Rho-kinase Contributes to Sustained RhoA Activation through Phosphorylation of p190A RhoGAP*§

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RhoA is transiently activated by specific extracellular signals such as endothelin-1 (ET-1) in vascular smooth muscle cells. RhoGAP negatively regulates RhoA activity: thus, RhoA becomes the GDP-bound inactive form afterward. Sustained activation of RhoA is induced with high doses of the extracellular signals and is implicated in certain diseases such as vasospasms. However, it remains largely unknown how prolonged activation of RhoA is induced. Here we show that Rho-kinase, an effector of RhoA, phosphorylated p190A RhoGAP at Ser1150 and attenuated p190A RhoGAP activity in COS7 cells. Binding of Rnd to p190A RhoGAP is thought to enhance its activation. Phosphorylation of p190A RhoGAP by Rho-kinase impaired Rnd binding. Stimulation of vascular smooth muscle cells with a high dose of ET-1 provoked sustained RhoA activation and p190A RhoGAP phosphorylation, both of which were prohibited by a Rho-kinase inhibitor. The phosphomimic mutation of p190A RhoGAP weakened Rnd binding and RhoGAP activities. Taken together, these results suggest that ET-1 induces Rho-kinase activation and subsequent phosphorylation of p190A RhoGAP, leading to prolonged RhoA activation.

RhoA small GTPase is the molecular switch for various extracellular signals and is implicated in a variety of biological functions, including cell contraction, cell migration, cell adhesion, cell cycle progression, and gene expression (1, 2). RhoA regulates these functions through its specific effectors such as Rho-kinase/ROCK/ROK and mDia (2). We previously found that Rho-kinase phosphorylates myosin phosphatase target protein 1 (MYPT1)3 of myosin phosphatase and thereby inactivates its phosphatase activity, resulting in an increase in the phosphorylation of myosin light chain followed by smooth muscle contraction (3–5). Rho-kinase increases the agonist-induced Ca2+ sensitivity and contributes to sustained contraction of smooth muscle (6).

RhoA cycles between the GTP-bound active and GDP-bound inactive conformations. This cycle is under the direct control of three groups of regulatory proteins: the guanine nucleotide exchange factors (GEFs), which catalyze the exchange of GDP for GTP to activate RhoA; GTPase-activating proteins (GAPs), which enhance the intrinsic GTPase activity of RhoA to promote hydrolysis of GTP to GDP to inactivate RhoA; and the guanine nucleotide dissociation inhibitors, which sequester the GDP-bound RhoA and may also regulate its intracellular localization (1, 2).

The typical RhoGEFs contain a catalytic Dbl homology domain and an adjacent pleckstrin homology domain. This Dbl homology-associated pleckstrin homology domain interacts with phospholipids, which may localize GEFs to the plasma membrane and activate GEF activity (7, 8). RhoA activation is often mediated by G protein-coupled receptors. Three RhoGEFs, which contain the regulator of G protein-signaling domains of these RhoGEFs and positively regulate their GEF activity (12). The typical RhoGAPs have a catalytic domain and various domains for protein-protein interaction. Recent studies suggest that RhoGAPs are regulated by various mechanisms, including protein–protein interaction, phospholipid interaction, phosphorylation, subcellular translocation, and proteolytic degradation (13, 14). However, the precise mechanisms that regulate RhoGAP activity remain elusive in many cases.

When the smooth muscle cells are stimulated with agonists such as ET-1, RhoA is transiently activated presumably through RhoA-specific GEFs such as leukemia-associated RhoGEF and inactivated later (11, 15). The RhoA-specific GAP appears to be responsible for RhoA inactivation under physiological conditions (16). Sustained RhoA/Rho-kinase activation occurs with GTPase-activating protein; FL, full length; RBD, Rho-binding domain; WT, wild-type; GFP, green fluorescent protein; GST, glutathione S-transferase; aa, amino acid(s); HA, hemagglutinin; Ab, antibody.

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§ The abbreviations used are: MYPT1, myosin phosphatase target protein-1; ET-1, endothelin-1; GEF, guanine nucleotide exchange factor; GAP, GTPase-activating protein; FL, full length; RBD, Rho-binding domain; WT, wild-type; GFP, green fluorescent protein; GST, glutathione S-transferase; aa, amino acid(s); HA, hemagglutinin; Ab, antibody.
high doses of ET-1 (17). Prolonged activation of RhoA/Rho-kinase is implicated in the pathogenesis of certain vascular diseases, including subarachnoid hemorrhage-induced cerebral vasospasm, coronary vasospasm, essential hypertension, and pulmonary hypertension (18, 19). For example, RhoA activity is higher in aortic smooth muscle cells derived from the stroke-prone spontaneously hypertensive rat than from the wild-type rat, although the expression levels of RhoA are not different between mutant and wild-type rats (20). Rho-kinase activity is up-regulated, and phosphorylation levels of MYPT1 are increased in the coronary spastic lesion in a porcine swine model (21). Subarachnoid hemorrhage induces sustained Rho-kinase activation in the canine basilar artery and subsequent cerebral vasospasm (22). Chronic hypoxia-induced pulmonary hypertension in rats is associated with an increase of RhoA activity in pulmonary artery (23). However, it remains largely unknown how prolonged activation of RhoA is induced.

