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Sphingosine 1-Phosphate Stimulates Tyrosine Phosphorylation of Focal Adhesion Kinase and Chemotactic Motility of Endothelial Cells via the G_i Protein-Linked Phospholipase C Pathway

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We have previously shown that sphingosine 1-phosphate (S1P) stimulates motility of human umbilical vein endothelial cells (HUVECs) (O.-H. Lee *et al., Biochem. Biophys. Res. Commun.* 264, 743–750, 1999). To investigate the molecular mechanisms by which S1P stimulates HUVEC motility, we examined tyrosine phosphorylation of p125 focal adhesion kinase ($p125^{FAK}$) which is important for cell migration. S1P induces a rapid increase in tyrosine phosphorylation of $p125^{FAK}$. Compared with other structurally related lipid metabolites such as sphingosine, C2-ceramide, and lysophosphatidic acid, S1P uniquely stimulated $p125^{FAK}$ tyrosine phosphorylation and migration of HUVECs. The effect of S1P on $p125^{FAK}$ tyrosine phosphorylation was markedly reduced by treatment with pertussis toxin or U73122, a phospholipase C (PLC) inhibitor. As a downstream signal of PLC, $p125^{FAK}$ tyrosine phosphorylation in response to S1P was totally blocked by depletion of the intracellular calcium pool. However, protein kinase C (PKC) inhibitor had no effect on the response to S1P. Finally, chemotaxis assays revealed that inhibition of PLC but not PKC significantly abrogated S1P-stimulated HUVEC migration. These results suggest that the G_i-coupled receptor-mediated PLC-Ca^{2+} signaling pathway may be importantly involved in S1P-stimulated focal adhesion formation and migration of endothelial cells.

Abbreviations used: ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cell; LPA, lysophosphatidic acid; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; PDGF, platelet-derived growth factor; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; PTX, pertussis toxin; S1P, sphingosine 1-phosphate; bFGF, basic fibroblast growth factor; $p125^{FAK}$, p125 focal adhesion kinase.

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S1P, a bioactive sphingolipid metabolite, is recognized as an extracellular mediator and an intracellular second messenger that regulate a wide range of biological responses such as cell growth, death, and differentiation (1, 2). Many of the functions of S1P are previously thought to be due to the second messenger action of S1P produced intracellularly by activation of sphingosine kinase, the enzyme which catalyzes the phosphorylation of sphingosine, in response to various stimuli (3, 4). Recently, EDG receptors such as EDG-1, EDG-3 and EDG-5/H218/AGR16 were identified as plasma membrane receptors for S1P, and the extracellular action mechanism of S1P has been delineated (1, 2). These receptors are shown to be associated with various G proteins, and modulate multiple signaling pathways including PLC activation, Ca^{2+} mobilization, Ras/mitogen activated protein kinase (MAPK) activation, and adenylate cyclase inhibition in various cell types (2).

S1P has been implicated in the regulation of cell motility (5). It has been shown to inhibit melanoma B16/F10 motility through a receptor-coupled extracellular action and in a pertussis toxin (PTX)-insensitive manner (6). Recently, it has been also reported that S1P inhibits chemoinvasiveness and chemotactic motility of MDA-MD-231 and MCF-7 cells (7). In human arterial smooth muscle cells, S1P interfered with the dynamics of platelet-derived growth factor (PDGF)-stimulated actin filament disassembly and assembly by acting intracellularly, resulting in a marked inhibition of chemotaxis toward PDGF (8). However, in contrast to the inhibitory effect of S1P on cell migration of these cell types, we have recently demonstrated that S1P potently stimulates chemotactic motility of HUVECs via PTX-sensitive G proteins (9). Thus, the mechanisms by which S1P either inhibits or stimulates

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cell motility in different cell types have become an intriguing issue.

Among a number of proteins engaged in regulating cell migration, $p125^{FAK}$ has been thought to modulate the dynamic changes in actin cytoskeleton organization that are a prerequisite for the promotion of cell migration (10, 11). Indeed, the role of $p125^{FAK}$ in cell migration has been demonstrated by studies in knockout mice and $p125^{FAK}$ overexpressed cell lines (12). In addition, it has been also reported that growth factors such as PDGF-BB and vascular endothelial growth factor stimulate tyrosine phosphorylation of $p125^{FAK}$ in vascular smooth muscle cells and HUVECs, respectively, and the resulting tyrosine phosphorylation is suggested to have a role in the migratory cell response to these growth factors (13, 14). Also, $p125^{FAK}$ tyrosine phosphorylation is shown to be stimulated by a variety of bioactive peptides and lipids that act through G protein-coupled receptors (15, 16).

In the present study, we investigated tyrosine phosphorylation of $p125^{FAK}$ in HUVECs. Our results revealed that S1P induced a rapid tyrosine phosphorylation of $p125^{FAK}$ via G_i protein-linked PLC-Ca^{2+} system in HUVECs. Also, we found that activation of PLC which may lie downstream of G_i protein is closely involved in stimulating chemotactic migration of HUVECs in response to S1P.

