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Targeting by Myosin Phosphatase-RhoA Interacting Protein Mediates RhoA/ROCK Regulation of Myosin Phosphatase

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Abstract Vascular smooth muscle cell contractile state is the primary determinant of blood vessel tone. Vascular smooth muscle cell contractility is directly related to the phosphorylation of myosin light chains (MLCs), which in turn is tightly regulated by the opposing activities of myosin light chain kinase (MLCK) and myosin phosphatase. Myosin phosphatase is the principal enzyme that dephosphorylates MLCs leading to relaxation. Myosin phosphatase is regulated by both vasoconstrictors that inhibit its activity to cause MLC phosphorylation and contraction, and vasodilators that activate its activity to cause MLC dephosphorylation and relaxation. The RhoA/ROCK pathway is activated by vasoconstrictors to inhibit myosin phosphatase activity. The mechanism by which RhoA and ROCK are localized to and interact with myosin light chain phosphatase (MLCP) is not well understood. We recently found a new member of the myosin phosphatase complex, myosin phosphatase-rho interacting protein, that directly binds to both RhoA and the myosin-binding subunit of myosin phosphatase in vitro, and targets myosin phosphatase to the actinomyosin contractile filament in smooth muscle cells. Because myosin phosphatase-rho interacting protein binds both RhoA and MLCP, we investigated whether myosin phosphatase-rho interacting protein was required for RhoA/ROCK-mediated myosin phosphatase regulation. Myosin phosphatase-rho interacting protein silencing prevented LPA-mediated myosin-binding subunit phosphorylation, and inhibition of myosin phosphatase activity. Myosin phosphatase-rho interacting protein did not regulate the activation of RhoA or ROCK in vascular smooth muscle cells. Silencing of M-RIP lead to loss of stress fiber-associated RhoA, suggesting that myosin phosphatase-rho interacting protein is a scaffold linking RhoA to regulate myosin phosphatase at the stress fiber. J. Cell. Biochem. 103: 1158–1170, 2008.

Key words: myosin phosphatase-rho interacting protein; p116RIP; myosin light chain; myosin phosphatase; myosin-binding subunit; smooth muscle; RhoA; ROCK

Blood vessel tone plays an important role in the determination of tissue perfusion and blood pressure. Vascular smooth muscle cells (VSMCs) in the blood vessel wall control blood vessel tone via their contractile properties. The contractile state of VSMCs is thus critical for normal blood vessel function and abnormalities in these cells play an important role in the pathogenesis of vascular diseases [Stemerman and Ross, 1972; Goldberg et al., 1979; Shepard and Vanhoutte, 1985; Stary, 1989; Fuster et al., 1992].

The contractile state of VSMCs is directly related to the phosphorylation state of the myosin regulatory light chain (MLC) [Hartshorne, 1987]. MLC phosphorylation is controlled by the opposing activities of the enzymes myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP; reviewed in [Somlyo and Somlyo, 1994]). MLCK is activated by calcium to phosphorylate MLC leading to

Abbreviations used: ROCK, Rho-associated coiled coil kinase; VSMC, vascular smooth muscle cell; MLC, myosin light chain; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; MBS, myosin-binding subunit; PKG, cGMP-dependent protein kinase; M-RIP, myosin phosphatase-rho interacting protein; RNAi, RNA interference; ERM, ezrin/radixin/moesin.

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VSMC contraction [Kamm and Stull, 1985; Taylor and Stull, 1988]. MLCP dephosphorylates MLC leading to VSMC relaxation and is also the target of signaling pathways that regulate VSMC contractility (reviewed in [Somlyo and Somlyo, 1998; Ito et al., 2004]).

MLCP is a heterotrimer consisting of a PPI catalytic subunit, a 130 kDa myosin-binding subunit (MBS) and a 20 kDa subunit of unknown function [Alessi et al., 1992; Shimizu et al., 1994; Shirazi et al., 1994; Takahashi et al., 1997]. The MBS was found to target PP1 to dephosphorylate MLC, thus providing specificity to MLCP via its targeting function [Alessi et al., 1992]. The MBS also contains protein–protein interaction domains that are important for MLCP regulation and targeting (reviewed in [Hartshorne, 1998]).

Both vasodilator and vasoconstrictor signaling pathways modulate MLCP activity to alter VSMC contractile state. Nitrovasodilators, via production of cGMP and activation of cGMP-dependent protein kinase (PKG), activate MLCP activity, augmenting MLC dephosphorylation [Wu et al., 1996; Lee et al., 1997; Etter et al., 2001]. We have shown previously that PKG interacts with the MBS of MLCP and this interaction is critical for activation of MLCP [Surks et al., 1999; Khatri et al., 2001; Surks and Mendelsohn, 2003]. Vasoconstrictors inhibit MLCP activity, increasing MLC phosphorylation [Somlyo and Somlyo, 2003; Hartshorne et al., 2004]. One pathway by which vasoconstrictors inhibit MLCP is via activation of the monomeric GTPase RhoA. RhoA binds to and activates its downstream effector ROCK [Leung et al., 1995; Matsui et al., 1996; Ishizaki et al., 1997]. RhoA-activated ROCK phosphorylates the MBS of MLCP in vitro [Kimura et al., 1996]. Phosphorylation of MBS on threonine 850 leads to ROCK-mediated MLCP inhibition [Kawano et al., 1999; Muranyi et al., 2005]. Using ROCK inhibitors and phosphospecific antibodies against the ROCK phosphorylation sites on MBS, multiple studies have demonstrated that vasoconstrictor agonists lead to phosphorylation and inhibition of MLCP via RhoA/ROCK, leading to VSMC contraction in vivo (reviewed in [Loirand et al., 2006]). Despite abundant data supporting a role for RhoA/ROCK in the regulation of MLCP, there is little information regarding the mechanism of the RhoA/ROCK-MLCP interaction and their targeting to the actinomyosin contractile stress fibers.

We recently identified a new member of the MLCP complex, myosin phosphatase-rho interacting protein (M-RIP) that binds directly to the leucine zipper domain at the carboxy-terminus of MBS [Surks et al., 2003]. The amino-terminus of M-RIP binds actin, and M-RIP is localized to actin filaments in VSMCs [Mulder et al., 2003; Surks et al., 2003; Koga and Ikebe, 2005]. Consistent with its MBS and actin binding, we and others have found that M-RIP targets MLCP to the actinomyosin contractile filament [Mulder et al., 2004; Koga and Ikebe, 2005; Surks et al., 2005], and M-RIP silencing uncouples MLCP from stress fibers, resulting in MLC phosphorylation [Surks et al., 2005]. Thus, one targeting function of M-RIP is to localize the MLCP complex to the actinomyosin contractile filament to dephosphorylate myosin.

