Rho-kinase is implicated in the phosphorylation of myosin light chain downstream of Rho, which is thought to induce smooth muscle contraction and stress fiber formation in non-muscle cells. Here, we examined the mode of action of inhibitors of Rho-kinase. The chemical compounds such as HA1077 and Y-32885 inhibited not only the Rho-kinase activity but also the activity of protein kinase N, one of the targets of Rho, but had less of an effect on the activity of myotonic dystrophy kinase-related Cdc42-binding kinase β (MRCKβ). The COOH-terminal portion of Rho-kinase containing Rho-binding (RB) and pleckstrin homology (PH) domains (RB/PH (TT)), in which point mutations were introduced to abolish the Rho binding activity, interacted with Rho-kinase and thereby inhibited the Rho-kinase activity, whereas RB/PH (TT) had no effect on the activity of protein kinase N or MRCKβ, suggesting that the COOH-terminal region of Rho-kinase is a possible negative regulatory region of Rho-kinase. The expression of RB/PH (TT) specifically blocked the stress fiber and focal adhesion formation induced by the active form of Rho or Rho-kinase in NIH 3T3 cells, but not that induced by the active form of MRCKβ or myosin light chain. Thus, RB/PH (TT) appears to specifically inhibit Rho-kinase in vitro.

There is mounting evidence that the small GTPase Rho plays crucial roles in the rearrangements of cytoskeleton and cell adhesion (1–3). Rho cycles between GDP-bound inactive and GTP-bound active forms, and the GTP-bound form binds to specific effectors and then exerts its biological functions. Numerous putative Rho effectors have been identified; PKN1 (4, 5), Rho-kinase/ROKα/ROCK II (6–8), myosin binding subunit of myosin phosphatase (9), mDia1 (10), citron (11), citron kinase (12), rhophilin, rhoteikin (11), Kv1.2 (13), and phospholipase D (14). The catalytic domain mutated at the ATP-binding site (kinase dead: CAT-KD) and the PH domain (PH) serve as dominant negative forms (16). The Rho-binding domain (RB) of Rho-kinase inhibits Rho-dependent activation of Rho-kinase in vitro and interferes with the Rho pathway in vivo (16). The catalytic domain mutated at the ATP-binding site (kinase dead: CAT-KD) and the PH domain (PH) serve as dominant negative forms in vivo in some cases (16), while they have no or a very weak effect on the Rho-kinase activity in vitro. It has been also reported that ROCK I, an isoform of Rho-kinase, that is kinase dead and cannot bind to Rho (named as KD-IA) functions as dominant negative form in the cells (17). Recently, we found that the COOH-terminal portion of Rho-kinase containing Rho-binding and PH domains (RB/PH (TT)), in which point mutations are introduced to abolish the Rho binding activity (15, 30), inhibits the lysophosphatidic acid-induced neurite retraction in neuroblastoma cells and cytotaxis in Xenopus eggs or in mammalian cells (21, 24), although PH has minimal effects in these cases. These results suggest that RB/PH (TT) functions as the dominant negative form of Rho-kinase by the different mechanism of inhibition from those of CAT-KD, RB, and PH.

Here, we examined the mode of action and the specificity of inhibitors of Rho-kinase. We found that RB/PH (TT) directly and specifically inhibited the kinase activity of catalytic fragment of Rho-kinase in vitro. The expression of RB/PH (TT) specifically blocked the stress fiber and focal adhesion formation induced by the active form of Rho or Rho-kinase in NIH 3T3 cells, but not that induced by the active form of MLC.

