GIT1 Mediates Thrombin Signaling in Endothelial Cells
Role in Turnover of RhoA-Type Focal Adhesions


Abstract—Thrombin mediates changes in endothelial barrier function and increases endothelial permeability. A feature of thrombin-enhanced endothelial hyperpermeability is contraction of endothelial cells (ECs), accompanied by formation of focal adhesions (FAs). Recently, a G protein–coupled receptor kinase-interacting protein, GIT1, was shown to regulate FA disassembly. We hypothesized that GIT1 modulates thrombin-induced changes in FAs. In human umbilical vein ECs (HUVECs), thrombin recruited GIT1 to FAs, where GIT1 colocalized with FAK and vinculin. Recruitment of GIT1 to FAs was dependent on activation of the small GTPase RhoA, and Rho kinase, as demonstrated by adenoviral transfection of dominant-negative RhoA and treatment with Y-27632. Thrombin stimulated GIT1 tyrosine phosphorylation with a time course similar to FAK phosphorylation in a Rho kinase– and Src-dependent manner. Depletion of GIT1 with antisense GIT1 oligonucleotides had no effect on basal cell morphology, but increased cell rounding and contraction of HUVECs, increased FA formation, and increased FAK tyrosine phosphorylation in response to thrombin, concomitant with increased endothelial hyperpermeability. These data identify GIT1 as a novel mediator in agonist-dependent signaling in ECs, demonstrate that GIT1 is involved in cell shape changes, and suggest a role for GIT1 as a negative feedback regulator that augments recovery of cell contraction. (Circ Res. 2004;94:1041-1049.)

Key Words: contractility ■ endothelium ■ focal adhesion kinase ■ thrombin

Increased endothelial permeability is a vascular reaction to many inflammatory and angiogenic stimuli. Endothelial barrier function is maintained principally by cytoskeletal elements that determine cell shape, facilitate cell adhesion to subendothelial matrix, and participate in formation of junctional complexes. Thrombin rapidly stimulates formation of focal adhesions (FAs) and actin stress fibers in ECs. FAs are sites where cells contact the extracellular matrix (ECM) and are anchoring sites for stress fibers, transient contractile bundles of actin filaments.2,3 Thrombin contraction is accompanied by formation of small gaps between cells and disturbed barrier function of the endothelial monolayer,4,5 the major cause of vascular leakage under inflammatory conditions.6 We and others identified three signaling pathways involved in thrombin-induced endothelial hyperpermeability:7:8: (1) Ca2+-dependent activation of myosin light chain kinase,4,5 (2) a RhoA/Rho kinase-signaling pathway in a Ca2+-independent manner,7–9,11 and (3) a protein tyrosine kinase pathway that disrupts intercellular junctions.8 RhoA is an essential component of a signaling pathway linking growth factors to formation of stress fibers and FAs.12,13 We demonstrated that RhoA, but not Rac, is activated by thrombin in ECs.7 We proposed a pathway for thrombin regulation of FAs in ECs. After RhoA activation, Rho kinase and c-Src stimulate integrin clustering14 and tyrosine phosphorylation of FAK.15 After integrin clustering, FAK becomes phosphorylated at many tyrosine residues.16 Phosphorylation of FAK-Y397, the apparent autophosphorylation site, creates a high-affinity binding site for SH2-domains of Src-family kinases. After binding to FAK, Src phosphorylates Y576 and Y577, located in the kinase activation domain, which enhances FAK catalytic activity.17 Based on studies in FAK−/−cell, FAK is important in FA turnover and cell motility.18

GIT1, a substrate for Src kinase,19 has drawn attention as a putative FAK substrate involved in disassembly of Rac/Cdc42-dependent FAs formed during cell spreading and migration.19,20 GIT proteins bind to p21-activated kinase (PAK), the downstream effector of Cdc42 and Rac, and a guanine nucleotide exchange factor termed PAK interacting exchange factor (PIX). By enhancing FA turnover, GIT1 might facilitate migration and spreading. Because the permeability-enhancing effects of thrombin are reversible and GIT1 regulates assembly of FAs, we hypothesized that GIT1 mediates recovery from thrombin-induced endothelial barrier injury.
Materials and Methods

Bovine aortic ECs (BAECs) and human umbilical vein ECs were cultured and transfected with adenoviruses and cDNAs as described previously. Design of antisense GIT1 oligonucleotides and transfection protocols were performed as described. Sources of reagents are listed in the expanded Materials and Methods section in the online data supplement available at http://circres.ahajournals.org. Western blot analyses were performed from ≥3 experiments as described. Densitometric analyses were performed by NIH image. For immunofluorescence, ECs were fixed and stained for FAK, and vinculin as described previously. Total gap surface area was then quantified from these pictures. For confocal microscopy, BAECs were cultured overnight on noncoated glass cover slips, immuno-stained, and imaged with an Olympus laser scanning microscope. For interference reflection microscopy (IRM) the microscope was adjusted to the IRM mode.

