Suppression of RhoA Activity by Focal Adhesion Kinase-induced Activation of p190RhoGAP

ROLE IN REGULATION OF ENDOTHELIAL PERMEABILITY*

The JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 4, pp. 2296 – 2305, January 27, 2006
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The interaction of endothelial cells with extracellular matrix proteins at focal adhesions sites contributes to the integrity of vascular endothelial barrier. Although focal adhesion kinase (FAK) activation is required for the recovery of the barrier function after increased endothelial junctional permeability, the basis for the recovery remains unclear. We tested the hypothesis that FAK activates p190RhoGAP and, thus, negatively regulates RhoA activity and promotes endothelial barrier restoration in response to the permeability-increasing mediator thrombin. We observed that thrombin caused a transient activation of RhoA but a more prolonged FAK activation temporally coupled to the recovery of barrier function. Thrombin also induced tyrosine phosphorylation of p190RhoGAP, which coincided with decrease in RhoA activity. We further showed that FAK was associated with p190RhoGAP, and importantly, recombinant FAK phosphorylated p190RhoGAP in vitro. Inhibition of FAK by adenoviral expression of FRNK (a dominant negative FAK construct) in monolayers prevented p190RhoGAP phosphorylation, increased RhoA activity, induced actin stress fiber formation, and produced an irreversible increase in endothelial permeability in response to thrombin. We also observed that p190RhoGAP was unable to attenuate RhoA activation in the absence of FAK activation induced by FRNK. The inhibition of RhoA by the C3 toxin (Clostridium botulinum toxin) restored endothelial barrier function in the FRNK-expressing cells. These findings in endothelial cells were recapitulated in the lung microcirculation in which FRNK expression in microvessel endothelia increased vascular permeability. Our studies demonstrate that FAK-induced down-modulation of RhoA activity via p190RhoGAP is a crucial step in signaling endothelial barrier restoration after increased endothelial permeability.

The vascular endothelial barrier, consisting of both the endothelial cell monolayer and underlying extracellular matrix (ECM) constituents to which endothelial cells adhere, is the key determinant of the transvascular exchange of liquid and plasma proteins (1–3). Endothelial cells contract within minutes in response to mediators increasing vascular permeability such as thrombin (4–6). This is followed by recovery during the next 2 h when the cells restore their shape and endothelial barrier function is re-established (4–6). Endothelial cell contracture depends on the ability of the cell to produce the actin-myosin-generated contractile force required for the formation of minute intercellular gaps (2, 3). RhoA was shown to be important in the engagement of the endothelial cell contractile machinery (7–10). Thrombin-induced activation of RhoA stimulated Rho kinase, in turn increasing myosin light chain phosphorylation, thus increasing myosin light chain phosphorylation (8, 11, 12). Although elements of the signaling pathways mediating increased endothelial permeability are better understood, the basis of the recovery of endothelial barrier function remains unclear.

Integrin receptors present in focal adhesion sites are responsible for the adhesive interactions of the endothelial cells with the ECM (1, 13). Focal adhesion kinase (FAK), a non-receptor protein-tyrosine kinase found in focal adhesions, mediates the formation of the focal adhesion complex (14). FAK was shown to bind to and phosphorylate multiple proteins such as paxillin and p130CAS upon activation of integrin receptors and thereby induced their translocation to focal adhesion sites (14). Permeability-increasing mediators thrombin, hydrogen peroxide, and vascular endothelial growth factor resulted in the phosphorylation and recruitment of FAK to focal adhesions (6, 15, 16), suggesting a role of FAK in the regulation of endothelial permeability (6, 7, 15). We showed that depletion of FAK in endothelial cells impaired the recovery of endothelial barrier function after thrombin challenge (6), suggesting the involvement of FAK in barrier restoration. Other studies showed that FAK was required for strengthening of the endothelial barrier in response to hyperosmolar stress (17), further supporting the role of FAK in maintaining endothelial barrier function. Because deletion of FAK in fibroblasts results in the activation of RhoA (18, 19), a requirement for increasing endothelial permeability (7–10), we surmised that a potentially important mechanism of FAK restoration of endothelial barrier function could involve the modulation of RhoA activity by FAK activation.