The murine homolog of M-RIP, p116RIP, was originally identified as a RhoA-binding protein [Gebbink et al., 1997]. We found previously that M-RIP binds directly to RhoA in vitro and colocalizes with MBS and RhoA on actinomyosin stress fibers in VSMCs [Surks et al., 2003]. The direct binding of RhoA and MBS to M-RIP occurs through separate and adjacent binding sites on M-RIP, and M-RIP can assemble a complex of all three proteins in vitro. Although our recent data support a role for M-RIP in targeting the MLCP complex in its entirety, we have not previously explored whether M-RIP also plays a role in targeting RhoA within the MLCP complex to mediate MLCP inhibition.

We therefore hypothesized a second targeting function of M-RIP, linkage of the RhoA/ROCK-signaling pathway to MBS to regulate MLCP. Using RNAi, M-RIP expression was silenced in A7r5 VSMCs. M-RIP silencing prevented RhoA/ROCK-mediated regulation of MLCP as measured by phosphorylation of MBS and MLCP activity. M-RIP silencing did not prevent activation of RhoA or ROCK. Less RhoA was localized to stress fibers following M-RIP silencing. These data support a critical regulatory role for M-RIP in the targeting of RhoA to regulate MLCP.

METHODS

Materials

1-Oleoyl-2-hydroxy-sn-3-phosphate sodium (LPA) was from Sigma, Y27632 was purchased

from Tocris. Antibodies used were as follows: anti-M-RIP, anti-ROCK1 and 2, anti-ERM, and phospho-ERM from BD Transduction Laboratories, anti-MBS from Covance, anti-MBS P-850 from Upstate Biochemical, rabbit anti-RhoA from Santa Cruz. Glutathione agarose beads were obtained from Sigma. Alexa Fluor 488-phalloidin and anti-mouse IgG-Cy3 were from Molecular Probes.

Cell Culture

A7r5 rat aortic smooth muscle cells were purchased from ATCC and maintained in Dulbecco's-modified Eagle's medium with 10% fetal bovine serum. For assays, cells were rinsed three times in PBS and maintained overnight in serum-free media.

RNA Interference

M-RIP and scrambled control oligonucleotides were designed as described [Surks et al., 2005]. Double stranded RNA oligonucleotides were purchased from Dharmacon. A7r5 cells were transfected at 30% confluency using 100 nM oligonucleotide and Lipofectamine 2000 according to the manufacturer's instructions. Cells were assayed 96 h after transfection. Silencing efficiency in A7r5 cells was assessed as described [Surks et al., 2005].

Fluorescence Microscopy

A7r5 cells were cultured on glass coverslips, fixed, and immunostained as described [Surks et al., 2003]. The cells were incubated first with primary antibody,1:400 anti-MRIP, 1:500 anti-RhoA, followed by secondary antibody 1:500 anti-mouse or rabbit IgG conjugated to Cy3 and Alexa Fluor 488-phalloidin. Cells were washed in PBS and mounted on glass slides with Slow Fade reagent (Molecular Probes). Images were captured using a Nikon Optiphot-2 fluorescence microscope and a spot charge-coupled device camera (Diagnostic Instruments Inc.). For RhoA localization following RNAi, 20 cells were examined for each condition over three experiments.

Stress Fiber Preparations

Purified stress fibers were prepared from A7r5 cells as described [Katoh et al., 2001; Surks et al., 2003]. Briefly, cells were grown on 100 mm dishes to confluence, washed with cold PBS, then extracted with 10 ml of TEA extraction buffer (2.5 mM TEA, 1 μg/ml leupeptin and pepstatin A, 20 μg/ml aprotinin) 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 X buffer (0.05% Triton X, 1 μg/ml each of leupeptin and pepstatin A, 20 μg/ml aprotinin in PBS) for 90 s while shaking. The Triton X was then removed by washing with 10 ml aprotinin-PBS (20 μg/ml aprotinin, 1 μg/ml of leupeptin and pepstatin A in PBS) for 8 min while shaking with one replacement of wash buffer. The remaining insoluble material was 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 (low speed pellet), and stress fibers were isolated by centrifugation of the supernatant at 100,000*g* for 1 h. The stress fiber pellet was boiled in protein sample buffer and subjected to SDS–PAGE and immunoblotting with the indicated antibodies.

MBS and ERM Phosphorylation Assays

Subconfluent A7r5 cells were serum-deprived overnight, and then treated with LPA (1 μM for 30 s for ERM phosphorylation or 10 min for MBS phosphorylation) and/or Y27632 (10 μM for 30 min). Following stimulation, trichloroacetic acid, EDTA, and dithiothreitol (DTT) were added (to 10%, 2 mM and 10 mM, respectively) to the cells on ice. The cells were scraped, centrifuged, and washed three times with cold acetone containing 2 mM DTT. After drying, the cell pellet was solubilized in SDS sample buffer, separated by SDS–PAGE and immunoblotted for MBS, MBS P-850, ERM, P-ERM, and M-RIP. MBS, MBS P-850, ERM, and P-ERM were quantitated by densitometry and expressed as the density of the phospho-protein divided by the density of the total protein for each sample.

MLCP Activity Assay

Preparation of recombinant chicken gizzard regulatory light chains and phosphorylation of the light chains was performed as described [Surks et al., 2005]. A7r5 cells were grown in 100 mm dishes after transfection with either scrambled control or M-RIP-specific siRNA. Cells at 50% confluency were serum-deprived overnight. Cells were treated with vehicle or LPA, with and without pretreatment with Y27632. The cells were then rinsed with cold PBS on ice. Cells from each dish were then lysed and proteins immunoprecipitated with both

MBS and nonimmune Ig, as described [Surks et al., 2005]. The immunopellets were incubated in phosphatase assay buffer with 10 μM [^{32}P]MLC for 20 min at 30°C as described [Surks et al., 2005]. The assay was terminated and the protein precipitated by the addition of trichloroacetic acid to 10% and bovine serum albumin to 0.1%. After a 10-min incubation on ice, the samples were microfuged and the supernatant was subjected to Cerenkov counting. The specific MLCP activity was obtained by subtracting the activity in the nonimmune IP from the activity from the MBS IP from each dish of cells. For each sample, the amount of MBS in the immunopellet was quantitated by immunoblotting and densitometry. The phosphatase activity was normalized to the amount of MBS recovered in each immunopellet.