In light of these observations, we hypothesized that highly activated RhoA/Rho-kinase can inhibit Rho-specific GAP and lead to sustained RhoA activation. Here we show that Rho-kinase phosphorylated p190A RhoGAP, the best characterized RhoA-specific GAP, at Ser1150 in vitro and in vivo. Phosphorylation of p190A RhoGAP by Rho-kinase appeared to attenuate its GAP activity.

**EXPERIMENTAL PROCEDURES**

**Materials and Chemicals**—The cDNA-encoding human p190A RhoGAP (KIAA1722, p190A) was obtained from the Kazusa DNA Research Institute (Chiba, Japan). Monoclonal anti-GFP antibody was purchased from Roche Diagnostics (Mannheim, Germany). Polyclonal anti-GFP antibody was from MBL (Nagoya, Japan). Monoclonal anti-p190A RhoGAP antibodies were from BD Biosciences Pharmingen (San Diego, CA) and Upstate Biotechnology (Lake Placid, NY). Monoclonal anti-RhoA antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal anti-HA antibody (12CA5) was from Boehringer (Ingelheim, Germany). Polyclonal anti-MYPT1-pT853 antibody was from Upstate Biotechnology. Polyclonal anti-total MYPT1 antibody was generated as previously described (24). A rabbit polyclonal antibody against p190A RhoGAP phosphorylated at Ser1150 was produced against the phosphopeptide Cys-Arg-Gly-Arg-Lys-Val-phospho-Ser1150-Ile-Val-Ser-Lys-Pro1155 by Biologica Co. (Nagoya, Japan). Y-27632, a Rho-kinase-specific inhibitor, was obtained from commercial sources. Glutathione S-transferase (GST) fusion proteins were produced in Escherichia coli BL21(DE3) and purified on glutathione-Sepharose 4B beads (GE Healthcare). GST-Rho-kinase-CAT (aa 6–553), a constitutively active form of Rho-kinase, and GST-p190A RhoGAP-4 + 5 (aa 953–1499) were produced in Sf9 cells with a baculovirus system and purified on glutathione-Sepharose 4B beads.

**Phosphorylation Assay**—The phosphorylation assay was performed as previously described (25). In brief, the kinase reaction of Rho-kinase for p190A RhoGAP was carried out in 50 μl of the reaction mixture (50 mM Tris/HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 5 mM MgCl2) containing 100 μM [γ-32P]ATP (1–20 GBq/mmol), purified GST-Rho-kinase-CAT (0.001–0.1 μM), and 1 μM purified GST-p190A RhoGAP fragments. After incubation for 10 min at 30 °C, the reaction mixtures were boiled in SDS sample
buffer and subjected to SDS-PAGE. The radiolabeled bands were visualized and estimated by an image analyzer (BAS2000, Fuji, Tokyo, Japan).

GAP Assay—The RhoA GAP assay was performed as previously described (26). Briefly, recombinant GST–RhoA was preloaded with 1 μM [γ-32P]GTP (222 TBq/mmol, PerkinElmer Life Sciences) in 25 μl of buffer containing 50 mM HEPS, pH 7.4, 50 mM NaCl, 0.1 mM dithiothreitol, 0.1 mM EGTA, 5 mM EDTA, and 1 mg/ml bovine serum albumin for 10 min at 30 °C before the addition of MgCl2 to a final concentration of 10 mM. An aliquot of [γ-32P]GTP-loaded GST–RhoA was mixed with the GAP assay buffer, which contained 25 mM HEPS, pH 7.5, 50 mM NaCl, 1 mM MgCl2, 0.1 mM dithiothreitol, 0.1 mM GTP, and 1 mg/ml bovine serum albumin in the presence of nonphosphorylated GST-p190A RhoGAP-4 + 5 or phosphorylated GST-p190A RhoGAP-4 + 5. The reaction was performed for 5 min at 30 °C and terminated by rapid addition of 5 ml of ice-cold buffer containing 50 mM HEPS, pH 7.5, 50 mM NaCl, and 1 mM MgCl2. The samples were then immediately deposited onto nitrocellulose filters. The radioactivity retained on the filter was then subjected to quantitative analysis by scintillation counting. RhoGAP activity was detected as the remainder of [γ-32P]GTP bound to GST–RhoA.