EXPERIMENTAL PROCEDURES

Materials and Chemicals—GST-Rho-kinase-CAT (6–553 amino acids), GST-MRCKb-CAT (1–550 amino acids), and GST-PKN-CAT (581–942 amino acids) were produced in Sf9 cells with a baculovirus system and...
purified on a glutathione-Sepharose column (6,16,26). MBP-RB/PB (TT) (941–1388 amino acids with mutations; N10367, R10377), MBP-RB (941–1075 amino acids), MBP-PH (1125–1388 amino acids), GST-RB, GST-PHI, and GST-coil y (941–1131 amino acids) were expressed in Escherichia coli and purified with amylose resin (New England Biotech) for MBP fusion proteins or with glutathione-Sepharose (Amersham Pharmacia Biotech) for GST fusion proteins, respectively. MLC was kindly provided by Dr. Ito (Mie University, Mie, Japan). Rsk kinase S6 substrate peptide (RRRLSSLRA) and PKC substrate peptide (RPPARKGSLRQKNVHEVK) were synthesized. \([\gamma^{32P}]ATP\) was purchased from Amersham Pharmacia Biotech. Stauroporine was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). HA1077 was kindly provided by Asahi Chemical Industry (Shizuoka, Japan). Y-32885 was synthesized (29). Tetramethyl rhodamine isothiocyanate-labeled phalloidin and anti-vinculin monoclonal antibody were purchased from Sigma. Anti-Myc polyclonal antibody was purchased from Santa Cruz Biotech Inc. (Santa Cruz, CA). All materials used in the nucleic acid study were purchased from Takara Shuzo Co. (Kyoto, Japan). Other materials and chemicals were obtained from commercial sources.

**Kinase Assay**—The kinase reaction for GST-Rho-kinase-CAT, GST-MRCK\(b\)-CAT, or GST-PKN-CAT was carried out in 50 \(\mu\)l of kinase buffer (50 \(\mu\)M Tris/HC1 at pH 7.5, 2 \(\mu\)M EDTA, 1 \(\mu\)M MgCl\(_2\), 5 \(\mu\)M GTP) containing 100 \(\mu\)M \([\gamma^{32P}]ATP (1–20 GBq/mmol), recombinant kinase (30 ng of Rho-kinase-CAT, 250 ng of MRCK\(b\)-CAT, or 60 ng of PKN-CAT), and 40 \(\mu\)M substrate peptide. Rsk kinase S6 substrate peptide and PKC substrate peptide were used for Rho-kinase and MRCK\(b\), and for PKN as substrates, respectively. After incubation for 10 min at 30 °C, the reaction mixtures were spotted onto a Whatman P81 paper and washed with 75 mM phosphoric acid three times. Incorporation of \(^{32}\)P into the substrates was assessed by scintillation counting.

The kinase reaction for GST-Rho-kinase-CAT was carried out in 50 \(\mu\)l of kinase buffer (50 \(\mu\)M Tris/HC1 at pH 7.5, 2 \(\mu\)M EDTA, 1 \(\mu\)M MgCl\(_2\), 5 \(\mu\)M GTP, 3 \(\mu\)M KCl) containing 100 \(\mu\)M \([\gamma^{32P}]ATP (1–20 GBq/mmol), recombinant kinase (6 ng of Rho-kinase-CAT or 50 ng of MRCK\(b\)-CAT), and purified MLC. After incubation for 10 min at 30 °C, the reaction mixtures were boiled in SDS sample buffer and subjected to SDS-PAGE. The radiolabeled bands were visualized by an image analyzer (Fuji). Afinity Chromatography—The cytosol fraction and membrane extract of bovine brain gray matter (50 g) were prepared (4). The cytosol fraction (4 ml; 200 mg of protein) or membrane extract (4 ml; 20 mg of protein) was preincubated with 2 ml of amylose resin. Half of the bovine cytosol or membrane extract was loaded onto a 0.25-ml amylose resin column (New England Biotech) for MBP fusion proteins or GST fusion proteins, respectively. MLC was kindly provided by Dr. Ito (Mie University, Mie, Japan). Y-32885 was synthesized (29). Tetramethyl rhodamine isothiocyanate-labeled phalloidin and anti-vinculin monoclonal antibody were purchased from Sigma. Anti-Myc polyclonal antibody was purchased from Santa Cruz Biotech Inc. (Santa Cruz, CA). All materials used in the nucleic acid study were purchased from Takara Shuzo Co. (Kyoto, Japan). Other materials and chemicals were obtained from commercial sources.

**Cell Culture and Microinjection**—NIH 3T3 cells were maintained in DMEM containing 10% calf serum. For expression of Rho\(\gamma\)-34, Rho-kinase-CAT, or MRCK\(b\)-CAT, cells were seeded at a density of 2 \(\times\) 10\(^5\) onto 13-mm glass coverslips. After 5 days, the cells were deprived of serum for 24 h in DMEM. Plasmid DNA was microinjected into nuclei and the cells were incubated for 24 h in DMEM. For expression of MLC or MLC\(\gamma\)-34, cells were seeded at a density of 2 \(\times\) 10\(^5\) onto 13-mm glass coverslips, and cultured in DMEM containing 10% calf serum. After 24 h, plasmid DNA was microinjected into nuclei, and the cells were incubated in DMEM containing 10% calf serum for 24 h, then deprived of serum for 24 h in DMEM. If necessary, the cells were treated with HA1077 or vehicle for 20 min after incubation.

