Regulation of vascular smooth muscle cell contractile state is critical for the maintenance of blood vessel tone. Abnormal vascular smooth muscle cell contractility plays an important role in the pathogenesis of hypertension, blood vessel spasm, and atherosclerosis. Myosin phosphatase, the key enzyme controlling myosin light chain dephosphorylation, regulates smooth muscle cell contraction. Vasoconstrictor and vasodilator pathways inhibit and activate myosin phosphatase, respectively. G-protein-coupled receptor agonists can inhibit myosin phosphatase and cause smooth muscle cell contraction by activating RhoA/Rho kinase, whereas NO/cGMP can activate myosin phosphatase and cause smooth muscle cell relaxation by activation of cGMP-dependent protein kinase. We have used yeast two-hybrid screening to identify a 116-kDa human protein that interacts with both myosin phosphatase and RhoA. This myosin phosphatase-RhoA interacting protein, or M-RIP, is highly homologous to murine p116RIP3. M-RIP is expressed in vascular smooth muscle, and is localized to actin myofilaments. M-RIP binds directly to the myosin binding subunit of myosin phosphatase in vivo in vascular smooth muscle cells by an interaction between coiled-coil and leucine zipper domains in the two proteins. An adjacent domain of M-RIP directly binds RhoA in a nucleotide-independent manner. M-RIP copurifies with RhoA and Rho kinase, colocalizes on actin stress fibers with RhoA and MBS, and is associated with Rho kinase activity in vascular smooth muscle cells. M-RIP can assemble a complex containing both RhoA and MBS, suggesting that M-RIP may play a role in myosin phosphatase regulation by RhoA.

Blood vessel tone is regulated by the contractile state of vascular smooth muscle cells in the blood vessel wall. Diseases characterized by abnormal vascular smooth muscle cell contraction include hypertension, blood vessel spasm, and atherosclerosis (1–5). Smooth muscle contraction is tightly coupled to myosin light chain phosphorylation (6), which in turn is regulated by the relative activities of myosin light chain kinase and myosin phosphatase. Myosin light chain kinase is activated by intracellular calcium and phosphorylates myosin light chains, leading to cell contraction (7, 8). Myosin phosphatase dephosphorylates myosin light chains, leading to smooth muscle cell relaxation (9). Myosin phosphatase activity, once thought to be constitutive, is now known to be highly regulated. Both vasoconstrictor signaling pathways, which lead to inhibition of myosin phosphatase, and cell contraction (reviewed in Ref. 10), and vasodilator signaling pathways, which lead to cell relaxation via activation of myosin phosphatase have been recently defined (11–15).

Myosin phosphatase is a heterotrimer consisting of a PP1 catalytic subunit, a 130-kDa myosin binding subunit (MBS)1 and a 20-kDa subunit of unknown function (9, 16–18). The MBS is a regulatory subunit that targets PP1 to its substrate, myosin light chain (9), and has multiple protein interaction domains, including ankyrin repeats at its amino terminus, and a leucine zipper domain at its carboxyl terminus. MBS binds PP1 and myosin light chain at its amino terminus and the M20 subunit and cGMP-dependent protein kinase 1α (cGK) at its carboxyl terminus (Ref. 11, reviewed in Ref. 19). The MBS-cGK interaction is necessary for NO/cGMP-mediated activation of myosin phosphatase (11).

In vascular smooth muscle, G-protein-coupled receptor agonists cause contraction in part by inhibition of myosin phosphatase activity (20). Several downstream signaling pathways that inhibit myosin phosphatase activity have been discovered recently, including RhoA/Rho kinase (21), protein kinase C activation of the inhibitory phosphoprotein CPI-17 (22), and arachidonic acid (23, 24). In addition, several kinases copurify with myosin phosphatase, including ZIP-like kinase (25), integrin-linked kinase (26), myotonic dystrophy-related kinase (27), and Raf-1 (28), each of which can phosphorylate MBS and inhibit myosin phosphatase activity. RhoA/Rho kinase has been the most extensively studied myosin phosphatase inhibitor. RhoA binds to a myosin phosphatase complex in vitro, and GTP-bound RhoA, in combination with its downstream effector Rho kinase, inhibits myosin phosphatase activity (21). Specific blockade of Rho kinase has been found to ameliorate hypertension in several rat models (29), as well as to prevent the response to vascular injury and blood vessel spasm in animal models (30–33). Furthermore, phosphorylation-specific antibodies against inhibitory sites on MBS demonstrate that phosphorylation correlates directly with contractile agonist-mediated myosin phosphatase inhibition (34, 35). Recently, a Rho kinase inhibitor has been found to be effective in preventing...
coronary artery spasm and treating myocardial ischemia in humans (36, 37).

