S1P induces FA remodeling in human pulmonary endothelial cells: role of Rac, GIT1, FAK, and paxillin

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Shikata, Yasushi, Konstantin G. Birukov, and Joe G. N. Garcia. S1P induces FA remodeling in human pulmonary endothelial cells: role of Rac, GIT1, FAK, and paxillin. J Appl Physiol 94: 1193–1203, 2003. First published December 13, 2002; 10.1152/japplphysiol.00690.2002.—Sphingosine 1-phosphate (S1P) enhances human pulmonary endothelial monolayer integrity via Rac GTPase-dependent formation of a cortical actin ring (Garcia et al. J Clin Invest 108: 689–701, 2001). The mechanisms underlying this response are not well understood but may involve rapid redistribution of focal adhesions (FA) as attachment sites for actin filaments. We evaluate the effects of S1P on the redistribution of paxillin, FA kinase (FAK), and the G protein-coupled receptor kinase-interacting proteins (GITs). S1P induced Rac GTPase activation and cortical actin ring formation at physiological concentrations (0.5 µM), whereas 5 µM S1P caused prominent stress fiber formation and activation of Rho and Rac GTPases. S1P (0.5 µM) stimulated the tyrosine phosphorylation of FAK Y576, and paxillin was linked to FA disruption and redistribution to the cell periphery. Furthermore, S1P induced a transient association of GIT1 with paxillin and redistribution of the GIT2-paxillin complex to the cell cortical area without affecting GIT2-paxillin association. These results suggest a role of FA rearrangement in S1P-mediated barrier enhancement via Rac- and GIT-mediated processes.

Rho; human pulmonary endothelium; barrier function; cytoskeleton

AN INCREASE IN VASCULAR PERMEABILITY is a main feature of inflammation and an essential component of tumor metastasis, angiogenesis, and atherosclerosis. Proteins and lipids released after platelet activation enhance the integrity of the microcirculation in vivo and in vitro (13, 27), whereas a reduction in circulating platelets in humans accelerates capillary leakage and tissue edema formation (20, 34). Impaired endothelial barrier function can be reversed by platelet infusions or systemic administration of platelet-released products (22).

Several platelet-derived lipids have been shown to be important signaling molecules that exert their effects by inducing endothelial cell activation (8). The platelet-released phospholipid sphingosine 1-phosphate (S1P) is a remarkably effective endothelial cell agonist that induces proliferation, calcium mobilization, adhesion molecule expression, and suppression of apoptosis (1, 8, 14, 16, 18). S1P is released by stimulated platelets to bind to G protein-coupled receptors, which are members of the endothelial differentiation gene family of receptors (18, 19, 42). Although the serum and plasma concentrations of S1P vary depending on the method used to measure them (23, 24, 46), S1P is assumed to be present in nanomolar to micromolar concentrations in human serum (1, 25).

We previously reported that the barrier-protective effect of S1P on human pulmonary endothelial monolayers is associated with the formation of a prominent cortical actin ring and showed a key role for the small GTPase Rac in this process (11). However, the exact mechanism by which the cortical actin ring interacts with membrane adhesion proteins to enhance the endothelial cell barrier remains to be elucidated. Recent findings suggest that assembly and disassembly of focal adhesions (FA) result in stress fiber formation and displacement, respectively (29), with phosphorylation of the FA kinase (FAK) potentially modulating increased endothelial cell-matrix adhesion. Paxillin, a major component of FA, is a multidomain adapter protein containing binding sites for various signaling molecules and structural proteins (3, 28, 37, 38, 43, 45). Paxillin facilitates signal transduction from the extracellular matrix and receptor-dependent agonists by recruiting specific molecules to FA, whereas the phosphorylation status of paxillin is believed to be important in determining paxillin binding partners (2, 5, 32, 36). Within the paxillin structure is an FAK docking site, and Y118 is a major site of FAK-catalyzed phosphorylation (2, 30, 34). It has been suggested that FAK activation might promote the disassembly of FA (9, 17, 26, 30), inasmuch as fak−/− knockout cell lines or transient transfection studies demonstrated FAK involvement in FA disassembly and redistribution (17, 26). Small GTPases (Rac and Rho) also regulate actin cytoskeletal remodeling and FA dynamics via ADP-ribosylation factor GTPase activation factors (ARF GAPs) (39), which interact with several signaling and cytoskeletal proteins, including paxillin. Among ARF GAP proteins, G protein-coupled receptor kinase-inter-
acting protein (GIT) 1 and paxillin kinase linker or GIT2 (PKL/GIT2) bind with paxillin directly and participate in signaling events at FA (21, 39, 44, 47). Furthermore, GIT1 may be engaged in the regulation of directional cell motility and disassembly of Rho-containing FA through displacement of paxillin (47). Although these results strongly suggest the important role of paxillin, FAK, and GITs in the process of redistribution of FA, their involvement in the formation of a prominent cortical actin ring after S1P stimulation is unclear.