**Immunofluorescent Staining**—The cells were fixed with 3.7% formaldehyde in PBS for 10 min, washed with PBS, and permeabilized with PBS containing 0.2% Triton X-100 for 10 min. After being washed with PBS three times, the cells were double stained with tetramethyl rhodamine isothiocyanate-labeled phalloidin and anti-Myc polyclonal antibody with fluorescein isothiocyanate-conjugated anti-rabbit antibody, or with anti-vinculin monoclonal antibody with fluorescein isothiocyanate-conjugated anti-mouse antibody and anti-Myc polyclonal antibody with Texas red-conjugated anti-rabbit antibody. After being washed three times with PBS, the cells were examined using a Zeiss axiophoto microscope.

**RESULTS**

**Chemical Compounds Inhibit the Rho-kinase Activity in Vitro**—We examined the effect of chemical compounds, which are thought to interact with the kinase domain of Rho-kinase and compete with ATP, on the activity of the catalytic domain of Rho-kinase (Rho-kinase-CAT). Stauroporine, HA1077, and Y-32885 inhibited the activity of Rho-kinase-CAT in a dose-dependent manner (Fig. 1), and the IC\(_{50}\) values were approximately 7 nM, 1.7 \(\mu\)M, and 0.4 \(\mu\)M, respectively, under the conditions. The \(K_i\) values for staurosporine, HA1077, and Y-32885 were calculated to be approximately 1.6 nM, 0.4 \(\mu\)M, and 0.1 \(\mu\)M, respectively. The \(K_i\) values for HA1077 and Y-32885 are roughly the same as reported previously (29). PKN is one of the Rho targets. PKN has sequence similarity to protein kinase C within the kinase domain and substrate specificity similar to that of protein kinase C (32). PKN is activated by the active form of Rho like Rho-kinase, but shows substrate specificity distinct from that of Rho-kinase. MRCK\(b\) was identified as a Cdc42 target molecule, and a member of the myotonic dystrophy kinase family like Rho-kinase. MRCK\(b\) shares sequence similarity with Rho-kinase within the kinase domain and shows substrate specificity similar to that of Rho-kinase (33). To test the specificity of the above compounds, we examined their effects on the activity of the catalytic domain of PKN (PKN-CAT) and the catalytic domain of MRCK\(b\) (MRCK\(b\)-CAT). These compounds inhibited the activity of PKN-CAT with similar doses as for Rho-kinase, whereas they had less inhibitory effect on that of MRCK\(b\)-CAT despite their similarity in primary structure (Fig. 1). Thus, it is likely that these compounds inhibit both Rho-kinase and PKN activities with a similar efficiency and cannot distinguish Rho-kinase and PKN under the conditions.

**The COOH-terminal Portion of Rho-kinase Inhibits the Rho-kinase Activity in Vitro**—We then examined whether the various fragments of Rho-kinase affect the Rho-kinase activity in vitro. We previously reported that RB or PH fragment expressed in \(E\). coli as a GST fusion protein had no or a very weak effect on the activity of Rho-kinase-CAT in vitro (16). Here, we found that the COOH-terminal portion of Rho-kinase contain-

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2 N. Nakamura, M. Fukata, Y. Fukata, N. Oshiro, M. Amano, S. Kuroda, T. Yano, M. Shiibata, M. Ikebe, Y. Matsuura, K. Okawa, A. Iwamatsu, and K. Kaibuchi, manuscript in preparation.

