Essential Role of Cofilin-1 in Regulating Thrombin-induced RelA/p65 Nuclear Translocation and Intercellular Adhesion Molecule 1 (ICAM-1) Expression in Endothelial Cells*

JULY 31, 2009• VOLUME 284 • NUMBER 31 • 21047

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Activation of RhoA/Rho-associated kinase (ROCK) pathway and the associated changes in actin cytoskeleton induced by thrombin are crucial for activation of NF-κB and expression of its target gene ICAM-1 in endothelial cells. However, the events acting downstream of RhoA/ROCK to mediate these responses remain unclear. Here, we show a central role of cofilin-1, an actin-binding protein that promotes actin depolymerization, in linking RhoA/ROCK pathway to dynamic alterations in actin cytoskeleton that are necessary for activation of NF-κB and thereby expression of ICAM-1 in these cells. Stimulation of human umbilical vein endothelial cells with thrombin resulted in Ser3 phosphorylation/inactivation of cofilin and formation of actin stress fibers in a ROCK-dependent manner. RNA interference knockdown of cofilin-1 stabilized the actin filaments and inhibited thrombin- and RhoA-induced NF-κB activity. Similarly, constitutively inactive mutant of cofilin-1 (Cof1-S3D), known to stabilize the actin cytoskeleton, inhibited NF-κB activity by thrombin. Overexpression of wild type cofilin-1 or constitutively active cofilin-1 mutant (Cof1-S3A), known to destabilize the actin cytoskeleton, also impaired thrombin-induced NF-κB activity. Additionally, depletion of cofilin-1 was associated with a marked reduction in ICAM-1 expression induced by thrombin. The effect of cofilin-1 depletion on NF-κB activity and ICAM-1 expression occurred downstream of IκBα degradation and was a result of impaired RelA/p65 nuclear translocation and consequently, RelA/p65 binding to DNA. Together, these data show that cofilin-1 occupies a central position in RhoA-actin pathway mediating nuclear translocation of RelA/p65 and expression of ICAM-1 in endothelial cells.

The nuclear factor κB (NF-κB)² represents a ubiquitously expressed family of transcription factor participating in various biological effects ranging from immune, inflammatory, and stress-induced responses to cell fate decisions such as proliferation, differentiation, apoptosis, and tumorigenesis (1, 2). The mammalian NF-κB family is comprised of five members: RelA (p65), RelB, c-Rel, NF-κB1 (p50 and its precursor p105), and NF-κB2 (p52 and its precursor p100). A characteristic feature of these proteins is the presence of a conserved N-terminal 300-amino acid Rel homology domain that contains nuclear localization signal and is involved in dimerization, sequence-specific DNA binding, and interaction with inhibitory IκB proteins. A distinguishing feature of RelA, RelB, and c-Rel from p50 and RelA/p65 subunits associated with IκBα, the prototype of a family of inhibitory proteins IκBs that keeps NF-κB in the cytoplasm by virtue of masking the nuclear localization signal of RelA/p65 (3, 4). Activation of NF-κB requires phosphorylation of IκBα on two specific serine residues (Ser32 and Ser36) by a macromolecular cytoplasmic IκB kinase (IKK) complex composed of the catalytic subunits IKKα and IKKβ and the regulatory subunit NEMO/IKKγ (5, 6). Phosphorylation triggers the ubiquitination of IκBα by the E3-SCFβ-TRCP ubiquitin ligase, which in turn marks it for degradation by the 26 S proteasome (7, 8). The unleashed NF-κB migrates to the nucleus to activate transcription of target genes encoding intercellular adhesion molecule-1 (ICAM-1) (9–14), an inducible endothelial adhesion protein that serves as a ligand for β2-integrins (CD11/CD18) present on the surface of leukocytes (15–17). Interaction of ICAM-1 with β2-integrins enables polymorphonuclear leukocytes to adhere firmly and stably to the vascular endothelium and to migrate across the endothelial barrier (18–20). We have shown that RelA/p65 is an essential regulator of endothelial ICAM-1 following stimulation of protease-activated receptor-1 by thrombin, a serine protease released during intravascular coagulation initiated by tissue injury or sepsis (21, 22). A key signal mediating RelA/p65 activation by thrombin involves stimulation of the small GTPase RhoA and its effector Rho-associated kinase (23, 24). Activated RhoA/ROCK leads to activation of IKKβ, which in turn mediates the release of RelA/p65 for its nuclear uptake and binding to the ICAM-1 promoter, secondary to phosphorylation and degradation of IκBα (24).

