Phosphorylation of Moesin by Rho-associated Kinase (Rho-kinase) Plays a Crucial Role in the Formation of Microvilli-like Structures*

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Rho-associated kinase (Rho-kinase), which is activated by the small GTPase Rho, phosphorylates moesin at Thr\textsuperscript{558} in vitro. Here, using a site- and phosphorylation state-specific antibody, we found that the expression of dominant active RhoA in COS7 cells induced moesin phosphorylation and the formation of microvilli-like structures at apical membranes where the Thr\textsuperscript{558} phosphorylated moesin accumulated, whereas the expression of dominant negative Rho-kinase inhibited both of these processes. The expression of dominant active Rho-kinase also induced moesin phosphorylation. When COS7 cells expressing moesin or moesin\textsuperscript{T558A} (substitution of Thr by Ala) were cultured under serum-depleted conditions, there were fewer microvilli-like structures, whereas microvilli-like structures remained in the cells expressing moesin\textsuperscript{T558D} (substitution of Thr by Asp). The expression of moesin\textsuperscript{T558A} inhibited the dominant active RhoA-induced formation of microvilli-like structures. These results indicate that Rho-kinase regulates moesin phosphorylation downstream of Rho in vivo and that the phosphorylation of moesin by Rho-kinase plays a crucial role in the formation of microvilli-like structures.

The ERM (ezrin, radixin, and moesin) family proteins function as cross-linking proteins between the plasma membranes and actin filaments (for reviews, see Refs. 1–3). The ERM family proteins are localized at the specific regions where actin filaments associate with plasma membranes such as microvilli at the brush border of the intestinal epithelial cells, microvilli-like structures at apical membranes (apical membrane protrusions), cleavage furrows, ruffling membranes, filopodia, and cell-cell adhesion sites (1–3). The NH\textsubscript{2}-terminal and COOH-terminal domains of the ERM family proteins are thought to directly bind to some integral membrane proteins such as CD44 (4) and actin filaments, respectively (5, 6). The NH\textsubscript{2}- and COOH-terminal domains of the ERM family proteins mask each other, presumably through the intramolecular head-to-tail association between the NH\textsubscript{2}- and COOH-terminal domains (7, 8). Once the ERM family proteins are activated, they appear to be translocated from the cytosol to the plasma membranes, where they serve as cross-linkers (1–3). Recent evidence suggests that the intramolecular suppression is released by acidic phospholipids such as phosphatidylinositol 4,5-diphosphate, and the small GTPase Rho enhances the formation of the ERM family proteins-CD44 complex (9). A permeable cell reconstitution assay showed that the ERM family proteins are essential for Rho- and Rac-induced cytoskeletal reorganization (10). The ERM family proteins are phosphorylated in growth factor-stimulated cells (11, 12). In thrombin-activated platelets moesin is phosphorylated at Thr\textsuperscript{558} (13). This phosphorylation is observed at filopodia in macrophages and thought to be required for the stable interaction of moesin with actin (14).

Accumulating evidence indicates that Rho regulates the formation of actin stress fibers, focal adhesions (15, 16), and microvilli-like structures at apical membranes (17), membrane ruffling (18, 19), neurite retraction (20, 21), and cell motility (22–24). Rho cycles between GDP-bound inactive and GTP-bound active forms, and the GTP-bound form binds to specific targets and then exerts its biological functions. Several Rho targets have been identified; protein kinase N (25, 26), Rho-kinase/ROK\alpha (27, 28), the MBS of myosin phosphatase (29) and p140mDia (30). p160ROCK is an isoform of Rho-kinase (31). Rho-kinase regulates the phosphorylation of MLC by the direct phosphorylation of MLC and by the inactivation of myosin phosphatase through the phosphorylation of MBS (29, 32, 33). In addition to MLC and MBS, Rho-kinase phosphorylates the ERM family proteins and adducin in vitro (34–36). Rho-kinase phosphorylates moesin at Thr\textsuperscript{558}, which is identical to the phosphorylation site in thrombin-activated platelets (34). The phosphorylation of moesin by Rho-kinase is thought to result in the inhibition of the head-to-tail suppression of moesin, leading to its activation (34). Rho-kinase regulates the formation of actin stress fibers and focal adhesions (37–39), smooth muscle contraction (40), c-fos expression (33), and neurite retraction (41–43). However, it has not been known whether Rho-kinase phosphorylates moesin in vivo, and the role of the phosphorylation of moesin remains to be clarified. Here we found that Rho-kinase phosphorylates moesin at Thr\textsuperscript{558} in vivo and that this phosphorylation plays a crucial role in the formation of microvilli-like structures.