Production of Rhotekin-RBD-GST Beads

GST-Rhotekin-RBD construct was a kind gift from Dr. Naoki Mochizuki. Bacteria transformed with the GST-Rhotekin-RBD construct were grown overnight in 100 ml of LB with 150 μg/ml ampicillin. The culture was then scaled up to 1 L of LB/ampicillin and expression was induced with 1 mM isopropyl-beta-D-thiogalactopyranoside. After 4 h incubation at 37°C, the cells were pelleted and stored at −80°C. The pellet was thawed in 35 ml of 20 mM Tris pH 8.0, 100 mM NaCl, 1.5 mM EDTA. To the bacterial lysate was added 0.1% sarkosyl, 0.25 mg/ml lysozyme, 20 mM dithiothreitol, 12 mM benzamidine, 2 mM phenylmethylsulfonyl fluoride, and 0.01 μg/ml each of aprotinin, leupeptin, and pepstatin A. The lysate was incubated on ice for 30 min, after which 5 mM EDTA and 1.4% sarkosyl were added. The lysate was then sonicated and centrifuged at 6,000*g* for 40 min. The supernatant was mixed with 2% Triton X and 75 μl of glutathione agarose beads and incubated 2 h at 4°C, rocking. The beads were washed three times with cold PBS and then resuspended in PBS as a 1:1 slurry. The beads were then aliquotted, snap frozen in liquid nitrogen, and stored at −80°C.

RhoA Activation Assay

The assay was based on the method of Ren and Schwartz [Ren and Schwartz, 2000]. A7r5 cells transfected with scrambled control or M-RIP-specific dsRNA were grown on 150 mm dishes and serum-deprived when subconfluent. Cells were treated with vehicle or LPA, rinsed with cold PBS, and lysed in 50 mM Tris pH 7.5, 1% Igepal, 300 mM NaCl, 10 mM $MgCl_2$, 2 mM phenylmethylsulfonyl fluoride, 0.01 μg/ml each of aprotinin, leupeptin, and pepstatin A. The lysate was microfuged for 5 min at 4°C, and the supernatant was added to 30 μg of Rhotekin-RBD GST beads. The lysate was incubated with beads for 45 min at 4°C, and then washed three times in 25 mM Tris pH 7.5, 40 mM NaCl, and 30 mM $MgCl_2$. The washed beads were then boiled in SDS sample buffer. Both the bead bound and the input RhoA were separated by SDS–PAGE and detected by immunoblotting with anti-RhoA antibodies. Rho-GTP was quantitated densitometrically by calculating the Rhotekin-bound RhoA as a fraction of the input RhoA.

Data Analysis

All data were plotted and analyzed using Sigma Plot 5.0 software, and *P*-value was determined using Student's *t*-test and ANOVA.

RESULTS

M-RIP Expression, Localization, and Silencing in A7r5 Smooth Muscle Cells

In A7r5 rat aortic smooth muscle cells, M-RIP is colocalized with actin filaments (Fig. 1A,B), similar to the localization of M-RIP in human aortic smooth muscle cells (Surks et al., 2005). M-RIP expression in A7r5 cells was silenced by transfection of dsRNA. Consistent with our previous data, M-RIP silencing in A7r5 cells did not alter the expression of the MBS of MLCP, RhoA, ROCK1, or ROCK2 (Fig. 1C). M-RIP silencing in A7r5 cells increased cell size and stress fiber number as shown in human VSMCs ([Surks et al., 2005], Fig. 1E). The M-RIP protein that remained after silencing retained its stress fiber localization (Fig. 1D,E).

M-RIP Regulates MLCP MBS Phosphorylation in Response to LPA

In A7r5 cells, the contractile agonist LPA inhibits MLCP activity in a ROCK-dependent manner [Surks et al., 2005; Muranyi et al., 2005]. LPA-induced phospho-threonine 850 was measured in cells following transfection with control or M-RIP-specific siRNA. In control RNAi cells, LPA treatment increased phospho-threonine 850 as expected (Fig. 2A, top left

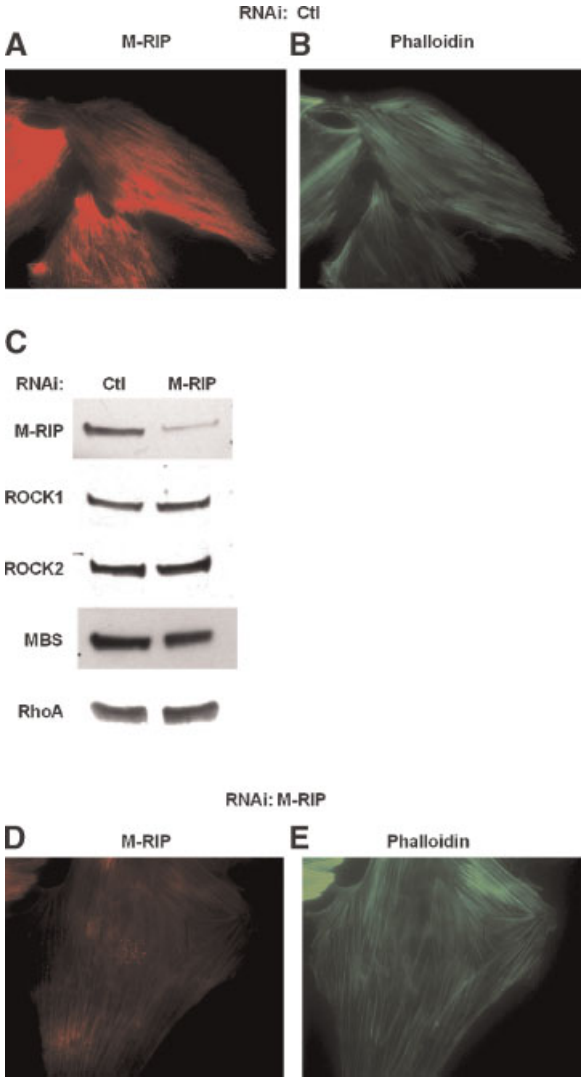


Fig. 1. M-RIP expression, localization, and silencing in A7r5 smooth muscle cells: (**A**,**B**) M-RIP expression and localization in A7r5 cells. A7r5 cells in culture 96 h following transfection with scrambled dsRNA were fixed and labeled with anti-M-RIP antibody (A) and phalloidin (B). **C**: A7r5 cells were transfected with scrambled (Ctl) or M-RIP-specific siRNA. Thirty μg of cell lysate was subjected to SDS–PAGE and immunoblotting with the indicated antibodies. **D**,**E**: A7r5 cells in culture 96 h after transfection with M-RIP-specific dsRNA were fixed and labeled with anti-M-RIP antibody (D) and phalloidin (E). The remaining M-RIP protein following silencing retains its stress fiber distribution. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