The GTPase-activating proteins (GAPs) stimulate the intrinsic GTPase activity on RhoGTPases and thus "switch off" the Rho cycle (20). p190RhoGAP was shown to inhibit preferentially RhoA activity (21). Studies showed that phosphorylation of p190RhoGAP on tyrosine residues (22–25) increased its GAP activity on RhoA (22–25). p190RhoGAP also associated with FAK (26), raising the possibility that FAK by phosphorylating p190RhoGAP could modulate RhoA activity and thereby influence endothelial permeability. Therefore, in the present study we addressed the role of FAK modulation of RhoA activity via p190RhoGAP in signaling endothelial barrier restoration. We used the FAK-related non-kinase (FRNK) construct, which competes with FAK for binding to focal adhesion sites and negatively regulates FAK-activated signaling pathways (27–31). We show herein that thrombin tran-
siently activated RhoA but induced a prolong phosphorylation of FAK that was temporally coupled to the recovery of barrier function. We also showed that thrombin induced the tyrosine phosphorylation of p190RhoGAP in association with decreased RhoA activity. We further showed that FAK associates with p190RhoGAP and directly phosphorylates it in vitro. Impairment of FAK function decreased p190RhoGAP phosphorylation and increased RhoA activity as well as actin stress fiber formation, which in turn resulted in increased endothelial permeability. We observed that p190RhoGAP was unable to attenuate RhoA activation in the absence of FAK activation. Thus, the results demonstrate the key role of FAK activation as a negative feedback mechanism that maintains endothelial barrier function by p190RhoGAP-mediated suppression of RhoA activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human α-thrombin was purchased from Enzyme Research Laboratories (South Bend, IN). Human pulmonary arterial endothelial (HPAE) cells and endothelial growth medium 2 were purchased from Clonetics (San Diego, CA). Trypsin was purchased from Invitrogen. FAK antibodies (Abs) were raised against a C terminus or N-terminal peptide, anti-RhoA Ab, anti-GFP Ab, anti-PY20, anti-PY99, anti-PY350, normal goat IgG, and protein A/G beads were purchased from Santa Cruz Biotechnology (San Diego, CA). Anti-phospho FAK (PY397 and PY576), anti-phospho-paxillin (PY31 and PY118), anti-Cdc42, anti-Rac, anti-p190RhoGAP, anti-paxillin Abs, and recombinant FAK were purchased from BIOSOURCE (Camarillo, CA). Alexa-Fluor 568 phalloidin and Prolong Antifade kit were purchased from Molecular Probes (Eugene, OR). Electrodes for transendothelial electrical resistance measurements were purchased from Applied Biophysics (Troy, NY). Rho activation kit containing GST-robekin-Rho binding domain beads was purchased from Cytokeleton (Denver, CO).

**Endothelial Cell Culture**—HPAE cells were cultured in a T-75 flask coated with 0.1% gelatin in endothelial growth medium 2 supplemented with 10% fetal bovine serum. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO2 and 95% air until they formed a confluent monolayer. Cells from each of the primary flasks were detached with 0.025% Trypsin/EDTA and plated on either 100 mm dishes for immunoprecipitation analysis, 8-well electrodes for electrical impedance measurements or on 12 mm coverslips for immunofluorescence microscopy as described below. In all experiments, the monolayer of HPAE was incubated for 1–2 h in MCDB 131 serum-free medium before treatment with inhibitors or agonists. In all experiments, cells were used between passages 4–8.

**Adenoviral Infection**—Green Fluorescent Protein (Ad-GFP) or GFP-tagged FRNK (Ad-GFP-FRNK) adenoviral constructs were generated as previously described (27). Cells grown to confluence in 100 mm dishes, 12 mm coverslips or ECIS electrodes were infected with 50 multiplicity of infection of Ad-GFP-FRNK or Ad-GFP overnight in serum containing medium, after which the media was replaced with fresh media with no virus. In all experiments, cells were used between 32 and 36 h post-virus infection.

**Immunoblotting**—Cells infected with Ad constructs were serum-deprived after which they were stimulated with 50 nM α-thrombin for the indicated times. Cell lysates were then electrophoresed on a polyacrylamide gel and transferred to nitrocellulose membranes for Western blotting with anti-phospho 576 FAK or anti-phospho-397 FAK or FAK Abs to determine FAK phosphorylation.

**Phosphorylation of p190RhoGAP**—Phosphorylation of p190RhoGAP was determined as described before (6). Briefly, HPAE cells were lysed after stimulation using radioimmunoprecipitation assay buffer (1% Triton-X, 150 mM NaCl, 10 mM Tris, 1 mM EDTA, 1 mM EGTA, 2 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 0.5% Nonidet P-40, and 2 μg/ml each of pepstatin A, leupeptin, and aprotinin) and centrifuged. The cleared lysate was incubated with anti-p190RhoGAP Abs for 2 h followed by the addition of protein A/G plus agarose beads overnight. The beads were then collected by centrifugation and washed three times with detergent-free radioimmunoprecipitation assay buffer. Protein from each sample was eluted by boiling the beads in SDS sample buffer, electrophoresed on 7.5% SDS-polyacrylamide gels, and transferred to nitrocellulose for analysis of p190RhoGAP phosphorylation using a mixture of PY20, PY99, and PY350 phosphoryrosine antibodies and protein content by Western blotting with p190RhoGAP antibody.

**Co-immunoprecipitation of FAK and p190RhoGAP**—Human pulmonary artery endothelial cells grown in 100-mm dishes were lysed and processed to assess the interaction of FAK with p190RhoGAP as described (32). Briefly, lysates containing an equal amount of protein were incubated with anti-rabbit or mouse IgG or incubated with anti-mouse p190RhoGAP Ab or anti-rabbit polyclonal FAK Abs for 3–4 h followed by the addition of protein A-agarose beads overnight at 4 °C. Beads were collected by centrifugation and washed 3× with lysis buffer without detergents after which proteins were eluted from the beads by boiling the samples suspended in Laemml sample buffer. Each sample was then electrophoresed on 7.5% SDS-PAGE gels and transferred to nitrocellulose for Western blotting with FAK or p190RhoGAP Abs.

**Measurement of GTPase Activity**—RhoA activity was measured using GST-robekin-Rho binding domain that specifically pulls down activated RhoA (9). Rac or Cdc42 activity was determined using GST-P21-activated kinase binding domain (33). Samples were electrophoresed on 12.5% SDS-polyacrylamide gel, transferred to nitrocellulose, and analyzed by Western blotting using the indicated Abs.

