Evidence for Actin Cytoskeleton-dependent and -independent Pathways for RelA/p65 Nuclear Translocation in Endothelial Cells*

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Activation of the transcription factor NF-κB involves its release from the inhibitory protein IkBα in the cytoplasm and subsequently, its translocation to the nucleus. Whereas the events responsible for its release have been elucidated, mechanisms regulating the nuclear transport of NF-κB remain elusive. We now provide evidence for actin cytoskeleton-dependent and -independent mechanisms of RelA/p65 nuclear transport using the proinflammatory mediators, thrombin and tumor necrosis factor α, respectively. We demonstrate that thrombin alters the actin cytoskeleton in endothelial cells and interfering with these alterations, whether by stabilizing or destabilizing the actin filaments, prevents thrombin-induced NF-κB activation and consequently, expression of its target gene, ICAM-1. The blockade of NF-κB activation occurs downstream of IkBα degradation and is associated with impaired RelA/p65 nuclear translocation. Importantly, thrombin induces association of RelA/p65 with actin and this interaction is sensitive to stabilization/destabilization of the actin filaments. In parallel studies, stabilizing or destabilizing the actin filaments fails to inhibit RelA/p65 nuclear accumulation and ICAM-1 expression by tumor necrosis factor α, consistent with its inability to induce actin filament formation comparable with thrombin. Thus, these studies reveal the existence of actin cytoskeleton-dependent and -independent pathways that may be engaged in a stimulus-specific manner to facilitate RelA/p65 nuclear import and thereby ICAM-1 expression in endothelial cells.

NF-κB2 is an ubiquitously expressed family of transcription factors controlling varied biological effects ranging from inflammatory, immune, and stress-induced responses to cell fate decisions such as proliferation, differentiation, tumorigenesis, and apoptosis (1–3). NF-κB dimers, typically a heterodimer of p50 and RelA/p65 subunits, are mostly sequestered in the cytoplasm by IkBα, the prototype of a family of inhibitory proteins IkBs that mask the nuclear localization signal of RelA/p65 (4, 5). Activation of NF-κB requires serine phosphorylation (Ser32 and Ser36) of IkBα by a macromolecular IkB kinase (IKK) complex (6–8). Phosphorylation marks IkBα for polyubiquitination by the E3-SCFβ-TrCP ubiquitin ligase leading to its degradation by the 26 S proteasome (9–11). The released NF-κB undergoes rapid nuclear translocation and subsequent binding to NF-κB responsive elements to activate transcription of target genes including intercellular adhesion molecule-1 (ICAM-1; CD54), an inducible endothelial adhesive protein that serves as a counter-receptor for β2-integrins (CD11/CD18) present on the surface of leukocytes (12–14). 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 (15–18). Studies (19–22) have shown that activation of NF-κB is essential for ICAM-1 expression in endothelial cells after stimulation with the proinflammatory cytokine tumor necrosis factor α (TNFα), and the procoagulant thrombin, a serine protease released during intravascular coagulation initiated by tissue injury or sepsis (23, 24).

Despite the fact that the nuclear translocation constitutes the key step of NF-κB activation, mechanisms underlying the nuclear transport of NF-κB are uncertain; it is likely that they may involve association of the liberated RelA/p65 with cytoplasmic structures such as actin cytoskeleton. The actin cytoskeleton is dynamic, and the rates of polymerization and depolymerization of actin are critical determinants of many cellular responses including transcripational regulation (25–28). We recently showed that the small GTPase RhoA, a key regulator of actin cytoskeleton (29–32), plays an essential role in mediating thrombin- but not TNFα-induced activation of NF-κB and the expression of ICAM-1 in endothelial cells via activation of its downstream effector Rho-associated kinase (ROCK) (33). These findings led us to compare the alterations in actin cytoskeleton induced by thrombin and TNFα and address the role of these alterations in mediating NF-κB activation and ICAM-1 expression in endothelial cells. Using a combination of fluorescence microscopy and biochemical approaches, we demonstrate that RelA/p65 associates with actin, accumulates in the nucleus, and induces ICAM-1 expression after thrombin stimulation of endothelial cells. Disruption of thrombin-induced alterations in actin cytoskeleton, either by stabilizing or destabilizing the actin filaments, interferes with
the association of RelA/p65 with actin, impairs the nuclear uptake of RelA/p65, and prevents the expression of ICAM-1. We also show that TNFα is incapable of inducing actin stress fiber formation comparable with thrombin and consequently, stabilization or destabilization of the actin filaments fails to inhibit TNFα-induced RelA/p65 nuclear uptake and ICAM-1 expression. These findings are consistent with the existence of actin cytoskeleton-dependent and -independent mechanisms that may be engaged in a stimulus-specific manner to facilitate RelA/p65 nuclear import and thereby ICAM-1 expression in endothelial cells.