We have studied S1P-induced activation of the FA proteins paxillin and FAK and investigated the interaction of paxillin and FAK with the regulatory small GTPases GIT1 and GIT2. Our results indicate that these proteins are active participants of FA assembly and demonstrate the novel interaction of FA proteins with members of the regulatory small GTPases. These studies extend our understanding of the potential mechanisms involved in S1P-induced FA remodeling and endothelial cell barrier enhancement.

MATERIALS AND METHODS

Reagents and antibodies. Chemicals and reagents, including synthetic S1P and phosphatase inhibitor cocktail set, were obtained from Sigma Chemical (St. Louis, MO), unless noted otherwise. Fetal bovine serum (FBS) was obtained from American Type Culture Collection (Manassas, VA). Cell culture medium (EBM-2) and growth supplements were obtained from Clonetics (Walkersville, MD). Alexa Fluor 488 anti-mouse IgG antibody and Texas red-phalloidin were purchased from Molecular Probes (Eugene, OR). The Rho activation assay kit (rabbit polyclonal anti-Rho antibody and glutathione S-transferase-tagged fusion protein corresponding to Rhotekin Rho-binding domain bound to glutathione-agarose), the Rac activation assay kit [mouse monoclonal anti-Rac antibody and p21-activated kinase (PAK)-1 (PAK-1) bound to agarose], mouse monoclonal anti-FAK antibody, and rabbit polyclonal anti-FAK (pY397) phosphospecific antibody were obtained from Upstate Biotechnology (Lake Placid, NY). Mouse monoclonal anti-GIT1, GIT2/PKL, Crk, paxillin, and paxillin Y118 phosphospecific antibodies were obtained from Transduction Laboratories (Lexington, KY). Rabbit polyclonal anti-FAK Y576 and Y925 phosphospecific antibodies were purchased from BioSource International (Camarillo, CA). Horseradish peroxidase (HRP)-linked anti-mouse and -rabbit IgG antibodies and HRP Western blot detection kit were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). The protease inhibitor cocktail set was obtained from Calbiochem. Y-27632, AG-1433, and vascular endothelial growth factor (VEGF) receptor (VEGFR) tyrosine kinase inhibitor were purchased from Calbiochem (La Jolla,
Polyvinylidene fluoride (PVDF) membrane was purchased from Millipore (Bedford, MA).

Human pulmonary artery endothelial cell culture. Human pulmonary artery endothelial cells (HPAEC) were obtained from Clonetics and cultured in EBM-2 complete medium containing 10% FBS. Endothelial cell cultures were maintained at 37°C in a humidified atmosphere and grown to contact-inhibited monolayers with typical cobblestone morphology. Cells from each primary flask were detached with 0.05% trypsin, resuspended in fresh culture medium, and passaged into 100-mm² dishes for Rho and Rac activity assay, 60-mm² dishes for Western blot and immunoprecipitation, or 12-well plates (with cover glasses) for immunofluorescent analysis. HPAEC were used at passages 5–8.

