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Identification and Characterization of the Unique Guanine Nucleotide Exchange Factor, SmgGDS, in Vascular Smooth Muscle Cells

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Abstract The guanine nucleotide exchange factor (GEF), SmgGDS, promotes nucleotide exchange by several GTPases in both the Ras and Rho families, especially by RhoA. Because RhoA plays an important role in regulating the contraction of vascular smooth muscle cells (VSMC), we examined the expression and function of SmgGDS in VSMC. SmgGDS is expressed in primary rat aortic smooth muscle (ASM) cells, primary bovine coronary artery smooth muscle (BCASM) cells, and the immortalized A7r5 line of rat ASM cells. Down regulation of SmgGDS expression by siRNA transfection resulted in a decrease of RhoA-GTP levels, enhanced cell spreading, and loss of the characteristic elongated morphology of VSMC. A similar morphology was also observed following treatment with the Rho-kinase inhibitor, Y27632. In contrast, cells with reduced RhoA expression exhibit an elongated shape. Subsequent immunofluorescent staining revealed a disruption of the myosin filament organization in the cells with reduced SmgGDS expression. Further studies analyzed the effect of SmgGDS siRNA transfection on the contraction of A7r5 cells and BCASM cells, which is also a Rho-regulated pathway. Transfection of SmgGDS siRNA or RhoA siRNA resulted in an impaired ability of the A7r5 and BCASM cells to undergo contraction in a collagen gel matrix. However, phosphorylation of the myosin-binding subunit of myosin phosphatase (MYPT1) or the light chain of myosin II (MLC) was not altered by downregulating expression of either SmgGDS or RhoA GTPase. Taken together these results identify SmgGDS as a novel regulator of myosin organization and contraction in VSMC. J. Cell. Biochem. 104: 1760–1770, 2008.

Key words: RhoA; guanine nucleotide exchange factors; smooth muscle; Smg GDS; SmgGDS; Rap1GDS1

Smooth muscle contraction is regulated by the formation of myosin-actin filaments, which is a Rho-dependent pathway. Previous studies have delineated the mechanism of agonist-induced VSMC contraction in the absence of free cytosolic Ca^{2+}. Following activation by RhoA-GTP, Rho-kinase phosphorylates threonine-696 (Thr696) and threonine-853 (Thr853) on myosin phosphatase (MYPT1), rendering MYPT1 inactive [Lai and Frishman, 2005]. Phosphorylation of Thr696 inhibits the phosphatase activity of MYPT1, while phosphorylation of Thr853 inhibits binding of the myosin phosphatase subunit to myosin II [reviewed in Ito et al., 2004]. In this state, MYPT1 is unable to dephosphorylate the light chain of myosin II (MLC). This effect triggers an increase in MLC phosphorylation, allowing for an increase in intrinsic ATPase action of myosin. The result of this activated signaling cascade is an increase in actomyosin cross-bridging [Lai and Frishman, 2005]. This Ca^{2+} independent pathway is thought to play a role in both agonist-induced contraction and preservation of vascular tone under pathological conditions.

The molecular events that activate RhoA leading to VSMC contraction have not yet been elucidated. It has been proposed that a Rho GEF may couple the activation of a

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heterotrimeric G-protein to the RhoA-induced contraction pathway [Hilgers and Webb, 2005]. Although there are approximately seventy Rho GEFs in the human genome which have been identified, only four are expressed in smooth muscle and include PDZ-RhoGEF, leukemia-associated RhoGEF (LARG), p115-RhoGEF, and Vsm-RhoGEF [Kozasa et al., 1998; Ogita et al., 2003; Ohtsu et al., 2005; Ying et al., 2006]. Due to the defined linkage between the Rho-signaling pathway and VSMC contraction, it is very important to identify regulators of Rho GTPases in VSMC. The limited number of known Rho GEFs in VSMC compared to those known in the human genome suggests that additional previously uncharacterized GEFs regulate RhoA activity in VSMC.

SmgGDS, a 61 kDa protein originally isolated from bovine brain cytosol, is the only GEF of the armadillo (ARM) family of proteins [Yamamoto et al., 1990; Williams, 2003]. SmgGDS is one of the most structurally and sequence unique GEFs, because it apparently lacks the DH-PH, Cdc25, and Docker domains that are typically found in other GEFs that activate Ras and Rho family members. Furthermore, unlike other known GEFs, SmgGDS displays a wide specificity for target proteins among both the Ras and Rho families. SmgGDS increases GTP-binding by many small GTPases, including RhoA [Cadwallader et al., 1994; Apolloni et al., 2000; van Hennik et al., 2003; Esufali and Bapat, 2004], Rac1 [Cadwallader et al., 1994; Apolloni et al., 2000], K-Ras4B [Cadwallader et al., 1994; Esufali and Bapat, 2004], Rap1A [Esufali and Bapat, 2004; Ueyama et al., 2005; ten Klooster et al., 2006], and Rap1B [Cadwallader et al., 1994; ten Klooster et al., 2006]. However, RhoA is the preferred substrate of SmgGDS [Orita et al., 1993; Chuang et al., 1994; Yaku et al., 1994]. Based on the important role of the RhoA GTPase in VSMC contraction and myosin dynamics, we hypothesized that SmgGDS plays a prominent role in these cellular processes. These studies are the first to identify SmgGDS expression in VSMC, and characterize its role in agonist-induced VSMC contraction through the Rho signaling pathway.