EXPERIMENTAL PROCEDURES

Materials and Chemicals—The cDNA encoding mouse moesin (1–577 aa) was kindly provided by Dr. S. Tsukita (Kyoto University, Kyoto, Japan). Anti-HA Ab (12CA5) was purchased from Boehringer Mannheim (Indianapolis, IN). Anti-CD44 Ab (F10-44-2) was purchased from Serotec Ltd (Oxford, United Kingdom). Anti-Myc Ab (9E10), TRITC-phalloidin, and LPA were purchased from Sigma. Other materials and chemicals were obtained from commercial sources.

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1 The abbreviations used are: Rho-kinase, Rho-associated kinase; MBS, myosin-binding subunit; MLC, myosin light chain; aa, amino acids; HA, hemagglutinin; Ab, antibody; TRITC, tetramethylrhodamine isothiocyanate; LPA, lysophosphatidic acid; CAT, catalytic domain; DMEM, Dulbecco’s modified Eagle medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline.

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**Plasmid Constructs**—The cDNA encoding mouse moesin was subcloned into pEF-BOS-HA to obtain pEF-BOS-HA-moesin. The CDNs of moesin-transfected and moesin-transfected, which substituted Ala or Asp for Thr at 558 amino acid residues, were generated with a site-directed mutagenesis kit (Stratagene, La Jolla, CA) and subcloned into pEF-BOS-HA. The cDNA encoding human Rac1V12, Cdc42V12, RhoA14, and RhoA19 were subcloned into pEF-BOS-Myc, pEF-BOS-Myc-CAT, pEF-BOS-Myc-RB, and pEF-BOS-Myc-RB/PH(PT) were constructed as described (38, 42).

**Production of Site- and Phosphorylation State-specific Antibody for Moesin**—A rabbit polyclonal antibody against moesin phosphorylated at Thr558 (anti-pT558) was raised as described (44). The phosphopeptide Cys-Arg1-Asp-Lys-Tyr-Lys-phospho Thr-Leu-Arg-Gln-Ile-Arg was chemically synthesized as an antigen and bound to the carrier protein, keyhole limpet hemocyanin at the NH2-terminal cysteine residue, by Peptide Institute Inc. (Osaka, Japan). The antiserum obtained was then affinity-purified against the phosphopeptide. The specific binding of the anti-pT558 to phosphorylated HA-moesin, but not to nonphosphorylated HA-moesin was confirmed.

**Cell Culture, Transient Transfection, and Microinjection**—COS7 cells were maintained in DMEM containing 10% FBS, streptomycin, and penicillin. The transfection of plasmids into COS7 cells was carried out by the standard DEAE-dextran method (33). The transfected cells were cultured in DMEM with 10% FBS for 24 h and then in serum-free DMEM for 24 h. For some experiments, the serum-depleted cells were stimulated with 6 μM LPA or 10% FBS for 15 min. For microinjection into nuclei, COS7 cells were seeded onto coverslips with a grid to mark the localization of microinjected cells. After microinjection, the cells were cultured in DMEM with 10% FBS for 24 h and then in serum-free DMEM for 24 h.

**In Vivo Phosphorylation of Moesin by Rho-kinase**—The transfected cells were treated with 10% (v/v) trichloroacetic acid. The resulting precipitates were subjected to an immunoblot analysis using the anti-HA Ab or anti-pT558, to determine the amount of expressed HA-moesin or the amount of phosphorylated HA-moesin, respectively.

