Sphingosine 1-Phosphate Stimulates Smooth Muscle Cell Differentiation and Proliferation by Activating Separate Serum Response Factor Co-factors*

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Sphingosine 1-phosphate (SIP) is a lipid agonist that regulates smooth muscle cell (SMC) and endothelial cell functions by activating several members of the SIP subfamily of G-protein-coupled Edg receptors. We have shown previously that SMC differentiation is regulated by RhoA-dependent activation of serum response factor (SRF). Because SIP is a strong activator of RhoA, we hypothesized that SIP would stimulate SMC differentiation. Treatment of primary rat aortic SMC cells with SIP activated RhoA as measured by precipitation with a glutathione S-transferase-rhotekin fusion protein. In SMC and 10T1/2 cells, SIP treatment up-regulated the activities of several transiently transfected SMC-specific promoters, and these effects were inhibited by the Rho-kinase inhibitor, Y-27632. SIP also increased smooth muscle α-actin protein levels in SMC but had no effect on SRF binding to the smooth muscle α-actin CArG B element. Quantitative reverse transcriptase-PCR showed that SIP treatment of SMC or 10T1/2 cells did not increase the mRNA level of either of the recently identified SRF co-factors, myocardin or myocardin-related transcription factor-A (MRTF-A). MRTF-A protein was expressed highly in SMC and 10T1/2 cultures, and importantly the effects of SIP were inhibited by a dominant negative form of MRTF-A indicating that SIP may regulate the transcriptional activity of MRTF-A. Indeed, SIP treatment increased the nuclear localization of FLAG-MRTF-A, and the effect of MRTF-A overexpression on smooth muscle α-actin promoter activity was inhibited by dominant negative RhoA. SIP also stimulated SMC growth by activating the early growth response gene, c-fos. This effect was not attenuated by Y-27632 but could be inhibited by the MEK inhibitor, U0126. SIP enhanced SMC growth through ERK-mediated phosphorylation of the SRF co-factor, Elk-1, as measured by gel-shifted Elk-1 activation assays. Taken together these results demonstrate that SIP activates multiple signaling pathways in SMC and regulates proliferation by ERK-dependent activation of Elk-1 and differentiation by RhoA-dependent activation of MRTF-A.

It is well established that SMC growth and differentiation are regulated by a complex array of local environmental cues including growth factors, contractile agonists, cell-cell and cell-matrix interactions, inflammatory stimuli, and mechanical stresses (see Refs. 1 and 2 for reviews). However, the cell signaling mechanisms by which these stimuli regulate the expression of the SMC differentiation marker genes such as SM α-actin, SM22, and SM myosin heavy chain are not well understood. The transcription factor, serum response factor (SRF), is clearly involved in regulating SMC-specific transcription, (1–7) but SRF is also known to regulate growth response genes such as c-fos, suggesting that signaling pathways exist that differentially regulate SRF-dependent growth and SRF-dependent differentiation (8, 9). Recent evidence indicates that the myocardin family of SRF co-activators is important for SMC differentiation and SRF-dependent gene regulation, but the signaling mechanisms that regulate their function are virtually unknown (10–14). We have shown that signaling through the small GTPase, RhoA, regulates SMC-specific transcription through SRF-dependent mechanisms, (15) and we hypothesize that signaling through RhoA may integrate some of the diverse signals that regulate SMC phenotype.

The lipid agonist, sphingosine 1-phosphate (SIP), has recently been shown to have interesting effects on vascular development and SMC growth and migration (see Refs. 18 and 19 for reviews). SIP is present in high concentrations in serum and platelets and has been shown to affect a variety of important vascular processes by binding specifically to the SIP subfamily of the G-protein-coupled Edg receptors (16). SIP receptors are found on SMC and endothelial cells (EC), and many studies have shown that SIP can stimulate growth and migration in these and other cell types (17–22). SIP has been shown to stimulate angiogenesis, enhance angiogenic responses to basic fibroblast growth factor, and differentiate EC cultures into endothelial tubes (23, 24). Interestingly, SIP induces vessel maturation in many model systems by increasing EC adhesive interactions and pericyte/SMC investment indicating that it may be important for the full development of larger vessels (25–27).