Immunofluorescence microscopy. HPAEC grown on gelatinized coverslips were rendered quiescent in EBM-2 containing 1% FBS for 20 h and incubated with S1P or vehicle control. If necessary, cells were preincubated with 5 μM Y-27632 for 30 min before stimulation. HPAEC were fixed in 3.7% paraformaldehyde in PBS for 15 min, washed three times with PBS, permeabilized with 0.25% Triton X-100 and 0.1% Tween 20 containing Tris-buffered saline (TBS-T) for 15 min, and blocked with 2% BSA in TBS-T for 30 min. Incubations with primary antibodies of interest were performed in blocking solution (2% BSA in TBS-T) for 1 h at room temperature. After three washes with TBS-T, cells were incubated with appropriate secondary antibodies conjugated to immunofluorescent dyes (Alexa 488 for green fluorescence or Alexa 546 for red fluorescence) in blocking solution for 1 h at room temperature. Actin filaments were visualized by staining cells with Texas red-conjugated phalloidin for 1 h at room temperature. After three washes with PBS, the coverslips were mounted using the Slow Fade kit (Molecular Probes). Analyses of immunofluorescent staining were performed using a microscope (Eclipse TE 300, Nikon) with a 60× objective lens and a digital camera (model DKC 5000, Sony).

Rac and Rho activation assay. Rac and Rho GTPase activation was assessed as previously described (11). Briefly, endothelial cells grown in 100-mm dishes and rendered quiescent in EBM-2 containing 1% FBS for 20 h were incubated with 0.5 or 5 μM S1P in the same medium for the indicated time periods. After they were washed with PBS, the cells were lysed in 500 μL of 1% SDS sample buffer (MLB) containing 25 mM HEPES, pH 7.5, 150 mM NaCl, 1% NP-40, 10 mM MgCl₂, 1 mM EDTA, 2% glycerol, 0.2 mM vanadate, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and 1:200 dilution of phosphatase inhibitor cocktail, homogenized by pipetting, and then briefly centrifuged to remove cell debris. For Rac activation assay, 200 μL of supernatants were incubated with 10 μg of agarose-conjugated p21-binding domain of human PAK-1 (residues 67–150) for 30 min. The agarose beads were washed three times with MLB, resuspended in 20 μL of 3× SDS sample buffer, and boiled for 5 min. The samples were then subjected to electrophoresis. Proteins were transferred to polyvinylidene difluoride membranes, blotted with antiphosphotyrosine monoclonal antibody (PY20), and then reprobed with anti-FAK (A) or antipaxillin (B) monoclonal antibody. Signal intensity of protein tyrosine phosphorylation was evaluated using Image Quant software. Three experiments were performed independently for FAK and paxillin stimulation with S1P, and Student’s t-test was used for statistical analysis. IP, immunoprecipitation; WB, Western blot.

Fig. 2. S1P stimulates tyrosine phosphorylation of focal adhesion (FA) kinase (FAK) and paxillin. Quiescent HPAEC were challenged with 0.5 μM S1P for 0–60 min and then lysed in cell lysis buffer. FAK and paxillin were immunoprecipitated and subjected to electrophoresis. Proteins were transferred to polyvinylidene difluoride membranes, blotted with antiphosphotyrosine monoclonal antibody (PY20), and then reprobed with anti-FAK (A) or antipaxillin (B) monoclonal antibody. Signal intensity of protein tyrosine phosphorylation was evaluated using Image Quant software. Three experiments were performed independently for FAK and paxillin stimulation with S1P, and Student’s t-test was used for statistical analysis. IP, immunoprecipitation; WB, Western blot.

A

IP: FAK, WB: PY20

0.5 μM S1P

0 10 30 60

(min)

% of FAK

PHOSPHORYLATION

TIME after S1P (min)

* p<0.05

(*)

B

IP: Paxillin, WB: PY20

0.5 μM S1P

0 10 30 60

(min)

% of Paxillin

PHOSPHORYLATION

TIME after S1P (min)

* p<0.05

** p<0.02

(*)

(*)