EXPERIMENTAL PROCEDURES

Materials—Human thrombin was purchased from Enzyme Research Laboratories (South Bend, IN). Polyclonal antibodies to IKKβ, p65/RelA, and β-actin, and a monoclonal antibody to ICAM-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). In addition, polyvinyldene difluoride membrane was from Millipore Corp. (Bradford, MA); cytochalasin D, latrunculin B, and jasplakinolide were from Calbiochem-Novabiochem Corp. (La Jolla, CA); plasmid maxi-kit was from Qiagen Inc. (Valencia, CA); 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 (34, 35) in gelatin-coated flasks. Cell cultures were as described (35) in gelatin-coated flasks using endothelial basal medium 2 with bullet kitTM additives. HUVEC used in the experiments were between 3 and 6 passages. After treatment, cells were lysed in radioimmune precipitation (RIPA) 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 immunoprecipitated with the indicated antibodies as described (33, 37). For immunoblotting, the precipitated proteins or 10 μg of 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 enhanced chemiluminescence (ECL) method as described (36).

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 (38). Permeabilized cells were rinsed 3 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 subsequent steps were carried out at room temperature and cells were rinsed 3 times in 1% bovine serum albumin/PBS between each of the steps. To localize F-actin filaments, cells were incubated with Alexa 488-phalloidin for 20 min at room temperature in a humid chamber. RelA/p65 was detected using a rabbit polyclonal anti-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). Images were obtained using fluorescence or an LSM 510 confocal microscope (Zeiss axioplasm).

Phalloidin staining was quantified using custom image processing routines written in MATLAB (Mathworks, Cambridge, MA). The routines use edge detection techniques to automatically identify cell boundaries, remove background fluorescence, and calculate the fluorescence for each cell. Averages and standard errors were computed over 3 or more images per condition for a minimum of 70 cells per condition.

Reporter Gene Constructs, Endothelial Cell Transfection, and Luciferase Assay—The plasmid pNF-κB-LUC containing 5 copies of consensus NF-κB sequences linked to a minimal E1B promoter-luciferase gene was purchased from Stratagene. Transfections were performed using the DEAE-dextran method (39) with slight modifications (22). 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, cells were incubated for 4 min with 10% dimethyl sulfoxide in serum-free endothelial basal medium 2. The cells were then washed 2 times with endothelial basal medium 2, 10% FBS 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 and Renilla luciferase activities using the Promega Biotech Dual Luciferase Reporter Assay System. The data were expressed as a ratio of firefly to Renilla luciferase activity.
Actin Cytoskeleton and RelA/p65 Nuclear Transport