SIP-dependent signaling is fairly complex because of the expression of multiple SIP receptor isoforms (SIP1 through SIP3) that couple to several different G-proteins (see Refs. 18, 19).
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32, and 33 for reviews). SIP receptors 1, 2, and 3 have been studied in a variety of heterologous systems to identify receptor-specific actions, and it is thought that the effects of SIP on cell function are mainly determined by receptor subtype expression (28, 29). SIP1, which is highly expressed in EC, couples almost exclusively to Gi and stimulates cell growth through activation of Ras/mitogen-activated protein kinase, cell survival through Akt, and cell migration through activation of Rac1 (21, 22, 24, 30, 31). In contrast, SIP2, which is expressed highly in adult medial SMC, couples only weakly to Gi, but strongly activates the small GTPase, RhoA, by coupling to G_{11}G_{12} (18, 32, 33). Activation of RhoA in SMC leads to increased actin polymerization and cellular contractility and can promote or inhibit SMC migration depending upon the model system studied (34, 35). In HEK293 cells, overexpression of SIP1 inhibited SIP- and insulin-like growth factor I-induced migration by increasing RhoA activation and inhibiting Rac (35). SIP3 can also activate RhoA under some conditions, and both SIP1 and SIP3 have been shown to stimulate phospholipase C by activating G_{3} (31, 36, 37). Some evidence suggests that SIP may also act as an intracellular signaling molecule, but little is known about its putative role as such (38).

The direct effects of SIP on SMC differentiation have not been addressed, yet several lines of evidence suggest that SIP signaling may be important for regulating this process. First, the expression pattern and G-protein-coupling properties of SIP1 and SIP2 correlate very well with the growth and migration properties of different SMC phenotypes. For example, although adult medial SMC express little if any SIP1, this growth-coupled receptor is expressed highly in medial SMC during development and in intimal SMC (18). In fact, overexpression of SIP1 in adult medial SMC can restore growth responsiveness of SMC to SIP (18). In contrast, SIP2 is highly expressed in medial SMC, and stimulation of this receptor inhibits SMC migration and stimulates SMC contraction (18, 39, 40). Second, SIP1 targeted mice die of hemorrhage around embryonic day 13.5 because of lack of vessel maturation with significant impairment of SMC investment of vessels (25). This SMC phenotype may be secondary to effects on EC (41), but SIP1 deletion may also have cell autonomous effects on SMC during development. Finally, SIP1 is a strong activator of RhoA in cells expressing SIP2 (33, 36). Because we have previously shown that SMC differentiation marker gene expression is positively regulated by RhoA-dependent activation of SRF (15), and medial SMC express high levels of SIP2, we hypothesized that SIP stimulation should enhance the differentiated SMC phenotype.

The goals of the current study were to determine the extent to which SIP regulates SMC differentiation and to identify the signaling mechanisms by which SIP stimulates changes in gene expression in SMC. Results demonstrate that SIP increases the expression of multiple SMC differentiation marker genes by RhoA-mediated activation of SRF. Interestingly, these effects may be mediated by the newly described SRF co-factor, myocardin-related transcription factor A (MRTF-A). SIP also moderately stimulated SMC proliferation, a process that was dependent upon ERK and involved activation of another SRF co-factor, Elk-1.

