EphA4-Mediated Rho Activation via Vsm-RhoGEF Expressed Specifically in Vascular Smooth Muscle Cells

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Abstract—Rho-kinase, an effector of Rho GTPase, increases the contractility of vascular smooth muscle by phosphorylating myosin light chain (MLC) and by inactivating MLC phosphatase. A wide variety of extracellular stimuli activate RhoA via G protein–coupled receptors. In the present study, we demonstrate a novel cell–cell interaction–mediated Rho activation signaling pathway in vascular smooth muscle cells (VSMCs). Among many receptor tyrosine kinases, the Eph family receptors are unique in that they require cell–cell interaction to engage their ligands, ephrin. We found that a novel VSMC-specific guanine nucleotide exchange factor (GEF) for Rho (Vsm-RhoGEF/KIAA0915) was expressed specifically in VSMCs of several organs including the heart, aorta, liver, kidney, and spleen, as examined by the immunohistochemical analysis using a specific antibody against Vsm-RhoGEF. Based on the association of Vsm-RhoGEF with EphA4 in quiescent cells, we tested whether EphA4 and Vsm-RhoGEF were expressed in the same tissue and further studied the molecular mechanism of Vsm-RhoGEF regulation by EphA4. Immunohistochemical analysis showed that EphA4 and Vsm-RhoGEF expression overlapped in VSMCs. Additionally, tyrosine phosphorylation of Vsm-RhoGEF induced by EphA4 upon ephrin-A1 stimulation enhanced the Vsm-RhoGEF activity for RhoA. The requirement of Vsm-RhoGEF for ephrin-A1–induced assembly of actin stress fibers in VSMCs was shown by the overexpression of a dominant-negative form of Vsm-RhoGEF and by the depletion of Vsm-RhoGEF using RNA interference. These results suggested that ephrin-A1–triggered EphA4-Vsm-RhoGEF-RhoA pathway is involved in the cell–cell interaction–mediated RhoA activation that regulates vascular smooth muscle contractility. (Circ Res. 2003;93: 23-31.)

Key Words: smooth muscle cells ■ Rho ■ Eph ■ ephrin ■ contraction

Vascular smooth muscle cell (VSMC) contractility regulates vascular tone to maintain blood circulation. Increased vascular smooth muscle contraction results in spasm and chronic contraction leads to hypertension, both of which contribute to cardiovascular pathology. Vascular contraction is regulated by actin–myosin II coupling in a Ca2+-dependent manner and a Ca2+-independent manner. The Rho GTPases play an important role in the Ca2+-independent vascular contraction, known as Ca2+ sensitization.

Myosin II is regulated by phosphorylation and dephosphorylation of the myosin regulatory light chain. The former is controlled by myosin light chain (MLC) kinase regulated by Ca2+/calmodulin, and the latter is regulated by MLC phosphatase (MLCP). Recently, RhoA has been shown to be involved in the inhibition of MLCP via the Rho effector molecule, Rho-kinase. The phosphorylation of MLCP inhibits the phosphatase activity and thereby activates MLC, resulting in contraction of smooth muscle. In addition to MLCP phosphorylation, Rho-kinase directly phosphorylates MLC and increases the contractility of myosin II. These data support that Rho activation is clinically involved in vasospastic angina and unfavorable smooth muscle contraction of atherosclerotic arteries.

The Rho GTPase functions as a molecular switch by cycling between a GTP-bound active form and a GDP-bound inactive form. This cycle is regulated by three classes of molecules: guanine nucleotide exchange factors (GEFs), guanine nucleotide dissociation inhibitors, and GTPase-activating proteins. GEFs initiate the exchange of GDP with GTP and promote the association of Rho with its effector molecules. Vasocostricators, including endothelin, angiotensin II, and urotensin II, induce VSMC contraction by activating RhoA via heterotrimeric GTP-binding protein-coupled receptors. G12/13 is responsible for this vasoconstrictor-mediated RhoA activation. The effectors of G12/13, RGS (regulator for G protein signaling) domain–containing RhoGEF family members, p115RhoGEF, PDZ-RhoGEF, and LARG, have been identified as GEFs for RhoA. Typical RhoGEF family members including RGS domain–containing RhoGEFs contain Dbl homology (DH) domains and pleckstrin homology (PH) domains. More than 60 RhoGEF family members containing DH-PH domains have been found in the human genome, yet most of them have not been characterized to date.
Eph family tyrosine kinase receptors consist of two groups: EphA group members respond to ephrin-A, which is anchored to the cell membrane by glycosylphosphatidylinositol, whereas EphB group members respond to ephrin-B, containing a transmembrane domain. Among them, only EphA4 can cross-respond to both ephrin-A and -B.12 Recently, the Eph-ephrin system has been found to be involved in vascular development and also in mediating intracellular signaling in vascular endothelial cells in angiogenesis.13–16

