

Rac1 Modulates Sphingosine 1-Phosphate-mediated Activation of Phosphoinositide 3-Kinase/Akt Signaling Pathways in Vascular Endothelial Cells*

Received for publication, September 22, 2005, and in revised form, November 3, 2005 Published, JBC Papers in Press, December 8, 2005, DOI 10.1074/jbc.M510434200

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Sphingosine 1-phosphate (S1P) is a platelet-derived sphingolipid that activates G protein-coupled S1P receptors and initiates a broad range of responses in vascular endothelial cells. The small GTPase Rac1 is implicated in diverse S1P-modulated cellular responses in endothelial cells, yet the molecular mechanisms involved in S1P-mediated Rac1 activation are incompletely understood. We studied the pathways involved in S1P-mediated Rac1 activation in bovine aortic endothelial cells (BAEC) and found that S1P-induced Rac1 activation is impaired following chelation of G protein $\beta\gamma$ subunits by transfection of β ARKct. Treatment with the Src tyrosine kinase inhibitor PP2 completely attenuated S1P-mediated Rac1 activation; however, pretreatment of BAEC with wortmannin, an inhibitor of phosphoinositide (PI) 3-kinase, had no effect on Rac1 activation while completely blocking S1P-induced Akt phosphorylation. We used Rac1-specific small interfering RNA (siRNA) duplexes to “knock down” endogenous Rac1 expression and found that siRNA-mediated Rac1 knockdown significantly impaired basal as well as S1P-induced phosphorylation of protein kinase Akt, as well as several downstream targets of Akt including endothelial nitric-oxide synthase and glycogen synthase kinase β . By contrast, S1P-induced phosphorylation of the mitogen-activated protein kinases ERK1/2 was unperturbed by siRNA-mediated Rac1 knockdown. We found that overexpression of the Rac1 guanine nucleotide exchange factor (GEF) Tiam1 markedly enhanced Rac1 activity, whereas a dominant negative Tiam1 mutant significantly attenuated S1P-mediated Rac1 activation. Taken together, these studies identify G protein $\beta\gamma$ subunits, Src kinase and the GEF Tiam1 as upstream modulators of S1P-mediated Rac1 activation, and establish a central role for Rac1 in S1P-mediated activation of PI 3-kinase/Akt/endothelial nitric-oxide synthase signaling in vascular endothelial cells.

Sphingosine 1-phosphate (S1P)² is a platelet-derived sphingolipid that activates a family of G protein-coupled S1P receptors (for review

see Refs. 1 and 2). Activation of S1P₁ receptors in the vascular endothelium triggers the activation of signaling cascades via pertussis toxin-sensitive G proteins (3). S1P₁ receptor-mediated activation of Rac1 GTPase appears to be required for many S1P-mediated cellular responses including proliferation, migration, cytoskeletal organization, trans-monomer permeability, adherens junction assembly, and morphogenesis (4–8). Rac1 is a member of the Rho family of small GTPases and functions as a molecular switch by cycling between an inactive GDP-bound form and an active GTP-bound form (9, 10). Activation of Rac1 is tightly regulated by guanine nucleotide exchange factors (GEFs) that promote dissociation of GDP from Rac1, the rate-limiting step in protein activation (11, 12). Once activated, GTP-bound Rac1 is slowly inactivated through its intrinsic GTPase activity, a process that is enhanced by interactions between Rac1 and specific GTPase-activating proteins (GAPs) (12). Both GEFs and GAPs are themselves activated in response to extracellular stimuli, and stimulus-specific activation of Rac1 by different GEFs may help to confer specificity to Rac1-mediated cellular responses.

There is a complex and incompletely understood relationship between Rac1 and its associated GEFs with inositol phosphates and phosphoinositide (PI) 3-kinases. The GEFs that activate Rho family proteins are characterized by a Dbl homology domain and by a pleckstrin homology domain that can bind phosphatidylinositol 4,5-bis-phosphate (PIP₂) or phosphatidylinositol 3,4,5-triphosphate (PIP₃) (12). Not only are Rac1 GEFs activated by phosphoinositides, but Rac1 itself can interact with and activate both PI 4,5-kinase and PI 3-kinase (13), suggesting a possible feedback loop between Rac1 activity and PIP₂ and PIP₃ synthesis. Furthermore the roles of lipids and lipid kinases in Rac1 signaling appear to be stimulus- and cell type-dependent. For example, we have recently shown that down-regulation of caveolin-1, the key scaffolding protein of caveolae membrane domains, enhances S1P-mediated activation of both Rac1 and PI 3-kinase/Akt pathways (14). These findings suggest the existence of common upstream modulators of S1P-mediated activation of Rac1 and of PI 3-kinase/Akt in endothelial cells. In the present study, we pursued both pharmacological approaches and siRNA-mediated protein knockdown methodologies to study the role of Rac1 in S1P-mediated PI 3-kinase/Akt signaling, and we have identified key pathways involved in Rac1 and Akt activation in response to S1P in endothelial cells.

EXPERIMENTAL PROCEDURES

Materials—Fetal bovine serum (FBS) was purchased from Hyclone (Logan, CT). Cell culture reagents and media were from Invitrogen, Lipofectamine 2000 transfection reagent was from Invitrogen, and FuGENE 6 transfection reagent was from Roche Applied Science. S1P and PP2 were purchased from BioMol (Plymouth Meeting, PA). Wortmannin was from Calbiochem. Polyclonal antibodies directed against

* This work was supported by National Institutes of Health Grants HL46457 and GM36259 (to T. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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² The abbreviations used are: S1P, sphingosine 1-phosphate; siRNA, small interfering RNA; Rac1 siRNA, small interfering RNA targeted to the bovine Rac1 mRNA; BAEC, bovine aortic endothelial cells; PI, phosphoinositide or phosphatidylinositol; eNOS, endothelial nitric-oxide synthase; β ARK, β -adrenergic receptor kinase; GSK3 β , glycogen synthase kinase β ; GEF, guanine nucleotide exchange factor; PAK, p21-activated kinase; ERK, extracellular signal-regulated kinase; VEGF, vascular endothelial growth factor; VEGFR2, VEGF receptor 2; HA, hemagglutinin; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; GST, glutathione S-transferase; ANOVA, analysis of variance.

phospho-eNOS (Ser¹¹⁷⁹), phospho-Akt (Ser⁴⁷³), Akt, phospho-GSK3 β (Ser⁹), phospho-p70S6 kinase (Thr³⁸⁹), p70S6 kinase, phospho-ERK1/2 (Thr²⁰²/Tyr²⁰⁴), and ERK1/2 were from Cell Signaling Technologies (Beverly, MA). Endothelial nitric-oxide synthase (eNOS) monoclonal antibody and glycogen synthase kinase 3 β (GSK3 β) monoclonal antibody were from Transduction Laboratories (Lexington, KY). Polyclonal antibodies for hemagglutinin (HA) epitope, β -adrenergic receptor kinase-1 (BARK-1), and Tiam1 antibody and the monoclonal antibody for VEGFR2 were from Santa Cruz Biotechnology (Santa Cruz, CA). Rac1 monoclonal antibody and Rac activation assay kit was from Upstate Biotechnology (Lake Placid, NY). Super Signal substrate for chemiluminescence detection and secondary antibodies conjugated with horseradish peroxidase were from Pierce. Tris-buffered saline and phosphate-buffered saline (PBS) were from Boston Bioproducts (Ashland, MA). Other reagents were from Sigma.