MATERIALS AND METHODS

Materials. S1P, sphingosine, C2-ceramide, lysophosphatidic acid (LPA), U73122, GF109203X, ionomycin, and BAPTA-AM were purchased from BIOMOL. Phorbol 12-myristate 13-acetate (PMA) was from Sigma. SB203580 was from Alexis. PTX was from Research Biochemicals International. U0126, which is a specific inhibitor of MAPK kinase (MEK), was a kind gift of Dr. J. M. Trzaskos (DuPont Merck Research Laboratories). M199, basic fibroblast growth factor (bFGF), and heparin were obtained from Life Technologies. Transwell plate was from Corning Costar. Antibody for $p125^{FAK}$ was from Upstate Biotechnology. Anti-phosphotyrosine antibody was from Transduction Laboratories.

Cell culture. HUVECs were isolated as described previously (17). The cells were grown onto a gelatin-coated 75-cm^2 flask in M199 with 20% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, 3 ng/ml bFGF, and 5 units/ml heparin at 37°C under 5% CO_2 and 95% air. The cells used in this study were from passages 2 to 7.

Immunoprecipitation. Confluent HUVECs were incubated for 6 h in M199 containing 1% FBS. The cells were treated with S1P, lysed at 4°C in 1 ml of a lysis buffer containing 10 mM Tris/HCl, pH 7.6, 5 mM EDTA, 50 mM NaCl, 50 mM β-glycerophosphate, 50 mM NaF, 0.1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 0.5% Nonidet P-40, and 1% Triton X-100. Lysates were clarified by centrifugation at 15,000*g* for 10 min, and the resulting supernatants were immunoprecipitated with 1 μg/ml of anti-$p125^{FAK}$ antibody for 3 h at 4°C, followed by the addition of protein A–agarose beads for 1 h at 4°C. Immunoprecipitates were washed three times with lysis buffer, solubilized in SDS–PAGE sample buffer, and further analyzed by Western blotting.

Western blotting. Immunoprecipitates from HUVECs were loaded into a 10% SDS–PAGE gel, and transferred to polyvinyldifluoride membrane. The blocked membranes were then incubated with the indicated antibody, and the immunoreactive bands were visualized using chemiluminescent reagent as recommended by Amersham Pharmacia Biotechnology. The signals of the bands were quantitated using a densitometer.

Chemotaxis assay. The chemotactic motility of HUVECs was assayed using a Transwell chamber with 6.5-mm-diameter polycarbonate filters (8 μm pore size). Briefly, the lower surface of the filter was coated with 10 μg of gelatin. S1P and PMA prepared in 600 μl of M199 with 1% FBS was placed in the lower wells. HUVECs were trypsinized and suspended at a final concentration of 1×10^6 cells/ml in M199 containing 1% FBS. Inhibitors were given to the cells for 30 min at room temperature before seeding. One hundred microliters of the cell suspension was loaded into each of the upper wells. The chamber was incubated at 37°C for 4 h. Cells were fixed and stained with hematoxylin and eosin. Nonmigrating cells on the upper surface of the filter were removed by wiping with a cotton swab, and chemotaxis was quantified by counting the cells migrated to the lower side of the filter with optical microscopy at ×200 magnification. Ten fields were counted for each assay. The data are presented as percentage ± SE of control. Each sample was assayed in duplicate, and the assays were repeated three times. Statistical comparisons between groups were performed using the paired Student's *t* test.

RESULTS

S1P Stimulates Tyrosine Phosphorylation of $p125^{FAK}$ in HUVECs

We examined the effect of S1P on tyrosine phosphorylation of $p125^{FAK}$ in HUVECs. Confluent cultures of HUVECs were treated with various concentration of S1P for 2 min, and anti-$p125^{FAK}$ immunoprecipitates were prepared and blotted with an anti-phosphotyrosine antibody. The stimulation of tyrosine phosphorylation of $p125^{FAK}$ by S1P was prominently observed at a concentration as low as 0.1 μM, gradually increasing in a dose-dependent manner, and reaching a near maximum at 1 μM (Fig. 1A). The stimulation of tyrosine phosphorylation of $p125^{FAK}$ was further examined by treating HUVECs with 5 μM S1P for various lengths of time. As shown in Fig. 1B, S1P induced a rapid and biphasic activation of $p125^{FAK}$ tyrosine phosphorylation which reached a peak of 3–4 fold over basal level after 2 min and rapidly declined thereafter.

The Effects of Sphingolipid Metabolites and LPA on $p125^{FAK}$ Tyrosine Phosphorylation and Migration of HUVECs

Since S1P is converted into other sphingolipid metabolites in cells, it is of interest to examine whether the specific structural attributes of S1P are required for the stimulation of HUVEC migration. Thus, we investigated the effects of sphingolipid metabolites, C2-ceramide and sphingosine, and LPA, which are structurally related to S1P, on HUVEC migration. As shown in Fig. 2A, all these lipids either did not affect or slightly inhibited chemotactic migration of HUVECs at 1 and 5 μM, while S1P strongly induced cell motility at

