

S1P₃-mediated Akt activation and crosstalk with platelet-derived growth factor receptor (PDGFR)

Linnea M. Baudhuin,^{*,†} Ying Jiang,^{*} Alexander Zaslavsky,^{*,†} Isao Ishii,[‡] Jerold Chun,[§] and Yan Xu^{*,†,||}

^{*}Department of Cancer Biology, The Lerner Research Institute, The Cleveland Clinic Foundation; [†]Department of Chemistry, Cleveland State University, Cleveland, Ohio 44115; [‡]Department of Molecular Genetics, National Institute of Neuroscience, Kodaira, Tokyo 187-8502, Japan; [§]Department of Molecular Biology, Scripps Research Institute, La Jolla, California 92037; and ^{||}Department of Gynecology and Obstetrics, The Cleveland Clinic Foundation, Cleveland, Ohio 44195

Linnea M. Baudhuin and Ying Jiang contributed equally to this work.

Current address of L. M. Baudhuin: Department of Laboratory Medicine and Pathology, Mayo Clinic and Foundation, 200 First St. SW, Rochester, MN 55905.

Corresponding author: Yan Xu, Department of Cancer Biology, Lerner Research Institute, Cleveland Clinic Foundation, 9500 Euclid Ave. Cleveland, OH 44195. E-mail: xuy@ccf.org

ABSTRACT

Akt plays a pivotal role in cell survival and tumorigenesis. We investigated the potential interaction between sphingosine-1-phosphate (S1P) and platelet-derived growth factor (PDGF) in the Akt signaling pathway. Using mouse embryonic fibroblasts (MEFs) from S1P receptor knockout mice, we show here that S1P₃ was required for S473 phosphorylation of Akt by S1P. In addition, S1P-stimulated activation of Akt, but not ERK, was blocked by a PDGF receptor (PDGFR)-specific inhibitor, AG1296, suggesting a S1P₃-mediated specific crosstalk between the Akt signaling pathways of S1P and PDGFR in MEFs. We investigated this crosstalk under different conditions and found that both Akt and ERK activation induced by S1P, but not lysophosphatidic acid (LPA), in HEY ovarian cancer cells required PDGFR but not epidermal growth factor receptor (EGFR) or insulin-like growth factor-I receptor (IGFR). Importantly, S1P induced a G_i-dependent tyrosine phosphorylation of PDGFR in HEY cells. This dependence on PDGFR in S1P-induced Akt activation was also observed in A2780, T47D, and HMEC-1 cells (which express S1P₃), but not in PC-3 or GI-101A cells (which do not express S1P₃), further supporting that S1P₃ mediates the crosstalk between S1P and PDGFR. This is the first report demonstrating a unique interaction between S1P₃ and PDGFR, in addition to demonstrating a specific role for S1P₃ in S1P-induced Akt activation.

Key words: sphingosine-1-phosphate • mouse embryonic fibroblasts • GPCR

The bioactive lysophospholipid S1P elicits many of its effects by binding to G protein-coupled receptors (GPCRs). Five GPCRs have been identified for S1P: S1P₁ (Edg-1), S1P₂ (Edg-5), S1P₃ (Edg-3), S1P₄ (Edg-6), and S1P₅ (Edg-8) (1–5). While S1P₁, S1P₂,

and S1P₃ are widely expressed, S1P₄ receptor is lymphoid specific and S1P₅ is expressed mainly in the brain (3). S1P₁, S1P₂, and S1P₃ mediate both overlapping and distinct signaling pathways and regulate important physiological processes such as blood vessel maturation, cardiac development, and in vivo angiogenesis (2, 6–12).

Phosphatidylinositol-3 kinase (PI3-K)/Akt signaling is involved in cell survival in many cellular systems and cancers, including ovarian cancer (13–15). The activation of Akt, an anti-apoptotic protooncogene, is mediated by PI3-K, after receptor stimulation. Activation of the Akt signaling pathway by S1P has been reported in a variety of cell types, including endothelial cells, hepatic myofibroblasts, and transfected CHO cells (16–20). In our recent work, we have shown that activation of Akt by both S1P and LPA is likely to be involved in cell survival and anti-apoptotic processes in ovarian cancer cells (21). We have demonstrated that among a number of stimuli tested, S1P, LPA, and PDGF, but not endothelin-1 (Et-1), thrombin, insulin, or EGF, required MEK and likely its downstream target, ERK, for activation of Akt in certain cellular systems, including HEY ovarian cancer cells (21). In addition, S1P and PDGF, but not LPA, were independent of Rho for Akt activation. Based on these results, we postulated that while LPA may have its own independent signaling pathway, S1P, PDGF, and their receptors may interact with each other (crosstalk), leading to Akt activation in HEY cells and potentially other cell types. Furthermore, based on the expression patterns of S1P₁₋₃ in various cell lines tested, we have suggested that among different S1P receptors, S1P₃ may play an important role in Akt activation induced by S1P (21).

The interactions between the signaling pathways of S1P and PDGF have been shown previously in two different models: the sequential and the integrative models. Hobson et al. (22) showed a sequential model with PDGF-induced cell motility that was dependent on S1P₁ expression and an interaction between S1P₁ and PDGF that was mediated via the production of S1P in HEK293 cells and MEFs. This same group has also shown that S1P₁ appears to be dispensable for mitogenic and survival effects, even those induced by its ligand S1P and by PDGF (23). In contrast, Alderton et al. (24) demonstrated an integrative model showing that when both PDGFR and S1P₁ were overexpressed in HEK293 cells, they formed a tethered complex, which led to more efficient stimulation of pro-mitogenic cellular events. Furthermore, they showed that tyrosine phosphorylation of G_{iα} by PDGFR was required for efficient ERK signaling induced by either PDGF or S1P (24). More recently this same group has provided more information on the integrative model by demonstrating that S1P acts via endogenous PDGF-β receptor-S1P₁ receptor complexes in airway smooth muscle cells to promote mitogenic signaling (25).

In this work, we further explored the signaling mechanisms of S1P-induced Akt activation and revealed a novel type of crosstalk between S1P and PDGFR. This crosstalk is mainly mediated by S1P₃ (a S1P receptor subtype different from the previously published studies; refs 22–25) and leads to Akt activation (a signaling pathway different from the cell motility and ERK activation pathways studied previously; refs 22–25). Importantly, we show here that S1P requires PDGFR to induce Akt phosphorylation in a cell type-dependent manner and S1P induces tyrosine phosphorylation of PDGFR in HEY ovarian cancer cells.

MATERIALS AND METHODS

Materials

S1P and oleoyl-LPA were purchased from Avanti Polar Lipids (Birmingham, AL) or Toronto Research Chemicals (Toronto, Canada). 18:1-LPA was dissolved in PBS, and S1P was dissolved in Tris-saline (50 mM Tris, pH 9.5, 145 mM NaCl) to 4 and 2 mM stock solutions, respectively. Pertussis toxin (PTX) was from Invitrogen (Rockville, MD), and LY294002 was from Biomol (Plymouth Meeting, PA). PDGF-BB was a kind gift from the laboratory of Dr. Paul DiCorleto, The Cleveland Clinic Foundation, and was also purchased from R&D Systems (Minneapolis, MN). EGF was from Calbiochem (La Jolla, CA), and IGF-I was a kind gift from the lab of Dr. Alan Wolfman, The Cleveland Clinic Foundation, or purchased from Calbiochem. AG1295, AG1296, AG1478, and AG1024 were obtained from Calbiochem. Neutralizing antibodies to PDGFR- α (2H7C5) and PDGFR- β (2A1E2) were kindly provided by Dr. Carol M. Sullivan, COR Therapeutics, Inc. (South San Francisco, CA). Anti-phospho-S473-Akt, anti-phospho-T308-Akt, anti-Akt, anti-phospho-ERK, and anti-ERK antibodies were obtained from Cell Signaling Technology (Beverly, MA).