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.

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2 The abbreviations used are: NF-κB, nuclear factor-kappa B; ICAM-1, intercellular adhesion molecule 1; ROCK, Rho-associated kinase; LIMK1, LIM kinase 1; IKK, IκB kinase; HUVEC, human umbilical vein endothelial cell; LUC, luciferase; RNAi, RNA interference; WT, wild type; PBS, phosphate-buffered saline; siRNA, small interfering RNA; CHiP, chromatin immunoprecipitation.

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We also showed that translocation of the released RelA/p65 to the nucleus requires dynamic alterations in the actin cytoskeleton and interfering with these alterations, whether by stabilizing or destabilizing the actin cytoskeleton using the drugs jasplakinolide or latrunculin B, respectively, inhibits nuclear accumulation of RelA/p65 and expression of ICAM-1 (25). Considered together, these data implicate RhoA/ROCK pathway in regulating NF-κB activation and ICAM-1 expression by a dual mechanism involving IKK-dependent release and actin cytoskeleton-dependent translocation of RelA/p65 to the nucleus.

Among the RhoA/ROCK effectors mediating reorganization of the actin cytoskeleton include the actin-depolymerizing factor/cofilin, a family of small (15–20 kDa) proteins that bind monomeric and filamentous actin (26, 27). Cofilin regulates actin dynamics by depolymerizing actin filaments at their pointed ends or by creating new filament barbed ends for F-actin assembly through their severing activity (28, 29). The status of actin polymerization/depolymerization depends on the Ser³ phosphorylation level of cofilin (30). The phosphorylation of cofilin on this residue renders it inactive and prevents it from binding to actin, thus facilitating actin polymerization (30). This phosphorylation event is catalyzed by LIM kinases (LIMK), which in turn are phosphorylated and activated by ROCK (31–34). The requirement of RhoA/ROCK in the regulation of actin dynamics and activation of NF-κB by thrombin (24, 25) led us to investigate the possibility that cofilin serves to link the RhoA/ROCK signaling to changes in actin dynamics and thus contributes in the mechanism of RelA/p65 nuclear translocation and ICAM-1 expression. Our data show that cofilin-1 occupies a central position in RhoA-actin pathway controlling nuclear translocation of RelA/p65 and expression of ICAM-1 in endothelial cells.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Human thrombin was purchased from Enzyme Research Laboratories (South Bend, IN). Polyclonal antibodies to RelA/p65, IkBα, and β-actin, and a monoclonal antibody to ICAM-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A rabbit polyclonal antibody to cofilin-1 was obtained from Cytoskeleton (Denver, CO), and a rabbit polyclonal antibody that detects cofilin-1 when phosphorylated at Ser³ was from Santa Cruz Biotechnology (Santa Cruz, CA). Y27632 was purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA). In addition, polyvinylidene difluoride membrane was from Millipore Corp. (Billerica, MA); plasmid maxi-kit was from Qiagen Inc.; and the protein assay kit and nitrocellulose membrane were from Bio-Rad. Alexa Fluor 488-phalloidin and Texas Red goat anti-rabbit IgG were purchased from Molecular Probes (Eugene, OR). All other materials were from VWR Scientific Products Corp. (Gaithersburg, MD).

**Cell Culture**—Human umbilical vein endothelial cell (HUVEC) cultures were established as described previously (35, 36) by using umbilical cords collected within 48 h of delivery. The cells were cultured in gelatin-coated flasks using endothelial basal medium 2 with Bullet™ kit additives (BioWhittaker, Walkersville, MD) as described (35). For treatment, the cells were washed twice with serum-free MCDB-131 medium and incubated in same serum-free medium for 0.5–1 h prior to thrombin challenge. The cells used in the experiments were between three and six passages.

