GIT1 Mediates VEGF-Induced Podosome Formation in Endothelial Cells

Critical Role for PLCγ

Jing Wang, Yoji Taba, Jinjiang Pang, Guoyong Yin, Chen Yan, Bradford C. Berk

Objective—We and others showed that tyrosine kinase receptors (TKRs) such as the epidermal growth factor receptor stimulate G protein–coupled receptor (GPCR) kinase-interacting protein 1 (GIT1) phosphorylation via c-Src, which is required for phospholipase C-γ (PLCγ) activation, indicating that GIT1 participates in TKR signaling. VEGF is the most important TKR in endothelial cells (ECs); essential for cell survival, migration, and angiogenesis. Podosomes, actin-rich structures, were found to contribute to EC migration, tissue invasion, and matrix remodeling, suggesting a role for podosomes in angiogenesis. Because GIT1 is a substrate of c-Src, and podosome formation is c-Src dependent, we hypothesized that GIT1 plays an important role in VEGF-induced EC podosome formation and cell migration.

Methods and Results—Exposure of ECs to VEGF for 30 minutes stimulated GIT1 colocalization with podosomes. Depletion of GIT1 by siRNA significantly decreased VEGF-induced podosome formation. A key role for PLCγ was suggested by several experiments. Double staining PLCγ and actin showed colocalization of PLCγ with podosomes. Podosome formation was dramatically reduced by PLCγ inhibitor U73122, Src inhibitor PP2, or expression of dominant negative small GTPases. Therefore, VEGF-induced EC podosome formation is dependent on Src, GIT1, PLCγ, and small GTPases. In addition, matrix metalloprotease 2 (MMP2) and MT-MMP1 were detected at sites of VEGF-induced podosomes. Depletion of GIT1 by siRNA also significantly inhibited VEGF-induced MMP2 activation and extracellular matrix (ECM) degradation. Therefore, GIT1 mediates VEGF-induced matrix metalloproteinase (MMP) activation and ECM degradation by regulating podosome formation. Finally, depletion of GIT1 by siRNA significantly decreased VEGF-induced cell migration.

Conclusions—These data indicate that GIT1 is an essential mediator for VEGF-induced EC podosome formation and cell migration via PLCγ. (Arterioscler Thromb Vasc Biol. 2009;29:00-00.)

Key Words: GIT1 • VEGF • PLCγ • podosomes • endothelial cells

Angiogenesis, the formation of new blood vessels, is a tightly coordinated process. Extracellular matrix (ECM) degradation and migration of endothelial cells (ECs) are prerequisites for angiogenesis, especially in wound healing. One of the key stimuli for angiogenesis is vascular endothelial growth factor (VEGF). VEGF binding to VEGF receptor-2 (VEGFR-2/Flk-1/KDR) stimulates several downstream signaling pathways, including the tyrosine kinase c-Src.1–3 In addition, it has been shown that VEGF-induced recruitment and subsequent activation of phospholipase Cγ1 (PLCγ1) is essential for angiogenesis.4–6 Mice nullizygous for PLCγ1 experience embryonic lethality attributable to significantly impaired vasculogenesis and erythropoiesis.7,8 Studies in zebrafish demonstrated that PLCγ1 is critically required for the function of VEGF and arterial development.9 These data suggest a critical physiological role for PLCγ1 in angiogenesis.