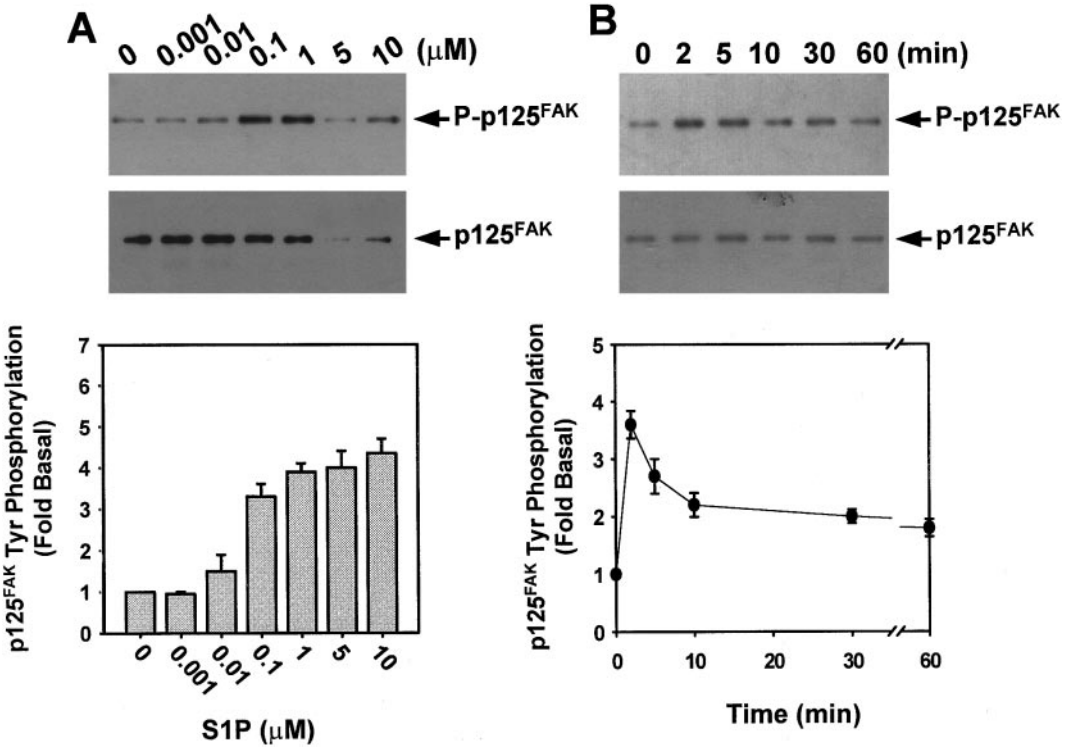

FIG. 1. S1P induces tyrosine phosphorylation of p125^{FAK} in HUVECs. (A) Dose response of S1P-induced p125^{FAK} tyrosine phosphorylation. Confluent HUVECs were treated with various concentration of S1P for 2 min. (B) Time course of S1P-induced p125^{FAK} tyrosine phosphorylation. HUVECs were stimulated for the indicated times with 5 μM S1P. Cell lysates were prepared and immunoprecipitated with anti-p125^{FAK} antibody. The immune complexes were analyzed by immunoblotting with anti-phosphotyrosine antibody to assay for p125^{FAK} tyrosine phosphorylation (P-p125^{FAK}) or anti-p125^{FAK} antibody for p125^{FAK} protein levels (p125^{FAK}). The graphs represent quantitative results from densitometric analysis. Values are the means of three separate experiments and expressed as fold-stimulation above unstimulated control.

the same concentration. And then, we compared the effect of these compounds on p125^{FAK} tyrosine phosphorylation. Confluent HUVECs were treated with 1 μM of each of these lipids for 2 min. In accordance with the results of cell migration, tyrosine phosphorylation of p125^{FAK} was uniquely stimulated by S1P, but not altered by C2-ceramide, sphingosine, and LPA (Fig. 2B). Thus despite structural similarity between these lipids, the roles of S1P in chemotaxis of HUVECs appears to be different from those of other lipids.

Characterization of the p125^{FAK} Signaling Pathway in HUVECs

To elucidate the p125^{FAK} signaling pathways induced by S1P in HUVECs, we investigated the effects of agents that interfere with activation of G_i protein or intracellular signaling routes on S1P-stimulated tyrosine phosphorylation of p125^{FAK}. As similar to the potent inhibitory effect of PTX on HUVEC chemotaxis (9), PTX (100 ng/ml for 6 h) dramatically abrogated S1P-induced p125^{FAK} tyrosine phosphorylation (Figs. 3A and 3C). These results suggest that the signaling pathway regulated by S1P in HUVECs is dependent on a G_i-coupled S1P receptor, and show clear difference with the p125^{FAK} pathways mediated by intracellular action of S1P in Swiss 3T3 fibroblasts and MDA-MB-231 cells (7, 18).

Previously, we demonstrated that S1P induced extracellular signal-regulated kinases (ERKs) and p38 MAPK activation in a PTX-sensitive manner. However, treatment of HUVECs with either the inhibitor U0126 of MEK, the upstream activator of ERKs, or the p38 MAPK inhibitor SB203580, did not influence S1P-induced p125^{FAK} tyrosine phosphorylation (Figs. 3A and 3C), although the roles of ERKs and p38 MAPK have been implicated in actin reorganization and cell movement in endothelial cells (19–21). These results suggest that S1P-induced phosphorylation of p125^{FAK} is independent of ERKs and p38 MAPK activation.