**Cell Lysis and Immunoblotting**—The cells were lysed in radioimmune precipitation buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.25 mM EDTA, pH 8.0, 1% deoxycholic acid, 1% Triton X-100, 5 mM NaF, 1 mM sodium orthovanadate supplemented with complete protease inhibitors (Sigma). Cell lysates were resolved by SDS-PAGE and transferred onto nitrocellulose (Bio-Rad) or polyvinylidene difluoride membranes, and the residual binding sites on the filters were blocked by incubating with 5% (w/v) nonfat dry milk in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20) for 1 h at room temperature or overnight at 4 °C. The membranes were subsequently incubated with the indicated antibodies and developed using an ECL method as described (37).

**Immunofluorescence**—Cells grown on coverslips were fixed in 3.7% paraformaldehyde/PBS and permeabilized with 0.1% Triton X-100 for 5 min at room temperature as described (25, 38). Permeabilized cells were rinsed three times with PBS and incubated in blocking solution (1% bovine serum albumin/PBS) for 30 min at room temperature to remove nonspecific binding of the antibody. All of the subsequent steps were carried out at room temperature, and the cells were rinsed three times in 1% bovine serum albumin/PBS between each of the steps. To localize F-actin filaments, the cells were incubated with Alexa Fluor 488-phalloidin for 20 min at room temperature in a humid chamber. RelA/p65 was detected using a rabbit polyclonal antibody to RelA/p65 antibody (C-20; Santa Cruz Biotechnology) and a secondary antibody conjugated to Texas Red (Molecular Probes, Eugene, OR). DNA was stained using Hoechst Dye in PBS to visualize nuclei. The coverslips were rinsed in PBS and mounted on the slide using Vectashield mounting media (Vector Laboratories, Lincolnshire, IL). The images were obtained using fluorescence or confocal microscope (Zeiss Axioplasm).

**RNAi Knockdown of Cofilin-1—SMARTpool siRNA duplexes specific for cofilin-1 and a nonspecific siRNA control were obtained from Dharmacon (Lafayette, CO). The cells were transfected with siRNA using DharmaFect1 siRNA transfection reagent (Dharmacon, Lafayette, CO) according to the manufacturer’s recommendations. Briefly, 50–100 nM siRNA was mixed with DharmaFect1 and added to cells that are 50–60% confluent. After 24–36 h, the cells were lysed to determine the level of cofilin-1 by immunoblotting.

**cDNA Constructs and Reporter Gene Assay**—The constructs pRK5-cof, pRK5-cofS3A, and pRK5-cofS3D encoding wild type (Cof-WT), phosphorylation-defective (CofS3A) and phosphomimic (Cof-S3D) forms of cofilin-1 respectively, were from Theo Rein (Max Planck Institute of Psychiatry, Munich, Germany) (39). The CofS3A was generated by replacing serine 3 with alanine, and this mutation rendered cofilin-1 constitutively active. The Cof-S3D was generated by replacing serine 3 with aspartate, and this mutation rendered cofilin-1 constitutively inactive. Expression vector encoding constitutively active form of RhoA (RhoA–CAT) is described elsewhere (24). The construct pNF-κB-LUC containing five copies of consensus NF-κB sequences linked to a minimal E1B promoter-luciferase gene was purchased from Stratagene (La Jolla, CA). Transfections were performed using the DEAE-dextran method essen-
tially as described (40). Briefly, 5 μg of DNA was mixed with 50 µg/ml DEAE-dextran in serum-free endothelial basal medium 2, and the mixture was added onto cells that were 60–80% confluent. We used 0.125 µg of pTKRluc plasmid (Promega, Madison, WI) containing Renilla luciferase gene driven by the constitutively active thymidine kinase promoter to normalize transfection efficiencies. After 1 h, the cells were incubated for 4 min with 10% dimethyl sulfoxide in serum-free endothelial basal medium 2. The cells were then washed two times with endothelial basal medium 2, 10% fetal bovine serum and grown to confluence. We achieved transfection efficiency of 16 ± 3 (mean ± S.D.; n = 3) in these cells. Cell extracts were prepared and assayed for firefly luciferase activity using the Promega Bio-Tech dual luciferase reporter assay system. The data were expressed as a ratio of firefly luciferase activity. For experiments examining the effect of cofilin-1 knockdown on NF-κB activity, the cells were first transfected with siRNA using DharmaFect1. After 12–16 h, the cells were again transfected with pNF-κB-LUC using DEAE-dextran method, and luciferase activity was determined as described above.