Despite strong evidence for RhoA/Rho kinase-mediated inhibition of myosin phosphatase, the molecular mechanism for this contractile pathway is not well understood. Activated RhoA and Rho kinase translocate to the cell membrane (38, 39), and have also been found colocalized with actin myofilaments (40, 41). The mechanism whereby RhoA and Rho kinase are targeted to and inhibit myosin phosphatase and thereby prevent dephosphorylation of myosin light chains in contractile myofibrils remains unclear.

We hypothesized that other signaling proteins regulate RhoA/Rho kinase-mediated inhibition of myosin phosphatase and searched for proteins that interact with both myosin phosphatase and RhoA using yeast two-hybrid screening methods. Here we report the identification and initial characterization of a protein that binds both MBS and RhoA. This myosin phosphatase-RhoA interacting protein (M-RIP) is a potential molecular link between RhoA signaling and myosin phosphatase regulation.

EXPERIMENTAL PROCEDURES

Materials—Vectors pGBT9 and pGAD424, yeast strain Y190, and human aorta Matchmaker cDNA library were from Clontech. Vector pCMV tag was from Stratagene. All enzymes were from New England Biolabs. Y27632 was from Tocris. pGEX vectors were from Pharmacia, pQE vectors from Qiagen, and pCI mammalian expression vector was from Promega. The TA cloning system was from Invitrogen. Antibodies against RhoA and Rho kinase were from Cell Signaling Technology, and antibodies against myosin phosphatase and RhoA using yeast two-hybrid screening methods. RhotekinRBD were kind gifts of Dr. Naoki Mochizuki. RhoA was cloned into pQE for expression of polyhistidine-tagged RhoA in bacteria. RhotekinRBD includes amino acids 1–99 of mouse Rhotekin (42), amplified by PCR from a mouse cDNA library, and ligated into pPCX-4T. GST-MBSLZ mutant was made as described (43). GFP-MBS was prepared by amplifying full-length human MBS from the Clontech human aorta library, followed by ligation into pEGFP. GFP-MBSLZ mutant was prepared by replacing the COOH-terminal domain of GFP-MBS with the COOH-terminal domain of the GST-MBSLZ mutant. All DNA constructs were fully sequenced in both directions.

Production of Anti-M-RIP Antiserum—cDNA encoding the 5′–480 bp of M-RIP was amplified from full-length M-RIP using PCR primers 5′-ATCGAATTCATGTCGGCAGCCAAGGAGAAC-3′ and 5′-CGATCTCAGAGTCAGCAGCGTCCGCAACCGTGATTTGTT-3′. The PCR product was ligated into pGEX and sequenced. The GST fusion protein of the NH2-terminal MBS (1–380) was made as described (11). GST-MBSLZ mutant was made as described (43). GFP-MBS was prepared by amplifying full-length human MBS from the Clontech human aorta library, followed by ligation into pEGFP. GFP-MBSLZ mutant was prepared by replacing the COOH-terminal domain of GFP-MBS with the COOH-terminal domain of the GST-MBSLZ mutant. All DNA constructs were fully sequenced in both directions.