3 M. Amano, K. Chihara, N. Nakamura, T. Kaneko, Y. Matsuura, and K. Kaibuchi, unpublished data.
ing the RB and PH domains (RB/PH (TT)), in which point mutations are introduced to abolish the Rho binding activity, inhibited the Rho-kinase activity. The RB/PH (TT) region expressed in E. coli as an MBP fusion protein (MBP-RB/PH (TT)) inhibited the kinase activity of Rho-kinase-CAT toward both Rsk kinase S6 peptide (RRRLSSSLRA) and MLC in a dose-dependent manner (Fig. 2, A and C). IC50 values of MBP-RB/PH (TT) were approximately 0.1 and 0.02 μM, respectively, under the conditions. MBP-RB/PH (TT) had no effect on the activity of MRCKβ-CAT or PKN-CAT (Fig. 2, A and C). Kinetic analyses revealed that MBP-RB/PH (TT) inhibited the activity of Rho-kinase-CAT toward S6 peptide in a competitive manner (Fig. 2B) and toward MLC in a noncompetitive-competitive mixed manner (Fig. 2D). We confirmed that MBP-RB/PH (TT) inhibited the activity of native Rho-kinase purified from bovine brain (data not shown). Since MBP-RB/PH (TT) inhibited the activity of Rho-kinase-CAT in a manner competitive with S6 peptide, it is possible that MBP-RB/PH (TT) interacts with the catalytic domain of Rho-kinase and thereby competes with S6 peptide. To prove this rationale, we examined whether MBP-RB/PH (TT) interacts with Rho-kinase. Bovine cytosol or membrane extract fraction containing Rho-kinase was loaded onto affinity beads coated with MBP or MBP-RB/PH (TT). The proteins bound to the affinity beads were eluted by the addition of 0.1 M NaCl and the eluates were subjected to immunoblot analysis with anti-Rho-kinase antibody. The band corresponding to Rho-kinase was detected in the eluate from the beads coated with MBP-RB/PH (TT), but not in that from the beads coated with MBP (Fig. 3), MBP-RB, or MBP-PH (data not shown). We also confirmed that Rho-kinase-CAT interacted with MBP-RB/PH (TT) under similar conditions (data not shown). Taken together, these results indicate that MBP-RB/PH (TT) interacts with the catalytic domain of Rho-kinase and thereby inhibits the kinase activity more specifically than do the chemical compounds.

To identify the minimal region in RB/PH fragment responsible for the inhibition, we examined whether various fragments within the RB/PH region inhibit the activity of Rho-kinase-CAT. Except RB/PH (TT), all fragments or combinations of fragments tested had no or very weak inhibitory effects on the activity of Rho-kinase-CAT as summarized in Fig. 4. These results suggest that the entire structure of RB/PH (TT) is necessary for the inhibition of the activity of Rho-kinase-CAT.

**Effects of HA1077 and RB/PH (TT) on the Cytoskeletal Organization Induced by Rho-kinase**—We then investigated the specificity of RB/PH (TT) and HA1077 in vivo by the nuclear injection into NIH 3T3 cells of the eDNA encoding RB/PH (TT) with the eDNA encoding activated Rho (RhoVal14), Rho-kinase-CAT, or MRCKβ-CAT. As previously reported, the expression of RhoVal14 (34), Rho-kinase-CAT (16), or MRCKβ-CAT (33) resulted in the formation of stress fibers and focal adhesions in serum-starved NIH 3T3 cells (Fig. 5). In contrast, coexpression of RB/PH (TT) with RhoVal14 or MRCKβ-CAT inhibited the formation of stress fibers in more than 50% of cells expressing RhoVal14 or Rho-kinase-CAT (Fig. 5). We examined the various ratios between RB/PH (TT) and Rho-kinase-CAT and confirmed that an RB/PH (TT) excess was required for inhibition of the Rho-kinase-CAT-induced stress fiber formation (data not shown). Most cells expressing both MRCKβ-CAT and RB/PH (TT) showed stress fibers (Fig. 5), although the number or
and/or GST-coil (941–1388 amino acids), GST-RB, GST-PH, (941–1075 amino acids), GST-RB, GST-PH, and/or GST-coil (941–1131 amino acids) were added into the kinase reactions of Rho-kinase-CAT. Numbers indicate the amino acid residues. Functional and structural domains of Rho-kinase are shown schematically. +, indicates the fragment with inhibitory effect.