**Immunofluorescence Analysis**—The transfected or microinjected COS7 cells were fixed with 3% formaldehyde in PBS for 10 min and treated with PBS containing 0.2% Triton X-100 for 10 min. After being washed with PBS three times, the cells were incubated with the anti-pT558 and anti-HA Ab or anti-Myc Ab for 1 h at room temperature. The cells were washed with PBS three times, followed by incubation with fluorescein isothiocyanate-conjugated anti-rabbit Ig Ab and Texas Red-conjugated anti-mouse Ig Ab. For the detection of the formation of microvilli-like structures, the cells were stained with an anti-CD44 Ab (F19-44-2) or TRITC-phalloidin together with anti-HA poly Ab or anti-Myc poly Ab. The cells were examined using a Zeiss axiophoto microscope or a confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany).

**RESULTS AND DISCUSSION**

We examined whether moesin was phosphorylated via the Rho/Rho-kinase pathway in COS7 cells by an immunoblot analysis with anti-pT558. pEF-BOS-HA-moesin, in which moesin was HA epitope-tagged at its NH2-terminal, was cotransfected into COS7 cells with the plasmids carrying the cDNA of Rho family members or Rho-kinase (Fig. 1). The level of HA-moesin phosphorylation at Thr558 was low in serum-depleted COS7 cells expressing HA-moesin alone. The expression of dominant active RhoA (RhoAV14) or constitutively active Rho-kinase (catalytic domain, CAT) induced moesin phosphorylation at Thr558 to severalfold. Dominant negative RhoA (RhoAN19) was inactive in this capacity. In the cells cotransfected with dominant active Rac1 (Rac1V12), the level of moesin phosphorylation at Thr558 slightly increased. Dominant active Cdc42 had no effects. Rho-kinase is composed of catalytic (CAT), coiled-coil (COIL), Rho-binding (RB), and pleckstrin-homology (PH) domains (28). RB (941–1075 aa) and RB/PH(PT) (941–1388 aa), which encompasses the RB and PH domains, work as the dominant negative forms of Rho-kinase (38, 43). RB binds to Rho and inhibits the Rho-dependent Rho-kinase activity (38). RB/PH(PT), which has point mutations in the RB domain and does not bind to Rho, inhibits the Rho-kinase activity without titoring out Rho in vitro.2 In the present study, the effect of RhoAV14 was suppressed by RB and RB/PH(PT) (Fig. 1). The anti-pT558 recognized the proteins corresponding to endogenous ezrin as well as endogenous moesin (Fig. 1). Because the sequence (553–563)aa around the phospho-Thr558 that was selected as the antigen is completely conserved among the ERM family proteins, the anti-pT558 may recognize Thr557-phosphorylated ezrin. RhoAV14 or CAT enhanced the phosphorylation of not only endogenous moesin but also that of endogenous ezrin to some extent (Fig. 1). Taken together, these results indicate that moesin is phosphorylated at Thr558 in COS7 cells in a Rho/Rho-kinase-dependent manner.

Next, we determined the subcellular localization of Thr558-phosphorylated moesin in COS7 cells by an indirect immunofluorescence analysis with anti-pT558 (Fig. 2). In serum-depleted COS7 cells expressing HA-moesin alone, the level of moesin phosphorylation at Thr558 was low, and phosphorylated moesin showed diffuse localization throughout the cytoplasm. When the cells were stimulated with 6 μM LPA or medium containing 10% FBS, the phosphorylation level of moesin was elevated and phosphorylated moesin was distributed into the microvilli-like structures at apical membrane. In the cells coexpressing RhoAV14, phosphorylated moesin specifically accumulated in the microvilli-like structures at apical membrane, which were similar to the LPA- or serum-induced apical structures (Fig. 2A). We confirmed that the LPA-, serum-, and RhoA-induced structures, where phosphorylated moesin accumulated, were specifically present in the apical membrane by scanning along the z axis of the cell with a confocal laser scanning microscope (data not shown). Fig. 2B shows the immunolocalization of total HA-moesin and phosphorylated moesin in RhoAV14-expressing cells by double-labeled immunofluorescence. A merged image revealed that part of the expressed HA-moesin was phosphorylated and that the phosphorylated HA-moesin was specifically incorporated into the microvilli-like structures. In the RhoAV14-expressing cells, the phosphorylated moesin was partly colocalized with CD44, which is one of the partners of the ERM family proteins and accumulates in the microvilli-like structures (data not shown). The coexpression of RB/PH(PT) completely abrogated the effects of RhoAV14.