Cell culture

HEY and OCC1 ovarian cancer cells were from Dr. G. Mills or Dr. R. Bast, MD Anderson (Houston, TX). T47D breast cancer cells were from ATCC (#HTB-133; Manassas, VA). GI-101A breast cancer cells were from the Goodwin Institute for Cancer Research, Inc. (Plantation, FL). An immortalized human microvascular endothelial cell line, HMEC-1 was from Centers for Disease Control and Prevention (Atlanta, GA). PC-3 prostate cancer cells were from Dr. Warren Heston, The Cleveland Clinic Foundation. All of the above cell lines were maintained in RPMI 1640 medium containing 10% fetal bovine serum (FBS). A2780 cells (also from Dr. G. Mills) were maintained in DMEM:F-12 (1:1) medium supplemented with 10% FBS. MEF wild-type, S1P₃ null, and S1P_{2/3} null cells were prepared as described previously (26, 27) and maintained in DMEM with 10% FBS. All cells were cultured in serum-free media for 24-48 h before lipid treatment.

S1P₃ receptor constructs, transfection, and infection

Human cDNAs of S1P₃ were kind gifts from the laboratory of Kevin R. Lynch, University of Virginia Health System. The entire ORF of S1P₃ was PCR amplified and cloned into the p3XFLAG-CMV10 mammalian expression vector (Sigma) at the Hind III and EcoR I sites to introduce FLAG-tagged sequence into the N terminus of the receptor. The sequences of the primers used for S1P₃ are as follows: sense, 5'-TGA CAA GCT TGC AAC TGC CCT CCC G-3', and antisense, 5'-CCG CGA ATT CAG TTG CAG AAG ATC CCA TTC-3'. The coding region of 3XFLAG-S1P₃ was PCR amplified and subcloned into the pMSCVpuro retrovirus vector (BD biosciences) at the Pme I and EcoR I sites, and the sequence of S1P₃ was confirmed by sequencing. The sequences of the primers used are as follows: sense, 5'-CCG AGT TTA AAC ATG GAC TAC AAA GAC CAT-3', and antisense, 5'-CCG CGA ATT CAG TTG CAG AAG ATC CCA TTC-3'. The S1P₃ construct was cotransfected with an amphotropic-packaging plasmid into 293T cells with a DNA-calcium phosphate coprecipitation method. The retrovirus supernatant was harvested every 3-4 h for a total of 3 days and stored at -80°C until use. MEFs at passages 3-5 were infected. The viral supernatant supplemented with polybrene (8 μ g/ml) was

added to the media of the cells on 6-well plates, and the plates were centrifuged (at 700 g) at 32°C for 1 h. The cells were cultured for 12 h, and then the infections were repeated two more times. Twenty-four hours after the third infection, cells were starved from serum for 24 h before S1P or PDGF stimulation.

Nonradioactive immunoprecipitation Akt kinase assay

The Akt kinase assay was performed with the Nonradioactive Akt Kinase Assay Kit (Cell Signaling Technology) according to the instructions of the manufacturer. All reagents were provided with the kit. Briefly, cells were treated with LPA or S1P, rinsed with ice-cold phosphate-buffered saline (PBS), and then lysed in cell lysis buffer. Immunoprecipitation was carried out using immobilized Akt 1G1 monoclonal antibody. The immunoprecipitate was then incubated with GSK-3 fusion protein and ATP in kinase buffer. Western analyses were used to determine the extent of GSK-3 phosphorylation by active Akt using a phospho-GSK-3 α/β (Ser21/9) antibody.

Western blot analysis

After treatment with LPA, S1P, or other stimuli, cells were rinsed with ice-cold PBS and then lysed in SDS sample buffer. Samples were electrophoresed through a 10-12% SDS polyacrylamide gel and then transferred to a PVDF membrane (Bio-Rad, Hercules, CA). Immunoblot analyses were carried out using the appropriate antibodies. Specific proteins were detected with the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Piscataway, NJ).

Determination of tyrosine phosphorylation of PDGFR

After treatment with S1P or PDGF-BB, cells were immediately placed on ice, rinsed with ice-cold PBS, and then lysed for 30 min on ice in 20 mM HEPES (pH 7.4) containing 0.5% Triton X-100, 150 mM NaCl, 12.5 mM β -glycerophosphate, 1.5 mM MgCl₂, 2 mM EGTA, 10 mM NaF, 2 mM DTT, 1 mM sodium orthovanadate, 1 mM PMSF, and 20 mM aprotinin. Lysates were cleared of cellular debris by centrifugation and then precleared with protein A-Sepharose beads (Amersham Pharmacia Biotech). The lysates were then subjected to immunoprecipitation with antihuman PDGF- β receptor antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and the immune complex was collected by incubation with protein A-Sepharose beads. The immunoprecipitates underwent 8% SDS-PAGE and were transferred to a PVDF membrane. The membrane was probed with antiphosphotyrosine antibody (clone 4G10; Upstate Biotechnology, Lake Placid, NY), and tyrosine phosphorylated PDGFR was detected by the ECL system.

RESULTS

S1P₃ was required for the PDGFR-dependent S473 Akt phosphorylation in MEFs

We have recently reported that both S1P and PDGF induce a MEK-dependent Akt activation only in S1P₃-expressing cells (21). Thus, we postulated that S1P₃ is required for the MEK (and ERK)-dependent Akt phosphorylation and proposed that a potential crosstalk exists between S1P and PDGF. To test this, we used S1P₃ null MEFs, which in addition to S1P₃, have no endogenous expression of S1P₄ and S1P₅ but have normal expression of S1P₁ and S1P₂ (26). In wild-type MEFs, which express S1P₁₋₃, S1P (1 μ M) and PDGF (10 ng/ml) induced

phosphorylation of both ERK and Akt (S473) ([Fig. 1A](#)). As expected, AG1296, a specific inhibitor of PDGFR, inhibited the phosphorylation of both Akt and ERK induced by PDGF-BB ([Fig. 1A](#)). Interestingly, phosphorylation of Akt, but not ERK, induced by S1P was also sensitive to AG1296 (at 50 μ M), suggesting that S1P requires PDGFR activity for Akt S473 phosphorylation in MEFs, while ERK phosphorylation by S1P is independent of PDGFR. On the other hand, we found that S1P was unable to induce Akt phosphorylation in S1P₃ null MEFs, but ERK activation by S1P was unaffected ([Fig. 1B](#)). The ability of PDGF to induce phosphorylation of ERK and Akt was not significantly affected in S1P₃ null cells ([Fig. 1B](#) and [E](#)), suggesting that PDGF was not dependent on S1P₃ to activate these downstream signaling molecules in MEFs. Reestablishment of S1P₃ expression (by infection of S1P₃ in a retroviral vector) restored the ability of S1P to induce Akt activation, which was sensitive to AG1296 ([Fig. 1C](#)), strongly suggesting that S1P₃ is required for S473 Akt phosphorylation in MEFs, and this activity is dependent on the activation of PDGFR. We confirmed this notion in S1P_{2/3} double null MEFs, where S1P was again unable to induce Akt phosphorylation, but ERK activation by S1P was unaffected (data not shown). S1P-induced Akt signaling could be restored by infecting S1P₃ alone in S1P_{2/3} double null MEFs ([Fig. 1D](#)), indicating the necessity of S1P₃ in Akt signaling and also suggesting that S1P₂ is unnecessary for the activity in MEFs. In both S1P₃ null and S1P_{2/3} double null MEFs, vector-only infected cells did not change cells responsiveness to either S1P or PDGF (data not shown). [Figure 1E](#) shows the mean densitometric traces of several experiments.

