p116\textsuperscript{Rip} was originally found to be a RhoA-binding protein, but its function has been unknown. Here, we clarify the function of p116\textsuperscript{Rip}. Two critical findings were made. First, we found that p116\textsuperscript{Rip} activates the GTPase activity of RhoA \textit{in vitro} and that p116\textsuperscript{Rip} overexpression in cells consistently diminished the epidermal growth factor-induced increase in GTP-bound RhoA. Second, p116\textsuperscript{Rip} activated the myosin light chain phosphatase (MLCP) activity of the holoenzyme. p116\textsuperscript{Rip} did not activate the catalytic subunit alone, indicating that the activation is due to the binding of p116\textsuperscript{Rip} to the myosin phosphatase targeting subunit MYPT1. Interestingly, the activation of phosphatase was specific to myosin as substrate, and p116\textsuperscript{Rip} directly bound to myosin, thus facilitating myosin/MLCP interaction. The gene silencing of p116\textsuperscript{Rip} consistently and significantly increased myosin phosphorylation as well as stress fiber formation in cells. Based upon these findings, we propose that p116\textsuperscript{Rip} is an important regulatory component that controls the RhoA signaling pathway, thus regulating MLCP activity and myosin phosphorylation in cells.

The actin-myosin system plays a fundamental role in the regulation of cell motility, including cell contractility, migration, division and shape changes. In vertebrates, the phosphorylation of the regulatory light chain RLC20 regulates conventional myosin (myosin II) for both motor activity and filament formation (1–3). The extent of phosphorylation of myosin II is controlled by the change in the myosin light chain kinase and myosin light chain phosphatase (MLCP) activities. There are two pathways that regulate myosin phosphorylation. One is a Ca\textsuperscript{2+}-dependent pathway. The increase in cytoplasmic Ca\textsuperscript{2+} caused by external stimuli activates Ca\textsuperscript{2+}/calmodulin-dependent myosin light chain kinase, thus increasing myosin phosphorylation. The other is the Ca\textsuperscript{2+}-independent pathway. This pathway regulates both myosin phosphorylation and dephosphorylation processes. It has been shown recently that several Ca\textsuperscript{2+}-independent kinases can phosphorylate myosin \textit{in vitro} (4–7). Among them, zipper-interacting protein kinase has been shown to play an important role in phosphorylating myosin in migrating mammalian cells (8). On the other hand, a number of studies have revealed that MLCP is regulated by a RhoA signaling pathway (9, 10).

MLCP consists of three subunits: a myosin phosphatase targeting subunit (MYPT1), a 20-kDa small subunit, and a catalytic subunit of the type 1 protein serine/threonine phosphatase family (11–13). The N-terminal one-third of the large subunit is composed of eight repeat sequences that correspond to the sequence for an ankyrin repeat, a motif that is found in proteins involved in tissue differentiation and cell cycle and cytoskeleton regulation (14). The domain responsible for the binding of MYPT1 to myosin has been studied. Ichikawa et al. (15) showed that the recombinant N-terminal two-thirds of MYPT1 contains the myosin-binding site because phosphorylated heavy meromyosin (HMM) and RLC20 bound to an affinity column made using this recombinant fragment. On the other hand, Johnson et al. (16) reported that the C-terminal 291 residues of the large subunit, but not the N-terminal fragment, bind to myosin. Although the identity of the myosin-binding domain of MLCP remains controversial, it is generally agreed that the large subunit is the myosin targeting subunit.

The interaction of the subunits of MLCP has been studied. Johnson et al. (16) showed that the 20-kDa subunit does not interact with the catalytic subunit, but does interact with the C-terminal 72 residues of MYPT1. Hirano et al. (17) reported that the catalytic subunit binds to MYPT1 at two sites: a relatively strong site in the N-terminal 38 residues and a weaker site in the ankyrin repeat (residues 39–295). The phosphorylated light chain-binding site is also assigned to the ankyrin repeat. The existence of several isoforms of the type I phosphatase catalytic subunit has been demonstrated (α1, α2, γ1, γ2, and δ) (18–20), with the catalytic subunit of MLCP identified as a δ-isofrom (11).

It has been postulated that the substrate specificity and regulation of protein phosphatases are governed by their regulatory subunits and that the regulatory subunits act as targeting subunits (21). For MLCP, it is known that its holoenzyme has higher activity than its catalytic subunit alone, suggesting that the binding of the regulatory subunits increases MLCP activity. Consistent with this finding, Gong et al. (22) showed that the inhibition of MLCP by a high arachidonic acid concentration is due to the dissociation of the catalytic subunit from the holoenzyme.