We found that KIAA0915 was closely related to ephexin. Ephexin, which contains DH and PH domains, has been shown to bind to EphA4 and exhibit GEF activity for RhoA, Rac1, and Cdc42 in neuronal cells.17 The DH and PH domains were conserved in KIAA0915, which we renamed Vsm-RhoGEF (vascular smooth muscle–specific RhoGEF), because it was expressed exclusively in VSMCs.

In this study, we investigated the function and the regulation of Vsm-RhoGEF in VSMCs. We demonstrate that Vsm-RhoGEF functions as a GEF for RhoA and that the GEF activity of Vsm-RhoGEF is regulated by the activation of EphA4 and the subsequent tyrosine phosphorylation of Vsm-RhoGEF. Collectively, our data suggest that the cell-cell contact-triggered ephrin-EphA4 interaction and the subsequent Vsm-RhoGEF activation may contribute to vascular constriction by regulating RhoA.

**Materials and Methods**

**Reagents and Antibodies**

Recombinant soluble mouse ephrin-A1-human Fc chimeric protein (ephrin-A1/Fc) was purchased from R&D Systems (Minneapolis, Minn). Ephrin-A1/Fc was prepared as described previously, and 1 μg/mL ephrin-A1/Fc chimera was used in the following experiments.16 Protein A- and G-Sepharose were from Calbiochem (La Jolla, Calif). Anti-EphA4, anti-RhoA, and anti-Cdc42 antibodies were from Santa Cruz Biotechnology (Santa Cruz, Calif); anti-FLAG antibody was from Sigma-Aldrich (St Louis, Mo); rhodamine-phalloidin was from Molecular Probes (Eugene, Ore); anti-Rac1 was from Transduction Laboratories (Lexington, Ky); anti-HA antibody was from Roche Diagnostics (Basel, Switzerland); and antiphosphotyrosine (PY100) antibody was from Cell Signaling Technology (Beverly, Mass). Anti-GFP (green fluorescent protein) was developed in our laboratory. Anti-Vsm-RhoGEF antibody was raised in rabbits against the synthetic peptide (EAVGPSSGTPNAPPP corresponding to the carboxy terminus of Vsm-RhoGEF) coupled to keyhole limpet hemacyanin.

**Expression Plasmids**

cDNA clone of Vsm-RhoGEF (KIAA0915) was obtained from Kazusa DNA Research Institute (Chiba, Japan). pCA-EGFP-Vsm-RhoGEF-WT, pCA-EGFP-Vsm-RhoGEF-DH-PH, and pCA-EGFP-Vsm-RhoGEF-PH were derived from pCAGGS eukaryotic expression vector and expressed enhanced green fluorescent protein (EGFP)-tagged wild type, DH-PH domains, and PH domain of Vsm-RhoGEF, respectively (Figure 1A).18 pCXN2-FLAG-INES-EGFP was derived from pCAGGS and contained an internal ribosome entry site (IRES) and the coding region of EGFP at the 3′ side.19 The DNA fragments encoding full-length or PH domain of Vsm-RhoGEF were amplified by polymerase chain reaction (PCR) and subcloned into pCXN2-FLAG-INES-EGFP vector. cDNA of RhoQL substituted at Gln63 for Leu was amplified by polymerase chain reaction (PCR) and ligated into pCXN2 vector. pCXN2-Rac1V12 and pCXN2-FLAG-Cdc42V12 were obtained from M. Matsuda (Osaka University, Suita, Japan). pCI-HA-EphA4 expressing HA-tagged EphA4 was obtained from M. Tanaka (Hamamatsu University, Shizuoka, Japan). All of the DNA fragments amplified by PCR were ligated into pCR.