Comparisons among treatment groups were performed with one-way analysis of variance and a post hoc Student Newman-Keuls comparison. Data are the mean±SEM. A value of *P*<0.05 was accepted as a significant difference. Comparisons between two groups were done by Student’s *t* test. For permeability assays, we performed a Wilcoxon ranking test on the absolute values of the slopes using SPSS.

Results

GIT1 Is Redistributed to FAs by Thrombin

To test whether thrombin redistributes GIT1 to FAs, HUVECs were stimulated with thrombin for 5 to 150 minutes, fixed, and stained for GIT1. Basally GIT1 was largely detected in perinuclear areas (Figure 1), as described previously for the related protein GIT2. Thrombin rapidly redistributed GIT1 into focal spots at the cell periphery. Redistribution of GIT1 started 5 minutes after stimulation and was maximal at 30 to 60 minutes (Figure 1). Cell rounding and contraction occurred forming large gaps between cells (Figure 1, times 30' and 60', arrows), as reported previously. After 120 minutes, a partial recovery toward basal morphology was observed. Cells formed new cell-cell interactions and the peripheral punctate GIT1 staining disappeared at sites of cell-cell contact (Figure 1, time 120', arrowheads). Similar results were obtained with BAECs (not shown).

The punctate pattern of GIT1 staining after stimulation with thrombin suggested recruitment of GIT1 to FAs. To test this possibility, HUVECs were double-stained for GIT1 and FAK (Figures 2A through 2F) and for GIT1 and vinculin, a marker for formation of FAs (Figures 2G through 2I). Basally FAK exhibited a diffuse staining pattern (Figure 2B) and little colocalization with GIT1 (Figure 2C). After stimulation with thrombin for 30 minutes, the punctate GIT1 staining pattern exactly matched the punctate staining patterns of both FAK (Figures 2D through 2F) and vinculin (Figures 2G through 2I). However, no interaction between GIT1 and either FAK could be demonstrated by immunoprecipitation experiments (online Figure 1S available in the online data supplement). This finding suggests that these interactions are either indirect or detergent sensitive.

To define the relationship between GIT1 and FAs, we also identified FAs by IRM. Preliminary experiments showed that BAECs provided much better IRM images than HUVEC. The advantage of BAEC was probably related to the fact that IRM requires plating on glass, and BAECs attach better to glass than HUVECs. FAs were readily detected in thrombin-stimulated BAECs using IRM (Figures 2K and 2M). The punctate GIT1 staining pattern (Figure 2J) matched the FA pattern very well (Figure 2L).

GIT1 Recruitment to FAs Depends on RhoA

GIT1 recruitment to focal complexes in migrating fibroblasts depends on interactions with βPIX and PAK3. However, in HUVECs stimulated with thrombin, no stoichiometric changes in either βPIX-GIT1 complexes or PAK3-GIT1 complexes were observed (online Figure 2S). Basally GIT1 formed a stable complex with paxillin consistent with previous reports for p95PKL. GIT1-paxillin interaction was also not affected by thrombin (not shown).

To assess the roles of Rac and RhoA in regulating thrombin changes in GIT1 localization, confluent HUVECs were infected with recombinant adenoviruses that express dominant-negative mutants N17Rac and N19RhoA. Infection with Ad-LacZ had no effect on GIT1 distribution in control or thrombin-stimulated HUVECs (compare Figures 3A and 3F with 3B and 3G). Infection with Ad-N17Rac resulted in appearance of a thin peripheral line of GIT1 in unstimulated cells, but did not prevent thrombin-induced EC rounding and redistribution of GIT1 in FAs (Figures 3C and 3H). Infection with Ad-N19RhoA prevented thrombin mediated increases in FAs and cell contraction to a large extent (Figures 3D and 3I). Occasionally, a few small FAs were observed after thrombin stimulation of Ad-N19RhoA–infected HUVECs. Pretreatment with the Rho kinase inhibitor Y-27632 completely prevented recruitment of GIT1 to FAs similar to N19RhoA (Figures 3E and 3J). These data indicate...
that thrombin-induced GIT1 redistribution occurs independently of Rac and requires RhoA and Rho kinase.

