Thrombin Inactivates Myosin Light Chain Phosphatase via Rho and Its Target Rho Kinase in Human Endothelial Cells*

(Received for publication, August 27, 1997, and in revised form, May 11, 1998)

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The role of Rho GTPase and its downstream targets Rho kinase and myosin light chain phosphatase in thrombin-induced endothelial cell contraction was investigated. The specific Rho inactivator C3-transferase from Clostridium botulinum as well as microinjection of the isolated Rho-binding domain of Rho kinase or active myosin light chain phosphatase abolished thrombin-stimulated endothelial cell contraction. Conversely, microinjection of constitutively active V14Rho, constitutively active catalytic domain of Rho kinase, or treatment with the phosphatase inhibitor tautomycin caused contraction. These data are consistent with the notion that thrombin activates Rho/Rho kinase to inactivate myosin light chain phosphatase in endothelial cells. In fact, we demonstrate that thrombin transiently inactivated myosin light chain phosphatase, and this correlated with a peak in myosin light chain phosphorylation. C3-transferase abolished the decrease in myosin light chain phosphatase activity as well as the subsequent increase in myosin light chain phosphorylation and cell contraction. These data suggest that thrombin activates the Rho/Rho kinase pathway to inactivate myosin light chain phosphatase as part of a signaling network that controls myosin light chain phosphorylation/contraction in human endothelial cells.

A variety of pathological conditions including the early stages of atherosclerosis, acute inflammation, and anaphylactic shock are associated with increased vascular permeability (1, 2). Thrombin generated under these pathological conditions induces endothelial cell contraction and increases vascular permeability through activation of a specific receptor that is coupled via heterotrimeric G-proteins of the Gq family to phospholipase Cβ that cleaves phosphatidylinositol-4,5-bisphosphate to yield inositol 1,4,5-trisphosphate. Inositol 1,4,5-trisphosphate, mobilizes Ca2+ from intracellular stores and thus increases intracellular Ca2+ concentration leads to activation of Ca2+/calmodulin-dependent myosin light chain kinase (MLCK),1 which phosphorylates Thr-18 and Ser-19 of the light chain of myosin II (MLC) (3). Phosphorylation induces a conformational change in MLC that enables actin-myosin interaction and activates the Mg2+-ATPase activity of myosin (6). Besides MLC kinases, myosin-associated MLC phosphatase (PP1M) also seems to play a major role in the control of MLC phosphorylation/dephosphorylation in endothelial cells. This is demonstrated by the finding that pharmacological inhibitors of protein phosphatase 1 (PP1) increased MLC phosphorylation and cell contraction (7), whereas microinjection of active PP1 decreased MLC phosphorylation and disturbed actin/myosin interaction (8). PP1 is composed of three components, a 37–38-kDa catalytic subunit, a 130-kDa regulatory subunit, and a 20-kDa subunit (9–11). Recently, it was shown that the regulatory subunit can be phosphorylated and inactivated by Rho kinase, a specific target protein of the GTPase Rho (12). Previous work had indicated that Rho mediates cell contraction induced by thrombin, but the Rho targets involved have not been identified thus far (13–15).

The Rho family of Ras-like GTPases that consists of more than 10 members has been implicated in actin cytoskeleton organization and cellular shape changes in a variety of cell types (16, 17). Rho-GTPases cycle between a GTP-bound active state and a GDP-bound inactive state, and this cycle is controlled by guanine nucleotide exchange factors and GTPase-activating proteins (18–20). ADP-ribosylation and inactivation of Rho by C3-transferase from Clostridium botulinum specifically inhibits the cellular effects of Rho (21–28). Microinjection of constitutively active V14Rho induced formation of stress fibers and focal adhesion sites as well as a contractile phenotype in fibroblasts (21, 28). It has also been reported that GTPγS and aluminum fluoride-mediated Ca2+ sensitization of smooth muscle contraction is mediated by Rho (29). A number of target proteins that interact with GTP-bound but not with GDP-bound Rho have been identified (16). These include the closely related Ser/Thr kinases ROKα/Rho kinase and ROKβ/p160 (30, 31, 33–37). ROKα/Rho kinase consists of multiple domains including a catalytic domain at the amino terminus, a coiled coil domain including the Rho binding domain (RBD), and a C-terminal pleckstrin homology domain (34). Microinjection of isolated Rho kinase domains into fibroblasts or HeLa