FIGURE 1. A, effects of Cyto D and Lat B on thrombin-induced actin stress fiber formation. Confluent HUVEC monolayers were left untreated (a) or challenged for 1 h with thrombin in the absence (b) and presence of 5 μM Cyto D (c) or 1 μM Lat B (d). Cells were then fixed, permeabilized, and stained with Alexa 488-labeled phallolidin to visualize the actin stress fibers by confocal microscopy. The bar graph represents the effects of Cyto D and Lat B on thrombin-induced phalloidin staining. Phalloidin staining was quantified as described under “Experimental Procedures.” Data are mean ± S.E. (n = 3 or more images containing a minimum of 70 cells per condition), * different from thrombin-stimulated controls (p < 0.05), # different from Cyto D and Lat B on thrombin-induced NF-κB activity. HUVEC were transfected with the NF-κB-LUC construct as described under “Experimental Procedures.” Cells were pretreated for 30–45 min with 5 μM Cyto D or 1 μM Lat B and then challenged with 5 units/ml thrombin for 6 h. Cell extracts were prepared and assayed for firefly and Renilla luciferase activities. Data are mean ± S.E. (n = 3 for each condition), * different from controls (p < 0.005); #, different from thrombin-stimulated controls (p < 0.005). C, TNFα fails to induce actin stress fiber formation comparable with thrombin. Confluent HUVEC monolayers were left untreated (a) or stimulated with 5 units/ml thrombin (b) or 100 units/ml TNFα (c) for 1 h. Cells were fixed, permeabilized, and stained with Alexa 488-labeled phallolidin to visualize the actin stress fibers by fluorescence microscopy. The bar graph represents thrombin- and TNFα-induced phalloidin staining. Phalloidin staining was quantified as described under “Experimental Procedures.” Data are mean ± S.E. (n = 3 or more images containing a minimum of 70 cells per condition), * different from controls (p < 0.005); # different from thrombin-stimulated controls (p < 0.005). D, effect of Cyto D on TNFα-induced NF-κB activity. HUVEC were transfected with the NF-κB-LUC construct as described under “Experimental Procedures.” Cells were pretreated with 5 μM Cyto D for 45 min and then challenged with 100 units/ml TNFα for 6 h. Cell extracts were prepared and assayed for firefly and Renilla luciferase activities. Data are mean ± S.E. (n = 3 for each condition), * different from controls (p < 0.005). E, effect of Lat B on RhoA<sup>Cat</sup>-induced NF-κB activity. HUVEC were transfected with NF-κB-LUC in combination with a construct encoding the constitutively active form of RhoA (RhoA<sup>Cat</sup>) as described under “Experimental Procedures.” pcDNA3 was used as vector alone control. Sixteen hours after transfection, cells were treated with 1 μM Lat B for 6 h. Cell extracts were prepared and assayed for firefly and Renilla luciferase activities. Data are mean ± S.E. (n = 3–6 for each condition), * different from vector controls (p < 0.005); # different from RhoA<sup>Cat</sup> controls (p < 0.001).

Cytoplasmic and Nuclear Extract Preparation—After treatments, cells were washed 2 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 phenylmethylsulfonfyl fluoride). After 15 min, Nonidet P-40 was added to a final concentration of 0.6%. Samples were centrifuged to collect the supernatants containing cytosolic proteins for determining IκBα degradation by Western blot analysis. The pelletted nuclei were resuspended in 50 μl of buffer B (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride). After 30 min at 4 °C, 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).
sequence present in the mouse Igκ light chain gene (41). The sequence motifs within the oligonucleotides are underlined.

Statistical Analysis—Data are expressed as mean ± S.E. Comparisons between experimental groups were made by Student’s t test. Differences in mean values were considered significant at p < 0.05.

RESULTS

Stabilization and Destabilization of Actin Filaments Selectively Inhibits Thrombin-induced NF-κB Activity and ICAM-1 Expression in Endothelial Cells—To address the role of actin cytoskeleton in the mechanism of thrombin-induced NF-κB activation in endothelial cells, we first evaluated the effect of destabilizing the actin filaments on NF-κB-dependent reporter gene activity. We used cytochalasin D (Cyto D) and latrunculin B (Lat B), the prototypic actin depolymerizing agents (42, 43), to prevent the formation of actin stress fibers induced by thrombin. Analysis by confocal microscopy of HUVEC stained with Alexa 488-labeled phalloidin revealed that thrombin induced the formation of actin stress fibers (F-actin) (Fig. 1A, panel b). Pretreatment of cells with Cyto D or Lat B prevented the formation of stress fibers induced by thrombin (Fig. 1A, panels c and d). We observed that the effect of Lat B on stress fiber formation was more pronounced than Cyto D (Fig. 1A, panels c and d), consistent with Lat B being more potent than Cyto D (43). Using the same approach as described above, we determined the effect of destabilization of actin filaments on the NF-κB activity. We found that destabilizing the actin cytoskeleton inhibited thrombin-induced NF-κB-dependent reporter activity (Fig. 1B). In parallel experiments, we determined the ability of TNFα, the prototypic inducer of NF-κB activation, to induce stress fiber formation in endothelial cells. Results showed that TNFα failed to induce stress fiber formation comparable with thrombin (Fig. 1C, panel b versus c). We also found that destabilization of actin filaments failed to inhibit TNFα-induced NF-κB-dependent reporter activity (Fig. 1D).

