Sphingosine 1-Phosphate and Platelet-derived Growth Factor (PDGF) Act via PDGFβ Receptor-Sphingosine 1-Phosphate Receptor Complexes in Airway Smooth Muscle Cells*

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Platelet-derived growth factor (PDGF) and sphingosine 1-phosphate (S1P) act via PDGFβ receptor-S1P, receptor complexes in airway smooth muscle cells to promote mitogenic signaling. Several lines of evidence support this conclusion. First, both receptors were co-immunoprecipitated from cell lysates with specific anti-S1P, antibodies, indicating that they form a complex. Second, treatment of airway smooth muscle cells with PDGF stimulated the phosphorylation of p42/p44 MAPK, and this phosphorylated p42/p44 MAPK associates with the PDGFβ receptor-S1P, receptor complex. Third, treatment of cells with antisense S1P1 receptor plasmid construct reduced the PDGF- and S1P-dependent activation of p42/p44 MAPK. Fourth, S1P and/or PDGF induced the formation of endocytic vesicles containing both PDGFβ receptors and S1P receptors, which was required for activation of the p42/p44 MAPK pathway. PDGF does not induce the release of S1P, suggesting the absence of a sequential mechanism. However, sphingosine kinase 1 is constitutively exported from cells and supports activation of p42/p44 MAPK by exogenous sphingosine. Thus, the presentation of sphingosine from other cell types and its conversion to S1P by the kinase exported from airway smooth muscle cells might enable S1P to act with PDGF on the PDGFβ receptor-S1P, receptor complex to induce biological responses in vivo. These data provide further evidence for a novel mechanism for G-protein-coupled receptor and receptor tyrosine kinase signal integration that is distinct from the transactivation of receptor tyrosine kinases by G-protein-coupled receptor agonists and/or sequential release and action of S1P in response to PDGF.

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*Sphingosine 1-phosphate (S1P)1 is a bioactive lysolipid that has been proposed to have both intracellular and extracellular actions (1). To date, five closely related G-protein-coupled receptors (GPCR), termed S1P1–S1P5 (2) and (formerly named EDG1, EDG5/AGR16/H218, EDG3, EDG6 and EDG8/ARG-1, respectively) have been identified as high affinity S1P receptors (3–9). Further characterization studies confirmed the S1P1 receptor to be a GPCR with high affinity for S1P that stimulates p42/p44 MAPK and inhibits adenylyl cyclase in cells (10–13). The S1P2 and S1P3 receptors also have high affinity for S1P (14) and are linked via Gα to phospholipase C and calcium mobilization and p42/p44 MAPK activation (14, 15) and via G12 and G13 to Rho guanine nucleotide factor and Rho activation. The S1P3 receptor is lymphoid specific and, in common with the S1P1 receptor, uses Gαi and G12 to signal (6, 8).

The S1P1 receptor is implicated in regulating smooth muscle cell migration, proliferation, and vascular maturation. Insight into the function of the S1P1 receptor was obtained by studies showing that disruption of the s1p1 gene by homologous recombination in mice results in extensive intra-embryonic hemorrhaging and intrauterine (16). This is caused by incomplete vascular maturation due to the failure of mural cells, vascular smooth muscle cells, and pericytes to migrate to arteries and capillaries and to reinforce them properly. Interestingly, the disruption of the PDGFβ receptor gene in mice results in a similar phenotype (17, 18). Hobson et al. (16) showed that PDGF-stimulated cell motility is S1P1 receptor-dependent in human embryonic kidney 293 cells (HEK 293 cells) and mouse embryonic fibroblasts. Therefore, a sequential model has been proposed in which PDGF stimulates S1P synthesis/release, which in turn binds to and activates the S1P1 receptor to induce activation of Rac, PKB, and cell motility (16). In contrast, we have reported that the PDGFβ receptor forms a complex with the S1P1 receptor in HEK 293 cells transfected with recombinant forms of these receptors (19, 20). We showed that the association of these recombinant receptor types enables more efficient tyrosine phosphorylation of Gαi by the PDGFβ receptor kinase, possibly via proximity-induced effects. We showed that the tyrosine phosphorylation of Gαi was required to stimulate the p42/p44 MAPK pathway by PDGF or S1P, consistent with an integrative signal model (19). Freedman et al. (21) recently confirmed the involvement of G1 in PDGFβ receptor signaling. We also reported that β-arrestin 1/GRK2 associate with the PDGFβ receptor-S1P1 receptor complex in HEK 293 cells (19) and that this might represent an important step regulating p42/p44 MAPK signaling. Freedman et al. (21) recently confirmed the association of GRK2 with the PDGFβ receptor.

The question as to which model, sequential versus integrative signaling, is the major mechanism of action of PDGF and S1P is very important and still remains to be fully investigated.
Immunoprecipitates were washed twice with buffer A containing 10 mM then combined with boiling sample buffer containing 62 mM Tris-HCl, once in buffer A without Nonidet P-40. The immunoprecipitates were their identity was confirmed to be smooth muscle by the presence of airway smooth muscle (ASM) cells has been described previously (23).

Construct after complex formation with LipofectAMINE2000 accord-
s medium supplemented with 0.1% (v/v) fetal
s modified Eagle

Acute treatment of ASM with 10 nm PDGF and 80 nm S1P led to the appearance of immunoreactive bands that co-migrated with authentic p42 MAPK, PKB, and p38 MAPK antibodies. Anti-phospho-p42 MAPK, anti-phospho-PKB, and anti-phospho-p38 MAPK antibodies were also used for Western blotting to establish the presence of phosphorylated forms of p42/p44 MAPK, PKB, and p38 MAPK in cells. Consistent with an integrative model, we have also established that these receptors are co-internalized together as a functional signaling unit to regulate the p42/p44 MAPK pathway.