G_i protein dissociates into $G\alpha_i$ and $\beta\gamma$ subunits upon receptor activation and the released $\beta\gamma$ subunit is known to stimulate PLC (22). To determine whether the stimulation of p125^{FAK} tyrosine phosphorylation by S1P was dependent on PLC, we used U73122, a specific inhibitor of PLC. At 5 μM, U73122 markedly blocked S1P-induced p125^{FAK} tyrosine phosphorylation, indi-

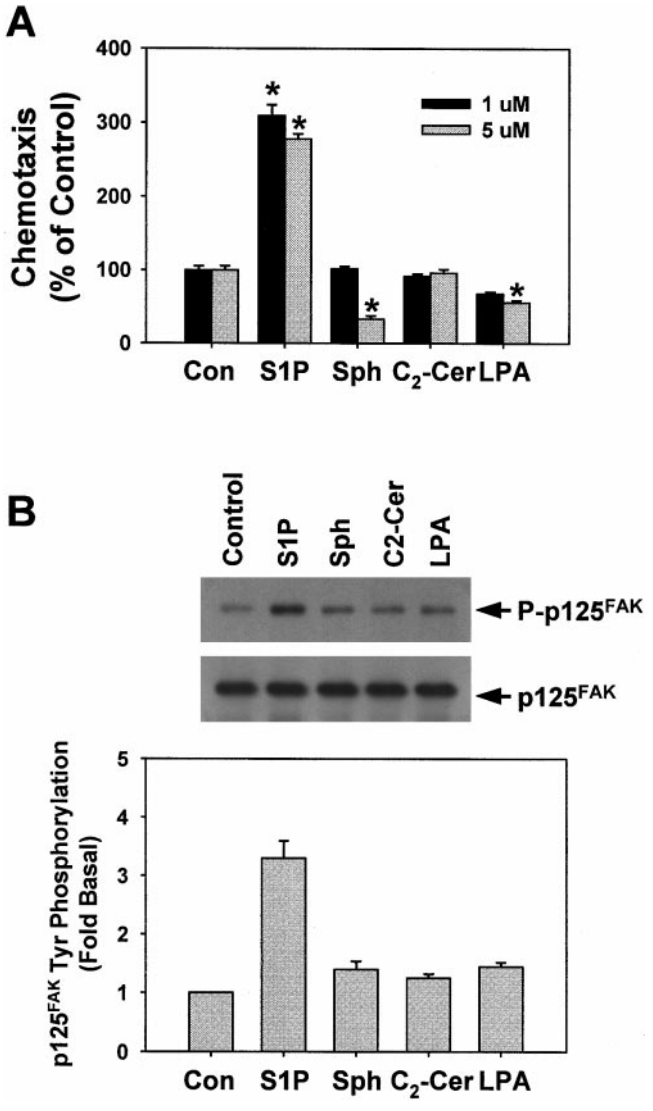

FIG. 2. Effects of S1P, sphingosine, C2-ceramide, and LPA on chemotactic motility and tyrosine phosphorylation of $p125^{FAK}$ in HUVECs. (A) Chemotaxis assays were performed as described under Materials and Methods. S1P, sphingosine (Sph), C2-ceramide (C2-Cer), and LPA were added to lower wells at the concentration of 1 or 5 μM. The basal migration (Con) in the absence of chemoattractant was 127.3 $\pm$ 7.0 cells/field. Results are expressed as percentage $\pm$ SE of control. Each sample was assayed in duplicate, and the assays were repeated three times. $*p < 0.01$ from control (Con). (B) HUVECs were treated for 2 min with S1P, sphingosine (Sph), C2-ceramide (C2-Cer), and LPA at the concentration of 1 μM. Cell lysates were immunoprecipitated with anti-$p125^{FAK}$ antibody. The immune complexes were analyzed by immunoblotting with anti-phosphotyrosine antibody to assay for $p125^{FAK}$ tyrosine phosphorylation (P-$p125^{FAK}$) or anti-$p125^{FAK}$ antibody for $p125^{FAK}$ protein levels ($p125^{FAK}$). The graphs represent quantitative results from densitometric analysis. Values are the means of three separate experiments and expressed as fold-stimulation above unstimulated control.

cating that activation of PLC by S1P is closely involved in the $p125^{FAK}$ signaling pathway (Figs. 3B and 3C).

Since PLC is known to generate inositol triphosphate and diacylglycerol, which activate intracellular Ca^{2+} mobilization and PKC, respectively, we evaluated the potential roles of PKC and an increase of intracellular Ca^{2+} in $p125^{FAK}$ tyrosine phosphorylation. The PKC inhibitor GF109203X had no significant effect on S1P-induced $p125^{FAK}$ tyrosine phosphorylation (Figs. 3B and 3C). In contrast, S1P-induced $p125^{FAK}$ tyrosine phosphorylation was completely abolished by the intracellular Ca^{2+} chelator BAPTA-AM (30 μM for 30 min), suggesting that an increase in intracellular Ca^{2+} may play an important role in S1P-induced $p125^{FAK}$ activation (Figs. 3B and 3C). Indeed, treatment with the Ca^{2+} ionophore ionomycin (1 μM) for 2 min was sufficient to stimulate tyrosine phosphorylation of $p125^{FAK}$ (Fig. 4A). Further, downregulation of S1P-induced $p125^{FAK}$ phosphorylation by PTX was significantly restored by ionomycin, and cotreatment of S1P with ionomycin further increased $p125^{FAK}$ tyrosine phosphorylation compared with S1P alone (Fig. 4B).