MATERIALS AND METHODS

Cell Culture

The primary BCASM cells were isolated as previously described [Li et al., 2000] and maintained in Medium 199 (Sigma) containing 10% FBS (Novacell), 1% antibiotic-antimycotic (Gibco), 10,000 U/μl nystatin (Sigma), 10 mg/ml gentamycin (Sigma), and 5 mg/ml tylosin (Sigma). BCASM cells were passaged every 3–5 days, and used between 10 and 15 passages from harvest. The A7r5 line of rat ASM cells was obtained from the American Type Culture Collection (ATCC, CRL-1444) and cultured in DMEM supplemented with 10% FBS (Biosource), 20 U/ml penicillin (Gibco), and 20 μg/ml streptomycin sulfate (Gibco). A7r5 cells were passaged every 3–5 days and used between 2 and 15 passages. Primary rat ASM cells were a generous gift from Dr. Mark Taubman (University of Rochester, NY) and were cultured as previously described [Bogdanov et al., 1998].

Transfection of Cells With siRNA

Multiple siRNA duplexes for both SmgGDS and RhoA were purchased from Dharmacon RNA Technologies (Lafayette, CO) and tested for maximal and specific suppression of their target proteins. We determined that SmgGDS expression was maximally and specifically suppressed by the siRNA duplex corresponding to SmgGDS 5′-GCAAAGAUGUUAUCAGCUG-3′, 5′-CAGCUGAUAACAUCUUUGCUU-3′, and RhoA expression was maximally and specifically suppressed by the siRNA duplex corresponding to RhoA 5′-AUGGAAAGCAGGUAGCGUUUU-3′, 5′-AACUCUACCUGCUUUCCAUUU-3′. A functional-non targeting siRNA that was bioinformatically designed by Dharmacon, Inc. to have $\geq$4 mismatches with known human and mouse genes was included as a control (siControl). A7r5 or BCASM cells were transfected with DharmaFECT2 reagent alone (mock transfection), 50 nM of siControl, 50 nM of siRNA specific for SmgGDS or 50 nM of siRNA specific for RhoA. Seventy-two hours post-transfection, the cells were then analyzed by immunostaining, Western blot, or contraction in a collagen gel matrix.

Immunostaining of Cells

The SA-2 human monoclonal autoantibody to the myosin heavy chain was used as described previously to determine the intracellular distribution of myosin [Williams et al., 1989]. Briefly, A7r5 or BCASM cells were plated on glass coverslips in a 24-well plate, grown to approximately 50% confluency and transfected with siRNA as described above. Seventy-two

hours post-transfection, the cells were fixed by immersion in ice-cold acetone and incubated (30 min, 24°C) in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA). The fixed cells were incubated (1 h, 24°C) with the SA-2 antibody, washed in PBS, and incubated (1 h, 24°C) with fluorescein-labeled anti-human IgM antibody (Southern Biotech, cat# 2020-02) diluted 1:100 in PBS, 1% BSA. The cells were mounted in 90% glycerol/0.1% p-phenylenediamine and examined using a Nikon Eclipse E600 fluorescence microscope.

Collagen Gel Contraction

Collagen gels were prepared by mixing Type I Collagen (Vitrogen-100 or Purecol), 10× PBS, and 0.1 M NaOH at a ratio of 8:1:1 (pH 7.4 ± 0.2). An equal volume of the collagen solution was then mixed with an equal volume of A7r5 or BCASM cells, with or without 10% FBS, to result in a final concentration of 6×10^5 cells/ml. A volume of 500 μl of the collagen/cell mixture was cast into each well of a 24-well plate that was pre-coated with 1% BSA/PBS. After polymerization for 60 min at 37°C, the collagen gels were allowed to undergo free contraction in the presence or absence of 10% serum and/or 3 μM of the Rho-kinase inhibitor, Y27632. The collagen gel images were recorded with a CCD camera and analyzed by NIH Image J software. The areas of the gels were measured with the Image J software, and the ratio of contraction was calculated as $(A_1-A_2)/A_2$, where A_1 is the area before contraction and A_2 is the area after contraction, as described in Li et al. [2003].

Western Blot Analysis of Lysates

Whole-cell lysates were prepared by solubilizing the cells in Laemmli sample buffer, and then boiled for 5 min. The whole cell lysates were subjected to SDS–PAGE and probed with a monoclonal antibody against SmgGDS (Becton Dickinson, cat# 624088), RhoA (Santa Cruz Biotechnology, cat# sc-418), MLC (Sigma, cat# m4401), GAPDH (Santa Cruz, cat# sc-32233) or polyclonal antibodies MLC-P18 (Santa Cruz, cat# 19848-R), MLC-P19 (Santa Cruz, cat# sc-19849-R), MYPT1-P696 (Upstate, cat# 07-251), MYPT1-P850 (Upstate, cat# 36-003), and MYPT1 (Santa Cruz, cat# 25618). After incubation with sheep α-mouse-HRP-IgG (Amersham Biosciences, cat# NA931V), donkey α-rabbit-HRP-IgG (Amersham Biosciences, cat# NA934V), or goat-α-mouse-HRP IgM (Santa Cruz, cat# sc-2064), the blots were developed using enhanced chemiluminescence (Perkin Elmer Lifesciences, Western Lightning) and imaged with X-ray film.

RhoA Activation

Activated RhoA levels were measured using a RhoA G-LISA Activation Assay Kit (colorimetric assay; Cytoskeleton, Inc., Denver, CO) according to the manufacturer's instructions. Briefly, cell lysates were prepared according to the specified instructions of the kit, (Cytoskeleton, Inc.). Equal amounts of total protein were added to Rho-GTP affinity wells in duplicate and incubated at 4°C for 30 min with vigorous shaking at 400 rpm. The samples were incubated with anti-RhoA antibody and secondary HRP-conjugated antibody for 45 min each with vigorous shaking at room temperature. Rho-GTP levels were determined following the incubation of the substrate solution, by measuring absorbance at 490 nm using an ELISA plate reader following subtraction of the absorbance from a sample containing only lysis buffer.