**EXPERIMENTAL PROCEDURES**

**Materials**—All biochemicals including PDGF were from Sigma. Sphingosine 1-phosphate was from BD Transduction Laboratories (Oxford, UK). Cell culture supplies and LipofectAMINETM 2000 were from Invitrogen. Anti-phospho-p42/p44 MAPK, anti-phospho-PKB, and anti-phospho-p38 MAPK antibodies were from New England Biolabs (UK). Anti-p42 MAPK, anti-PKB, anti-horseradish peroxidase-linked anti-phosphorytosine and anti-p38 MAPK antibodies were from BD Transduction Laboratories. Anti-PDGFB/β receptor antibodies were from Santa Cruz (Santa Cruz, CA). Reporter horseradish peroxidase–anti-rabbit/mouse IgG antibodies were from Diagnostics Scotland (Car-

**Cell Culture**—The preparation of primary cultures of guinea pig airway smooth muscle (ASM) cells has been described previously (25). Their identity was confirmed to be smooth muscle by the presence of α-actin using smooth muscle-specific mouse anti-α-actin monoclonal antibodies. Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum and 10% (v/v) horse serum. Cells were routinely used at passage 3–4. Cells were placed in Dulbecco’s modified Eagle’s medium supplemented with 0.1% (v/v) fetal calf serum and 10% (v/v) horse serum for 4 h before exposure to PDGF. HEK 293 cells were maintained in culture as previously reported by us (19).

**Transfection**—ASM cells were transiently transfected with anti-sense/sense S1P1 receptor, sense PDGFB receptor, FLAG-tagged hSk1WT, or FLAG-tagged hSk1α plasmid constructs as required. Cells at 90% confluence were placed in minimum Eagle’s medium containing 2% fetal calf serum and transfected with 2 µg of plasmid construct after complex formation with LipofectAMINE2000 according to the manufacturer’s instructions. The cDNA-containing media was removed after incubation for 24 h at 37 °C, and the cells were incubated for a further 24 h in serum-free medium before agonist additions.

**Immunoprecipitation**—The medium was removed, and cells were lysed in ice-cold immunoprecipitation buffer (1 ml) containing 20 mM Tris/HCl, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 1% (v/v) Tris/HCl, 1% (v/v) horse serum, 0.5 µM sodium orthovanadate, 0.2 mM phenylmethylsulfonyl fluoride, leupeptin, anti-pain pepstatin, and aprotinin (all protease inhibitors were at 10 µg/ml), pH 8, for 10 min at 4 °C. The material was harvested and centrifuged at 22,000 × g for 5 min at 4 °C, and 200 µl of cell lysate supernatant (equalized for protein, 0.5–1 mg/ml) was taken for immunoprecipitation with antibodies (5 µg of anti-S1P1 receptor antibodies and 40 µl of 1 µg immunoprecipitation buffer and 1 part protein AV (Sephacel CL4B). After agitation for 2 h at 4 °C, the immune complex was collected by centrifugation at 22,000 × g for 15 s at 4 °C. Immunoprecipitates were washed twice with buffer A containing 10 mM Hepes, pH 7, 100 mM NaCl, 0.2 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 20 µg/ml aprotinin, and 0.5% (v/v) Nonidet P-40 and once in buffer B without Nonidet P-40. The immunoprecipitates were then boiled in sample buffer containing 10% (v/v) glycerol, 3.75% (v/v) mercaptoethanol, and 0.05% (v/v) bromphenol blue. The samples were then subjected to SDS-PAGE and Western blotting.

**Immunoblotting**—Immunoblotting was performed as described by us previ-

ous (19). Immunoreactive proteins were visualized using enhanced chemiluminescence detection.

**SIP Measurements**—Cells grown to confluence on multiple 6-well plates (1.2 × 106 cells/well) were serum-starved overnight and equili-

bactin to 37 °C for at least 1 h before stim-

ulation. [3H]Sphingosine was resuspended with phosphate-buffered sa-

line containing fatty acid-free bovine serum albumin (2 mg/ml) by sonication for 10 min. The reactions were started by the addition of [3H]sphingosine (222,000 dpm/well, final concentration 5–10 nM) and various agonists for the periods of time indicated.

Stimulation of cells was stopped by removal of the medium and the addition of 0.5 ml of ice-cold methanol. Cells were then scraped into glass vials, and an equal volume of chloroform was added. Samples were vortexed vigorously and maintained at room temperature for at least 20 min to allow extraction of cellular lipids. The samples were dried using a SpeedVac centrifuge. Samples were then re-dissolved in 80 µl of CHCl3/CH3OH (19:1, v/v) and resolved by TLC using silica gel G60 L68D TLC plates (Whatman) and solvent system CHCl3/CH3OH: CH4COOH:H2O (25:10:1, v/v). A [3H]SIP standard was resolved in parallel. Sample profiles were obtained by excising 1-cm fractions of the silica and quantification of the radioactivity by liquid scintillation counting.