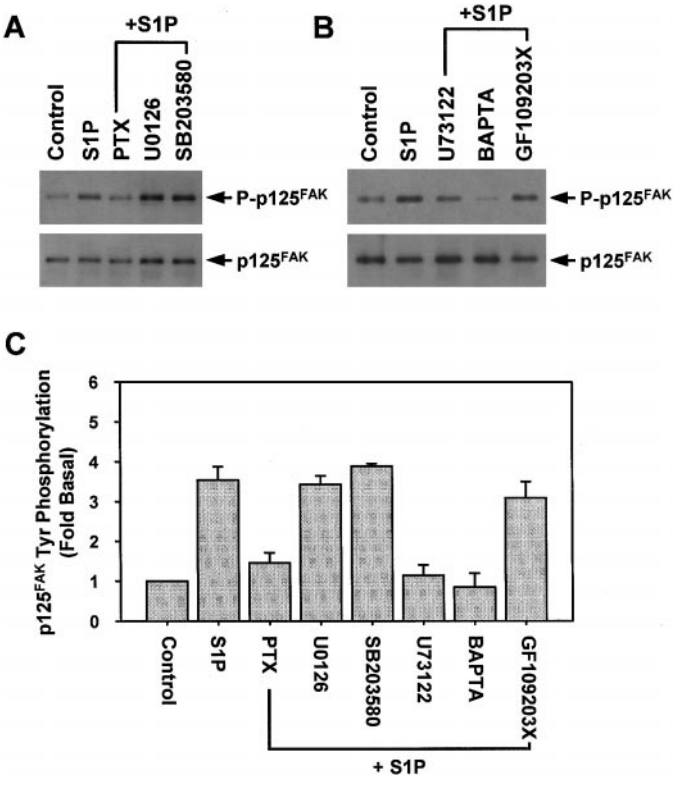

FIG. 3. S1P-induced tyrosine phosphorylation of $p125^{FAK}$ in HUVECs is sensitive to the inhibitors of G_i protein and PLC. (A, B) HUVECs were pretreated with PTX (100 ng/ml for 6 h), U0126 (5 μM for 30 min), SB203580 (10 μM for 30 min), U73122 (5 μM for 30 min), BAPTA-AM (30 μM for 30 min), or GF109203X (2 μM for 30 min). Then, HUVECs were incubated for 2 min with 5 μM of S1P. Cell lysates were prepared and immunoprecipitated with anti-$p125^{FAK}$ antibody. The immune complexes were analyzed by immunoblotting with anti-phosphotyrosine antibody to assay for $p125^{FAK}$ tyrosine phosphorylation (P-$p125^{FAK}$) or anti-$p125^{FAK}$ antibody for $p125^{FAK}$ protein levels ($p125^{FAK}$). (C) The densities of the bands corresponding to phosphorylated and non-phosphorylated $p125^{FAK}$ were quantitated using a densitometer. The results are expressed as fold-stimulation above control.

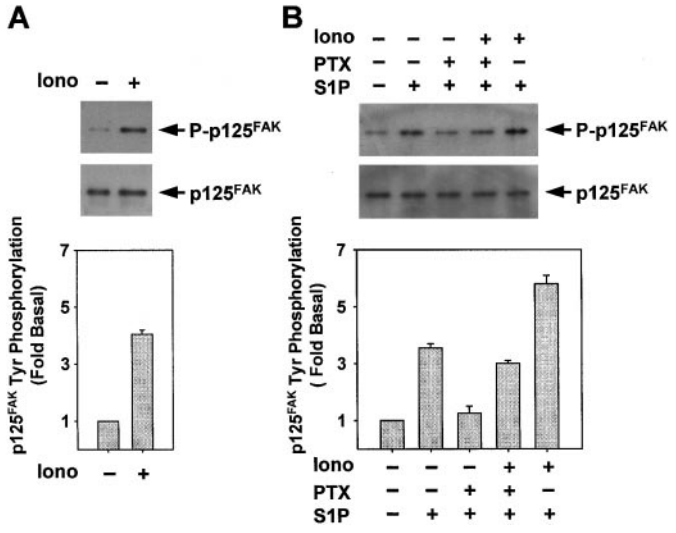

FIG. 4. The role of Ca^{2+} in the tyrosine phosphorylation of $p125^{FAK}$ in HUVECs. (A) HUVECs were exposed to ionomycin (1 μM) for 2 min. (B) HUVECs were incubated with or without 100 ng/ml of PTX for 6 h, and stimulated by 5 μM S1P alone or cotreatment with 5 μM S1P plus 1 μM ionomycin (Iono) for 2 min. Cell lysates were then prepared and immunoprecipitated with anti-$p125^{FAK}$ antibody. The immune complexes were analyzed by immunoblotting with anti-phosphotyrosine antibody to assay for $p125^{FAK}$ tyrosine phosphorylation (P-$p125^{FAK}$) or anti-$p125^{FAK}$ antibody for $p125^{FAK}$ protein levels ($p125^{FAK}$). The graphs represent quantitative results from densitometric analysis. Results are expressed as fold-stimulation above unstimulated control.