**Actin Stress Fiber Alterations and Focal Adhesion Formation**—After infection with Ad constructs, cells were serum-deprived. Cells were then stimulated with 50 nM thrombin for indicated times, rinsed quickly with ice cold Hanks’ balanced salt solution, and fixed with 2% paraformaldehyde. Cells were then labeled with anti-FAK Ab (N-terminal) or stained with phalloidin as previously described (6).

**In Vitro Phosphorylation of p190RhoGAP by FAK**—HPAE cells were lysed using radioimmunoprecipitation assay buffer as described above and centrifuged. The cleared lysate was incubated with anti-p190RhoGAP Ab or anti-mouse IgG for 2 h followed by the addition of protein A/G plus-agarose beads overnight. Immunocomplexes were then washed 2× with radioimmunoprecipitation assay buffer and once with kinase buffer (50 mM Tris, pH 7.4, 10 mM MnCl2, 1 mM dithiothreitol). Beads were then incubated in the kinase buffer with the addition of 8 mM ATP and recombinant FAK for 30 min at 30 °C. The kinase reactions were then stopped by the addition of SDS sample buffer. After boiling for 5 min, the samples were resolved on SDS-PAGE and analyzed by Western blotting using indicated Abs.

**Reporter Gene Constructs, Endothelial Cell Transfection, and Luciferase Assay**—We showed that thrombin-induced generation of serum response elements (SRE) requires RhoA activity (9). We, therefore, used SRE reporter gene activity to address the role of p190RhoGAP in regulating FRNK-induced RhoA activity. Transfections were performed using the DEAE-dextran method as described (9). Briefly, DNA (5 μg total) was mixed with 50 μg/ml DEAE-dextran in serum-free MCDB131 medium, and the mixture was added onto confluent cells. pTKRUC plasmid (0.1 μg) (Promega Corp., Madison, WI) containing Renilla luciferase gene driven by the constitutively active thymidine kinase promoter was added to normalize the transfection efficiencies. After 1 h cells were incubated for 4 min with 10% dimethyl sulfoxide...
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(Me2SO) in serum-free medium, washed twice with endothelial growth medium-containing 10% fetal bovine serum, and grown to confluence. After 6 h of stimulation with 50 nM α-thrombin, cell extracts were prepared and assayed for luciferase activity using the Dual Luciferase Reporter Assay system (Promega). SRE-luciferase activity was expressed as the ratio of firefly and Renilla luciferase activity.

Transendothelial Electrical Resistance—The time course of endothelial cell retraction, a measure of increased endothelial permeability, was assessed according to described procedures (4).

Microvessel Permeability Studies in Mouse Lung—Cationic liposomes were prepared using the method described by us (34). The DNA-liposome complex (50 µg of plasmid DNA/100 µl of liposome mixture) was slowly injected into the right jugular vein of male CD1 mice followed by ligation of the vein and closure of the neck using sutures. Forty-eight hours after injection of pCMV-vector or pCMV-GFP-FRNK cDNA, we measured microvessel permeability in the lung by determining microvascular filtration coefficient \( K_{f,c} \) and isogravimetric lung water determinations as described (35). Briefly, according to an approved protocol of the University of Illinois at Chicago Animal Care Committee, male mice weighing 25–30 g were anesthetized with a combination of 10 mg/ml ketamine, 0.25 mg/ml xylazine, and 0.25 mg/ml acepromazine, and isolated mouse lungs were prepared and perfused as described (35). All lung preparations underwent a 20-min equilibration perfusion, and lungs that were not isogravimetric at the end of this period were discarded. After a 20-min equilibration perfusion, outflow pressure was elevated by 10 cm of H2O for 2 min. The lung wet weight increase over this time, which reflects the net fluid accumulation, was continuously recorded. At the end of each experiment, lung dry weight was determined.

Statistical Analysis—Comparison between experimental groups was made using analysis of variance and Bonferroni correction t test program using statistical software. Differences in mean values were considered significant at \( p < 0.05 \).

RESULTS

GFP-FRNK Expression Inhibits FAK Phosphorylation and Its Translocation to Focal Adhesions—We determined the effects of FRNK expression on FAK function in endothelial cells by measuring FAK phosphorylation and translocation to focal adhesions. HPAE cells infected with adenoviral-GFP-FRNK (Ad-GFP-FRNK) or control vector (Ad-GFP) were left unstimulated or stimulated with thrombin for the indicated times. Translocation of FAK to focal adhesions was examined by confocal microscopy after immunostaining HPAE cells with Ab specific for the N terminus of FAK. Because tyrosine phosphorylation at Tyr-397 and Tyr-576 sites primarily regulates HPAE cells with Ab specific for the N terminus of FAK. Because tyrosine phosphorylation at Tyr-397 and Tyr-576 sites primarily regulates FAK activation (36, 37), we used site-specific phosphotyrosine Abs to determine FAK phosphorylation.