Cytoplasmic and Nuclear Extract Preparation—After treatments, the cells were washed two times with ice-cold Tris-buffered saline and resuspended in 400 μl of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride). After 15 min, Nonidet P-40 was added to a final concentration of 0.6%. The samples were centrifuged to collect the supernatants containing cytosolic proteins for determining IκBα degradation by immunoblotting. The pelleted nuclei were resuspended in 50 μl of buffer B (20 mM HEPES, pH 7.9, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride). After 30 min at 4 °C, the lysates were centrifuged, and supernatants containing the nuclear proteins were transferred to new vials. Protein concentration of the extract was measured using a Bio-Rad protein determination kit (Bio-Rad).

Chromatin Immunoprecipitation (ChIP) Assay—ChIP assays were performed as described (41-43). Briefly, cellular proteins and DNA were cross-linked by adding formaldehyde to the growth media to a final concentration of 0.1%. The cells were harvested in ice-cold phosphate-buffered saline and lysed with SDS buffer (50 mM Tris, 10 mM EDTA, and 1% w/v SDS). The lysates were sonicated and precleared with salmon sperm DNA/protein A-agarose (Upstate Biotechnologies, Inc., Lake Placid, NY) and then incubated overnight at 4 °C with salmon sperm DNA/protein A-agarose with and anti-RelA/p65 antibody. Immunoprecipitated complexes were serially washed three times each with low salt (20 mM Tris, 150 mM NaCl, 2 mM EDTA, 0.1% (v/v) SDS, and 1% (v/v) Triton X-100); high salt (20 mM Tris, 500 mM NaCl, 2 mM EDTA, 0.1% (v/v) SDS, and 1% (v/v) Triton X-100); LiCl wash (10 mM Tris, 250 mM LiCl, 1 mM EDTA, 1% (v/v) deoxycholate, and 1% (v/v) Nonidet P-40); and TE buffer (20 mM Tris and 2 mM EDTA). Washed complexes were eluted with freshly prepared elution buffer (1% SDS and 100 mM NaHCO₃) and the Na⁺ concentration was adjusted to 200 mM, followed by incubation at 37 °C to reverse protein/DNA cross-links. DNA was purified utilizing a PCR purification kit (Qiagen) and then amplified across the human ICAM-1 promoter region using forward (5'-ACCTTAGGCAGGTG-

FIGURE 1. Thrombin induces phosphorylation of cofilin in a ROCK-dependent manner. Confluent HUVEC monolayers were challenged with thrombin (5 units/ml) for the indicated time periods (A) or pretreated with Y27632 for 1 h prior to challenge with thrombin for 15 min (B). Total cell lysates were separated by SDS-PAGE and immunoblotted with an anti-phospho-cofilin-1 (Ser³) antibody. Cofilin-1 levels were used to monitor loading. The results are representative of two or three separate experiments.

RESULTS

Thrombin Induces Cofilin-1 Phosphorylation/Inactivation via a ROCK-dependent Pathway—To understand the role of cofilin-1 in thrombin-induced stress fiber formation, we first evaluated the ability of thrombin to inactivate cofilin by monitoring the phosphorylation of cofilin-1 at Ser³. Western blot analysis showed that thrombin induced cofilin-1 phosphorylation in a time-dependent manner. The increase in cofilin-1 phosphorylation was evident within 1 min (data not shown) and peaked between 5 and 15 min after thrombin challenge (Fig. 1A). Phosphorylation of cofilin-1 began to decline at 30 min and returned to base line by 2–4 h after thrombin stimulation (Fig. 1A). We next determined whether phosphorylation of cofilin-1 by thrombin requires the activation of RhoA effector ROCK. Pretreatment of cells with Y27632, a relatively specific inhibitor of ROCK inhibited basal as well as thrombin-induced cofilin-1 phosphorylation (Fig. 1B). We also determined the involvement of ROCK effector LIMK1 in this response. We found that RNAi knockdown of LIMK1 was effective in preventing both the basal and thrombin-induced cofilin-1 phosphorylation (supplemental Fig. S1).