Plasmids—Full-length wild type murine Akt1 cDNA, epitope-tagged with Myc peptide (Akt-myc) and subcloned into pUSEamp, was from Upstate Biotechnology. cDNA encoding the C-terminal fragment peptide of bovine β -adrenergic receptor kinase-1 (BARKct, described in Ref. 15), subcloned into pRK5 was kindly provided by Dr Robert J. Lefkowitz (Duke University). Wild type Rac1, dominant negative Rac1 mutant (N17Rac1), and constitutively active Rac1 mutant (V12Rac1) cDNAs, epitope-tagged with HA peptide and subcloned into pcDNA3.1+, were obtained from University of Missouri-Rolla cDNA Resource Center. cDNAs encoding full-length wild type human Tiam1 and the GEF inactive mutant Tiam1-QK (Q1191A,K1195A, described in Ref. 16) subcloned into pFlag-CMV2 were kindly provided by Dr Michael Greenberg (Harvard Medical School).

siRNA Preparation—Small interfering RNA duplex oligonucleotides were purchased from Dharmacon, Inc. (Lafayette, CO). We designed a Rac1 siRNA duplex corresponding to bases 78–96 from the open reading frame of the bovine Rac1 mRNA: 5'-UGC GUU UCC UGG AGA AUA UdTdT-3' (14). For targeting of the VEGFR2 receptor (KDR), we designed a duplex siRNA corresponding to bases 199–217 from the open reading frame of bovine VEGFR2 mRNA: 5'-GGU CUC CGU UUA UUG CUU C-dTdT-3'. The RNA sequence used as a negative control for siRNA activity was: 5'-GCG CGC UUU GUA GGA UUC G-dTdT-3'.

Cell Culture and Transfections—Bovine aortic endothelial cells (BAEC) were obtained from Cambrex (Walkersville, MD) and maintained in culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with FBS (10% v/v) as described (17). Cells were plated onto 0.2% gelatin-coated culture dishes and studied prior to cell confluence between passages 5 and 9. In some cultures, BAEC were transfected with indicated plasmids (0.5 μ g/plasmid) using FuGENE 6, following the instructions provided by the supplier, and were analyzed 48 h after transfection. siRNA transfections were carried out as we have described previously in detail (14). BAEC maintained in DMEM/10% FBS were transfected with siRNA (30 nM) using Lipofectamine 2000 (0.15% v/v), following the protocols provided by the manufacturer. Fresh medium was added 5 h post-transfection, and experiments were conducted 48 h after transfection.

Drug Treatment, Cell Lysates, and Immunodetection—Culture medium was changed to serum-free medium, and incubations proceeded overnight prior to all experiments (18, 19).

S1P was solubilized in methanol and stored at -20°C ; the same volume of methanol was used as a vehicle control, and the final concentration of methanol did not exceed 0.4% (v/v). Wortmannin and PP2 were solubilized in dimethyl sulfoxide and kept at -20°C ; where indicated, dimethyl sulfoxide 0.1% (v/v) was used as vehicle control.

After drug treatments, BAEC were washed with PBS and incubated for 10 min in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EDTA, 2 mM Na₃VO₄, 1 mM NaF, 2 μ g/ml leupeptin, 2 μ g/ml antipain, 2 μ g/ml soybean trypsin inhibitor, and 2 μ g/ml lima trypsin inhibitor). Cells were harvested by scraping and then centrifuged for 5 min at 4°C . For immunoblot analyses, 20 μ g of cellular protein was resolved by SDS-PAGE, transferred to nitrocellulose membranes, and probed with antibodies using protocols provided by the suppliers. Densitometric analyses of the Western blots were performed using a ChemImager 4000 (Alpha-Innotech).

Rac Activity Assay—BAEC in 100-mm dishes were stimulated with S1P in the presence or absence of inhibitors, and cells were then washed with ice-cold PBS and lysed in lysis buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 10 mM MgCl₂, 1 mM EDTA, 10% glycerol, 2 mM Na₃VO₄, 1 mM NaF, 2 μ g/ml leupeptin, 2 μ g/ml antipain, 2 μ g/ml soybean trypsin inhibitor, and 2 μ g/ml lima trypsin inhibitor). Pull-down of GTP-bound Rac was performed by incubating the cell lysates with GST fusion protein corresponding to the p21-binding domain of PAK-1 bound to glutathione-agarose (Upstate Biotechnology) for 1 h at 4°C following instructions provided by the suppliers. The beads were washed three times with lysis buffer, and the protein bound to the beads was eluted with Laemmli buffer and analyzed for the amount of GTP-bound Rac by immunoblotting using a Rac monoclonal antibody (Upstate Biotechnology).

Migration Assay—Cell migration was assayed using a Transwell cell culture chamber containing polycarbonate membrane inserts with 8- μ m pore size (Corning Costar Corp.) BAEC were transfected with control or Rac1-specific siRNA and maintained in DMEM supplemented with 0.4% FBS for 18 h before the experiments. Experiments were carried out 48 h after transfection. Polycarbonate filters were coated with 0.2% gelatin. Cells were briefly incubated with trypsin to obtain a single-cell suspension, and 5×10^4 cells in DMEM/0.4% FBS were added to the upper chamber. The bottom chamber was filled with 600 μ l of medium, and the assembly was incubated at 37°C for 1 h. S1P (100 nM) was added to the lower chamber, and the chambers were incubated at 37°C for 3 h to allow cell migration. Membranes were washed with PBS, and cells were fixed in 3.7% formaldehyde for 10 min, permeabilized in 0.1% Triton X-100 in PBS for 3 min, and stained with 4',6-diamino-2-phenylindole dihydrochloride (DAPI, Molecular Probes, Eugene, OR). Cells that did not migrate through the membrane were gently removed from the upper surface, and the membranes were mounted between slide and coverslips using the SlowFade[®] Antifade Kit (Molecular Probes Inc.) Cell migration was scored in four random fields, and each group was carried out in duplicate.