S1P is dependent on PDGFR for Akt and ERK activation in HEY ovarian cancer cells

As described above, we revealed a new type of interaction between S1P and PDGFR in Akt activation in MEFs. To determine whether this interaction was also present in other cell types and to further study the nature of the interaction, we examined the PDGFR dependence of S1P-induced activation of Akt and ERK in HEY cells. HEY cells express relatively high levels of S1P₁ and S1P₃ (21). We found that AG1296 (10 μ M) pretreatment of HEY cells resulted in a complete inhibition of S1P- and PDGF-induced Akt activation (measured by an Akt enzymatic activity; [Fig. 2A](#)). To confirm that PDGFR activity was required for the Akt activation induced by S1P, we examined the effects of AG1296, 2A1E2 (a neutralizing antibody to PDGFR- β), and 2H7C5 (a neutralizing antibody to PDGFR- α) on Akt S473 and T308 phosphorylation. We performed titration analyses of 2A1E2 and 2H7C5 to determine the optimal concentration to use in our system (data not shown). A combination of 2A1E2 and 2H7C5, both at 0.3 nM, completely blocked PDGF-BB-induced Akt activation/phosphorylation, and this concentration was used in our subsequent studies. The dependence on PDGFR activity for Akt activation was S1P specific, since a similar lipid signaling molecule, LPA, was not dependent on PDGFR for Akt phosphorylation at both S473 and T308 ([Fig. 2B](#)). We also found that S473 phosphorylation of Akt induced by endothelin-1 and thrombin in HEY cells was not affected by pretreatment with AG1296 (data not shown). In addition, the dependence on a tyrosine kinase receptor by S1P was specific to PDGFR, since AG1478 and AG1024 (specific inhibitors of EGFR and IGFR, respectively) had no effect on S1P-induced Akt activation ([Fig. 2C](#)).

In contrast to MEFs, we demonstrated that PDGFR was also required for ERK phosphorylation induced by S1P in HEY cells, as determined by Western blot analysis ([Fig. 2D](#)). This is consistent with our previous observation that MEK and ERK are upstream of Akt in S1P-induced Akt activation in HEY cells (21). These results suggest that upstream signaling mechanisms leading to Akt activation may be different in different cell types.

S1P induces tyrosine phosphorylation of PDGFR

To assess directly the involvement of PDGFR, and whether S1P could actually stimulate tyrosine phosphorylation of PDGFR, HEY cells were treated with S1P or PDGF, and tyrosine phosphorylation of PDGFR- β was assessed. As expected, PDGFR was strongly phosphorylated by PDGF ([Fig. 3A](#)). S1P also induced phosphorylation of PDGFR, although its kinetics was slower ([Fig. 3A](#)). Phosphorylation of PDGFR by S1P occurred as early as 1 min and maximized at 20 min ([Fig. 3A](#)). Treatment of cells with PTX attenuated S1P-, but not PDGF-induced PDGFR tyrosine phosphorylation, suggesting that the former occurred in a G_i -dependent manner and the latter did not require G_i ([Fig. 3B](#)). S1P- and PDGF-induced tyrosine phosphorylation of PDGFR was insensitive to LY294002 ([Fig. 3B](#)), a specific inhibitor of PI3-K, indicating that PI3-K activation is not required for PDGFR phosphorylation. Furthermore, since Akt activation by S1P and PDGF requires PI3-K, it is likely that PI3-K is downstream of S1P-induced PDGFR activation in this system.

PDGFR-dependent Akt activation by S1P is cell line specific

To test whether the requirement of PDGFR activity for S1P-induced Akt activation is cell type specific, we examined Akt S473 phosphorylation in the presence or absence of AG1296 in six cancer cell lines. In addition to HEY ovarian cancer cells, S1P phosphorylated Akt in a PDGFR-dependent manner in a number of other cell lines: A2780 (an ovarian cancer cell line), T47D (a breast cancer cell line), and an immortalized human microvascular endothelial cell line, HMEC-1 ([Fig. 4A](#), and data not shown). However, in PC-3 prostate and GI-101A breast cancer cells, S1P did not require activation of PDGFR, as demonstrated by pretreatment with both AG1296 and the neutralizing antibodies against PDGFRs ([Fig. 4B](#) and data not shown). Interestingly, we found that all PDGFR-dependent cell lines express $S1P_3$ and all PDGFR-independent cell lines express no or very low levels of $S1P_3$ (21). These results suggest that PDGFR-dependent phosphorylation of Akt by S1P is mediated by $S1P_3$ specifically. Other S1P receptors, such as $S1P_1$, and $S1P_2$ may mediate PDGFR-independent Akt activation by S1P in a cell type-dependent manner.

DISCUSSION

The work presented here focuses on $S1P_1$, $S1P_2$, and $S1P_3$, which are widely expressed. The three newly identified potential S1P receptors, *gpr3*, *gpr6*, and *gpr12* are constitutively active (28), and their role in Akt signaling remains to be investigated. Whether these receptors are expressed in MEFs has not been reported.

S1P receptor subtype-dependent Akt activation

Specific S1P receptor subtypes can regulate important physiological processes such as blood vessel maturation (7), cardiac development (8), and angiogenesis in vivo (9). S1P stimulates or inhibits numerous cellular functions and signaling pathways, which appear to be cell type, receptor subtype, and signaling pathway dependent, leading to different cellular functions (2, 3, 11, 12, 29). For example, S1P stimulates and inhibits cell migration of endothelial cells and nonendothelial cells, respectively (30). While $S1P_1$ and $S1P_3$ act as typical chemotactic receptors, $S1P_2$ may uniquely act as a chemorepellant receptor (31). All three S1P receptors, $S1P_1$, $S1P_2$, and $S1P_3$, can mediate activation of PI3-K by S1P in S1P receptor transfected CHO

cells (31). However, the downstream signaling molecules can be differentially regulated in a receptor subtype-dependent manner. It has been shown that while S1P₁ and S1P₃ positively regulate Rac, S1P₂ negatively regulates Rac, resulting in opposing effects on cell migration (31). Rosenfeldt et al. (23) showed that S1P₁ is required for cell motility but not for mitogenicity and survival effects induced by either S1P or PDGF. We have previously shown that MEFs from S1P₃ null mice show significant decreases in phospholipase C (PLC) activation, slight decreases in adenylyl cyclase inhibition, and no change in Rho activation upon S1P stimulation (26). In contrast, S1P₂ null MEFs show a significant decrease in Rho activation but no change in PLC activation, calcium mobilization, or adenylyl cyclase inhibition. Double null (S1P₃ and S1P₂ null) MEFs displayed a complete loss of Rho activation (mediated through G_{12/13}) and a significant decrease in PLC activation and calcium mobilization (mediated via G_q), with no change in adenylyl cyclase inhibition (26, 27). Overall, PLC activation by S1P appears to be mediated by S1P₃. The relationship of S1P-induced PLC and Akt activation remains to be investigated.