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* This study was supported by Deutsche Forschungsgemeinschaft Grants Ae11/5–1 and SFB413, by August Lenz Stiftung, and by Wilhelm Sander Stiftung. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: MLCK, myosin light chain kinase; HUVEC, human umbilical vein endothelial cells; MLC, myosin light chain; MBS, myosin binding subunit of myosin light chain phosphatase; PP1, protein phosphatase 1; PP2, protein phosphatase 2; PP1C, catalytic subunit of PP1; PP1M, myosin-binding PP1; RBD, Rho-binding domain of Rho kinase; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; GTPγS, guanosine 5’-O-(thio)triphosphate.
cells indicated that Rho kinase is the target protein by which Rho forms stress fiber and focal adhesions (36, 37). Consistent with the involvement of Rho kinase in contractile events, overexpression of constitutively active V14Rho in fibroblasts caused phosphorylation of the MBS, inactivation of myosin phosphatase, and an increase in MLC phosphorylation (12).

Here, we provide evidence that thrombin uses the Rho/Rho kinase pathway to inactivate PP1M in human endothelial cells. Inactivation of PP1M seems to be coordinated with activation of Ca\(^{2+}\)-calmodulin-dependent MLCK to maximally increase MLC phosphorylation in the early phase of thrombin-induced endothelial cell contraction.

**EXPERIMENTAL PROCEDURES**

**Materials**—The inhibitors okadaic acid, KT5926, and tautomycin were from Calbiochem (Bad Soden, Germany); all other materials not specifically indicated were from Sigma.

**Cell Culture**—Human umbilical vein endothelial cells (HUVEC) were obtained and cultured as described previously (24). Briefly, cells were harvested from umbilical cords were plated onto collagen-coated (24 h, 100 \(\mu\)g/ml collagen G; Biochrom, Berlin, Germany) plastic culture flasks and cultured in endothelial growth medium (Promo Cell, Heidelberg, Germany), containing endothelial cell growth supplement/haptenin and 10% fetal calf serum. For all experiments, cells were plated at a density of 2 \(\times\) 10\(^5\) cells/cm\(^2\) and grown to confluency for 10 days. For all experiments, cells were plated at a density of 2 \(\times\) 10\(^5\) cells/cm\(^2\) and grown to confluency for 10 days.

**Measurement of Endothelial Permeability**—Horseradish peroxidase diffusion through HUVEC monolayers was determined as described previously with some modifications (38). Briefly, cells were plated (2 \(\times\) 10\(^4\) cells/cm\(^2\)) on collagen-coated polyethylene terephthalate cell culture inserts (3-\(\mu\)m pore size, Becton Dickinson), which were set into 24-well Falcon companion TC plates (Becton Dickinson). HUVEC were cultured for 10 days with medium changes every 2 days. For thrombin stimulation, medium was replaced with 500 \(\mu\)l of medium containing thrombin. For controls, thrombin was omitted, but otherwise were cultured identically. After 15 min of stimulation, 500 \(\mu\)l of medium was filled into the lower compartment, and the medium in the upper compartment was replaced with fresh medium containing horseradish peroxidase (0.34 mg/ml, IV-A type, 44,000 \(M_r\); Sigma, Deisenhofen, Germany). After 1 min, 60 \(\mu\)l of medium was collected from the lower compartment and mixed with 880 \(\mu\)l of reaction buffer (50 mM Na\(_2\)PO\(_4\), 5 mM (NH\(_4\))\(_2\)SO\(_4\), and 100 mM (NH\(_4\))\(_2\)SO\(_4\); pH 6.0) to solution (0.6 \(\mu\)mol of medium in H\(_2\)O). The reaction was allowed to proceed for 15 min at room temperature, and absorbance was measured at 470 nm.