Inhibition of ROCK Prevents Whereas RNAi Knockdown of Cofilin-1 Promotes Stress Fiber Formation—The involvement of ROCK in cofilin-1 phosphorylation/inactivation prompted us to assess its role in stress fiber formation induced by thrombin. The effect of ROCK inhibition on stress fiber formation was analyzed by fluorescence microscopy. HUVECs stained with Texas Red labeled-phalloidin showed increased formation of actin stress fibers in cells stimulated with thrombin (Fig. 2, panel c versus panel a). Inhibiting ROCK by Y27632 impaired
the basal as well as thrombin-induced stress fiber formation Fig. 2 (panels b and d). To determine whether ROCK promotes stress fiber formation by its ability to phosphorylate and thereby inactivate cofilin-1, we assessed the effect of depleting cofilin-1 on stress fiber formation. To this end, the cells were transfected with siRNA targeting cofilin-1 (cofilin-siRNA) or control siRNA and analyzed for cofilin-1 expression by immunoblotting. The cells transfected with cofilin-siRNA showed a marked depletion of cofilin-1 compared with cells transfected with control siRNA (Fig. 3A). Analysis of fluorescence microscopy of HUVECs stained with Alexa-488 labeled-phalloidin showed that depletion of cofilin-1 augmented basal as well as thrombin-induced stress fiber formation (Fig. 3B, panels c and d versus panels a and b), consistent with the actin depolymerizing function of cofilin. We also assessed the effect of overexpressing cofilin-1 on stress fiber formation by thrombin. The results showed that overexpression of cofilin impaired thrombin-induced stress fiber formation (supplemental Fig. S2; green cells in panel d versus b), as expected.

RNAi Knockdown of Cofilin-1 Inhibits Thrombin-induced NF-κB Activity and ICAM-1 Expression—We next examined whether stabilization of the actin filaments induced by cofilin-1 knockdown had an effect on NF-κB activity induced by thrombin. HUVECs were transfected with pNF-κB-LUC in combination with cofilin-siRNA or control siRNA. The results showed that thrombin challenge of cells transfected with control siRNA resulted in increased NF-κB-dependent reporter activity and that this response was inhibited in the cells transfected with cofilin-siRNA (Fig. 4A). In view of the essential role of NF-κB in ICAM-1 transcription (10, 12), we determined whether depletion of cofilin-1 produces a similar effect on ICAM-1 expression. We found that depleting cofilin-1 was also effective in inhibiting thrombin-induced ICAM-1 expression (Fig. 4B), consistent with its effect on NF-κB activity (Fig. 4A).

In reciprocal experiments, we addressed the effect of overexpression of cofilin-1, likely to destabilize the actin filaments, on NF-κB activity. Transfection of cells with a construct encoding wild type cofilin-1 (Cof1-WT) resulted in inhibition of thrombin-induced NF-κB activity (Fig. 5). Similarly, expression of phosphorylation-defective cofilin-1 mutant (Cof1-S3A), a constitutively active form of cofilin-1 implicated in destabilizing the actin filaments (44), also inhibited thrombin-induced NF-κB activity (Fig. 5). Expression of a constitutively inactive mutant of cofilin-1 (Cof1-S3D), known to stabilize the actin filaments (26, 39), also inhibited NF-κB activity by thrombin (Fig. 5). Collectively, these results indicate that the dynamic reorganization of actin cytoskeleton induced by thrombin is
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Effects of cofilin-1 knockdown on thrombin-induced NF-κB activity and ICAM-1 expression.