RESULTS

SmgGDS is Expressed in Different Vascular Beds

Previous studies by Kaibuchi et al. [1991] demonstrated that SmgGDS mRNA is ubiquitously expressed in various tissues. However, expression of SmgGDS protein in VSMC has not been previously reported. In order to evaluate the expression of SmgGDS in different vascular beds, equal numbers of primary rat ASM, primary BCASM cells, and the immortalized A7r5 line of rat ASM cells were analyzed by Western blotting with a monoclonal antibody specific to SmgGDS. The SmgGDS protein, observed to possess a molecular weight of approximately 61 kDa, was found to be expressed in all three cell types (Fig. 1). A lower band corresponding to approximately 58 kDa was also detected in these samples. Current studies are directed towards determining whether this lower band represents a modified form of SmgGDS.

Knockdown of SmgGDS Expression in A7r5 and BCASM Cells

In order to study the functional effects of SmgGDS expression in VSMC, siRNA transfection was used to downregulate the expression

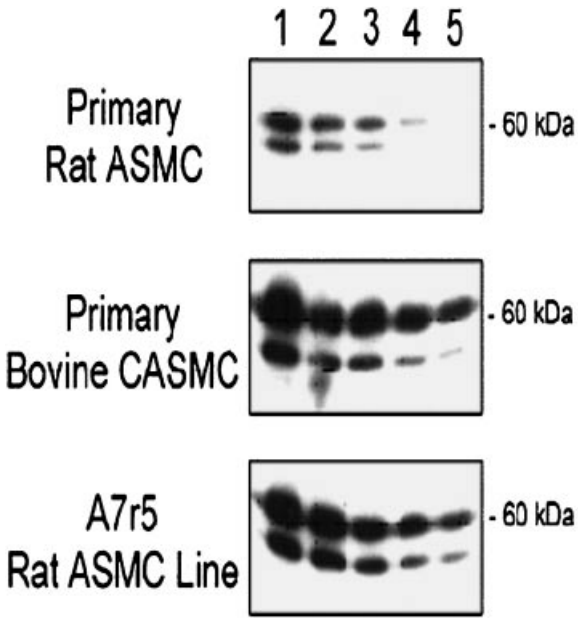

Fig. 1. SmgGDS is expressed in VSMC. Equal numbers of cells from cultures of primary rat ASM cells (**top panel**), primary BCASM cells (**middle panel**) or A7r5 immortalized rat ASM cells (**bottom panel**) were lysed in sample buffer and subjected to SDS–PAGE followed by Western blotting using serial doubling dilutions (beginning at a dilution of 1:50 in Lane 1) of a monoclonal anti-SmgGDS antibody.

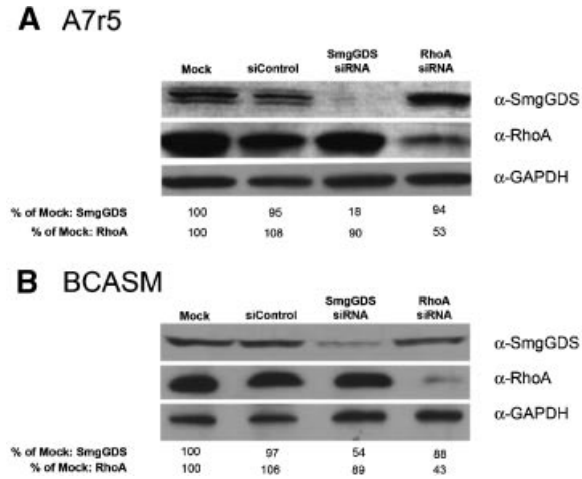

Fig. 2. SmgGDS siRNA and RhoA siRNA Specifically Down-regulate Expression of SmgGDS and RhoA Proteins. A7r5 (**A**) and BCASM (**B**) cells were transfected with reagent alone (Mock), 50 nM of siControl, 50 nM of SmgGDS siRNA (SmgGDS siRNA) or 50 nM of RhoA siRNA (RhoA siRNA). Seventy-two hours post-transfection the cell lysates were collected and subjected to SDS–PAGE followed by Western blotting with monoclonal anti-SmgGDS antibody, monoclonal anti-RhoA and monoclonal anti-GAPDH antibodies. The ratio of the O.D. of SmgGDS (or RhoA) to the O.D. of GAPDH was calculated for each sample, and this ratio was normalized relative to the ratio obtained with mock transfected cells. A representative blot of three independent experiments is shown.

of either SmgGDS or RhoA protein. To this end, A7r5 and BCASM cells were transfected with siRNA specific for either SmgGDS or RhoA. As a control, a functional but non-targeting siRNA (siControl) was also used. Seventy-two hours post-transfection, the cell lysates were analyzed for protein levels by Western blotting with either a monoclonal anti-SmgGDS antibody, or monoclonal anti-RhoA antibody. Anti-GAPDH antibody was also used as a loading control. Densitometry analysis indicated that SmgGDS expression levels were reduced by approximately 80% in A7r5 cells and 50% in BCASM cells following transfection of SmgGDS siRNA, relative to Mock transfected cells (Fig. 2). RhoA protein expression was diminished by approximately 50% in both A7r5 and BCASM cells following transfection of RhoA siRNA (Fig. 2). Transfection of siControl did not significantly alter the expression of either SmgGDS or RhoA levels (Fig. 2).