In view of our findings that the RhoA/ROCK pathway, a critical regulator of actin cytoskeleton (29–32), controls thrombin- but not TNFα-induced NF-κB activation in endothelial cells (33), we determined whether destabilization of the actin filaments also interferes with NF-κB-dependent reporter activity induced by RhoA. Expression of constitutively active RhoA mutant (RhoA<sup>cat</sup>) induced NF-κB activity in the absence of thrombin challenge. Exposure of cells to Lat B inhibited RhoA<sup>cat</sup>-induced NF-κB activity (Fig. 1E). These data are consistent with selective regulation by RhoA and the actin cytoskeleton of thrombin-induced NF-κB activation in endothelial cells.

In reciprocal experiments, we used jasplakinolide (Jas), the classic actin cytoskeleton stabilizing agent (44), to assess the effect of stabilizing the actin filaments on NF-κB activity. Intriguingly, pretreatment of cells with Jas also prevented

![FIGURE 2. Effect of Jas on thrombin-induced NF-κB activity. HUVEC were transfected with NF-κB-LUC construct as described under “Experimental Procedures.” Cells were pretreated with 1 μM Jas for 30–45 min prior to challenge with 5 units/ml thrombin (A) or 100 units/ml TNFα (B) for 6 h. Cell extracts were prepared and assayed for firefly and Renilla luciferase activities. Data are mean ± S.E. (n = 3 for each condition). *<i>p</i> < 0.005; #, different from thrombin-stimulated controls (<i>p</i> < 0.001).

![FIGURE 3. A and B, effects of stabilization and destabilization of actin filaments on thrombin-induced ICAM-1 mRNA expression. Confluent HUVEC monolayers were pretreated with 1 μM Lat B (A) or 1 μM Jas (B) and then challenged with 5 units/ml thrombin for 3 h. Total RNA was isolated and analyzed by reverse transcriptase-PCR for ICAM-1 mRNA expression. GAPDH mRNA expression was used as an internal control. Results are representative of two experiments. C and D, effects of stabilization and destabilization of actin filaments on thrombin-induced ICAM-1 protein expression. Confluent HUVEC monolayers were pretreated with the indicated concentrations of Cyto D (C) or Jas (D) and then challenged with 5 units/ml thrombin for 6 h. Total cell lysates were immunoblotted with an antibody to ICAM-1. Actin levels were used to monitor loading. The bar graphs represent the effect of 5 μM Cyto D (C) or 1 μM Jas (D) on thrombin-induced ICAM-1 protein expression. ICAM-1 protein expression normalized to actin level is expressed as -fold increase relative to the untreated control. Data are mean ± S.E. (n = 3–7 for each condition). *<i>p</i> < 0.05; #, different from thrombin-stimulated controls (<i>p</i> < 0.05).
thrombin-induced NF-κB-dependent reporter activity (Fig. 2A), but had only a small effect on the TNFα response (Fig. 2B).

Because NF-κB is an essential regulator of ICAM-1 transcrip- tion (21), we determined if the effects of stabilization/de- stabilization of the actin filaments on NF-κB activity are repro- duced on thrombin- and TNFα-induced ICAM-1 gene transcrip- tion in endothelial cells. Analysis by reverse transcriptase-PCR showed that destabilizing/stabilizing the actin filaments inhibited thrombin-induced ICAM-1 mRNA expression (Fig. 3, A and B). We also determined the effect of destabilizing/stabilizing the actin filaments on thrombin-induced ICAM-1 protein expression. Western blot analysis showed that thrombin challenge of HUVEC resulted in increased ICAM-1 protein expression and that this response was inhibited in cells pretreated with varying concentrations of Cyto D or Jas (Fig. 3, C and D). We observed that a low dose of 5 μM Cyto D or 1 μM Jas was sufficient to inhibit ICAM-1 protein expression by thrombin (Fig. 3, C and D). In contrast, destabilizing/stabilizing the actin filaments failed to inhibit TNFα-induced ICAM-1 expression even at higher concentrations of Cyto D (15 μM) and Jas (4 μM) (Fig. 4, A and B). The ineffectiveness of Jas and Cyto D in preventing TNFα-induced ICAM-1 expression is consistent with the findings of Vandenberg et al. (45).