Cell Growth Curves—BAEC in 12-well dishes (10^5 cells/well) were transfected with control or Rac1-specific siRNA, and cell growth was measured by counting the number of cells in three independent wells for each condition every 24 h using a hemocytometer. At every time point the culture medium was replaced in all samples that were not counted.

RESULTS

S1P-mediated Rac1 Activation Is Impaired by Sequestration of G β Subunits but Is Unaffected by PI 3-Kinase Inhibition—We first used pharmacological inhibitors to analyze the role of G_i proteins and PI 3-kinase in S1P-mediated activation of Rac1 and protein kinase Akt. BAEC were stimulated with S1P following treatment with pertussis toxin, with the PI 3-kinase inhibitor wortmannin, or with vehicle (Fig. 1A). Activation of Rac1 was measured by pull-down of active GTP-Rac1 from cell lysates using a GST-fused p21-binding domain of PAK-1; active and total Rac1 were then detected in immunoblots probed with a

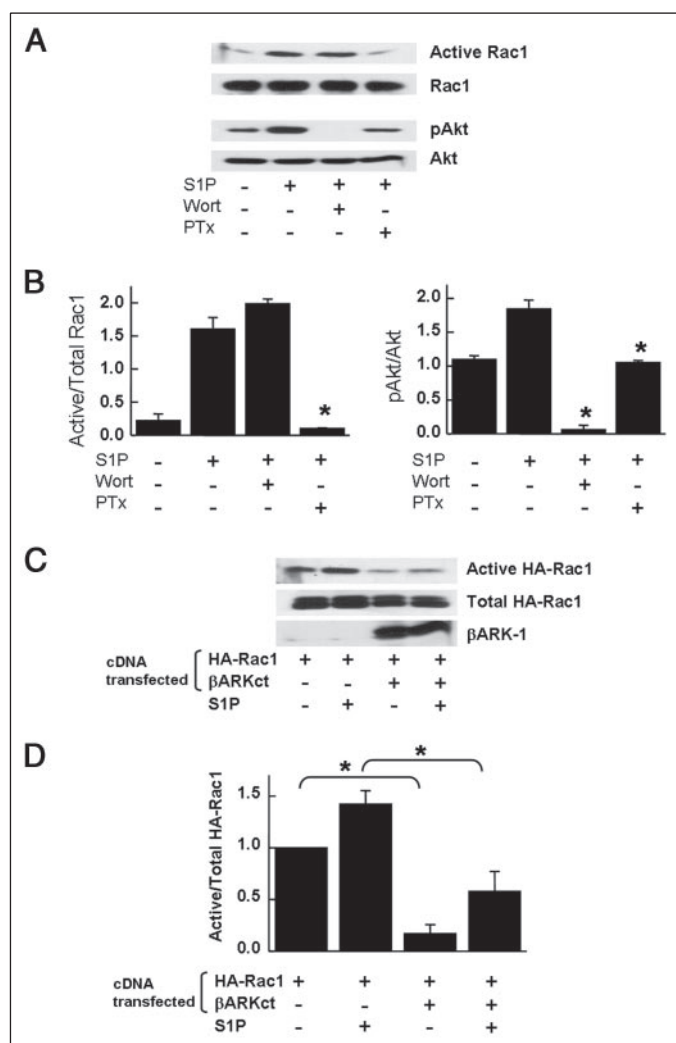


FIGURE 1. Effect of wortmannin, pertussis toxin, and β ARKct overexpression on S1P-induced Rac1 activation. A, for this immunoblot, BAEC were treated with S1P (100 nM) for 5 min, and Rac1 activity in the cell lysates was measured by pull-down of GTP-Rac1 using the GST-p21-binding domain of PAK-1. Active and total Rac1 were detected in immunoblots using an anti-Rac1-specific antibody. Phospho-Akt and total Akt in the cell lysates were detected in immunoblots using specific antibodies as shown. In some experiments, prior to S1P stimulation the cells were treated with wortmannin (*Wort*; 500 nM) for 30 min, with pertussis toxin (*PTx*; 20 ng/ml) overnight, or with vehicle as noted. B, densitometric analysis of pooled data showing the ratio between active and total Rac1 or phosphorylated Akt and total Akt both under basal conditions and after treatment with S1P in the presence or absence of inhibitors. Each data point represents the mean \pm S.E. derived from three independent experiments; in the absence of inhibitors, the addition of S1P induced a significant increase in Rac1 activity and Akt phosphorylation ($p < 0.001$). *, $p < 0.01$ versus S1P-treated cells (ANOVA). C, BAEC were cotransfected with a plasmid encoding HA-tagged wild type Rac1 and a plasmid encoding β ARKct or an empty vector as control. 48 h after transfection, cells were stimulated with S1P (100 nM) for 5 min and activation of HA-Rac1 was measured by pull-down of GTP-Rac1 using the GST-p21-binding domain of PAK-1. Active and total HA-Rac1 were detected in immunoblots probed with an anti-HA epitope antibody. D, densitometric analyses from pooled data plotting the ratio between active and total HA-Rac1 under basal conditions and after stimulation with S1P. Each data point represents the mean \pm S.E. derived from three independent experiments. *, $p < 0.05$ (ANOVA) versus cells that were not transfected with β ARKct.

Rac1-specific antibody. Akt phosphorylation was detected in cell lysates in immunoblots probed with a phospho-Akt-specific antibody. As shown in Fig. 1A, pretreatment with pertussis toxin completely blocked S1P-induced Rac1 activation as well as Akt phosphorylation, consistent with previous reports showing that S1P₁ receptors are coupled to G_i proteins (3). Pretreatment of BAEC with the PI 3-kinase inhibitor wortmannin completely blocked S1P-induced Akt phosphorylation, but S1P-mediated Rac1 activation was not significantly affected by this

treatment, suggesting that activation of Rac1 by S1P does not require PI 3-kinase stimulation.

Having previously observed that G $\beta\gamma$ subunits are required for S1P-induced Akt phosphorylation (18), we now analyzed whether S1P-mediated Rac1 activation is also mediated by G $\beta\gamma$ subunits. BAEC were cotransfected with plasmids encoding HA-tagged Rac1 plus β ARKct, a previously described chelator of G $\beta\gamma$ subunits (15), or with an empty vector. After S1P stimulation, HA-Rac1 activation was analyzed in the cell lysates by pull-down of GTP-HA-Rac1. Active and total HA-Rac1 were detected in immunoblots using a specific antibody against the HA epitope. As shown in Fig. 1, C and D, overexpression of β ARKct significantly impaired basal ($80 \pm 19\%$ reduction compared with cells not expressing β ARKct, $n = 3$, $p < 0.05$) as well as S1P-mediated Rac1 activation ($76 \pm 23\%$, $n = 3$, $p < 0.05$), suggesting an important role for G protein $\beta\gamma$ subunits in the modulation of Rac1 activity in BAEC.