The G protein–coupled receptor kinase (GRK)-2 interacting protein 1 (GIT1) was originally identified by its binding to GRK-2.10 GIT1 has 5 functional domains, including a zinc finger domain responsible for ARF-GAP activity, three ankyrin repeats, a SpaII homology domain (SHD), a synaptic localization domain (SLD), and a conserved carboxyl-terminal region that interacts with paxillin (PBS).11 A major GIT1 function is regulation of cytoskeletal dynamics during cell spreading and migration by interacting with specific binding partners and targeting them spatially.12,13 Our previous studies have demonstrated an important role for GIT1 in signal transduction mediated by tyrosine kinase receptors and angiostatin II (Ang II), especially in activation of PLCγ, MEK1-ERK1/2, and FAK.12,14 Specifically, we showed that GIT1 was a substrate for c-Src that undergoes tyrosine phosphorylation in response to Ang II and EGF in vascular smooth muscle.12 GIT1 associates with PLCγ via the PLCγ Src homology 2 and 3 domains, and this interaction is required for PLCγ activation.14 To determine the physiological importance of GIT1 in vivo, our laboratory generated
GIT1 traditional knockout mice. The GIT1-KO mouse phenocopies the VEGF120 mouse\textsuperscript{15,16} and resembles the PLC\textgamma KO mouse which having a vascular phenotype,\textsuperscript{8} suggesting that GIT1 deficiency may abrogate VEGF-PLC\textgamma signaling. Thus we propose that GIT1 is a novel regulator of PLC\textgamma function that mediates PLC\textgamma activation by c-Src in response to VEGF.

Podosomes are dynamic actin-rich adhesion structures, shown to play a role in tissue invasion and cell migration by regulating MMP activity and ECM degradation.\textsuperscript{17–19} Podosome formation is regulated by several signaling pathways, including Rho family GTPases, actin regulatory pathways, protein tyrosine phosphorylation, and the microtubule system. Activation of c-Src is central to podosome formation.\textsuperscript{19}

It has been shown that formation of podosomes increases polarization and motility of ECs,\textsuperscript{18,20,21} suggesting a key role in angiogenesis. Because GIT1 is a c-Src substrate and plays an important role in EC adhesion and migration,\textsuperscript{12,22} we hypothesized that GIT1 will mediate VEGF-induced EC migration by affecting EC podosome formation and ECM degradation.

**Methods**

**Cell Culture and Transient Transfection with siRNA**

Human umbilical vein endothelial cells (HUVECs) were isolated as described previously and maintained in Medium 200 (Cascade Biologics) with low serum growth supplement. Cells were used at passages 2 to 4. HUVECs were seeded onto 35-mm dishes 24 hours before transfection, and transiently transfected with 100 nmol/L control siRNA or GIT1 siRNA per dish at 90% confluence with Lipofectamine 2000 reagent in OptiMEM medium. GIT1 siRNA was described previously and ordered from Ambion. Control siRNA was purchased from Qiagen. After 2 hours, 5% serum medium was added.

**Cell Lysate Preparation**

Cells were rinsed with ice-cold phosphate-buffered saline (PBS; 150 mmol/L NaCl, 20 mmol/L Na\textsubscript{2}PO\textsubscript{4}, pH 7.4) on ice and harvested in lysis buffer (150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 5 mmol/L NaF, 1 mmol/L Na\textsubscript{3}VO\textsubscript{4}, plus 1:1000 protein inhibitor cocktail (PIC, Sigma) and clarified by centrifugation. The protein concentration was determined by the Bradford assay (Bio-Rad).

**Western Analysis**

Total cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes, and the membranes were incubated with appropriate primary antibodies: GIT1 (Santa Cruz), Actin (Santa Cruz). After washing and incubating with secondary antibodies, immunoreactive proteins were visualized by the Odyssey infrared imaging system (LI-COR Biotechnology).

**Immunofluorescence and Podosome Analysis**

HUVECs were cultured in serum-free medium 200 overnight and then stimulated with 50 ng/mL VEGF. Cells were fixed with 4% formaldehyde for 10 minutes, permeabilized with 0.05% Triton for 5 minutes, and blocked with 10% normal goat serum for 1 hour. Cells were incubated with GIT1 antibody (invitrogen) or PLC\textgamma antibody (BD) followed by Alexa Fluor 488 antirabbit IgG for green fluorescence and 2.5 μg/mL TRITC-labeled phallolidin for red fluorescence. Podosomes were identified as big phallolidin positive ring. The number of cells containing podosomes were counted and analyzed by fluorescence microscope. Data were shown as percentage of podosome positive cells per 100 cells.