**[3H]Thymidine Incorporation**—[3H]Thymidine incorporation was as described previously by us (24).

**RESULTS**

The Existence of Functional PDGFβ Receptor-S1P1 Receptor Complexes in ASM Cells—We set out to investigate whether the PDGFB receptor forms a complex with the S1P1 receptor in cultured ASM cells and whether this represents a functional integrative signaling unit. ASM cells were chosen for several reasons. First, these cells express high levels of S1P1 receptor (molecular mass 45 kDa) were co-immunoprecipitated from cell

p42/p44 MAPK, PKB, and p38 MAPK Assays—The phosphorylated forms of p42/p44 MAPK, PKB, and p38 MAPK were detected by Western blotting cell lysates with the respective anti-phospho-specific anti-

bodies. Anti-p42 MAPK, PKB, and p38 MAPK antibodies were also used for Western blotting to establish equal loading of protein in each sample.

**Blotting**—Immunoblotting was performed as described by us previ-

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**RESULTS**

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nositide 3-kinase, which lies up-stream of p42/p44 MAPK acti-

vation (24, 25). Fourth, a combination of submaximal SIP and PDGF-AB induced a synergistic activation of p42/p44 MAPK (24). Fifth, SIP is co-mitogenic with PDGF-AB (24). These data are consistent with the possibility that the S1P1 receptor might be associated with and exert cross-talk regulation with the PDGF receptor.

**Fig. 1a** shows that the native PDGFB receptor is indeed associated with native S1P1 receptors in ASM cells. Thus, the PDGFB receptor (molecular mass 180 kDa) and S1P1 receptor (molecular mass 45 kDa) were co-immunoprecipitated from cell lysates with anti-S1P1 receptor antibodies. Neither receptor was immunoprecipitated when the antibodies were omitted from the procedure. Stimulation of ASM cells with PDGF-AB
Fig. 1. The association of S1P₁ receptors with PDGFβ receptors and phosphorylated p42/p44 MAPK. ASM cells were pre-treated with the c-Src inhibitor PP2 (10 μM, 10 min) before stimulation with or without PDGF-AB (10 ng/ml, 10 min) as indicated. In certain cases, cells were transfected with PDGFβ receptor plasmid construct. a, Western blots (WB), showing the co-immunoprecipitation of PDGFβ receptor (molecular mass 180 kDa) and S1P₁ receptor (molecular mass 45 kDa) from ASM cell lysates using anti-S1P₁ receptor antibodies (Ab). Anti-S1P₁ receptor immunoprecipitates were resolved on SDS-PAGE and Western-blotted with anti-PDGFB receptor and anti-S1P₁ receptor antibodies. ASM cells in which recombinant PDGFβ receptor (PDGFβR) were transfected were also used to show that endogenous S1P₁ receptors are limiting for the binding of PDGFβ receptor. b, Western blots showing the PDGF-dependent phosphorylation of p42/p44 MAPK and association with the PDGFβ receptor-S1P₁ receptor complex. Also shown is the effect of pre-treating cells with the c-Src inhibitor, PP2, on receptor-associated phosphorylated p42/p44 MAPK and PDGFβ receptor-S1P₁ receptor complexes. Anti-S1P₁ receptor immunoprecipitates were resolved on SDS-PAGE and Western-blotted with anti-PDGFB receptor and anti-phosphorylated p42/p44 MAPK antibodies. This figure also shows a Western blot of the corresponding effects of PP2 on the PDGF-dependent phosphorylation of p42/p44 MAPK and PDGFβ receptor levels in total cell lysates. These are representative results of an experiment performed three times. IP, immunoprecipitation; T/IF, transfection.

Fig. 2. PDGF and S1P stimulation of p42/p44 MAPK in ASM cells. ASM cells were pretreated with antisense S1P₁, plasmid construct (24 h) before stimulation with PDGF-AB (10 ng/ml, 10 min) or S1P (5 μM, 10 min) or phorbol ester (PMA, 1 μM, 10 min). a, Western blots showing the effect of an antisense S1P₁ plasmid construct on PDGF or S1P- or PMA-dependent activation of p42/p44 MAPK. b, Western blots showing the effect of antisense S1P₁, plasmid construct on S1P₁ receptor expression. Blots were stripped and reprobed with antibodies that react with p42 MAPK to ensure equal protein loading. These are representative of an experiment performed 3–4 times. C, control.

PDGF stimulation significantly increased p42/p44 MAPK activation when compared with control, PMA stimulation increased the activation of p42/p44 MAPK compared with control and had no significant effect on PDGF stimulation. PP1 treatment significantly reduced the activation of p42/p44 MAPK. The effect of antisense S1P₁ plasmid construct significantly reduced the activation of p42/p44 MAPK when compared with control. Western blots showing the effect of antisense S1P₁, plasmid construct on S1P₁ receptor levels in total cell lysates. These are representative of an experiment performed three times.
tisense S1P1 receptor plasmid construct reduced S1P1 receptor (molecular mass 45 kDa) expression (Fig. 2b), thereby also validating the specificity of the anti-S1P1 receptor antibody for S1P1 receptor. The antisense construct had no effect on cell viability under the conditions used (data not shown). Finally, S1P does not cause the release of PDGF, based on previous results obtained by us, showing that S1P does not stimulate PDGF receptor tyrosine phosphorylation (24).