SmgGDS Regulates A7r5 and BCASM Cell Morphology and Myosin Organization

We observed that cells with reduced SmgGDS expression exhibit enhanced spreading, and no longer have the rectangular shape that is characteristic of the VSMC morphology of A7r5 and BCASM cells (Fig. 3B,F). Enhanced cell spreading similarly occurred in non-transfected cells treated with 3 μM of the Rho-kinase inhibitor Y27632 (Fig. 3D,H). In contrast, cells with reduced RhoA expression exhibited an abnormally elongated shape (Fig. 3C,G). The unique morphology induced by SmgGDS siRNA compared to RhoA siRNA suggests that SmgGDS regulates VSMC morphology by controlling the activities of multiple small GTPases in addition to RhoA. Since Rho-kinase is known to be activated by other isoforms of Rho-GTPase, such as RhoB, and RhoC [Leung et al., 1996; Wheeler and Ridley, 2004], it is possible that the unique morphology induced by Y27632 compared to RhoA siRNA is due to the inactivation of both RhoB- and RhoC-mediated regulation of Rho kinase in the Y27632-treated cells.

Since RhoA regulates cytoskeletal organization, the effects of SmgGDS siRNA and RhoA siRNA were evaluated by immunostaining with SA2, a human monoclonal antibody against the myosin heavy chain [Williams et al., 1989].

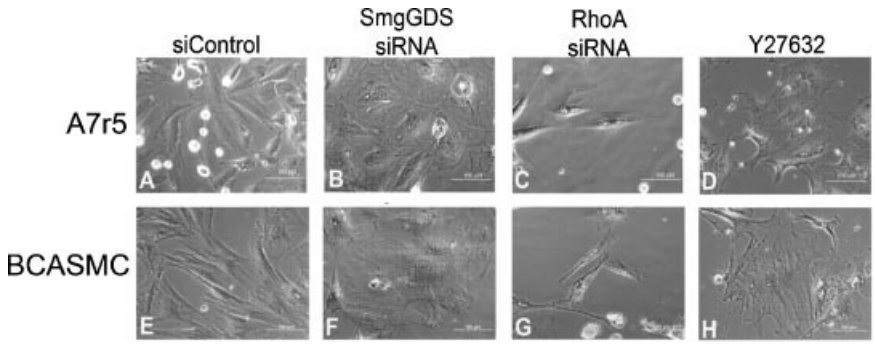

Fig. 3. Downregulation of SmgGDS Expression Alters Cellular Morphology. A7r5 (**top**) or BCASM (**bottom**) cells were transfected with 50 nM of siControl (**A**,**E**), 50 nM of SmgGDS siRNA (**B**,**F**), or 50 nM of RhoA siRNA (**C**,**G**), or treated with 3 μM Y27632 (**D**,**H**) for 60 min. Seventy-two hours post-transfection the cells were visualized by Phase Contrast microscopy. All images are shown at the same magnification and are representative of three independent experiments.

Previous studies have shown that diminishing cellular levels of RhoA-GTP results in a decrease in actomyosin crossbridging, thereby altering myosin organization [reviewed in Lai and Frishman, 2005]. Reduced SmgGDS expression disrupted myosin filament organization in both the A7r5 and BCASM cells (Fig. 4B,F). The myosin heavy chain was observed to change from a filamentous organization to a homogenous distribution. These changes are consistent with inactivation of the Rho signaling pathway [Pearce et al., 2004; Kuzuya et al., 2004]. As expected, reduced RhoA expression (Fig. 4C,G) or inactivation of Rho kinase with Y27632 (Fig. 4D,H) also disrupted myosin organization, similar to the alterations observed with SmgGDS siRNA.

VSMC Contraction is Significantly Diminished by Silencing Expression of SmgGDS

Smooth muscle contraction is regulated by the formation of myosin-actin filaments, which is a Rho-regulated pathway. Our findings demonstrating that loss of SmgGDS expression results in the disruption of myosin organization (Fig. 4) suggest that the absence of SmgGDS reduces the formation of myosin-actin filaments. Since vascular smooth muscle contraction is dependent on the formation of myosin-actin filaments, it is likely that the loss of SmgGDS expression will also inhibit the contractile ability of A7r5 and BCASM cells. The collagen gel contraction assay is widely used as a reliable and consistent method of analyzing SMC contraction because collagen gels contract over time in a predictable and consistent manner [Kuzuya et al., 2004; Pearce et al., 2004; Defawe et al., 2005; Ivanov et al., 2005; Ngo et al., 2006]. Therefore, 72 h following transfection of siControl, SmgGDS siRNA, or RhoA siRNA, the A7r5 and BCASM cells were evaluated for contractility in a collagen gel matrix. Agonist-induced contraction was stimulated with 10% serum, and resulted in a

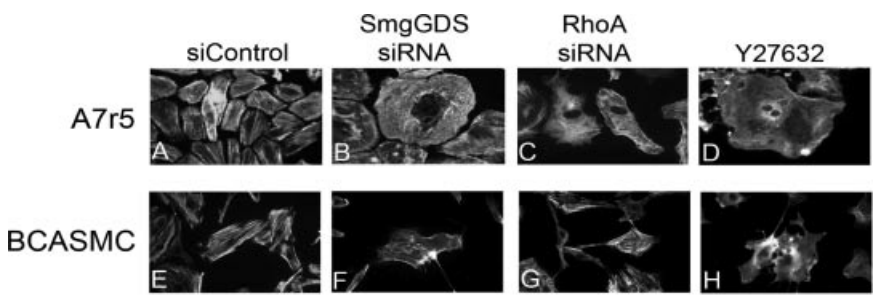

Fig. 4. Downregulation of SmgGDS Alters Myosin Organization. A7r5 (**top**) or BCASM (**bottom**) cells were transfected with 50 nM of siControl (**A**,**E**), 50 nM of SmgGDS siRNA (**B**,**F**), 50 nM of RhoA siRNA (**C**,**G**), or treated with 3 μM Y27632 (**D**,**H**) for 60 min. Seventy-two hours post-transfection the cells were stained with the SA-2 human monoclonal IgM antibody against myosin heavy chain and visualized by fluorescence microscopy. All images are shown at the same magnification, and are representative of three independent experiments.

significant increase of the contraction ratio of siControl-transfected cells (Fig. 5). In contrast, treatment of cells with 3 μM of Y27632 almost completely inhibited contraction (Fig. 5) which is consistent with inactivation of the Rho-signaling pathway through the inhibition of Rho-kinase. Transfection of either SmgGDS siRNA or RhoA siRNA resulted in a significantly smaller contraction ratio than in siControl-transfected cells (Fig. 5), suggesting that downregulation of either SmgGDS or RhoA decreases the ability of the A7r5 and BCASMC to undergo agonist-induced contraction.