A critical finding that sheds light on the linkage between RhoA and its downstream cascade was the discovery of Rho-
Regulation of Myosin Phosphorylation by p116Rip

dependent protein kinase (referred to as Rho kinase). Rho kinase was cloned from various tissues (25–25). The kinase (~160 kDa) is composed of several domains, including a catalytic domain, a coiled-coil domain, a pleckstrin homology domain, and a cysteine-rich zinc finger motif that is homologous to the cysteine-rich domains of protein kinase C (24, 25). Interestingly, it was shown that MYPT1 can be phosphorylated by Rho kinase, which results in a decrease in MLCP activity in vitro (26). Rho kinase phosphorylates MYPT1 at two sites in vitro, i.e. Thr641 and Thr799, with Thr641 responsible for the inhibition of MLCP activity (27). This suggests that activation of the RhoA signaling pathway would phosphorylate MYPT1 by Rho kinase, thus down-regulating MLCP and increasing myosin motor function. It may be asked whether an external stimulus induces the phosphorylation of MYPT1 at Thr641. Quite recently, it was shown that agonist-induced stimulation in smooth muscle, the phosphorylation of MYPT1 at Thr641 was unchanged, whereas RLC20 phosphorylation was significantly increased (28). This suggests that MYPT1 phosphorylation at Thr641 is not responsible for the change in myosin phosphorylation activity, thus inactivating RhoA activity. The gene silencing of p116Rip was performed by p116Rip FACtS, previously found to be a RhoA-interacting protein (29). It was reported that overexpression of p116Rip attenuates lysophosphaticid acid-induced cell shape change, suggesting that p116Rip might inhibit RhoA activity. The mechanism by which p116Rip attenuates the lysophosphaticid acid-induced cell shape change is unclear. Quite recently, Surks et al. (30) reported that p116Rip interacts with MYPT1. To date, the function of p116Rip has not been understood. Here, we found that p116Rip activates MLCP activity. The activation is achieved by p116Rip facilitation of the binding of MYPT1 to myosin. Furthermore, we found that p116Rip has RhoGAP activity, thus inactivating RhoA activity. The gene silencing of p116Rip consistently and significantly increased myosin phosphorylation in cells. Based upon these findings, we propose that p116Rip is an important regulatory component that controls the RhoA signaling pathway, thus regulating MLCP activity and myosin phosphorylation in cells.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Analysis—Yeast two-hybrid screening was performed using the Matchmaker Two-Hybrid System 3 (Clontech) according to the manufacturer’s instructions. Yeast AH109 strains were sequentially transformed with the gBKBT7 vector, coding for full-length rat MYPT1 with a leucine zipper fused in-frame to the DNA-binding domain of Gal4, and then with a human aorta cDNA library constructed in the pACT2 vector, coding for the activation domain of Gal4. Initial transformants were selected as being positive on synthetic complete plates lacking tryptophan/leucine/histidine/adenine and containing 15-Bromo-4-chloro-3-indolyl-alpha-methylsulfonyl fluoride, and 10 μM leucine/valine. The cells were allowed to grow to a late-log phase, and the samples were centrifuged at 14,000 × g for 5 min at 4 °C. Supernatants were incubated with protein A-Sepharose for 1 h at 4 °C, and the resin was centrifuged to remove nonspecific binding proteins. The supernatants were incubated overnight with control IgG, anti-MYPT1 antibody, or anti-p116Rip antibody at 4 °C and then incubated with protein A-Sepharose for 1 h at 4 °C. The resin was precipitated, and the bound proteins were resolved by SDS-PAGE.

Quantitative Analysis of the Binding of p116Rip to MYPT1 or HMM—A fixed amount of FLAG-tagged p116Rip (0.25 μg) was mixed with various amounts of MYPT1 (0.125–3 μg) or HMM (10 μg) in 30 mM Tris-Cl (pH 7.5), 100 mM NaCl, and 0.5% Nonidet P-40 for 1 h at 4 °C. The amount of MYPT1 or HMM bound to p116Rip was determined by SDS-PAGE.

Measurement of Protein Expression Levels in Cells—The homogenates of COS-7 cells were prepared as described above. Protein concentration was measured using a DC protein assay kit (Bio-Rad). The samples were mixed with 2.5–10 μg of myosin light chain (MLC) with 15–25 ng of purified MLCP, and the data was fit by linear regression. Approximately 40 μg of the total COS-7 cell lysates, which fell on the linear portion of the standard curves of p116Rip and MYPT1, was subjected to SDS-PAGE, followed by Western blotting.

