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 (p125FAK) which is important for cell migration. S1P induces a rapid increase in tyrosine phosphorylation of p125FAK. Compared with other structurally related lipid metabolites such as sphingosine, C2-ceramide, and lysophosphatidic acid, S1P uniquely stimulated p125FAK tyrosine phosphorylation and migration of HUVECs. The effect of S1P on p125FAK tyrosine phosphorylation was markedly reduced by treatment with pertussis toxin or U73122, a phospholipase C (PLC) inhibitor. As a downstream signal of PLC, p125FAK 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.

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 chemoinsaviveness 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

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; p125FAK, p125 focal adhesion kinase.

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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 protein-linked PLC-Ca\(^{2+}\)-system in HUVECs. Also, we found that activation of PLC which may lie downstream of G 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 BAFTA-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 Biochemicals International). U0126, which is a specific inhibitor of MAPK kinase (MEK), was a kind gift of Dr. J. M. Trzaskos (DuPont Biochemicals International). U0126, which is a specific inhibitor of MAPK kinase (MEK), was a kind gift of Dr. J. M. Trzaskos (DuPont Biochemicals International). U0126, which is a specific inhibitor of MAPK kinase (MEK), was a kind gift of Dr. J. M. Trzaskos (DuPont Biochemicals International). U0126, which is a specific inhibitor of MAPK kinase (MEK), was a kind gift of Dr. J. M. Trzaskos (DuPont Biochemicals International). 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the same concentration. And then, we compared the effect of these compounds on p125FAK 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 p125FAK 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 p125FAK Signaling Pathway in HUVECs

To elucidate the p125FAK signaling pathways induced by S1P in HUVECs, we investigated the effects of agents that interfere with activation of G\textsubscript{i} protein or intracellular signaling routes on S1P-stimulated tyrosine phosphorylation of p125FAK. As similar to the potent inhibitory effect of PTX on HUVEC chemotaxis (9), PTX (100 ng/ml for 6 h) dramatically abrogated S1P-induced p125FAK tyrosine phosphorylation (Figs. 3A and 3C). These results suggest that the signaling pathway regulated by S1P in HUVECs is dependent on a G\textsubscript{i}-coupled S1P receptor, and show clear difference with the p125FAK 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 p125FAK 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 p125FAK is independent of ERKs and p38 MAPK activation.

G\textsubscript{i} protein dissociates into G\textsubscript{i\alpha} and G\textsubscript{i\beta\gamma} subunits upon receptor activation and the released G\textsubscript{i\beta\gamma} subunit is known to stimulate PLC (22). To determine whether the stimulation of p125FAK tyrosine phosphorylation by S1P was dependent on PLC, we used U73122, a specific inhibitor of PLC. At 5 μM, U73122 markedly blocked S1P-induced p125FAK tyrosine phosphorylation, indi-

![FIG. 1. S1P induces tyrosine phosphorylation of p125FAK in HUVECs. (A) Dose response of S1P-induced p125FAK tyrosine phosphorylation. Confluent HUVECs were treated with various concentration of S1P for 2 min. (B) Time course of S1P-induced p125FAK tyrosine phosphorylation. HUVECs were stimulated for the indicated times with 5 μM S1P. Cell lysates were prepared and immunoprecipitated with anti-p125FAK antibody. The immune complexes were analyzed by immunoblotting with anti-phosphotyrosine antibody to assay for p125FAK tyrosine phosphorylation (P-p125FAK) or anti-p125FAK antibody for p125FAK protein levels (p125FAK). The graphs represent quantitative results from densitometric analysis. Values are the means of three separate experiments and expressed as fold-stimulation above unstimulated control.](image-url)
cating that activation of PLC by S1P is closely involved in the p125FAK signaling pathway (Figs. 3B and 3C). Since PLC is known to generate inositol triphosphate and diacylglycerol, which activate intracellular Ca²⁺ mobilization and PKC, respectively, we evaluated the potential roles of PKC and an increase of intracellular Ca²⁺ in p125FAK tyrosine phosphorylation. The PKC inhibitor GF109203X had no significant effect on S1P-induced p125FAK tyrosine phosphorylation (Figs. 3B and 3C). In contrast, S1P-induced p125FAK tyrosine phosphorylation was completely abolished by the intracellular Ca²⁺ chelator BAPTA-AM (30 μM for 30 min), suggesting that an increase in intracellular Ca²⁺ may play an important role in S1P-induced p125FAK activation (Figs. 3B and 3C). Indeed, treatment with the Ca²⁺ ionophore ionomycin (1 μM) for 2 min was sufficient to stimulate tyrosine phosphorylation of p125FAK (Fig. 4A). Further, downregulation of S1P-induced p125FAK phosphorylation by PTX was significantly restored by ionomycin, and cotreatment of S1P with ionomycin further increased p125FAK tyrosine phosphorylation compared with S1P alone (Fig. 4B).

**FIG. 2.** Effects of S1P, sphingosine, C2-ceramide, and LPA on chemotactic motility and tyrosine phosphorylation of p125FAK 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 ± 7.0 cells/field. Results are expressed as percentage ± 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-p125FAK antibody. The immune complexes were analyzed by immunoblotting with anti-phosphotyrosine antibody to assay for p125FAK tyrosine phosphorylation (P-p125FAK) or anti-p125FAK antibody for p125FAK protein levels (p125FAK). The graphs represent quantitative results from densitometric analysis. Values are the means of three separate experiments and expressed as fold-stimulation above unstimulated control.

**FIG. 3.** S1P-induced tyrosine phosphorylation of p125FAK in HUVECs is sensitive to the inhibitors of G 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), and 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-p125FAK antibody. The immune complexes were analyzed by immunoblotting with anti-phosphotyrosine antibody to assay for p125FAK tyrosine phosphorylation (P-p125FAK) or anti-p125FAK antibody for p125FAK protein levels (p125FAK). (C) The densities of the bands corresponding to phosphorylated and non-phosphorylated p125FAK were quantitated using a densitometer. The results are expressed as fold-stimulation above control.
Taken together, these results suggest that S1P-induced p125FAK 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.

**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 p125FAK and the p125FAK 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 p125FAK 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 p125FAK tyrosine phosphorylation in HUVECs. Consistently with the suggested role of p125FAK for endothelial cell migration, our data showed that S1P induces a rapid increase in the tyrosine phosphorylation of the focal adhesion-associated protein p125FAK in HUVECs. Apparently, compared with other structurally related...
lipids at 1 μM concentration, S1P uniquely activated migration and p125FAK tyrosine phosphorylation of HUVECs. Also, p125FAK 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 protein-coupled receptors to stimulate p125FAK tyrosine phosphorylation and motility of HUVECs.

In our previous study, we suggested the role of the Gi 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 Gi 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 p125FAK phosphorylation and endothelial cell migration. Also, intracellular Ca\(^{2+}\) mobilization but not PKC is an important downstream signal of PLC in the S1P-induced p125FAK 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-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 Gi 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, forskolin, 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 Gi protein-coupled S1P receptor, presumably EDG-1, and the PLC-Ca\(^{2+}\) signaling pathway is crucially involved in S1P-induced p125FAK 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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