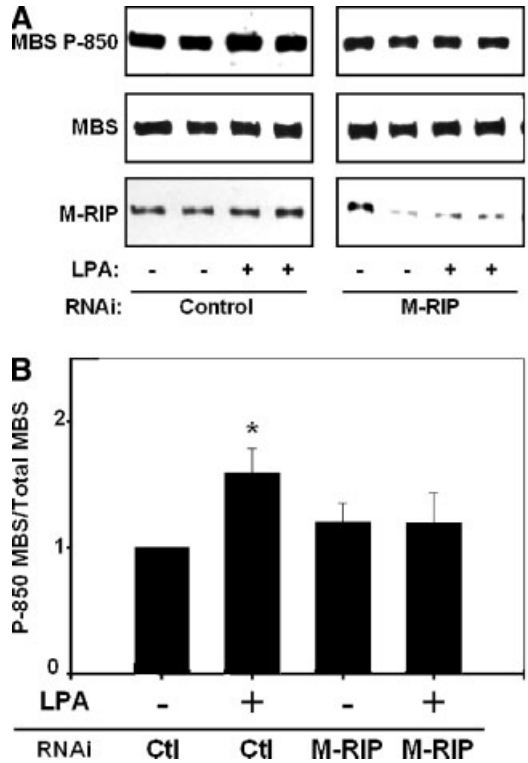


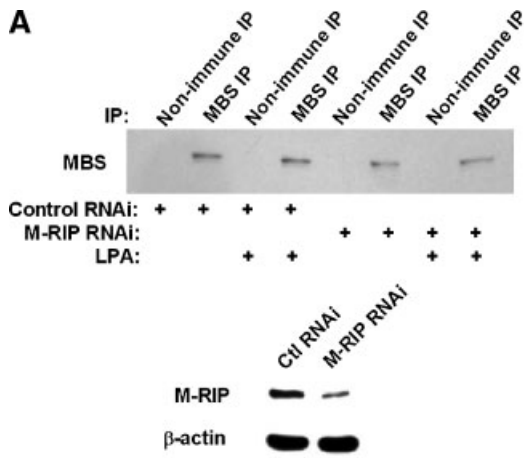
Fig. 2. M-RIP regulates MLCP MBS P-850 in response to LPA: (**A**) Immunoblot showing increased threonine 850 phosphorylation in response to LPA in control siRNA transfected but not in M-RIP siRNA-transfected cells (representative of three independent experiments). **B**: Pooled data from three experiments showing MBS phosphothreonine-850 with LPA stimulation in control (Ctl) siRNA-transfected cells (*$P < 0.05$), and in M-RIP siRNA-transfected cells. The slight increase in basal phospho-850 in M-RIP RNAi-transfected cells was not statistically significant.

panel, Fig. 2B). However, when M-RIP expression was silenced, LPA did not lead to an increase in phospho-threonine 850 (Fig. 2A, top right panel, Fig. 2B). These experiments indicate that M-RIP is required for RhoA/ROCK-mediated phosphorylation of MBS threonine 850, the phosphorylation event that is associated with MLCP inhibition by RhoA/ROCK.

M-RIP Regulates MLCP Activity in Response to LPA

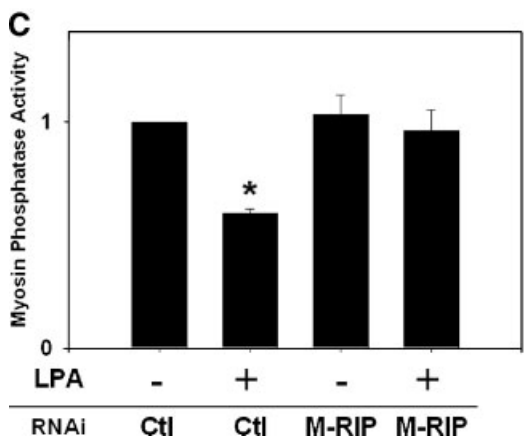
To further explore the role of M-RIP in RhoA/ROCK-mediated regulation of MLCP, we directly tested intracellular MLCP activity. Immunoprecipitation of MBS recovered PP1 phosphatase activity that was regulated by RhoA and ROCK signaling in the cell (data not shown and [Surks et al., 2005]). We recently found that basal MLCP activity was unaffected by M-RIP silencing [Surks et al., 2005]. To examine the role of M-RIP in the regulation of MLCP activity by RhoA/ROCK, we studied cells treated with and without LPA following control or M-RIP RNAi. Figure 3A and B show data from a representative experiment. Anti-MBS antibodies specifically immunoprecipitated

MBS (Fig. 3A). As shown in Figure 3B, when the MLCP-specific counts were normalized to the amount of MBS in the immunopellet, LPA was found to inhibit MLCP activity in the control RNAi cells, but not in the M-RIP RNAi cells. Pooled data from four experiments is shown in Figure 3C. LPA treatment inhibited MLCP activity by 41% in the control RNAi cells. In the M-RIP RNAi cells, there was no inhibition of MLCP activity by LPA. These data further support that M-RIP is required for RhoA/ROCK-mediated regulation of MLCP activity in smooth muscle cells.



B

RNAi	LPA Tx	CPM MBS-NI	MBS Densit.	CPM/ MBS	% of Control
Control	-	1623	35.8	45.3	100
Control	+	1076	37.5	28.7	63
M-RIP	-	1470	28	52.6	116
M-RIP	+	1579	30.6	51.6	114



Silencing of M-RIP Expression Does Not Affect RhoA Activation by LPA

We next tested the mechanism whereby M-RIP regulates MLCP activity. M-RIP may regulate MLCP by controlling the protein composition of the MLCP complex via its scaffolding function, or it may regulate the activation of RhoA and ROCK. We previously found that basal RhoA activation state was not affected by M-RIP silencing, but did not test whether M-RIP affected RhoA activation. We therefore measured basal and LPA-activated RhoA in A7r5 cells following M-RIP silencing. LPA stimulation of A7r5 cells lead to RhoA activation in both control and M-RIP RNAi cells (Fig. 4A). The level of RhoA activation was the same in control and M-RIP-silenced cells (1.7-fold activation, $P = 0.03$ for control, $P = 0.005$ for M-RIP RNAi, n = 3, Fig. 4B). These data indicate that M-RIP silencing does not affect RhoA activation by LPA and suggests that regulation of RhoA activation state is not a mechanism whereby M-RIP regulates MLCP activity.