**Immunofluorescence**—For fluorescence staining, HUVEC were plated (2 \(\times\) 10\(^5\) cells/cm\(^2\)) on Eppendorf Collatex glass coverslips (Eppendorf, Hamburg, Germany) coated with 100 \(\mu\)g/ml collagen G (24 h) and grown to confluency for 10 days. To label F-actin, cells were fixed for 1 min, 60 \(\mu\)l of medium was collected from the lower compartment and mixed with 880 \(\mu\)l of reaction buffer (50 mM Na\(_2\)PO\(_4\), 5 mM (NH\(_4\))\(_2\)SO\(_4\), and 100 mM (NH\(_4\))\(_2\)SO\(_4\); pH 6.0) to solution (0.6 \(\mu\)mol of medium in H\(_2\)O). The reaction was allowed to proceed for 15 min at room temperature, and absorbance was measured at 470 nm.

**Measurement of Cytosolic Ca\(^{2+}\) Concentration**—Cytosolic [Ca\(^{2+}\)] was measured as described previously (41). Cells grown on collagen-coated glass coverslips were loaded with 10 mM fura-2 AM (2.5 \(\mu\)g/ml) in the presence of 0.1 mM Ca\(^{2+}\)-free PBS (Sigma), and 200 \(\mu\)l of medium containing thrombin was added. After 1 min, the cells were washed with fresh medium and resuspended in HEPES buffer (20 mM HEPES, 120 mM NaCl, 2.7 mM KCl, 1.4 mM MgSO\(_4\), 0.5 mM Ca\(_{2+}\), 1.4 mM KH\(_2\)PO\(_4\), 25 mM NaHCO\(_3\), 10 mM glucose, pH 7.4). HUVEC were cultured for 10 days. After a double excitation measurement at the wavelengths 340 and 380 nm, the emission wavelength set at 510 nm and the emission wavelength rapidly alternating between 340 and 380 nm. [Ca\(^{2+}\)]\(_i\) was quantified by applying the equation [Ca\(^{2+}\)]\(_i\) = \(K_c/R - R_{max}/(R_{max} - R)\times(S_0/S_0)\).
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RESULTS

Inactivation of Rho by C3-transferase Blocks Thrombin-induced Endothelial Cell Contraction—To test whether Rho is involved in endothelial cell contraction, we measured the thrombin-stimulated increase of transendothelial horseradish peroxidase diffusion in the absence and presence of the specific Rho inactivator C3-transferase from C. botulinum (45, 46). The results presented in Fig. 1 demonstrate that confluent monolayers of HUVEC show a low transendothelial diffusion of horseradish peroxidase. Stimulation with thrombin (0.1–1 unit/ml) dose dependently increased horseradish peroxidase diffusion in the absence and presence of the specific Rho inactivator C3-transferase (C3) from C. botulinum for 24 h and stimulated with thrombin (Th; 0.1–1 units/ml; 15 min). Transendothelial diffusion of horseradish peroxidase was determined spectrophotometrically as described under "Experimental Procedures." Each bar represents the mean ± SD of three to six experiments.

Thrombin Inactivates PP1M Activity via Rho Kinase—To obtain evidence that the Rho target protein Rho kinase mediates the thrombin/Rho effect on contraction, we microinjected isolated RBD of Rho kinase, which has been shown to inhibit interaction of Rho with Rho kinase (37). Microinjection of RBD blocked thrombin-induced cell contraction similar to C3-transferase (Fig. 2d). Conversely, when we microinjected the recombinant catalytic domain of Rho kinase, endothelial cells contracted and showed a shape change (Fig. 2g) similar to stimulation with thrombin (Fig. 2b), microinjection of V14RhoA (Fig. 2f), or treatment with the PP1M inhibitor tautomycin (not shown). These data suggest that Rho kinase is the Rho target protein by which thrombin exerts its effects on cell contraction. To investigate whether inhibition of the Rho kinase target protein PP1M contributes to thrombin-induced endothelial cell contraction, we microinjected the constitutively active catalytic domain of PP1. As can be seen in Fig. 2e, microinjection of PP1 inhibited the thrombin-induced cell contraction similar to C3-transferase (Fig. 2c) or RBD (Fig. 2d). These data are consistent with the idea that thrombin uses a pathway that involves activation of Rho and Rho kinase as well as inactivation of PP1M to regulate cell contraction.