**Cells and Transfection**

Rat aortic smooth muscle cells (A7r5 cells) were purchased from American Type Culture Collection (Manassas, Va). Human coronary artery smooth muscle cells (HCASMCs) were from Cascade Biolog-
ics (Portland, Ore). HCASMCs were maintained in HuMedia-SG2 (Kurabo, Osaka, Japan) supplemented with a growth additive set as described previously.\textsuperscript{19} 293T cells and A7r5 cells were cultured in DMEM (Invitrogen) supplemented with 10% FBS, 2 mmol/L L-glutamine and 15 mmol/L NaHCO\textsubscript{3}. 293T cells were transfected by calcium phosphate method, and A7r5 cells were transfected by using LipofectAMINE 2000 reagent (Invitrogen). A7r5 cells were starved for more than 6 hours before the ephrin-A1/Fc stimulation in DMEM/F-12 (Invitrogen) without phenol red supplemented with 2 mmol/L L-glutamine, 10 mmol/L HEPES, 15 mmol/L NaHCO\textsubscript{3}, and 0.5% bovine serum albumin fraction V.

**Immunoprecipitation and Immunoblotting**

Cells were washed with PBS three times and lysed in lysis buffer (150 mmol/L NaCl, 20 mmol/L Tris hydrochloride, pH 7.5, 1.5 mmol/L MgCl\textsubscript{2}, 1 mmol/L Na\textsubscript{3}VO\textsubscript{4}, 1% Triton X-100, 10 mmol/L NaF, and protease inhibitor cocktail (Roche Applied Science). Lysates were precleared by centrifugation at 15 000 \textit{g} for 10 minutes, and immunoprecipitated by antibodies, indicated in the figure, and protein A- or G-Sepharose. Immunoprecipitates were subjected to SDS-PAGE and immunoblotting with antibodies as indicated in the figure. Protein isolation of each organ from Wister-Kyoto rats was performed according to the method as previously described.\textsuperscript{20} Briefly, each organ from a rat was cleaned, pulverized in liquid nitrogen, and homogenated. Equivalent amounts of protein from each organ were separated on SDS-PAGE and transferred to PVDF membrane for immunoblotting with anti-Vsm-RhoGEF. Proteins reacting with primary antibodies were visualized by an enhanced chemiluminescence system (Amersham Biosciences UK, Buckinghamshire, UK) for detecting peroxidase-conjugated and species-matched secondary antibodies and analyzed with an LAS-1000 system (Fuji Film, Tokyo, Japan).

**Immunohistochemical Analysis**

Rats were sacrificed by overdose injection of pentobarbital intraperitoneally. The immunohistochemical study followed the protocol as described previously.\textsuperscript{21} The formalin-fixed paraffin-embedded sections of rat organs were deparaffinized, heated by pressure cooker in 10 mmol/L sodium citrate pH 6.0 for 3 minutes for antigen unmasking, and cooled in water. After washing in 0.02% Tween-20/PBS, they were treated with 0.3% H\textsubscript{2}O\textsubscript{2} methanol and normal goat serum to quench endogenous peroxidase activity and thereafter incubated with anti-Vsm-RhoGEF (1:1000) or anti-EphA4 (1:200) at 4°C overnight. After incubation with the biotinylated goat anti-rabbit IgG and peroxidase-labeled streptavidin, immunoreactive products were visualized by 3,3'-diaminobenzidine tetrahydrochloride. For comparison of immunostaining, counterstaining was also performed with hematoxylin. All animal procedures were performed according to the Guide for the Care and Use of Laboratory Animals (NIH, revision 1996).

**Detection of GTP-Bound RhoA, Rac1, and Cdc42**

GTP-bound RhoA, Rac1, and Cdc42 was detected by the pull-down assay as reported previously.\textsuperscript{22,23} 293T cells transfected with the plasmids indicated in the figure or ephrin-A1/Fc–stimulated A7r5 cells were lysed in lysis buffer. Cleared lysates were incubated with glutathione \textit{S}-transferase (GST)-Rho-binding domain of Rhotekin or GST-Rac/Cdc42 binding domain of PAK for Rac1 or Cdc42, respectively. GST-bound small GTPases collected on glutathione-agarose beads were subjected to SDS-PAGE followed by immunoblotting with anti-RhoA, anti-Rac1, or anti-Cdc42 antibody. Quantitative analyses of immunoblots were performed using Image Gauge version 3.4X software included in an LAS-1000 system. Relative intensity compared with the control was calculated and expressed as an average with standard deviation (SD). Statistically significant difference among each group was evaluated by the Student’s \textit{t} test.