Stabilization and Destabilization of the Actin Filaments Prevents Thrombin-induced NF-κB DNA Binding Activity Independent of IκBα Degradation—We next asked if stabilization or destabilization of the actin filaments inhibits thrombin-induced NF-κB activity by influencing the DNA binding of NF-κB. We first verified the identity of NF-κB complex activated by thrombin in endothelial cells. Electrophoretic mobility supershift assay showed that anti-RelA/p65 antibody produced a strong supershift (Fig. 5A), whereas anti-p50 antibody caused only a faint supershift (data not shown). These data confirm our previous observation that thrombin-induced NF-κB complexes are predominantly composed of RelA/p65 homodimer (21). Importantly, NF-κB complex activated by TNFα in endothelial cells is also predom- inantly composed of RelA/p65 homodimer (19). In subsequent experiments, we found that destabilizing and stabilizing the actin filaments both reduced the DNA binding of RelA/p65 in response to thrombin challenge (Fig. 5, B and C). In contrast, TNFα-induced NF-κB DNA binding was refractory to stabilizing or destabilizing the actin filaments (Fig. 5, D and E). To address the possibility that the impaired DNA binding of RelA/p65 was due to inhibition of IκBα degra- dation, we examined the effect of stabilization/destabilization of the actin filaments on IκBα degradation induced by thrombin. Contrary to
The impaired translocation of RelA/p65 to the nucleus following stabilization or destabilization of the actin cytoskeleton prompted us to examine whether actin associates with RelA/p65 to facilitate RelA/p65 nuclear translocation. We analyzed the immunoprecipitates of actin from control and thrombin-challenged cells to determine the association of actin with RelA/p65 in endothelial cells. Results showed a time-dependent association of actin with RelA/p65 with maximal association occurring at 1 h after thrombin challenge (Fig. 9A).

**FIGURE 5.** A, thrombin-induced NF-κB complex is predominantly composed of RelA/p65. Confluent HUVEC monolayers were left untreated (lane 1) or challenged with thrombin (5 units/ml) for 1 h (lanes 2 and 3). Nuclear extracts were incubated in the absence (lanes 1 and 2) or presence (lane 3) of an anti-RelA/p65 antibody at room temperature prior to addition of radiolabeled NF-κB probe. SS, supershift. Results are representative of two experiments.

B and C, effects of stabilization and destabilization of actin filaments on thrombin-induced DNA binding of RelA/p65. Confluent HUVEC monolayers were pretreated with 5 μM Cytodex or 1 μM Lat B (B) or 1 μM Jas (C) for 30 min prior to challenge with thrombin (5 units/ml) for 1 h. Nuclear extracts were prepared and assayed for DNA binding of RelA/p65 by electrophoretic mobility shift assay as described under “Experimental Procedures.” The bar graphs represent the effect of 5 μM Cytodex or 1 μM Lat B (B) or 1 μM Jas (C) on thrombin-induced DNA binding of RelA/p65. RelA/p65 DNA binding is expressed as -fold increase relative to the untreated control. Data are mean ± S.E. (n = 3–4 for each condition). *, different from controls (p < 0.05).

D and E, effects of stabilization and destabilization of actin filaments on TNF-α-induced IκBα degradation. Confluent HUVEC monolayers were pretreated with 5 μM Cytodex or 1 μM Lat B (D) or 1 μM Jas (E) for 30 min prior to challenge with 100 units/ml TNF-α for 1 h. Cytoplasmic extracts were separated by SDS-PAGE and immunoblotted with anti-IκBα antibody. Actin levels were used to monitor loading. The same extracts were re-electrophoresed and immunoblotted with an anti-histone H3 antibody to assess the purity of the cytoplasmic extracts. The bar graphs represent the effect of 5 μM Cytodex or 1 μM Lat B (D) or 1 μM Jas (E) on TNF-α-induced IκBα degradation. IκBα level normalized to actin level is expressed relative to the untreated control set at 1. Data are mean ± S.E. (n = 4–6 for each condition). *, different from controls (p < 0.05).
Actin Cytoskeleton and RelA/p65 Nuclear Transport

**DISCUSSION**

The present study implicates a novel role for the actin cytoskeleton in facilitating thrombin-induced nuclear transport of RelA/p65, and thereby promoting ICAM-1 expression in endothelial cells. We demonstrate that thrombin alters the actin cytoskeleton in endothelial cells and that interfering with these alterations, whether by stabilizing or destabilizing the actin filaments, prevents thrombin-induced NF-κB activation and ICAM-1 expression. The effects of preventing thrombin-induced changes in the actin cytoskeleton occur downstream of IkBα degradation, and are associated with impaired nuclear translocation of RelA/p65. We further show that thrombin induces the association of RelA/p65 with actin and that this interaction is sensitive to stabilization and destabilization of actin filaments. In parallel studies, TNFα fails to induce stress fiber formation comparable with thrombin and stabilization or destabilization of actin filaments fails to inhibit TNFα-induced RelA/p65 nuclear localization, and the resultant ICAM-1 expression. These data support the notion that specific alterations in the actin cytoskeleton as induced by thrombin in endothelial cells are necessary for RelA/p65 interaction with actin, RelA/p65 nuclear localization, and thereby expression of ICAM-1.