Akt plays a very important role in cell survival and tumorigenesis. S1P₃-mediated cell survival has been shown in rat hepatoma cells (32) but not in human umbilical vein endothelial cells (HUVEC) (9) or Schwann cells, although LPA mediates cell survival in Schwann cells through a G_i-dependent Akt activation, indicating variability of signaling based on cell type examined (33). Activation of the Akt signaling pathway by S1P has been reported in various cell types, including endothelial cells, hepatic myofibroblasts, and transfected CHO cells (16–18, 31). With the use of an antisense approach in HUVEC, both S1P₁ and S1P₃ (which are endogenously expressed in these cells) (9) have been shown to be involved in S1P-induced NO production (34). This activity has been related to phosphorylation of the endothelial isoform of nitric oxide synthase (eNOS) by Akt (35–37). Banno et al. (6, 19, 20) have shown that S1P₃, when overexpressed in CHO cells, mediates Akt activation (measured by S473 phosphorylation of Akt). However, they did not address whether this activation is S1P₃ specific.

Our results not only are consistent with and support the findings of others, but we have also added important new insights to the cell type-, receptor subtype-, and signaling pathway-dependent cellular effects of S1P. We show here that S1P₃ is required for S1P-induced Akt but not ERK activation in MEFs. All other S1P receptors, including S1P₁ and S1P₂ (which are expressed in S1P₃ null MEFs), are either not sufficient or not required for Akt phosphorylation in MEFs. Since S1P₃ expression resumes the Akt activation induced by S1P in both S1P₃ null and S1P_{2/3} double knockout MEFs, it suggests that S1P₂ is unnecessary for S1P-induced Akt phosphorylation in MEFs. Whether S1P₃ is sufficient for the activity remains to be addressed and will require complete removal of all other S1P receptors, including S1P₁, S1P₂, S1P₄, S1P₅, and possibly even the newly identified S1P receptors, gpr3, 6, and 12 (38). In contrast to MEFs, we have found that S1P may be able to induce Akt phosphorylation via other S1P receptors in a number of epithelial cell lines, including a prostate cell line, PC-3, and a breast cell line, GI-101A. These cells express low or nondetectable (via quantitative PCR) levels of S1P₃ (21). However, since these cells are not S1P₃-“null,” it cannot be ruled out that even at very low levels of expression, S1P₃ may play some role in Akt signaling. Similarly, it has been suggested that S1P₁ mediates Akt activation (measured by S473 phosphorylation) in bovine lung microvascular endothelial cells (BLMVECs). However, this conclusion was derived from the expression data (S1P₁ is highly expressed in these cells) but not functional subtype receptor based analyses. Thus, the role and involvement of each S1P receptor subtype in Akt signaling remain to be further addressed in BLMVECs.

Due to the lack of antibodies highly effective at detecting low levels of endogenous S1P receptors, almost all published work and the results presented here are based on the mRNA expression levels of these receptors, which may or may not correlate with the protein levels of these receptors in cells. More importantly, the levels of functionally active receptors cannot be easily accessed. Therefore, caution should be taken when interpreting the data presented here or in other publications. In our opinion, the work presented here using knockout MEFs provides the best evidence for the S1P₃-mediated and PDGFR-dependent Akt activation. Antisense- and/or short-interfering RNA (siRNA)-based approaches to knock down endogenous expression of the receptor may provide useful information for the role of a particular receptor. However, complete removal of a receptor at the protein and the functional levels is hard to assess as mentioned above. Moreover, in light of a recent report on the nonspecific effects of siRNA (39), special caution needs to be taken when using these approaches.

Nevertheless, a highly interesting finding revealed through this work is that while S1P₁, S1P₂, and S1P₃ all potentially mediate Akt signaling in a cell type-dependent manner, the PDGFR-dependent Akt phosphorylation induced by S1P was only observed in S1P₃-expressing (measured by the mRNA levels) cells among all cell types (fibroblasts, epithelial, or endothelial cells) tested. This suggests that among S1P₁₋₃, the PDGFR-dependent Akt activation is potentially mediated by S1P₃ specifically. Together, our data, combined with the findings of others, suggest that each individual S1P receptor may have differential preferences to specific signaling pathways.

Crosstalk between S1P and PDGFR and a novel type of interaction between S1P and PDGFR

Interactions between the signaling pathways of S1P and PDGF have been shown previously in two different models as noted above. The S1P₁-dependent PDGF action shown by Spiegel's group (22, 23) may be cell type specific, since Kluk et al. (40) have recently shown that S1P₁ was critical for S1P-induced, G_i-dependent migration, but not for PDGF-BB-induced, receptor tyrosine kinase-dependent chemotaxis in vascular smooth muscle cells. The major differences between the sequential model by Spiegel's group (22, 23) and the integrative model by Pyne's group (24, 25) are that the former suggests that the production of S1P induced by PDGF is the mediator of the interaction, and PDGF is unidirectionally dependent on S1P₁ to induce cell motility, while the latter suggests that a physical interaction between S1P₁ and PDGFR, but not S1P production, plays the central role and the signaling efficiencies of both S1P and PDGF are increased (bi-directional) ([Fig. 5](#)). In studies from Pyne's group (24, 25), PDGF did not induce S1P production in either HEK293 or smooth muscle cells. Our work presented here adds important and interesting new information to models of interactions of S1P-PDGF signaling. We show here that 1) another S1P receptor subtype (S1P₃, rather than S1P₁) is involved in the interaction between S1P and PDGF signaling; 2) a "reciprocal" requirement exists, i.e., S1P-induced Akt phosphorylation via S1P₃ requires PDGFR; and 3) S1P can activate PDGFR by induction of tyrosine phosphorylation of PDGFR ([Fig. 5](#)). Transactivation of tyrosine kinase receptors by GPCRs has drawn a great deal of attention in recent years. Collectively, the published data and our novel observations may not necessarily be mutually exclusive and the interactions between S1P and PDGF are likely to be signaling pathway, S1P receptor subtype, and cell type specific. These interactions between S1P and PDGFR may play important physiological and pathological regulatory roles.

ACKNOWLEDGEMENTS

We would like to express our sincere appreciation to Kelly L. Cristina for technical support in the preparation of this manuscript and also to Dr. Bryan R. G. Williams for critical reading of this manuscript. We would also like to thank Dr. Kevin Lynch for the receptor cDNAs. This work was supported in part by a National Institutes of Health Grant RO1 CA89228-01A2, a Lynn Cohen Foundation grant, a Charlotte Geyer Foundation grant (to Y. Xu), and a National Institute of Mental Health grant (to J. Chun).

REFERENCES

1. Chun, J., Goetzl, E. J., Hla, T., Igarashi, Y., Lynch, K. R., Moolenaar, W., Pyne, S., and Tigyi, G. (2002) International Union of Pharmacology. XXXIV. Lysophospholipid receptor nomenclature. *Pharmacol. Rev.* **54**, 265–269
2. Fukushima, N., Ishii, I., Contos, J. J., Weiner, J. A., and Chun, J. (2001) Lysophospholipid receptors. *Annu. Rev. Pharmacol. Toxicol.* **41**, 507–534
3. Kluk, M. J., and Hla, T. (2002) Signaling of sphingosine-1-phosphate via the S1P/EDG-family of G- protein-coupled receptors. *Biochim. Biophys. Acta* **1582**, 72–80
4. Goetzl, E. J., and An, S. (1998) Diversity of cellular receptors and functions for the lysophospholipid growth factors lysophosphatidic acid and sphingosine 1-phosphate. *FASEB J.* **12**, 1589–1598
5. Spiegel, S., and Milstien, S. (2000) Functions of a new family of sphingosine-1-phosphate receptors. *Biochim. Biophys. Acta* **1484**, 107–116
6. Banno, Y., Takuwa, Y., Akao, Y., Okamoto, H., Osawa, Y., Naganawa, T., Nakashima, S., Suh, P. G., and Nozawa, Y. (2001) Involvement of phospholipase D in sphingosine 1-phosphate-induced activation of phosphatidylinositol 3-kinase and Akt in Chinese hamster ovary cells overexpressing EDG3. *J. Biol. Chem.* **276**, 35622–35628
7. Liu, Y., Wada, R., Yamashita, T., Mi, Y., Deng, C. X., Hobson, J. P., Rosenfeldt, H. M., Nava, V. E., Chae, S. S., Lee, M. J., et al. (2000) Edg-1, the G protein-coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation. *J. Clin. Invest.* **106**, 951–961
8. Im, D. S., and Ungar, A. R., and Lynch, K. R. (2000) Characterization of a zebrafish (*Danio rerio*) sphingosine 1-phosphate receptor expressed in the embryonic brain. *Biochem. Biophys. Res. Commun.* **279** 139-143
9. 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) Vascular endothelial cell adherens junction assembly and morphogenesis induced by sphingosine-1-phosphate. *Cell* **99**, 301–312