GTP–Rho Pulldown Assay—The activity of RhoA was determined by pulldown assay with the GST–Rho-binding domain of Rhotekin (GST–Rhotekin-RBD) as previously described (27). Briefly, the cells were washed with ice-cold phosphate-buffered saline and lysed in 500 μl of lysis buffer (50 mM Tris/HCl, pH 7.5, 1 mM EDTA, 10 mM MgCl2, 500 mM NaCl, 0.5% Nonidet P-40, 0.1 mM (p-aminodiphenyl)methanesulfonyl fluoride, 2.5 μg/ml aprotinin, 2.5 μg/ml leupeptin) containing 20 μg of GST–Rhotekin-RBD. The lysates were centrifuged at 20,000 × g for 3 min at 4 °C, and the supernatants were incubated with glutathione-Sepharose 4B beads for 30 min at 4 °C. The beads were washed with an excess of lysis buffer and then eluted with SDS-sample buffer. The eluates were subjected to SDS-PAGE, followed by immunoblot analysis with anti-GFP antibody or anti-RhoA antibody.

Rnd1-binding Assay—COS7 cells were transiently transfected with pEF-BOS-HA-Rnd1. The cells were washed with phosphate-buffered saline and lysed with lysis buffer (20 mM Tris/HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 1 mM dithiothre-
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itol, 1% Nonidet P-40, 0.2 μM Calcium A, 1 mM sodium orthovanadate, 0.1 mM (p-aminophenyl)methanesulfonyl fluoride, 2.5 μg/ml aprotonin, 2.5 μg/ml leupeptin. The lysates were centrifuged at 20,000 × g for 20 min at 4 °C, and the supernatants were incubated with glutathione-Sepharose 4B beads coated with 200 or 400 pmol of GST, 100 or 200 pmol of phosphorylated GST-p190A RhoGAP-4, and 100 or 200 pmol of nonphosphorylated GST-p190A RhoGAP-4 for 1 h at 4 °C. The beads were washed, and the eluates were subjected to SDS-PAGE, followed by immunoblot analysis with anti-HA antibody.

Cell Culture and Agonist Stimulation—Human aortic smooth muscle cells were obtained from Takara Bio Inc. (Shiga, Japan) and cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Human aortic smooth muscle cells at the sixth passage were transfected with GFP-p190A RhoGAP-FL by using the Nucleofector system (Axima, Cologne, Germany). 24 h after transfection, the cells were starved for serum of 8 h and then stimulated with 1 μM ET-1. For the experiments with inhibitors, the cells were pretreated with inhibitors 30 min before ET-1 stimulation.

Measurement of Cell Size—To measure the cell size, human aortic smooth muscle cells were transfected with plasmids by using the Nucleofector system and then seeded on glass coverslips coated with fibronectin (BD Biosciences Pharmingen). 24 h after transfection, the cells were fixed with 3.0% formaldehyde in phosphate-buffered saline for 10 min and then treated with phosphate-buffered saline containing 0.1% Triton X-100 for 10 min. The cell area was measured with a laser scanning confocal microscope (model LSM510, Carl Zeiss, Oberkochene, Germany).

RESULTS

Counteraction of p190A RhoGAP Function by Rho-kinase—p190A and p190B RhoGAPs are ubiquitously expressed in various tissues and display GAP activity exclusively toward RhoA in vivo (28, 29). p190RhoGAP activity accounts for ~60% of the total RhoGAP activity detected in whole cell extracts in fibroblasts (30). Inhibition of p190RhoGAP activity is sufficient to promote RhoA activation in fibroblasts (30). Knockdown of p190A RhoGAP activity using siRNA increases RhoA activity in spreading microvascular endothelial cells (31). Thus, p190RhoGAP appears to account for the majority of RhoGAP activity.

Hence, we first examined whether Rho-kinase affects the p190A RhoGAP function in intact cells. GFP-RhoA was transfected into COS7 cells, and the amount of the GTP-bound form of GFP-RhoA was monitored by pulldown assay (Fig. 1). The expression of GFP-p190A RhoGAP-FL decreased the amount of GTP-bound GFP-RhoA in COS7 cells, suggesting that GFP-p190A RhoGAP-FL acts as RhoAGAP. The amount of GTP-bound GFP-RhoA was greater in the cells expressing GFP-p190A RhoGAP-FL and GFP-Rho-kinase-CAT than that in the cells expressing GFP-p190A RhoGAP-FL alone. This result suggests that Rho-kinase counteracts the GAP activity of p190A RhoGAP in COS7 cells.