**GIT1 Is Phosphorylated on Thrombin Stimulation**

With a Similar Time Course as FAK

Thrombin rapidly induced phosphorylation of GIT1 on tyrosine residues, as shown by immunoblotting of immunoprecipitated GIT1 with an anti-phosphotyrosine antibody (Figure 4A). Thrombin did not induce tyrosine phosphorylation of GIT2 (not shown). Thrombin stimulated GIT1 phosphorylation in a time-dependent manner with a maximum at 30 to 60 minutes after thrombin treatment of BAECs (Figure 4A) and HUVECs (185 ± 9% compared with basal level at 30 minutes, n=11; see also Figure 4B). Under the same conditions, thrombin increased overall FAK tyrosine phosphorylation with a similar time course compared with GIT1 (Figures 4C

**Figure 2.** GIT1 colocalizes with FAK and vinculin in FAs. A through I, HUVECs were preincubated for 1 hour in RPMI+1% HSA and stimulated for 30 minutes with 1 U/mL thrombin (D, E, F, G, H, and I) or sham-treated (A, B, and C). Cells were double-stained either for GIT1 and FAK (A, B, C, D, E, and F) or for GIT1 and vinculin (G, H, and I). A, D, and G show GIT1 staining; B and E show FAK staining; and G shows vinculin staining. C and F show the merge of the GIT1 and FAK staining; I shows the merge of the GIT1 and vinculin staining. Similar results were observed in 3 independent experiments. Bar=20 μm. J through M, BAECs were preincubated for 1 hour in Medium 199+1% HSA and stimulated for 30 minutes with 1 U/mL thrombin. Cells were stained for GIT1 and IRM performed. J, GIT1 staining. K, same as M but inverted and colored in green. L, merge of J and K. M, IRM. Bar=20 μm.
and 4D). Using phosphospecific antibodies, both FAK Y397 (SH2-binding site) and Y576 (in the kinase domain) were phosphorylated on stimulation with thrombin (Figure 4E).

Rho Kinase and Src Act as Upstream Signaling Mediators of GIT1 and FAK Phosphorylation

Pretreatment for 30 minutes with the Rho kinase inhibitor Y-27632 (10 μmol/L) reduced basal GIT1 tyrosine phosphorylation and almost completely inhibited stimulation by thrombin (Figure 5A).

In Src-transformed NIH 3T3 cells GIT1 phosphorylation has been shown to be significantly higher compared with normal NIH 3T3 cells.4,20 This finding suggests that Src kinases might act as upstream regulators of GIT1 phosphorylation, and is consistent with our data in fibroblasts and vascular smooth muscle cells.21,26 Pretreatment of HUVECs with 10 μmol/L PP2, a specific inhibitor of Src kinases, prevented thrombin-induced GIT1 tyrosine phosphorylation in HUVECs by densitometry is shown. *P<0.05, thrombin-stimulated vs control cells. C, Immunoblot showing tyrosine phosphorylation of FAK in HUVECs after exposure to 1 U/mL thrombin. Cells were preincubated for 1 hour in RPMI 1% HSA and stimulated with thrombin. Membranes were reprobed with a GIT1 antibody (lower) to ensure equal loading. D, Quantification of thrombin-induced GIT1 tyrosine phosphorylation in HUVECs by densitometry is shown. *P<0.05, thrombin-stimulated cells vs control cells. E, Thrombin enhances phosphorylation of FAK at the Y397 and Y576 sites. HUVECs were stimulated with thrombin as indicated above and lysates were immunoblotted using phosphospecific FAK antibodies. Membranes were reprobed with a FAK antibody to ensure equal loading. Similar results were obtained in 3 independent HUVEC cultures.

Figure 4. Thrombin induces tyrosine phosphorylation of GIT1 and FAK in ECs. A, Immunoblot showing tyrosine phosphorylation of GIT1 in BAECs after exposure to 1 U/mL thrombin for the indicated times. Cells were preincubated for 1 hour in medium 199 +1% HSA and stimulated with thrombin. Immunoprecipitation was performed with a GIT1 antibody. Normal mouse IgG was used as a negative control for nonspecific IP of GIT1. Membranes were then immunoblotted (IB) with a 4G10 antibody (top). Membranes were reprobed with GIT1 antibody (lower) to ensure equal loading. 1/10 of control whole cell lysate (WCL) was used as a positive control for GIT1 immunoblotting. Similar results were obtained in HUVEC cultures. B, Quantification of thrombin-induced GIT1 tyrosine phosphorylation in HUVECs by densitometry is shown. *P<0.05, thrombin-stimulated vs control cells. C, Immunoblot showing tyrosine phosphorylation of FAK in HUVECs after exposure to 1 U/mL thrombin. Cells were preincubated for 1 hour in RPMI 1% HSA and stimulated with thrombin. Immunoprecipitation was performed with FAK antibody or with normal mouse IgG (NS) as a negative control for nonspecific IP of FAK. Membranes were then immunoblotted (IB) with a 4G10 antibody (top). Membranes were reprobed with FAK antibody (lower) to ensure equal loading. D, Quantification of thrombin-induced FAK tyrosine phosphorylation in HUVECs by densitometry is shown. *P<0.05, thrombin-stimulated cells vs control cells. E, Thrombin enhances phosphorylation of FAK at the Y397 and Y576 sites. HUVECs were stimulated with thrombin as indicated above and lysates were immunoblotted using phosphospecific FAK antibodies. Membranes were reprobed with a FAK antibody to ensure equal loading. Similar results were obtained in 3 independent HUVEC cultures.