Thrombin Inactivates PP1M Activity via Rho—We reasoned that if Rho kinase is in fact activated by thrombin, an inhibition of PP1M activity should be detected. We therefore determined PP1M activity in cells stimulated with thrombin for different time periods by assaying dephosphorylation of phosphatase substrate. As can be seen in Fig. 3a, stimulated cells showed a significant phosphatase activity compared with control. These results indicate that thrombin produced a transient decrease of PP1M activity between 30 s and 3 min, which was followed by a return to base-line values after 5 min (Fig. 3c).

In the C3-transferase–treated cells, the thrombin-induced decrease in PP1M activity was abolished, further supporting the notion that Rho regulates PP1M. To unambiguously demonstrate that [32P]phosphorylation buffer (250 mM Tris-HCl, pH 8.2, 16.7 mM MgCl2, 1.67 mM ATP, 0.83 mM CaCl2, 133 mM 2-mercaptoethanol) for 1 h at 30°C. Reaction was stopped with 90% ammonium persulfate solution (4°C). Reaction tube was then kept on ice for 1 h and subsequently centrifuged at 12,000 × g for 10 min. The resulting protein pellet was resuspended with ammonium persulfate solution (45°C saturated). The protein pellet was washed four times in this way. Protein solution was then concentrated to a final concentration of 3 mg/ml using Amicon Centricon-30 concentrators.

Phosphatase activities of myosin-enriched cell fractions were then quantified by measuring release of radioactive from [32P]phosphorylase a in the presence of myosin-enriched cell fractions. For this purpose, myosin-enriched fractions were diluted with 30 μl of assay buffer (50 mM Tris, 0.1 mM EDTA, 28 mM β-mercaptoethanol, 6.25 mM caffeine, pH 7.0) and mixed with 20 μl of radioactive phosphatase substrate. Reaction was allowed to proceed for 10 min at 30°C and stopped with ice-cold 20% trichloroacetic acid. Samples were then incubated on ice for 10 min and centrifuged at 12,000 × g for 3 min. The radioactivity released in the supernatant was measured using a Wallack 1410 liquid scintillation counter. To prove that phosphorylase b dephosphorylation in fact measures MLC-phosphatase, we also used smooth muscle [32P]-MLC as a substrate (7, 9). MLC (0.8 mg/ml) was phosphorylated for 1 h at room temperature with MLCK (50 μg/ml) in buffer containing 50 mM Tris-HCl, pH 7.4, 5 mM magnesium acetate, 0.1 mM CaCl2, 0.1 mg/ml calyculin A, 0.1 μM wortmannin, 0.1 μM Rho inactivator C3-transferase (24 h, 5 μg/ml). To investigate whether inhibition of the Rho kinase target protein PP1M contributes to thrombin-induced endothelial cell contraction, microinjected isolated RBD of Rho kinase, which has been shown to inhibit interaction of Rho with Rho kinase (37). Microinjection of RBD blocked thrombin-induced cell contraction similar to C3-transferase (Fig. 2d). Conversely, when we microinjected the recombinant catalytic domain of Rho kinase, endothelial cells contracted and showed a shape change (Fig. 2g) similar to stimulation with thrombin (Fig. 2b), microinjection of V14RhoA (Fig. 2f), or treatment with the PP1M inhibitor tautomycin (not shown). These data suggest that Rho kinase is the Rho target protein by which thrombin exerts its effects on cell contraction.
FIG. 2. Thrombin-induced endothelial cell contraction is mediated by Rho, Rho kinase, and PP1. HUVEC were not stimulated (a), stimulated with thrombin (1 unit/ml, 15 min) (b), pretreated with C3-transferase (24 h, 5 μg/ml), and then stimulated with thrombin (c),
Phosphorylation of the light chain of myosin II (MLC) is a crucial mechanism by which thrombin signals are converted into the mechano-chemical force for cell contraction (47, 48). To investigate whether the observed thrombin-induced inhibition of PP1M is relevant for MLC phosphorylation, we stimulated control and C3-pretreated (24 h, 5 μg/ml) endothelial cells for different times with thrombin (1 unit/ml) and separated un-, mono-, and diphosphorylated MLC on 10% urea polyacrylamide gels. Densitometric quantitation of these MLC forms revealed that thrombin caused phosphate incorporation into MLC with a peak after 1 min (Fig. 3c). The level of phosphorylated MLC then dropped to a plateau above base line. In cells pretreated with C3, the thrombin-stimulated peak in MLC phosphorylation was essentially abolished (Fig. 3c). These data suggest that inhibition of PP1M activity via Rho/Rho kinase is an essential mechanism by which thrombin yields a peak level in MLC phosphorylation.