Taken together, these results suggest that S1P-induced $p125^{FAK}$ tyrosine phosphorylation in HUVECs is mediated extracellularly via G_i-coupled receptor(s) and subsequent downstream signals involve the PLC-Ca^{2+} pathway but not PKC.

Activation of PLC Is Involved in S1P-Stimulated Chemotaxis of HUVECs

Based on the signaling pathways of $p125^{FAK}$ phosphorylation induced by S1P, we investigated the role of PLC in S1P-stimulated HUVEC migration. Treatment of HUVECs with U73122 inhibited S1P-stimulated chemotaxis in a dose-dependent manner, with 97% inhibition at 5 μM (Fig. 5A). However, the PKC inhibitor GF109203X did not affect S1P-induced HUVEC chemotaxis. Consistently, there is no significant effect of the PKC activator PMA on chemotaxis under this condition (Fig. 5B). Taken together, these results suggest that activation of PLC but not PKC is responsible for HUVEC chemotaxis by S1P.

DISCUSSION

We have recently demonstrated that S1P strongly stimulates motility of endothelial cells, which is one of critical step in angiogenesis (9). Cell migration is accompanied by some sort of organized linkage connecting the cell membrane and cytoskeleton which is represented as the focal adhesion (23). It has been reported that the tyrosine phosphorylation of Y397 in $p125^{FAK}$ and the $p125^{FAK}$ activity are required for endothelial cell migration (24, 25), and the phosphotyrosine concentrations were elevated in the focal adhesions of HUVEC migrating into wound (25). Recently, Owen *et al.* have also reported that both the Y397 autophosphorylation and tyrosine phosphorylation of activation loop sites in $p125^{FAK}$ are critical for cell spreading and migration responses (26). To further examine the signaling mechanism by which S1P stimulates endothelial cell migration, we investigated the effect of S1P on $p125^{FAK}$ tyrosine phosphorylation in HUVECs. Consistently with the suggested role of $p125^{FAK}$ for endothelial cell migration, our data showed that S1P induces a rapid increase in the tyrosine phosphorylation of the focal adhesion-associated protein $p125^{FAK}$ in HUVECs. Apparently, compared with other structurally related

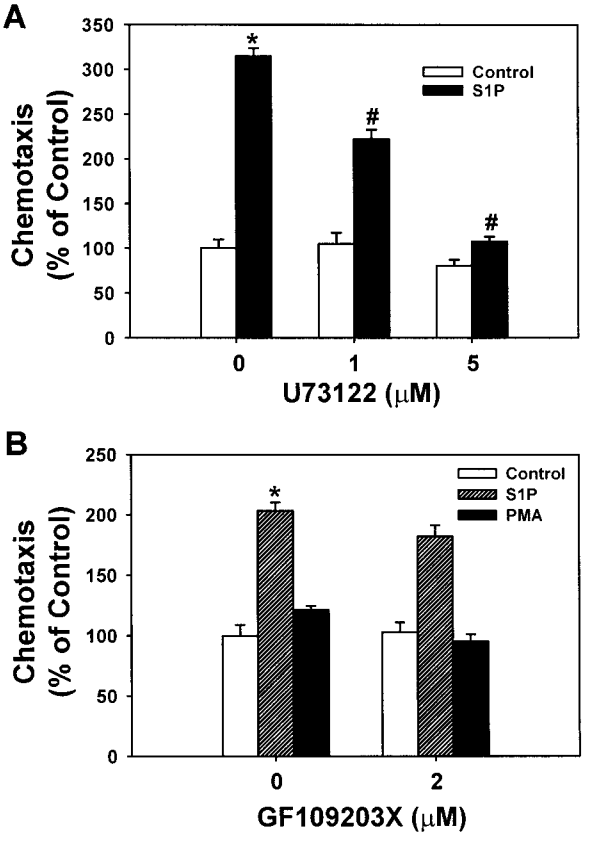

FIG. 5. Activation of PLC but not PKC is involved in S1P-induced chemotactic motility of HUVECs. S1P-induced chemotactic motility of HUVECs was determined in the absence and presence of the PLC inhibitor U73122 (A) and the PKC inhibitor GF109203X (B). Cells were preincubated for 30 min with or without U73122 (1, 5 μM) or GF109203X (2 μM) prior to treatment with 5 μM S1P or 100 nM PMA. Results are expressed as percentage $\pm$ SE of control. Each sample was assayed in duplicate, and the assays were repeated three times. *$p < 0.01$ from control; #$p < 0.01$ from S1P.

lipids at 1 μM concentration, S1P uniquely activated migration and $p125^{FAK}$ tyrosine phosphorylation of HUVECs. Also, $p125^{FAK}$ phosphorylation was occurred in a PTX-sensitive manner. Therefore, these results strongly suggest that the characteristic structural moiety of S1P is required for binding to G_i protein-coupled receptors to stimulate $p125^{FAK}$ tyrosine phosphorylation and motility of HUVECs.