Thrombin stimulation resulted in dephosphorylation of Src Y529 (a measure of Src activation), which was maximal at 10 minutes and restored after 30 minutes (Figure 5E and data not shown). Phosphorylation of Y529 promotes intramolecular interactions of the Src COOH terminus with the SH2 domain, effectively inhibiting kinase activity.30 Thus, dephosphorylation at Y529 demonstrates Src activation by thrombin. Interestingly, this Src activation was not affected by inhibition of Rho kinase (Figure 5E), indicating that thrombin activates Src independently of RhoA-signaling (see Discussion and Figure 8).

GIT1 Is Involved in Thrombin-Induced Cell Rounding

To study the role of GIT1 in thrombin signaling in greater detail, we used antisense GIT1 oligonucleotides and siRNA to deplete GIT1 protein.21,31 Transfection of ECs reached >95% efficiency as demonstrated by transfection of FITC-labeled control oligonucleotides (Figure 6A). Transfection of 200 nmol/L antisense GIT1 oligonucleotides significantly reduced GIT1 protein expression (Figure 6B, top; 56±6% reduction compared with nontransfected cells, n=4, P<0.05). Treatment with scrambled (Figure 6B) or sense (not shown) oligonucleotides did not alter GIT1 expression. Levels of FAK (Figure 6B, bottom) eNOS (not shown) and ERK1/2 (not shown) were not altered. GIT1 siRNA was even inhibition with Y-27632 (Figure 5C) and PP2 (Figure 5D), similar to reported data.5,15

Thrombin-induced FAK Y576 phosphorylation was also dependent on Rho kinase and Src kinase activity as shown by
more effective in decreasing GIT1 protein expression (Figure 6C, top; 92±6% reduction) and did not alter FAK expression (Figure 6C, bottom). We measured the effect of GIT1 depletion on FAs in BAEC using IRM. In cells depleted of GIT1, thrombin significantly increased FA density (from 3.2±0.9% in control and 2.5±0.2% in sense oligonucleotide-treated cells to 9.4±1.0% in antisense GIT1 oligonucleotide-treated cells; *P<0.05; online Figure 3S). These results suggest that GIT1 plays a role in thrombin-mediated regulation of FAs.

To test whether GIT1 has a functional role in thrombin-induced EC contraction, we assayed the effect of antisense GIT1 oligonucleotides on subcellular localization of vinculin in HUVECs. The morphology of GIT1-depleted HUVEC monolayers was similar to control monolayers. Vinculin appeared as a thin line at the cell periphery (Figures 7A and 7C). Cells remained closely attached to each other. Treatment with sense GIT1 oligonucleotides did not alter thrombin-induced FA formation, and similar to control monolayers small gaps between cells formed (Figures 7D and 7E). GIT1 depletion had no effect on recruitment of vinculin to FAs. However, GIT1 depletion dramatically potentiated thrombin-induced cell rounding (Figure 7F), although cells remained firmly attached to their matrix. To quantitate this effect, we measured gap surface area after thrombin, which significantly increased in GIT1 depleted cells (fold-induction): 10.1±3.4 in control cells, 10.6±2.7 in sense-treated cells, and 19.5±5.9 in antisense-treated cells (mean±SD of 9 pictures from three independent experiments). Only antisense-treated cells differed significantly from sense and control (*P<0.01).

Finally, we tested effects of GIT1 overexpression on endothelial stress fibers. Cells were transfected with GIT1 cDNA or LacZ. After 48 hours, cells were stimulated with thrombin for 30 minutes (online Figure 4S available in the online data...
supplement), fixed, and stained for actin with rhodamine-phalloidin. GIT1-transfected cells clearly showed fewer stress fibers on thrombin stimulation compared with LacZ-transfected cells. These data further support the role of GIT1 in EC shape.

The finding that GIT1 depletion augments EC rounding suggests a role for GIT1 in endothelial processes dependent on cell contraction. Thrombin-induced endothelial barrier disruption depends on Rho kinase–mediated cell contraction. Stimulation with thrombin induced a rapid increase in passage of the tracer molecule HRP across EC monolayers followed by recovery of barrier function after 1.5 hours (Figures 7G and 7H). Whereas GIT1 depletion had no effect on basal endothelial barrier function, thrombin-stimulated HRP passage was significantly prolonged (Figure 7G recovery, Figure 7H, compare solid and open bars).

