that one or more of the RGS-RhoGEFs mediated the effects of S1P on RhoA in SMC and that this family of RhoA activators was important for the regulation of SMC phenotype.

To begin to examine the role of the RGS-RhoGEFs in SMC, we tested whether ectopic expression of these proteins in 10T1/2 cells could upregulate SMC-specific promoter activity. As shown in Figure 3, overexpression of LARG strongly increased (8- to 16-fold) the activities of the SM MHC, SM α-actin, and SM22 promoters. The effects of PRG were somewhat less robust (∼4- to 7-fold), but this difference may reflect slightly lower PRG expression levels in our experiments (Figure 3, inset). Somewhat surprisingly, expression of p115 had only minor effects on SM MHC and SM22 promoter activity and no significant effect on SM α-actin promoter activity. Coexpression of C3 toxin completely inhibited the transcriptional effects of all 3 RGS-RhoGEFs, strongly supporting the involvement of RhoA in this response (data not shown). Because the effects of RhoA signaling on SMC-specific transcription are thought to be due to alterations in actin polymerization that regulate MRTF nuclear localization, we also tested the effects of the RGS-RhoGEFs on these parameters. As shown in Figure 3, overexpression of either LARG, PRG, or p115 in 10T1/2 cells enhanced actin

Figure 2. Gα12 and Gα13 increased SMC transcription. SMCs were cotransfected with the indicated promoter-luciferase construct along with Gα12(Q231L), Gα13(Q226L), Goq(Q209L), or empty expression vector (EV). Luciferase activity was measured 24 hours after transfection. *P<0.05 vs EV.

Figure 1. S1P stimulated SMC differentiation marker gene expression through S1PR2. A, Mouse aortic SMC were serum starved for 24 hours and then treated with S1P (10 μmol/L) for 24 hours. Cell lysates were separated on an SDS-PAGE gel, transferred to nitrocellulose, and probed with antibodies to the indicated SMC differentiation marker genes. MHC indicates myosin heavy chain. B, Western blot analyses were performed on SMCs that were serum starved and then treated with S1P (10 μm), SEW2871 (5 μm) (stimulates only S1PR1), or FTY-720 (10 μm) (stimulates all S1P receptors except S1PR2). S1P marker expression represents a 24-hour agonist treatment. MAPK activation control represents 12.5-minute agonist treatment. C, Western blot analyses were performed on SMC treated with S1P for 24 hours pretreatment with JTE-013. D, 10T1/2 cells transfected with the indicated promoter-luciferase construct were treated with S1P±pretreatment with the S1PR2 antagonist, JTE-013. Luciferase activity was measured 24 hours after treatment with JTE-013.

and is expressed relative to untreated cells. DMSO indicates dimethyl sulfoxide. *P<0.05 vs S1P treated in the absence of JTE-013.
polymerization (Figure 3B) and localization of GFP-MRTF-A to the nucleus (Figure 3C).

Effects of S1P Were Mediated by LARG

To identify the RGS-RhoGEFs that were activated by S1P in SMC, we used an assay described by the Burridge laboratory that employs a nucleotide-free variant of RhoA (G17A) to precipitate activated RhoGEFs from cell lysates.19 As shown in Figure 4A, treatment of SMC with S1P resulted in a dramatic increase in the amount of LARG present in GST-RhoA(G17A) precipitates, suggesting that S1P activates LARG in this model. LARG was also activated by 10% serum to a somewhat lesser extent. We observed little to no activation of PRG or p115 by S1P or serum.

We next used a siRNA approach to test whether LARG was required for S1P-mediated activation of RhoA in SMC. In parallel experiments, we also knocked-down all 3 RGS-RhoGEFs in combination to examine potential compensatory effects within this family. We consistently achieved greater than 90% knockdown of LARG when compared with SMC transfected with a control siRNA that targets GFP, and we observed no upregulation of either PRG or p115 in LARG knockdown cells (Figure 4B). As measured by the Rhotekin-based assay (Cytoskeleton, Inc.), knockdown of LARG in SMC inhibited S1P-dependent RhoA activation by approximately 65% (Figure 4C). In addition, depletion of LARG was as effective as the triple knockdown, providing further evidence that LARG is the major GEF within this family that mediates the activation of RhoA by S1P. LARG knockdown had no effect on S1P-mediated or platelet-derived growth factor-BB-mediated activation of MAPK (Figure 4D).