thickness of stress fibers decreased in a few cases. Coexpression of COIL (amino acids 421 to 701) of Rho-kinase did not affect the Rho\(^{Val-14}\), Rho-kinase-CAT-, or MRCK\(^{β-}\)-induced stress fiber formation (data not shown). Twenty-four h after the injection, treatment of the cells expressing Rho\(^{Val-14}\) with HA1077 for 20 min disrupted the actin stress fibers. Treatment of the cells expressing Rho-kinase-CAT with HA1077 also resulted in disruption of stress fibers, although the inhibitory effect by HA1077 on stress fibers induced by Rho-kinase-CAT was weaker than on those induced by Rho\(^{Val-14}\) at the same concentration. This may be explained by the notion that higher concentrations of HA1077 are required to inhibit overexpressed Rho-kinase-CAT efficiently. HA1077 did not affect the stress fibers induced by MRCK\(^{β-}\).

The coexpression of RB/PH (TT) with Rho\(^{Val-14}\) or Rho-kinase-CAT also inhibited the formation of focal adhesions, which were visualized as vinculin accumulation at the tip of stress fibers with an arrowhead-like shape, but had no effect on focal adhesion formation induced by MRCK\(^{β-}\)-CAT (Fig. 6). The vinculin accumulation at the tips occasionally remained after the treatment of the cells expressing Rho\(^{Val-14}\) with HA1077 (Fig. 6), although the stress fiber structure was mostly disrupted under the same conditions. The vinculin accumulation at the tips in the cells expressing Rho\(^{Val-14}\) or Rho-kinase-CAT became less prominent after treatment with HA1077, while the staining pattern of vinculin in the cells expressing MRCK\(^{β-}\)-CAT did not change after treatment. These results indicate that RB/PH (TT) and HA1077 inhibit the activity of Rho-kinase in vivo but not that of MRCK\(^{β-}\), as expected from the results of the *in vitro* experiments.

Effects of HA1077 and RB/PH (TT) on the Cytoskeletal Organization Induced by Activated MLC—The replacement of MLC by recombinant MLC\(^{T18D,S19D}\) in myosin II partially activates the ATPase activity and forms myosin filaments in *vitro* (35–37). We have recently found that the expression of MLC\(^{T18D,S19D}\) induces neurite retraction in N1E-115 neuroblastoma cells and stress fiber formation in NIH 3T3 cells (21).

Since the contractility driven by myosin II activation is presumed to play a critical role in focal adhesion formation (38), we examined whether the expression of MLC\(^{T18D,S19D}\) is sufficient for the formation of focal adhesion. The expression of MLC\(^{T18D,S19D}\) but not MLC, conferred not only stress fibers but also vinculin accumulation at the tip of stress fibers in NIH 3T3 cells (Fig. 7). We further examined the effect of RB/PH (TT) or HA1077 on the MLC\(^{T18D,S19D}\)-induced stress fibers and vinculin accumulation at the tip. Stress fibers and vinculin accumulation at the tip remained in the cells coexpressing RB/PH (TT) with MLC\(^{T18D,S19D}\), although levels were slightly weakened (Fig. 7). HA1077 had a minimal effect on both stress fibers and vinculin accumulation at the tip. Thus, it is likely that RB/PH (TT) and HA1077 do not inhibit stress fiber and focal adhesion formation induced by active form of MLC.

**DISCUSSION**

**Inhibitory Mechanism and Specificity of Chemical Compounds—**HA1077 and Y-32885 have been shown to inhibit the Rho-kinase activity (29). We here found that these compounds inhibited the PKN activity at similar doses as Rho-kinase, whereas they had a less inhibitory effect on the MRCK\(^{β-}\) activity. The IC\(_{50}\) values of HA1077 and Y-32885 for PKN-CAT were almost the same as those for Rho-kinase-CAT under the above conditions, although \(K\) values for PKN-CAT were higher than those for Rho-kinase-CAT depending on the \(K_m\) values for ATP.\(^3\) These compounds affected the activity of not only Rho-kinase but also PKN at the same concentrations. Thus, it is unlikely that these compounds can distinguish Rho-kinase and PKN under the conditions used. HA1077 is an isoquinolinesulfonic acid derivative. A similar isoquinolinesulfonic acid derivative known as H-89 interacts with the ATP-binding site of protein kinase A through its isoquinoline ring and thereby inhibits the kinase activity (39). Y-32885 is a compound containing pyridine moiety. Since both HA1077 and Y-32885 inhibit the Rho-kinase activity in a manner competitive with ATP, they may interact with the ATP-binding site of the catalytic domain of Rho-kinase. The ATP-binding sites are well conserved among protein kinases (40). Thus, it is possible that these compounds interact with protein kinases other than Rho-kinase and PKN, and inhibit their kinase activities. One has to be careful when using these compounds as specific probes for Rho-kinase, although they are convenient and useful.