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2 M. Amano, K. Chihara, N. Nakamura, Y. Matsuura, and K. Kaibuchi, manuscript in preparation.
The accumulation of phosphorylated moesin in microvilli-like structures was not observed in the cells transfected with RhoAN19 (Fig. 2A). We found that RB/PH(TT) and RhoAN19 also inhibited the LPA- or serum-induced moesin phosphorylation and accumulation of moesin into the microvilli-like structures (data not shown). Rac1V12 failed to induce the localization of phosphorylated moesin in microvilli-like structures, but induced membrane ruffling-like structures, where phosphorylated moesin accumulated. Cdc42V12 did not show any morphological effects under the conditions. The expression of CAT induced moesin phosphorylation. The phosphorylated moesin by CAT accumulated not only in microvilli-like structures but also in large dot-like structures (Fig. 2A) where CD44 and actin filaments accumulated (data not shown). Since CAT is known to induce the formation of both actin stress fibers and

**Fig. 2.** Localization of Thr558-phosphorylated moesin in COS7 cells. A, an indirect immunofluorescence analysis with the anti-pT558 was performed in COS7 cells. pEF-BOS-HA-moesin (4 μg) was cotransfected with pEF-BOS vector (12 μg) (a–c), pEF-BOS-Myc-RhoAV14 (4 μg) (d), pEF-BOS-Myc-RhoAN19 (4 μg) (e), pEF-BOS-Myc-RbPHTT (8 μg) (f), pEF-BOS-Myc-RhA (4 μg) (g), and pEF-BOS-Myc-Rac1V12 (4 μg) (h). Total amount of plasmids was compensated with pEF-BOS vector. The cells were treated with 6 μM LPA (b) or medium containing 10% FBS (c) for 15 min, respectively. Phosphorylated moesin was labeled by anti-pT558. The arrowheads indicate the transfected cells. The arrows in g indicate the large dot-like structures. Bar, 10 μm. B, pEF-BOS-HA-moesin (4 μg) was cotransfected with pEF-BOS vector (12 μg) (a–c), pEF-BOS-Myc-RhoAV14 (4 μg) (d–f), or pEF-BOS-Myc-RhA (4 μg) (g) and pEF-BOS-Myc-Rac1V12 (4 μg) (h). Total amount of plasmids was compensated with pEF-BOS vector. The arrowheads indicate the injected cells. Bar, 10 μm. B, the ratios of the cells displaying microvilli-like structures where CD44 was enriched per the transfected cells. COS7 cells were transfected with pEF-BOS-HA vector (12 μg), pEF-BOS-HA-moesin (12 μg), pEF-BOS-HA-moesin T558A (12 μg), or pEF-BOS-HA-moesin T558D (12 μg) in the absence or presence of pEF-BOS-Myc-RhA (4 μg). Total amount of plasmids was compensated with pEF-BOS vector. Open column, in the absence of RhoAV14; striped column, in the presence of RhoAV14. The results shown are representative of three independent experiments.

**Fig. 3.** Effects of moesin mutants on the formation of microvilli-like structures in COS7 cells. A, the patterns of anti-CD44 Ab and TRITC-phalloidin staining in HA-moesin T558D-expressing cells. CD44 and F-actin were doubly labeled in COS7 cells into which pEF-BOS vector (0.1 mg/ml) (a–c) or pEF-BOS-HA-moesin T558D (0.1 mg/ml) (d–f) were microinjected in the absence of RhoAV14. CD44 is shown in green (a, c, d, and f). F-actin is shown in red (b, c, e, and f). c and f show merged images of a and b, and d and e, respectively. The arrowheads indicate the injected cells. Bar, 10 μm. B, the ratios of the cells displaying microvilli-like structures where CD44 was enriched per the transfected cells. COS7 cells were transfected with pEF-BOS-HA vector (12 μg), pEF-BOS-HA-moesin (12 μg), pEF-BOS-HA-moesin T558A (12 μg), or pEF-BOS-HA-moesin T558D (12 μg) in the absence or presence of pEF-BOS-Myc-RhA (4 μg). Total amount of plasmids was compensated with pEF-BOS vector. Open column, in the absence of RhoAV14; striped column, in the presence of RhoAV14. The results shown are representative of three independent experiments.