To determine the effect of GIT1 on FAK phosphorylation, we measured thrombin-stimulated FAK tyrosine phosphorylation in GIT1-depleted cells. GIT1 was depleted both by antisense GIT1 oligonucleotides and GIT1 siRNA (Figures 6C and 7I). Antisense and siRNA treatment did not significantly alter basal FAK expression (Figure 6C) or phosphorylation (not shown). GIT1-depletion dramatically increased FAK Y397 phosphorylation and total FAK phosphorylation (Figure 7I). The stimulation was greater with siRNA, likely due to greater depletion of GIT1 expression by siRNA (Figure 6C). Interestingly, there was minimal effect of GIT1 depletion on thrombin-stimulated phosphorylation of FAK Y576.

**Discussion**

The major finding of the present study is that GIT1 is a novel mediator for thrombin signal transduction in ECs modulating thrombin-induced changes in cell shape and EC barrier function. Specifically, we show that GIT1 is recruited to FAs in a RhoA-dependent manner. GIT1 is phosphorylated on tyrosine residues in an agonist-dependent manner that requires Rho kinase, Src, and FAK activation. Finally, an important role for GIT1 in EC rounding and endothelial barrier recovery was demonstrated by GIT1 depletion. Based
on these findings, we propose a model for thrombin-induced changes in EC function mediated by GIT1 (Figure 8). Specifically, binding of thrombin to its receptor initiates a F-actin-dependent EC contraction involving activation of RhoA and Rho kinase. GIT1 then translocates to FAs, where in concert with FAK and Src, it modulates FA (dis)assembly contributing to changes in cell shape. An intact F-actin cytoskeleton is necessary for GIT1 recruitment to FAs, as disruption of the F-actin cytoskeleton with cytochalasin prevented GIT1 recruitment (not shown). We propose that GIT1 acts as a negative feedback regulator of EC contraction and enhances recovery of cell shape by promoting turnover of FAs. The present study extends results of Shikata et al. who showed that thrombin and sphingosine-1-phosphate induced FA rearrangement in association with FAK tyrosine phosphorylation and GIT1 redistribution to the cell periphery.

The effects of thrombin to disrupt endothelial barrier function have been well described. However, much less is known regarding the process for recovery of normal barrier function. Understanding this process is important as currently no clinical treatments are available to improve disturbed barrier function in patients suffering from vascular leakage. In an attempt to unravel the underlying mechanisms of barrier recovery we focused on GIT1, a protein recently proposed to promote FA turnover.

Thrombin induced a transient tyrosine phosphorylation of GIT1 similar to our recent results with angiotensin II and EGF. Previous reports have described increased GIT1 phosphorylation induced by integrin clustering as well as by overexpression of FAK or Src. Remarkably, GIT1 phosphorylation had a similar time course as FAK phosphorylation and GIT1 translocated to Fas, suggesting a mechanistic link between phosphorylation, redistribution to FAs, and changes in cell shape (Figure 8).

We propose that coordinate tyrosine phosphorylation of GIT1 and FAK is important in cell shape change and recovery of barrier function. FAK has been demonstrated to be tyrosine-phosphorylated on thrombin stimulation in a RhoA-dependent manner. In this study, we identify Y397 and Y576 as sites of FAK phosphorylation induced by thrombin. Phosphorylation at Y397 creates a SH2-binding site for several different signaling and adapter proteins, including Src-family kinases, which are activated on binding to FAK. Phosphorylation of FAK Y576 is highly correlated with FAK activation. The coordinate phosphorylation of FAK Y397 and Y576 by thrombin, yet divergent effects of GIT1 depletion on these same tyrosines, suggest specific roles for GIT1 in activation of kinases (eg, Src) and phosphatases that regulate FAK function. Future studies will be required to define these roles. A role for FAK in thrombin-induced changes in endothelial barrier function has been suggested previously. Several recent studies show that FAK activation promotes barrier recovery similar to the present findings for GIT1.

A dramatic finding in the present study was recruitment of GIT1 to FAs in response to thrombin. Our data indicate that Rho kinase is necessary for GIT1 recruitment, although this is not specific for GIT1. Interestingly, PKL was not recruited to FAs on RhoA activation, suggesting specialized functions for different GIT1 family members. Preliminary data indicate that Src is not necessary for GIT1 recruitment to FAs, although Src activity is essential for GIT1 tyrosine phosphorylation. These findings support a model for thrombin-stimulated FA formation downstream of Rho kinase, but upstream of Src (see Figure 8). Based on these data, we propose that Src-dependent GIT1 phosphorylation is not necessary for recruitment of GIT1 to FAs, but probably occurs after recruitment of GIT1 to FAs. So, thrombin activates two different signaling pathways that converge at FAs. This explains the apparently contradictory findings that GIT1 phosphorylation is Rho kinase- and Src-dependent, whereas Src activation occurs independently of Rho kinase.