Preparation of Fusion Proteins—GST and His6 fusion proteins were grown in bacteria overnight at 37 °C in LB with 150 µg/ml ampicillin. The culture was diluted 10-fold with LB/Amp, and incubated an additional 1 h. Isopropyl-1-thio-β-D-galactopyranosidase was added to a final concentration of 0.1 mM for GST fusion proteins and 1 mM for His6 fusion proteins, and the cells were incubated 4 h, pelleted, and frozen. MBS fusion proteins, the bacterial pellet, was thawed and resuspended in 35 ml of 20 mM Tris, pH 8, 100 mM NaCl, 1.5 mM EDTA, 0.1% Sarkosyl, 0.25 mg/ml lysozyme, 2 mM PMSF, 10 mM benzamidine, 20 mM dithiothreitol, and 0.01 µg/ml each of antipain, leupeptin, and pepstatin A. The cell lysate was incubated on ice for 15 min, then EDTA and Sarkosyl were added to final concentrations of 5 mM and 1.4%, respectively. The lysate was sonicated, centrifuged, and to the supernatant was added 20 ml of 10% Triton X-100 and glutathione-agarose beads. This was incubated for 2 h at 4 °C, following which the beads were washed with cold PBS. GST fusion proteins were either stored at 4 °C bound to glutathione-agarose beads, or eluted from beads in 50 mM Tris, pH 8.0, and 15 mM glutathione, snap frozen, and stored at −80 °C. For His6 fusion proteins, the bacterial pellet was resuspended in 10 mM Tris, 1 mM EDTA, and 10 mM imidazole, 0.01 µg/ml each of aprotinin, leupeptin, and pepstatin A, 2 mM PMSF. Lysozyme was added to a final concentration of 1 mg/ml and incubated for 30 min on ice. The lysate was then sonicated and centrifuged at 12,500 rpm for 20 min. The supernatant was incubated with 0.5 ml of Ni-NTA beads (Qiagen) for 2 h, then washed with 2× binding buffer, then washed at 4 °C, and harvested. The beads were either eluted from the beads in 50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM MgCl2, 1 mM dithiothreitol, and 250 mM imidazole, snap frozen, and stored at −80 °C.

Purification and Loading of His6,RhoA—The procedure was based on that of Diekmann and Hall (45). Briefly, His6,RhoA was purified as described above for His6 fusion proteins. Eluted His6,RhoA was dialyzed in 10 mM Tris, pH 7.5, 150 mM NaCl, 2 mM MgCl2, and 0.1 mM dithiothreitol, then snap frozen. Two µg of purified His6,RhoA was loaded in 50 mM Tris, pH 7.5, 5 mM EDTA, and 0.5 mM bovine serum albumin with either 200 µM GTP•S or GDP•S for 15 min at room temperature.
followed by the addition of MgCl₂ to a final concentration of 60 mM.

**Solubility Assay**—Subconfluent cultured human aortic smooth muscle cells were rinsed twice with cold PBS and lysed in 50 mM Tris, pH 7.5, 1 mM EDTA, 0.5% Nonidet P-40, 2 mM PMSF, 0.01 μg/ml each of aprotinin, leupeptin, and pepstatin A, and 250 or 350 mM NaCl. Lysates were incubated 1 h at room temperature, then centrifuged at 14,000 rpm for 20 min. The supernatant fraction was precleared with protein A beads, then used for immunoprecipitation with polyclonal anti-M-RIP antibody as described below. An equivalent percentage of each fraction was analyzed by anti-M-RIP immunoblot.

**FIG. 1. Identification, cloning, and structure of M-RIP.** A, amino acid sequence of M-RIP. The human M-RIP sequence is shown aligned with murine p116RIP3. B, predicted domains from translated full-length M-RIP cDNA. PH, pleckstrin homology domain; PPPP, proline-rich domain. The amino acid numbers corresponding to each domain are shown. Above is shown the corresponding regions of M-RIP encoded by yeast two-hybrid clones 6 and 11.
Human: 837 AQALEARQALRQCQRENQELNANHQLNLNNLAEAEITRLRTLLTGGGGEATGSPAQQK 896
AQALEARQALRQCQRENQELNANHQLNLNNLAEAEITRLRTLLTGGGGE+TG PL QGK
Mouse: 837 AQALEARQALRQCQRENQELNANHQLNLNNLAEAEITRLRTLLTGGGGESTGQLPTQGK 896

Human: 897 DAVELELVRVKESIQQIKQEIISSSSSLKDELQTALRDKYYSADKDYK0 KIYELTSIAKAD 956
DAVELELVRVKESIQQIKQEIISSSSSLKDELQTALRDKYYSADKDYK0 KIYELTSIAKAD
Mouse: 897 DAVELELVRVKESIQQIKQEIISSSSSLKDELQTALRDKYYSADKDYK0 KIYELTSIAKAD 956

Human: 957 DISRLKQELKAATEALGKPSDSATVSGYDIMS5KSNPDFFLKKDRSCVTRQLRNI RO S KSV 1016
DISRLKQELKAATEALGKSP+ TVSGYDIMS5KSNPDFFLKKDRSCVTRQLRNI RO S KSV
Mouse: 957 DISRLKQELKAATEALGKPSPEGTTVSGYDIMS5KSNPDFFLKKDRSCVTRQLRNI RO S KSV 1016