MATERIALS AND METHODS

Cell Culture, Transient Transfections, and Reporter Assays—SMCs from rat thoracic aorta were isolated, cultured, and transfected as described previously (42, 43). In short, cells were maintained in 24-well plates in 10% serum and were transfected 24 h after plating at 70–80% confluency using the transfection reagent Superfect (Qiagen) as per protocol. The SM22 promoter (from −450 to +88), the SM α-actin promoter (from −2560 to +2784), the SM myosin heavy chain promoter (from −4,200 to +11,600) and the c-fos promoter (from −356 to +109) used in this study have been described previously (1, 4, 5, 44). The CARG mutant SM α-actin promoter has been described previously (45) and was a generous gift from Gary Owens (University of Virginia). For co-transfection experiments 100–250 ng of N19RhoA, MRTF-A, dominant negative MRTF-A, or empty pcDNA vector was included with promoter/reporter constructs. Prior to agonist treatment SMC were placed in completely serum-free media for 48 h. 10T1/2 cells were plated in 0.2% charcoal-treated serum (to remove active serum lipids). Cells were transfected with sphingosine 1-phosphate for 24 h, and luciferase assays were performed using the Steady-Glo system (Promega). Sphingosine 1-phosphate used in these experiments was obtained from Matreya and was maintained in 4 mg/mL fatty acid-free bovine serum albumin, which was used as a vehicle control. In some experiments cells were pretreated with latrunculin B (0.5 μg/mL) for 10 min prior to the addition of SIP. Relative promoter activities are expressed as the means ± S.E. computed from a set of at least three separate transfection experiments. We did not cotransfect a viral promoter/Lac Z construct as a control for transfection efficiency because we have previously shown that such constructs exhibit unknown and variable squelching effects on the SM-specific promoters, presumably because of competition for common transcription factors (46). Moreover, we have found that inclusion of such controls are unnecessary in that variations in transfection efficiency between independent experimental samples is routinely very small (<10%) (46).

RhoA and ERK Activity Assays—The pull-down assay used to measure RhoA activity has been previously described (47). In short, cell lysates were rotated with 40 μg of a GST-rhotekin Rho binding domain fusion protein immobilized to glutathione-Sepharose 4B beads (Amersham Biosciences) for 45 min at 4 °C in binding buffer (50 mM Tris, pH 7.6, 500 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% Triton X-100, 0.5 mM MgCl2). Beads were washed three times (50 mM Tris, pH 7.6, 150 mM NaCl, 1% Triton X-100, 0.5 mM MgCl2) and resuspended in 2× Laemmli buffer. Proteins were separated on a 15% SDS-PAGE and transferred to 0.2 μm polyvinylidene difluoride (Bio-Rad). After blocking in 3% bovine serum albumin/TBST (20 mM Tris-HCl, 500 mM NaCl, 0.05% Tween-20, pH 7.4) for 2 h at room temperature, blots were probed with 2 μg/mL rhotekin antibody (26C4) (Santa Cruz Biotechnology) overnight at 4 °C. Loading controls (typically 10%) are taken from each lysate sample prior to pull downs. For ERK assays, equal amounts of S1P-treated and control cell lysates were run on a 10% SDS-PAGE and transferred to nitrocellulose. ERK activity was then measured using an activation-specific antibody (Cell Signaling). In some experiments the MEK inhibitor U0126 was added 10 min prior to SIP addition.

MRTFA Western Blots and Constructs—Cell lysates were prepared from rat aortic SMC, 10T1/2 cells, NIH3T3 fibroblasts, and A7r5 rat fibroblasts. Seventy-five μg of protein was separated on a 10% SDS-PAGE, transferred to nitrocellulose, and probed for MRTFA with a polyclonal antibody was a generous gift of Richard T. biomean (Cancer Research UK, London, UK). The WT and ΔN MRTFA constructs were a generous gift of Da-Zhi Wang (University of North Carolina, Chapel Hill, NC). The dominant negative MRTFA mutation (lacking the trans-activation domain) was generated by sub-cloning amino acids 1–631 into pcDNA3.1.