10. Kluk, M. J., and Hla, T. (2001) Role of the sphingosine 1-phosphate receptor EDG-1 in vascular smooth muscle cell proliferation and migration. *Circ. Res.* **89**, 496–502
11. Siehler, S., and Manning, D. R. (2002) Pathways of transduction engaged by sphingosine 1-phosphate through G protein-coupled receptors. *Biochim. Biophys. Acta* **1582**, 94–99
12. Pyne, S., and Pyne, N. J. (2002) Sphingosine 1-phosphate signalling and termination at lipid phosphate receptors. *Biochim. Biophys. Acta* **1582**, 121–131
13. Marte, B. M., and Downward, J. (1997) PKB/Akt: connecting phosphoinositide 3-kinase to cell survival and beyond. *Trends Biochem. Sci.* **22**, 355–358
14. Liu, A. X., Testa, J. R., Hamilton, T. C., Jove, R., Nicosia, S. V., and Cheng, J. Q. (1998) AKT2, a member of the protein kinase B family, is activated by growth factors, v-Ha-ras, and v-src through phosphatidylinositol 3-kinase in human ovarian epithelial cancer cells. *Cancer Res.* **58**, 2973–2977
15. Yuan, Z. Q., Sun, M., Feldman, R. I., Wang, G., Ma, X., Jiang, C., Coppola, D., Nicosia, S. V., and Cheng, J. Q. (2000) Frequent activation of AKT2 and induction of apoptosis by inhibition of phosphoinositide-3-OH kinase/Akt pathway in human ovarian cancer. *Oncogene* **19**, 2324–2330
16. Morales-Ruiz, M., Lee, M. J., Zoellner, S., Gratton, J. P., Scotland, R., Shiojima, I., Walsh, K., Hla, T., and Sessa, W. C. (2001) Sphingosine-1-phosphate activates Akt, nitric oxide production and chemotaxis through a Gi-protein/phosphoinositide 3-kinase pathway in endothelial cells. *J. Biol. Chem.* **276**, 19672–19677
17. Lee, M. J., Thangada, S., Paik, J. H., Sapkota, G. P., Ancellin, N., Chae, S. S., Wu, M., Morales-Ruiz, M., Sessa, W. C., Alessi, D. R., et al. (2001) Akt-mediated phosphorylation of the G protein-coupled receptor EDG-1 is required for endothelial cell chemotaxis. *Mol. Cell* **8**, 693–704
18. Davaille, J., Li, L., Mallat, A., and Lotersztajn, S. (2002) Sphingosine 1-phosphate triggers both apoptotic and survival signals for human hepatic myofibroblasts. *J. Biol. Chem.* **277**, 37323–37330
19. Banno, Y., Fujita, H., Ono, Y., Nakashima, S., Ito, Y., Kuzumaki, N., and Nozawa, Y. (1999) Differential phospholipase D activation by bradykinin and sphingosine 1-phosphate in NIH 3T3 fibroblasts overexpressing gelsolin. *J. Biol. Chem.* **274**, 27385–27391
20. Banno, Y., Takuwa, Y., Yamada, M., Takuwa, N., Ohguchi, K., Hara, A., and Nozawa, Y. (2003) Involvement of phospholipase D in insulin-like growth factor-I-induced activation of extracellular signal-regulated kinase, but not phosphoinositide 3-kinase or Akt, in Chinese hamster ovary cells. *Biochem. J.* **369**, 363–368
21. Baudhuin, L. M., Cristina, K. L., Lu, J., and Xu, Y. (2002) Akt activation induced by lysophosphatidic acid and sphingosine-1-phosphate requires both mitogen-activated protein kinase kinase and p38 mitogen-activated protein kinase and is cell-line specific. *Mol. Pharmacol.* **62**, 660–671

22. Hobson, J. P., Rosenfeldt, H. M., Barak, L. S., Olivera, A., Poulton, S., Caron, M. G., Milstien, S., and Spiegel, S. (2001) Role of the sphingosine-1-phosphate receptor EDG-1 in PDGF-induced cell motility. *Science* **291**, 1800–1803
23. Rosenfeldt, H. M., Hobson, J. P., Maceyka, M., Olivera, A., Nava, V. E., Milstien, S., and Spiegel, S. (2001) EDG-1 links the PDGF receptor to Src and focal adhesion kinase activation leading to lamellipodia formation and cell migration. *FASEB J.* **15**, 2649–2659
24. Alderton, F., Rakhit, S., Kong, K. C., Palmer, T., Sambhi, B., Pyne, S., and Pyne, N. J. (2001) Tethering of the platelet-derived growth factor beta receptor to G- protein-coupled receptors. A novel platform for integrative signaling by these receptor classes in mammalian cells. *J. Biol. Chem.* **276**, 28578–28585
25. Waters, C., Sambhi, B. S., Kong, K. C., Thompson, D., Pitson, S. M., Pyne, S., and Pyne, N. J. (2002) Sphingosine 1-phosphate and platelet-derived growth factor act via platelet-derived growth factor beta receptor-sphingosine 1-phosphate receptor complexes in airway smooth muscle cells. *J. Biol. Chem.* **278**, 6282–6290
26. Ishii, I., Friedman, B., Ye, X., Kawamura, S., McGiffert, C., Contos, J. J., Kingsbury, M. A., Zhang, G., Brown, J. H., and Chun, J. (2001) Selective loss of sphingosine 1-phosphate signaling with no obvious phenotypic abnormality in mice lacking its G protein-coupled receptor, LP(B3)/EDG-3. *J. Biol. Chem.* **276**, 33697–33704
27. Ishii, I., Ye, X., Friedman, B., Kawamura, S., Contos, J., Kingsbury, M., Yang, A., Zhang, G., Brown, J., and Chun, J. (2002) Marked perinatal lethality and cellular signaling deficits in mice null for the two sphingosine 1-phosphate (S1P) receptors, S1P(2)/LP(B2)/EDG-5 and S1P(3)/LP(B3)/EDG-3. *J. Biol. Chem.* **277**, 25152–25159
28. Uhlenbrock, K., Gassenhuber, H., and Kostenis, E. (2002) Sphingosine 1-phosphate is a ligand of the human gpr3, gpr6 and gpr12 family of constitutively active G protein-coupled receptors. *Cell. Signal.* **14**, 941–953
29. Yang, A. H., Ishii, I., and Chun, J. (2002) In vivo roles of lysophospholipid receptors revealed by gene targeting studies in mice. *Biochim. Biophys. Acta* **1582**, 197–203
30. Panetti, T. S. (2002) Differential effects of sphingosine 1-phosphate and lysophosphatidic acid on endothelial cells. *Biochim. Biophys. Acta* **1582**, 190–196
31. Takuwa, Y. (2002) Subtype-specific differential regulation of Rho family G proteins and cell migration by the Edg family sphingosine-1-phosphate receptors. *Biochim. Biophys. Acta* **1582**, 112–120
32. An, S., Zheng, Y., and Bleu, T. (2000) Sphingosine 1-phosphate-induced cell proliferation, survival, and related signaling events mediated by G protein-coupled receptors Edg3 and Edg5. *J. Biol. Chem.* **275**, 288–296
33. Weiner, J. A., and Chun, J. (1999) Schwann cell survival mediated by the signaling phospholipid lysophosphatidic acid. *Proc. Natl. Acad. Sci. USA* **96**, 5233–5238