Human: 1017 IEQVSWDT 1024
IEQVSWD
Mouse: 1017 IEQVSWDN 1024

B

Co-immunoprecipitation Assays—Confluent cultured human aortic smooth muscle cells were firstly in PBS, then lysed in buffer A (50 mM Tris, pH 7.6, 7 mM MgCl2, 2 mM EDTA, 2 mg/ml n-dodecyl-B-maltoside, 0.4 mg/ml cholesteryl hemisuccinate, 0.6 x NaCl, 10 mM sodium molybdate, 2 mM PMSF, and 0.01 µg/ml each of aprotinin, leupeptin, and pepstatin A) for MBS immunoprecipitations and in buffer B (40 mM Tris, pH 7.5, 0.275 M NaCl, 4 mM EDTA, 2% Triton X-100, 20% glycerol, 50 mM b-glycerol phosphate, 2 mM PMSF, and 0.01 µg/ml each of aprotinin, leupeptin, and pepstatin A) for M-RIP and cGMP-dependent protein kinase immunoprecipitations. Cell lysates were incubated 1 h at room temperature, then centrifuged at 14,000 rpm for 10 min at 4 °C. The supernatant was pre-cleared with protein A beads and incubated overnight with polyclonal anti-M-RIP, anti-MBS, or anti-cGMP-dependent protein kinase 1. Protein A beads were added, and the lysates were incubated 2 h. The beads were washed three times with buffer C (50 mM Tris, pH 7.6, 7 mM MgCl2, 2 mM EDTA), then proteins were eluted in SDS sample buffer and analyzed by SDS-PAGE and immunoblotting as above. For co-immunoprecipitation of MBS with FLAG-M-RIP domains, COS-1 cells were transfected by electroporation, and lysates were prepared with buffer A as above after 48 h. Immunoprecipitations were performed as above except that M2 antibody was used for immunoprecipitation, and protein G beads were used to collect antigen-antibody complexes.

Fusion Protein Interaction Assays—For fusion protein interactions with proteins from cell lysates, confluent cells were lysed twice with ice-cold PBS, lysed in buffer A, incubated for 1 h at room temperature, then centrifuged at 14,000 rpm for 20 min at 4 °C. The supernatant was mixed with GST fusion proteins prebound to glutathione–agarose beads. After overnight incubation at 4 °C, the beads were washed with buffer C, eluted with SDS sample buffer, and bound proteins were analyzed by immunoblotting. For fusion protein interactions with purified proteins, fusion proteins immobilized on beads were incubated in buffer A for 1 h with purified soluble protein previously eluted from beads. After incubation, the beads were washed three times with buffer C, bound proteins were eluted in SDS sample buffer and analyzed by immunoblotting with anti-GST antibodies.

Stress Fiber Preparation—This procedure was adapted from Katoh et al. (40). Human aortic smooth muscle cells were cultured on two 100-mm dishes to near confluence, washed with cold PBS, then extracted with 10 ml of triethanolamine extraction buffer (2.5 mM triethanolamine, 1 µg/ml leupeptin and pepstatin A, 20 µg/ml aprotinin, 10 mM NaCl) for 30 min, shaking, with replacement of extraction buffer every 2–3 min. Remaining cell components were then further extracted using 10 ml of Triton buffer (0.5% Triton X-100, 1 µg/ml leupeptin and pepstatin A, 20 µg/ml aprotinin) for Triton extractions or 10 ml of glycerol buffer (50% glycerol, 1 µg/ml each of leupeptin and pepstatin A, 20 µg/ml aprotinin) for glycerol extractions for 5 min while shaking, with replacement of extraction buffer twice. Triton or glycerol was then removed by washing with 10 ml of aprotinin/PBS (20 µg/ml aprotinin, 1 µg/ml leupeptin and pepstatin A in PBS) for 10 min while shaking with one replacement of wash buffer. Remaining insoluble material was then scraped in aprotinin/PBS, and homogenized with a Z-shaped 21-gauge needle. The insoluble debris was pelleted at 1,000 × g for 5 min, and stress fibers were isolated by centrifugation of the supernatant at 100,000 × g for 1 h. The stress fiber pellet was boiled in 0.1 ml of protein sample buffer and subjected to SDS-PAGE and immunoblotting with indicated antibodies. For immunostaining of purified stress fibers, human coronary artery smooth muscle cells were grown on coverslips and glycerol-extracted as described above. Stress fibers were then fixed and immunostained as described below. All antibody dilutions were prepared with 1:100.