Protein Phosphatase and Kinase Assay—After incubation of smooth muscle myosin with 1 μM microcystin LR for 10 min at room temperature, myosin (5 mg/ml) was phosphorylated with 10 μM/ml myosin light chain kinase were prepared from frozen turkey gizzards as described previously (32, 33). Xenopus oocyte calcium was expressed in Escherichia coli and purified as described previously (34, 35). Recombinant MLCP was prepared as described previously (34, 35). HMM was prepared by α-chymotryptic hydrolysis of gizzard myosin (36). Recombinant MLCP holoenzyme was purified by co-infecting Sf9 cells with viruses expressing rat MYPT1 with a leucine zipper, rat p116Rip, and rat M21 as described previously (37). The expressed MLCP holoenzyme was purified by affinity chromatography. HMM was incubated in buffer containing 30 mM Tris-Cl (pH 7.5) and 150 mM NaCl. The bound proteins were analyzed by Western blotting or Coomassie Blue staining. For the binding of p116Rip to myosin, 10 μg of FLAG-tagged p116Rip and 17.5 μg of myosin were incubated in buffer containing 30 mM Tris-Cl (pH 7.5), 50 mM NaCl, and 0.5% Nonidet P-40 for 1 h at 4 °C. The bound proteins were determined as described above.

Immunoprecipitation—COS-7 cells attached to the culture dish were washed three times with cold phosphate-buffered saline and scraped into lysis buffer containing 50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 10 μM leucine/valine. The cells were lysed through a 26-gauge needle. The samples were centrifuged at 14,000 × g for 5 min at 4 °C. Supernatants were incubated with protein A-Sepharose for 1 h at 4 °C, and the resin was centrifuged to remove nonspecific binding proteins. The supernatants were incubated overnight with control IgG, anti-MYPT1 antibody, or anti-p116Rip antibody at 4 °C and then incubated with protein A-Sepharose for 1 h at 4 °C. The resin was precipitated, and the bound proteins were resolved by SDS-PAGE.
chain kinase and 10 μg/ml calmodulin in buffer containing 0.2 mM [γ-32P]ATP, 150 mM KCl, 1 mM MgCl2, 0.2 mM CaCl2, 1 mM dithio-rietol (DTT), 30 mM Tris-HCl (pH 7.5), and 0.1 μM microcystin LR at 25 °C for 30 min. The phosphorylated myosin was precipitated by adding 8 volumes of salt-free buffer containing 15 mM MgCl2, 1 mM DTT, and 30 mM Tris-HCl (pH 7.5). After centrifugation at 14,000 × g for 5 min, the precipitated myosin was dissolved with high salt buffer (0.4 mM KCl, 1 mM DTT, and 30 mM Tris-HCl (pH 7.5)). The precipitation-solubilization cycle was repeated again. The phosphorylated myosin was finally dissolved in high salt buffer at a concentration of 25 μM. MLC20 was also phosphorylated as described previously (34). The phosphorylated MLC20 was precipitated with 10% trichloroacetic acid. The precipitates were washed three times with water and dissolved in 50 mM NaCl, 30 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 1 mM DTT. Protein phosphatase assays were carried out at 25 °C using [γ-32P]ATP-labeled myosin or MLC20 (final concentration of 3 or 0.75 μM, respectively) as substrate in the presence of 100 mM NaCl, 30 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.2 mM MgCl2 bovine serum albumin, and 1 mM MLCP or 20 mM PP1. MLCP at 10 mM was used when phospho-MLC20 was used as substrate. The reactions were terminated by the addition of 10% trichloroacetic acid. After sedimentation of the proteins at 5000 × g for 5 min, Cerenkov counting was performed to determine the radioactivity of the supernatant containing the liberated 32P. A protein kinase assay was performed using FuGENE 6 (Roche Applied Science).