Inhibition of Src Kinase Impairs S1P-mediated Rac1 Activation and Akt Phosphorylation—It has been reported previously that G $\beta\gamma$ subunits can interact with Src tyrosine kinase to activate members of the Rho GTPase family (20, 21). We explored the role of Src tyrosine kinase in S1P-mediated Rac1 activation in endothelial cells. BAEC were stimulated with S1P in the presence or absence of the Src inhibitor PP2, and activation of Rac1 was measured in the cell lysates by pull-down of GTP-Rac1 as described above. As shown in Fig. 2, pretreatment of BAEC with the Src inhibitor PP2 completely abolished S1P-mediated Rac1 activation. We also observed that the Src kinase inhibitor impaired S1P-mediated Akt phosphorylation in BAEC. By contrast to the marked inhibition by PP2 of Rac1 or Akt activation, this Src inhibitor had no substantive effect on S1P-induced ERK1/2 phosphorylation (Fig. 2B). Previous reports have suggested that Src-mediated transactivation of the vascular endothelial growth factor receptor 2 (VEGFR2, also known as KDR) is required for S1P-mediated Akt/eNOS phosphorylation (22). To further explore a role for VEGFR2 in S1P-mediated Rac1 activation, we designed a duplex siRNA specific for the bovine isoform of VEGFR2 and analyzed the effects of siRNA-mediated VEGFR2 knockdown on S1P-induced Akt and Rac1 activation (Fig. 2C). As shown in this figure, transfection with VEGFR2-specific siRNA efficiently down-regulated the expression of VEGFR2 ($\sim 90\%$), with no effect on the expression levels of Rac1 or Akt. BAEC treated with control or VEGFR2-specific siRNA were stimulated with S1P, and activation of Rac1 and phosphorylation of Akt was analyzed in the cell lysates. We observed that, despite a nearly complete abrogation of VEGFR2 expression, S1P-mediated Rac1 activation and Akt phosphorylation were not affected (Fig. 2C). These data support a role for Src kinase on S1P-mediated Akt and Rac1 activation but argue strongly against a requirement for Src-mediated VEGFR2 transactivation in this process.

Effect of Rac1 Mutants on Akt Phosphorylation—We were intrigued by our observations that S1P-mediated Rac1 and Akt activation in BAEC shared similar upstream modulators, and thus we further explored the relationship between these signaling molecules. Because blocking PI 3-kinase/Akt signaling with wortmannin did not inhibit S1P-induced Rac1 activation (Fig. 1A), it seemed improbable to us that PI 3-kinase/Akt would act upstream of Rac1 in S1P-mediated signaling. To confirm this hypothesis using an alternative experimental approach, we analyzed the effects of a dominant negative (N17Rac1) or a constitutively active (V12Rac1) Rac1 mutant (23) on Akt phosphorylation. BAEC were cotransfected with plasmids encoding Myc-tagged Akt (Myc-Akt) and HA-tagged dominant negative mutant of Rac1 (HA-N17Rac1) or an HA-tagged constitutively active mutant of Rac1 (HA-V12Rac1). The transfected Myc-tagged Akt can be distinguished by its slower mobility on SDS-PAGE relative to the endogenous wild type Akt

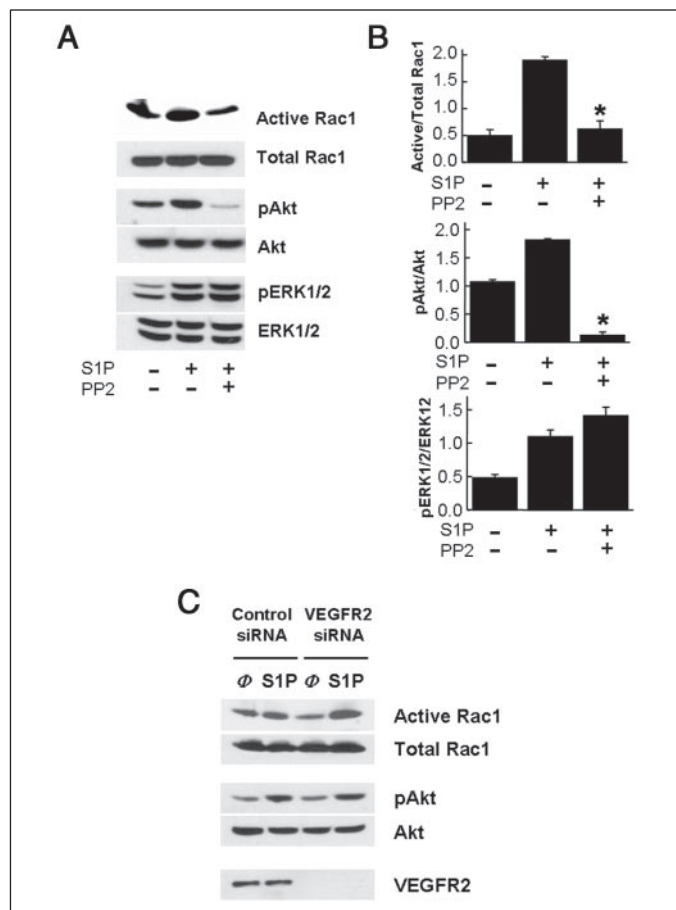


FIGURE 2. Effects of Src inhibitor PP2 and of VEGFR2 siRNA on S1P-induced Rac1 activation and Akt phosphorylation. *A*, BAEC were treated with S1P (100 nM) for 5 min, and Rac1 activity in the cell lysates was measured by pull-down of GTP-Rac1 using the GST-p21-binding domain of PAK-1. Active and total Rac1 were detected in immunoblots using an anti-Rac1-specific antibody. Phospho-Akt and total Akt and phospho-ERK1/2 and total ERK1/2 in the cell lysates were detected in immunoblots using specific antibodies as shown. In some experiments, prior to S1P stimulation cells were treated with PP2 (10 μ M) for 30 min or vehicle as noted. *B*, densitometric analysis of pooled data showing the ratio between active and total Rac1, phosphorylated and total Akt, and phosphorylated and total ERK1/2 under basal conditions and after treatment with S1P in the presence or absence of PP2. Each data point represents the mean \pm S.E. derived from three independent experiments; in the absence of inhibitors, the addition of S1P induced a significant increase in Rac1 activity and Akt and ERK1/2 phosphorylation ($p < 0.001$). *, $p < 0.001$ versus S1P treated cells (ANOVA). *C*, BAEC were transfected with control or VEGFR2 siRNA, and 48 h after transfection cells were stimulated with S1P (100 nM) for 5 min. Rac1 activation was measured as described above. Precipitated Rac1 and total Rac1 in the cell lysates were detected in immunoblots probed with a monoclonal antibody directed against Rac1. Cell lysates were resolved on SDS-PAGE and analyzed in immunoblots probed with phospho-Akt and total Akt-specific antibodies. VEGFR2 expression was analyzed by immunoblot with an anti-VEGFR2-specific antibody. ϕ , vehicle.