Fig. 1A shows the expression of FRNK in endothelial cells infected with Ad-GFP-FRNK. Western blotting with GFP antibody recognized GFP-FRNK as a 67-kDa protein in cells, whereas GFP protein (29 kDa) was recognized in the Ad-GFP-infected cells. We found that FAK was basally phosphorylated at Tyr-397 and Tyr-576 (Fig. 1B). Thrombin exposure of endothelial cells further increased the phosphorylation of FAK at both sites, which attained its maximum level at 10 min and remained phosphorylated up to 2 h (Fig. 1, B–C). Overexpression of FRNK reduced the basal phosphorylation of FAK at both sites, and it did not increase further after stimulation of cells with thrombin (Fig. 1, B–C). The reductions in basal and thrombin-induced FAK phosphorylation were not due to a decrease in the total amount of FAK in GFP-FRNK-expressing cells because endogenous levels of FAK were similar in Ad-GFP- and Ad-GFP-FRNK-infected cells. Thrombin also stimulated the translocation of FAK to focal adhesions, which appeared as dense puncta in the Ad-GFP-infected cells (Fig. 1D, upper panel). However, FAK failed to translocate to focal adhesions in response to thrombin in the FRNK-expressing cells (Fig. 1D, lower panel). FRNK-infected cells demonstrated numerous puncta, without co-localizing with FAK (Fig. 1E). These findings indicate that FRNK expression inhibited FAK phosphorylation and displaced the endogenous FAK from focal adhesion. Because FRNK phosphorylates paxillin at sites Tyr-31 and Tyr-118 (38), we next determined the effect of FRNK on paxillin phosphorylation at these sites using phosphorylation site-specific Abs. In Ad-GFP-infected cells, paxillin was basally phosphorylated, and phosphorylation further increased after thrombin stimulation (Fig. 1F). However, the basal phosphorylation of paxillin was reduced in Ad-GFP-FRNK-infected cells, and this did not increase in response to thrombin (Fig. 1F).

The endogenous paxillin levels were similar in the GFP- or GFP-FRNK-expressing cells. Together, these findings demonstrate that FRNK inhibited the FAK catalytic activity.

FRNK Expression Increases Permeability of Endothelial Monolayers and Lung Microvessels—We next determined the effects of FRNK on endothelial barrier function in naïve endothelium and after challenge with thrombin. Endothelial barrier function was determined by changes in transendothelial electrical resistance (TER) in monolayers after 1 h of infection with Ad-GFP-FRNK or Ad-GFP without or with thrombin stimulation (Fig. 2). Endothelial monolayers with comparable basal TER values (~4.0 ohms/cm²) were infected with GFP or FRNK adenovirus (Fig. 2A). After infection, a significant decrease in endothelial barrier function was evident at 16 h post-adenoviral infection in the cells expressing Ad-GFP-FRNK, whereas TER remained stable in cells treated with Ad-GFP (Fig. 2A). In another study we determined the effect of FAK inhibition on the recovery of the thrombin-induced increase in endothelial permeability. Before thrombin application, absolute TER values post-34 h of infection in Ad-GFP-infected cells was 4.2 ± 0.75 ohms/cm², whereas it decreased to 2.2 ± 0.24 ohms/cm² in Ad-GFP-FRNK-infected cells. TER values were then normalized to examine the effects thrombin challenge. As shown in Fig. 2B, thrombin decreased TER in Ad-GFP-infected cells, which fully recovered within an hour. Thrombin also decreased TER to the same extent within 10 min in the FRNK-expressing cells, but in contrast to control cells, this response was not restored in 2 h (Fig. 2B). We also determined the temporal relationship between endothelial barrier recovery and changes in FAK phosphorylation in the GFP- or GFP-FRNK-expressing cells (Fig. 3). FAK remained phosphorylated at Tyr-397 as well as Tyr-576 for the duration of recovery period in the GFP-infected cells (Fig. 3A). Moreover, the increase in FAK phosphorylation was coupled to the recovery of endothelial barrier function (Fig. 3A). However, both these responses were markedly reduced in the FRNK-expressing cells (Fig. 3B). These findings demonstrate the requirement of FAK activation in maintaining basal endothelial barrier function and, importantly, in promoting the recovery of endothelial barrier function after thrombin challenge.

Because endothelial permeability in monolayers may not always reflect vessel wall permeability in the intact microcirculation (39), in another series of experiments we determined the role of FAK in regulating endothelial barrier integrity in pulmonary microvessels (Fig. 4). We measured changes in wet weight and microvessel liquid permeabil-
ity \((K_{f,c})\) in perfused mouse lungs isolated after 48 h injection with cationic liposomes either conjugated with control vector (CMV cDNA) or GFP-FRNK cDNA. Expression of FRNK in lungs was detected by Western blotting with anti-rabbit GFP Ab. We observed a 67-kDa band indicative of GFP-FRNK in the mouse lungs injected with GFP-FRNK cDNA (Fig. 4, inset). FRNK-expressing lungs demonstrated a significant increase in \(K_{f,c}\) and gained water as compared with lungs expressing the vector alone (Fig. 4, A–B). There were no significant differences in pulmonary arterial pressure changes between the various groups (data not shown). The inhibition of FAK by FRNK disrupted normal barrier function seen in the intact microvasculature is consistent with the endothelial monolayer studies.

Expression of FRNK Activates RhoA—Based on the finding that increased RhoA activity is crucially involved in increasing endothelial permeability \((3, 9, 10)\), we addressed the possibility that activation of RhoA is responsible for disruption of endothelial barrier in the FRNK-expressing endothelial cells. In addition, we determined the effects of FRNK on activation of other monomeric RhoGTPases, Rac, and Cdc42. We used the GST pull-down assay to determine Rho, Rac, and Cdc42 activities. As shown in Fig. 5, in Ad-GFP-infected cells, RhoA activity
was barely detectable in unstimulated cells. Thrombin induced a transient activation of RhoA in GFP-expressing cells. However, the loss of FAK function by FRNK resulted in an increase in basal RhoA activity, and the response was sustained after thrombin stimulation (Fig. 5A). Overexpression of FRNK had no effect on endogenous levels of RhoA, as the total amount of RhoA was similar in GFP- or GFP-FRNK-expressing cells. FRNK also had no effect in increasing the basal activities of either Rac or Cdc42 (Fig. 5, B–C).