**Rho Is Not Involved in Thrombin-stimulated Ca^{2+} Mobilization in Endothelial Cells**—It is well established that thrombin elevates intracellular Ca^{2+} concentration and thereby activates Ca^{2+}/calmodulin-dependent MLCK (48). Interestingly, C3-transferase was shown to inhibit thrombin-stimulated Ca^{2+} mobilization in fibroblasts (32). To test whether this is the mechanism by which C3-transferase prevents thrombin-induced MLC phosphorylation in HUVEC, we loaded control or C3-treated endothelial cells with the Ca^{2+} indicator fura-2 AM and determined cytosolic-free Ca^{2+} concentration using fluorescence spectrometry. We found that the C3 treatment affected neither basal Ca^{2+} concentration nor the thrombin-stimulated increase in peak (after 30 s) or plateau (after 3 min) Ca^{2+} concentration (Fig. 6). We conclude that Rho is not involved in the thrombin-induced cytosolic Ca^{2+} increase in endothelial cells.

**C3-transferase Does Not Prevent Thrombin-induced Release of Catenins from the Cytoskeleton**—In tightly confluent HUVEC, the VE-cadherin/catenin-based adherens junctions are associated with the actin cytoskeleton to stabilize the endothelial barrier. It has been suggested that thrombin-induced increase in endothelial permeability might be partly because of a release of catenins from the Triton X-100 insoluble cytoskeletal cell fraction (43, 44). To exclude that the effect of C3-transferase on thrombin-induced increase in endothelial permeability was the result of prevention of this shift, we performed detergent solubility assays of catenins. As shown in Fig. 7, we found that plakoglobin and β-catenin are associated with the Triton X-100 insoluble fraction in confluent HUVEC. After thrombin treatment, both β-catenin and plakoglobin (γ-catenin) lost their association with the cytoskeleton and shifted to the cytoplasm. This shift was not prevented by C3-transferase treatment. This result indicates that prevention of the release of catenins from the cytoskeleton is not the mechanism by which C3-transferase inhibits thrombin-induced increase in endothelial permeability.