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Electrophoresis in 12.5% gel and transferred to PVDF membrane. Protein was detected by Western blot using mouse monoclonal anti-Rac antibody. To determine the extent of Rho activation, 200 μl of supernatants were incubated with 20 μg of Rhotekin Rho-binding domain (residues 7–89) bound to glutathione-agarose for 45 min. The glutathione-agarose beads were washed three times with MLB and resuspended in 20 μl of 3× SDS sample buffer. The samples were then subjected to electrophoresis in 12.5% gel and transferred to PVDF membrane. Rho protein was detected by Western blot using rabbit polyclonal anti-Rho antibody. For total Rho and Rac detection, 5 μl of the original cell lysates were subjected to electrophoresis in 12.5% gel and recognized by Western blot using anti-Rho and anti-Rac antibody, respectively.

**Western blot analysis.** HPAEC grown on 60-mm2 dishes were rendered quiescent in EBM-2 containing 1% FBS for 20 h and then stimulated with 0.5 μM S1P dissolved in the same medium for the indicated time periods. After the cells were washed briefly with PBS, they were lysed with 300 μl of cell lysis buffer containing 10 mM Tris (pH 7.4), 1% Triton X-100, 0.5% NP-40, 150 mM NaCl, 1 mM EDTA, 0.2 mM EGTA, 0.2 mM vanadate, 0.2 mM PMSF, and 0.5% phosphatase inhibitor cocktail for each dish. Total cell lysates were cleared by centrifugation and boiled with the same amount of 3× SDS sample buffer for 5 min, and each 15 μl of lysates were then subjected to 7.5% SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred to PVDF membranes by electrotransfer. The blots were subsequently blocked with 5% BSA in PBS containing 0.1% Tween 20 (PBS-T) at room temperature for 1 h and then incubated at 4°C overnight with primary rabbit polyclonal anti-FAK Y576, Y576, and Y925 phosphospecific antibodies (1:1,000 dilution). After the membrane was washed three times for 10 min with PBS-T, it was incubated with 1:3,000 dilution of HRP-linked anti-rabbit IgG secondary antibody at room temperature for 1 h. The blots were then visualized with the enhanced chemiluminescence Western blot detection system. To reprobe membranes with anti-FAK antibody, membranes were incubated in reprobing buffer containing 62.5 mM Tris (pH 6.8), 2% deoxycholate, and 100 mM mercaptoethanol at 4°C for 30 min. After they were washed with PBS-T four times (each for 10 min), membranes were incubated with anti-FAK antibody (1:1,000 dilution) at room temperature for 1 h and then visualized by enhanced chemiluminescence.

**Immunoprecipitation and coimmunoprecipitation analysis.** For immunoprecipitation, 70 μl of cell lysates were diluted with 100 μl of the same buffer, incubated with 2 μg of the appropriate antibody (antipaxillin or anti-FAK antibody) at 4°C for 1 h, and then incubated with protein G-agarose 4B for 1 h. Agarose beads were then collected by centrifugation, washed three times with the same buffer, resuspended in 20 μl of 3× SDS sample buffer, and boiled for 5 min. Proteins were separated and incubated with antiphosphotyrosine antibody (PY20, 1:1,000 dilution) as described for Western blot analysis. For coimmunoprecipitation analysis, cell lysis buffer was replaced with coprecipitation buffer containing 50 mM Tris (pH 7.8), 1% NP-40, 20 mM EDTA, 0.2 mM vanadate, and 0.2 mM PMSF (35); paxillin was immunoprecipitated; and proteins were separated as described for Western blot analysis. The resulting membranes were blotted with appropriate antibodies [anti-GIT1 (1:250 dilution), anti-GIT2 (1:500 dilution), and anti-FAK (1:1,000 dilution) antibodies], and the amount of coprecipitated proteins was analyzed using Image Quant software.