Quantitative PCR—RNA was prepared using the RNeasy kit with RNase-free DNase (Qiagen) per the manufacturer’s instructions, and RNA was quantified by Ribogreen Assay (Molecular Probes). One μg of RNA was used in a QPCR reaction containing 5 units of Reverse-iT RTase blend (Abgene) and 2× Absolute QPCR mix (Abgene). For rat myocardin amplification and detection, 0.05 μg of forward (5′-CGGATTCCTTCACTTGTGAGAGGC-3′) and 0.05 μg of reverse (5′-GTCTCATGTCCATGCGTACA-3′) primers and 0.7 μM myocardin Taqman probe (FAM-CCGATCTTTGTCCTGTACCA-TAMRA) were used in each reaction. MRTFA was amplified and detected using 0.05 μg of forward (5′-CAACAGCCTCCTGTCGGCAG-3′) and 0.05 μg of reverse (5′-GTTGGGAACTGGAAGCTGAGT-3′) primers with 1.0 μM MRTFA Taqman probe (FAM-CTGGCCCAGATGGGACCAC-TAMRA). GAPDH was amplified and detected as a reference gene using 0.025 μg of forward (5′-ATGGGTGTTGAAACCGAGA-3′) and 0.025 μg of reverse (5′-GGCTATGCTGGTCGATGTC-3′) primers with 1 μM GAPDH Taqman probe (TET-TGATCCTTGCAAC-CACACTCGTAG-TAMRA). Each sample was done in triplicate; reactions were analyzed using an ABI Prism 7700 sequence detector. Cycling parameters were as follows: one cycle at 48 °C for 30 min, one cycle at 95 °C for 10 min and 40 cycles (95 °C for 15 s, 60 °C for 1 min). All expression data were normalized to expression of GAPDH.

Electrophoretic Mobility Shift and Elk-1 Activation Assays—Whole
cell extracts were prepared by scraping SMC in lysis buffer (20 mM HEPES, pH 7.9, 5 mM EDTA, 5 mM NaF, 0.1 μg/ml okadaic acid, 10% glycerol, 0.4 mM KCl, 0.4% Triton X-100, 1 mM dithiothreitol, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM benzamidine). Lysates were left on ice for 5–10 min, and cell debris was pelleted and discarded. Supernatants were then frozen in liquid N₂ and stored at −80 °C for later use. Binding reactions were set up by combining 25 μg of lysate, a 32P-labeled oligonucleotide probe (20,000 cpm), and 0.25 μg dI-dC in binding buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 100 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol).

Reactions were incubated for 30 min before loading on non-denaturing 5% polyacrylamide gel that was pre-run at 170V for 1 h. Electrophoresis was performed at 170V in 0.5× TBE (45 mM Tris borate, 1 mM EDTA). Gels were dried and exposed to film for 24–72 h at −80 °C. For supershift studies, 1 μl of SRF or Elk-1 antibody (both from Santa Cruz Biotechnology) was added after 20 min of incubation. For measuring Elk-1 activation, S1P-treated and control lysates were run on a 10% SDS-PAGE and transferred to nitrocellulose. Phosphorylation of Elk-1 at Ser-383 was measured by probing with a phosphospecific antibody (Cell Signaling).
RESULTS

Medial SMC are known to express high levels of S1P2, a Gα13-coupled receptor that signals through RhoA (18). Because we had shown previously that activation of RhoA stimulates the expression of the SMC differentiation marker genes (15), we hypothesized that S1P would increase SMC differentiation marker gene expression. Results shown in Fig. 1 demonstrate that S1P increased the expression of several SMC differentiation marker genes in SMC by activating RhoA. S1P stimulated a transiently transfected SM22-luciferase promoter by more than 3-fold in primary SMC, and this effect was blocked by treatment with the Rho-kinase inhibitor, Y-27632 but not by the MEK inhibitor UO126 (Fig. 1A). The multipotent mouse embryonic 10T1/2 cell line has also been used to study SMC-specific transcription because several SMC-specific markers can be up-regulated by stimulation with TGF-β (48). S1P stimulated the SM22 promoter in 10T1/2 cells by greater than 4-fold. Fig. 1B demonstrates that S1P also significantly up-regulated the SM α-actin and SM myosin heavy chain promoters to a similar extent in both SMC and 10T1/2. Importantly, the SM22 promoter, SM α-actin promoter, and SM myosin heavy chain promoters used in these studies were shown to direct SMC-specific regulation of these genes in transgenic models (1, 3, 49) suggesting that S1P may be an important regulator of SMC phenotype in vivo. S1P also stimulated the expression of endogenous SM α-actin as determined by Western blot analysis (Fig. 1C). To determine whether the effects of S1P were mediated by an SRF dependent mechanism, we transfected a SM α-actin promoter construct that contained mutations to the CARG cis-element that mediate SRF binding. As shown in Fig. 1D, the CARG mutant construct was not activated by S1P. To measure the effects of S1P on the activation of RhoA in SMC, we used a GST-rhotekin fusion protein to precipitate GTPT-bound RhoA out of SMC lysates following treatment with S1P (Fig. 1E). RhoA activation peaked between 2.5 and 5 min following S1P treatment and was sustained at substantial levels at 15 min. Taken together, these results indicate that S1P up-regulates SMC differentiation marker gene expression through RhoA/Rho-kinase-dependent activation of SRF.