RhoAN19 also inhibited the LPA- or serum-induced moesin phosphorylation and accumulation of moesin into the microvilli-like structures (data not shown). Rac1V12 failed to induce the localization of phosphorylated moesin in microvilli-like structures, but induced membrane ruffling-like structures, where phosphorylated moesin accumulated. Cdc42V12 did not show any morphological effects under the conditions. The expression of CAT induced moesin phosphorylation. The phosphorylated moesin by CAT accumulated not only in microvilli-like structures but also in large dot-like structures (Fig. 2A) where CD44 and actin filaments accumulated (data not shown). Since CAT is known to induce the formation of both actin stress fibers and
large dot-like aggregates of F-actin (38), the large dot-like structures consisting of phosphorylated moesin, CD44 and F-actin might appear in cells expressing CAT.

To understand the role of the phosphorylation of moesin by Rho-kinase in the formation of microvilli-like structures, we produced the moesin mutants, HA-moesinT558A and HA-moesinT558D (substitution of Thr by Ala or Asp). HA-moesinT558A was not phosphorylated by Rho-kinase in vitro (data not shown). HA-moesinT558D was expected to mimic Thr558-phosphorylated moesin. To assess the formation of microvilli-like structures, anti-CD44 Ab staining was used as a marker for these structures. In the unstimulated cells into which the control vector was introduced, neither anti-CD44 Ab staining nor TRITC-phalloidin staining showed any specific accumulation in the microvilli-like structures (Fig. 3, A and B). When cells expressing HA-moesin or HA-moesinT558A were cultured under serum-depleted conditions in the absence of RhoAV14, there were few microvilli-like structures (Fig. 3, A and B). HA-moesinT558D-expressing cells microvilli-like structures remained (Fig. 3, A and B). CD44 and F-actin were doubly labeled, the merged image showed that, in HA-moesinT558D-expressing cells, CD44 was colocalized with F-actin at microvilli-like structures (Fig. 3A). HA-moesinT558D may substitute for endogenous moesin and maintain the microvilli-like structures. When RhoAV14 was coexpressed with the HA-moesin or moesin mutants in COS7 cells, microvilli-like structures appeared in the cells expressing HA-moesin or HA-moesinT558D (Fig. 3B). It should be noted that the coexpression of HA-moesinT558D with RhoAV14 enhanced the formation of microvilli-like structures by about 2-fold. In contrast, the expression of HA-moesinT558A inhibited the formation of the RhoAV14-induced microvilli-like structures (Fig. 3B). Essentially similar results were obtained when the TRITC-phalloidin was used to assess microvilli-like structures instead of the anti-CD44 Ab (data not shown). HA-moesinT558A may inhibit the function of endogenous moesin as the dominant negative form in vivo. Taken together, these results indicate that the phosphorylation of moesin at Thr558 by the Rho/Rho-kinase pathway plays a crucial role in the formation of microvilli-like structures.

Here, we noted that the expression of Rac1V12 in COS7 cells induced moesin phosphorylation and membrane ruffling, where Thr558-phosphorylated moesin accumulated. Myotonic dystrophy kinase-related gdc42-binding kinase (MRCK), which has homology with Rho-kinase within the kinase domain, was recently identified as a target for Cdc42 (45). We recently found that MRCKβ, which is one of the isoforms of MRCK, binds GTP-bound Rac as well as GTP-bound Cdc42 and that MRCKβ phosphorylates moesin in vitro.3 Thus, it is possible that MRCK participates in the phosphorylation of moesin downstream of Cdc42 or Rac1.

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