(Fig. 3). This distinctive feature of Myc-tagged Akt permits us to restrict our analyses of Akt phosphorylation to the small subset of BAEC that undergoes plasmid transfection. Phosphorylation of Akt in the cell lysates was detected by immunoblotting using a phospho-Akt-specific antibody. As expected, there was only a nominal effect of the Rac1 mutants on phosphorylation of endogenous Akt, consistent with the known low plasmid transfection efficiency in these cells. However, analysis of the slower migrating transfected Myc-Akt construct proved to be informative. As shown in Fig. 3, overexpression of a dominant negative mutant of Rac1 (N17Rac1) attenuated the phosphorylation of Myc-Akt in transfected BAEC.

siRNA-mediated Down-regulation of Rac1 Impairs S1P-induced Akt Signaling—We next used siRNA approaches to explore the role of endogenous Rac1 in S1P-mediated Akt activation in BAEC. In the con-

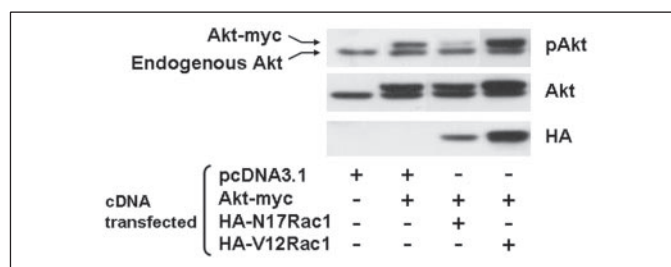


FIGURE 3. Effect of Rac1 mutants on Akt phosphorylation. BAEC were cotransfected with plasmids encoding Myc-tagged Akt (*Akt-myc*) and HA-tagged dominant negative mutant of Rac1 (*HA-N17Rac1*) or HA-tagged constitutively active mutant of Rac1 (*HA-V12Rac1*). 48 h after transfection cell lysates were resolved by SDS-PAGE, and phosphorylation of Akt was analyzed in immunoblots probed with phospho-Akt-specific antibody. Total Akt and Rac1 mutant proteins were detected using anti-Akt and anti-HA epitope antibodies as shown.

text of prior studies of caveolin regulation, we had previously designed and validated a siRNA duplex targeted against bovine Rac1 mRNA (Rac1 siRNA); these experiments suggested that Rac1 knockdown impairs Akt phosphorylation (14). The present studies have analyzed in greater detail the effect of Rac1 knockdown on S1P-induced Akt phosphorylation in BAEC. As shown in Fig. 4A, transfection of BAEC with Rac1 siRNA efficiently and specifically reduces expression of Rac1 protein by $\sim 90\%$. In time course experiments (Fig. 4), we found that siRNA-mediated Rac1 knockdown significantly attenuates basal and S1P-induced Akt phosphorylation compared with control siRNA-treated cells, suggesting that Rac1 is required for S1P-induced Akt activation. By contrast, we observed that siRNA-mediated Rac1 knockdown had no effect on S1P-induced ERK1/2 phosphorylation (Fig. 4), indicating a differential effect of Rac1 on ERK1/2 versus Akt activation by S1P.

We next analyzed whether Rac1 knockdown would affect the phosphorylation pattern of signaling proteins downstream of Akt. We analyzed the kinase Akt substrates GSK3 β and eNOS, as well as the downstream Akt effector, p70S6 kinase (24). BAEC were transfected with control or Rac1 siRNA, and 48 h after transfection cells were stimulated with S1P. Phosphorylations of Akt and its downstream targets were analyzed in immunoblots using phospho-specific antibodies. As shown in Fig. 5, the S1P-promoted increase in phosphorylation of eNOS, GSK3 β , and p70S6 kinase was significantly attenuated in Rac1 siRNA-treated cells. Rac1 knockdown inhibited S1P-mediated phosphorylation of eNOS ($33 \pm 10\%$), GSK3 β ($50 \pm 15\%$), and p70S6 kinase ($94 \pm 30\%$) when compared with control siRNA-treated cells (in each case $n = 3$, $p < 0.05$).

Rac1 Knockdown Impairs Endothelial Cell Growth and S1P-mediated Endothelial Cell Migration—S1P-mediated activation of Rac1 and the PI 3-kinase/Akt pathway has been correlated with a broad range of cellular effects including endothelial cell proliferation, migration, and endothelial barrier enhancement (4, 8, 25). To directly analyze the effect of Rac1 knockdown on S1P-induced endothelial cell migration we used a Transwell cell culture chamber. As shown in Fig. 6, S1P induced cell migration was significantly reduced in Rac1 siRNA-treated cells ($41 \pm 15\%$, $n = 3$, $p < 0.01$ relative to control siRNA-treated cells). We also analyzed the effect of Rac1 knockdown on endothelial cell growth (Fig. 6B) and found that siRNA-mediated down-regulation of Rac1 significantly reduced the growth rate of BAEC ($48 \pm 8\%$ decrease in Rac1 siRNA-treated cells compared with control siRNA-treated BAEC, $p < 0.001$).

Overexpression of an Inactive Mutant of Tiam1 Impairs S1P-mediated Rac1 Activation—A key step in the activation of the small GTPases of the Rho family is the recruitment of specific GEFs that allow the exchange of GDP for GTP, promoting the formation of GTP-bound