Silencing of M-RIP Expression Does Not Affect ROCK Activation by LPA

To test whether M-RIP regulates MLCP by mediating RhoA activation of ROCK, we measured the phosphorylation of another RhoA/ROCK substrate in the cell. The ezrin/radixin/moesin family of proteins link membrane-signaling events with the cytoskeleton and are phosphorylated by RhoA/ROCK at residues

Fig. 3. M-RIP regulates MLCP activity in response to LPA: (**A**) Immunoblot data from a representative MLCP assay showing the MBS immunoblot of the nonimmune and MBS immunoprecipitations from cells transfected with control or M-RIP siRNA followed by vehicle or LPA stimulation. The **lower panel** shows the expression of M-RIP from the same control and M-RIP RNAi cells with β-actin shown as a loading control. **B**: Data from the representative MLCP assay shown in (A). Control and M-RIP siRNA-transfected cells were treated without (−) and with (+) LPA. The total CPM released by MLCP-specific phosphatase activity (MBS IP counts minus nonimmune IP counts) is shown (CPM MBS-NI). The densitometry of the MBS band from the MBS IP is shown, followed by the CPM normalized to MBS (CPM/MBS). The last column shows the phosphatase data adjusted to the control sample (control RNAi, LPA−) which was assigned a value of 100%. **C**: Pooled data from four independent experiments showing MLCP activity in cells transfected with scrambled (Ctl) and M-RIP siRNA and then treated with and without LPA. Activity in the control-treated scrambled RNAi cells was given the value of 1. There is a 41% reduction in MLCP activity in scrambled RNAi cells treated with LPA ($P < 0.001$, n = 4), but no reduction in MLCP activity in M-RIP RNAi cells treated with LPA.

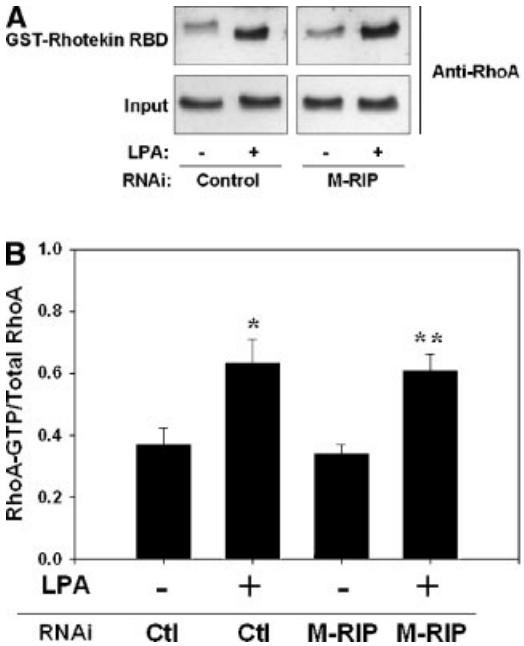


Fig. 4. Silencing of M-RIP expression does not affect RhoA activation by LPA: (**A**) Representative immunoblot showing binding of RhoA-GTP from untreated (−) and LPA-treated (+) cells to GST-Rhotekin-RBD beads in the **upper panels**, and input RhoA from each cell lysate in the **lower panels**. The **left panels** are from cells transfected with scrambled (control) siRNA, and the **right panels** are from cells transfected with M-RIP siRNA. **B**: Pooled data from three independent experiments showing the ratio of Rhotekin-bound to input RhoA in cells with and without LPA stimulation following transfection with either scrambled (Ctl) or M-RIP siRNA. *$P = 0.03$. **$P = 0.005$.

T567 (ezrin), T564 (radixin), and T558 (moesin) in response to LPA stimulation [Matsui et al., 1998]. Using a phospho-specific antibody that recognizes these phosphorylation events, we found that LPA stimulation of A7r5 cells lead to phosphorylation of ERM that was prevented by pretreatment of the cells with Y27632 (3.8-fold increase, $P < 0.001$, n = 4, Fig. 5A,B), as has been described in other cell types [Fukata et al.,