Kinase Assay—Subconfluent human aortic smooth muscle cells were lysed and M-RIP was immunoprecipitated as described for the solubility assay above, with 350 mM NaCl. The M-RIP and nonimmune IPs were washed with Rho kinase assay buffer (adapted from Feng et al. (46), 20 mM Tris, pH 7.5, 100 mM KCI, 0.1 mM dithiothreitol, 5 mM MgCl2, 1 mM EDTA, 1 µM okadaic acid) with or without 1 µM Y27632. After the addition of 20 µCi of [γ-32P]ATP, the samples were incubated for 20 min at 30 °C. The reaction was stopped by the addition of protein sample buffer, the samples were boiled 5 min and then analyzed by SDS-PAGE and autoradiography. The phosphobands were quantitated by densitometry using an Alpha Innotech image analyzer, and graphed using SigmaPlot 5.

Immunofluorescence Staining—Human coronary artery smooth muscle cells were plated on coverslips in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. The coverslips were washed with PBS twice, then fixed in 3.7% paraformaldehyde. The cells were permeabilized with 0.3% Triton X-100 and 10% donkey serum, then

Fig. 1—continued
blocked in PBS with 10% donkey serum. The cells were incubated with 1:400 anti-RIP (Transduction Laboratories) in 10% donkey serum in PBS for 1 h. After washing in PBS, the cells were incubated in 1:500 donkey anti-mouse IgG conjugated to Cy3 (Jackson ImmunoResearch) and phalloidin-fluorescein isothiocyanate (Sigma) or Alexa Fluor 488-phalloidin (Molecular Probes) in PBS for 1 h. Cells were washed in PBS and mounted on glass slides with SlowFade (Molecular Probes).

**RESULTS**

**Identification and Cloning of M-RIP**—To identify proteins involved in regulation of myosin phosphatase, base pairs 2043–3090 of human MBS (MBS-Cterm) were used as bait in a yeast two-hybrid screen of a human aorta library. Two clones, 6 and 11, encoded 3' regions of a cDNA with high homology to a murine RhoA-interacting protein, p116RIP3 (47). When retransformed into yeast, both clones interacted with MBS-Cterm but not with Gal4 DNA-binding domain alone (data not shown). Clone 6 was homologous to bp 1617–3072 and clone 11 was homologous to bp 2109–3072 of murine p116RIP3.

A human aorta library was next probed for the 5' sequence of human p116RIP3. PCR using the human aorta library as template yielded a 1,686-bp product that was highly homologous to the 5' sequence of murine p116RIP3. The full-length human clone was constructed by overlap extension PCR using yeast phalloidin (Fig. 2A). This human cDNA is hereafter called M-RIP.

Analysis of the M-RIP cDNA predicts a protein of 1,024 amino acids with multiple protein interaction domains, including a pair of pleckstrin homology domains flanking two polyproline motifs, and three carboxyl-terminal coiled-coil domains (Fig. 1B). Sites for myristoylation as well as for phosphorylation by protein kinase C, cyclic nucleotide-dependent protein kinases, and tyrosine kinases are also present in the M-RIP protein.

**Detection and Localization of M-RIP in Vascular Smooth Muscle Cells**—Specific M-RIP antisera were raised and tested first by immunoblotting of lysates from COS-7 cells transfected with full-length M-RIP cDNA. Anti-M-RIP recognized a specific band of 125 kDa in untransfected COS-7 cells that was augmented by overexpression of full-length M-RIP (Fig. 2A). A parallel immunoblot of the same lysates in which anti-M-RIP was preabsorbed with immunogen failed to identify any M-RIP band (data not shown). Anti-M-RIP was next used to probe lysates from two different human arterial smooth muscle cell lines. Anti-M-RIP identified a specific 125-kDa band in these lysates, supporting that M-RIP is expressed in human vascular smooth muscle cells (Fig. 2B). M-RIP from vascular smooth muscle cells was completely insoluble at 250 mM NaCl, but could be solubilized and immunoprecipitated under high ionic strength conditions (Fig. 2C).