We employed two reciprocal approaches to address the role of actin cytoskeleton in the mechanism of thrombin-induced NF-κB activation and ICAM-1 expression in endothelial cells. These included stabilization and destabilization of the actin filaments to prevent the alterations in actin cytoskeleton that occur in response to thrombin challenge of endothelial cells. Destabilization of the actin filaments was achieved using Cyto D and Lat B, the classic actin-depolymerizing drugs with distinct modes of action (42, 43). Cyto D, a cell permeable fungal toxin, binds to the barbed end of actin filaments, which inhibits both the association and dissociation of subunits at that end, and thus causes the disruption of actin filaments and inhibition of actin polymerization (42). Lat B, a marine toxin isolated from the Red Sea sponge Latrunculia magnifica, associates only with actin monomers in a 1:1 ratio, thereby preventing them from

reciprocal experiments, the immunoprecipitates of RelA/p65 also showed the interaction of actin with RelA/p65 in cells challenged with thrombin (Fig. 9B). In control IgG immunoprecipitates, we failed to detect the presence of RelA/p65 or actin (Fig. 9, A and B), indicating the specificity of the interaction. These results are consistent with a previous report (46) showing the interaction of RelA/p65 with actin containing structures. We also determined whether interfering with thrombin-induced alterations in the actin cytoskeleton by stabilizing or destabilizing the actin filaments influences the association of RelA/p65 with actin. Pretreatment of cells with Cyto D, Lat B, or Jas prevented thrombin-induced association of actin with RelA/p65 (Fig. 9B).

**FIGURE 7.** A and B, effects of stabilization and destabilization of actin filaments on thrombin-induced nuclear translocation of RelA/p65. Confluent HUVEC monolayers were pretreated with 1 μM Lat B or 5 μM Cyto D (A) or 1 μM Jas (B) for 30 min prior to challenge with 5 units/ml thrombin for 1 h. Nuclear extracts were separated by SDS-PAGE and immunoblotted with anti-RelA/p65 antibody. The same extracts were re-electrophoresed and immunoblotted for histone H3, a nuclear marker, to monitor loading. The bar graphs represent the effect of 5 μM Cyto D or 1 μM Lat B (A) or 1 μM Jas (B) on thrombin-induced nuclear accumulation of RelA/p65. RelA/p65 nuclear translocation is expressed as fold increase relative to the untreated control. Data are mean ± S.E. (n = 3–4 for each condition). *, different from controls (p < 0.05); #, different from thrombin-stimulated controls (p < 0.05). C and D, effects of stabilization and destabilization of actin filaments on TNFα-induced nuclear translocation of RelA/p65. Confluent HUVEC monolayers were pretreated with 1 μM Lat B (C) or 1 μM Jas (D) for 30 min prior to challenge with 100 units/ml TNFα for 1 h. Nuclear extracts were separated by SDS-PAGE and immunoblotted with anti-RelA/p65 antibody. The same extracts were re-electrophoresed and immunoblotted for histone H3, a nuclear marker, to monitor loading. The bar graphs represent the effect of 5 μM Cyto D (A) or 1 μM Jas (B) on TNFα-induced nuclear translocation of RelA/p65. RelA/p65 nuclear translocation is expressed as fold increase relative to the untreated control. Data are mean ± S.E. (n = 3–4 for each condition).
Using these agents, we found that disruption of thrombin-induced actin filaments resulted in a marked inhibition of NF-H9260B-dependent reporter activity and ICAM-1 expression. These findings prompted us to investigate if stabilizing the actin cytoskeleton by Jas, a macrocyclic peptide isolated from the marine sponge Jaspis johnstoni (44), influences thrombin-induced NF-H9260B activity and ICAM-1 expression. Intriguingly, stabilizing the actin cytoskeleton by this approach also inhibited NF-H9260B-dependent reporter activity and ICAM-1 expression by thrombin. Considered together, these data raise the possibility that specific reorganization of the actin cytoskeleton as induced by thrombin in endothelial cells is essential for NF-H9260B activation and ICAM-1 expression.