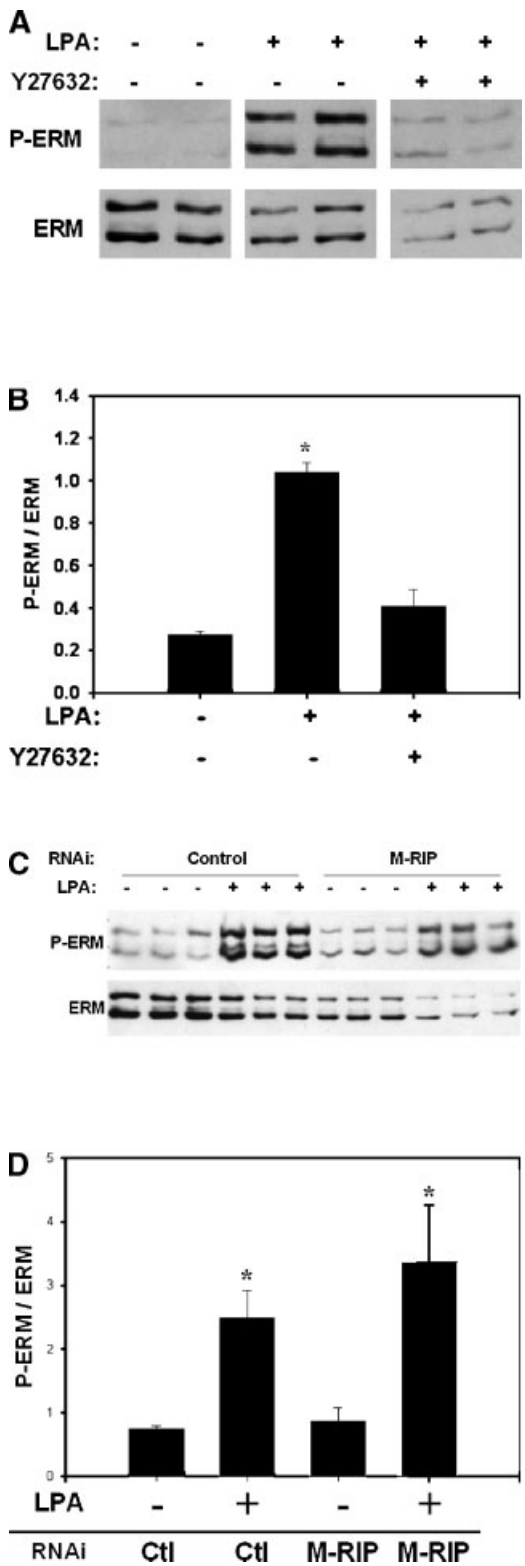


Fig. 5. Silencing of M-RIP expression does not affect cellular ROCK activity: (**A**) Phospho-ERM assay from A7r5 cells treated with vehicle (−), LPA for 30 s or pretreated with Y27632 for 30 min prior to LPA treatment for 30 s. The phospho-ERM and total ERM immunoblots are shown. **B**: Pooled data from four assays showing the proportion of phospho-ERM in control (−), LPA, and Y27632 followed by LPA-treated cells. LPA caused a 3.8-fold increase in phospho-ERM ($P < 0.001$). There was no significant difference in the level of phospho-ERM between control-treated cells and cells treated with Y27632 followed by LPA. **C**: Phospho-ERM assay from A7r5 cells transfected with control or M-RIP siRNA and treated with vehicle (−) or LPA (+) for 30 s. **D**: Pooled data from four phospho-ERM assays of control and M-RIP RNAi cells treated with vehicle (−) or LPA (+). LPA treatment leads to significantly increased phospho-ERM in both control and M-RIP RNAi cells (2.9-fold increase for control RNAi cells, 3.6-fold increase for M-RIP RNAi cells, $P < 0.05$ by ANOVA, n = 4). The difference in phospho-ERM between control RNAi and M-RIP RNAi cells treated with LPA was not statistically significant.

1998; Matsui et al., 1998]. These data indicate that ERM proteins are substrates of RhoA/ROCK in A7r5 cells and phosphorylation of ERM reflects LPA-mediated RhoA and ROCK activity. LPA treatment increased phospho-ERM in both control and M-RIP RNAi cells (2.9 and 3.6-fold increases, respectively, $P < 0.05$, $n = 4$). The LPA-mediated increase in phospho-ERM in cells following transfection with M-RIP siRNA was not significantly different from cells transfected with control siRNA (Fig. 5C,D). The similar level of ROCK-mediated phosphorylation of ERM despite M-RIP silencing supports that M-RIP does not regulate overall ROCK activation by RhoA in the cell.

M-RIP Targets RhoA to Actinomyosin Stress Fibers

In keeping with its role in targeting MLCP to actinomyosin stress fibers and binding to RhoA, we hypothesized that M-RIP would localize RhoA to stress fibers. Examination of purified stress fiber fractions from A7r5 cells revealed that substantially less RhoA was localized in the stress fiber fraction when M-RIP expression was silenced (Fig. 6A). Quantitation of three separate experiments confirmed that M-RIP silencing resulted in a 47% reduction in stress fiber-associated RhoA ($P = 0.002$, $n = 3$, Fig. 6B). We also tested localization of RhoA to stress fibers in intact A7r5 cells by immunofluorescence microscopy. Immunolabeling with a nonimmune antibody did not specifically label the smooth muscle cells (Fig. 6C). Anti-RhoA antibody revealed RhoA localized primarily in a cytoplasmic perinuclear location, but also, to a lesser extent, colocalized with actin-myosin stress fibers in 90% of scrambled RNA-transfected cells studied (Fig. 6D). In contrast, following M-RIP RNAi transfection, RhoA colocalized with stress fibers in 50% of cells examined, with a larger fraction of RhoA localizing in a perinuclear distribution (Fig. 6E). These data support that M-RIP plays a role in targeting RhoA to actin stress fibers in VSMCs. A hypothesis for M-RIP targeting of RhoA and MLCP is shown in Figure 7.

DISCUSSION

The regulation of MLCP by RhoA and ROCK is a well-established mechanism by which vasoconstrictor agonists modulate VSMC contractile state. Although there are abundant data implicating RhoA and ROCK in MLCP regulation, there is little information about the mechanism by which RhoA and ROCK are targeted to and interact with MLCP. We and others have recently described a new member of the MLCP complex, M-RIP, that is associated with the actinomyosin contractile apparatus and can bind directly to both the MBS of MLCP and to RhoA [Surks et al., 2003, 2005; Mulder et al., 2004; Koga and Ikebe, 2005]. Furthermore, M-RIP is an anchoring protein that localizes MLCP to stress fibers to dephosphorylate myosin [Mulder et al., 2004; Koga and Ikebe, 2005; Surks et al., 2005].

We hypothesized that M-RIP plays a role in RhoA/ROCK-mediated regulation of MLCP by targeting RhoA to interact with MLCP (Fig. 7). The present data support this hypothesis by showing that M-RIP silencing prevents RhoA/ROCK-mediated phosphorylation of the MBS of MLCP as well as RhoA/ROCK-mediated inhibition of MLCP activity. However, M-RIP silencing did not prevent activation of RhoA and ROCK in the cell. These data, coupled with our previous observation that M-RIP can assemble a complex including RhoA and MLCP, support that M-RIP regulates MLCP by targeting RhoA and ROCK to interact with and inhibit MLCP activity. Thus, M-RIP has at least two targeting functions in the cell; stress fiber localization of the MLCP complex and colocalization of RhoA and MCLP. Data from our group and others support that silencing of M-RIP uncouples MLCP from stress fibers [Mulder et al., 2004; Koga and Ikebe, 2005; Surks et al., 2005] and also prevents RhoA signaling to MLCP as shown in Figure 7.

To further support a role for M-RIP in targeting RhoA, two complementary immunolocalization studies were performed. Whole cell immunofluorescence labeling of RhoA following M-RIP silencing showed loss of RhoA from stress fibers. Because most of the cellular RhoA is in the cytoplasm, the cells were overexposed to highlight the stress fiber-associated RhoA. In a second method to examine the localization of RhoA, purified stress fiber fractions were prepared. Immunoblot of these fractions also revealed that less RhoA remained associated with stress fibers after M-RIP silencing, supporting a role for M-RIP in targeting RhoA to actin stress fibers.