Cultured human coronary artery smooth muscle cells were immunostained with anti-M-RIP (Fig. 2D, left panel) and with phalloidin (Fig. 2D, middle panel) to label actin fibers. M-RIP localized primarily in a filamentous pattern in the cytoplasm, similar to the distribution of actin filaments. Overlay of the two images revealed that M-RIP colocalized with actin myofila-

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2 smart.embl-heidelberg.de.
3 cubic.bioc.columbia.edu/predictNLS.
4 H. Surks, unpublished data.
and the leucine zipper domain of MBS.

Direct binding between M-RIP and MBS was also tested (Fig. 4F). GST-MBSLZ bound HisCC2, but not HisCC1 or HisCC3, demonstrating that the CC2 of M-RIP binds directly to MBS, whereas GST-MBSLZ mutant did not interact with any of the coiled-coil domains, confirming that the MBS leucine zipper domain mediates the interaction.

**M-RIP-RhoA Interactions**—Recombinant HisRhoA was used to test whether M-RIP binds RhoA. Both GDP– and GTP–RhoA bound M-RIP expressed in COS-1 cells (Fig. 5A). In direct binding assays, RhoA, loaded in vitro with GDP or GTP, was incubated with GST-Rhotekin Rho-binding domain (RhotekinRBD, positive control), GST-M-RIP-(545–823), or GST-M-RIP-(545–878). GST-RhotekinRBD bound Rho-GTP preferentially (48), whereas both GST-M-RIP-(545–823) and GST-M-RIP-(545–878) bound HisRhoA, without preference for GDP or GTP binding state of the low molecular weight G-protein (Fig. 5B).

**M-RIP Assembles a Complex including the Myosin Binding Subunit and RhoA**—The data above support that RhoA binds an M-RIP domain that includes amino acids 545–823 (Fig. 5B), whereas MBS binds a domain that includes amino acids 823–878 (Fig. 4, B and C). An M-RIP construct containing both domains was expressed in COS-1 cells and used to test the hypothesis that this region of M-RIP assembles a ternary complex of M-RIP with MBS and RhoA (Fig. 5C). RhoA bound equally well to GST-M-RIP-(545–823) and GST-M-RIP-(545–878), although MBS did not bind to GST-M-RIP-(545–823). These data support that amino acids 545–878 of M-RIP, via adjacent RhoA- and MBS-binding domains, mediates binding of both RhoA and MBS, and that MBS binding is not required.

**Fig. 2. Detection and localization of M-RIP in vascular smooth muscle cells.** A, 20 μg of lysate from COS-7 cells without (−) and with (+) overexpression of recombinant M-RIP. An anti-M-RIP immunoblot was performed using polyclonal anti-M-RIP. B, immunoblot using polyclonal anti-M-RIP of 20 μg of lysate from cultured aortic and coronary artery smooth muscle cells. C, aortic smooth muscle cells lysed in buffer containing 250 or 350 mM NaCl. Equal proportions of the soluble supernatant and insoluble pellet, as well as an M-RIP immunoprecipitate from the supernatant fraction were separated by SDS-PAGE and M-RIP was identified by immunoblot. D, coronary artery smooth muscle cells immunostained with anti-MRIP-Cy3 (left panel) and phallodin–fluorescein isothiocyanate (middle panel). An overlay of the M-RIP and phallodin immunostained cells is shown on the right panel.

**Fig. 3. In vivo interaction between M-RIP and myosin phosphatase.** A, M-RIP interacts with MBS. Top row: nonimmune, MBS, and M-RIP immunoprecipitates (IP) from vascular smooth muscle cells lysed in buffer B, immunoblotted with anti-MBS. Bottom row: nonimmune, MBS, and M-RIP immunoprecipitates from vascular smooth muscle cells lysed in buffer A, immunoblotted with anti-M-RIP. 5% of input lysate is shown on the left. B, M-RIP interacts with PP1. Nonimmune, MBS, and M-RIP immunoprecipitates from vascular smooth muscle cells lysed in buffer B, and immunoblotted with anti-PP1. 5% of input lysate is shown on the left. C, M-RIP interacts with cGK. Nonimmune, cGK, and M-RIP immunoprecipitates from vascular smooth muscle cells were lysed in buffer B, and immunoblotted with anti-cGK. 5% of input lysate is shown on the left.
M-RIP Binds Myosin Phosphatase and RhoA