Several studies have shown that S1P can stimulate SMC proliferation, but questions remain as to the specific S1P receptors involved and the precise signaling pathways that mediate this response. Data presented in Fig. 2A indicate that S1P moderately stimulated SMC proliferation in a dose-dependent fashion and that this effect was completely inhibited by the MEK inhibitor, U0126. Fig. 2B demonstrates that S1P activated ERK in a dose- and time-dependent manner and that S1P-induced ERK activation was completely inhibited by U0126. Fig. 2B also demonstrates that S1P is a relatively strong activator of ERK in that concentrations of S1P as low as 100 nM activated ERK as efficaciously as 20 ng/ml platelet-derived growth factor-β. Importantly, SMC proliferation was only slightly inhibited by Y-27632 indicating that if Rho-kinase signaling is involved in the S1P-induced growth response it plays only a minor role. S1P also stimulated the expression of a transiently transfected c-fos promoter by 2.5-fold, an effect not attenuated by Y-27632 (Fig. 2C). These data further substantiate that RhoA signaling (at least through Rho-kinase) does not regulate S1P-induced cell proliferation in our cell culture model.

The results presented so far indicate that S1P regulates SMC differentiation through activation of RhoA and SMC growth by activation of ERK signaling. Studies have shown that one potential mechanism for agonist-induced effects on SMC-specific transcription is increased SRF binding to the SMC-specific promoters (50). To test this, we performed gel shift analyses with an SRF-binding cis-element (CARG B) that has been shown to be required for expression of SM α-actin (1). To test whether S1P affected SRF binding in a gene-specific manner, we also included a shift probe for the c-fos SRE in these experiments. Fig. 3A demonstrates that S1P treatment did not increase SRF binding to the CARG B element. S1P also had no effect on SRF binding to the c-fos SRE CARG element (Fig. 3B, lower arrow) but did increase the presence of a higher order complex by 2 h (Fig. 3B, small arrow). The higher order complex was supershifted with an antibody to Elk-1 (Fig. 3B, seventh lane) indicating that S1P treatment of SMC results in

FIG. 2. S1P stimulated SMC proliferation by activating ERK. A, SMC were serum-starved for 48 h and then treated with S1P for 24 h. In some experiments, Y-27632 or U-0126 were added just prior to S1P treatment. Cell proliferation was measured indirectly by adding WST-1 tetrazolium salt (Roche Applied Science) per the manufacturer's instructions. B, SMC were treated with the indicated concentration of S1P or 20 ng/ml platelet-derived growth factor-β. At 10 and 60 min following treatment, cells were lysed and equal amounts of protein were separated on SDS-PAGE. Blots were probed with a phospho-specific antibody to ERK 1 and 2. The effects of U0126 on ERK activation are shown in the bottom panel. C, SMC and 10T1/2 were transfected with 0.75 μg of c-fos promoter luciferase reporter construct, serum-starved for 48 h, and then treated with S1P or Y-27632. Promoter activities are expressed as -fold over untreated set to 1.
Elk-1 activation and ternary complex formation at the c-fos SRE. S1P had no effect on SRF protein levels at any time point based upon Western blot analysis (data not shown). To further demonstrate that S1P induces Elk-1 activation, we used a phospho-specific antibody to Elk-1 Ser-383 to probe control and S1P-treated lysates. Fig. 3C demonstrates that S1P significantly up-regulated Elk-1 phosphorylation by 2 h, a time course of phosphorylation that closely paralleled the increase in higher order complex formation observed in gel shifts. These results indicate that S1P stimulates SMC growth by activating early response gene expression through ERK-dependent activation of the SRF cofactor, Elk-1.