RESULTS

Binding of p116Rip to MYPT1—The interaction between p116Rip and MYPT1 was found by yeast two-hybrid screening (Fig. 1A). The entire coding region of rat MYPT1 was used as bait, and the positive clones were subjected to α-gal assay to eliminate false positive clones. Two of the positive clones, TM3 and TM15, encoded bp 1840–3111 and 1459–3111 of human p116Rip, respectively (Fig. 1A). The binding between p116Rip, RhoA, and MYPT1 was studied. GFP-tagged p116Rip expressed in COS-7 cells was immunoprecipitated with an anti-GFP antibodies. The immunoprecipitated complex was analyzed by SDS-PAGE, followed by Western blot analysis (Fig. 1B). RhoA was co-immunoprecipitated with GFP-p116Rip using anti-GFP antibodies, but not control IgG (Fig. 1B, left panels). Co-immunoprecipitation of p116Rip with GFP-MYPT1 using anti-GFP antibodies was also observed with the cell lysates expressing GFP-tagged MYPT1 (Fig. 1B, right panels). These results support the interaction between p116Rip and MYPT1. To further address this issue, we examined the binding of endogenous p116Rip and MYPT1.

The COS-7 cell lysates were immunoprecipitated with anti-p116Rip antibody (Fig. 1C, left panels). Both MYPT1 and RhoA were co-immunoprecipitated. On the other hand, when anti-MYPT1 antibodies were used for the immunoprecipitation, both p116Rip and RhoA were co-immunoprecipitated (Fig. 1C, right panels). To determine whether co-immunoprecipitation of the three proteins resulted from the direct binding of these proteins, we expressed full-length MYPT1 and p116Rip using the baculovirus expression system, and the isolated proteins were subjected to a binding assay. Full-length p116Rip flanked with a FLAG tag at the N-terminal end was incubated with MYPT1, and anti-FLAG antibody-agarose was added to the mixture. After the reaction was washed exhaustively to eliminate the unbound proteins, FLAG-p116Rip was eluted with FLAG peptide. MYPT1 co-eluted with FLAG-p116Rip, indicating the direct binding between p116Rip and MYPT1 (Fig. 1D). There was no nonspecific binding of MYPT1 to anti-FLAG antibody-agarose (Fig. 1D). Fig. 1E also shows the direct binding between p116Rip and RhoA. We examined RhoA/Q63L, an active form, and RhoA(T19N), an inactive form, for binding to p116Rip. Both the active and inactive forms of RhoA were added to FLAG-p116Rip with anti-FLAG antibody-agarose and eluted with FLAG peptide as described above. A similar amount of RhoA was eluted, indicating that p116Rip binds to RhoA irrespective of the activity of RhoA. The binding of wild-type RhoA to p116Rip was also observed (data not shown).

Preparation and Transfection of Small Interfering RNA (siRNA)—The selected sequences were submitted to a BLAST search to ensure that only the p116Rip gene was targeted. p116Rip siRNA (AATTGCCAGCGACGGTCTCT, corresponding to bp 203–221 of p116Rip) and p116Rip siRNA2 (AATTGACTTGTCCGCATGT, corresponding to bp 1290–1308 of p116Rip) were used. A pair of 67-nucleotide complementary oligonucleotides were synthesized separately with the addition of a BamHI site to the 5'-end and an EcoRI site to the 3'-end. The annealed 67-bp DNA fragment with p116Rip siRNA was cloned into the pSIREN-DNR-DeRed vector (BD Knockout RNAi Systems, Clontech), which has two promoter regions for producing siRNA and DeRed, respectively. Negative control oligonucleotides were obtained from the RNAi-ready pSIREN-DNR-DeRed express kit (Clontech). Transfection of pSIREN vectors into HeLa cells was performed using FuGENE 6 (Roche Applied Science).