Our finding that inhibiting Src with PP2 prevents thrombin-stimulated GIT1 phosphorylation is consistent with our data for Src in angiotensin II- and EGF-induced signaling to GIT1. In line with this observation other investigators found that the catalytic activity of Src-family kinases promotes turnover of FAs during cell motility. Inhibition of Rho kinase also decreased basal GIT1 phosphorylation. This indicates some basal RhoA/Rho kinase activity in ECs similar to that we previously observed with respect to myosin light chain phosphorylation. These findings suggest that the balance of Rho kinase and Src activity is critical for GIT1 function and EC shape. The precise role of Rho-kinase in basal and thrombin-stimulated GIT1 phosphorylation will require further study.

To study the role of GIT1 in thrombin-induced EC contraction we used GIT1 depletion by antisense GIT1 oligonucleotide and GIT1 siRNA treatment. Overexpression of GIT1 is not a suitable tool, as other investigators have shown that overexpressing GIT1 alters basal cell morphology. GIT1 depletion experiments revealed a negative regulatory role for GIT1 in thrombin-induced cell contraction. The enhanced thrombin-induced cell rounding of GIT1-depleted cells is most likely the result of stabilization of FAs, whose turnover is reduced in the absence of GIT1. In BAECs, we observed
that thrombin increased the number of FAs by more than 3-fold when GIT1 was depleted, further supporting this concept. It is important to note that thrombin-induced contact may directly increase FA assembly, and inhibiting contact with Y27632 may reduce FA formation. Thus, there is a complex interplay between contact and FA dynamics.

The mechanisms by which GIT1 regulates RhoA and Rac dependent events in ECs remain unclear. Recently, it was shown that FAK suppresses RhoA activity to promote FA turnover. FA-fibroblasts adopt a nonpolarized circular shape after adhesion, unlike FA cells that have a more elongated polygonal morphology. Ren et al demonstrated that this is the result of the absence of a negative feedback by FAK on RhoA activity. This suggests that GIT1 could reverse EC contact via downregulation of RhoA activity. Our unpublished data, however, did not show a decrease in thrombin-induced RhoA activity in GIT1-depleted cells, excluding downregulation of RhoA activity as the likely mechanism.

The finding that GIT1 depletion augments EC rounding suggested a role for GIT1 in EC processes dependent on cell contraction, such as disruption of endothelial barrier function. Indeed, we observed that GIT1 depletion prolonged thrombin-stimulated endothelial hyperpermeability. In conclusion, these data identify GIT1 as a novel mediator in thrombin-induced signaling, EC contraction, and endothelial permeability and suggest a role for GIT1 in EC migration and angiogenesis.

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**Abbreviations**

ARF – ADP ribosylation factor  
BAEC – bovine aortic endothelial cell  
BLMVEC – bovine lung microvascular cell  
EC — endothelial cell  
ECM – extracellular matrix  
FA – focal adhesions  
FAK – focal adhesion kinase  
GIT1 – G protein coupled receptor interacting protein 1  

HUVEC – human umbilical vein endothelial cell

**Materials and Methods**

Anti-vinculin antibody and bovine \( \alpha \)-thrombin were from Sigma (St. Louis, MO). Human serum albumin (HSA) was from Sanguin CLB (Amsterdam, The Netherlands). ToPro3 and FITC-labeled anti-mouse IgG were from Vector Laboratories (Burlingame, CA). Texas Red-labeled anti-rabbit IgG was from ICN Pharmaceuticals, Inc (Costa Mesa, CA). PP2 was from Calbiochem (La Jolla, CA). Y-27632 was provided by Welfide Corporation. FITC-labeled secondary antibodies were from Vector Laboratories (Burlingame, CA). 4G10 was from Upstate (Lexington, KY). Rabbit anti-phosphoserine and anti-phosphothreonine antibodies were from Zymed (San Francisco, CA). Src antibody was a kind gift from Dr. Kawakatsu (UCSF)\(^1\). Negatively pre-adsorbed and affinity-purified polyclonal antibodies to peptides
corresponding to the sequences surrounding FAK tyrosine-phosphorylation sites Y397 and Y576 were from BioSource International (Camarillo, CA).
**EC culture and evaluation of barrier function**

Bovine aortic ECs and human umbilical vein ECs were cultured as described previously\(^3,4\).

One hour before all experiments medium was replaced with Medium 199 + 1% HSA. Barrier function was evaluated by measuring the transfer of HRP across HUVEC monolayers grown on fibronectin-coated polycarbonate filters of the Transwell system as described previously (Costar)\(^5\).