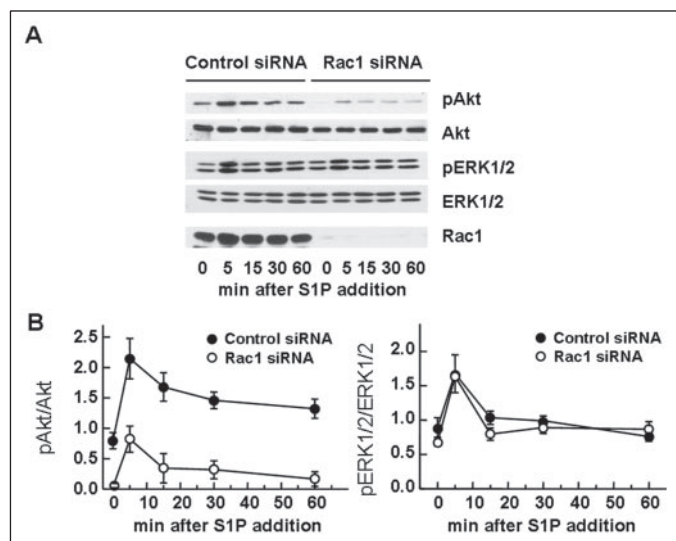


FIGURE 4. siRNA-mediated down-regulation of Rac1 in BAEC inhibits S1P-mediated Akt phosphorylation. A, BAEC transfected with control or Rac1-specific siRNA were treated with S1P (100 nM) for the indicated times. Cell lysates were resolved on SDS-PAGE and analyzed in immunoblots probed with specific antibodies directed against phospho-Akt, phospho-ERK1/2, and Akt and ERK1/2. Rac1 expression was analyzed by immunoblot with an anti-Rac1-specific antibody. Shown are the representative results of an experiment that was repeated three times with equivalent results. B, pooled data plotting the ratio between the signal intensity of phospho-Akt and total Akt or phospho-ERK1/2 and ERK1/2 at each time point as determined by densitometry. Each point in the graph represents the mean \pm S.E. of three independent experiments.

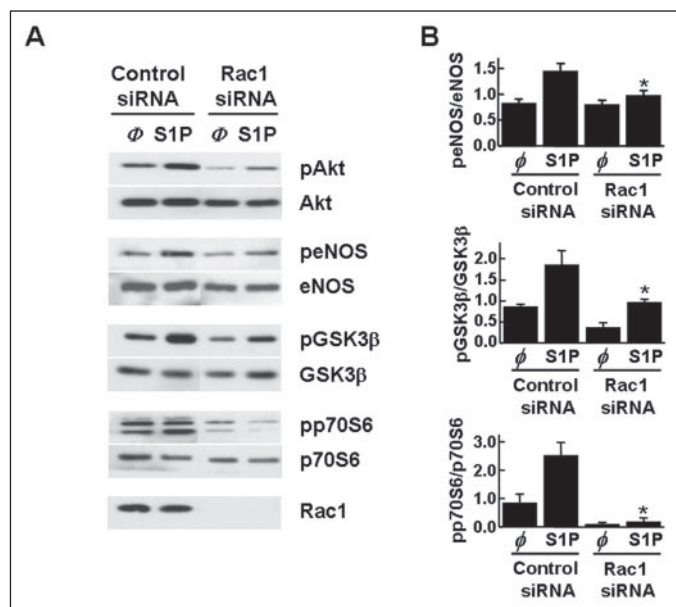


FIGURE 5. siRNA-mediated down-regulation of Rac1 impairs S1P-induced phosphorylation of Akt downstream targets. A, BAEC transfected with control or Rac1 siRNA were stimulated with S1P (100 nM) for 5 min. Cells were lysed, and phosphorylation of protein kinase Akt, eNOS, GSK3 β , and p70S6 kinase was analyzed in the cell lysates in immunoblots probed with phospho-specific antibodies as shown. B, densitometric analysis of pooled data showing the ratio between phosphorylated and total eNOS, GSK3 β , and p70S6 kinase in control and Rac1 siRNA-transfected cells, both under basal conditions and after treatment with S1P. Each data point represents the mean \pm S.E. derived from three to five independent experiments; the addition of S1P induced a significant increase in phosphorylation of Akt targets in control siRNA-transfected cells ($p < 0.001$). *, $p < 0.05$ for Rac1 versus control siRNA-transfected cells (ANOVA). ϕ , vehicle.

active state of these GTPases. Although several molecules have been shown to have GEF activity toward Rac1, including DOCK180 (26), Vav2 (27), and Tiam1 (28), the specific GEF(s) involved in S1P-mediated Rac1 activation has not been yet described. Tiam1 is a ubiquitously

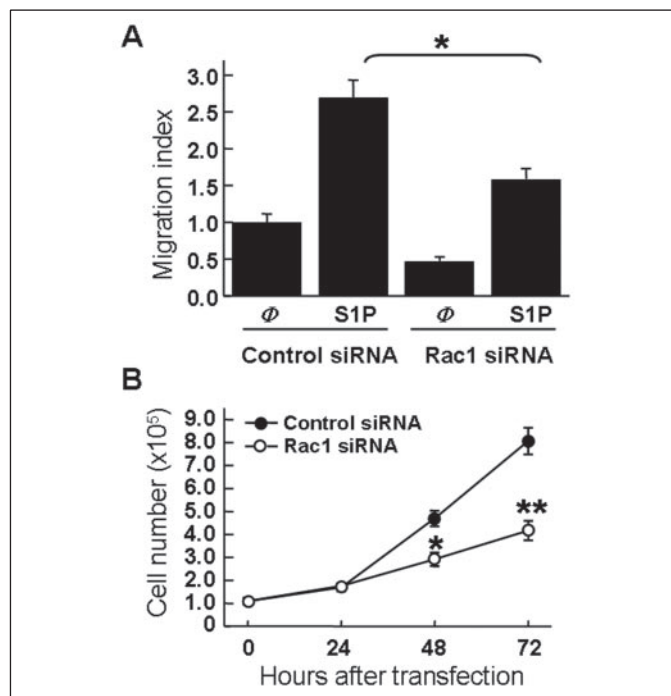


FIGURE 6. siRNA-mediated Rac1 down-regulation impairs endothelial cell migration and proliferation. A, S1P-induced endothelial cell migration was measured in BAEC transfected with control or Rac1 siRNA using a Transwell cell culture chamber as described under "Experimental Procedures." Migration index represents the number of migratory cells/number of migratory cells in vehicle-treated control siRNA-transfected BAEC (ϕ). Each data point represents the mean \pm S.E. from three independent experiments. For each experimental condition, the addition of S1P induced a significant increase in endothelial cell migration ($p < 0.001$). *, $p < 0.01$ versus control siRNA-treated cells (using ANOVA). B, growth curves of BAEC transfected with control or Rac1 siRNA. Each data point represents the mean \pm S.E. from six independent measurements. *, $p < 0.05$; **, $p < 0.001$ versus control siRNA-treated cells (using ANOVA).

expressed Rac1 GEF and has been found in mouse endothelial cells (29). Moreover, Tiam1 modulates the Rac1 activation mediated by lysophosphatic acid receptors (30), which are closely related to S1P receptors. We therefore analyzed the effect of Tiam1 overexpression in the activation of Rac1 in response to S1P in bovine aortic endothelial cells. In our preliminary experiments (not shown), we found that Tiam1 is expressed in BAEC, although the commercially available Tiam1 antibody did not yield a robust signal in immunoblots analyzed in these bovine cells. We cotransfected BAEC with plasmids encoding HA-tagged Rac1 plus either FLAG-tagged wild type Tiam1 or a FLAG-tagged Tiam1 dominant negative mutant termed Tiam1-QK (16) and then measured the activation of HA-Rac1 by pull-down of GTP-HA-Rac1; active and total HA-Rac1 were detected in immunoblots probed with anti-HA antibody. As shown in Fig. 7, overexpression of Tiam1 significantly enhanced HA-Rac1 activity both in the basal state and following S1P stimulation; the addition of S1P did not further enhance HA-Rac1 activation in cells already overexpressing Tiam1. In contrast, overexpression of the dominant negative mutant Tiam1-QK inhibited S1P-mediated HA-Rac1 activation (Fig. 7), suggesting that Tiam1 might be a GEF that is centrally involved in S1P-mediated activation of Rac1 in endothelial cells.