Immunofluorescence Staining and Image Processing—In order to analyze the localization of two different anti-rabbit polyclonal antibodies were used for the independent localization of p116Rip and MYPT1 as described previously (39). Briefly, COS-7 cells were incubated with anti-rabbit MYPT1 polyclonal antibody, followed by Cy5-conjugated anti-rabbit IgG and unlabeled anti-rabbit IgG to block anti-rabbit MYPT1 antibody. This blocking step assured that all free sites for anti-rabbit IgG interaction were covered before the second series of polyclonal and secondary labeled antibodies. After this blocking step, cells were incubated sequentially with anti-rabbit p116Rip polyclonal antibody and fluorescein isothiocyanate-conjugated anti-rabbit IgG. As a control, fluorescein isothiocyanate labeling was observed after the deletion of the second polyclonal antibody, providing a measure of the efficacy of the blocking step. Actin filaments were stained with phalloidin-conjugated Alexa Fluor 546 during the incubation with the secondary antibody. Immunocytochemistry was performed as described previously (8, 31, 40). Fluorescence images were viewed using a DM IRB laser scanning confocal microscope (Leica) controlled by TCS SP II systems (Leica). All images were taken with the same laser output to directly compare the fluorescence signal intensities. Images were processed using Adobe® Photoshop® Version 7.0 software.
was also supported by the results shown in Fig. 1, where immunoprecipitation of the endogenous proteins by anti-p116<sup>Rip</sup> antibody yielded both MYPT1 and RhoA. It should be noted that the binding of RhoA to p116<sup>Rip</sup> was weaker than that of MYPT1 to p116<sup>Rip</sup> and that although the majority of the added MYPT1 co-eluted from the resin with p116<sup>Rip</sup>, only part of the added RhoA was recovered with p116<sup>Rip</sup>. Fig. 1G shows the binding of MYPT1 to p116<sup>Rip</sup> as a function of MYPT1 concentration. The extent of binding showed a saturation curve, and a maximum binding stoichiometry of 0.83 mol of
MYPT1/mol of p116Rip with an apparent dissociation constant (K_d) of 1.0 × 10^-6 M was obtained. Fig. 1H shows the expression levels of p116Rip and MYPT1 in COS-7 cells. Total cell homogenates were subjected to SDS-PAGE, followed by Western blotting, and the signals were normalized to estimate the expression levels of these proteins using the isolated p116Rip and MYPT1 of known concentration as standards. Based upon the results, we estimated the molar ratio of the expression level of p116Rip to that of MYPT1 to be 1:2. It was reported previously (30) that p116Rip binds to the leucine zipper motif of MYPT1, suggesting that it binds to the isoform with the leucine zipper. It should be noted that although p116Rip interacted with RhoA, it did not directly bind to Rho kinase (Fig. 1I).

Fig. 1J shows the localization of endogenous p116Rip and MYPT1 in COS-7 cells, and p116Rip localization at stress fibers agrees with previous reports (30, 41). Consistent with the in vitro data shown above, MYPT1 and p116Rip co-localized well. The results support that the binding of p116Rip to MYPT1 takes place in cells.

Effect of p116Rip on MLCP Activity—Because MYPT1 is a regulatory subunit of MLCP, we examined whether the binding of p116Rip to MYPT1 affects phosphatase activity. As shown in Fig. 2A, p116Rip significantly activated the myosin phosphatase activity of the MLCP holoenzyme in a concentration-de-
dependent manner. On the other hand, p116<sup>Rip</sup> did not affect the phosphatase activity of the PP1<sub>6</sub> catalytic subunit (Fig. 2A). These results suggest that the binding of p116<sup>Rip</sup> to MYPT1 alters the interaction between MYPT1 and myosin, thus activating phosphatase activity.

It is known that Rho kinase phosphorylates MYPT1 and that the phosphorylation of MYPT1 at Thr<sup>641</sup> inhibits MLCP activity (27). We examined whether the binding of p116<sup>Rip</sup> to MYPT1 affects the phosphorylation of MYPT1 by Rho kinase. MYPT1 was phosphorylated by Rho kinase in the presence or absence of p116<sup>Rip</sup>, and the phosphorylation of Thr<sup>641</sup> was monitored using phosphorylation site-specific antibodies (28). As shown in Fig. 2B, the phosphorylation of MYPT1 at Thr<sup>641</sup> was not affected by p116<sup>Rip</sup> binding.

Because it is known that MLCP activity is affected by the binding of MYPT1 to the catalytic subunit, we examined the effect of p116<sup>Rip</sup> on the association of MYPT1 with the catalytic subunit. p116<sup>Rip</sup> was added to the MLCP holoenzyme and immunoprecipitated with anti-MYPT1 antibodies. All of the catalytic subunit was co-immunoprecipitated regardless of the presence of p116<sup>Rip</sup> (Fig. 2C), suggesting that p116<sup>Rip</sup> does not interfere with the association of MYPT1 and PP1<sub>6</sub>.