**Confocal Images**

A7r5 cells transfected with plasmids indicated in the figure or stimulated with preclustered ephrin-A1/Fc for the indicated time were washed with PBS three times and fixed by 2% paraformalde-
hyde at room temperature for 30 minutes, followed by permeabilization with 0.05% Triton X-100 for 10 minutes. Permeabilized cells were incubated with rhodamine-phalloidin to detect actin filaments. Cells were imaged by a confocal microscope, BX50WI controlled by Fluoview (Olympus, Tokyo, Japan).

RNA Interference
Small, interfering RNAs (siRNA), 5'-AAGAUCAUUG-AGCGCUGCAGC-3' and 5'-GCUGCAGCGCUCAAUGAU-3' (Dharmacon Research, Lafayette, Colo) were annealed and introduced into A7r5 cells by using LipofectAMINE 2000 reagent (Invitrogen). The rat RNA sequence corresponding to human Vsm-RhoGEF was derived from a partial cDNA sequence we obtained by RT-PCR using a rat cDNA library as a template. Scrambled double-stranded RNA used as a negative control was also obtained from Dharmacon Research.

Results
Vsm-RhoGEF Associates With EphA4
Vsm-RhoGEF (KIAA0915) is structurally related to ephexin, which associates with EphA4 in neuronal cells.17 We thus tested whether Vsm-RhoGEF associated with EphA4. Schematic illustration of Vsm-RhoGEF and its truncated forms used for the following experiments is shown in Figure 1A. Carboxy-terminally HA-tagged EphA4 was coimmunoprecipitated with EGFP-tagged Vsm-RhoGEF in 293T cells expressing both proteins (Figure 1B, lane 5). EGFP used as a negative control did not coimmunoprecipitate with EphA4. Although we examined the association between Vsm-RhoGEF and EphB2, EphB2 receptors were not coimmunoprecipitated with EGFP-tagged Vsm-RhoGEF (data not shown). To examine the region required for the association with EphA4, we constructed truncated forms of Vsm-RhoGEF and examined whether these could associate with EphA4 in 293T cells. Full length, the DH-PH domains, or the PH domain of Vsm-RhoGEF were expressed in 293T cells with HA-tagged EphA4. We found that the EGFP-tagged tandem DH-PH domains were coimmunoprecipitated with EphA4, whereas PH domain alone was not coimmunoprecipitated with EphA4 (Figure 1C). These results suggest that the association between Vsm-RhoGEF and EphA4 requires either the DH or the DH/PH domains of Vsm-RhoGEF. To determine whether the PH domain is required for binding to EphA4, we tested whether overexpression of the PH domain of Vsm-RhoGEF inhibited the association of Vsm-RhoGEF with EphA4 (Figure 1D). PH domain perturbed the association of Vsm-RhoGEF with EphA4, indicating that both DH domain and PH domain are required for the association of Vsm-RhoGEF with EphA4.

Vsm-RhoGEF Is Coexpressed and Associated With EphA4 in Vascular Smooth Muscle
We proceeded to examine the tissue distribution of Vsm-RhoGEF and the localization of Vsm-RhoGEF by immuno-
histochemical analysis using an anti-Vsm-RhoGEF antibody developed in our laboratory. Proteins from rat tissues were separated by SDS-PAGE and analyzed by immunoblotting with anti-Vsm-RhoGEF antibody. Immunoreactive bands were detected in the samples obtained from the heart and the aorta at the expected size of Vsm-RhoGEF (Figure 2A). Immunohistochemical analysis using anti-Vsm-RhoGEF detected Vsm-RhoGEF in the vascular smooth muscle of all examined organs including heart, liver, kidney, aorta, and spleen (Figure 2B). The staining was specific for Vsm-RhoGEF since it was abolished by the preabsorption with the peptide used for the immunization (data not shown). Cardiomyocytes showed weak positive immunoreaction to anti-Vsm-RhoGEF, which was consistent with the immunoblot analysis (Figure 2A); however, other parenchymal cells or blood cells did not show any immunoreactivity. We further examined the expression of EphA4 using serial sections used in those examined for Vsm-RhoGEF expression (Figure 2C). Notably, EphA4 was similarly expressed in the vascular smooth muscle that expressed Vsm-RhoGEF. Furthermore, immunoreactivity for EphA4 was found in cardiomyocytes and glomeruli in the kidney. Vascular endothelium was immunopositive for EphA4 but not for Vsm-RhoGEF, as indicated by the arrowhead (Figures 2B and 2C, bottom middle panels). These results suggested that the EphA4-Vsm-RhoGEF complex may function in VSMCs.