RNAi Knockdown of Cofilin-1 Inhibits RhoA-induced NF-κB Activity—We previously showed that activation of RhoA/ROCK and alterations in actin cytoskeleton are required for thrombin-induced NF-κB activation (24, 25). These findings together with observations that activation of ROCK is required for cofilin-1 phosphorylation and actin stress fiber formation (Fig. 2) led us to investigate the possibility that cofilin-1 couples RhoA/ROCK to actin cytoskeleton in controlling thrombin-induced NF-κB activation. For this purpose, we determined the effects of depleting cofilin-1 on NF-κB activity induced by RhoA. Expression of constitutively active form of RhoA (RhoA-CAT) was capable of inducing NF-κB activity in the absence of thrombin challenge, and depletion of cofilin-1 inhibited this response (Fig. 6). Together, these findings suggest that engagement of the RhoA/ROCK/cofilin-1/actin pathway is a critical determinant of NF-κB activation following thrombin challenge of endothelial cells.

RNAi Knockdown of Cofilin-1 Inhibits Thrombin-induced Recruitment of RelA/p65 to Endogenous ICAM-1 Promoter but Fails to Prevent IκBα Degradation—We next determined whether the reduced NF-κB activity following depletion of cofilin-1 was due to inhibition of NF-κB DNA binding activity. It should be noted that thrombin-induced NF-κB complexes are predominantly composed of RelA/p65 homodimer (10). Electrophoretic mobility supershift assay showed that cofilin-1 knockdown substantially reduced the DNA binding of RelA/p65 in response to thrombin challenge (Fig. 7A). Additionally, we used ChIP assay to investigate the RelA/p65 binding to the endogenous ICAM-1 promoter following thrombin challenge of endothelial cells. We found that thrombin stimulation resulted in recruitment of RelA/p65 to the endogenous ICAM-1 promoter, which was inhibited upon cofilin-1 knockdown (Fig. 7B). In control ChIP experiments using IgG, we failed to detect the occupancy of cofilin-1 inhibits RelA/p65 occupancy of the ICAM-1 promoter by influencing IκBα degradation, we evaluated the effect of cofilin-1 knockdown on this response. Intriguingly, IκBα degradation was insensitive to depletion of cofilin-1 (Fig. 7C).

RNAi Knockdown of Cofilin-1 Inhibits Nuclear Translocation of RelA/p65—We tested the possibility that reduced DNA binding of RelA/p65 following cofilin-1 depletion is a result of impaired translocation of RelA/p65 to the nucleus. Analysis of nuclear extracts by immunoblotting showed that depletion of cofilin-1 inhibited the nuclear uptake of RelA/p65 by thrombin (Fig. 8A). To further verify the suppressive effect of cofilin-1 knockdown on RelA/p65 nuclear accumulation, we employed fluorescence microscopy to analyze the cells. We noted the presence of RelA/p65 primarily in the cytoplasm of unstimulated cells, irrespective of whether they were transfected with control siRNA or cofilin-siRNA, as expected (Fig. 8B, panels a and b, red staining). Stimulation
with thrombin caused nuclear localization of RelA/p65 in cells transfected with control siRNA as indicated by the pink versus blue nuclei (Fig. 8B, panel c versus panel a). In contrast, thrombin failed to induce RelA/p65 nuclear translocation in cells transfected with coflin-siRNA as indicated by blue versus pink nuclei (Fig. 8B, panel d versus panel c).

**DISCUSSION**

We have recently shown that activation of NF-κB and expression of ICAM-1 induced by thrombin in endothelial cells is mediated by Rho GTPase and requires alterations in actin dynamics. In the present study, we define a central role of coflin-1 in Rho-actin pathway mediating nuclear translocation of RelA/p65 and ICAM-1 expression. Our experiments show that thrombin induces phosphorylation of coflin-1, rendering it inactive to promote stress fiber formation via Rho effector ROCK. Consistent with this, depletion of coflin-1 caused stabilization of actin filaments. Stabilizing the actin filaments by this approach impaired thrombin- and RhoA-induced NF-κB activity. The effect of coflin-1 knockdown on NF-κB activity occurred downstream of IkBα degradation and was a result of impaired RelA/p65 nuclear translocation and, consequently, RelA/p65 binding to ICAM-1 promoter. Together, these data are consistent with the notion that dynamic changes in actin cytoskeleton induced by thrombin via coflin-1 phosphorylation/inactivation by RhoA/ROCK pathway are necessary for nuclear translocation of RelA/p65 and expression of ICAM-1 in endothelial cells.