In Vitro Phosphorylation of p190A RhoGAP by Rho-kinase—We then examined whether p190A RhoGAP is phosphorylated by Rho-kinase in vitro. To make a full length of p190A RhoGAP, we transiently transfected COS7 cells with GFP-p190A RhoGAP-FL, and immunoprecipitated GFP-p190A RhoGAP-FL from cell lysates with a polyclonal anti-GFP antibody. The immunoprecipitated GFP-p190A RhoGAP-FL was effectively phosphorylated by GST-Rho-kinase-CAT in vitro (Fig. 2B). We also found that Rho-kinase phosphorylated p190B RhoGAP (data not shown).

To determine the phosphorylation sites of p190A RhoGAP by Rho-kinase, p190A RhoGAP was divided into five fragments, including GST-p190A RhoGAP-1 (aa 1–333), p190A RhoGAP-2 (aa 334–637), p190A RhoGAP-3 (aa 632–952), p190A RhoGAP-4 (aa 953–1208), and p190A RhoGAP-5 (aa 1209–1499) (Fig. 2A). These fragments were produced from E. coli and then purified. GST-p190A RhoGAP-4 and p190A RhoGAP-5 were effectively phosphorylated by GST-Rho-kinase-CAT, whereas GST-p190A RhoGAP-1, GST-p190A RhoGAP-2, and GST-p190A RhoGAP-3 were not phosphorylated (Fig. 2C). To identify potential phosphorylation sites in p190A RhoGAP, liquid chromatography tandem mass spectrometry was performed and three potential phosphorylation sites in p190A RhoGAP-4 were identified, namely Ser1150, Thr1173, and Ser1174 (Fig. 2D). To determine the major phosphorylation sites, we substituted Ser1150, Thr1173, or Ser1174 with Ala to produce GST-p190A RhoGAP-4-S1150A, GST-p190A RhoGAP-4-T1173A, and GST-p190A RhoGAP-4-S1174A. However, the single Ala substitution did not affect the
phosphorylation efficiency (data not shown). Then, Ser\textsuperscript{1150}, Thr\textsuperscript{1173}, and Ser\textsuperscript{1174} were simultaneously substituted with Ala to produce GST-p190A RhoGAP\textsuperscript{-4-AAA}. The phosphorylation efficiency of GST-p190A RhoGAP\textsuperscript{-4-AAA} was substantially reduced compared with the wild type (Fig. 2E), suggesting that at least two potential phosphorylation sites among these three sites are efficient phosphorylation sites.

The phosphorylation sites in p190A RhoGAP\textsuperscript{-5} were not identified by liquid chromatography tandem mass spectrometry. To identify potential phosphorylation sites in p190A RhoGAP\textsuperscript{-5}, we produced the additional deletion mutants, including GST-p190A RhoGAP\textsuperscript{-5N1} (aa 1222–1499), p190A RhoGAP\textsuperscript{-5N2} (aa 1229–1499), and p190A RhoGAP\textsuperscript{-5N3} (aa 1249–1499) (Fig. 2A). GST-p190A RhoGAP\textsuperscript{-5N1} was highly phosphorylated and GST-p190A RhoGAP\textsuperscript{-5N2} was moderately phosphorylated by GST-Rho-kinase-CAT, whereas GST-p190A RhoGAP\textsuperscript{-5N3} was not phosphorylated (supplemental Fig. S1A), suggesting that phosphorylation sites exist in aa 1222–1228 and aa 1229–1248. Because (R/K)XX(S/T) or (R/K)X(S/T) (X is any amino acid) is the consensus phosphorylation sequence by Rho-kinase, the potential phosphorylation sites in p190A-5 are Thr\textsuperscript{1226} in aa 1222–1228, and Ser\textsuperscript{1236} and Thr\textsuperscript{1241} in aa 1229–1248 (Fig. 2D), suggesting that Thr\textsuperscript{1226} is the putative major phosphorylation site. To determine the major phosphorylation sites in p190A RhoGAP\textsuperscript{-5}, we produced GST-p190A RhoGAP\textsuperscript{-5-T1226A/S1236A} (GST-p190A RhoGAP\textsuperscript{-5-AA}) and GST-p190A RhoGAP\textsuperscript{-5-T1226A/T1241A}. GST-p190A RhoGAP\textsuperscript{-5-AA} was minimally phosphorylated by GST-Rho-kinase-CAT (Fig. 2F). The degree of phosphorylation of GST-p190A RhoGAP\textsuperscript{-5-T1226A/T1241A} by GST-Rho-kinase-CAT was approximately half (supplemental Fig. S1B). Taken together, these results suggest that Rho-kinase phosphorylates p190A RhoGAP presumably at a minimum of five sites, including Ser\textsuperscript{1150}, Thr\textsuperscript{1173}, Ser\textsuperscript{1174}, Thr\textsuperscript{1226}, and Ser\textsuperscript{1236} in vitro.