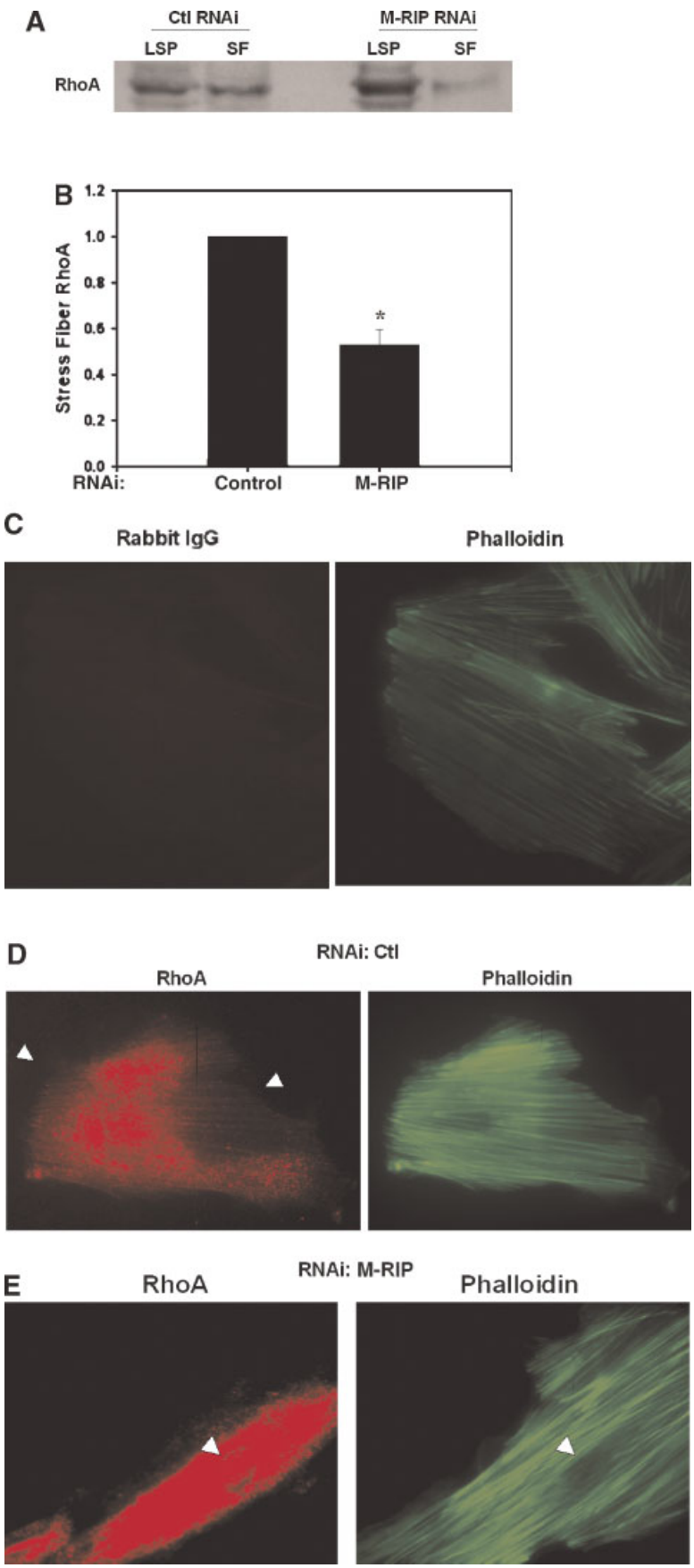

A
Ctl RNAi
M-RIP RNAi
LSP
SF
LSP
SF
RhoA
B
Stress Fiber RhoA
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
RNAi:
Control
M-RIP
C
Rabbit IgG
Phalloidin
D
RNAi: Ctl
RhoA
Phalloidin
E
RNAi: M-RIP
RhoA
Phalloidin

Fig. 6.

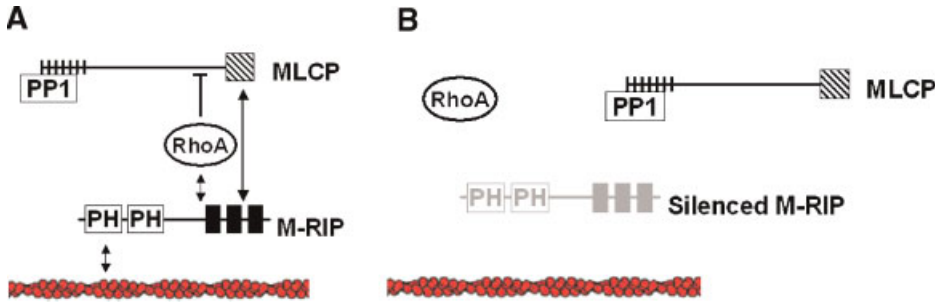


Fig. 7. Schematic representation of MLCP complex targeting by M-RIP. **A**: M-RIP binds actin filaments via its amino-terminal domain. M-RIP colocalizes RhoA and MBS by direct binding to both proteins. Colocalization of RhoA and MLCP permits regulation of MLCP activity by RhoA. **B**: Silencing of M-RIP expression leads to dissociation of MBS and RhoA from stress fibers, and loss of MLCP regulation by RhoA. PH = pleckstrin homology domain. The solid squares represent coiled coil domains on M-RIP. The hatched box is the leucine zipper domain on MBS. The vertical lines on MLCP represent ankyrin repeats. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Previous studies of RhoA function in smooth muscle and fibroblasts have shown that most cellular RhoA is soluble, but that upon activation, a fraction of GTP-bound RhoA is targeted to the cell membrane by isoprenylation of its carboxy-terminus. However, more recently, we and others have found that RhoA and ROCK are also localized at stress fibers [Katoh et al., 2001; Chen et al., 2002; Surks et al., 2003; Kawabata et al., 2004]. The mechanism of this localization has been unclear, but we have now shown that M-RIP can target both MLCP and RhoA to stress fibers. Because of its interactions with actin at the amino-terminus and MBS and RhoA at the carboxy-terminus, M-RIP not only targets MLCP and RhoA to stress fibers to regulate MLC phosphorylation state, but also colocalizes RhoA with MBS to facilitate regulation of MLCP activity by RhoA/ROCK (Fig. 7). RhoA and ROCK activity are known to promote stress fiber formation. However, M-RIP silencing, which prevents RhoA from regulating MLCP, increases stress fiber number in smooth muscle cells. This apparent discrepancy can be explained by the targeting by M-RIP of the MLCP complex to the cytoskeleton. Thus loss of M-RIP prevents MLCP regulation by RhoA, but also uncouples the MLCP complex from actinomyosin. This is also illustrated by the blunted ability of the ROCK inhibitor Y27632 to dissociate stress fibers when M-RIP is silenced [Surks et al., 2005].