**Fig. 3. Co-internalization of PDGFβ receptors and S1P1 receptors in endocytic vesicles in response to S1P and/or PDGF.** A, photomicrographs showing immunofluorescent staining of ASM cells (transfected with Myc-tagged S1P1 receptor) with anti-Myc tag and anti-PDGFβ receptor antibodies. Stimulation of ASM cells with PDGF (10 ng/ml, 5 min) is shown. Panels 1–3 are control cells; panels 4–6 are PDGF-stimulated cells. Panels 1 and 4 are immunostained with PDGFβ receptor antibodies (green) to detect endogenous receptor; panels 2 and 5 are immunostained with anti-Myc tag antibodies (red), and panels 3a and 6a are merged panels showing localization of both receptors (yellow). Panel 3b is a magnified area showing control cells, whereas panel 6b is a magnified area showing discrete endocytic vesicles containing both receptors formed in response to PDGF. B, photomicrographs showing immunofluorescent staining of HEK 293 cells (transfected with Myc-tagged S1P1 receptor and PDGFβ receptor) with anti-Myc tag and anti-PDGFβ receptor antibodies. Stimulation of HEK 293 cells with PDGF (10 ng/ml, 5 min) or S1P (5 μM, 5 min) or both. Panels a–d are control cells; panels e–h are S1P-stimulated cells; panels i–l are PDGF-stimulated cells; panels m–p are PDGF plus S1P-stimulated cells. Panels a, e, i, and m are immunostained with anti-PDGFβ receptor antibodies (green); panels b, f, j, and n are immunostained with anti-Myc tag antibodies (red); panels c, g, k, and o are merged panels showing co-localization of both receptors (yellow); panel d is a magnified area showing co-localization of both receptors to the plasma membrane in control cells. Panels h, l, and p are magnified areas of respective cells showing discrete endocytic vesicles containing both receptors formed in response to PDGF and/or S1P.

**Co-internalization of PDGFβ Receptor-S1P1 Receptor Complexes in Endocytic Vesicles**—One prediction of the proposed PDGFβ receptor-S1P1 receptor complex association model is that stimulation of cells with PDGF is expected to cause the co-internalization of both PDGFβ and S1P1 receptors into the same endocytic vesicles. ASM cells were transfected with Myc-tagged S1P1 receptor to capture PDGFβ receptor and to allow detection of S1P1 receptors with anti-Myc tag antibodies. Un-
under these conditions, stimulation of cells with PDGF promoted the formation of endocytic vesicles containing both PDGFβ receptor and Myc-tagged S1P1 receptor (yellow vesicles, Fig. 3A, panels 6a and 6b). Inhibitors of clathrin-mediated endocytosis (e.g., concanavalin A and monodansylcadaverine) blocked the internalization of the PDGFβ receptor-S1P1 receptor complex in response to PDGF (data not shown). We excluded the possibility that PDGF elicits the release of S1P (see later), which might have acted on S1P1 receptors, causing their recruitment to the same endocytic vesicles as those containing PDGFβ receptors. The treatment of cells with S1P also induced co-internalization of S1P1 and PDGFβ receptors, although the sensitivity of detection was considerably reduced (data not shown). This is explained by the fact that overexpression of recombinant S1P1 receptors probably results in the expression of a significant amount of non-associated S1P1 receptor, which might effectively buffer the PDGFβ receptor-S1P1 receptor complex pool from S1P.

We have previously shown that recombinant forms of the PDGFβ receptor and S1P1 receptor form functional associated signaling complexes in HEK 293 cells (19). Because the levels of the PDGFβ receptor-S1P1 receptor complex are very high in these cells due to overexpression of the two proteins, we predicted that this system might show increased sensitivity in terms of co-internalization of both receptors in response to S1P. Thus, overexpression of both receptors to increase their association might surmount the buffering effect of non-associated S1P1 receptors on S1P observed in ASM cells. Consistent with this, we found that the stimulation of transfected HEK 293 cells with S1P induced an increase in the number of endocytic vesicles containing both recombinant PDGFβ receptors and Myc-tagged S1P1 receptors. PDGF also induced co-internalization of both receptors to the same endocytic vesicles (Fig. 3B, yellow vesicles, panels h and l).

PDGF also promoted the formation of endocytic vesicles containing PDGFβ receptors that were not associated with Myc-tagged S1P1 receptors (green vesicles, Fig. 3B, panel l). These endocytic vesicles might contain recombinant PDGFβ receptor that is either free or associated with endogenous S1P1 receptors, for which transcript is expressed in HEK 293 cells (data not shown).

A combination of S1P and PDGF produced an increase in the number of endocytic vesicles containing both recombinant PDGFβ receptors and Myc-tagged S1P1 receptors compared with that induced with each agent alone (Fig. 3B, yellow vesicles, panel p). This is entirely consistent with a model in which combined stimulation of cells with PDGF and S1P will increase the number of PDGFβ receptor-S1P1 receptor complexes occupied with either both ligands, leading to an increased number of ligand-bound PDGFβ receptor-S1P1 receptor complexes being internalized within common endocytic vesicles. These findings represent an entirely novel mechanism for co-internalization of receptor tyrosine kinase/GPCRs.

**PDGF-stimulated S1P Formation and p42/p44 MAPK Activation**—The existence of a PDGFβ receptor-S1P1 receptor complex in ASM cells and the functional evidence provided here support a model in which S1P and PDGF use an integrative signaling mechanism in these cells. Nevertheless, it was necessary to formally evaluate whether part of the response to PDGF is mediated via the release of S1P, which might act on S1P1 receptors in a sequential manner. We therefore compared the effect of (i) different PDGF isoforms and (ii) sphingosine kinase inhibitors and recombinant wild type and a dominant negative mutant hSK1 on PDGF-stimulated S1P formation, p42/p44 MAPK, PKB, and p38 MAPK activation.

First, we found that there was a correlation between the ability of PDGF-BB to stimulate S1P formation and to activate p42/p44 MAPK or to promote [3H]thymidine incorporation in ASM cells. PDGF-BB (10 ng/ml) caused a rapid and transient increase in [3H]S1P production in ASM cells (300% increase in S1P above basal at peak 3 min of cell stimulation). However, neither PDGF-AA nor PDGF-AB stimulated S1P formation (Fig. 4a).