Expression of FRNK Induces Actin Stress Fiber Formation—Because Rho activation promotes gap formation between endothelial cells by forming actin stress fibers, we determined alterations in actin stress fibers in the GFP- or FRNK-expressing endothelial cells. Cells infected

FIGURE 2. Effect of expression of FRNK on endothelial permeability. Cells plated on gold electrodes were infected with either Ad-GFP or Ad-GFP-FRNK to measure the time course of changes in TER across naïve endothelium (A) or after the addition of 50 nM thrombin post-34-h infection (B). Data represent means ± S.E. of changes in TER from multiple experiments. In B, the effects of FAK inhibition on endothelial barrier recovery post-thrombin challenge were examined after normalization of TER values post-34-h GFP or GFP-FRNK infection. TER values are calculated as change in resistance over value at time 0 (n = 7). *, values different from Ad-GFP transfected monolayer (p < 0.05).

FIGURE 3. Relationship between FAK phosphorylation and endothelial barrier recovery in GFP- and GFP-FRNK-expressing cells. Thrombin-induced FAK phosphorylation shown in Fig. 1C was recalculated as % increase over value at time 0 and plotted against recovery of endothelial barrier function after thrombin challenge shown in Fig. 2B. TER recovery was calculated as % increase in resistance over the value that caused maximum decrease in resistance after thrombin challenge under each condition. The top panel shows data from GFP-expressing cells, whereas the bottom panel shows data from FRNK-expressing cells.
with GFP or GFP-FRNK were left unstimulated or stimulated with thrombin. Cells were then fixed and stained with phalloidin, and formation of actin stress fibers was determined by confocal imaging. As shown in Fig. 6, the unstimulated GFP-expressing cells had fewer actin stress fibers that were increased after stimulation with thrombin (Fig. 6, left panel). However, stress fibers were markedly increased in the GFP-FRNK-expressing cells without thrombin stimulation (Fig. 6, right panel). Numerous gaps between cells were also found in the FRNK-expressing cells (Fig. 6, right panel).

Inhibition of RhoA Reverses the FRNK-induced Increase in Endothelial Permeability—To determine whether increased activation of RhoA is causally related to the increased endothelial permeability in FRNK-expressing cells, we used the C3 toxin to inhibit RhoA function. After 5 h of FRNK infection cells were left untreated or treated with 20 μg/ml C3 toxin. Changes in TER were measured in unstimulated cells, after which they were exposed to thrombin (Fig. 7, A–B). Inhibition of RhoA prevented the FRNK-induced increase in endothelial permeability in naïve endothelium, and furthermore, it restored the recovery of barrier function after thrombin stimulation (Fig. 7, A–B).

Thrombin Induces p190RhoGAP Phosphorylation via FAK—Because p190RhoGAP can inhibit RhoA activity (21) and p190RhoGAP is phosphorylated on tyrosine residues (22–25) and associates with FAK (26), we addressed the possibility that FAK activation of p190RhoGAP regulates RhoA activity. We first determined whether thrombin phosphorylates p190RhoGAP. HPAE cells were stimulated with thrombin for the indicated times and lysed. Lysates from lungs were immunoblotted with anti-GFP Ab to detect expression of FRNK in lungs. Lysates were also immunoblotted with anti-FAK Ab to control for protein loading (bottom). GFP-Ab detected a 67-kDa band in GFP-FRNK-transfected lungs. *, values different from CMV-transfected microvessels (p < 0.05) (n = 7).
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thrombin resulted in increased p190RhoGAP phosphorylation at 10 min, which stayed elevated for 30 min (Fig. 8A). The time course of p190RhoGAP phosphorylation coincided with a decrease in RhoA activity (Fig. 5A, left panel).

To determine whether thrombin-induced phosphorylation of p190RhoGAP requires FAK, Ad-GFP- or Ad-GFP-FRNK-infected HPAE cells were stimulated without or with thrombin for 30 min. p190RhoGAP was immunoprecipitated from cell lysates to determine the tyrosine phosphorylation of p190RhoGAP. Fig. 8B shows that inhibition of FAK inhibited the phosphorylation of p190RhoGAP, indicating the requirement of FAK in regulating thrombin-induced p190RhoGAP phosphorylation.

We next addressed the possibilities that FAK associates with p190RhoGAP and that FAK phosphorylates p190RhoGAP in vitro. Using a co-immunoprecipitation assay, we observed that p190RhoGAP interacts with FAK (Fig. 8C). To determine that p190RhoGAP is a substrate for FAK, lysates from endothelial cells were immunoprecipitated with control IgG or p190RhoGAP Ab, and these immunocomplexes were used for in vitro kinase assay. We show that recombinant FAK phosphorylated the p190RhoGAP immunoprecipitated from endothelial cells (Fig. 8D). However, p190RhoGAP was not phosphorylated in the absence of recombinant FAK. In addition, the phosphorylation of p190RhoGAP was not observed when recombinant FAK was incubated with immunocomplexes obtained using control IgG (Fig. 8D). Thus, these results demonstrate that FAK is directly capable of phosphorylating p190RhoGAP in vitro.