Silencing of M-RIP expression completely prevents LPA-mediated MLCP regulation, but only partially reduces RhoA localization to stress fibers. This discrepancy may be explained by RhoA binding to stress fiber-associated proteins other than MLCP. RhoA has been shown to bind to mDia [Watanabe et al., 1997] and to filamin A [Ohta et al., 1999]. These interactions could explain the residual stress fiber localization of RhoA after M-RIP silencing, and the lack of MLCP regulation by this residual RhoA.

Fig. 6. M-RIP targets RhoA to actinomyosin stress fibers: (**A**) Purified stress fiber preparations from A7r5 cells transfected with control (Ctl RNAi) siRNA or M-RIP (M-RIP RNAi) siRNA. Equal aliquots from the low speed pellets (LSP) and stress fiber pellets (SF) were loaded on a 10% protein gel and following transfer were immunoblotted for RhoA. **B**: The proportion of RhoA in the stress fiber fraction was calculated by densitometry for control siRNA and M-RIP siRNA-transfected cells. The proportion of RhoA in the stress fiber fraction in the control siRNA-transfected cells was assigned a value of 1. There is a 47% reduction in stress fiber-associated RhoA in M-RIP siRNA-transfected cells compared with control siRNA-transfected cells ($P = 0.002$, $n = 3$). **C–E**: Whole-cell immunolabeling of A7r5 cells. The cells were overexposed to reveal the faint immunolabeling of RhoA on stress fibers. Each panel in C–E depicts a single cell with both RhoA and phalloidin labeling, and are shown at the same exposure and magnification. C: Whole cell immunolabeling with rabbit nonimmune IgG (**left panel**, same concentration used for anti-RhoA antibody) and phalloidin (**right panel**) showing no specific staining with nonimmune antibody. D: Whole cell immunolabeling of a representative A7r5 cell 96 h after transfection with scrambled dsRNA showing phalloidin (**right panel**) and RhoA (**left panel**). There is perinuclear cytoplasmic staining of RhoA, as well as colocalization of RhoA with actin stress fibers peripherally (arrowheads). E: Whole cell immunolabeling of a representative A7r5 cell 96 h after transfection with M-RIP-specific dsRNA showing phalloidin (**right panel**) and RhoA (**left panel**). Perinuclear RhoA is more pronounced and peripheral colocalization of RhoA with actin stress fibers is not apparent. The arrowheads denote the position of the nucleus. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

The loss of MLCP regulation when M-RIP is silenced also raises the question as to whether M-RIP regulates RhoA or ROCK activity. Koga and Ikebe recently showed that p116RIP silencing in Hela cells lead to RhoA activation, consistent with p116RIP having a GTPase-activating protein function [Koga and Ikebe, 2005]. However, direct testing of this question in smooth muscle cells showed that M-RIP silencing did not affect basal or LPA-activated RhoA activity. This raises the possibility that M-RIP may associate with a GTPase-activating protein in a cell type-specific manner.

We similarly tested whether M-RIP plays a role in ROCK activation in the cell. Phosphorylation of ERM proteins by ROCK is a well-described signaling event that can be monitored by the use of phospho-specific antibodies. ERM phosphorylation has been widely used to monitor ROCK activation state in the cell [Hattori et al., 2004; John et al., 2004]. Our control experiments verified that LPA rapidly leads to ROCK-mediated ERM phosphorylation in smooth muscle cells. M-RIP silencing had no significant effect on ERM phosphorylation, supporting that M-RIP does not regulate ROCK activation state. Overall, the RhoA and ROCK activity data support that M-RIP regulates MLCP primarily via its targeting functions.

There are two major phosphorylation sites for ROCK on MBS in vitro, threonines 696 and 850 [Feng et al., 1999; Kawano et al., 1999]. Initially both sites were found to be ROCK phosphorylation sites in vivo, but more recently, threonine 850 was found to be the ROCK phosphorylation site in vivo in VSMCs [Muranyi et al., 2005]. Phosphorylation of MBS at threonine 850 has been found to both inhibit MLCP activity [Muranyi et al., 2005], and to inhibit MBS binding to myosin [Velasco et al., 2002]. In our preliminary experiments, we found that treatment of VSMCs with LPA did not result in increased threonine 696 phosphorylation (data not shown), consistent with other recent studies [Kitazawa et al., 2003; Wilson et al., 2005]. However, LPA induced a 1.6-fold increase in phospho-threonine 850 in the scrambled control RNAi-transfected cells, and the magnitude of increase in phosphorylation at this site as well as the level of basal phosphorylation were similar to that described in another report using the same cell line [Muranyi et al., 2005]. Silencing of M-RIP prevented the LPA-mediated increase in phospho-threonine 850, supporting that M-RIP is required for RhoA/ROCK-mediated MLCP regulation.

M-RIP silencing prevented both RhoA-mediated MBS phosphorylation and MLCP inhibition, but interestingly, did not decrease basal MBS phosphorylation or MLCP activity. Although M-RIP can bind RhoA directly, M-RIP does not bind directly to ROCK [Koga and Ikebe, 2005]. This raises the possibility that ROCK may be targeted to the MLCP complex by a different mechanism that does not involve M-RIP. This hypothesis could explain how M-RIP silencing would prevent agonist-mediated increases in phosphorylation by preventing RhoA localization to MLCP, yet leave basal MBS phosphorylation intact since ROCK localization would be unaffected. We are currently investigating this hypothesis.

Regulation of MLCP activity by vasoconstrictor agonists and nitrovasodilators is a critical determinant of VSMC contractile state and blood vessel tone. RhoA/ROCK, activated by contractile agonists, is a widely recognized pathway whereby MLCP activity is inhibited to promote MLC phosphorylation and VSMC contraction. ROCK inhibitors are now promising new therapeutics for cardiovascular disease, underscoring the clinical importance of this pathway [Masumoto et al., 2002; Mohri et al., 2003; Kishi et al., 2005; Vicari et al., 2005]. Although the mechanism whereby RhoA and ROCK interact with MLCP to regulate its activity has been obscure, our data now support that M-RIP is required for RhoA/ROCK-mediated regulation of MLCP, and that a function of M-RIP is to target the MLCP signaling complex to the actinomyosin contractile filament and to colocalize RhoA and MLCP to facilitate regulation of MLCP activity (Fig. 7). Thus M-RIP is an essential targeting protein for localizing the MLCP complex within the cell and for linking the RhoA/ROCK pathway to MLCP regulation.

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