34. Kwon, Y., Min, J., Kim, K., Lee, D., Billiar, T., and Kim, Y. (2001) Sphingosine 1-phosphate protects human umbilical vein endothelial cells from serum-deprived apoptosis by nitric oxide production. *J. Biol. Chem.*, **276**, 10627-10633
35. Fulton, D., Gratton, J. P., McCabe, T. J., Fontana, J., Fujio, Y., Walsh, K., Franke, T. F., Papapetropoulos, A., and Sessa, W. C. (1999) Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature* **399**, 597-601
36. Dimmeler, S., Fleming, I., Fisslthaler, B., Hermann, C., Busse, R., and Zeiher, A. M. (1999) Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* **399**, 601-605
37. Kou, R., Igarashi, J., and Michel, T. (2002) Lysophosphatidic acid and receptor-mediated activation of endothelial nitric-oxide synthase. *Biochemistry* **41**, 4982-4988
38. Van Brocklyn, J. R., Graler, M. H., Bernhardt, G., Hobson, J. P., Lipp, M., and Spiegel, S. (2000) Sphingosine-1-phosphate is a ligand for the G protein-coupled receptor EDG-6. *Blood* **95**, 2624-2629
39. Sledz, C. A., Holko, M., de Veer, M. J., Silverman, R. H., and Williams, B. R. (2003) Activation of the interferon system by short-interfering RNAs. *Nat. Cell Biol.* **5**, 834-839
40. Kluk, M. J., Colmont, C., Wu, M. T., and Hla, T. (2003) Platelet-derived growth factor (PDGF)-induced chemotaxis does not require the G protein-coupled receptor S1P1 in murine embryonic fibroblasts and vascular smooth muscle cells. *FEBS Lett.* **533**, 25-28

Received April 23, 2003; accepted October 16, 2003.

Fig. 1

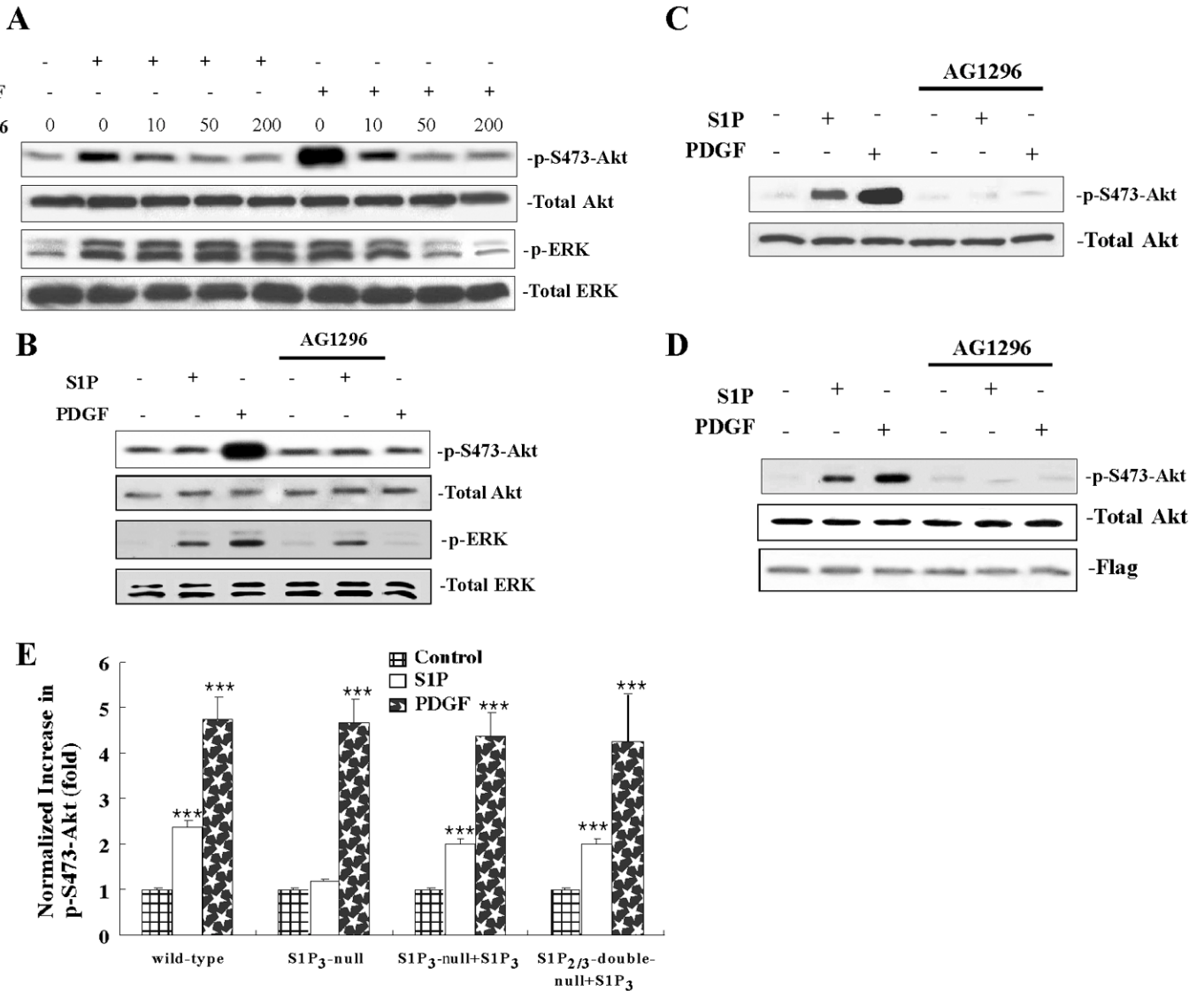


Figure 1. S1P₃ and PDGFR activity are required for Akt phosphorylation induced by S1P. *A*) Wild-type MEFs were stimulated with S1P (1 μ M) and PDGF-BB (10 ng/ml) for 10 and 5 min, respectively. Western blot analyses were performed as described in Materials and Methods using antibodies against phosphorylated Akt (p-S473-Akt), phosphorylated ERK (p-ERK), or total Akt and total ERK. AG1296 (30 min pretreatment) at concentrations indicated was used to block the PDGFR activity. *B*) S1P₃ null MEFs were stimulated by S1P and PDGF-BB as described above with or without AG1296 (50 μ M) pretreatment. *C*) pMSCV-S1P₃ was infected into S1P₃ null MEFs as described in Materials and Methods. Stimulation by S1P and PDGF-BB and Western blot analyses were performed as described above. *D*) Same as in *C* except that pMSCV-S1P₃ was infected into S1P_{2/3}-double null MEFs. Expression of S1P₃ was confirmed by detecting Flag-tag using an α -M2 antibody. *E*). Graph of mean densitometric traces of several experiments. *** $P < 0.001$; Student's *t* test.