**Inhibitory Mechanism and Specificity of RB/PH (TT)—**We here found that RB/PH (TT) inhibited the Rho-kinase activity in *vitro*, but not the activities of PKN and MRCK\(^{β-}\). RB/PH (TT) inhibited the Rho-kinase activity in a manner competitive with S6 peptide. We also found that Rho-kinase bound to MBP-RB/PH (TT). Taken together, these results indicate that MBP-RB/PH (TT) interacts with the catalytic domain of Rho-kinase and competes with S6 peptide, and thereby inhibits the kinase activity. It should be noted that RB/PH (TT) inhibited the activity of Rho-kinase-CAT toward MLC in a noncompetitive-competitive mixed manner. Both RB/PH (TT) and MLC are thought to interact with the active center of Rho-kinase-CAT.
Nevertheless, MLC does not efficiently compete with MBP-RB/PH (TT) under the conditions in which S6 peptide competes with MBP-RB/PH (TT). These results raise the possibility that

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**FIG. 6. Effect of RB/PH (TT) and HA1077 on the focal adhesion formation.** A, pEF-BOS-HA-Rho\(^{Val-14}\) (a, d, and g), pEF-BOS-myc-Rho-kinase-CAT (b, e, and h), or pEF-BOS-myc-MRCK\(\beta\)-CAT (c, f, and i) (0.4 mg/ml) were injected into nuclei of serum-starved NIH 3T3 cells with pEF-BOS-myc vector (a-c, g–h) or with pEF-BOS-myc-Rho-kinase-RB/PH (TT) (d-f) (0.8 mg/ml). The cells were incubated for 24 h after injection in the serum-depleted medium, and the cells were fixed and doubly stained by anti-vinculin antibody and anti-Myc or anti-HA polyclonal antibody. For g–i, the cells were treated with 100 \(\mu\)M HA1077 for 20 min before fixation. The arrows show the injected cells. Scale bar, 20 \(\mu\)m. B, the percentage of cells with vinculin accumulation in Myc- or HA-positive cells is indicated. Data are mean ± S.E. of at least triplicate determinations.

Nevertheless, MLC does not efficiently compete with MBP-RB/PH (TT) under the conditions in which S6 peptide competes with MBP-RB/PH (TT). These results raise the possibility that
MLC interacts with the catalytic domain of Rho-kinase with at least two sites, including the active center. MBP-RB/PH (TT) may mask the MLC-binding sites of the catalytic domain. Further studies are necessary for understanding how the catalytic domain recognizes its substrates.

Protein kinases such as protein kinase C are usually composed of catalytic and regulatory domains (41). The regulatory domain interacts with and masks the catalytic domain to inactivate the kinase activity in the resting state. Upon stimulation, the regulatory domain dissociates from the catalytic domain and subsequently induces the activation of the kinase. The active form of Rho interacts with the Rho-binding domain of Rho-kinase, and thereby activates it (6). Rho-kinase becomes constitutively active when the COOH portion containing the RB and PH domains is deleted (15–17). We here found that RB/PH (TT) interacts with Rho-kinase and inhibits the kinase activity of Rho-kinase-CAT. Thus, it is likely that the RB and PH domains interact with the catalytic domain and inactivate the activity in the resting state, and that the active form of Rho interacts with the RB domain, alters the conformation of Rho-kinase, and thereby cancels the inhibition by the RB and PH domains in response to extracellular signals such as lysophosphatidic acid.

We also found that both RB and PH domains are required to inhibit the activity of Rho-kinase-CAT. The regulatory domains of protein kinase such as protein kinase C usually contain a pseudosubstrate region that is thought to mimic substrates and interact with the active center of the catalytic domain. Consensus amino acid sequences for phosphorylation sites by Rho-kinase are (R/K)X0–2(S/T). There are several potential pseudosubstrate regions in RB and PH domains. We cannot explain at present why both RB and PH domains are required for the inhibition of the kinase activity. The regulatory subunit of protein kinase A has been shown to inhibit the protein kinase A activity by its interaction with the catalytic subunit through the pseudosubstrate region and an additional binding site (42, 43). Thus, it is possible that RB/PH (TT) interacts with the catalytic domain of Rho-kinase through at least two sites including the putative pseudosubstrate region.