**ECM Degradation Assay**

Fluorescein isothiocyanate (FITC)-gelatin was prepared as described previously.\textsuperscript{23} After transfection with control siRNA or GIT1 siRNA for 48 hours, cells were seeded on FITC-gelatin–coated coverslips. After treatment with VEGF, colocalization of dark areas and podosomes was visualized by merging FITC-gelatin (green) and F-Actin (red) images.

**MMP Activity by Zymography**

HUVECs were plated in 60-mm-diameter culture dishes. MMP activity was detected in both supernatants and cell lysates. Gelatinolytic activity was assayed by SDS-PAGE, in 8% polyacrylamide gels containing 1 mg/mL gelatin as described.\textsuperscript{24} Supernatants were concentrated by using centrifugal filter device (Millipore). Cell lysates and concentrated supernatants were obtained by adding sample buffer (50 mmol/L Tris-HCl, 2% SDS, 0.1% bromophenol blue, 10% glycerol, pH 6.8). After run at 25 mA, gels were then incubated in 2.5% Triton X-100 for 60 minutes to remove SDS followed by overnight incubation in developing buffer (50 mmol/L Tris-HCl, 0.2 mol/L NaCl, 5 mmol/L CaCl\textsubscript{2}, 0.02% Brij-35 pH 7.6). Then, gels were stained for 30 minutes in 0.5% Coomassie Blue G-250, 30% methanol, 10% glacial acetic acid, and destained for 30 minutes in 30% methanol, 10% glacial acetic acid.

**Migration Assay**

A wound healing assay was performed.\textsuperscript{23} HUVECs were grown on 35-mm dishes, the monolayer was scratched with a sterile disposable rubber policeman, and the edge labeled with a traced line. After injury, the cells were gently washed with normal medium without serum. EC migration from the edge of the injured monolayer was quantified by measuring the area between the wound edges before and after injury using light microscopy and the computer program ImageJ.

**Statistical Analysis**

Data are shown as mean±SE. Differences between mean values were analyzed using the Student's t test. P<0.05 was considered statistically significant.

**Results**

**GIT1 Colocalizes With VEGF-Induced Podosomes and Is Required for Podosome Formation**

HUVECs cultured overnight in serum-free medium exhibited podosomes in 20±2% of cells. After treatment with VEGF for 30 minutes, there was a significant increase in podosomes to 35±1% of cells, consistent with other reports.\textsuperscript{26} To evaluate the role of GIT1 in VEGF-induced podosome formation, we used GIT1 siRNA to deplete GIT1 specifically. After transfection with GIT1 siRNA for 48 hours, GIT1 expression was reduced by 70%, whereas control siRNA had no effect (Figure 1A). Treatment with GIT1 siRNA and control siRNA did not alter cell viability and cell morphology (data not shown). After transfection with control siRNA or GIT1 siRNA, HUVECs were treated with VEGF for different times, and podosome formation was measured. Podosome formation was significantly increased by VEGF from 21±1% to 33±2% at 30 minutes in cells treated with control siRNA (Figure 1B). In contrast, podosome formation was dramatically reduced in GIT1 siRNA treated cells (Figure 1B, 19±1% in GIT1siRNA treated cells at 30 minutes versus 33±2% in control siRNA treated cells). To test whether GIT1 colocalized with podosomes, we double stained HUVECs with antibodies to GIT1 and F-actin. In control conditions, there were few podosomes and minimal colocalization of
GIT1 and F-actin (Figure 1C through 1E). In response to VEGF, podosomes rapidly appeared as assayed by F-actin, and GIT1 colocalized with F-actin in podosomes (Figure 1F through 1H). Higher magnification revealed that podosomes aggregated and formed large ring-like structures (Figure 1I through 1K). These results indicated that GIT1 colocalized with podosomes and was required for podosome formation.