Fig. 2

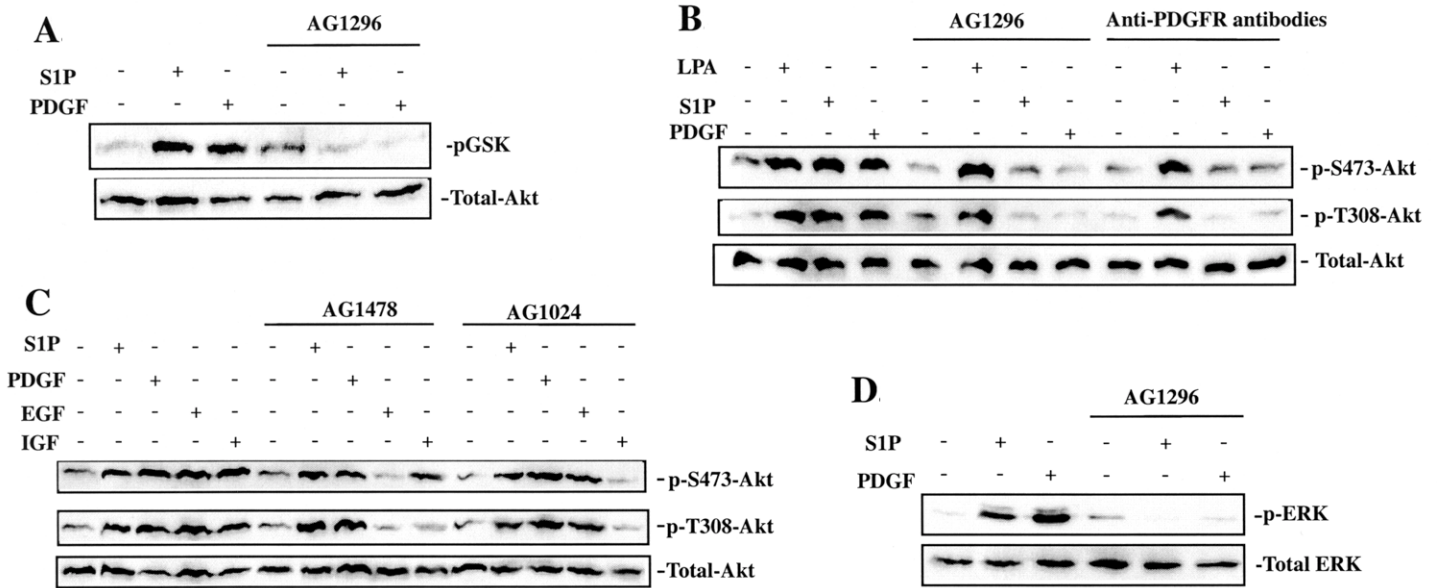


Figure 2. S1P activates Akt in a PDGFR-dependent manner. HEY cells were pretreated with AG1296 (10 μ M), AG1478 (200 nM), AG1024 (10 μ M) for 30 min, or 2A1E2 and 2H7C5 (0.3 nM, neutralizing antibodies against PDGFR) for 15 min, and then treated with S1P (1 μ M) for 20 min (Akt activation/phosphorylation) or 5 min (ERK phosphorylation), PDGF (10 ng/ml), EGF (10 ng/ml), or IGF-I (20 ng/ml) for 5 min. **A**) Nonradioactive immunoprecipitation Akt kinase assay. **B-D**) Western blot analyses using antibodies against phosphorylated Akt (p-S473-Akt, p-T308-Akt), phosphorylated ERK (p-ERK), or total Akt. Western analyses are representative examples of at least 3 independent experiments.

Fig. 3

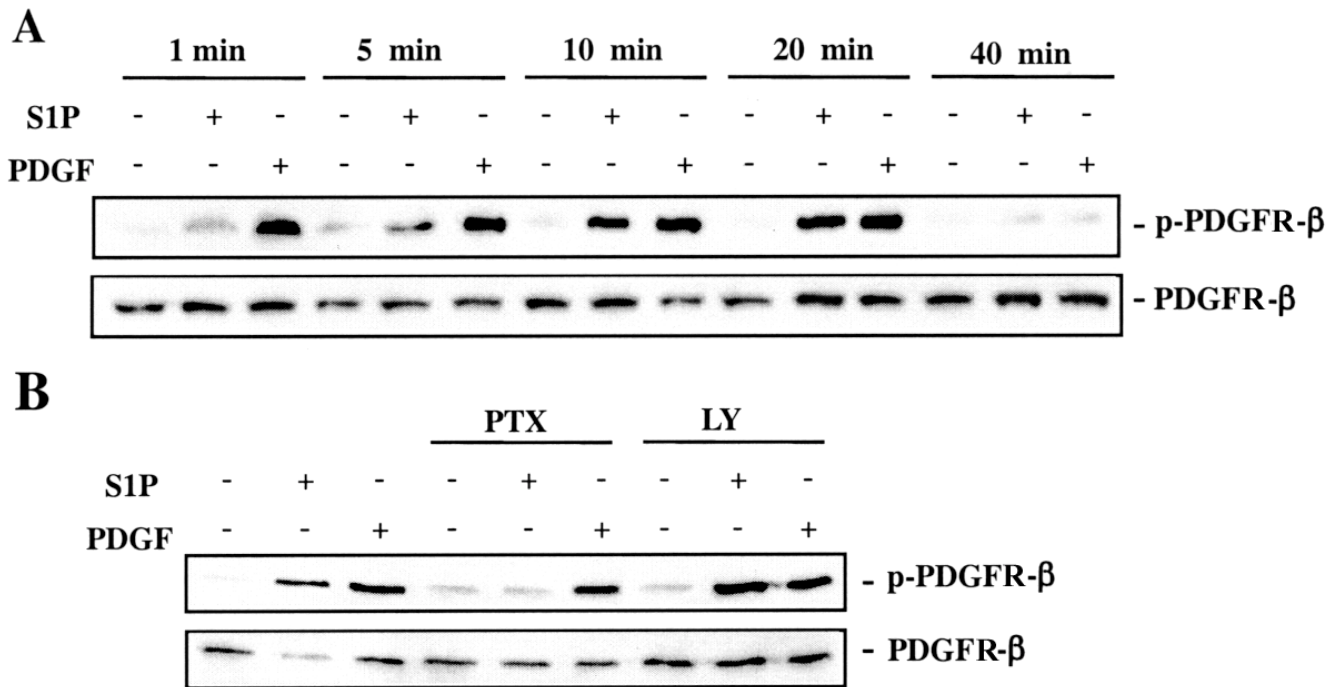


Figure 3. S1P induces phosphorylation of PDGFR- β in a time-dependent manner that requires G_i .

Immunoprecipitation of PDGFR- β , followed by Western blot with anti-phosphotyrosine antibodies (clone 4G10) in HEY cells. **A**) Cells were treated with S1P (1 μ M) or PDGF (10 ng/ml) for the indicated time points. **B**) Cells were pretreated with LY294002 (LY, 10 μ M) for 30 min, or PTX (100 ng/ml) for 16 h before treatment with S1P (1 μ M) or PDGF (10 ng/ml) for 20 min. Western analyses are representative examples of at least 3 independent experiments.