DISCUSSION

We have pursued pharmacological approaches along with protein overexpression experiments and siRNA-mediated knockdown methods to study the molecular mechanisms involved in S1P-signaling to Rac1 and PI 3-kinase/Akt. Through these studies we have explored the upstream modulators of S1P-mediated Rac1 activation and have iden-

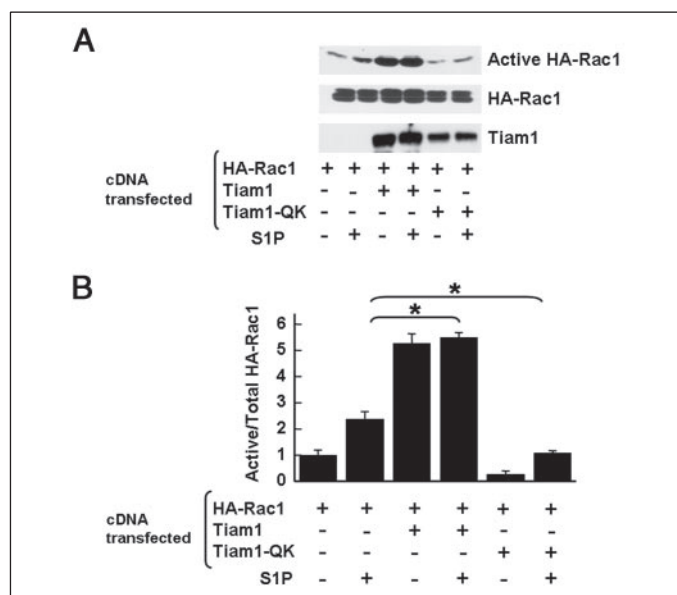


FIGURE 7. A Tiam1 inactive mutant inhibits S1P-induced Rac1 activation. *A*, BAEC were cotransfected with plasmids encoding HA-tagged wild type Rac1 and FLAG-tagged wild type Tiam1 or the inactive GEF mutant Tiam1-QK. 48 h after transfection, cells were stimulated with S1P (100 nM) for 5 min, and activation of HA-Rac1 was measured as described under "Experimental Procedures." Active and total HA-Rac1 were detected in immunoblots probed with an anti-HA epitope antibody. *B*, densitometric analysis from pooled data plotting the ratio between active and total HA-Rac1. Each data point represents the mean \pm S.E. derived from three independent experiments. *, indicates $p < 0.05$ versus S1P-treated control cells (ANOVA).

tified Rac1 as an important and specific modulator of S1P-dependent PI 3-kinase/Akt signaling in endothelial cells.

S1P₁ receptor-mediated responses are modulated by pertussis toxin-sensitive heterotrimeric G proteins (3, 17, 31), which consist of G α and G $\beta\gamma$ subunits with distinct molecular targets. We have previously reported that S1P-mediated Akt phosphorylation is dependent on G $\beta\gamma$ subunits (18). The present studies provide new information indicating that G protein $\beta\gamma$ subunits may be directly involved in proximal signaling from S1P₁ to Rac1 (Fig. 1). Our experiments show that sequestration of G $\beta\gamma$ subunits profoundly impairs S1P-induced Rac1 activation, suggesting a critical role for G $\beta\gamma$ subunits in S1P-mediated activation of both Rac1 and PI 3-kinase/Akt signaling. However, abrogation of G $\beta\gamma$ signaling by β ARKct overexpression did not completely block S1P-induced Rac1 activation, suggesting that additional mechanisms based on G_i-mediated signaling might be also involved in Rac1 activation by S1P.

G proteins previously have been found to interact with the non-receptor tyrosine kinase Src and thereby to activate specific Rac1-specific GEFs (20, 21). We found that the Src inhibitor PP2 completely blocks S1P-mediated Rac1 activation and Akt phosphorylation (Fig. 2), suggesting a key role for Src in S1P-mediated signal transduction. It seems plausible to us that G protein $\beta\gamma$ subunits signaling directly to Src may be involved in S1P-mediated signaling to Rac1 and kinase Akt. However, a previous report has suggested that S1P signaling to Akt in endothelial cells instead involves Src-mediated transactivation of the VEGF receptor, VEGFR2 (22). Because VEGF stimulation also activates Rac1 in endothelial cells (32), it might seem plausible that Src-mediated transactivation of VEGFR2 in response to S1P could be responsible for Rac1 activation. Our data suggest that this mechanism is quite unlikely; siRNA-mediated VEGFR2 knockdown did not affect S1P-mediated Rac1 activation or Akt phosphorylation (Fig. 2C) under conditions in which VEGF-induced Akt phosphorylation was abrogated (38). These results suggest that transactivation of VEGFR2 is not required for S1P-mediated Rac1 activation or Akt phosphorylation but, rather, indicate

that both S1P and VEGF signaling pathways are dependent on Src activity.

We found that the PI 3-kinase inhibitor wortmannin completely blocks S1P-mediated Akt phosphorylation without affecting Rac1 activation (Fig. 1). This observation suggests that Rac1 activation is independent of PI 3-kinase stimulation and may indicate further that Rac1 is a key upstream modulator of S1P-induced PI 3-kinase/Akt signaling in these cells. Further evidence for the key role of Rac1 in modulation of PI 3-kinase/Akt was provided by studies using constitutively active and dominant negative Rac1 mutants transfected into cultured endothelial cells (Fig. 3). We found that overexpression of a constitutively active mutant of Rac1 in BAEC significantly enhanced Akt phosphorylation, whereas an inactive mutant of Rac1 inhibited Akt phosphorylation. Taken together, these results suggest that activation of Rac1 may be necessary and sufficient to promote Akt phosphorylation in endothelial cells. This hypothesis is further supported by experiments in which we used siRNA to selectively knockdown endogenous Rac1 in BAEC (Fig. 4). We showed that siRNA-mediated Rac1 knockdown significantly impaired basal as well as S1P-mediated Akt phosphorylation. In contrast to the marked inhibitory effect of Rac1 siRNA on kinase Akt, we found that Rac1 siRNA had no substantive effect on ERK1/2 phosphorylation, indicating that Rac1 selectively modulates S1P signaling pathways in these cells. siRNA-mediated Rac1 knockdown inhibited S1P-induced phosphorylation of several effectors downstream of Akt, including eNOS, GSK3 β , and p70S6 kinase (Fig. 5). siRNA-mediated Rac1 knockdown also significantly impaired endothelial cell growth and S1P-induced endothelial cell migration (Fig. 6), indicating that Rac1 plays an important role in modulating key cellular processes. This observation is consistent with previous studies that explored the effects of Rac1 mutants on endothelial cell functions (10, 32).