PLCγ Colocalizes and Mediates Podosome Formation Induced by VEGF

We have shown that interaction between GIT1 and PLCγ is required for EGF-induced PLCγ activation.14 Recently, a key role for PLCγ in cell spreading was reported, which required PLCγ association with a βPIX/GIT1 complex and activation of small GTPases.27 Therefore, we hypothesized that GIT1 mediated VEGF-induced podosome formation and requires PLCγ activation. As shown in Figure 2A, PLCγ colocalized with F-actin in podosomes in response to VEGF (50 ng/mL for 30 minutes). To examine whether PLCγ plays a critical role in podosome formation, HUVECs were pretreated with the PLCγ inhibitor U73122. As shown in Figure 2B, VEGF-stimulated podosome formation was dramatically reduced by U73122 (from 30±2% to 18±1%). These data suggested that VEGF-induced podosome formation requires GIT1-PLCγ colocalization and activation.

VEGF-Induced Podosome Formation Is c-Src- and Small GTPase-Dependent

We next hypothesized that VEGF-induced podosome formation is dependent on c-Src and small GTPases based on 3

![Figure 1](image1.png)

**Figure 1.** GIT1 mediates VEGF-induced podosomes. A, ECs were transfected with 100 nmol/L control siRNA or GIT1 siRNA using Lipofectamine 2000 for 48 hours, and cell lysates were immuno-blotted with GIT1 antibody. Actin blot shows equal loading. B, HUVECs were transfected with 100 nmol/L siRNA as in A, then treated with VEGF for varying times. Podosomes were identified as large phalloidin positive rings. Percentage of podosome positive cells (%) was determined. (#P<0.05 vs control siRNA at time 0 minutes; &P<0.05 vs control siRNA at 15 and 30 minutes.) C through K, HUVECs were seeded in 2% gelatin coated dishes. After cells were treated with 50 ng/mL VEGF for 30 minutes, cells were stained for GIT1 (Invitrogen, green) and F-actin (Sigma, red). Arrow shows podosomes. Bar represents 10 μm.

![Figure 2](image2.png)

**Figure 2.** PLCγ mediates VEGF-induced podosome formation. A, Colocalization of PLCγ with podosomes. HUVECs were treated with 50 ng/mL VEGF for 30 minutes. Then cells were stained with PLCγ antibody (BD, green) and F-actin (Sigma, red). Arrow shows podosomes. Bar represents 10 μm. B, VEGF-induced podosome formation is dependent on PLCγ. HUVECs were pretreated with 5 μmol/L U73122 for 30 minutes, then stimulated with 50 ng/mL VEGF. Percentage of podosome positive cells (%) was determined. (#P<0.05 vs no VEGF treatment control; &P<0.05 vs no U73312 treatment control).
reports: (1) transforming growth factor (TGF)-β-induced podosome formation is dependent on Src and small GTPases,28 (2) Src is required for N-WASP–induced podosome formation in HUVECs,26 and (3) Src and small GTPases are essential for PMA-induced podosome formation in HUVECs.29 To test our hypothesis, we pretreated HUVECs with Src inhibitor PP2 or expressed dominant negative small GTPases (DN-GTPase), before treatment with VEGF, then cells were stained for podosomes. Percentage of podosome positive cells (%) was determined. (P<0.05 vs no VEGF treatment control; #P<0.05 vs no PP2 treatment control.) B, HUVECs were infected with Ad.DN small GTPases (Rho, RhoA, Cdc42) before treatment with VEGF, then cells were stained for podosomes. Percentage of podosome positive cells (%) was determined. (P<0.05 vs LacZ no VEGF treatment control; #P<0.05 vs LacZ VEGF treatment control.) C, At the same time, cell lysates were used for Western blotting to confirm the expression of adenovirus. Actin blots show equal loading. 

Role of GIT1 in VEGF-Induced MMP2 Activation
A major function of podosomes is to regulate MMP activity and ECM degradation. By dual immunofluorescence, we found that MMP2 colocalized with F-actin in VEGF-induced podosomes (Figure 4D through 4F). Because MT1-MMP, which can cleave pro-MMP2 into active MMP2, has been shown to colocalize with podosomes in ECs, we also analyzed the presence of MT-MMP1 in VEGF-induced podosomes, and observed significant colocalization (Figure 4J through 4L).