**Statistical analysis.** Statistical analysis was performed with Student’s t-test.

**RESULTS**

Concentration-dependent Rac and Rho activation in HPAEC stimulated by S1P. Studies using fibroblast cultures suggest that S1P may be an important agonist for Rho-dependent stress fiber formation (41). In con-

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**Fig. 3.** FAK Y576 is a major tyrosine residue phosphorylated after S1P stimulation. Total cell lysates prepared from S1P-stimulated HPAEC were probed with 3 site-specific antiphospho-FAK antibodies (A, B, and C, respectively). Repробing with anti-FAK antibody revealed equal amounts of total protein loadings in each lane. Results are representative of 3 independent experiments.
In contrast, our recent work in human and bovine endothelial cells revealed strong evidence of S1P-induced Rac activation without stress fiber formation. To investigate this differential stimulation of Rho family GTPases, we explored the concentration-dependent effect of S1P on Rac and Rho activation. Quiescent HPAEC were treated with a physiological dose of 0.5 μM or a 10-fold-higher dose (5 μM) of S1P for 10 min. Similar to our previous reports (11), Fig. 1A demonstrates the preferential Rac activation in HPAEC stimulated with 0.5 μM S1P, with little evidence of Rho GTPase activation. In contrast, increased S1P concentrations (5 μM) induced Rac and Rho GTPase activation. Inasmuch as Rac and Rho are prominent regulators of the actin cytoskeleton, we next investigated the concentration-dependent effect of S1P stimulation (0.5 or 5 μM) on actin cytoskeleton reorganization and stained HPAEC monolayers to visualize F actin (10 min). As shown in Fig. 1B and consistent with the differential Rac and Rho stimulation, 0.5 μM S1P induced the enhancement of actin staining within the cortical ring, whereas 5 μM S1P produced diffuse and prominent stress fiber formation with evidence of paracellular gap formation. To investigate whether Rho is involved in the cortical

Fig. 4. S1P promotes dissociation of FAK with paxillin and transient association of G protein-coupled receptor kinase-interacting protein (GIT1) with paxillin. HPAEC culture was stimulated with 0.5 μM S1P for 0–60 min, and cell lysates were subjected to coprecipitation with antipaxillin antibody. A: FAK, GIT1, and GIT2 (A1, A2, and A3, respectively) in immunoprecipitates. Reprobing membranes with antipaxillin antibody revealed equal amounts of paxillin immunoprecipitated (A4). Blots are representative of 3 independent experiments. Amounts of immunoprecipitated proteins over time were quantified and analyzed statistically. B: amount of coimmunoprecipitated proteins (GIT1, GIT2, and FAK) at 0, 10, 30, and 60 min; value under nonstimulated condition was assumed to be 100%. Values are means ± SD. *P < 0.05.
actin ring formation induced by 0.5 μM S1P, the HPAEC monolayer was pretreated with Y-27362, a specific inhibitor of Rho kinase, which is a downstream effector of Rho, and stimulated with 0.5 μM S1P. Y-27362 did not inhibit cortical actin ring formation, suggesting that Rho is not involved in this event (Fig. 1B).

S1P stimulates tyrosine phosphorylation of FAK and paxillin. The mechanisms by which barrier-regulatory agonists produce alterations in vascular permeability involve an intimate but poorly understood interaction between the actin cytoskeleton and membrane adhesive complexes such as the adherens junction and FA complex (6). To explore the effect of S1P on FA components, confluent HPAEC were stimulated with 0.5 μM S1P, and activation of protein tyrosine phosphorylation was evaluated by immunoprecipitation of FAK and paxillin followed by Western blot analysis with antiphosphotyrosine antibody (PY20) (see MATERIALS AND METHODS). S1P challenge at concentrations that selectively activate Rac GTPase induced the sustained tyrosine phosphorylation of FAK and paxillin, which reached maximum levels by 30 min and remained elevated for 60 min (Fig. 2). Several FAK tyrosine residues display specific functions after phosphorylation. Y397 is the major autophosphorylation site and provides the docking site for Src, phosphatidylinositol 3-kinase, phospholipase C-γ, and Grb7 (30, 31), whereas Y576 is located in the activation loop of the catalytic domain and is the regulatory site of phosphorylation that enhances catalytic activity (29). Phosphorylation of Y925 represents the FAK docking site for the Grb2 SH2 domain, which is associated with activation of the mitogen-activated protein kinase pathway by FAK (33). To determine the major site of FAK tyrosine phosphorylation, total cell lysates were subjected to electrophoresis and blotted with site-specific antiphospho-FAK antibodies (Fig. 3). S1P failed to induce an increase in FAK phosphorylation at Y397 or Y925 (Fig. 3, A and C); however, a substantial increase in FAK phosphorylation at Y576 was observed (Fig. 3B), consistent with significant FAK activation and enhanced catalytic activity.