In our previous study, we suggested the role of the G_i protein-coupled receptor EDG-1 in S1P stimulation of endothelial cell migration, with supporting evidence that S1P-induced HUVEC chemotaxis was highly sensitive to PTX and HUVECs expressed the high level of EDG-1 transcript (9). Similarly, others have recently shown that S1P potently stimulates the migration of EDG-1 or EDG-3-transfected CHO cells in a PTX-sensitive manner while it is ineffective in stimulating the migration of the vector- or AGR16-transfected cells (27). These observations further infer that the G_i protein signaling pathway triggered by the S1P receptor EDG-1 may play an essential role in mediating chemotaxis of endothelial cells.

Our present data further showed that PLC activation was one of essential signals that mediate both $p125^{FAK}$ phosphorylation and endothelial cell migration. Also, intracellular Ca^{2+} mobilization but not PKC is an important downstream signal of PLC in the S1P-induced $p125^{FAK}$ signaling pathway in HUVECs. Most recently, Lee *et al.* have reported that S1P induces a robust calcium response in HUVECs, which was inhibited approximately 90% by PTX, and suggested that EDG-1, a G_i-coupled S1P receptor, is mainly responsible for S1P-induced calcium increase in HUVECs (28). Taken together, these observations indicate that Ca^{2+} signal linked to the G_i protein-PLC system importantly participates in inducing endothelial cell motility in response to S1P.

However, it is notable that the reciprocal roles of S1P, inhibition or stimulation of cell migration, were shown in various cell types, nevertheless one of the universal actions of S1P is shown to be the activation of PLC-Ca^{2+} signal. Actually, in CHO cells expressing an equal number of S1P receptors, EDG-1, EDG-3, and EDG-5, it has been shown that all these receptors have the intrinsic activity to activate PLC-Ca^{2+} system (27). Ancellin *et al.* noted that a number of cell types expressed one or more of S1P receptors (29) in a varied level. Accordingly, it seems difficult to generalize the motogenic action of S1P in other cell types simply based on the activation of PLC and the subsequent increase in intracellular Ca^{2+}. However, several lines of previous evidence revealed the significant role of Ca^{2+} in endothelial cell migration (30). Indeed, a recent study clearly showed that vascular endothelial growth factor-induced endothelial cell migration required increase in intracellular Ca^{2+} and the subsequent generation of nitric oxide by endothelial nitric oxide synthase which is a Ca^{2+}/calmodulin-dependent enzyme (31). Such signaling cascade may be responsible for the specific action of S1P in endothelial cell migration. Another possibility is the synergistic contribution of a signal mediated by the $G\alpha_i$ subunit released upon receptor activation. In various EDG-1 transfected cells, it has been shown that EDG-1 induced the inhibition of cAMP accumulation (27, 29, 32). Since endothelial cells predominantly express the EDG-1 transcript, S1P is likely to downregulate the activity of adenylate cyclase in endothelial cells. Thus, such S1P-mediated inhibition of cAMP accumulation may positively contribute to cell migration because cAMP is implicated as an inhibitory factor of chemotactic response in various cells (33–35). This possibility is in part supported by our observation that an activator of adenylate cyclase, folskolin, significantly inhibited S1P-induced HUVEC migration in a dose-dependent manner (36), although the alternation of endothelial cAMP levels upon S1P treatment has yet been determined. Possible resolutions of these chemotactic signalings are awaiting for a future study.

In conclusion, our present findings in HUVECs provide in part the molecular basis of chemotactic signaling in response to S1P in intact cells. Our data revealed that the S1P-induced chemotactic signal in endothelial cells is mediated mainly via a G_i protein-coupled S1P receptor, presumably EDG-1, and the PLC-Ca^{2+} signaling pathway is crucially involved in S1P-induced $p125^{FAK}$ tyrosine phosphorylation and migration of endothelial cells. Further, we suggest that such signaling events may be correlated with the mechanism by which S1P stimulates angiogenesis.

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REFERENCES

1. An, S., Goetzl, E. J., and Lee, H. (1998) *J. Cell. Biochem. Suppl.* **30/31,** 147–157.
2. Goetzl, E. J., and An, S. (1998) *FASEB J.* **12,** 1589–1598.
3. Kolesnick, R., and Golde, D. W. (1994) *Cell* **77,** 325–328.
4. Olivera, A., and Spiegel, S. (1993) *Nature* **365,** 557–560.
5. Sadahira, Y., Ruan, F., Hakomori, S., and Igarashi, Y. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 9686–9690.
6. Yamamura, S., Yatomi, Y., Ruan, F., Sweeney, E. A., Hakomori, S., and Igarashi, Y. (1997) *Biochemistry* **36,** 10751–10759.
7. Wang, F., Nohara, K., Olivera, A., Thompson, E. W., and Spiegel, S. (1999) *Exp. Cell Res.* **247,** 17–28.
8. Bornfeldt, K. E., Graves, L. M., Raines, E. W., Igarashi, Y., Wayman, G., Yamamura, S., Yatomi, Y., Sidhu, J. S., Krebs, E. G., Hakomori, S., and Ross, R. (1995) *J. Cell Biol.* **130,** 193–206.
9. Lee, O.-H., Kim, Y.-M., Lee, Y. M., Moon, E.-J., Lee, D.-J., Kim, J.-H., Kim, K.-W., and Kwon, Y.-G. (1999) *Biochem. Biophys. Res. Commun.* **264,** 743–759.