All of the PDGF subtypes induced the activation of p42/p44 MAPK (fold activations of p42 MAPK (normalized for p42 MAPK levels) were: control, 1 ± 0.29; PDGF-AA, 5.35 ± 0.42; PDGF-AB, 8.82 ± 1.06; PDGF-BB, 10.52 ± 0.6, n = 3–4, p < 0.05 for PDGF-BB versus PDGF-AB. Therefore, the rank order activation of p42/p44 MAPK (at 10 ng/ml PDGF) was PDGF-BB > PDGF-AB > PDGF-AA (Fig. 5a, left bottom panel). This was correlated with PDGFββ receptor auto-tyrosine

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**Fig. 4.** The PDGF stimulation of S1P production in ASM cells. a, dose response of PDGF-stimulated S1P production in ASM cells. Cells were incubated with PDGF-AA (triangles) or PDGF-AB (circles) or PDGF-BB (squares) or EGF (diamonds) and [3H]sphingosine for 3 min. Results are expressed as percentage of [3H]S1P formed above control cells and are the means ± S.D. for n = 3 experiments. Basal [3H]S1P formed was 2000–6000 dpm/1.2 × 10⁶ cells. b, the effect of tDHS on basal (triangles) and PDGF-BB-stimulated (squares) S1P production after 3 min. c, the effect of tDHS on [3H]sphingosine uptake in control (triangles) and PDGF-BB-stimulated cells (squares). In b and c, results are expressed as dpm of [3H]S1P or dpm of [3H]sphingolipids formed per 1.2 × 10⁶ cells, respectively, and are means ± S.D. (n = 3).
FIG. 5. PDGF stimulation of p42/p44 MAPK, p38 MAPK, PKB, PDGF receptor tyrosine phosphorylation, and [$^{3}H$]thymidine incorporation in ASM cells. ASM cells were treated with or without PDGF-BB or PDGF-AA or PDGF-AB (all at 10 ng/ml) for 3 min. a, Western blots showing the effect of PDGF-BB, PDGF-AB, and PDGF-AA on p42/p44 MAPK activation or PDGF receptor tyrosine phosphorylation (left panel). The right panel shows the effect of the different PDGF sub types on [$^{3}H$]thymidine incorporation (PDGF-AA (triangles), PDGF-AB (circles), and PDGF-BB (squares)). b, Western blots showing the effect of dHDS (10 μM, 10 min) or N,N-dimethylsphingosine (DMS, 10 μM, 10 min) on PDGF-BB-dependent activation of p42/p44 MAPK, PKB, or p38 MAPK activation. The analysis with anti-FLAG antibodies proved that recombinant hSK1WT and hSK1G82D (molecular mass 45 kDa) are overexpressed in transfected cells. d, Western blots showing the effect of FLAG-tagged hSK1WT or hSK1G82D on PDGF-BB-dependent activation of p42/p44 MAPK, PKB, or p38 MAPK activation. The analysis with anti-FLAG antibodies proved that recombinant hSK1WT and hSK1G82D (molecular mass 45 kDa) are overexpressed in transfected cells. e, Immunofluorescent staining with anti-FLAG antibodies of ASM cells transfected with FLAG-tagged hSK1WT and hSK1G82D to demonstrate highly efficient transfection of the cell population. f and vi, FLAG-tagged hSK1WT-transfected; v and vi, FLAG-tagged hSK1G82D-transfected; c, histogram showing the increase in hSK1WT activity in transfected ASM cells; f, histogram showing the effect of vector (open bars), hSK1WT (filled bars), and hSK1G82D (hatched bars) on PDGF-AA-, PDGF-AB-, and PDGF-BB-stimulated [$^{3}H$]thymidine incorporation. Blots were stripped and reprobed with antibodies that react with p42 MAPK (a–c) or PDGFαβ receptors (a) to ensure equal protein loading. These are representative results of an experiment performed 3–9 times.
phosphorylation (Fig. 5a, left mid-panel) and PDGF-stimulated [3H]thymidine incorporation (Fig. 5a, right panel). Fig. 4a shows the dose response of S1P production (peak at 3 min of cell stimulation) induced by PDGF-BB and the lack of effect of PDGF-AA and PDGF-AB. Half-maximal and maximal stimulation of [3H]S1P production was observed at 1.5 ± 0.6 and 6 ng/ml PDGF-BB, respectively (Fig. 4a). We therefore further investigated whether the increased efficacy of PDGF-BB on p42/p44 MAPK activation could be accounted for by potential release of S1P by PDGF-BB.