**In Vivo Phosphorylation of p190A RhoGAP by Rho-kinase**—To examine the phosphorylation state of p190A RhoGAP by Rho-kinase in vivo, we prepared rabbit polyclonal antibodies that specifically recognize p190A RhoGAP phosphorylated at respective phosphorylation sites. Among them, the anti-p190A RhoGAP-pS1150 antibody specifically recognized GST-p190A

![Phosphorylation of p190A RhoGAP by Rho-kinase](image)
Phosphorylation of p190A RhoGAP by Rho-kinase

RhoGAP-4 phosphorylated by GST-Rho-kinase-CAT in a dose-dependent manner, but not phosphorylated GST-p190A RhoGAP-4-S1150A (Fig. 3A), indicating that the antibody specifically recognized GST-p190A RhoGAP-4 that was phosphorylated at Ser$^{1150}$. The anti-p190A RhoGAP-pT1173 and pS1174 antibodies only slightly recognized phosphorylated p190A RhoGAP, suggesting that Thr$^{1173}$ and Ser$^{1174}$ are not major phosphorylation sites. Alternatively, these antibodies did not work well on phosphorylated p190A RhoGAP, although they recognized the antigen phosphopeptides (data not shown). The anti-p190A RhoGAP-pT1226 and -pS1236 antibodies could recognize the phosphorylated p190A RhoGAP in vitro in a manner similar to that of anti-p190A RhoGAP-pS1150 antibody (data not shown).
To examine whether Rho-kinase phosphorylates p190A RhoGAP in intact cells, GFP-p190A RhoGAP-FL was exogenously co-transfected with GFP-Rho-kinase-CAT into COS7 cells. Co-transfection of Rho-kinase-CAT resulted in an increase of phosphorylated GFP-p190A RhoGAP-FL at Ser\(^{1150}\) (Fig. 3B). Treatment of the cells with Y-27632 inhibited phosphorylation of GFP-p190A RhoGAP-FL by GFP-Rho-kinase-CAT. GFP-Rho-kinase-CAT failed to phosphorylate GFP-p190A RhoGAP-FL-S1150A. Under the same conditions, phosphorylation of endogenous p190A RhoGAP at Ser\(^{1150}\) was not detected, presumably because the expression level of p190A RhoGAP was low in COS7 cells. Taken together, these results indicate that Rho-kinase can phosphorylate p190A RhoGAP at Ser\(^{1150}\) in COS7 cells. Similarly, the immunoblot analysis, through the use of the anti-p190A RhoGAP-pT1226 and -pS1236 antibodies, revealed that Rho-kinase can phosphorylate p190A RhoGAP at Thr\(^{1226}\) and Ser\(^{1236}\) in COS7 cells (data not shown).

Effects of Phosphorylation of p190A RhoGAP by Rho-kinase on Its GAP Activity—Does phosphorylation of p190A RhoGAP by Rho-kinase affect p190A RhoGAP functions? To examine the effects of phosphorylation on the GAP activity of p190A RhoGAP, we tried to produce and purify the full length of p190A RhoGAP from E. coli and insect cells, but the procedure failed. We then prepared nonphosphorylated and phosphorylated GST-p190A RhoGAP-4+5, which includes the identified five phosphorylation sites and the RhoGAP catalytic domain, and performed an in vitro GAP assay. Hydrolysis of GTP-bound GST-RhoA was accelerated by using purified GST-p190A RhoGAP-4+5 in a dose-dependent and time-dependent manner (supplemental Fig. S2). The GAP activity of GFP-p190A RhoGAP-4+5 was not dramatically affected by phosphorylation (Fig. 4, A and B).

We here found that Rho-kinase appeared to inhibit the p190A RhoGAP activity in COS7 cells (Fig. 1). How does Rho-kinase regulate the GAP activity of p190A RhoGAP in intact cells? Small GTPase Rnd is a member of the distinct subgroup of the Rho family GTPases and regulates the organization of actin cytoskeleton (32). Expression of Rnd inhibits the formation of the stress fibers in response to lysophosphatidic acid stimulation in fibroblasts (33), suggesting that Rnd antagonizes the action of RhoA. Consistently, Rnd binds to p190A RhoGAP and increases its GAP activity toward GTP-bound RhoA, resulting in RhoA inactivation (34, 35). This observation prompted us to examine whether phosphorylation of p190A RhoGAP affects its interaction with Rnd1. HA-Rnd1 efficiently interacted with GST-p190A RhoGAP-4 in the pulldown assay, and phosphorylation of GST-p190A RhoGAP-4 by Rho-kinase attenuated its interaction with Rnd1 (Fig. 5). Thus, it is conceivable that Rho-kinase suppresses the GAP activity of p190A RhoGAP by inhibiting the interaction with Rnd1 through phosphorylation.