The requirement of coflin-1 phosphorylation in actin stress fiber formation provides further insight into the mechanisms by which thrombin regulates actin dynamics in endothelial cells. Studies have established thrombin as a strong inducer of stress fiber formation in endothelial cells (45–47); however, the role of coflin in this response has remained to be clarified. Therefore, we examined the effect of thrombin on coflin-1 phosphorylation and demonstrate that thrombin promotes coflin-1 phosphorylation via activation of ROCK. Accordingly, inhibition of ROCK impaired the ability of thrombin to induce stress fiber formation. These results suggest that phosphorylation of coflin via ROCK plays an important role in actin cytoskeleton reorganization in response to thrombin. To corroborate these results, we used another strategy whereby coflin-1 was depleted by RNAi approach to ascertain whether knockdown of coflin-1 augments stress fiber formation. Indeed, we observed an augmentation both in basal and thrombin-induced stress fiber formation in cells depleted of coflin-1. Because activation of Rho is implicated in thrombin-induced stress fiber formation in endothelial cells (46, 47), our findings are consistent with involvement of RhoA/ROCK in coflin-1 phosphorylation. Recently, Gorovoy et al. (48) showed an important role of LIMK1 in the mechanism of thrombin-induced actin polymerization in endothelial cells. Given that LIMK lies downstream of RhoA/ROCK in phosphorylating coflin (32, 33, 49), we also examined the effect of depletion of LIMK1 on coflin phosphorylation. Depletion of LIMK1 inhibited coflin phosphorylation, indicating that thrombin engages RhoA/ROCK/LIMK in mediating coflin phosphorylation to alter the actin dynamics in endothelial cell. Considering that
thrombin controls several actin-driven responses in endothelial cells as well as other cell types (46, 47, 50–52), our data are of broader significance and suggest that cofilin-1 phosphorylation may be an important regulator of essential cellular functions known to be under control of actin dynamics.

We recently identified a novel function of actin cytoskeleton in controlling thrombin-induced NF-κB activity and ICAM-1 expression in endothelial cells (25). We showed that thrombin causes dynamic reorganization of actin cytoskeleton in endothelial cells and that this event is essential for NF-κB activity because interfering with actin reorganization, either by stabilizing or destabilizing the actin cytoskeleton, impairs thrombin-induced NF-κB activity and ICAM-1 expression (25). Therefore, we sought to determine whether cofilin-1 contributes in thrombin-induced NF-κB activity expression in endothelial cells. We explored this possibility by assessing the ability of phosphorylation-defective cofilin-1 (Cof1-S3A) mutant to modulate NF-κB activity. It should be noted that replacing serine 3 with alanine (S3A) renders the cofilin-1 mutant constitutively active and has been shown to destabilize the actin cytoskeleton by promoting actin depolymerization (39). Destabilizing the actin cytoskeleton by expressing Cof1-S3A inhibited NF-κB-dependent reporter activity in response to thrombin. Similarly, overexpression of cofilin-1 (Cof1-WT) impaired actin filament formation, consistent with its function to destabilize the actin cytoskeleton (26, 53, 54), and mimicked the effect of Cof1-S3A in inhibiting thrombin-induced NF-κB-dependent reporter activity. These results are in agreement with our earlier data obtained with cytochalasin D and latrunculin B, two classic actin-depolymerizing drugs with distinct modes of action (25).