Knockdown of SmgGDS Expression Significantly Diminishes RhoA-GTP

Previous in vitro and in vivo studies have shown RhoA is a preferred intracellular target of the guanine nucleotide exchange activity of SmgGDS [Strassheim et al., 2000]. In order to determine whether SmgGDS activates RhoA in VSM cells, we transfected BCASM cells with siRNA specific for either SmgGDS or RhoA. Forty-eight hours post-transfection, serum-free media was added to the cells to decrease RhoA-GTP to basal levels. After 24 h of serum-starvation, 10% serum was added to activate RhoA. After 5 min of serum activation, the lysates were collected and analyzed for levels of GTP-bound RhoA. In the absence of serum, there was no difference in the levels of RhoA-GTP following transfection of siControl, SmgGDS siRNA, and RhoA siRNA (Fig. 6). Activation with 10% serum significantly increased RhoA activity in siControl-transfected cells, but not in cells transfected with RhoA siRNA or SmgGDS siRNA. Following activation with 10% serum, Rho-GTP levels were significantly reduced in the RhoA siRNA-transfected cells compared to the control cells (Fig. 6). In the presence of serum, RhoA-GTP levels were 1.5-fold lower in the cells transfected with SmgGDS siRNA compared to siControl-transfected cells (Fig. 6). These results suggest that SmgGDS plays an

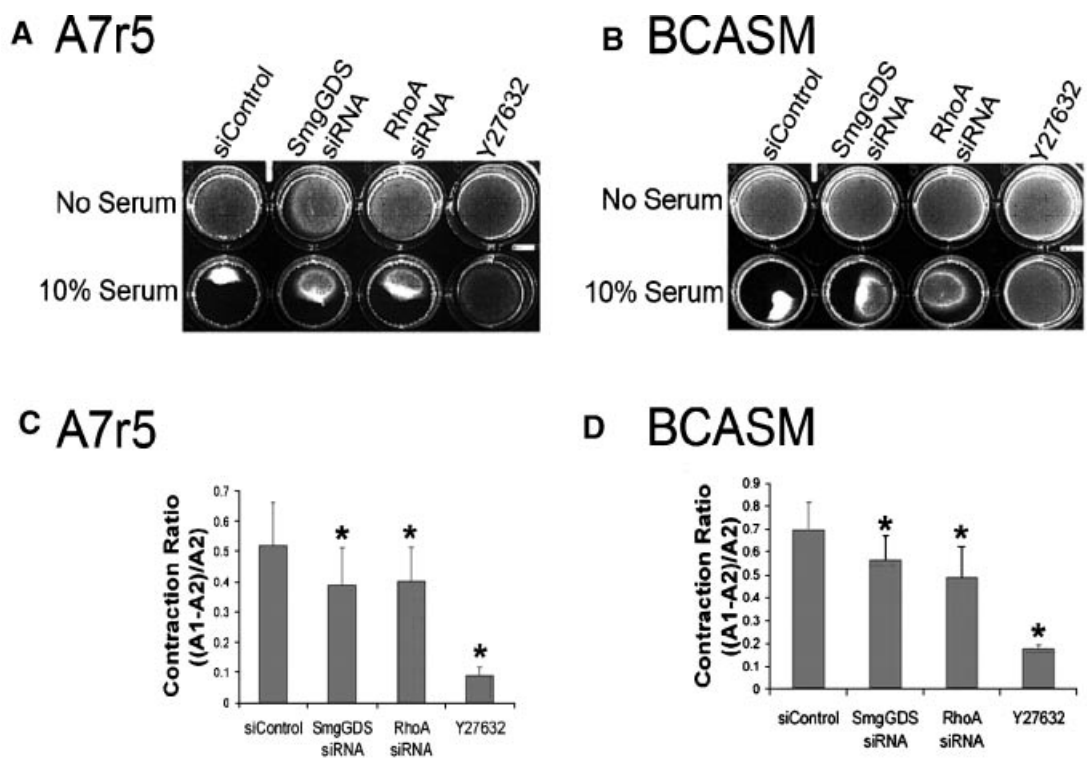

Fig. 5. Transfection of SmgGDS siRNA and RhoA siRNA significantly inhibits contraction of A7r5 and BCASM cells. A7r5 (**A**,**C**) or BCASM (**B**,**D**) cells were transfected with 50 nM of siControl, 50 nM of SmgGDS siRNA, 50 nM of RhoA siRNA, or treated with 3 μM Y27632 for 60 min. Seventy-two hours post-transfection, 3×10^5 A7r5 or BCASM cells were mixed with Type I Collagen (2.5 mg/ml) in the absence or presence of 10% serum. The Y27632 treated cells were incubated with medium containing 3 μM Y27632 during the assay. Images (A,B) are representative results from quadruplicate (C) and triplicate (D) experiments. Results are expressed as the ratio of contraction as defined in the Materials and Methods Section. The error bars indicate standard error of the mean. $^*P < 0.05$, one-way ANOVA, compared to results obtained using siControl-transfected cells.