Phosphorylation of p190A RhoGAP in Cultured Vascular Smooth Muscle Cells—To understand the physiological functions of p190A RhoGAP, we confirmed the expression profile of p190A RhoGAP in various rat tissues and found that p190A RhoGAP was highly expressed in brain, lung, and aorta (supplemental Fig. S3A). We also found that p190A RhoGAP was highly expressed in primary human aortic smooth muscle and endothelial cells (supplemental Fig. S3B).

Then, we monitored phosphorylation of endogenous p190A RhoGAP in human aortic smooth muscle cells. The basal phosphorylation level of p190A RhoGAP at Ser\(^{1150}\) was not detected (Fig. 6A). ET-1 is known to activate the Rho/Rho-kinase pathway (17). Stimulation of smooth muscle cells by ET-1 induced phosphorylation of endogenous p190A RhoGAP at Ser\(^{1150}\) (Fig. 6A). Treatment of the cells with Y-27632 inhibited ET-1-induced phosphorylation of p190A RhoGAP. As a positive control, the MYPT1 phosphorylation level was monitored. Phosphorylation of MYPT1 at Thr\(^{853}\) decreases the activity of myosin phosphatase in vascular smooth muscle cells: this phosphorylation is used as an indicator of the activity of Rho-kinase (17). ET-1 induced phosphorylation of MYPT1 at Thr\(^{853}\), whereas Y-27632 completely inhibited this phosphorylation. Taken together, these results suggest that ET-1 provoked phosphorylation of endogenous p190A RhoGAP at Ser\(^{1150}\) in a Rho-kinase dependent fashion in cultured vascular smooth muscle cells.

Of note, the immunoblot analysis using the anti-p190A RhoGAP-pT1226 and -pS1236 antibodies revealed that p190A RhoGAP was phosphorylated upon treatment with ET-1, but these phosphorylations were not dramatically inhibited by Y-27632, suggesting that these sites are not major phosphorylation sites by Rho-kinase in aortic smooth muscle cells (data not shown).

A high concentration of ET-1 has been shown to induce activation of Rho/Rho-kinase and subsequent MYPT1 phosphorylation, thereby resulting in the sustained contraction of vascular smooth muscle cells.

**FIGURE 6. Phosphorylation of p190A RhoGAP by Rho-kinase in cultured vascular smooth muscle cells.** A, phosphorylation of endogenous p190A RhoGAP in cultured vascular smooth muscle cells. The cells were incubated with 20 μM Y-27632 or DMSO for 30 min, and then treated with 1 μM ET-1 for 3 min. Endogenous p190A RhoGAP was immunoprecipitated with anti-p190A RhoGAP Ab (Upstate). The amounts of phosphorylated p190A RhoGAP were determined by immunoblot analysis with anti-p190A RhoGAP-pS1150 Ab. The amounts of phosphorylated and total MYPT1 were examined as a positive control. B, effect of Rho-kinase inhibitor on ET-1-induced sustained RhoA activation. After serum depletion for 8 h, the cells were incubated with 20 μM Y-27632 or DMSO for 30 min, and then stimulated with 1 μM ET-1 for the indicated periods of time. The cells were lysed with lysis buffer, and the lysates were incubated with GST-Rhotekin-RBD to precipitate the GTP-bound active form of RhoA. The eluates were analyzed by immunoblotting with anti-RhoA Ab. C, ET-1-induced sustained phosphorylation of p190A RhoGAP in cultured vascular smooth muscle cells. The cells were transiently transfected with GFP-p190A RhoGAP-FL with the use of the Nucleofector system. After serum depletion for 8 h, the cells were incubated with 20 μM Y-27632 or DMSO for 30 min, and then stimulated with 1 μM ET-1 for the indicated periods of time. The cells were subjected to SDS-PAGE followed by immunoblot analysis with anti-p190A RhoGAP-pS1150 Ab, anti-p190A RhoGAP Ab (Pharmingen), anti-MYPT1-pT853 Ab, and anti-MYPT1 Ab. D, effect of ET-1 antagonists on RhoA activity. The cells were starved for serum of 8 h and then stimulated with 1 μM ET-1 for 3 min after treatment with 1 μM BQ-123 or 1 μM BQ-788 for 30 min. The cells were lysed with lysis buffer, and the lysates were incubated with GST-Rhotekin-RBD to precipitate the GTP-bound active form of RhoA. The eluates were analyzed by immunoblotting with anti-RhoA Ab. E, effect of ET-1 antagonists on phosphorylation of p190A RhoGAP. The cells were transiently transfected with GFP-p190A RhoGAP-FL. Twenty-four hours after transfection, the cells were starved for serum of 8 h and then stimulated with 1 μM ET-1 for 3 min after treatment with BQ-123 (1 μM, 0.1 μM) or BQ-788 (1 μM, 0.1 μM) for 30 min. The cell lysates were subjected to immunoblot analysis with anti-p190A RhoGAP-pS1150 Ab and anti-p190A RhoGAP Ab (Pharmingen). These results are representative of three independent experiments.
We found that a high concentration of ET-1 induced sustained RhoA activation (supplemental Fig. S4), whereas Y-27632 suppressed ET-1-induced sustained RhoA activation (Fig. 6B and supplemental Fig. S4), suggesting that Rho-kinase is involved in prolonged RhoA activation. Because the sensitivity of the anti-p190A RhoGAP-pS1150 antibody is relatively low, we transiently transfected cultured vascular smooth muscle cells with GFP-p190A RhoGAP-FL to examine whether ET-1 induces sustained phosphorylation of p190A RhoGAP in a Rho/Rho-kinase-dependent manner (Fig. 6C). ET-1 induced sustained phosphorylation of GFP-p190A RhoGAP-FL at Ser1150, and Y-27632 effectively inhibited ET-1-induced phosphorylation of GFP-p190A RhoGAP-FL. Under the same conditions, ET-1 induced sustained phosphorylation of MYPT1 at Thr1831, whereas Y-27632 completely inhibited this phosphorylation (Fig. 6C). Other vasoconstrictors such as angiotensin II, acetylcholine, and thrombin slightly induced phosphorylation of p190A RhoGAP (data not shown). These results suggest that ET-1 induces sustained phosphorylation of p190A RhoGAP at Ser1150 in a Rho/Rho-kinase-dependent manner in cultured vascular smooth muscle cells.