Our gel shift and SRF Western blot data indicated that mechanisms unrelated to changes in SRF expression and DNA binding are important for S1P-induced stimulation of SMC-specific gene expression. It has recently been shown that the myocardin family of SRF co-factors can dramatically up-regulate the expression of the SMC-specific genes and that myocardin-targeted mice fail to develop SMC (45, 51, 52). Myocardin is expressed in rat aortic SMC, and reverse transcriptase-PCR confirmed low but detectable levels of myocardin message in our primary SMC cultures. Reverse transcriptase-PCR also showed that SMC and 10T1/2 cells express significant levels MRTF-A message, and Western analysis showed that SMC, 10T1/2, and A7r5 cells express substantial amounts of MRTF-A protein (Fig. 4A). Therefore, S1P-induced up-regulation of either myocardin or MRTF-A could explain the effects observed in these experiments. To test this, we used quantitative reverse transcriptase-PCR to measure the effects of S1P on myocardin and MRTF-A mRNA levels. As shown in figure 4B, S1P slightly decreased myocardin mRNA levels and had no effect on MRTF-A indicating that other mechanisms must be responsible for the effects of S1P on SMC-specific transcription.

A recent study in NIH3T3 fibroblasts by Miralles et al. demonstrated that MRTF-A activity was regulated by RhoA signaling (12). This was a very important finding because it was the first evidence to suggest that transactivation of SRF-dependent genes by a myocardin family member could be achieved through changes in cell signaling and not by increased transcription factor expression. These authors demonstrated that activation of RhoA caused MRTF-A to translocate from the cytoplasm to the nucleus where it was then able to regulate a variety of SRF-dependent structural genes.

To more directly test whether the effects of S1P on SMC differentiation marker gene expression were dependent upon MRTF-A, we co-transfected 10T1/2 cells with a C-terminal deletion of MRTF-A that acts as a dominant negative. Previous studies have shown that C-terminal deletions of the transactivation domain of MRTF-A act as dominant negative mutations (13). Results show that dominant negative MRTF-A attenuated S1P-induced up-regulation of the SM22 promoter by nearly 80% (Fig. 5A). To provide additional support that the effects of MRTF-A were mediated by RhoA signaling we co-transfected cells with MRTF-A and a dominant negative N19RhoA. Fig. 5B demonstrates that overexpression of MRTF-A resulted in a ~200-fold increase in SM22 promoter activity and that expression of N19RhoA inhibited transactivation by MRTF-A in a dose-dependent manner. To directly assess the effects of S1P on MRTF-A localization, we expressed FLAG-tagged MRTF-A in SMC, serum-starved the cells for 48 h, treated the cells with S1P for 1 h, and then immunostained the cells using an anti-FLAG antibody. Over 50 control and S1P-treated MRTF-A-expressing cells were counted and scored in three separate localization categories: nuclear, cytoplasmic, and diffuse. Representative micrographs of each of these categories are shown in Fig. 5C. In serum-starved cells MRTF-A localized to the nucleus in 44% of the cells, to the cytoplasm in 37%, and as a diffuse pattern in 19% (Fig. 5D). After 1 h of S1P treatment the percentage of cells with nuclear staining increased to 88%, whereas the fraction that showed cytoplasmic or diffuse staining decreased to 7 and 5%, respectively. Taken together these data indicate that the effect of S1P on SMC-specific transcription may be mediated by RhoA-dependent nuclear translocation of MRTF-A.
SIP Stimulates SMC Differentiation