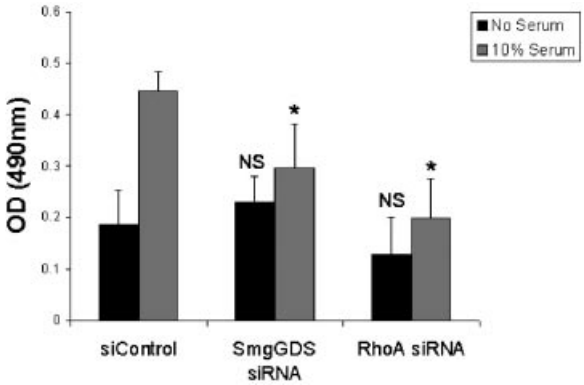

Fig. 6. RhoA-GTP levels are significantly diminished by silencing SmgGDS expression. BCASM cells were transfected with 50 nM of siControl, 50 nM of SmgGDS siRNA or 50 nM of RhoA siRNA for 48 h. Serum-free media was then added to the cell cultures and incubated for an additional 24 h. Activation of RhoA was stimulated by the addition of 10% serum for 5 min. Cell lysates were then collected and analyzed for Rho-GTP by the G-LISA assay (Cytoskeleton, Inc.). Representative results from triplicate experiments are shown. $*P < 0.05$, in paired Student's *t*-test, compared to siControl-transfected cells treated with serum. NS, not significant in paired student's *t*-test, compared to siControl-transfected cells in the absence of serum.

essential role in the activation of RhoA in VSMC.

Downregulation of SmgGDS Does not Alter MYPT1 or MLC Phosphorylation

In order to determine whether the decrease in collagen gel contraction by SmgGDS siRNA was due to alterations in the level of active MYPT1 or MLC, we next investigated the phosphorylation state of both MYPT1 and MLC following siRNA transfection of BCASM cells, in the absence (Fig. 7A,B) and presence of 10% serum (Fig. 7C,D). The addition of 10% serum to the cells resulted in an approximately 10-fold increase in phosphorylated MYPT1 and MLC proteins. Lysates were analyzed by Western blotting with anti-MYPT1-P696 antibody and anti-MYPT1-P850 antibody (MYPT1-P853 is human equivalent to bovine MYPT1-P850), which recognize MYPT1 phosphorylated at threonine-696 or threonine-850, respectively, and anti-MYPT1 antibody which recognizes both phosphorylated and non-phosphorylated MYPT1. Lysates were also blotted with

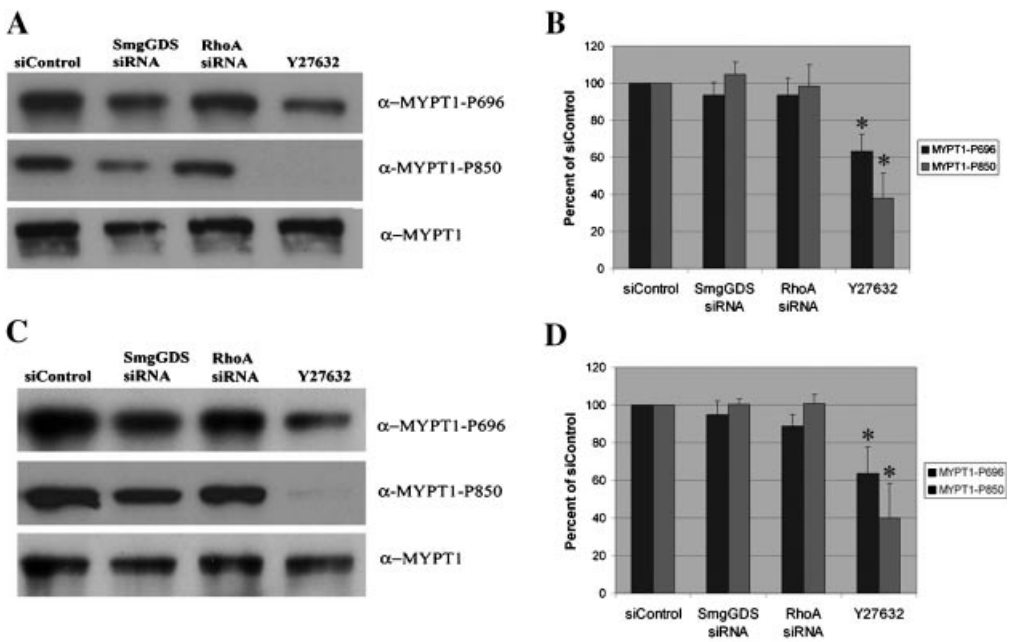

Fig. 7. Phosphorylation of MYPT1 is not detectably altered following silencing of SmgGDS or RhoA. BCASM cells were transfected with reagent alone (Mock), 50 nM of siControl, 50 nM of SmgGDS siRNA, or 50 nM of RhoA siRNA. Forty-eight hours post-transfection, the media was replaced with either serum-free media (**A**,**B**), or media with 10% serum (**C**,**D**). Seventy-two hours post-transfection the cell lysates were collected and subjected to SDS–PAGE followed by Western blotting with polyclonal anti-MYPT1-P696 (top blot), anti-MYPT1-P850 (middle blot), and anti-total MYPT1 (bottom blot). The ratio of the O.D. of MYPT1-P696 (or MYPT1-P850) to the O.D. of GAPDH was calculated for each sample, and this ratio was normalized relative to the ratio obtained with siControl transfected cells. A representative blot of three independent experiments is shown. The error bars indicate standard error of the mean. $*P < 0.05$, one-way ANOVA, compared to results obtained using siControl-transfected cells.