Importantly, knockdown of LARG in SMC significantly inhibited S1P-dependent activation of the SM22 and SM α-actin promoters (Figure 5A) and S1P-dependent activation of endogenous SMC differentiation marker gene expression as measured by quantitative RT-PCR (Figure 5B). Western blot analysis also demonstrated that LARG knockdown significantly inhibited S1P-mediated increases in SM22, SM MHC, and SM α-actin protein levels by 45%, 52%, and 27%, respectively (see Figure 5C). In good agreement with the results from the RGS-RhoGEF and RhoA activity assays, single knockdowns of PRG or p115 had no effect on S1P-dependent SMC differentiation marker gene expression (data not shown). Taken together, these results strongly indicate that S1P activates RhoA in SMC by activating LARG and that this signaling mechanism promotes SMC differentiation marker gene expression.

![Figure 3](image1.png)

Figure 3. RGS-RhoGEF overexpression increased SMC transcription, stress fiber formation, and MRTF-A localization. A, 10T1/2 cells were cotransfected with the indicated promoter-luciferase construct along with LARG, PRG, p115, or empty expression vector (EV). Luciferase activity was measured 24 hours after transfection. *P<0.05 vs EV. B, 10T1/2 cells expressing the indicated flag-tagged RGS-RhoGEF were stained with phalloidin to visualize actin polymerization. C, 10T1/2 cells were cotransfected with the indicated RGS-RhoGEF and GFP-MRTFA. Cell counts (>100 cells per condition) demonstrated that RGS-RhoGEF expression increased the percentage of cells that exhibited mainly nuclear MRTF-A localization (see lower right hand corner of micrographs in the middle column). DAPI indicates 4',6-diamidino-2-phenylindole.

![Figure 4](image2.png)

Figure 4. S1P-induced RhoA activation in SMC was mediated by LARG. A, SMCs were starved for 24 hours and then treated with S1P or 10% serum for 0, 3.5, or 9 minutes. Cell lysates were incubated with GST-RhoA(G17A) coated beads, and precipitates were analyzed for the presence of the indicated RGS-RhoGEF by Western blotting. The amount of RGS-RhoGEF protein present in cell lysates is presented at right. Min indicates minutes. B, Western blot analysis demonstrating significant knockdown of all 3 RGS-RhoGEFs using our siRNA transfection protocol. NTC indicates nontargeted control siRNA. C, RhoA activation was measured in control, LARG, and triple knockdown cells using the G-LISA assay (Cytoskeleton, Inc.) and is expressed relative to vehicle-treated cells. At least 90% RGS-RhoGEF knockdown was ensured by Western blot analysis in all experiments (data not shown). *P<0.05 vs S1P-treated NTC. D, Western blot analysis of control and LARG knockdown SMCs serum starved for 24 hours, treated for 12.5 minutes, and probed for phosphorylated and total MAPK. PDGF indicates platelet-derived growth factor.
Phenotypically modulated SMCs exhibit increased migration, and it is well known that RhoA plays an important, yet complicated, role in the regulation of this process. RhoA stimulates the formation of the stress fibers and focal adhesion complexes, that promote firm cell adhesion. However, RhoA also regulates actin-myosin-based contractility that is required for trailing edge retraction, and RhoA activity has been detected at the leading edge where it likely contributes to membrane protrusion by stimulating linear actin polymerization. The mechanisms that regulate this shifting balance between cellular adhesion and cell movement are incompletely understood, and studies have shown that RhoA can inhibit or promote cell migration depending on cell context. Given our demonstration that LARG promoted the differentiated SMC phenotype, we hypothesized that it may also inhibit SMC migration. To test this directly, we measured serum-induced migration in control and LARG knockdown SMC using a transwell assay. As shown in Figure 6, migration was significantly increased in LARG knockdown SMC cells, suggesting that LARG is a limiting factor in this assay. We also assessed the effects of LARG on SMC migration in a scratch wound assay using live cell imaging. As shown in Figure 6C (see also supplemental data), wound closure by SMC transfected with control siRNA was much slower than that in LARG knockdown SMC, which was virtually complete by 9 hours. Because wound closure can also be affected by changes in cell proliferation, we used 5-bromodeoxyuridine incorporation assays to determine cell proliferation indices in control and LARG knockdown SMC. As shown in Figure 6B, knockdown of LARG did not affect SMC proliferation, strongly suggesting that the effects of LARG observed in the scratch wound model were due to decreased SMC migration.