**RB/PH (TT) Inhibits the Rho-kinase Activity in Vivo**—We found that RB/PH (TT) inhibited both the RhoVal14- and Rho-kinase-CAT-induced formation of stress fibers and focal adhesions in NIH 3T3 cells, but not those induced by MRCKβ-CAT. We recently showed that RB/PH (TT) inhibits the serum-
induced MLC phosphorylation in NIH 3T3 cells (21), the RhoVal-14-induced moesin phosphorylation as well as microvilli formation in COS7 cells (23), and the 12-O-tetradecanoylphorbol-13-acetate-induced adducin phosphorylation as well as membrane ruffling in Madin-Darby canine kidney cells (25). We also found that α-adducinT445A,T480A (phosphorylation sites of Thr by Rho-kinase are replaced by Ala), which is not phosphorylated by Rho-kinase, inhibits the 12-O-tetradecanoylphorbol-13-acetate-induced membrane ruffling in Madin-Darby canine kidney cells, and that α-adducinT450D,T480D (phosphorylation sites of Thr by Rho-kinase are replaced by Asp), which might mimic the phosphorylated α-adducin, counteracts the inhibitory effect of RB/PH (TT) on the 12-O-tetradecanoylphorbol-13-acetate-induced membrane ruffling. Taken together, these results indicate that RB/PH (TT) specifically inhibits the Rho-kinase activity in vivo, and that RB/PH (TT) is a useful probe to analyze the functions of Rho-kinase.

HA1077 disrupted the organized actin stress fibers in the cells expressing RhoVal-14 or Rho-kinase-CAT under the conditions in which HA1077 had little effects on the MRCKα-CAT-induced stress fibers. Disorganized actin filaments remained in most of the cells expressing RhoVal-14 but not in the cells expressing Rho-kinase-CAT after treatment with HA1077. Disorganized actin filaments were also observed in the cells expressing both RhoVal-14 and RB/PH (TT). These results suggest that HA1077 inhibits the Rho-kinase activity more efficiently than the MRCKα activity in vivo, and that actin polymerization is induced by activated Rho in a fashion independent of Rho-kinase. Although HA1077 disrupted most actin stress fibers, a small size of vinculin accumulation sometimes remained in the cells expressing RhoVal-14 or Rho-kinase-CAT. Because HA1077 was applied 24 h after the injection of plasmids to the cells, in which stress fibers and focal adhesions were formed before treatment with HA1077, inhibition of the Rho-kinase may result in the breakdown of vinculin accumulation at the tip.

Activated MLC Maintains Stress Fibers and Focal Adhesions Independent of Rho/Rho-kinase—Burridge and co-worker (38) have proposed that the contractility driven by Rho plays a critical role in focal adhesion formation based on the observation that inhibition of contractility leads to inhibition of the Rho-induced stress fibers and focal adhesions. We here found that the expression of MLCCT18D,S19D, but not of MLC, induced not only stress fiber formation but also focal adhesion formation in NIH 3T3 cells, and that the MLCCT18D,S19D-induced focal adhesion was not inhibited by RB/PH (TT) or HA1077. MLCCT18D,S19D shows ATPase activity and forms myosin filaments when reconstituted with myosin heavy chain in vitro (35–37). MLCCT18D,S19D as well as phosphorylated MLC is preferentially incorporated into myosin fibers associated with actin stress fibers in fibroblasts (21, 44), suggesting that MLCCT18D,S19D mimics the phosphorylated MLC. MLCCT18D,S19D appears to exert the specific functions of Rho-kinase. Indeed, the expression of MLCCT18D,S19D in COS7 cells or NIH 3T3 cells did not induce microvilli formation, which was induced by the expression of RhoVal-14 or Rho-kinase-CAT through the phosphorylation of moesin (23). On the other hand, the expression of moesinT558D induced microvilli formation but not stress fiber formation. Taken together, these results indicate that myosin II activation plays a critical role in focal adhesion formation downstream of Rho-kinase.

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