**Immunoprecipitation and immunoblotting**

For immunoprecipitations, cells were lysed in RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris-HCl, pH 8.0) with inhibitor (0.5ug/ml leupeptin, 1mM EDTA, 1ug/ml pepstain A, 0. 2mM PMSF). Monoclonal anti-GIT1, anti-FAK, anti-p95PKL antibodies (Transduction Laboratories, Lexington, KY) or \(\text{Ph}\)PIX (L. Lim, Singapore\(^6\)) were used for immunoprecipitation. For immunoblotting we used, anti-phosphotyrosine antibody (4G10, Upstate Biotechnology, Lake Placid, NY), anti-GIT1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-FAK-397 or anti-FAK-576 antibody (Biosource, Camarillo, CA), and Protein A/G agarose (Santa Cruz Biotechnology, Santa Cruz, CA). Analysis of autoradiograms was performed by scanning densitometry and processing with NIH image analysis software. For some experiments we used the LI-COR (Lincoln, NE) Odyssey infrared imaging system and proprietary densitometry software as described by the manufacturer.

**Transfection of EC with adenoviral vectors, antisense oligonucleotides, and siRNA**

EC were transfected with adenoviruses as previously described\(^7\). Design of antisense GIT1 oligonucleotides and transfection protocols were carried out as described\(^8\). Phosphorothioated sense (S), scrambled, or anti-sense (AS) nucleotides (200 nM) corresponding to the GIT1
sequence (S-GIT1: 5’-GAAGTACTCGACGACGTGGAAAC-3’; scrambled-GIT1: 5’-AACGGTACGTGATCGGCGACaaA-3’; AS-GIT1: 5’-CTTCATGAGCTGGCTGCACCTTTG-3’, synthesized and purified by Operon technologies, Alameda, CA) were transfected into EC. 2.5 µg/mL Lipid G (Sequitur, Natick, MA) was used as a vehicle according to the manufacturers’ protocol. Cells were transfected twice at respectively 72 and 36 hours before cell lysis. Under these conditions, transfection efficiency was > 95 % as judged by parallel controls with FITC-labeled oligonucleotides (Sequitur, Natick, MA). The reduction in GIT1 expression was dose-dependent with a maximum at 200 nM, which was chosen for all further experiments. GIT1 siRNA sequences were designed as described 9. 20mM GIT1-directed siRNA (AAGCTGCTAAGAAGAAGCTGC) and a control non-silencing siRNA (AATTCTCCGAACGTGTCACT), synthesized and purified by Xeragon (Qiagen, Valencia, CA), were transfected into ECs. Oligofectamine (Invitrogen, Carlsbad, CA) was used as the vehicle according to the manufacturer’s protocol. Cells were stimulated with 1U/ ml thrombin and lysed 72 hours after transfection. One hour before all experiments, the medium was replaced with Medium 199 +1% human serum albumin. Western blot analysis using the anti-GIT1 antibody was used to confirm suppression of GIT1 expression by the GIT1-directed siRNA.

**Immunocytochemistry and confocal microscopy**

EC were fixed and stained for FAK (Transduction Labs), and vinculin (Sigma) as described previously 3,10. Total gap surface area was quantified from these pictures as previously described 10. For confocal microscopy. BAEC were cultured overnight on noncoated glass cover slips. They were fixed with 2% formaldehyde in PBS for 10 minutes at room temperature, washed thoroughly with PBS and blocked with 5% horse serum/1% bovine serum albumin for 10 minutes at room temperature. They were treated with rabbit anti-GIT1
antibody (30 μg/mL; BioSource International (Camarillo, CA)) for 60 minutes at room
temperature, washed in PBS for a total of 20 minutes with several changes, and incubated
with fluorescein labeled anti-rabbit-IgG for 30 minutes at room temperature. Immunostained
cells were washed in PBS as before and mounted in Vectashield and sealed. Confocal
microscopy was performed using an Olympus laser scanning microscope (Fluoview 300)
using a 60X objective lens (NA 1.4, UplanApo, oil).

**Interference Reflection microscopy (IRM).**

Endothelial cells were cultured overnight on uncoated glass surface and fixed in 3.7%
formaldehyde for 10 minutes at room temperature. After a brief wash with PBS, they were
sealed in acrylic. Reflection interference images were obtained using a 60X objective lens
(NA 1.4, UplanApo, oil) mounted on an Olympus laser scanning microscope (Fluoview 300)
adjusted for the IRM mode.

**Statistical Analysis**

All blots are representative of at least three to four experiments. Comparisons among
treatment groups were performed with one-way analysis of variance and a post hoc Student
Newman-Keuls comparison. P< 0.05 was accepted as a significant difference. Comparisons
between 2 groups were done by Students’ t-test. For analysis of permeability assays (figure 7)
we performed a Wilcoxon ranking test on the absolute values of the slopes using SPSS.
References


Figure Legends

Figure 1S. Thrombin does not induce a stable interaction between GIT1 and FAK in HUVEC.