**DISCUSSION**

A major goal of our laboratory has been to identify the signaling mechanisms that control the transcription of the SMC differentiation marker genes. The focus of the present study was to determine whether SIP regulates SMC differentiation and to identify the signaling mechanisms by which SIP stimulates changes in gene expression in SMC. Because we had previously shown that RhoA is an important regulator of SMC-specific transcription, and SIP has been shown to stimulate RhoA activation through SIP-coupled activation of Go13, we hypothesized that SIP would up-regulate SMC differentiation marker gene expression. The data presented indicate that: 1) SIP stimulated expression of multiple SMC differentiation markers in primary SMC cultures and in 10T1/2 cells in a RhoA-dependent fashion. 2) SIP stimulated SMC proliferation and c-fos expression. This effect was not dependent upon RhoA but was dependent upon ERK activation of Elk1 and the formation of the ternary complex at the c-fos SRE. 3) The effects of SIP on SMC-specific transcription were mediated by RhoA-dependent activation of the SRF co-factor, MRTF-A.

SIP signaling in the vasculature is certainly very complex. Five separate SIP receptors have been identified that not only differ in their patterns of expression but also in their coupling to very different G-proteins. A number of studies have shown that SIP regulates cell proliferation and migration, and the current studies support a role for the involvement of SIP in regulating SMC growth by stimulating ERK-dependent early response gene expression. Importantly, we demonstrate for the first time that SIP also stimulates SMC differentiation by up-regulating the transcription of several SMC differentiation marker genes. This effect did not require ERK activity but was mediated by activation of RhoA. These data suggest that the effects of SIP on SMC-specific transcription are mediated by the SIP2 receptor that is expressed highly in differentiated SMC and is coupled to Go13, a well known activator of RhoA signaling. The observation that SIP stimulated cell proliferation through activation of ERK but not RhoA suggests that the growth and differentiation pathways stimulated by SIP diverge. The observations that SIP2 couples very weakly to Go13, and that SIP1 has been detected in adult SMC at very low levels may explain the effects of SIP on ERK-dependent SMC growth (18, 32). Alternatively, SIP has been shown to transactivate several receptor tyrosine kinases such as Flk-1 and platelet-derived growth factor-ββ, and a similar mechanism could also be involved in these studies (48, 53).

The observation that SIP stimulates SMC growth and differentiation provides evidence that these processes are not necessarily mutually exclusive. RhoA and SRF are clearly involved in regulating both of these important SMC functions, and it will be critical to further clarify the specific signals that differentiate these pathways. In our hands RhoA is more important for signaling SMC differentiation, whereas growth is mainly regulated through ERK. However, RhoA activation has also been shown to be required for growth factor-induced expression of c-fos, and recent evidence suggests that RhoA activation may be involved in the growth response of SMC to AI, thrombin, and mechanical stretch (54–56). Taken together, these results suggest that although basal RhoA activity is required for SMC growth (perhaps through basal activation of SRF), additional signaling pathways are probably more important for regulating this process. In support of this idea, expression of constitutively active RhoA only potentiated SMC proliferation in the presence of activated Ras, (56) and additional
signaling through the stress-activated protein kinase/c-Jun NH2-terminal kinase pathway was required for Rho-mediated regulation of c-fos (57). The current findings are also very interesting in regard to the observed vascular phenotypes of several targeted mouse models in which genes in the S1P signaling pathway have been deleted. Deletion of S1P1 in the mouse resulted in hemorrhagic death at around embryonic day 13 because of incomplete investment of vessels with SMC and/or pericytes suggesting that S1P is important for this process (25). Although SMC phenotype was not assessed rigorously in this model, at least some SMα-actin/FLAG cells were associated with maturing vessels in S1P1−/− mice indicating that the S1P1 may regulate the migration and proliferation of SMC as opposed to initial SMC determination. Although some SMα-actin positive cells were associated with maturing vessels in S1P1−/− mice indicating that the S1P1 may regulate the migration and proliferation of SMC as opposed to initial SMC determination. Although it is impossible to completely rule out a cell autonomous effect of S1P1 deletion on SMC differentiation, we believe that the current studies indicate that it is unlikely that signaling through S1P1 is essential for this process. Interestingly, recent studies have shown that EC-specific deletion of S1P4 resulted in a similar SMC phenotype to that observed in S1P1-null mice suggesting that the effects of S1P1 deletion on SMC investment are mediated by EC-SMC interactions (41).