To confirm the coexpression and association of Vsm-RhoGEF with EphA4, we performed coimmunoprecipitation experiments using VSMCs. Both HCASMCs and rat aortic smooth muscle cells expressed EphA4 and Vsm-RhoGEF, as shown by the immunoblots detecting endogenous EphA4 and Vsm-RhoGEF (Figure 3A). Neither EphA4 nor Vsm-RhoGEF was detected in 293T cells used as a negative control. Vsm-RhoGEF was coimmunoprecipitated by anti-EphA4 in A7r5 cells, but not by normal rabbit serum used as a negative control (Figure 3B), indicating that Vsm-RhoGEF associates with EphA4 in VSMCs.

Tyrosine Phosphorylation of Vsm-RhoGEF Is Induced by Activation of EphA4 Upon Ephrin-A1 Stimulation

To understand the molecular mechanism of Vsm-RhoGEF regulation by EphA4, we examined EphA4 activation–dependent tyrosine phosphorylation of Vsm-RhoGEF. HA-tagged EphA4 in 293T cells was autophosphorylated irrespective of preclustered ephrin-A1/Fc stimulation (Figure 4A, lanes 3 and 4, indicated by arrow). Furthermore, we found that...
EGFP-tagged Vsm-RhoGEF was phosphorylated on tyrosine residues when it was cotransfected with HA-tagged EphA4 (Figure 4A, lanes 5 and 6, indicated by double arrow). These data suggested that the tyrosine phosphorylation of Vsm-RhoGEF depends on that of EphA4. Thus, we tested whether phosphorylation of Vsm-RhoGEF is dependent on ephrin-A1–induced EphA4 phosphorylation in A7r5 cells. Both EphA4 and Vsm-RhoGEF were tyrosine-phosphorylated upon ephrin-A1 stimulation (Figure 4B).

EphA4 Activation Upon Ephrin-A1 Stimulation Induces RhoA Activation in VSMCs
The DH-PH–containing GEFs have guanine nucleotide exchange activity for members of the Rho family GTPases including Rho, Rac, and Cdc42. We expected that Vsm-RhoGEF would function as a GEF for RhoA, regulating actin–myosin II coupling, based on its specific expression in VSMCs. Indeed, when EphA4 and Vsm-RhoGEF were coexpressed in 293T cells, GTP-bound RhoA was increased, as demonstrated by pull-down assay using GST-Rhotekin (Figure 5A, lane 4). These results and the results shown in Figure 4A indicated that EphA4 phosphorylation induces the phosphorylation of Vsm-RhoGEF and enhance its GEF activity for RhoA.

We then tested whether Vsm-RhoGEF induces the assembly of actin stress fiber, which is a typical consequence of RhoA activation. A7r5 cells overexpressing Vsm-RhoGEF exhibited increased assembly of actin stress fibers compared with untransfected cells (Figure 5B). Moreover, we examined the guanine nucleotide exchange activity of Vsm-RhoGEF for Rac1 and Cdc42 by pull-down assay using GST-PAK (Figure 5C). Vsm-RhoGEF exhibited GEF activity for neither Rac1 nor Cdc42, indicating that Vsm-RhoGEF functions as a specific GEF for RhoA.

Ephrin-A1 Induces RhoA Activation in A7r5 Cells
To examine whether ephrin-A1 induces RhoA activation and the subsequent assembly of actin stress fibers, we stimulated A7r5 cells with preclustered ephrin-A1/Fc. GTP-bound RhoA was increased in a time-dependent manner, as shown in Figure 6A. This increase in GTP-bound RhoA reached a maximum at 20 minutes after ephrin-A1 stimulation (Figure 6A, top and bottom). These data indicate that Vsm-RhoGEF functions as a GEF for RhoA downstream of EphA4 when both EphA4 and Vsm-RhoGEF are phosphorylated upon ephrin-A1 stimulation (Figures 4B and 6A).