HUVEC were grown to confluence. Immunoblot of GIT1 and FAK in HUVEC after exposure to 1 U/mL thrombin for the indicated times. Cells were preincubated for 1 hr in medium 199 + 1% HSA and stimulated with thrombin for the indicated times. GIT1 was immunoprecipitated (IP) from whole cell lysates (WCL). GIT1 expression in the lysates was measured by western blotting with GIT1 antibody (IB: GIT1, left panel). FAK expression in the lysates was measured by western blotting with FAK antibody (IB: FAK, right panel). Normal mouse IgG (NS) was used as a negative control for nonspecific IP of GIT1. 1/10 of control whole cell lysate (WCL) was used as a positive control for immunoblotting.

Figure 2S. Thrombin does not alter the stoichiometry of GIT1 and βPIX interactions in HUVEC.

HUVEC were grown to confluence. Immunoblot of GIT1 and βPIX in HUVEC after exposure to 1 U/mL thrombin for the indicated times. Cells were preincubated for 1 hr in medium 199 + 1% HSA and stimulated with thrombin for the indicated times. GIT1 or βPIX was immunoprecipitated (IP) from whole cell lysates (WCL). βPIX expression in the lysates was measured by western blotting with FAK antibody (IB: βPIX, upper panel). GIT1 expression in the lysates was measured by western blotting with GIT1 antibody (IB: GIT1, lower panel). Normal mouse IgG (NS) was used as a negative control for nonspecific IP of GIT1 and βPIX. 1/10 of control whole cell lysate (WCL) was used as a positive control for immunoblotting.

Figure 3S. IRM analysis of FAs in BAEC after GIT1 depletion with antisense GIT1 oligonucleotides.
GIT1 antisense oligonucleotides (B, and D) were transfected into BAECs as described in Materials & Methods or cells were left untreated (A, and C). 72 hours after transfection, cells were preincubated for 1 hour in medium 199 + 1% HSA and stimulated with 1 U/mL thrombin for 30 min (C, and D) or sham-treated (A, and B). Cell were fixed and IRM images were made. Bar = 20 μm. E-H: Black & white images were converted into pseudocolor images using the color table included in figure (bottom panel) with Adobe Photoshop software. Note that the blacker areas (= red pseudocolor) represent regions of closer contact with the substrate consistent with FAs. Adobe Photoshop software was also used to calculate cell surface area. Focal adhesions were quantitated by measuring red pixel intensity in pseudocolor images. Red pixel intensity was expressed as percentage red pixels of total pixels in a certain cell surface area. In addition, we counted the number of focal adhesions. GIT1-depletion did not affect the number of FAs per cell surface area under basal conditions, but significantly enhanced the thrombin-induced FA formation (171 ± 5% compared with 102 ± 10% sense oligonucleotide-treated cells; number of FAs per cell surface area expressed as a percentage of thrombin-stimulated control cells, n=4, p<0.05). Focal adhesions were quantitated by counting black spots manually in 4 different IRM images of about 10 cells each. The number of focal adhesions was divided by the total cell surface area in each image.

**Figure 4S. Overexpression of GIT1 in bovine lung microvascular endothelial cells (BLMVECs) reduces stress fiber formation.**

BLMVECs (from VEC Technologies) were cultured in MCDB-131 media (Gibco) supplemented with 10% Fetal Bovine Serum, recombinant human Epidermal Growth Factor (Sigma), hydrocortisone (Sigma), crude Endothelial Cell Growth Factor (home made), heparine (Leo Pharma BV), L-Glutamine and antibiotic/antimycotic. Cells were seeded on uncoated glass cover slips and grown to 80% confluency. LacZ (middle row) or GIT1 (lower
row) plasmid DNAs were transfected into cells using lipofectamine/Plus (from Invitrogen) according to the manufacturer’s protocol, resulting in a 50% transfection efficiency as was evidenced by parallel β-GAL staining or cells were left untreated (upper row). Final amount of DNA used for transfection of a 2 cm²-dish was 0.5 μg. After 48 hr cells were washed 3x with DMEM and incubated for 1 hr with DMEM/1% albumin (no other additions). Subsequently, cells were stimulated with 1 U/mL thrombin for 30 min (right column) or sham-treated (left column) and fixed with 2% formaldehyde in PBS for 10 min. Cells were stained for actin with Rhodamine-Phalloidin (Molecular Probes). GIT1-transfected cells marked with an asterisk clearly show fewer stress fibers upon thrombin stimulation compared to untreated or LacZ-transfected cells.
Figure 1S
Figure 2S
Control
basal

Antisense

Control

Antisense

3.2 ± 0.9 % to 9.4 ± 1.0%, P<0.05

Figure 3S
Figure 4S