10. Schaller, M. D., and Parsons, J. T. (1994) *Curr. Opin. Cell Biol.* **6,** 705–710.
11. Abedi, H., and Zachary, I. (1995) *Cardiovasc. Res.* **30,** 544–556.
12. Ilic, D., Furuta, Y., Kanazawa, S., Takeda, N., Sobue, K., Nakatsuji, N., Nomura, S., Fujimoto, J., Okada, M., and Yamamoto, T. (1995) *Nature* **377,** 539–544.
13. Cospedal, R., Abedi, H., and Zachary, I. (1999) *Cardiovasc. Res.* **41,** 708–721.
14. Abedi, H., and Zachary, I. (1997) *J. Biol. Chem.* **272,** 15442–15451.
15. Zachary, I., Sinnett-Smith, J., and Rozengurt, E. (1992) *J. Biol. Chem.* **267,** 19031–19034.
16. Rodriguez-Fernandez, J. L., and Rozengurt, E. (1998) *J. Biol. Chem.* **273,** 19321–19328.
17. Jaffe, E. A., Nachman, R. L., Becker, C. G., and Minick, C. R. (1973) *J. Clin. Invest.* **52,** 2745–2756.
18. Wang, F., Nobes, C. D., Hall, A., and Spiegel, S. (1997) *Biochem. J.* **324,** 481–488.
19. Li, S., Kim, M., Hu, Y. L., Jalali, S., Schlaepfer, D. D., Hunter, T., Chien, S., and Shyy, J. Y. (1997) *J. Biol. Chem.* **272,** 30455–30462.
20. Lin, T. H., Aplin, A. E., Shen, Y., Chen, Q., Schaller, M., Romer, L., Aukhil, I., and Juliano, R. L. (1997) *J. Cell Biol.* **136,** 1385–1395.
21. Rousseau, S., Houle, F., Landry, J., and Huot, J. (1997) *Oncogene* **15,** 2169–2177.
22. Clapham, D. E., and Neer, E. J. (1997) *Annu. Rev. Pharmacol. Toxicol.* **37,** 167–203.
23. Huttenlocher, A., Sandborg, R. R., and Horwitz, A. F. (1995) *Curr. Opin. Cell Biol.* **7,** 697–706.
24. Cary, L. A., Chang, J. F., and Guan, J. L. (1996) *J. Cell Sci.* **109,** 1787–1794.
25. Romer, L. H., McLean, N., Turner, C. E., and Burridge, K. (1994) *Mol. Biol. Cell.* **5,** 349–361.
26. Owen, J. D., Ruest, P. J., Fry, D. W., and Hanks, S. K. (1999) *Mol. Cell. Biol.* **19,** 4806–4818.
27. Kon, J., Sato, K., Watanabe, T., Tomura, H., Kuwabara, A., Kimura, T., Tamama, K., Ishizuka, T., Murata, N., Kanda, T., Kobayashi, I., Ohta, H., Ui, M., and Okajima, F. (1999) *J. Biol. Chem.* **274,** 23940–23947.
28. Lee, M.-J., Thangada, S., Claffey, K. P., Ancellin, N., Liu, C. H., Kluk, M., Volpi, M., Sha'afi, R. I., and Hla, T. (1999) *Cell* **99,** 301–312.
29. Ancellin, N., and Hla, T. (1999) *J. Biol. Chem.* **274,** 18997–19002.
30. Masiero, L., Lapidos, K. A., Ambudkar, I., and Kohn, E. C. (1999) *J. Cell Sci.* **112,** 3205–3213.
31. Papapetropoulos, A., Garcia-Cardena, G., Madri, J. A., and Sessa, W. C. (1997) *J. Clin. Invest.* **100,** 3131–3139.
32. Okamoto, H., Takuwa, N., Gonda, K., Okazaki, H., Chang, K., Yatomi, Y., Shigematsu, H., and Takuwa, Y. (1998) *J. Biol. Chem.* **273,** 27104–27110.
33. Harvath, L., Robbins, J. D., Russell, A. A., and Seamon, K. B. (1991) *J. Immunol.* **146,** 224–232.
34. Dai, Y., Holgate, S. T., Church, M. K., and Shute, J. K. (1994) *J. Leukocyte Biol.* **56,** 776–783.
35. Elferink, J. G. R., and Van Uffelen, B. E. (1996) *Gen. Pharmacol.* **27,** 387–393.
36. Lee, D.-J., Lee, O.-H., and Kwon, Y.-G., unpublished observation.