**FIGURE 8.** A, effects of Lat B and Jas on thrombin-induced nuclear accumulation of RelA/p65. Confluent HUVEC monolayers grown on coverslips were left untreated (a) or challenged for 1 h with 5 units/ml thrombin in the absence (b) and presence of 1 μM Lat B (c) or 1 μM Jas (d). Cells were then fixed, permeabilized, and stained with anti-RelA/p65 antibody and a secondary antibody conjugated with Texas Red as described under “Experimental Procedures.” DNA was stained using Hoechst Dye to visualize the nucleus. Coverslips were mounted on the slide and analyzed by fluorescence microscopy. Results are representative of three experiments. B, effects of Lat B and Jas on TNF-α nuclear accumulation of RelA/p65. Confluent HUVEC monolayers grown on coverslips were left untreated (a) or challenged for 1 h with 100 units/ml TNF-α-induced in the absence (b) and presence of 1 μM Lat B (c) or 1 μM Jas (d). Cells were then fixed, permeabilized, and stained with anti-RelA/p65 antibody and a secondary antibody conjugated with Texas Red as described under “Experimental Procedures.” DNA was stained using Hoechst Dye to visualize the nucleus. Coverslips were mounted on the slide and analyzed by fluorescence microscopy. Results are representative of two experiments.

**FIGURE 9.** A, thrombin induces association of actin with RelA/p65. Confluent HUVEC monolayers were challenged with 5 units/ml thrombin for the indicated times. Total cell lysates were subjected to immunoprecipitation (IP) with an antibody to actin or IgG. The immunoprecipitates were then immunoblotted with an antibody to RelA/p65 or actin. TL, total lysate. The bar graph represents the association of actin with RelA/p65 at 1 h after thrombin challenge. RelA/p65-actin association is expressed as -fold increase relative to the untreated control. Data are mean ± S.E. (n = 3 for each condition); *, different from controls (p < 0.05). B, stabilization or destabilization of actin filaments prevents thrombin-induced association of actin with RelA/p65. Confluent HUVEC monolayers were pretreated with 1 μM Lat B or Jas for 45 min prior to challenge with 5 units/ml thrombin for 1 h. Total cell lysates were immunoprecipitated with an antibody to RelA/p65 or IgG as indicated. The corresponding bar graph represents the effects of Lat B and Jas on thrombin-induced association of actin with RelA/p65 at 1 h after thrombin challenge. RelA/p65-actin association is expressed as -fold increase relative to the untreated control. Data are mean ± S.E. (n = 3–4 for each condition); *, different from controls (p < 0.05); #, different from thrombin-stimulated controls (p < 0.05).
Actin Cytoskeleton and RelA/p65 Nuclear Transport

actin cytoskeleton by the approaches described above had no effect on TNFα-induced NF-κB activation and ICAM-1 expression, further support this possibility. These results also indicate that the inhibitory effects of Cyto D, Lat B, or Jas on thrombin responses cannot be ascribed to possible toxic effects of the drugs.

Interestingly, we noted that whereas destabilizing the actin cytoskeleton had no effect, stabilizing the actin cytoskeleton by Jas slightly reduced TNFα-induced NF-κB activity. Given that Jas was ineffective even at higher concentrations in preventing ICAM-1 expression (Fig. 4B), the slight inhibition of TNFα-induced NF-κB activity is not likely due to a low concentration (1 μM) of Jas used (Fig. 2B). One possible explanation for this discrepancy could be the less efficient shutting of the NF-κB-LUC plasmid to the nucleus as stabilizing the actin cytoskeleton is implicated in hindering the translocation of plasmid toward nucleus (47).