**Discussion**

A growing body of evidence indicates that S1P regulates vascular function by controlling the growth, migration, contraction, and cell-cell interactions of endothelial cells and SMC. The identification of the S1P-dependent signaling mechanisms involved has been complicated by the expression of multiple S1P receptors in the vessel wall and the promiscuous coupling of those receptors to G proteins that have dramatically different effects on cell function. We were the first to demonstrate that S1P increased SMC differentiation in a RhoA-dependent manner, and the current study supports previous studies implicating S1PR2 in this response. More importantly, we show that S1PR2-dependent activation of RhoA in SMC is mediated by LARG and that this RGS-RhoGEF is a key component in the control of SMC phenotype. The identification of this signaling mechanism provides significant insight into the control of SMC function and into the vascular/SMC phenotypes observed in a variety of knockout models.

The analysis of S1P receptor subtype-specific knockout mouse models has yielded important information on the role of S1P receptor signaling in the vasculature. S1PR1-deficient mice die around embryonic day 13.5 and have a defect in SMC investment of the dorsal aorta. However, because a similar phenotype was observed in endothelial cell-specific S1PR1 knockouts, this effect was likely secondary to defects in endothelial cell tube maturation and not to defects in SMC differentiation per se. Vascular abnormalities were not observed in S1PR2 deficient mice, but the earlier lethality and increased hemorrhage observed in S1PR1/ S1PR2 and S1PR2/S1PR3 double knockouts suggests that S1PR2 may have an independent role in the establishment and maintenance of a mature vasculature. Although it has been difficult to determine whether SMC differentiation during development was affected by the loss of S1PR2, Shimizu et al have directly implicated S1PR2 in the...
Interestingly, neural crest cell-specific deletion of G<sub>12/13</sub> in the differentiation of cardiac neural crest cells into SMC. expression,43,44 also resulted in defective outflow tract SMC contraction and SMC differentiation marker gene expression.45

Our demonstration that S1PR2 signals through LARG may have important implications on the control of vascular tone. Extensive evidence indicates that RhoA regulates SMC contraction by inhibiting myosin phosphatase,46 and several in vitro studies have shown that S1P constricts vessels by a RhoA-dependent mechanism.47,48 Adult S1PR2-deficient mice have relatively normal systemic blood pressure, but flow measurements showed decreased resistance in mesenteric and renal vascular beds especially in the presence of adrenergic stimulation.49 It has been postulated that the deafness observed in S1PR2-deficient mice may be due to dilation of the spiral modiolar artery that supplies blood to the inner ear.50 LARG knockout mice also have relatively normal blood pressure but were shown to be less susceptible to salt-sensitive hypertension.51 Thus, we feel that S1PR2-dependent activation of LARG could serve a critical role in blood pressure regulation and that targeting this pathway could be beneficial in the treatment of hypertension. S1P also affects vascular tone by increasing intracellular calcium, an effect most likely mediated by S1P3.52 Interestingly, recent studies have demonstrated that calcium may also be important for SMC differentiation marker gene expression and that cross-talk between calcium and RhoA may be involved.16,23 In our model, overexpression of LARG in SMC did not activate the calcium/calcineurin-dependent intereleukin 2 promoter, suggesting that LARG does not directly activate calcium signaling (see supplemental Figure 1). However, it is possible that these 2 pathways act in parallel to regulate SMC-specific transcription or that they intersect further downstream.