To explore the role of GIT1 in MMP2 activation, we analyzed the effect of GIT1 depletion on VEGF-induced gelatinase activity. Both culture supernatants and cell lysates were run on gelatin-containing polyacrylamide gels. After treatment with VEGF, active MMP2 was detected in both supernatants and cell lysates (Figure 5A). GIT1 siRNA significantly decreased MMP2 activation induced by VEGF (Figure 5A). Because GIT1 siRNA strongly inhibited VEGF-induced podosome formation (Figure 1B), we propose that GIT1 mediates MMP activation by regulating podosome formation.

Role of GIT1 in VEGF-Induced ECM Degradation
Proteolytic activity is associated with podosomes in v-Src-transformed fibroblasts, tumor cells, osteoclasts, and ECs.30,31 To determine whether VEGF-induced podosomes in ECs are able to locally degrade ECM, we performed an in vitro ECM degradation assay as described previously.24 As shown in Figure 5B, after treatment with VEGF, cells displayed podosomes assayed by F-actin. ECM degradation, visualized as dark areas in the fluorescent ECM (FITC-gelatin), colocalized with podosomes (red fluorescence). To explore the role of GIT1 in ECM degradation, we performed the same experiment after cells were treated with GIT1 siRNA. As shown in Figure 5C, GIT1 siRNA dramatically decreased ECM degradation by 48±6%. These results suggest GIT1 is required for VEGF-induced ECM degradation by regulating podosome formation.

GIT1 Mediates VEGF-Induced EC Migration by Affecting Podosome Formation
We previously showed that GIT1 was required for cell migration of vascular smooth muscle cells (VSMCs), HEK293 cells, and HeLa cells.12 Recent studies of podosomes in ECs suggested a key role for podosomes in migration, tissue invasion, and ECM degradation.26,28 To study the role of GIT1 in EC migration, we used a scratch wound healing assay and depleted GIT1 with siRNA. We found that VEGF induced a significant increase in the recovered area from 22±1% to 47±2% in control siRNA transfected HUVECs (Figure 6B). Transfection with GIT1 siRNA completely inhibited cell migration with a recovered area of only 20±3% (Figure 6B). Immunostaining for F-actin showed that the migrating cells in the control siRNA group were podosome positive. In contrast, the cells in the GIT1 siRNA group were podosome negative (Figure 6C). These results suggest that GIT1 mediates EC migration in response to VEGF by enhancing podosome formation.

Discussion
The major finding of this study is that GIT1 is a key mediator of VEGF stimulated podosome formation in endothelial cells. Specifically, we define a novel role for GIT1 to mediate EC migration and ECM degradation by activating PLCγ, and regulating podosome formation. GIT1 has been previously shown to regulate migration of SMCs and HEK293 cells.32–34 We propose that VEGF stimulates GIT1-PLCγ translocation
to the site where podosomes will form, and then recruits Src, which phosphorylates and activates PLCγ. Functionally, GIT1 mediates cell invasion and migration by increasing podosome formation and ECM degradation. Evidence to support this concept includes: (1) GIT1 knockdown inhibits EC podosome formation induced by VEGF (Figure 1); (2) GIT1 colocalizes with podosomes induced by VEGF (Figure 1); (3) PLCγ colocalizes with podosomes induced by VEGF (Figure 2); (4) Inhibiting PLCγ with U73122 blocks podosome formation induced by VEGF (Figure 2); (5) VEGF-induced podosome formation is also dependent on Src and small GTPase (Figure 3); (6) Downregulation of GIT1 by siRNA significantly inhibited VEGF-induced MMP activation and ECM degradation (Figures 4 and 5); and (7) GIT1-mediated VEGF-induced cell migration correlated with podosome formation (Figure 6).