Fig. 4. Characterization of binding between M-RIP and myosin phosphatase. A, similar quantities of GST fusions of the three coiled-coil domains of M-RIP (CC1, CC2, and CC3) and the amino-terminal leucine/isoleucine zipper domain of cGMP-dependent protein kinase Iα incubated with vascular smooth muscle cell lysate and probed for MBS binding. Molecular weight size markers in kDa are shown on the right, and 5% of input lysate is shown on the left. B, similar quantities of GST fusion proteins of the M-RIP Rho-binding domain (GST-M-RIP-(545–823)), CC2 domain (GST-M-RIP-(728–878)), and Rho-binding domain extended to include the COOH-terminal CC2 domain (GST-M-RIP-(545–878)) incubated with vascular smooth muscle cell lysate and immunoblotted for MBS. The upper panel shows the anti-MBS immunoblot. The same M-RIP domains were expressed in COS-1 cells as FLAG-tagged proteins and immunoprecipitated with M2 antibody. The MBS immunoblot of the immunopellets is shown in the middle panel, and the total cellular MBS content from each lysate used for immunoprecipitation is shown in the lower panel. Molecular size markers in kDa are shown on the right. C, the domains of M-RIP tested for MBS binding are shown schematically on the left with the corresponding amino acid numbers. The dashed box represents the Rho-binding domain. MBS binding to each domain is shown by a minus (−) or plus (+) on the right. D, COOH-terminal 180 amino acids of MBS, which includes the leucine zipper domain (GST-MBSLZ), and the same domain in which all four leucines in the leucine zipper were mutated to alanines (GST-MBSLZ mutant), incubated with the COS-7 cell lysate overexpressing FLAG-tagged M-RIP, and probed for M-RIP binding with anti-FLAG (M2). Molecular weight markers are shown on the right, and 5% of input lysate is shown on the left. E, COS-1 cells transfected with GFP, GFP-MBS, or GFP-MBS LZ mutant were lysed and incubated with GST or GST-M-RIP-(545–878). Bound MBS, GFP-MBS, and GFP-MBSLZ mutant were detected with anti-MBS antibodies. The positions of MBS and GFP-MBS are shown by the arrowheads on the right. F, purified protein interaction assay in which similar quantities of His6CC1, CC2, and CC3 bound to Ni-NTA beads were incubated with purified soluble GST-MBSLZ or GST-MBSLZ mutant. Bound GST-MBSLZ and GST-MBSLZ mutant were detected using anti-GST antibodies.
for the binding of RhoA to M-RIP (Fig. 5C).

**M-RIP Is Associated with RhoA/Rho Kinase in Vivo**—RhoA and Rho kinase have been shown to colocalize with actin stress fibers, and are present in stress fiber preparations made by glycerol extraction, but are lost from stress fibers extracted with Triton X-100 (40, 41). When Triton- and glycerol-extracted stress fibers were examined with immunoblot, both contained actin, MBS, and M-RIP, whereas glycerol-extracted stress fibers, but not Triton-extracted stress fibers, also contained RhoA and Rho kinase (Fig. 6A). Immunostaining of glycerol-extracted vascular smooth muscle cells revealed colocalization of M-RIP, MBS, and RhoA with actin stress fibers (Fig. 6B). Because of the requirement of detergent and high ionic strength conditions for M-RIP solubilization, RhoA could not be detected in the M-RIP immunopellet by immunoblot. However, the M-RIP immunopellet contained kinase activity (Fig. 6C) that was inhibited 45% (p = 0.005, n = 3) by the Rho kinase inhibitor Y27632 (Fig. 6D) supporting an association between M-RIP and RhoA/Rho kinase in vivo.

**DISCUSSION**

Myosin phosphatase activity is regulated by both contractile agonists and nitrovasodilators. RhoA and its downstream effector Rho kinase mediate contractile agonist-induced myosin phosphatase inhibition, but the mechanisms whereby RhoA interacts with myosin phosphatase remain unclear. Activated RhoA and Rho kinase translocate to the cell membrane, and a subpopulation of RhoA/Rho kinase has been identified on actin stress fibers (40, 41). The mechanism whereby RhoA/Rho kinase localize to actin stress fibers is unknown.