S1P-induced alterations in paxillin association with FAK, GIT1, and GIT2. We next explored whether S1P alters paxillin association with activated FAK and
ARF GAP proteins such as GIT1 and GIT2. Confluent HPAEC were stimulated with 0.5 μM S1P, and immunoprecipitation of paxillin was performed. Under basal conditions, paxillin appears to be stably associated with FAK and GIT1; however, S1P challenge produced clear dissociation of FAK from paxillin in a time-dependent manner (Fig. 4, A1 and B), suggesting the disassembly of FAK- and paxillin-containing FA. S1P induced a transient increase in paxillin-GIT1 association that was maximal at 10 min (Fig. 4, A2 and B). In contrast to GIT1, the amount of GIT2 that coprecipitated with paxillin was not significantly altered by S1P challenge (Fig. 4, A3 and B).

S1P-induced redistribution of FAK and paxillin to the cell cortical area. The redistribution of FAK and paxillin was also monitored by immunofluorescent microscopy (see MATERIALS AND METHODS). In quiescent cells, FAK (Fig. 5A) stains diffusely in the cytoplasm as well as at sites of stress fiber attachment to the randomly arranged FA. Paxillin staining was similar to FAK staining in nonstimulated cells (Fig. 6A) and also associated with randomly distributed FA. The addition of 0.5 μM S1P induced a dramatic redistribution of FAK and paxillin to the cell periphery (Figs. 5B and 6B), which occurred in a time frame consistent with the dramatic S1P-induced enhancement of the cortical actin ring (Fig. 5E) and persisted for up to 30 min after S1P challenge (Figs. 5C and 6C). Examination of the intracellular localization of phosphopaxillin after S1P stimulation using antiphosphopaxillin Y118 antibody (Fig. 6, D–F) revealed immunofluorescent staining in a pattern similar to total paxillin distribution at 10 min (Fig. 6E). However, phosphopaxillin staining appeared within the cortical area of the cell (Fig. 6F) at 30 min. These results suggest that translocation of activated paxillin (phosphopaxillin) results in formation of a new protein complex independently of FAK at later time points (30 min), and the dissociation of FAK and paxillin detected by coimmunoprecipitation analysis (Fig. 4A) appears to support this observation.

S1P induces redistribution of GIT1 and GIT2. As mentioned previously, the small GTPases Rac and Rho play an important role in regulation of cytoskeletal remodeling and cell contact rearrangements (11, 41). Recently, a new group of small GTPase regulators, GIT proteins, have been described; they may participate in...

Fig. 6. Effect of S1P on intracellular distribution of paxillin and phosphopaxillin. HPAEC were treated with 0.5 μM S1P and stained with antipaxillin monoclonal antibody (A–C) and antiphosphopaxillin Y118 antibody (D–F). Immunofluorescent staining exhibited a similar distribution of paxillin as noted for FAK (Fig. 5) after 10 and 30 min of incubation with S1P (B and C). However, after 30 min of incubation (0.5 μM S1P), phosphopaxillin staining was observed as dotlike structures, which decreased in number centrally but were markedly enhanced at the cell periphery (C). Arrows, paxillin and phosphopaxillin redistribution to the cell periphery after S1P stimulation (B and C, and E and F, respectively).
the disassembly of preexisting cell adhesions (21, 39, 44, 47). We examined the role of the two members of the GIT family, GIT1 and GIT2, in S1P-mediated FA remodeling. S1P stimulation enhanced obvious, albeit transient, localization of GIT1 to the cell cortical area, which peaked at 10 min (Fig. 7B) and returned to the level observed in quiescent cells by 60 min (Fig. 7D). These results are consistent with the GIT1/paxillin coimmunoprecipitation data (Fig. 4B). A related member of the GIT family, GIT2, stained diffusely in quiescent HPAEC (Fig. 7E) and also redistributed to the cell cortical area within 10 min after incubation with S1P (Fig. 7F). Interestingly, this localization was observed up to 60 min after S1P challenge (Fig. 7H).