Our data indicate that S1P activates the SMC differentiation marker genes through activation of RhoA through S1P2 (or perhaps S1P3), but it is currently unclear whether this signaling pathway is required for differentiation of SMC in vivo. The S1P2 and S1P3 receptors have been deleted individually in the mouse resulting in fairly normal phenotypes (although seizures were reported by one group) (36, 58, 59). Importantly, neither of these deletions by themselves completely inhibited S1P-induced RhoA activity indicating that both of these receptors probably couple to Gq11 to activate RhoA (36). S1P2/S1P3 double knock-out mice did show perinatal lethality, and importantly embryonic fibroblasts isolated from these lines were not able to activate RhoA in response to S1P (36). The precise cause of death in the double knock-out mice is unclear, so it will be important to analyze these mice for SMC phenotypes. It is currently unknown whether signaling through S1P4 or S1P5 can compensate for loss of other receptors in this family and the
Interestingly, deletion of Gsm/H9251 relative to non S1P-treated cells. Ciferase activity was measured after 24 h, and all values are expressed in micromolar. Latrunculin B (LB), and then treated with S1P. LB cells were serum-starved, pre-treated for 10 min with the actin depolymerizing agent, latrunculin B (LB), and then treated with S1P. Luciferase activity was measured after 24 h, and all values are expressed relative to non S1P-treated cells. B, 10T1/2 cells were co-transfected with SM α-actin/luciferase and either full-length MRTF-A or an MRTF-A variant that lacked the N-terminal actin binding domain. Cells were treated with LB and assayed for luciferase after 12 h.

The observations that S1P had no effect on MRTF-A expression levels and that the effects of S1P on promoter activity were maximal by 3 h indicate that signal-induced changes in the transcriptional activity of MRTF-A may be involved. A recent study in NIH 3T3 cells has demonstrated that MRTF-A activity is regulated by RhoA-dependent changes in nuclear translocation (12). Our studies in SMC and 10T1/2 cells indicate that the mechanisms that regulate MRTF-A nuclear localization may be dependent upon cell type because a substantial number of serum-starved cells contained MRTF-A in the nucleus (44%). Nevertheless, S1P treatment caused a substantial rise in the percentage of cells showing nuclear localization (to 88%), a result that may explain the effects of S1P on SMC differentiation marker gene expression. Our data also suggest that S1P regulates MRTF-A localization through its effects on RhoA-dependent actin polymerization and support the idea that changes in actin dynamics are ultimately sensed by the actin binding site contained in the N terminus of MRTF-A.

Interestingly, S1P has recently been shown to stimulate SMC contraction by activating RhoA (39, 62, 63). Because many of the SMC-specific differentiation marker genes code for contractile or contractile-associated proteins, it is interesting to postulate that short-term regulation of the SMC contractile force by S1P (and possibly other agonists) may be coupled to long-term regulation of SMC contractile-associated gene expression. In addition, many of the environmental factors that control SMC phenotype including the contractile agonists, mechanical stretch, matrix interactions, and serum/growth factors also affect RhoA activity, and signaling through this pathway may be a common mechanism by which these factors contribute to the regulation of SMC phenotype.

In summary, the data presented here indicate that S1P regulates SMC differentiation through its effects on MRTF-A. The control of MRTF-A localization by RhoA signaling in SMC (and other cell types) may be an important link in the signaling pathway by which SRF-dependent cell type-specific genes are regulated by extrinsic factors. The observation that S1P also regulates SMC growth by activating ERK suggests that sub-
type-specific expression of S1P receptors may be an important determinant of SMC phenotype. Because SMC differentiation and changes in SMC phenotype are critical during blood vessel development and cardiovascular pathophysiology, respectively, further studies to identify the mechanisms by which S1P, RhoA, and MRTF-A regulate these processes will be important.

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