The protein described here, M-RIP, is expressed in human vascular myocytes, and is bound to myosin phosphatase in these cells. Immunostaining additionally shows that M-RIP colocalizes with actin myofilaments, consistent with a recent report showing p116RIP3 binding to actin (49). M-RIP is thus localized to the contractile filament where myosin light chain phosphorylation regulates the contractile state, suggesting a role for M-RIP in myosin phosphatase regulation. The amino terminus of M-RIP contains adjacent pleckstrin homology domains and polyproline motifs, a structural combination also found on Bruton’s tyrosine kinase where it mediates binding to both actin and Gα12 (50, 51). This region of p116RIP3 has recently been shown to mediate actin binding and actin bundling activity in vitro (49). Whereas p116RIP3 has been noted to be present in the cell nucleus (47), we could not detect endogenous M-RIP in the nucleus of vascular smooth muscle cells, implying either a difference in localization between the murine and human homologs, or cell type-specific nuclear localization.

The COOH-terminal domain of M-RIP interacts with both MBS and RhoA. The RhoA-binding domain of M-RIP overlaps the amino-terminal 95 amino acids of CC2, which raised the possibility that M-RIP interacts with MBS indirectly via RhoA. However, we have found that both RhoA and MBS bind M-RIP directly to separate adjacent domains (amino acids 545–823 for RhoA and 823–878 for MBS). Our data support a model where M-RIP binding brings MBS and RhoA into proximity (Fig. 7).

The COOH-terminal domain of M-RIP contains a leucine zipper domain that mediates binding to a leucine/isoleucine zipper in cGMP-dependent protein kinase 1α (43). The present study shows that the MBS leucine zipper domain also binds the COOH-terminal 55 amino acids of the M-RIP CC2 domain. Thus the same domain of MBS binds to both proteins. Our data support that MBS can bind M-RIP and cGMP-dependent protein kinase 1α simultaneously because M-RIP can interact with cGMP-dependent protein kinase 1α, as shown by coimmunoprecipitation, with evidence of direct binding, suggesting that the interaction occurs indirectly with MBS as the intermediary protein. It will be of interest in future studies to explore the mechanism of this multimeric interaction.

p116RIP3 was initially described as a RhoA-binding protein (45), although a subsequent study was unable to confirm binding to RhoA (49). In contrast, using both purified protein interaction studies and cell lysates, we find that M-RIP binds RhoA directly and independently of nucleotide binding state. The high ionic strength and detergents required to solubilize and immunoprecipitate M-RIP would be expected to disrupt binding to RhoA and Rho kinase (40). However, using a glycerol
extraction method shown to preserve RhoA and Rho kinase localization to stress fibers (40) we found that M-RIP copurified with RhoA and Rho kinase. Immunostaining of glycerol-extracted coronary myocytes confirmed that stress fiber architecture and colocalization of M-RIP, MBS, and RhoA with these structures was preserved. Furthermore, despite the presence of detergent and high ionic strength, Rho kinase activity could be detected in the M-RIP immunopellet because of the high sensitivity of autoradiography. Coupled with the in vitro direct binding studies, these data strongly support that M-RIP interacts with RhoA in vivo.

The M-RIP interaction occurs without preference for the nucleotide binding state of RhoA, whereas Kimura et al. (21) found that only GTP-RhoA interacted with myosin phosphatase. If M-RIP links RhoA to myosin phosphatase, it is unclear how GTP dependence of the RhoA-M-RIP-myosin phosphatase interaction occurs. Further study is required to determine whether a complex interaction between M-RIP, RhoA, and MBS establishes GTP dependence of the interaction, or whether a Rho kinase phosphorylation event stabilizes the complex.

Both our study of M-RIP and a recent report of p116 RIP3...
M-RIP Binds Myosin Phosphatase and RhoA

Fig. 7. Model of M-RIP interactions with RhoA and MBS. M-RIP, MBS, and RhoA are shown schematically. Direct binding interactions are shown by arrows. LZ, leucine zipper; MBS-BD, MBS-binding domain; Rho-BD, Rho-binding domain; PH, pleckstrin homology domain. The hatched square depicts the Rho-binding domain. Amino acid numbers for the interacting domains are shown in parentheses.

As indicated in these proteins colocalize with actin filaments. Interestingly, Mulder et al. (49) show that p116RIP is colocalized with both stress fibers and lamellipodia, has actin bundling properties and regulates the myosin phosphorylation state. RhoA into proximity (Fig. 7). The binding to myosin phosphatase and RhoA and localization to actin myofilaments suggest that M-RIP may target RhoA to the myosin phosphatase complex to regulate the myosin phosphorylation state.

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