p190RhoGAP Requires FAK Activation for Inhibiting Rho Activity—Because the above findings indicate that p190RhoGAP is a FAK substrate, we overexpressed wt-p190RhoGAP in HPAE cells and using the SRE reporter gene activity as the read-out for RhoA activation we addressed the role of p190RhoGAP in mediating the FAK-induced regulation of RhoA activity. HPAE cells were transfected with SRE-luciferase reporter gene construct together with wt-p190RhoGAP. Thrombin stimulation resulted in a significant increase in RhoA activity, whereas this failed to occur in HPAE cells co-transfected with wt-p190RhoGAP (Fig. 9A). In another study, we determined the effects of wt-p190RhoGAP on RhoA activity in the FRNK-expressing cells (i.e. in the absence of FAK activation) (Fig. 9B). Consistent with the results of the pull-down assay (Fig. 5A), expression of FRNK alone increased RhoA activity by 3-fold, which slightly increased further after thrombin challenge (Fig. 9B). In contrast with control cells, wt-p190RhoGAP partially reduced RhoA activity in the FRNK-expressing cells (Fig. 9B). p190RhoGAP-induced inhibition of RhoA activity was significantly lower in FRNK-expressing cells than control cells (Fig. 9C). These findings indicate that a functional FAK is needed for the p190RhoGAP-induced inhibition of RhoA activity.

DISCUSSION

FAK activation has been shown to strengthen the endothelial barrier in response to inflammatory mediators and osmotic stress by promoting the interaction of endothelial cells with the underlying ECM proteins (6, 17). The increase in endothelial permeability in response to mediators such as thrombin is typically a reversible event (4–6). Because RhoA plays a key role in signaling the increase in endothelial permeability (7–10), we surmised that it would be necessary to inactivate RhoA signaling to reverse the permeability response. In the present study we have addressed the possible role of FAK in inactivating RhoA and thereby restoring endothelial barrier function. Our results demonstrate that FAK maintains barrier function by suppressing RhoA activity via the GAP, p190RhoGAP. We showed that thrombin induced rapid and short-lived activation of RhoA as well as phosphorylation of p190RhoGAP in a FAK-dependent manner. The phosphorylation of p190RhoGAP coincided with a decrease in RhoA activity. We also observed that FAK associated with p190RhoGAP, and it was required for the phosphorylation of p190RhoGAP induced by thrombin. Importantly, FAK was shown to phosphorylate directly p190RhoGAP in vitro. Inhibition of FAK activation by dominant negative FAK construct, FRNK, resulted in the activation of RhoA and actin stress fiber formation in endothelial cells. Overexpression of p190RhoGAP prevented the thrombin-induced RhoA activation, but it only partly reduced RhoA activation in the absence of FAK activation, suggesting the requirement of FAK activation in inactivating RhoA. The increase in RhoA activity secondary to the loss of FAK function was a critical factor in inducing the increase in endothelial permeability since barrier function was rescued by preventing RhoA activation using the C3 toxin. Thus, our data show that FAK, by activating p190RhoGAP, negatively regulates RhoA activity and thereby serves to modulate endothelial barrier function.

Endothelial cells attach to the ECM by focal adhesions consisting of integrins and other focal adhesion proteins (13). The formation of focal adhesions is a crucial factor regulating endothelial permeability by altering the cell-ECM attachments (2, 3, 5). FAK localizes to the focal adhesions and controls their turnover (40, 41). FAK is activated upon phosphorylation at Tyr-397 (42), creating a binding site for Src kinase to further phosphorylate FAK at S76 within the FAK kinase domain. Phosphorylation of FAK at these sites is important in the formation of focal adhesions since it regulates the catalytic activity of FAK and is required for the recruitment of other proteins such as paxillin at focal adhesions (41). In the present study we showed that FAK was basally phosphorylated at Tyr-397 and Tyr-576 but that the inhibition of phosphorylation...
as induced by the expression of FRNK resulted in a leaky endothelial barrier. This finding is different from a previous observation showing that transient transfection of HPAE cells with FAK antisense oligonucleotide had no significant effect on basal endothelial barrier function (6). We speculate that this difference is likely attributable to the higher transfection efficiency with adenoviral vector (≈ 95%) in the present study. To validate the above observations in endothelial monolayers, we also showed that FRNK expression resulted in a leaky mouse pulmonary microvascular barrier. Transfection of purified FRNK protein in pig coronary vessel, however, was shown to have no effect on basal endothelial permeability (43). Because endothelial cells show tissue- and organ-as well as cell-specific heterogeneity in barrier function (44), the effects of FAK inhibition on barrier function may differ within endothelial cells subtypes from different species. Endothelial-specific deletion of

FIGURE 7. Inhibition of RhoA by C3 toxin inhibits FRNK-mediated endothelial barrier dysfunction. Cells plated on gold electrodes were infected with Ad-GFP or Ad-GFP-FRNK for 5 h, after which they were left untreated or treated with 20 μg/ml C3 toxin for 18 h, and changes in TER was determined with or without 50 nM thrombin stimulation. Data represent the means ± S.E. of TER from multiple experiments calculated as % change in resistance over value at time 0 (n = 7). *, values different from C3 transfected monolayer (p < 0.05).