It is important to note from our previous studies that the 50% inhibitory effect of pertussis toxin on PDGF-stimulated p42/p44 MAPK activation in ASM cells was achieved with PDGF-AB (25). Therefore, PDGF-AB in part uses a classical G-protein-coupled receptor signaling mechanism that does not involve S1P release, since PDGF-AB does not induce S1P formation. Nevertheless, we formally proceeded with studies to evaluate the effect of SK inhibitors and overexpression of recombiant wild type/dominant negative hSK1 on PDGF-BB-stimulated responses. First, the SK inhibitor tDHS dose dependently inhibited the PDGF-BB-stimulated [3H]S1P production as well as reducing basal S1P levels (Fig. 4b). Using 1 μM tDHS, basal [3H]S1P levels were reduced by ~70%, whereas the response to PDGF-BB was completely suppressed. tDHS did not affect [3H]sphingosine uptake into sphingolipids of control or PDGF-BB-treated cells (Fig. 4c). However, neither tDHS nor N,N-dimethylsphingosine (DMS, SK inhibitors) (at 10 μM, which ablated S1P formation) had a significant effect on the PDGF-BB-dependent stimulation of p42/p44 MAPK, p38 MAPK, or PKB (Fig. 5a) (fold activations of p42 MAPK (normalized for p42 MAPK levels) were: control, 0.73 ± 0.56; PDGF-BB, 9.94 ± 1.33; tDHS, 1.24 ± 0.39; PDGF-BB plus tDHS, 8.95 ± 2.7; DMS, 1.18 ± 0.27; PDGF-BB plus DMS, 9.45 ± 0.02, n = 5 for tDHS and n = 3 for DMS). Higher concentrations of tDHS (30 μM) consistently inhibited the PDGF-BB-dependent activation of p42/p44 MAPK. We have previously shown that this is due to its action on protein kinase C, which is required for PDGF-dependent stimulation of p42/p44 MAPK in ASM cells (26). Second, neither FLAG-tagged hSK1WT nor FLAG-tagged dominant negative hSK1G82D modulated PDGF-BB-stimulated p42/p44 MAPK (fold activations of p42 MAPK (normalized for p42 MAPK levels) were: control, 1.38 ± 1.4; PDGF-BB, 11.8 ± 2.2; hSK1WT, 1.04 ± 0.26; PDGF-BB plus hSK1WT, 11.8 ± 3.7; hSK1G82D, 1.12 ± 0.17; PDGF-BB plus hSK1G82D, 10.9 ± 3.9, n = 9), PKB, or p38 MAPK activation (Fig. 5c). Overexpression of FLAG-tagged hSK1WT or FLAG-tagged hSK1G82D in transfected cells was confirmed by Western blot analysis with anti-FLAG antibodies (Fig. 5d). Western blot showing export of hSK1 from ASM cells (Fig. 6a) shows that FLAG-tagged hSK1WT was exported into the medium. The transfection of FLAG-tagged hSK1WT into ASM cells did not compromise cell integrity, as assessed by trypan blue exclusion (data not shown), and did not cause leakage of other proteins of similar molecular mass to hSK1, such as p42/p44 MAPK (Fig. 6a).

The exported SK is capable of converting sphingosine to S1P. This was supported by data showing that a low concentration of exogenous sphingosine stimulated p42/p44 MAPK activation in ASM cells (Fig. 6b). The activation of p42/p44 MAPK by sphingosine was significantly increased in ASM cells transfected with S1P receptor (data not shown). These findings suggest that export of endogenous SK may convert sphingosine to S1P, which can subsequently stimulate p42/p44 MAPK. Indeed, the sphingosine-dependent activation of p42/p44 MAPK was abrogated by overexpression of FLAG-tagged hSK1G82D (Fig. 6b). However, a role for exported SK in mediating part of the action of PDGF in ASM cells was excluded on the basis that PDGF does not promote export of hSK1 (data not shown) and SK inhibitors/hSK1G82D did not abrogate PDGF receptor signaling (Fig. 5).

**DISCUSSION**

We have presented evidence that directly supports a model for signal integration by PDGFβ receptor-S1P1 receptor complexes in ASM cells. We have also demonstrated that PDGF does not promote the release of S1P to stimulate p42/p44 MAPK, PKB, or p38 MAPK in a sequential manner in these cells. The sequential model may be operative in other cell types where PKB is very sensitive to low levels of S1P release. hSK1WT is constitutively exported from ASM cells and is not regulated by PDGF. Interestingly, the enzyme converts exogenous sphingosine to S1P, which in turn stimulates the p42/p44 MAPK pathway.

Several lines of evidence were obtained to support the exist-
ence of a functionally active native PDGFβ receptor-S1P₁ receptor complex that can support integrative signaling by SIP/ PDGF in ASM cells. First, the PDGFβ receptor and S1P₁ were co-immunoprecipitated from cell lysates with specific anti-S1P₁/PDGFβ receptor antibodies. Second, the treatment of ASM cells with PDGF stimulated the phosphorylation of p42/p44 MAPK, and this phosphorylated p42/p44 MAPK associates with the PDGFβ receptor-S1P₁ receptor complex. To our knowledge, this is the first report showing that phosphorylated p42/p44 MAPK is present in a complex with receptor in response to growth factor. Current models have suggested that after ligand binding and the initiation of signaling the receptor is subsequently disconnected from events such as association of activated Raf-MEK (Raf-MAPK kinase 1) with p42/p44 MAPK. The close association of phosphorylated p42/p44 MAPK with the PDGFβ receptor-S1P₁ receptor complex and attendant protein components is entirely compatible with an integrative signaling response to PDGF and SIP. Third, the treatment of cells with antisense S1P₁ receptor plasmid construct (which lowered S1P₁ receptor expression) reduced the PDGF- and S1P-dependent activation of p42/p44 MAPK. These findings support the requirement for cross-talk regulation between both receptors. They also suggest that the S1P₁ receptor might be partially constitutively active and capable of releasing active G-protein subunits for tyrosine phosphorylation by the PDGFβ receptor kinase, as observed in HEK 293 cells (19). Fourth, S1P and PDGF promoted the co-internalization of PDGFβ receptor and S1P₁ receptor in the same endocytic vesicles. The ability of SIP to induce internalization of the PDGFβ receptor is important as it establishes further evidence for close association between the S1P₁ receptor and the PDGFβ receptor. This model is, therefore, distinct from the sequential model, in which PDGF causes the release of SIP that subsequently acts at S1P₁ receptors in a manner that is completely independent of the PDGF receptor. Indeed, the results presented here clearly show an interaction between SIP and PDGF receptors that is independent of PDGF-stimulated SIP₁ release from cells.