We further examined whether ephrin-A1 induced the assembly of actin stress fibers in A7r5 cells. Cells serum-starved for 6 hours were stimulated with preclustered ephrin-A1/Fc for the time indicated at the bottom of the figure. The most increased assembly of stress fibers was found 30 minutes after stimulation (Figure 6B, bottom middle panel). This prominent stress fiber formation followed the RhoA activation with an approximate 10-minute delay (Figures 6A and 6B).

Vsm-RhoGEF Is Required for Ephrin-A1–Induced Assembly of Actin Stress Fibers in A7r5 Cells
To investigate whether Vsm-RhoGEF is essential for ephrin-A1–induced actin stress fiber formation, we overexpressed the PH domain of Vsm-RhoGEF to inhibit the association of Vsm-RhoGEF with EphA4 (Figure 1D). A7r5 cells expressing the mutant marked by IRES-driven EGFP expression exhibited fewer actin stress fibers than untransfected cells before ephrin-A1 stimulation (Figure 7A, top). This reduction in stress fibers in cells transfected with a dominant-negative mutant remained unchanged in response to preclustered ephrin-A1/Fc (Figure 7A, bottom). These observations suggest that Vsm-RhoGEF is involved in the regulation of actin stress fiber formation even in unstimulated cells. In addition, we performed an RNA interference experiment to examine the effect of Vsm-RhoGEF on actin stress fiber and RhoA activation. A7r5 cells transfected with double-stranded small-
interference RNA specific to Vsm-RhoGEF exhibited less actin stress fiber and became shrunken, which paralleled the reduction of Vsm-RhoGEF (Figure 7B). Furthermore, A7r5 depleted of Vsm-RhoGEF did not show the increase in GTP-bound RhoA upon ephrin-A1 stimulation. These results indicate that Vsm-RhoGEF is required for ephrin-A1–dependent RhoA activation, which is a prerequisite for regulating actin stress fiber in A7r5 cells.

**Discussion**

RhoA is involved in Ca\(^{2+}\)-independent vascular smooth muscle contraction via Rho-kinase. Most circulating vasoconstrictives, including angiotensin II, endothelin, and vasopressin, are suggested to induce smooth muscle contraction partly in a Ca\(^{2+}\)-independent Rho–Rho-kinase–dependent manner via G\(_{12/13}\) and partly in a Ca\(^{2+}\)-dependent manner via G\(_{q11}\). We demonstrated in the present study a novel Rho activation pathway triggered by the ephrin-A1–EphA4 interaction, which induces Vsm-RhoGEF phosphorylation in VSMCs.

Ephrin-Eph signaling is required not only for embryonic vascular development but also for angiogenesis by modulating endothelial cell migration and/or proliferation. We have previously demonstrated that human aortic endothelial cells express EphB1 and that EphB1 activation causes membrane ruffling, a hallmark of increased cell motility. Steinle et al. reported that EphB4 activation upon ephrin-B2 stimulation promotes endothelial cell migration via phosphatidylinositol 3-kinase. Conversely, ephrin-B1 upon EphB1 stimulation is shown to trigger angiogenesis, which was demonstrated by a corneal angiogenesis assay. These data implicate the Eph-ephrin system in the regulation of vasculature. To date, only two reports demonstrated that ephrin-B2 is expressed in arterial vascular smooth muscle; however, the physiological function of the Eph-ephrin system in VSMCs is unknown. We have shown for the first time that EphA4 is expressed in VSMCs in addition to vascular endothelial cells. Furthermore, we have found that an EphA4–associating molecule, Vsm-RhoGEF/KIAA0915, is specifically expressed in VSMCs.