Fig. 4

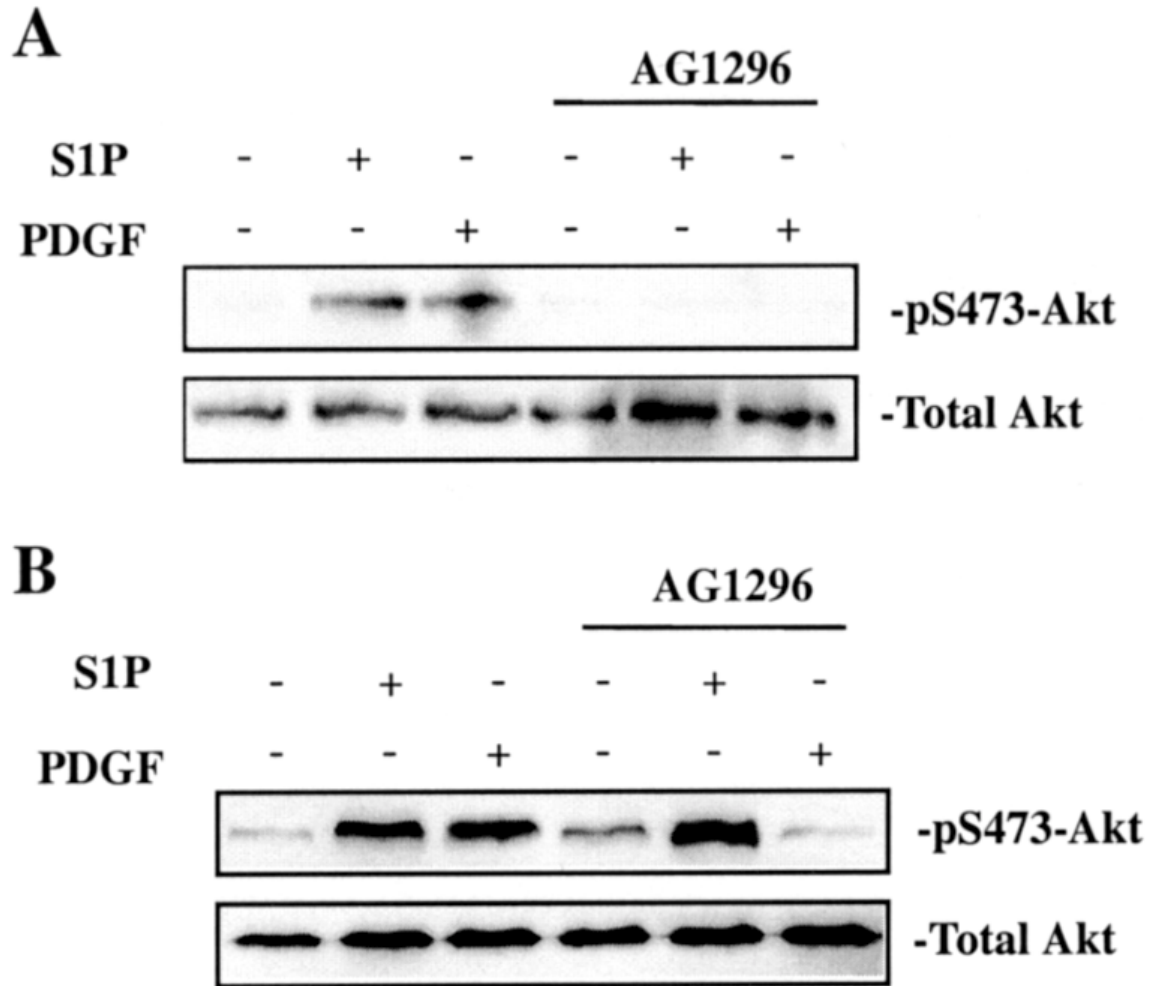


Figure 4. Effect of PDGFR inhibition on Akt activation in T47D and GI-101A cells treated with S1P. Western analyses of cell lysates probed with p-S473-Akt and total Akt antibodies. Cells were pretreated with AG1296 (10 μ M) for 30 min followed by treatment with S1P (1 μ M) for 20 min or PDGF (10 ng/ml) for 5 min. Western analyses are representative examples of at least 3 independent experiments. *A*) T47D cells; *B*) GI-101A cells.

Fig. 5

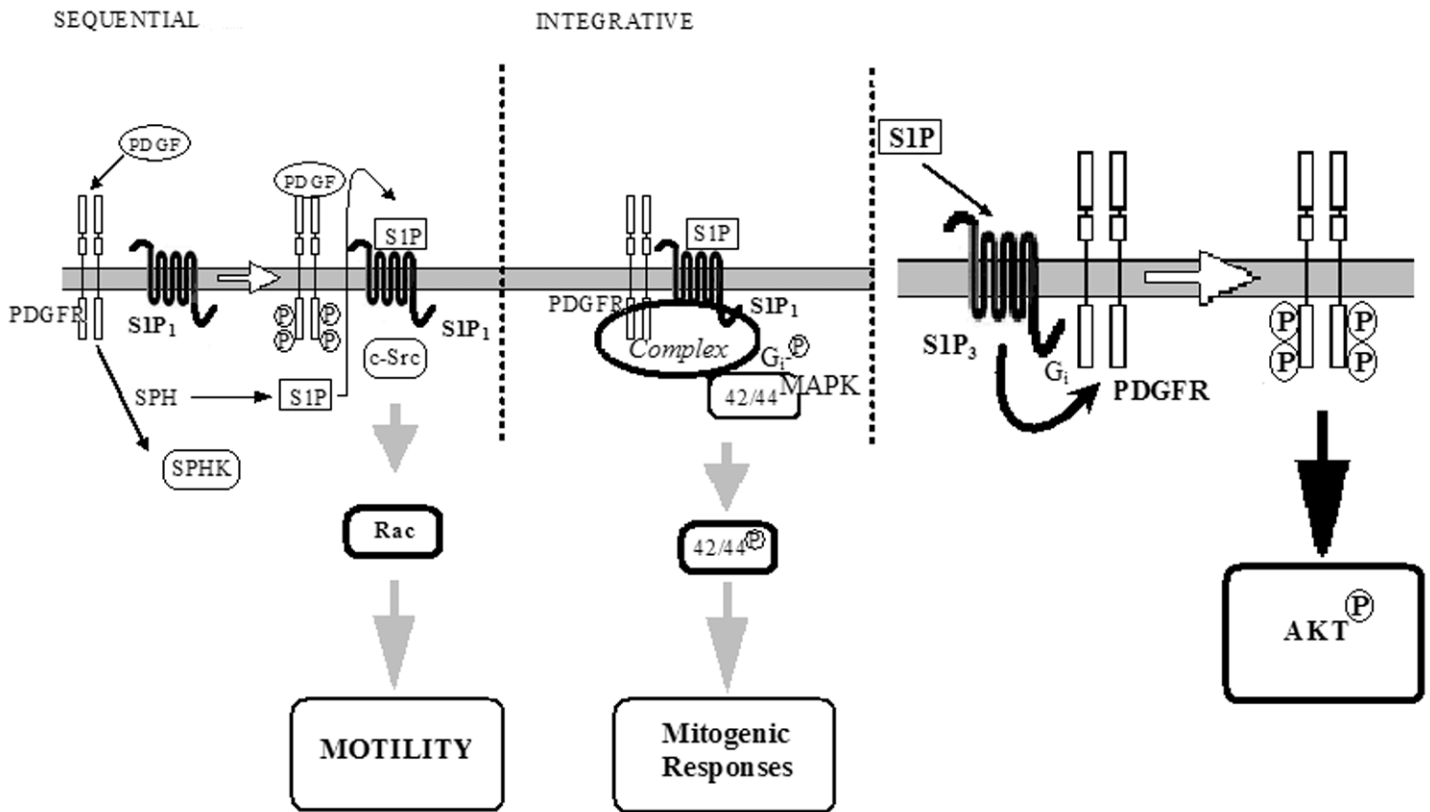


Figure 5. Schematic diagram of different types of interactions between S1P receptors and PDGFR. Left panel shows the “sequential” model proposed by Spiegel’s group (18). SPH, sphingosine; SPHK, sphingosine kinase. Middle panel illustrates the “integrative” model proposed by Pyne’s group (20, 21). Right panel summarizes results shown in this communication. Different S1P receptor subtypes are involved in different models.