Part of the PDGF-stimulated response in ASM cells is insensitive to pertussis toxin and to S1P₁ receptor antisense plasmid construct. Thus, a fraction of the response does not involve Gᵢ or S1P₁ receptors. This is an interesting finding and is supported by other evidence that suggests that the GPCR input into signaling by receptor tyrosine kinases is dependent upon both receptor density and ligand concentration. Thus, high receptor tyrosine kinase density or ligand concentration has been shown to surmount the requirement for GPCR input (28, 29). These findings suggest that receptor tyrosine kinases use at least two distinct signaling pathways to stimulate p42/p44 MAPK, one of which requires G-protein, whereas the other is probably initiated by auto-tyrosine phosphorylation of the growth factor receptor (28, 29). Indeed, our previous findings showed that the G-protein-dependent activation of p42/p44 MAPK in response to PDGF occurs via a mechanism that does not require PDGF-dependent tyrosine phosphorylation of the PDGFβ receptor (19). Moreover, we showed that overexpression of Gᵢ suppresses PDGFβ receptor tyrosine phosphorylation while increasing PDGF-stimulated activation of p42/p44 MAPK (19). This is supported by studies from Freedman et al. (21), who have recently shown that GRK2-catalyzed phosphorylation of the PDGFβ receptor suppresses PDGF-stimulated auto-tyrosine phosphorylation of the PDGFβ receptor. Thus, G-protein input may switch growth factor receptor signaling such that stimulation of p42/p44 MAPK is less dependent upon recruitment of proteins to tyrosine phosphates on the growth factor receptor and is more dependent upon a Gᵢ/GRK2/β-arrestin I-mediated pathway. Taken together with the findings presented in Alderton et al. (19) and in this current paper we propose that PDGFβ receptor endocytic signaling mediated by G-protein might be initiated by GRK2 and β-arrestin I that are recruited indirectly to the PDGFβ receptor via its association with the S1P₁ receptor. In this regard, Watterson et al. (30) have recently shown constitutive association of GRK2 with the S1P₁ receptors.

Endocytosis of the PDGFβ receptor signal complexes is required for activation of p42/p44 MAPK (31), and our results suggest that this is driven by a Gᵢ,PCR-dependent mechanism. Thus, increased internalization of PDGFβ receptor-S1P₁ receptor complexes in response to combined stimulation with S1P and PDGF will improve the efficiency with which PDGF (and vice versa, SIP) stimulates the p42/p44 MAPK pathway.

We have also reported that PDGF stimulates a pertussis toxin-sensitive tyrosine phosphorylation of the Grb-2-associated binding protein, Gab1, in ASM cells (31). This appears to be dependent upon Gᵢ, and c-Src (25, 31). In previous studies, we have shown that c-Src is activated by PDGF, and this can be blocked by pretreating ASM cells with pertussis toxin (25). Furthermore, there is an indication that GRK2 is regulated by c-Src (21), thereby providing a functional link between the PDGFβ receptor and GRK2. The tyrosine phosphorylation of Gab1 promotes the binding of phosphoinositide 3-kinase 1a (PI3K1a), which in turn induces association of dynamin II to the PI3K1α-Gab1-Grb-2 complex. This process appears to be essential for clathrin-mediated endocytosis of PDGF receptor signal complexes that include Raf-MAPK kinase 1 for re-localization with and activation of cytoplasmic p42/p44 MAPK (31). In this regard, dynamin II might function to promote “pinching off” of the endocytic vesicles containing active PDGFβ receptor-S1P₁ receptor signal complexes for subsequent activation of p42/p44 MAPK in the cytoplasm.

Others report co-internalization of GPCR-receptor tyrosine kinase complexes in response to GPCR agonist. For instance, Maudsley et al. (32) show that isoprenaline induces transactivation of the EGF receptor, resulting in EGF receptor tyrosine phosphorylation and complex formation with β-adrenergic receptors. Under these conditions, isoprenaline induces the co-internalization of β-adrenergic receptors and EGF receptors to the same endocytic vesicles. However, Maudsley et al. (32) report that EGF alone does not induce internalization of the β-adrenergic receptor. This contrasts with our findings, where we show that PDGF induces co-internalization of both PDGFβ receptor and the GPCR, S1P₁. In addition, the internalization of the PDGFβ receptor in response to SIP₁ is significant because this does not involve PDGF release. We conclude that integrative signaling and co-internalization of both PDGFβ receptor and SIP₁ receptor in response to PDGF and/or SIP₁ is a novel important mechanism. It is clearly a different mechanism compared with receptor tyrosine kinase transactivation by GPCR agonists.

The formation of a complex between the PDGFβ receptor and S1P₁ receptor may be a prototypical example for other growth factor receptors. This is supported by several studies showing that a number of growth factors use classic GPCR-mediated signaling pathways to stimulate p42/p44 MAPK in mammalian cells (33, 34).

Finally, we show that hSK1 is constitutively exported and can support activation of p42/p44 MAPK by exogenous sphingosine. The export of hSK1 is not regulated by PDGF and, therefore, does not contribute to signaling by this growth factor. However, it is possible that the presentation of sphingosine on other cell types and its conversion to SIP by exported kinase from ASM cells might enable SIP₁ to act with PDGF on the PDGFβ receptor-S1P₁ receptor complex to induce biological
responses in vivo. This would also represent a novel form of intercellular communication.