**DISCUSSION**

Our data suggest an additional signal pathway by which thrombin regulates myosin light chain phosphorylation and the subsequent increase in endothelial cell contraction/vascular permeability. We propose that thrombin activates Rho, which then interacts with its target Rho kinase that in turn inactivates PP1M, most likely by phosphorylation of the 130-kDa regulatory subunit (12). The transient inactivation (within 0.5–5 min) of PP1M demonstrated here most likely produces the peak in MLC phosphorylation seen within the first 5 min of thrombin stimulation. Others have found a similar peak of MLC phosphorylation in thrombin-stimulated cells (49). Interestingly, the peak in MLC phosphorylation also correlates with the transient peak in intracellular Ca^{2+} elevation obtained after thrombin stimulation (41). After 5 min of thrombin stimulation, MLC phosphatase activity reversed to near base-line values and, in parallel MLC phosphorylation, dropped to a plateau. This plateau MLC phosphorylation correlates with a plateau in intracellular Ca^{2+} concentration (41). Taken together, these data suggest that the Rho-induced inhibition of MLC phosphatase activity is coordinated with a peak in Ca^{2+} mobilization to produce maximal MLC phosphorylation. Along this line, it has been reported that GTPγS- and aluminum fluoride-mediated Ca^{2+} sensitization of smooth muscle cell contraction is dependent on Rho (29). In these smooth muscle cells, myosin light chain phosphatase was inhibited by a Triton-soluble membrane-bound effector, which was not Rho. This effector could be Rho kinase.

We demonstrated that C3-transferase could completely block thrombin-stimulated MLC phosphorylation and cell contraction as well as the increase in endothelial permeability. We want to emphasize that this does not contradict the idea that Rho-induced inhibition of MLC phosphatase is mainly responsible for the transient peak in MLC phosphorylation. As shown...
in Fig. 5, C3-transferase by itself increased MLC phosphatase activity in unstimulated cells, most likely by inhibiting a basal Rho activity. This somewhat artificially elevated MLC phosphatase activity could blunt MLC kinase activity brought about by the thrombin-induced Ca$^{2+}$ signal. The fact that the MLC phosphatase inhibitor tautomycin completely reversed the C3-transferase effect on cell contraction also supports this idea.

Besides phosphorylating and inactivating PP1M, it was demonstrated that Rho kinase can directly phosphorylate MLC in vitro, i.e., can act as a MLC kinase (50). At present, we have no indication that this mechanism is relevant in endothelial cells.

Recently, it was reported that C3-transferase inhibited lysophosphatidic acid-induced MLC phosphorylation and contraction in fibroblasts. It was speculated that this inhibition is because of enhanced myosin phosphatase activity (13). Our results obtained in thrombin-stimulated endothelial cells seem to support this notion. Furthermore, we noticed that contraction induced by thrombin, V14Rho, or active Rho kinase precedes formation of stress fibers, which is consistent with the idea that contraction drives stress fiber formation (13). We noticed, however, that stress fibers were not as efficiently pro-

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**FIG. 3.** Thrombin-induced inactivation of PP1M correlates with an increase in MLC phosphorylation. a, HUVEC were stimulated with thrombin (1 unit/ml), and at indicated time points phosphorylase b phosphatase activity was determined as described under "Experimental Procedures." Asterisk indicates phosphatase activity in C3-pretreated cells. Values represent mean ± S.E. of three to four experiments. The inset shows a representative time course of phosphatase activity using $^{32}$P-MLC substrate (n = 3). b, HUVEC were pretreated without (−) or with (+) 5 μg/ml C3-transferase (C3) from C. botulinum for 24 h and stimulated with thrombin (1 unit/ml) for different time periods. Un- ($P_0$), mono- ($P_1$), and di- ($P_2$)-phosphorylated MLCs were separated by urea gel electrophoresis and Coomassie stained. Specific bands were quantified by densitometric analysis. Stoichiometry phosphate incorporation was calculated as described under "Experimental Procedures." Results are representative of three experiments ± S.E. Thrombin maximally increased MLC phosphorylation within the first 2 min. This peak in MLC phosphorylation was prevented by C3-transferase. c, The protein level of the catalytic subunit (PP1C) and the regulatory subunit (MBS) of PP1C in the myosin fractions was determined by Western blot. A representative experiment is shown.