**DISCUSSION**

We have reported that the lung vascular barrier-protective effect of 0.1–1 μM S1P requires Rac GTPase-dependent formation of a prominent cortical actin ring (11). Rac involvement in barrier-protective cytoskeletal remodeling was further supported by overexpression of

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**Fig. 7.** S1P induces redistribution of GIT1 and GIT2. HPAEC were incubated with 0.5 μM S1P for 0–60 min and then subjected to immunofluorescent staining with anti-GIT1 or anti-GIT2 monoclonal antibodies. S1P enhanced transient localization of GIT1 within the cell cortical area, which peaked at 10 min (B) and returned to the basal punctate pattern of staining by 60 min (A and D). GIT2 was stained diffusely in quiescent HPAEC (E) and redistributed to the cell cortical actin ring after 10 min of incubation with S1P (F). Unlike GIT1, this peripheral redistribution of GIT2 staining persisted for up to 60 min (H).
the constitutively active Rac construct, which resulted in a similar enhancement of the cortical actin ring (11) and inhibition of shear stress-induced cortical actin remodeling by a Rac dominant-negative construct (4). In contrast, exposure of a fibroblast cell line to elevated S1P doses (5 μM) induced significant stress fiber formation, which was prevented by microinjection of the Rho inhibitor botulinum C3 transferase exoenzyme (41). Vouret-Craviari et al. (40) also reported that treatment of human umbilical vein endothelial cells with 500 nM S1P induced actin translocation to a cell periphery within 1 min without any activation of Rho. Together with the previous report of Rho activation with 5 μM S1P in fibroblasts (41), these findings strengthen our hypothesis that S1P can induce dose-dependent activation of Rac and Rho, resulting in the translocation of actin to a cell periphery and stress fiber formation, respectively.

We attempted to explore the basis for these differences and demonstrated concentration-dependent S1P effects on the activation of these Rho family GTPases and actin remodeling. Low concentrations of S1P (0.5 μM) induced preferential Rac activation with enhancement of cortical actin cytoskeleton and barrier protection, whereas elevated S1P concentration (5 μM) induced predominant Rho activation and significant stress fiber formation (Fig. 1).

One possible mechanism underlying the differential activation of small GTPase proteins induced by 0.5 and 5 μM S1P is the transactivation of receptors for other agonists. The higher concentration (5 μM) of S1P might stimulate some unknown receptor(s) leading to Rho GTPase activation, whereas the lower concentration (0.5 μM) might not be capable of this signal induction. It has been reported that S1P induces CrkII phosphorylation via transactivation of VEGFR in human umbilical vein endothelial cells (7). CrkII is an adapter protein containing an SH2 domain that can interact with paxillin, suggesting its role in FA remodeling and actin redistribution (7). Migration of the phosphorylated CrkII on SDS-polyacrylamide gel is slower than migration of the nonphosphorylated form, and this mobility change has been used for the determination of CrkII phosphorylation (15). In HPAEC, 20 μM AG-1433 (VEGFR-2 inhibitor) and 5 μM VEGFR tyrosine kinase inhibitor (VEGFR-1 and -2 inhibitor) inhibited the VEGF-induced mobility change of Crk on SDS-polyacrylamide gel. Furthermore, 0.5 and 5 μM S1P induced mobility change of Crk on SDS-polyacrylamide gel, and these changes were inhibited by pretreatment of cells with 20 μM AG-1433 and 5 μM VEGFR tyrosine kinase inhibitor (unpublished observations). These results suggest that CrkII does not participate in the differential actin redistribution by the different concentrations of S1P, whereas it is possible that the transactivation of receptors other than VEGFR is induced by 5 μM S1P.