FIGURE 8. Thrombin phosphorylates p190RhoGAP in a FAK-dependent manner. A, Western blot showing thrombin induced p190RhoGAP phosphorylation. After serum deprivation, HPAE cells were left unstimulated or stimulated with 50 nM thrombin for the indicated times. Lysates were immunoprecipitated (IP) with anti-p190RhoGAP Ab as described under “Experimental Procedures.” Proteins bound to protein A/G-Sepharose beads were eluted from the beads by boiling them, after which they were electrophoresed and transferred to nitrocellulose membrane for analysis of tyrosine phosphorylation (top) or p190RhoGAP content by Western blotting (bottom). Data are representative of three independent experiments. p190, phosphorylated p190RhoGAP. B, p190RhoGAP phosphorylation in Ad-GFP- or Ad-GFP-FRNK-expressing cells after thrombin challenge. Cells were stimulated without or with thrombin for 30 min. Lysates were immunoprecipitated with anti-p190RhoGAP Ab as described under “Experimental Procedures” to determine tyrosine phosphorylation (top) or p190RhoGAP content by Western blotting (bottom). Results are representative of at least three experiments. C, Western blot (WB) showing association of FAK with p190RhoGAP. Cells lysates were incubated with control IgG, anti-p190RhoGAP, or anti-FAK Abs followed by the addition of protein A/G-Sepharose overnight at 4 °C. Protein complexes on beads were boiled, electrophoresed, transferred to nitrocellulose, and Western-blotted with anti-FAK or anti-p190RhoGAP antibody to detect p190RhoGAP interaction with FAK. These experiments were repeated at least two times. D, Western blot showing p190RhoGAP phosphorylation by purified FAK in vitro. Cells lysates were incubated with control IgG or anti-p190RhoGAP Abs followed by the addition of protein A/G-Sepharose overnight at 4 °C. Protein complexes on beads were incubated without or with recombinant FAK in kinase buffer containing ATP at 30 °C for 30 min. The kinase reaction was terminated by the addition of SDS sample buffer, after which samples were electrophoresed and transferred on nitrocellulose for analysis of p190RhoGAP phosphorylation by FAK (PY, top) or p190RhoGAP (p190, bottom) content by Western blotting. rFAK, recombinant FAK; −, absent; +, present.
FAK in mice led to embryonic lethality at E13.5 due to the formation of a defective vasculature and ensuing hemorrhage and edema (45), supporting our conclusion that normal FAK function is required for maintenance of endothelial barrier integrity.

We further showed that the increase in endothelial permeability induced by FRNK was the result of augmentation of RhoA activity and the formation of actin stress fibers. These findings are in accord with previous observations in which deletion of FAK in fibroblasts resulted in the activation of RhoA (18, 19). RhoA is known to increase endothelial monolayer permeability by disrupting intercellular adhesion (e.g. adherens junctions) and reorganizing the cell-ECM attachment sites (3, 7, 46, 47). Taken together, these data suggest the crucial role of FAK in maintaining endothelial barrier function by down-modulating RhoA activity.

Studies have shown that endothelial cells retract in response to inflammatory mediators, and this is followed by restoration of the endothelial barrier function (4–6). We observed that thrombin induced a short-lived activation of RhoA, whereas the activation of FAK was relatively prolonged and paralleled the recovery of barrier function. Inhibition of FAK phosphorylation by FRNK expression had no effect on thrombin-induced endothelial retraction, but it significantly impaired the restoration of barrier function. Endothelial cell contractility secondary to RhoA activation is capable of triggering the phosphorylation of FAK and translocating it to focal adhesions (7–10). FAK activation was not observed in the absence of RhoA activation or actin stress fiber formation (6, 7, 47, 48). Studies also showed that thrombin-induced sustained activation of FAK requires Src (47, 48). Because we observed that thrombin induced transient activation of RhoA, it is possible that the sustained activation of FAK induced by Src (rather than RhoA per se) is responsible for restoring barrier function.

Although our data implicate the crucial role of FAK-induced down-modulation of RhoA activity in restoring endothelial barrier function, the effect of FAK in regulating endothelial permeability may be more complex. Studies have shown that FAK induces the localization of vascular endothelial cadherin at adherens junctions (17), raising the possibility that FAK can also restore endothelial barrier function by a mechanism involving the re-annealing of adherens junctions. Additionally, studies have shown that activation of GIT1 and Cdc42 is required for restoring endothelial barrier function (33, 48). The relative contributions of FAK-induced down-modulation of RhoA activity in restoring endothelial barrier function as compared with these other pathways is unknown, but it is likely that multiple mechanisms are engaged to correct endothelial barrier defect.

An important aspect of our studies involves delineating the mechanism of FAK regulation of RhoA activation. Studies have suggested a critical role of p190RhoGAP in regulating RhoA activation through its GAP domain (21–25). A recent study in endothelial cells showed that