It is critical to determine whether the activation of the MLCP-induced dephosphorylation of myosin by p116<sup>Rip</sup> is due to the activation of the enzyme itself or to the change in the interaction between myosin and MYPT1. To address this question, we examined the effect of p116<sup>Rip</sup> on the MLCP-induced dephosphorylation of isolated MLC20. Quite interestingly, the dephosphorylation rate of MLC20 was not significantly increased in the presence of p116<sup>Rip</sup> (Fig. 2D), suggested that the increase in the MLCP-induced dephosphorylation of myosin by p116<sup>Rip</sup> resulted from the p116<sup>Rip</sup>-induced changes in the interaction between MLCP and myosin. Therefore, we examined whether p116<sup>Rip</sup> binds directly to myosin or strengthens the binding between MYPT1 and myosin. FLAG-tagged p116<sup>Rip</sup> was mixed with myosin, and the complex was precipitated with anti-FLAG antibody-agarose. As shown in Fig. 2E, myosin was pulled down by p116<sup>Rip</sup>. On the other hand, myosin was not precipitated by the resin without p116<sup>Rip</sup>. Fig. 2F shows the binding of HMM to p116<sup>Rip</sup> as a function of HMM concentration. We used HMM to estimate the binding stoichiometry; we did not use whole myosin because myosin forms filaments under physiological ionic conditions, and the binding of one molecule of myosin in the filament to p116<sup>Rip</sup> coprecipitates a number of myosin molecules with the p116<sup>Rip</sup>-bound resin. This is avoided using the soluble fragment of myosin, HMM. The extent of binding showed a saturation curve, and a maximum binding stoichiometry of 1.1 mol of head HMM/mol of p116<sup>Rip</sup> with an <i>K<sub>d</sub></i> of 1.0 × 10<sup>-6</sup> M was obtained. It is known that the phosphorylation of myosin at MLC20 Ser<sup>19</sup> changes the conformation of myosin (42, 43). Therefore, we examined the effect of myosin phosphorylation on the binding of p116<sup>Rip</sup> to myosin. There was no significant change in binding upon MLC20 phosphorylation (Fig. 2F). It should be noted that myosin could be precipitated by high speed centrifugation, but not by low speed centrifugation (100 × g) used for the experiment. These results clearly demonstrate that p116<sup>Rip</sup> directly binds to myosin.

**Effect of p116<sup>Rip</sup> on RhoA GTPase Activity**—We next examined whether p116<sup>Rip</sup> influences RhoA function. It has been shown previously that RhoA activity depends upon the nucleotide to which it is bound (44). GTP-RhoA and GDP-RhoA are the active and inactive forms, respectively. The regulatory proteins (GTPase-activating protein and GDP-GTP exchange factor) control the relative presence of the two forms. The GTPase-activating protein favors the production of the GDP-bound form, whereas the GDP-GTP exchange factor induces the exchange of the bound GDP with GTP to facilitate the production of the GTP-bound form (44). We first determined the effect of p116<sup>Rip</sup> on the rate of GTP hydrolysis by measuring the decrease in the GTP-bound RhoA concentration. As shown in Fig. 3A, p116<sup>Rip</sup> significantly decreased the amount of GTP-bound RhoA in a p116<sup>Rip</sup>-concentration-dependent manner. This result suggests that p116<sup>Rip</sup> enhances the GTPase activity of RhoA, i.e. GTPase-activating protein activity. We then examined the RhoGEF activity of p116<sup>Rip</sup>. RhoA was incubated with GDP, and then [γ<sup>32</sup>PGTP]-S was added in the presence or absence of p116<sup>Rip</sup>. The production of GTP-RhoA was monitored as described under “Experimental Procedures.” p116<sup>Rip</sup> had no influence on the exchange of GTP for GDP (data not shown).

**p116<sup>Rip</sup> Inhibits RhoA Activity in Cells**—As p116<sup>Rip</sup> increased the GTPase activity of RhoA, we anticipated that p116<sup>Rip</sup> would inactivate RhoA by destabilizing the GTP-bound form of RhoA. To investigate whether p116<sup>Rip</sup> affects RhoA activity in cells, we examined the effect of p116<sup>Rip</sup> on RhoA activity by measuring the amount of GTP-RhoA in a rhotekin binding assay. HeLa cells were transfected with GFP-p116<sup>Rip</sup> or GFP alone. The protein expression of GFP-p116<sup>Rip</sup> was confirmed by Western blot analysis (Fig. 3B). p116<sup>Rip</sup> overexpression did not affect the RhoA expression level (Fig. 3B). After...
These results are consistent with the finding that p116Rip acts as an activator of RhoA GTPase activity, suggesting that p116Rip inhibits RhoA activity in cells.