anti-MLC-P18 and anti-MLC-P19 antibodies, which recognize MLC phosphorylation at serine-18 or serine-19, respectively, and anti-MLC antibody which recognizes both phosphorylated and non-phosphorylated MLC. Transfection with either SmgGDS siRNA or RhoA siRNA did not significantly diminish phosphorylation of either threonine-696 or threonine-850 of MYPT1 (Fig. 7). Similar results of MYPT1 phosphorylation were also observed with A7r5 cells (data not shown). Similarly, phosphorylation of threonine-18 or serine-19 of MLC was also not altered by either SmgGDS or RhoA siRNA in BCASM cells (Fig. 8) and A7r5 cells (data not shown). However, treatment with 3 μM Y27632 did result in a significant reduction in phosphorylation of MYPT1-Thr696, MYPT1-Thr850, MLC-Ser18, and MLC-Ser19 (Figs. 7 and 8), which is consistent with the significant decrease in contraction of both A7r5 and BCASM cells treated with Y27632 shown in Figure 5. Therefore, although both SmgGDS siRNA and RhoA siRNA decrease collagen gel contraction, it does not appear to be due to changes in the levels of MYPT1 or MLC phosphorylation. It is also interesting to note that downregulation of RhoA, which has traditionally been viewed as an important regulator of MLC activity, did not affect MLC phosphorylation in these studies. This observation supports previous studies that demonstrate agonist-induced contraction in fibroblast fibers and rat cerebral arteries may occur independently of MLC phosphorylation [Nobe et al., 2003; Seasholtz, 2003; Corteling et al., 2007].

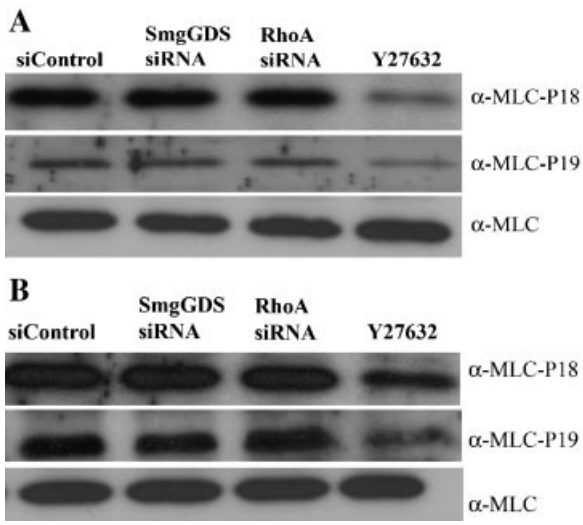

Fig. 8. Phosphorylation of MLC is not detectably altered following silencing of SmgGDS or RhoA. BCASM cells were transfected with reagent alone (Mock), 50 nM of siControl, 50 nM of SmgGDS siRNA, or 50 nM of RhoA siRNA. Forty-eight hours post-transfection the media was replaced with either serum-free media (**A**) or media with 10% serum (**B**). Seventy-two hours post-transfection the cell lysates were collected and subjected to SDS–PAGE followed by Western blotting with polyclonal anti-MLC-P18 (top blot) anti-MLC-P19 (middle blot), and monoclonal anti-MLC (bottom blot) antibodies. The ratio of the O.D. of MLC-P18 (or MLC-P19) to the O.D. of GAPDH was calculated for each sample, and this ratio was normalized relative to the ratio obtained with mock transfected cells. A representative blot of three independent experiments is shown. The high levels of background observed with the anti-MLC-P18 and anti-MLC-P19 antibodies precluded analysis of these blots by densitometry.

DISCUSSION

These studies are the first to identify the expression of the Rho GEF, SmgGDS, in VSMC. We found that SmgGDS is required for serum-induced activation of RhoA (Fig. 6) and participates in serum-induced contraction of VSMC (Fig. 5). These results indicate that SmgGDS is an important activator of RhoA and participates in the RhoA signaling pathway that regulates VSMC contraction. In striking contrast to PDZ-RhoGEF, p115-RhoGEF, Vsm-RhoGEF, and LARG-RhoGEF, SmgGDS lacks the DH-PH domain as well as other domains known to promote guanine nucleotide exchange [reviewed in Quilliam et al., 2002]. This lack of structural similarity with all other known GEFs indicates that SmgGDS activates RhoA through a unique mechanism. Current studies in our laboratory are directed towards delineating how SmgGDS uniquely controls activation of the Rho-signaling pathway in VSMC.

We found that many of the effects of SmgGDS downregulation in VSMC are mimicked by downregulation of RhoA, consistent with the ability of SmgGDS to act as a major regulator of RhoA activity. Downregulation of SmgGDS or RhoA expression results in a similar disruption of myosin organization and a similar reduction in VSMC contraction. However, while SmgGDS siRNA-transfected cells exhibited increased cell spreading, the RhoA siRNA-transfected cells appeared more elongated. The different morphologies induced by downregulating SmgGDS compared to downregulating RhoA may occur because SmgGDS can activate other small GTPases in addition to RhoA [reviewed in Quilliam et al., 2002]. The spread morphology of cells that have downregulated SmgGDS may

reflect the loss of signaling by many small GTPases in addition to RhoA, whereas the elongated morphology of cells that have downregulated RhoA may reflect the loss of RhoA signaling while other GTPase signaling pathways remain intact.

Several of the effects induced by downregulating SmgGDS or RhoA differ from those induced by treating the cells with the Rho kinase inhibitor Y27632. Although downregulation of SmgGDS or RhoA induces significant loss of contraction, neither treatment inhibits contraction to the same extent as treatment with Y27632. Downregulating Smg GDS or RhoA also does not detectably inhibit phosphorylation of MYPT1 or MLC, in contrast to the dramatic loss of MYPT1 phosphorylation and MLC phosphorylation in cells treated with Y27632. The diminished MYPT1/MLC phosphorylation and loss of contraction in Y27632-treated cells are consistent with previous reports that Y27632 inhibits the ability of Rho kinase to phosphorylate MYPT1, which leads to reduced MLC phosphorylation and diminished contraction [Kitazawa et al., 2003; Maeda et al., 2003; Bi et al., 2005]. Our observation that downregulating SmgGDS or RhoA expression diminishes contraction even in the absence of detectable changes in the phosphorylation of MYPT1 or MLC is particularly interesting. This finding suggests that SmgGDS and RhoA are able to regulate VSMC contraction through a mechanism that does not involve changes in MYPT1 or MLC phosphorylation.