![Figure 9. Effects of p190RhoGAP on SRE reporter gene activity.](image-url)
p190RhoGAP interacts with protein kinase Cθ, and this association may be important for regulating RhoA activity and basal endothelial barrier function (49). Intriguingly, p190RhoGAP is propitiably localized as it is capable of associating with FAK (26) and is phosphorylated on tyrosine residues in response to growth factor stimulation of fibroblasts or integrin ligation (22–25). Tyrosine phosphorylation of p190RhoGAP was shown to promote the rapid disassembly of RhoA-mediated actin stress fiber formation (23). These findings led us to investigate the potential role of p190RhoGAP in regulating the FAK modulation of RhoA activity. Although there was some degree of p190RhoGAP phosphorylation basally, the exposure of endothelial cells to thrombin resulted in a marked increase in phosphorylation. The time course of p190RhoGAP phosphorylation occurring between 10 and 30 min after thrombin coincided with the period of RhoA inactivation. FAK also associated with p190RhoGAP in endothelial cells. Additionally, FAK activation was required for the thrombin-induced phosphorylation of p190RhoGAP since the response was abrogated in FAK-inhibited cells. In vitro studies showed that FAK was directly capable of phosphorylating p190RhoGAP. Although Src is capable of activating p190RhoGAP (23, 24), our findings show that the GAP is also a FAK substrate; thus, the results support the hypothesis that FAK activation of p190RhoGAP can suppress RhoA activity. The finding that RhoA is constitutively active in FAK-null fibroblasts (18, 19), despite increased Src activity (50), is consistent with our hypothesis. Furthermore, our finding that overexpression of wt-p190RhoGAP prevented the thrombin-induced RhoA activation but failed to attenuate RhoA activity in the absence of FAK activation provides additional support for the concept that p190RhoGAP activity requires normal FAK function. Thus, our results demonstrate a novel role of FAK in mediating the p190RhoGAP-dependent down-regulation of RhoA activity in endothelial cells and, thus, modulating the increase in endothelial permeability.

In conclusion, we describe herein the key role of FAK in promoting endothelial barrier recovery through its ability to inhibit RhoA activity via p190RhoGAP. We propose a model whereby thrombin activates FAK by inducing RhoA-mediated actin-myosin cross-bridging. FAK in turn phosphorylates p190RhoGAP, which increases the GAP activity to switch-off the RhoA cycle. Thus, the p190RhoGAP-mediated suppression of RhoA activity promotes cell-ECM attachment and intercellular adhesion, resulting in the restoration of endothelial permeability.

Acknowledgments—We thank Drs. J. T. Parsons and K. Burridge for the GFP-FRNK and p190RhoGAP cDNA constructs. We also thank Dr. Arun Roy for valuable discussions.

REFERENCES

1. Lampugnani, M. G., Resnati, M., Dejana, E., and Marchisio, P. C. (1991) J. Cell Biol. 112, 479–490
2. Lum, H., and Malik, A. B. (1994) J. Physiol. 479, 1233–1241
3. Deneault, J. A., and Garcia, J. G. (2003) J. Appl. Physiol. 95, 1487–1500
4. Tiruppathi, C., Malik, A. B., Del Vecchio, P. J., Keese, C. R., and Giaever, I. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 7919–7923
5. Mow, A. B., Van Engelenhour, J., Bodiner, J., Kamath, J., Keese, C. R., Giaever, I., Shabsy, S., and Shabsy, D. M. (1996) J. Clin. Invest. 97, 1020–1027
6. Mehta, D., Tiruppathi, C., Sandoval, R., Minshall, R. D., Holminst, M., and Malik, A. B. (2002) J. Physiol. (Lond.) 539, 779–789
7. Carbajal, J. M., and Schaeffer, R. C., Jr. (1999) Am. J. Physiol. 277, C955–C964
8. van Nieuw Amerongen, G. P., Natarajan, K., Yin, G., Hoefen, R. J., Osawa, M., Haen-deker, K., and Schlaepfer, D. D. (2002) J. Biol. Chem. 277, L150–L158
9. Kouklis, P., Konstantoulaki, M., Vogel, S., Brommann, M., and Malik, A. B. (2004) Circ. Res. 94, 159–166
10. Hultin, S., Eneström, P., and Gudmundsson, V. (2005) J. Cell Biol. 169, 113, 993–1005
11. Parsons, J. T. (2003) J. Cell Sci. 116, 1409–1416
12. Chen, H. C., Appelbu, P. A., Isoda, H., and Guan, J. L. (1996) J. Biol. Chem. 271, 26329–26334
13. Wu, M. H., Guo, M., Yuan, S. Y., and Granger, H. J. (2003) J. Physiol. (Lond.) 552, 691–699
14. Gebb, S., and Stevens, T. (2004) Microvasc. Res. 68, 1–12
15. Shen, T. L., Park, A. Y., Alcaraz, A., Peng, X., Jang, I., Koni, P., Flavell, R. A., Gu, H., and Guan, J. L. (2005) J. Cell Biol. 169, 941–952
16. Wojcik-Strohl, B., Potempa, S., Eichholtz, T., and Ridley, A. J. (2001) J. Cell Sci. 114, 1343–1355
17. Carbazal, J. M., Gratzin, M. L., Yu, C. H., and Schaeffer, R. C., Jr. (2000) Am. J. Physiol. Cell Physiol. 279, C195–C204
18. van Nieuw Amerongen, G. P., Natarajan, K., Yin, G., Hoefen, R. J., Osawa, M., Haendelker, J., Ridley, A. J., Fujiiwara, K., van Hinsbergh, V. W., and Berk, B. C. (2004) Circ. Res. 94, 1041–1049
19. Harrington, E. O., Shannon, C. J., Morin, N., Rovett, H., Murphy, C., and Lu, Q. (2005) Exp. Cell Res. 308, 407–421
20. Sieg, D. J., Ille, D., Jones, K. C., Damsky, C. H., Hunter, T., and Schlaepfer, D. D. (1998) EMBO J. 17, 5933–5947