PLCγ has been reported to associate with GIT1-βPIX complex (p21-activated kinase interacting exchange factor), which requires both GIT1 and β-PIX. Tyrosine phosphorylation of the βPIX/GIT1 complex is essential for the interaction with PLCγ, the subsequent activation of PLCγ, and the progression to an elongated cell morphology.
Depletion of βPIX, GIT1, or PLCγ shows that all 3 components of the complex are necessary to promote cell spreading, and overexpression of individual components is not sufficient to replace deficiencies in the other components of the PLCγ/GIT1/βPIX complex. However, constitutively active forms of Cdc42 or Rac1 were able to rescue the elongation of these cells, suggesting that PLCγ, with complexes containing GIT1 and βPIX, is essential for cell spreading and motility by activating Cdc42 and Rac1. Data from several labs, including ours, demonstrate important roles for at least 3 small GTPases in podosome formation (Figure 3). The apparent redundancy in effects of inhibiting a single GTPase (Figure 3) limits our ability to comment on the specific and hierarchical nature of their roles in podosome formation. We can only suggest that there are likely interdependent pathways for the small GTPases. In contrast, we show that both GIT1 and PLCγ localize in podosomes (Figures 1 and 2) and are required for VEGF-induced podosome formation (Figures 1 and 2). Therefore we propose that PLCγ is a critical component of a PLCγ-GIT1-PIX complex that mediates activation of small GTPases and is required for podosome formation. The present study demonstrates that VEGF increases PLCγ activation, which is GIT1 dependent. We previously showed that GIT1-mediated activation of PLCγ by angiotensin II and epidermal growth factor in SMCs, which is dependent on GIT1 tyrosine phosphorylation via c-Src. Therefore, we propose that VEGF-stimulated Src-dependent tyrosine phosphorylation of GIT1 creates a scaffold to mediate localization and activation of PLCγ, regulation of podosome formation, and cell migration by stimulating small GTPases.

Several small GTPases have been reported to mediate podosome formation in different cell types, and Rac1 had the greatest effect (Figure 3). It is possible that the Rac1 had the greatest effect (Figure 3). It is possible that the Rac1 had the greatest effect (Figure 3). It is possible that the Rac1 had the greatest effect (Figure 3). It is possible that the Rac1 had the greatest effect (Figure 3). It is possible that the prominent role for Rac1 depends on PLCγ-mediated calcium dependent events, because PLCγ has been reported to regulate cell spreading by increasing intracellular calcium. Specifically, calcium-activated proteases, calpains, associate with βPIX, and cleavage of βPIX by calpains is an early event required for Rac activation in some cellular systems. Therefore, GIT1 may act as a scaffold to link PLCγ with βPIX, which is small GTPase GEF. The role of PLCγ in the complex could be to increase local calcium concentration which causes calpain activation and subsequent Rac1 stimulation by the GEF function of βPIX.

PIX has been shown to play a central role in podosome formation, dependent in part on its GEF activity. A PIX–PAK complex was shown to function both upstream and downstream of small GTPases and generate localized feedback loops that could regulate podosome formation. Our laboratory previously showed that GIT1 interacted with MEK1 directly and acted as a scaffold to mediate ERK1/2 activation in focal adhesions. Recently, a MEK1–ERK1/2–caldesmon signaling cascade was shown to regulate PKC-mediated podosome dynamics in A7r5 cells. Based on these data we suggest that GIT1 serves as a scaffold to facilitate the localization and activation of PLCγ, small GTPases, PI(3)K, and ERK1/2, thereby promoting podosome formation.

Besides podosomes, filopodia and lamellipodia are also important structures for endothelial cell migration and angiogenesis. Recently, bone morphogenetic protein-6 was shown to be a potent stimulator of angiogenesis by regulating filopodial assembly in endothelial tip cells. Specifically these authors found that myosin-X translocated into filopodia, stimulated filopodial motility, and increased EC migration and angiogenesis. GIT1 may also regulate lamellipodia and filopodia formation by mediating Rac activation attributable to the Arf-GAP activity of GIT1. Our data demonstrate that...
GIT1 is essential for podosome formation by mediating PLCγ activation. These data suggest that GIT1 may be involved in functions of several macromolecular structures that regulate EC migration and angiogenesis.

In summary, we showed that a c-Src–GIT1–PLCγ signaling pathway is required for VEGF-mediated podosome formation and cell migration. Because podosomes play an important role in cell invasion and migration, these findings suggest a novel function for GIT1 in ECs as a mediator of angiogenesis and tissue remodeling.

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Disclosures

None.

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