S1P elicits a broad range of PI 3-kinase/Akt-modulated cellular responses in vascular endothelial cells including NO release and vascular relaxation (17, 33), endothelial cell migration (5, 7), adherens junction assembly, and vascular morphogenesis (4, 34). Rac1 has been implicated in several of these responses, as we also have shown in this study (Fig. 6), but the interrelationships between Rac1 and PI 3-kinase/Akt pathways remain incompletely understood. Cross-talk between Rac1 and PI 3-kinase/Akt appears to vary between different cell types and may also be differentially regulated in a receptor-specific fashion. Thus, there are situations in which Rac1 may modulate PI 3-kinase activity (13, 35) and yet other examples in which activation of PI 3-kinase is required for the activation of Rac1 by GEFs (12). The present studies provide an interesting counterpoint to a recent study that explored the control of S1P₁ receptor signaling by kinase Akt (7). In this previous report, Lee *et al.* (7) showed that S1P-mediated activation of PI 3-kinase/Akt leads to the direct phosphorylation of the S1P₁ receptor by Akt; inactivation of the Akt-modified phosphorylation site on the S1P₁ receptor was associated with impaired S1P-mediated Rac1 activation in Chinese hamster ovary cells. Although it is difficult to make a direct comparison of signaling pathways studied in different cell types, we feel that, taken together, the current study and the previous report (7) suggest a model wherein S1P-mediated Rac1 activation may be required for the activation of PI 3-kinase/Akt (Figs. 3 and 4) but that sustained activation of S1P₁ signaling may be dependent on Akt-dependent phosphorylation in order to maintain longer term Rac1-mediated responses such as cell migration or angiogenesis.

In vascular endothelial cells, we previously reported that the S1P₁ receptor is targeted to plasmalemmal caveolae (17) and demonstrated that siRNA-mediated knockdown of caveolin-1 enhances both Akt phosphorylation and Rac1 activation by S1P, providing evidence that

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these two pathways share common upstream modulators (14). S1P has been shown previously to modulate vascular relaxation by inducing Akt-mediated eNOS phosphorylation and nitric oxide release (17, 33). The present studies provide new evidence that Rac1 GTPase can modulate NO signaling in endothelial cells by regulating agonist-mediated eNOS phosphorylation, suggesting a role for this GTPase in the S1P-mediated control of vascular tone. It is interesting to note that Rac1 is known to stimulate superoxide production in phagocyte and non-phagocyte cells by binding and activating NADPH oxidase (10). The present studies suggest that Rac1 is also intimately involved in nitric oxide signaling and may identify a new role of Rac1 in differential modulation of oxidative and nitrosative signaling pathways in vascular endothelial cells.

As for other Rho family GTPases, Rac1 activation is promoted by GEFs that facilitate the exchange of GDP for GTP. Several GEFs have been shown to activate Rac1 (11, 26–28), but the GEF involved in S1P-mediated Rac1 activation has not been clearly established in previous studies. We found (Fig. 7) that overexpression of Rac1-GEF Tiam1 dramatically increased Rac1 activity in endothelial cells, consistent with Tiam1 acting as a GEF for Rac1 (16, 36). More importantly, when we overexpressed a dominant negative Tiam1 mutant, activation of Rac1 by S1P was significantly attenuated, suggesting that Tiam1 is involved in S1P-mediated activation of Rac1 in endothelial cells. Targeting of Tiam1 to the plasma membrane is required for activation of this GEF (28), and a previous report (4) has shown that S1P promotes the translocation of Tiam1 as well as Rac1 to regions of cell-cell contacts in cultured endothelial cells. Interestingly, activation of Rac1 in response to lysophosphatidic acid is impaired in embryonic fibroblast derived from Tiam1 knock-out mice (30), supporting a broader role for Tiam1 in lysolipid receptor-mediated activation of Rac1. In a recent study Singleton *et al.* (37) have also proposed that Tiam1 is the GEF involved in S1P-mediated Rac1 activation. These authors (37) showed that S1P promoted the recruitment of Tiam1 to caveolae domains and enhanced the association of Tiam1 with the S1P₁ receptor; in these studies siRNA-mediated Tiam1 knockdown blocked S1P-mediated Rac1 activation in human pulmonary artery endothelial cells. Taking their results together with ours, which pursued complementary experimental approaches, it seems plausible that the GEF Tiam1 is centrally involved in S1P-mediated activation of Rac1 in endothelial cells. However, the molecular mechanisms involved in Tiam1/Rac1 activation might be divergent in different vascular endothelial cells. For example, Singleton *et al.* (37) reported that S1P-mediated Tiam1/Rac1 activation appears to be dependent on PI 3-kinase activity, which is in contrast to our findings (Fig. 1) showing that PI 3-kinase inhibition failed to block S1P-modulated Rac1 activation. Further studies of S1P-mediated Tiam1 activation, including analyses of phosphoinositide binding and/or Tiam1 phosphorylation, may help to clarify the mechanisms involved in Rac1 activation by Tiam1 in response to S1P.

These studies have shown that S1P-mediated activation of Rac1 is dependent on Gβγ subunits, as well as Src tyrosine kinase, and that Tiam1 is a plausible candidate as the GTP exchange factor that stimulates Rac1 in these cells. Most importantly, we have found that activation of Rac1 is a critical proximal event in S1P-mediated PI 3-kinase/Akt signaling. These studies provide new insights into the molecular mechanisms involved in S1P signaling in endothelial cells and establish evidence for a key role of Rac1 in the control of PI 3-kinase/Akt/nitric oxide signaling in the vascular endothelium.

Acknowledgments—We thank Dr. Michael Greenberg for the gift of wild type and inactive Tiam1 cDNAs constructs and Dr. Robert Lefkowitz for the gift of plasmid cDNA encoding βARKct.

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