Potential mechanisms of endothelial cell barrier dysfunction include increases in contractile forces, decreases in intercellular junctional connections, and reductions in endothelial cell-extracellular matrix adhesive forces (12) associated with dramatic cytoskeletal rearrangements (6, 9). Several reports suggest that FA assembly promotes stress fiber formation,
whereas FA disassembly leads to stress fiber dissolution (29). These findings strongly suggest the role of FA remodeling in the regulation of endothelial cell barrier function. Paxillin, a major component of FA, is a multidomain adapter protein containing binding sites for various signaling molecules and structural proteins (3, 37, 38, 43, 47). Paxillin plays a pivotal role in facilitating signal transduction from the extracellular matrix and soluble agonists by recruiting specific molecules to FA, with the phosphorylation status of paxillin apparently important for interaction with cytosolic binding partners (2, 3, 32, 36). Paxillin contains a docking site for FAK, and Y118 is a major site of FAK-catalyzed phosphorylation (2, 3, 36). FAK is reported to promote the disassembly of FA in fibroblast models (8, 17, 26, 30), and gene knockout experiments using fak–/– fibroblast cell lines further demonstrate an important role of FAK in FA disassembly (17, 26).

We have demonstrated S1P-induced tyrosine phosphorylation of FAK and paxillin (Fig. 2), with FA phosphorylation occurring at Y576, a site within the activation loop of the catalytic domain that enhances FAK catalytic activity (Fig. 3B). The major FAK auto-phosphorylation site, Y297, was modestly phosphorylated in quiescent cells and did not significantly change after S1P challenge (Fig. 3A). Finally, Y295, which represents the FAK docking site for the Grb2 SH2 domain, was not phosphorylated after S1P challenge (Fig. 3C), suggesting that S1P stimulation does not induce the formation of an FAK-Grb2-mitogen-activated protein kinase complex, which is dependent on Y295 phosphorylation. However, activated FAK may clearly be involved in other signaling events (30).

Recently, several ARF GAP proteins have been reported to interact with key signaling and cytoskeletal proteins, including paxillin (21, 39, 44, 47), and to participate in the regulation of cell motility, membrane trafficking, and organelle structure (39). Among these ARF GAP proteins, GIT1 and PKL/GIT2 bind paxillin through defined paxillin-binding sites and participate in signaling events at FA. GIT1 may be engaged in the regulation of directional cell motility and disassembly of Rho-containing FA through displacement of paxillin (47), whereas GIT2 may deliver paxillin to FA and mediate lamellipodia formation through regulation of Rac activity (21, 39). Considering that GIT1 is involved in disassembly of the FA complex and GIT2 is engaged in Rac-containing protein complex formation (39), GITs appear to play a pivotal role in the promotion of cell barrier integrity by S1P.

As noted earlier, S1P stimulation induces a pronounced cytoskeletal remodeling that may serve as a signal to significant remodeling of cell contacts (41). Our results suggest that S1P redistributes FAK and paxillin together with similar patterns at early times (10 min), whereas at later times (30 min), phosphopaxillin (Fig. 6) and GIT2 (Fig. 7) appear in the cell cortical area. Paxillin is required for recruitment of GIT2 to Rac-dependent focal complexes at the cell leading edge (44). Our results providing evidence of a stable association of GIT2 with paxillin (Fig. 4) suggest the formation of Rac-dependent complexes containing paxillin as well as other proteins independently from FAK catalytic activity at the cell cortical area contributing to barrier enhancement.

In summary, we have demonstrated S1P-induced, spatially defined redistribution of FAK and paxillin to the cell cortical area in a time-dependent manner and revealed early FAK/paxillin colocalization followed by dissociation of this complex. Although the precise role of FAK, paxillin, and GIT protein involvement in the barrier-protective effect of S1P on the endothelial cell monolayer remains to be completely elucidated, the results of this study indicate a pivotal role of these molecules in the formation of the cortical actin ring related to endothelial cell barrier enhancement (Fig. 8). Together these results suggest that the sustained duration of the barrier enhancement produced by S1P may involve significant FA remodeling via FAK, PAK, and GIT proteins.

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