Gene Silencing of p116Rip Increases MLC20 Phosphorylation—Because p116Rip affected both MLCP and RhoA activities, we anticipated that p116Rip would be involved in the regulation of myosin II phosphorylation in cells. To address this issue, we studied the effect of the elimination of p116Rip on myosin phosphorylation in cells. We attempted to eliminate the expression of p116Rip by transfecting the p116Rip-specific siRNA into cells. Two sequences were chosen for the production of siRNA, siRNA1 and siRNA2. HeLa cells were transfected with the pSIREN-DNR-DsRed vector containing either p116Rip siRNA sequence or control sequence (see “Experimental Procedures”). The transected cells could be identified by the expression of DsRed. The transfection efficiency was ~80%. The cells were harvested and subjected to Western blot analysis to evaluate the efficiency of gene silencing. Both siRNA1 and siRNA2 efficiently eliminated p116Rip expression, whereas the p116Rip siRNAs did not affect the expression of MYPT1 or MLC20 (Fig. 4A). An important finding is that p116Rip siRNA significantly increased the phosphorylation of MLC20 as revealed by Western blotting using the antibodies specific to phospho-Ser19 of MLC20. The transfection efficiency was ~80%, it was calculated that nearly 30% of the myosin in the siRNA-transfected cells was phosphorylated.

The increase in MLC20 phosphorylation upon elimination of p116Rip was also shown by immunostaining of the cells with the phosphorylation site-specific antibodies to MLC20. As shown in Fig. 4C, the elimination of p116Rip by siRNA markedly increased MLC20 phosphorylation in both the cell cortical region and stress fibers. On the other hand, the signal intensity and localization probed by antibodies recognizing MLC20 regardless of its phosphorylation state were the same in the siRNA-transfected and control cells. Fig. 5 shows the effect of p116Rip elimination by siRNA on stress fiber formation. For the cells treated with two different siRNAs for p116Rip, stress fiber formation was significantly increased compared with the control cells. Note that the non-siRNA-transfected cells on the same coverslip did not show any increase in stress fiber formation. The number of cells showing stress fibers was significantly increased upon siRNA transfection (Fig. 5B). The fraction of untransfected cells showing stress fibers was the same as that of control siRNA-transfected cells (Fig. 5B). It should be noted that although ~20% of the control cells showed clear stress fibers, the number of stress fibers was clearly lower than in p116Rip siRNA-transfected cells. It is known that RhoA controls stress fiber formation (45, 46). It is also known that an increase in MLC20 phosphorylation stabilizes stress fibers (47, 48). Therefore, these results are consistent with the finding that p116Rip has RhoGAP activity and MLCP-activating function.

DISCUSSION

We have found that p116Rip binds to both RhoA and MYPT1. Quite recently, Surks et al. (30) demonstrated an interaction between p116Rip and MYPT1 using yeast two-hybrid screening. However, the function of p116Rip has not been clarified. Our results are consistent with this recent report and further show that p116Rip can bind to both RhoA and MYPT1 simultaneously, thus forming a ternary complex. It was shown previously that the RhoA-binding region and MYPT1-binding region
reside in the first coiled-coil region and the second/third coiled-coil region of p116Rip, respectively (30). Our results are consistent with this finding and suggest that MYPT1 and RhoA do not interfere with each other in their binding to p116Rip.

The critical finding of this study is that p116Rip increases RhoA GTPase and MLCP activities. It is anticipated that the activation of RhoA GTPase activity increases GDP-RhoA, an inactive form, thus decreasing the GTP-bound form of RhoA. In support of this finding, the rhotekin binding assay revealed that the expression of p116Rip inhibits RhoA activation by epidermal growth factor in cells. This result suggests that p116Rip down-regulates RhoA activity in cells, thus forming the inactive form of RhoA. This is consistent with the RhoGAP activity of p116Rip. We also observed that the elimination of p116Rip by p116Rip-specific siRNA resulted in an increase in stress fiber formation. Because it is known that the activation of RhoA increases stress fiber formation (45, 46), the elimination of p116Rip with RhoGAP activity increases GTP-bound RhoA, thus increasing stress fiber formation.

Another important finding of this study is that the binding of p116Rip to MYPT1 activates MLCP activity. The elimination of p116Rip by the p116Rip-specific siRNA consistently increased MLC20 phosphorylation. This result supports the notion that the p116Rip-induced activation of MLCP takes place in vivo. The activation of the myosin light chain dephosphorylation activity is due to the binding of p116Rip to MYPT1 since p116Rip did not activate the PP1 catalytic subunit. Quite interestingly, p116Rip increased MLCP-catalyzed myosin dephosphorylation, but not isolated MLC20 dephosphorylation. Consistent with this finding, we have found that p116Rip directly binds to myosin. Taking these results into account, it is thought that p116Rip interacts with both myosin and MYPT1 simultaneously, facilitating MLCP to access the phosphorylation site of myosin at MLC20 and enhancing the dephosphorylation of myosin. It is known that MYPT1 or the MLCP holoenzyme binds to myosin with moderate binding activity. On the other hand, it is also known that MLCP activity is tightly associated with myosin under physiological ionic conditions and is barely removed from myosin preparations (49). Theoretically, MLCP should be easily removed from myosin if the binding of MLCP to myosin is moderate. Our findings suggest that p116Rip enhances the binding of MLCP to myosin, and it is plausible that the tight association of MLCP activity in myosin preparations may be due to the presence of p116Rip, although the amount of p116Rip and MLCP present in myosin preparations is negligible. It was reported previously that p116Rip can bind to actin with a dissociation constant of 0.5 μM (41). p116Rip consistently showed a discrete localization at the stress fibers and in the cell.
cortical region, where actin and myosin are the major components. Therefore, p116Rip functions as a scaffolding protein that holds MLCP at the actomyosin structure in cells, thus promoting the efficiency and specificity of MLCP against myosin dephosphorylation.