In reciprocal experiments, we determined the effect of phosphorylation mimic cofilin-1 (Cof1-S3D) mutant in which negatively charged phosphate is mimicked by aspartate. Replacing serine 3 with aspartate generates a constitutively inactive mutant of cofilin-1 that stabilizes actin cytoskeleton by impairing actin depolymerization (26, 53, 54). Stabilizing actin cytoskeleton by this approach also caused inhibition of NF-κB-dependent reporter activity induced by thrombin. To further validate these data, we evaluated whether knockdown of cofilin-1 produces a similar effect on the thrombin response. We found that siRNA-mediated silencing of cofilin-1 resulted in stabilization of the actin cytoskeleton and inhibition of NF-κB activity. We also assessed whether the impaired NF-κB activity following cofilin-1 knockdown leads to

![Figure 7](http://www.jbc.org/)
We addressed the mechanism by which cofilin-1 controls NF-κB activity and thereby ICAM-1 expression. Activation of NF-κB involves its release secondary to degradation of IκBα (4, 56). The released NF-κB then migrates to the nucleus, where it binds to the promoter and activates the transcription of its target genes including ICAM-1 (10, 12). We began the analysis of NF-κB signaling pathway by evaluating the effect of cofilin-1 knockdown on thrombin-induced IκBα degradation. We found that IκBα degradation was refractory to cofilin-1 depletion. The insensitivity of IκBα degradation excludes the possibility that depletion of cofilin-1 exerts its inhibitory effect on NF-κB activity and ICAM-1 expression by interfering with thrombin activation of its receptor, protease-activated receptor-1. Intriguingly, cofilin-1 depletion caused a substantial reduction in thrombin-induced RelA/p65 binding to the ICAM-1 promoter in the nucleus. The decreased RelA/p65 binding to the ICAM-1 promoter in the face of IκBα degradation raised the possibility of impairment in the translocation of RelA/p65 to the nucleus. Indeed, we found that cofilin-1 knockdown was associated with impaired nuclear translocation of the released RelA/p65 after thrombin stimulation. It should be stressed that these results are in accord with our previous report that changes in actin dynamics are crucial for the nuclear transport of RelA/p65 (25). Thus, our findings support the notion that cofilin-1-dependent changes in actin dynamics control NF-κB activity by facilitating the nuclear translocation of RelA/p65.

The requirement of functional and dynamic actin cytoskeleton in RelA/p65 nuclear translocation is consistent with previous studies documenting an important role in actin dynamics in agonist-mediated endocytosis of β-adrenergic and the β isoform of thromboxane A2 receptors (23, 57). These studies showed that internalization of these receptors was prevented by both the agents that destabilize (latrunculin B) or stabilize (jasplakinolide) the actin cytoskeleton (23, 57). Consistently, expression of Cof1-WT or Cof1-S3A each inhibited β-adrenergic receptor internalization induced by isoproterenol (23). Lin et al. (58) showed that shear stress engages Rho-ROCK-LIMK-Cofilin pathway to activate sterol regulatory element-binding proteins in endothelial cells and that this response is inhibited in the presence of Cof1-WT or Cof1-S3A. Extensive studies by Treisman and co-workers (59, 60) have established a crucial role of actin dynamics in regulating the nuclear uptake of MAL, the myocardin-related coactivator of serum response factor. However, unlike RelA/p65 nuclear translocation or endocytosis of β-adrenergic and thromboxane A2 receptors (23, 25, 57), nuclear accumulation of MAL, which is retained in the cytoplasm through its association with G-actin, is facilitated by signals and agents that deplete G-actin by inducing F-actin formation. Accordingly, inhibition of actin polymerization by latrunculin B prevents MAL translocation to the nucleus and consequently, serum response factor activity (59 – 61). Importantly, microtubule dynamics is also implicated in the nuclear accumulation of the tumor suppressor protein p53 (62). Studies by Giannakakou et al. (62) showed that p53 associates with microtubules and that this association is important for p53 nuclear localization after DNA damage. Upon disrupting the normal microtubule dynamics, whether by stabilizing or destabilizing the microtubule cytoskeleton, this association is lost,
In conclusion, our results identify cofilin-1 as a critical determinant of thrombin-induced RelA/p65 nuclear translocation and thereby ICAM-1 expression by virtue of controlling the actin dynamics downstream of RhoA/ROCK pathway. Thus, the specific targeting of cofilin-1 may be a useful strategy for dampening the thrombin-activated inflammatory responses associated with intravascular coagulation.

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J. Biol. Chem. 2009, 284:21047-21056.
doi: 10.1074/jbc.M109.016444 originally published online May 29, 2009

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