The residual S1P-dependent RhoA activity observed in LARG-deficient SMC could be due to incomplete knockdown of LARG expression but could also reflect the contributions of S1PR3 coupling to G<sub>q</sub>. G<sub>q</sub> has been shown to activate RhoA and SRF-dependent transcription through a separate family of RhoGEFs that includes Trio, Duet, and p63RhoGEF.52 In support of this idea, Ishii et al detected residual S1P-dependent RhoA activity in S1PR2-deficient fibroblasts but no S1P-dependent RhoA activity in fibroblasts isolated from S1PR2/S1PR3 double knockouts. The increased lethality and hemorrhage observed in the S1PR2/S1PR3 double knockout mice also supports this concept.40 Surprisingly, G<sub>q</sub> did not stimulate SMC-specific transcription in SMCs, but it is possible that these RhoGEFs are not highly expressed in these cells. Compensatory RhoA activation may also explain the lack of a significant SMC phenotype in S1PR2 and LARG knockout mice during development and why SMC phenotypes have been revealed only under conditions of vascular stress (ie, artery ligation or salt-induced hypertension).

Although all 3 RGS-RhoGEFs are expressed in SMC and are sufficient to increase actin polymerization and GFR-MRTF-A localization when overexpressed in 10T1/2 cells, we detected little to no RGS-RhoGEF activity in serum-starved SMC, and only LARG was activated by S1P. The latter result supports previous studies demonstrating that specific G<sub>12/13</sub>-coupled agonists activate specific RGS-RhoGEFs. For example, activation of RhoA by thrombin in PC-3 prostate cancer cells was primarily mediated by LARG,53 whereas activation of RhoA by lysophosphatidic acid in HEK293 cells was mediated by...
PRG.\textsuperscript{54} Although the molecular interactions within the agonist-receptor-\(\alpha_{12/13}\) complex that mediate this specificity are currently unknown, agonist-specific RGS-RhoGEF activation could provide an additional level of control over RhoA activity and could help integrate multiple RhoA-dependent signals. Further supporting differential activation of the RGS-RhoGEFs, Guilluy et al\textsuperscript{55} recently demonstrated in SMC (using the G17ARhoA pull-down assay) that angiotsin II treatment specifically activated p115. These authors also described a novel mechanism of p115 activation that was mediated by JAK2-dependent tyrosine phosphorylation. In our experiments, p115 did not activate SMC-specific promoter activity as strongly as LARG or PRG but did result in increased actin polymerization and MRTF-A nuclear localization. These data suggest that additional signals might be required to fully activate MRTF-A-dependent transcription or that the RGS-RhoGEFs might act on separate pools of RhoA that differentially affect MRTF-A activity. Interestingly, qualitative assessment of p115-expressing cells revealed a more cortical pattern of actin polymerization. p115 lacks the PDZ domain present in LARG and PRG, revealing a more cortical pattern of actin polymerization. Thus, this difference significantly affects p115 activation, function, and/or localization has not been directly tested in our model.

Our demonstration that LARG decreases SMC migration provides mechanistic insight into the control of cell migration by RhoA and helps explain the anti-migratory effects of S1PR2-dependent signaling observed in several other models.\textsuperscript{56,57} Importantly, our results are in excellent agreement with the previous demonstration that S1PR2-deficient SMCs exhibit increased migration\textsuperscript{41} and strongly suggest that this effect is due, at least in part, to decreased LARG activity. Ong et al\textsuperscript{58} have recently shown that expression of LARG in breast and colorectal cancer cells markedly inhibited migration, providing further evidence that LARG inhibits this process. The precise mechanisms for the anti-migratory effects of LARG are not completely understood but may involve increased cell adhesion. For example, Dubash et al\textsuperscript{59} have shown that siRNA depletion of LARG decreases stress fiber and focal adhesion formation. Clearly, additional studies will be necessary to identify the spatial and temporal patterns of RhoA activation that are necessary for cell migration and to determine whether LARG (and other RhoGEFs) activate RhoA within specific cellular compartments.

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**Disclosures**
None.

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