Analysis of NF-κB signaling pathway revealed that stabilizing or destabilizing the actin filaments did not prevent thrombin-induced IkBα degradation, a requirement for RelA/p65 translocation to the nucleus (5, 48). The lack of effect of Cyto D, Lat B, or Jas on IkBα degradation excludes the possibility that these drugs exert their inhibitory effect on NF-κB activation by interfering with thrombin activation of its receptor, protease-activated receptor-1 (PAR-1). Despite the normal release of RelA/p65 from IkBα in the cytoplasm, we observed a marked decrease in thrombin-induced NF-κB binding to DNA in the nucleus after stabilization or destabilization of the actin filaments. The reduced nuclear binding of NF-κB in the face of IkBα degradation pointed to a possible impairment in the nuclear accumulation of the released RelA/p65. Indeed, we found that stabilizing or destabilizing the actin filaments interfered with thrombin-induced nuclear transport of the released RelA/p65. In contrast, stabilization or destabilization of the actin filaments failed to prevent nuclear translocation of RelA/p65 induced by TNFα. Taken together, these data underscore the importance of actin dynamics in facilitating RelA/p65 nuclear transport following thrombin challenge of endothelial cells.

These findings led us to investigate whether thrombin induces nuclear uptake of RelA/p65 by promoting association of RelA/p65 with actin and whether this interaction is disrupted by stabilization and destabilization of the actin filaments. Co-immunoprecipitation studies showed that thrombin induced the association of RelA/p65 with actin in a time-dependent manner. Interestingly, the time course of RelA/p65-actin interaction paralleled the time course of IkBα phosphorylation/degradation as well as RelA/p65 nuclear localization (36) by thrombin. Thus, it is likely that the release of RelA/p65 from IkBα is a prerequisite for its association with actin; however, this remains to be addressed. We further observed that stabilizing or destabilizing the actin filaments prevented the association of RelA/p65 with actin, and impaired the translocation of RelA/p65 to the nucleus. Collectively, these data support a role of actin-RelA/p65 interaction in facilitating thrombin-induced RelA/p65 nuclear translocation in endothelial cells.

Our findings are consistent with previous reports describing a crucial role of actin dynamics in controlling the nuclear accumulation of MAL, the myocardin-related coactivator of serum response factor (26). However, several lines of evidence suggest that the mechanisms by which actin dynamics control serum-induced MAL or thrombin-induced RelA/p65 nuclear localization are different. For example, MAL is constitutively associated with G-actin in the cytoplasm and this association is disrupted upon actin polymerization induced by serum stimulation. Thus, the signals and agents that deplete G-actin by inducing F-actin formation promote MAL nuclear accumulation, whereas inhibition of actin polymerization prevents MAL translocation to the nucleus and consequently, serum response factor activity (26, 27, 49). Unlike MAL, the association of RelA/p65 with actin is contingent upon specific alterations in actin dynamics as induced by thrombin, and the agents that interfere with these alterations prevent RelA/p65 distribution from the cytoplasm to the nucleus. Another example supporting this concept is provided by the role of microtubule dynamics in the nuclear accumulation of the tumor suppressor protein p53 (50).

The agonist-specific effects of actin dynamics on RelA/p65 nuclear translocation and ICAM-1 expression is consistent with the selective regulation of thrombin-induced NF-κB activation and the resultant ICAM-1 expression in endothelial cells by the RhoA/ROCK pathway (33). We showed that thrombin activation of the RhoA/ROCK pathway 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 IkBα (33). Given that the RhoA/ROCK pathway is also implicated in thrombin-induced changes in actin dynamics (29–32) and that these changes are associated with nuclear translocation of RelA/p65 downstream of IkBα degradation, our findings are consistent with a model wherein the RhoA/ROCK pathway regulates thrombin-induced NF-κB activation and ICAM-1 expression by a dual mechanism involving IKK-dependent release and actin-dependent transport of RelA/p65 to the nucleus (Fig. 10). Such a model finds further support from our observation that destabilizing the actin prevents NF-κB activity induced by constitutively active RhoA. It is, however, not clear how RelA/p65-actin interaction promotes RelA/p65 nuclear accumulation. Intriguingly, we have found that inhibition of myosin light chain kinase selectively prevents thrombin-induced ICAM-1 expression in endothelial cells.3 These data raise the possibility of actin-myosin interaction mediating thrombin-induced RelA/p65 translocation and ICAM-1 expression in endothelial cells; however, additional studies are required to address this possibility.

The lack of effect of stabilizing/destabilizing the actin filaments on TNFα-induced RelA/p65 nuclear translocation and ICAM-1 expression raises the possibility that RelA/p65 liberated by TNFα may interact with other cytoplasmic structures

3 F. Fazal, unpublished observation.
Actin Cytoskeleton and RelA/p65 Nuclear Transport

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Actin Cytoskeleton and RelA/p65 Nuclear Transport