We found that Vsm-RhoGEF associates with EphA4. Since ephexin, structurally related to Vsm-RhoGEF, contains DH and PH domains and associates with EphA4, we tested whether the DH and PH domains of Vsm-RhoGEF were required for its association with EphA4. Both DH and PH domains of Vsm-RhoGEF are necessary for its association with EphA4, as shown in Figure 1. Previously, an intramolecular interaction of PH domain and DH domain has been...
shown in Vav, one of the RhoGEF family members.\textsuperscript{29} This intramolecular folding of DH and PH domains may occur and be required for the association of Vsm-RhoGEF with EphA4. Indeed, overexpression on the PH domain of Vsm-RhoGEF perturbed the association with EphA4 (Figure 1D). The PH domain alone functioned as a dominant-negative peptide, as shown in Figure 7A. PH domain functions as a membrane-domain alone functioned as a dominant-negative peptide, as perturbed the association with EphA4 (Figure 1D). The PH domain of Vsm-RhoGEF associated with EphA4 affects basal contraction via RhoA irrespective of ephrin. Upon ephrin-EphA4 interaction triggered by cell-cell contact including T lymphocyte–VSMC and platelet–VSMC, EphA4 becomes autophosphorylated and induces tyrosine phosphorylation of Vsm-RhoGEF and the subsequent enhancement of GEF activity for RhoA, thereby inducing vascular smooth muscle contraction.

Vsm-RhoGEF functioned as a GEF for Rho and regulated actin stress fiber formation in VSMCs. Vsm-RhoGEF activity depends on its tyrosine phosphorylation, as shown in Figure 5A. Stimulation with preclustered ephrin-A1/Fc induces the phosphorylation of both EphA4 and Vsm-RhoGEF, thereby increasing the GEF activity of Vsm-RhoGEF. We previously reported that the activity of C3G, a GEF for Rap1, is increased upon phosphorylation at Tyr504.\textsuperscript{34} Furthermore, the GEF activity of PDZ-RhoGEF and LARG, which link GTP to RhoA, is enhanced by tyrosine phosphorylation by nonreceptor tyrosine kinases, FAK and Tec, respectively.\textsuperscript{35,36} Thus, the tyrosine phosphorylation–dependent regulation of Vsm-RhoGEF is similar to other GEFs.

We observed that the reduction of Vsm-RhoGEF expression in A7r5 cells using RNA interference resulted in cell shrinkage and less stress fiber formation. These results suggested that basal RhoA activity regulated by Vsm-RhoGEF contributes to the maintenance of cell shape by regulating stress fiber formation and/or focal adhesion assembly.\textsuperscript{11} Vsm-RhoGEF–regulated RhoA activity may also control basal muscular contractility by modulating the phosphorylation of both MLC and MLCP via Rho-kinase in vascular smooth muscle.\textsuperscript{1} To address this issue, it will be interesting to see the phenotype of Vsm-RhoGEF gene-disrupted mice.

In conclusion, we have demonstrated a novel RhoA regulatory signaling pathway in VSMCs. Ephrin triggers EphA4–Vsm-RhoGEF–mediated RhoA activation in VSMCs. The GEF activity of Vsm-RhoGEF depends on its tyrosine phosphorylation after ephrin-A1–mediated EphA4 tyrosine phosphorylation (Figure 8). These results suggest that the EphA4–Vsm-RhoGEF-RhoA pathway may play a role in the regulation of blood pressure, atherogenesis, and thrombosis-triggered spasm.