There are three different types of ET receptors, ETA, ETB, and ETC. ETB receptors are classified into two subtypes, ETB1 and ETB2. ETA and ETB2 receptors are expressed in vascular smooth muscle, and both receptors mediate vascular smooth muscle contraction (36). To examine which types of ET receptors are involved in phosphorylation of p190A RhoGAP, vascular smooth muscle cells transfected with GFP-p190A RhoGAP-FL were treated with a selective ETA or ETB antagonist. BQ-123, a selective ETA antagonist, inhibited ET-1-induced RhoA activation and phosphorylation of p190A RhoGAP, whereas BQ-788, a selective ETB antagonist, minimally affected ET-1-induced RhoA activation and phosphorylation of p190A RhoGAP (Fig. 6, D and E). These results suggest that ET-1 induces p190A RhoGAP phosphorylation via RhoA activation through ETA receptor in cultured vascular smooth muscle cells.

Because the activity of p190A RhoGAP is thought to be regulated through the tyrosine phosphorylation by Src family kinases and Abl family kinases, we examined whether ET-1 affects the tyrosine phosphorylation state of p190A RhoGAP. Sodium orthovanadate, an inhibitor of phosphotyrosine phosphatase, increased the tyrosine phosphorylation level of GFP-p190A RhoGAP-FL. Under the same conditions, ET-1 did not induce the tyrosine phosphorylation of p190A RhoGAP (supplemental Fig. S5). These results suggest that ET-1 did not induce the tyrosine phosphorylation of p190A RhoGAP in cultured vascular smooth muscle cells.

Effect of Phosphomimic Mutant of p190A RhoGAP on Vascular Smooth Muscle Cell Size—To explore the effect of phosphorylation of p190A RhoGAP in vitro, we replaced three putative phosphorylation sites in p190A RhoGAP-4 with Glu to produce phosphomimic mutant of p190A RhoGAP. The binding of phosphomimic mutant to Rnd1. COS7 cells were transiently transfected with pEF-BOS-HA-Rnd1. The cell lysates were incubated with glutathione-Sepharose 4B beads coated with 200 pmol of GST, GST-p190A RhoGAP-4-WT, and GST-p190A RhoGAP-4-EEE for 1 h at 4 °C. The beads were washed, and the eluates were subjected to SDS-PAGE, followed by immunoblot analysis with anti-HA Ab. GST fusion proteins were visualized by silver staining. The result is representative of three independent experiments. B, effect of GFP-p190A RhoGAP-FL-5E on RhoA in COS7 cells. The activity of RhoA was monitored by pulldown assay with the GST-Rhotekin-RBD. The eluates were analyzed by immunoblotting with anti-GFP Ab (top). The ratio of GFP-RhoA-GTP to total GFP-RhoA is shown (bottom). Data represent the means ± S.E. of four independent experiments. C, effect of phosphomimic mutant of p190A RhoGAP on vascular smooth muscle cell size. The area of cells transfected with the indicated constructs was measured. Data are indicated as mean ± S.D. (n > 100 in each experiment), and these results are representative of three independent experiments. The asterisk indicates a significant difference (p < 0.01) from the value of GFP-p190A RhoGAP-FL-WT.
GST-p190A RhoGAP-4-EEE, the phosphomimic mutants. We first examined the effect of the Glu substitution on Rnd1 binding to p190A RhoGAP. HA-Rnd1 associated with GST-p190A RhoGAP-4-WT in the pulldown assay, whereas it associated less efficiently with GST-p190A RhoGAP-4-EEE (Fig. 7A), suggesting that the Glu substitution weakens the binding of Rnd1 to p190A RhoGAP.