Although p116Rip was originally found to be a RhoA-interacting protein, we found that the binding of p116Rip to RhoA is rather weak. This is consistent with the finding that RhoA and p116Rip do not show significant co-localization in cells. It is known that the active form of RhoA (i.e., GTP-RhoA) translocates from the cytosol to the membrane (25, 50). A question is how p116Rip localized in the actomyosin-based cytoskeleton such as stress fibers interacts with GTP-RhoA to induce GTP hydrolysis, resulting in inactive or inactivated RhoA. Since the overexpression of p116Rip in HeLa cells resulted in the inactivation of RhoA, the RhoGAP function of p116Rip should operate in the cellular environment. Although GTP-RhoA translocates to the membrane, it is reasonable to think that GTP-RhoA is in the equilibrium between the membrane-bound and the cytosolic molecules. Cytosolic GTP-RhoA encounters p116Rip and is converted to GDP-RhoA, thus reducing GTP-RhoA on the membrane in the equilibrium between the membrane and cytosolic molecules. The net result is a decrease in the membrane-bound active RhoA molecules.

Previously, Surks et al. (30) reported that immunoprecipitates from cell lysates using anti-p116Rip antibodies contained protein kinase activity that was inhibited by Y27632 and claimed that p116Rip bound to Rho kinase, although the presence of Rho kinase in the immunoprecipitates was not directly shown. In the present study, we found that p116Rip does not bind directly to Rho kinase. Therefore, it is likely that if Rho kinase was in the immunoprecipitates of p116Rip, the kinase was co-immunoprecipitated with p116Rip via RhoA bound to p116Rip.

It is known that the RhoA pathway plays an important role in the regulation of myosin phosphorylation (9, 10). The activation of RhoA activates Rho kinase, which results in the inhibition of MLCP activity, thus increasing myosin phosphorylation (26). Therefore, it is anticipated that p116Rip facilitates the decrease in myosin phosphorylation by diminishing RhoA-dependent MLCP inhibition.

Our findings provide the following scenario for the function of p116Rip. p116Rip binds to GTP-RhoA to accelerate GTP hydrolysis, thus forming GDP-RhoA. The GDP-RhoA produced associates with the guanine nucleotide dissociation inhibitor, resulting in the dissociation of p116Rip from the RhoA-GDI complex. The dissociated p116Rip becomes available for binding to GTP-RhoA to initiate another cycle.

Based upon our findings, we propose a model explaining the function of p116Rip as follows (Fig. 6). p116Rip co-localizes with actomyosin structures such as stress fibers and cortical actin in cells, where p116Rip forms a complex with MYPT1 and myosin. p116Rip holds MLCP with myosin, thus facilitating efficient and specific dephosphorylation of myosin in cells. As a result, myosin phosphorylation at this cytoskeletal structure decreases. On the other hand, p116Rip facilitates the GTP hydrolysis of RhoA to produce GDP-RhoA. GDP-RhoA then forms a complex with RhoGDI in the cytosol. p116Rip shifts the equilibrium of GTP-RhoA and GDP-RhoA toward the GDP-bound inactive form. Because Rho kinase phosphorylates MYPT1, which inactivates MLCP activity (26), it is anticipated that the inactivation of RhoA by p116Rip also contributes to the increase in MLCP activity, thus reducing myosin phosphorylation. Therefore, it is postulated that p116Rip contributes to the decrease in myosin phosphorylation via activation of the myosin dephosphorylation activity of MLCP and the inactivation of the RhoA pathway.
p116Rip Decreases Myosin II Phosphorylation by Activating Myosin Light Chain Phosphatase and by Inactivating RhoA
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