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References

7. Scherer EQ, Herzog M, Wangemann P. Endothelin-1–induced vaso- 
spasms of spiral modiolar artery are mediated by Rho-kinase–induced 
Ca2+ sensitization of contractile apparatus and reversed by calcitonin 
G. Human uterotonin II–induced contraction and arterial smooth muscle 
cell proliferation are mediated by RhoA and Rho-kinase. Circ Res. 2001; 
88:1102–1104.
9. Hart MJ, Jiang X, Kozasa T, Roscoe W, Singer WD, Gilman AG, 
Hart MJ, Jiang X, Kozasa T, Roscoe W, Singer WD, Gilman AG, 
Gilman AG. Human urotensin II receptor signaling mediates 
endothelial cell proliferation are mediated by RhoA and Rho-kinase. 
12. Eph Nomenclature Committee. Unified nomenclature for Eph family 
receptors and their ligands, the ephrins. Cell. 1997;90:403–404.
13. Wang HU, Chen ZF, Anderson DJ. Molecular distinction and angiogenic 
and ephrins provide a mechanism to support platelet aggregation once cell-
mediated migration, attachment and angiogenesis. J Cell Sci. 2002;115: 
3073–3081.
mediators of vascular development. Trends Cardiovasc Med. 2000;10: 
183–188.
B4 receptor signaling mediates endothelial cell migration and prolif-
2002;277:43830–43835.
Matsuda M, Mochizuki N. Adaptor protein Crk is required for ephrin-
Bazalakova M, Neve RL, Corfas G, Debant A, Greenberg ME. EphA 
receptors regulate growth cone dynamics through the novel guanine 
18. Niwa H, Yamamura K, Miyazaki J. Efficient selection for high-
expressing transfectants with a novel eukaryotic vector. Gene. 1991;108: 
193–199.
N. Sphingosine 1-phosphate induces membrane ruffling and increases 
motility of human umbilical vein endothelial cells via vascular endothel-
ial growth factor receptor and CrkII. J Biol Chem. 2002;277: 
23747–23754.
defines enhanced spontaneous and agonist-induced contraction in aorta 
of deoxycorticosterone acetate-salt hypertensive rats. Circ Res. 2002;91: 
360–369.
Tanaka S, Takahashi H, Nagashima K. Expression of JC virus agno-
protein in progressive multifocal leukoencephalopathy brain. Acta Neu-
Membrane recruitment of DOCK180 by binding to PtdIns(3,4,5)P3. 
23. Reid T, Furuyashiki T, Ishizaki T, Watanabe G, Watanabe N, Fujisawa K, 
Mori N, Madaule P, Narumiya S. Rhoetkin, a new putative target for Rho 
bearing homology to a serine/threonine kinase, PKN, and rhophilin in the 
Risau W, Kleinf R. Roles of ephrinB ligands and EphB receptors in 
cardiovascular development: demarcation of arterial/venous domains, 
vascular morphogenesis, and sprouting angiogenesis. Genes Dev. 1999; 
Chen J, Daniel TO. Ephrin-B1 transduces signals to activate integrin-
ediated migration, attachment and angiogenesis. J Cell Sci. 2002;115: 
3073–3081.
27. Gale NW, Baluk P, Pan L, Kwan M, Holash J, DeChiara TM, McDonald 
DM, Yancopoulos GD. Ephrin-B2 selectively marks arterial vessels and 
neovascularization sites in the adult, with expression in both endothelial 
G, Isner J, Folkman J, Gimbome MA Jr, Anderson DJ. Expression of 
ephrinB2 identifies a stable genetic difference between arterial and 
venous vascular smooth muscle as well as endothelial cells, and marks 
substrates of microvessels at sites of adult neovascularization. Dev Biol. 
2001;230:139–150.
29. Das B, Shu X, Day GI, Han J, Krishna UM, Falck JR, Broek D. Control 
of intramolecular interactions between the pleckstrin homology and Dbl 
homology domains of Vav and Sos1 regulates Rac binding. J Biol Chem. 
2002;277:15074–15081.
30. Whitehead JP, Lambert QT, Glaiven JA, Abe K, Rossman KL, Mahon 
GM, Trzaskos JM, Kay R, Campbell SL, Der CJ. Dependence of Dbl and 
Dbs transformation on MEK and NF-κB activation. Mol Cell Biol. 1999; 
19:7759–7770.
31. Prevost N, Wouffe D, Tanaka T, Brass LF. Interactions between Eph 
ikines and ephrins provide a mechanism to support platelet aggregation once cell-
to-cell contact has occurred. Proc Natl Acad Sci U S A. 2002;99:9219–9224.
32. Aasheim HC, Munthe E, Funderud S, Smeland EB, Breike K, Log-
tenberg T. A splice variant of human ephrin-A4 encodes a soluble 
molecule that is secreted by activated human B lymphocytes. Blood. 
34. Ichiba T, Hashimoto Y, Nakaya M, Kuraishi Y, Tanaka S, Kurata T, 
Mochizuki N, Matsuda M. Activation of C3G guanine nucleotide 
1999;274:14376–14381.
35. Chikumi H, Fukuhara S, Gutkind JS. Regulation of G protein-linked 
guanylate nucleotide exchange factors for Rho, PDZ-RhoGEF, and LARG 
by tyrosine phosphorylation: evidence of a role for focal adhesion kinase. 
through tyrosine-phosphorylated leukemia-associated RhoGEF. Proc 
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