Recent studies support the view that RhoA can regulate VSMC contraction without affecting phosphorylation of MLC [Corteling et al., 2007]. These studies illustrated the inability of RhoA siRNA to decrease MLC phosphorylation despite drastically reducing UTP-induced contraction in cerebral arteries. RhoA was subsequently shown to be involved in facilitating the formation of F-actin, to which phosphorylated MLC binds, initiating arterial constriction [Corteling et al., 2007]. These findings clearly define the importance of an intact actin-myosin cytoskeleton for vascular contraction and support our results demonstrating that downregulation of SmgGDS or RhoA expression results in a decrease in contraction without altering phosphorylation of MYPT1 or MLC.

Even though downregulating SmgGDS or RhoA expression apparently does not alter the Rho kinase-mediated phosphorylation of MYPT1, it is possible that downregulating SmgGDS or RhoA impairs the ability of Rho kinase to phosphorylate other substrates needed for VSMC contraction (Fig. 9). Activated Rho-kinase can induce phosphorylation of a number of different target proteins in addition to MYPT1 which are able to affect cytoskeletal organization and contraction. For example, previous studies have demonstrated that Rho-kinase phosphorylates LIM kinase, which leads to the inhibition of the actin-severing protein cofilin. Inhibition of cofilin promotes actin polymerization and cytoskeletal reorganization [Arber et al., 1998; Ohashi et al., 2000]. Other studies have identified elongation factor-1α (eF1A), a co-factor for polypeptide elongation, as a target of Rho-kinase. Phosphorylation of elongation factor-1α reduces its ability to bind actin, thereby reducing F-actin bundling activity [Izawa et al., 2000]. The cytoskeletal regulatory proteins ezrin/radixin/moesin (ERM) are also phosphorylated by Rho-kinase and play critical roles in cytoskeletal cellular processes [Matsui et al., 1998; Yonemura et al., 2002;

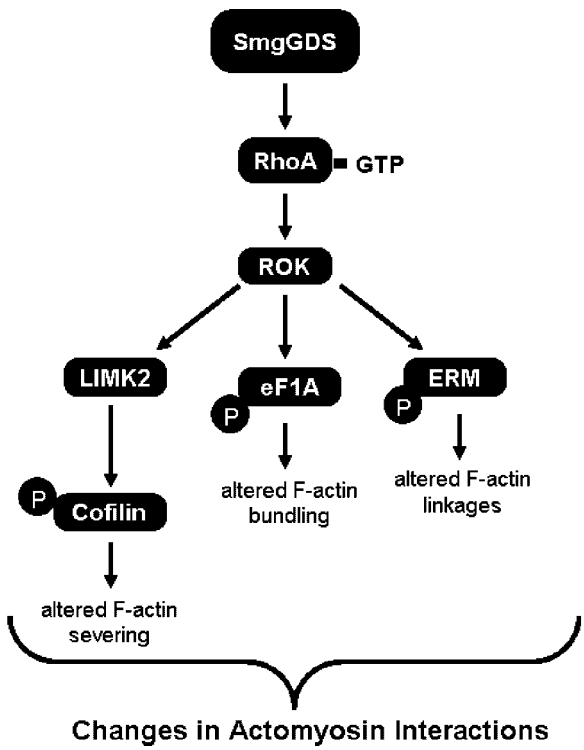

Fig. 9. Model of regulation of actomyosin interactions by SmgGDS. Alterations in actomyosin interactions may occur in a SmgGDS dependent manner through several Rho-kinase (ROK) signaling pathways.

Hughes and Fehon, 2007]. Thus, there are a number of different mechanisms in which altering SmgGDS or RhoA expression could potentially regulate Rho-kinase activity and the phosphorylation of downstream substrates involved in VSMC contraction.

Because Rho-kinase is activated by RhoA, RhoB, and RhoC [reviewed in Hughes and Fehon, 2007], treatment with Y27632 is expected to inhibit all Rho kinase pathways that are mediated by RhoA, RhoB, and RhoC. In contrast, downregulation of RhoA is expected to inhibit only those Rho kinase pathways which are mediated by RhoA, and not affect Rho kinase pathways mediated by RhoB or RhoC. Several studies have shown the importance of RhoB and RhoC in the activation of Rho kinase and the phosphorylation of MLC [Leung et al., 1996; Wheeler and Ridley, 2004; Conway et al., 2004]. We found that the VSMC examined in this study express RhoA, RhoB, and RhoC (data not shown). Thus, some of the observed differences induced by Y27632 compared to those induced by downregulating RhoA expression may be due to the unique ability of Y27632 to inhibit all RhoA-, RhoB-, and RhoC-mediated pathways involving Rho kinase.

These results identify SmgGDS as unique regulator of RhoA GTPase activity in VSMC. The significant reduction in serum-induced RhoA activation and contraction when SmgGDS expression is down-regulated indicates that SmgGDS provides a critical point of regulation for agonist-induced RhoA activation and contraction. SmgGDS may also regulate other GTPases in addition to RhoA in VSMC, based on the differences in cell morphology following transfection of SmgGDS siRNA and RhoA siRNA. The ability of SmgGDS to regulate myosin organization and agonist-induced contraction suggests that this novel GEF may play an important role in pathological diseases such as asthma and hypertension. Current studies are focused to determine how SmgGDS regulates RhoA and other GTPase substrates in VSMC to promote contraction.

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