We next replaced five putative phosphorylation sites with Glu to produce GFP-p190A RhoGAP-FL-5E to examine the effect of phosphorylation of p190A RhoGAP on RhoA in intact cells. We found that the amount of GTP-bound GFP-RhoA was greater in the cells expressing GFP-p190A RhoGAP-FL-5E than that in the cells expressing GFP-p190A RhoGAP-WT, suggesting that GFP-p190A RhoGAP-5E shows a weaker GAP activity than wild type (Fig. 7B).

It was previously demonstrated that the constitutively active form of RhoA (RhoA Val14) induces the formation of stress fibers and decreases the size of vascular smooth muscle cells through contraction, whereas the dominant negative form of RhoA (RhoA Asn19) weakens the formation of stress fibers and increases the cell size (37). We employed this assay to compare the effects of p190A RhoGAP and GFP-p190A RhoGAP-FL-5E on cell contraction. The vascular smooth muscle cells were transiently transfected with GFP-RhoA Asn19 or GFP-p190A RhoGAP-FL-WT, and the cell sizes were measured. The expression of GFP-p190A RhoGAP-FL-WT increased the cell size under these conditions, with GFP-RhoA Asn19 showing increased cell size (Fig. 7C), suggesting that basal RhoA activity is necessary for maintaining cell contractility. GFP-p190A RhoGAP-FL-5E showed a weaker effect than that of GFP-p190A RhoGAP-FL-WT (p < 0.01), suggesting that the GFP-p190A RhoGAP-FL-5E mutant mimics the phosphorylation state and shows weaker activity to inactivate RhoA.

**DISCUSSION**

Here we show that Rho-kinase phosphorylated p190A RhoGAP in a cell-free system and identified putative phosphorylation sites, including Ser1150. Rho-kinase phosphorylated p190A RhoGAP at Ser1150 in intact cells. Phosphorylation by Rho-kinase of the p190A RhoGAP fragment containing the GAP domain did not affect GAP activity toward RhoA in a cell-free system. However, we found that constitutively active Rho-kinase could counteract p190A RhoGAP activity in intact cells. The binding of Rnd to p190A RhoGAP is thought to enhance its GAP activity (35). Phosphorylation of p190A RhoGAP by Rho-kinase inhibited its binding to Rnd. The phosphomimic mutant of p190A RhoGAP showed the weaker Rnd binding and RhoGAP activities. Thus, it is conceivable that Rho-kinase phosphorylates p190A RhoGAP and thereby inhibits its binding to Rnd, resulting in inactivation of p190A RhoGAP and subsequent prolonged RhoA activation.

The primary action of ET-1 in vivo is to increase blood pressure and vascular tone. ET-1 was shown to activate Gq/G13, and to induce Ca^{2+} mobilization and subsequent phosphorylation of myosin light chain in cultured aortic smooth muscle cells (38). High concentrations of ET-1 cause long-lasting vasoconstriction (17). The transient contractile phase is mediated by G_q and Ca^{2+}/calmodulin-dependent activation of myosin light chain kinase (39). On the other hand, the sustained phase is mediated by G_{13}-dependent activation of the Rho/Rho-kinase signaling pathway (39). Consistently, high concentrations of ET-1 transiently induce Ca^{2+} mobilization in smooth muscle cells, which lasts for a minute (17).

Here we show that high concentrations of ET-1 induced sustained RhoA activation and p190RhoGAP phosphorylation in cultured vascular smooth muscle cells under the conditions in which ET-1 induced sustained MYPT1 phosphorylation. Y-27632 inhibited ET-1-induced phosphorylation of MYPT-1 as well as p190A RhoGAP and partly prohibited sustained RhoA activation, suggesting that ET-1 causes prolonged activation of the RhoA/Rho-kinase pathway. We also found that expression of p190A RhoGAP weakens the cell contractility, whereas the phosphomimic 5E mutant showed weaker activity than that of the wild type. Thus, it is possible that ET-1 induces the Rho/Rho-kinase activation and subsequent phosphorylation of p190A RhoGAP, thereby constituting the positive feedback loop to amplify Rho activation and promote contraction of vascular smooth muscle cells. ET-1 is thought to be involved in the pathogenesis of various cardiovascular diseases such as essential hypertension, pulmonary hypertension, and coronary vasospastic angina (40). The next challenge will be to explore whether p190A RhoGAP phosphorylation participates in these cardiovascular diseases.

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