**FIG. 4.** Inhibition of PP1M by tautomycin restores thrombin-stimulated increase in permeability in C3-treated cells. Cells pretreated for 24 h without or with 5 μg/ml C3-transferase (C3) were stimulated with thrombin (Th; 1 unit/ml) where indicated. Where indicated, cells were pretreated for 15 min with tautomycin (Taut; 6 nM) and then stimulated with thrombin as described under "Experimental Procedures." Transendothelial diffusion of horseradish peroxidase was measured as described under "Experimental Procedures." Values represent mean ± S.D. of two experiments.

**FIG. 5.** C3-transferase enhances PP1C activity in a dose-dependent manner. HUVEC were incubated for 24 h with indicated concentrations of C3-transferase. PP1M activity was measured as described under "Experimental Procedures." Values represent mean ± S.D. of two to three experiments.

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FIG. 6. Thrombin-induced Ca2+ mobilization is not affected by C3-transferase. HUVEC were pretreated for 24 h without (−) or with (+) 10 μg/ml C3-transferase (C3) from C. botulinum, loaded with fura 2 and supplemented with thrombin (1 unit/ml). Basal, peak (after 30 s), and plateau (after 3–5 min) levels of cytosolic Ca2+ concentration ([Ca2+]i) were quantified spectrophotometrically. Bars represent the mean ± SD of three experiments. Differences between control and C3-treated cells were not significant.

FIG. 7. Thrombin-induced release of plakoglobin and β-catenin from the cytoskeleton is not mediated by Rho. HUVEC pretreated with or without 5 μg/ml C3-transferase were stimulated with thrombin, and TX-100 insoluble cell fractions were separated and analyzed by Western blot. A representative experiment is shown. Plakoglobin and β-catenin were associated with the cytoskeleton, and C3-transferase did not influence this association. Thrombin caused a release from the cytoskeleton of plakoglobin after 1 min and of β-catenin after 5 min. These shifts were not influenced by C3-transferase.

duced by Rho kinase as by V14Rho, indicating that additional Rho targets contribute to efficient stress fiber formation.

Endothelial cells flatten and spread out when Rho is inactivated and contract when Rho is activated. A similar behavior has been found in neuronal cells (15), human and mouse macrophages (51, 52), and HeLa cells (36). In contrast, other cells including NIH 3T3 fibroblasts and Vero cells round up when Rho is inactivated and spread out when it is activated (53, 54). Presumably, this behavior depends on the relative importance of Rho-dependent focal adhesion/integrin cluster formation versus Rho-dependent contractility in the respective cell type.

In fibroblasts, Rho seems to directly trigger Ca2+ mobilization, most likely by providing phosphatidylinositol 4,5-bisphosphate through stimulation of a PI(5) kinase activity (32). The reason why we did not find an effect of Rho inhibition on Ca2+ mobilization in endothelial cells might lie in the recruitment of different target proteins by Rho, depending on the cell type (16).

Fig. 8 depicts the presumptive signal pathway by which thrombin induces cell contraction. The Ca2+-triggered activation of MLCK induces in concert with inhibition of MLC phosphatase by Rho/Rho kinase an increase in MLC phosphorylation and finally cell contraction.

The mechanism by which Rho is activated by thrombin in HUVEC remains to be determined. One possibility is that thrombin activates Rho via G13 and the epidermal growth factor receptor tyrosine kinase, as was recently shown for lysophosphatidic acid in fibroblasts (55).

Here, we describe a pathway involving Rho/Rho kinase by which thrombin inactivates PP1M and thus controls MLC phosphorylation and contraction in human endothelial cells. This pathway is coordinated with the well established Ca2+/-calmodulin-dependent pathway of MLC kinase activation and presumably with yet another pathway regulating adherens junction disassembly. A complex signaling network of thrombin-controlled increase in endothelial permeability is evolving.

Acknowledgments—We thank Barbara Böhlig for expert technical assistance, Manfred Schliwa (Institut für Zellbiologie, LMU München) for help with microinjection, Alan Hall for providing V14Rho, Wolfgang Siess for helpful discussions, Jürgen Heesemann (Institut für Medizinische Mikrobiologie, LMU München) for support, and Markus Bauer for help with densitometry.

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