There is a possibility that a subfraction of ASM cells undergoes apoptosis in culture. This raises the intriguing question, Are cell death mechanisms associated with selective export of SK1 from cells, thereby reducing its ability to promote cell survival via an intracellular action? This is possible if there is hindered supply of sphingosine presented from other cells. However, contact with other cells that present sphingosine might effectively convert the export of SK into a cell survival action, since S1P (acting at cell surface S1P receptors) increases PDGF-stimulated p42/p44 MAPK activation and DNA synthesis in ASM cells (24).

In summary, our findings provide important information on integration of G-protein-mediated signals by receptor tyrosine kinases in mammalian cells by a mechanism that differs from sequential regulation and/or transactivation of receptor tyrosine kinases by GPCR agonists.

REFERENCES
1. Pyne, S., and Pyne, N. J. (1999) Biochem. J. 249, 385–402
2. Chun, J., Goetzl, E. J., Hla, T., Igarashi, Y., Lynch, K., Moolenaar, W., Pyne, S., and Tigi, G. (2002) Pharmacol. Rev. 54, 265–269
3. Hla, T., and Maciag, T. (1990) J. Biol. Chem. 265, 9308–9313
4. Okazaki, H., Ishizaka, N., Sakurai, T., Kurokawa, K., Goto, K., Kamada, M., and Takewa, Y. (1993) Biochem. Biophys. Res. Commun. 190, 1104–1109
5. Graler, M. H., Bernhardt, G., and Lipp, M. (1998) Genomics 53, 164–169
6. Im, D. S., Heise, C. E., Ancellin, N., O’Donnell, W. W., Geuze, H. J., and Lee, N. H. (1999) Mol. Cell. Neuror. 14, 141–152
7. Glickman, M., Malek, E. L., Kvitik-Black, A. E., Jacob, H. J., and Lee, N. H. (1999) Mol. Cell. Neurosci. 14, 141–152
8. Yamazaki, Y., Kon, J., Sato, K., Tomura, H., Sato, M., Yoneya, T., Okazaki, H., Okajima, F., and Ohta, H. (2000) Biochem. Biophys. Res. Commun. 268, 583–589
9. Lee, M-J., Evans, M., and Hla, T. (1996) J. Biol. Chem. 271, 11272–11279
10. Lee, M-J., Van Brocklyn, J. R., Thanjada, S., Liu, C. H., Hand, A. R., Menzeleea, R., Spiegel, S., and Hla, T. (1998) Science 276, 1552–1555
11. Kon, J., Sato, K., Watanabe, T., Tomura, H., Kurosawa, A., Kimura, T., Tamaka, K-L., Ishizaka, T., Murata, N., Kamada, T., Kobayashi, I., Ohta, H., U., M., and Okajima, F. (1999) J. Biol. Chem. 274, 23940–23947
12. Windh, R. T., Lee, M-J., Hla, T., An, S., Barr, A. J., and Manning, D. R. (1999) J. Biol. Chem. 274, 27351–27358
13. Van Brocklyn, J. R., Tu, Z., Edsall, L., Schmidt, R. R., and Spiegel, S. (1999) J. Biol. Chem. 274, 4626–4632
14. Ancellin, N., and Hla, T. (1999) J. Biol. Chem. 274, 18997–19002
15. Buhl, A. M., Johnson, N. L., Dhanasekaran, N., and Johnson, G. L. (1995) J. Biol. Chem. 270, 24631–24634
16. Hobson, J. P., Rosenfeldt, H. M., Barak, L. S., Oliveira, A., Poulton, S., Caron, M. C., Milsten, S., and Spiegel, S. (2001) Science 291, 1800–1803
17. Lindahl, P., Johansson, B. R., Leveen, P., and Betsholtz, C. (1997) Science 277, 242–245
18. Hellstrom, M., Kano, M., Lindahl, P., Abramsson, A., and Betsholtz, C. (1999) Development 126, 3047–3055
19. Alderton, F., Rakshit, S., Choi, K., Palmer, T., Sambhi, B., Pyne, S., and Pyne, N. J. (2001) J. Biol. Chem. 276, 28578–28585
20. Pyne, S., and Pyne, N. J. (2002) Biochim. Biophys. Acta 1582, 121–131
21. Freedman, N. J., Kim, L., Murray, J. P., Exum, S. T., Brian, L., Wu, J.-H., and Peppel, K. (2002) J. Biol. Chem. 277, 48261–48269
22. Pitson, S. M., Moretti, P. A. B., Zehol, J. R., Game, J. R., Vadas, M. A., D'Andrea, R. J., and Wattenberg, B. W. (2000) J. Biol. Chem. 275, 33945–33950
23. Pyne, S., and Pyne, N. J. (1993) Biochem. Pharmacol. 45, 593–603
24. Rakshit, S., Conway, A. M., Tate, R., Bower, T., Pyne, N. J., and Pyne, S. (1999) Biochem. J. 338, 643–649
25. Conway, A. M., Rakshit, S., Pyne, S., and Pyne, N. J. (1999) Biochem. J. 337, 171–177
26. Tolman, D., Conway, A. M., Rakshit, S., Pyne, N. J., and Pyne, S. (1999) Cell Signal. 11, 349–354
27. Ancellin, N., Collomont, C., Su, J., Li, O., Mittereder, N., Chae, S. S., Stefansson, S., Liu, G., and Hla, T. (2002) J. Biol. Chem. 277, 6667–6675
28. Luttrell, L. M., Van Biesen, T., Hawes, B. E., Koch, W. J., Tshara, K., and Lefkowitz, R. J. (1995) J. Biol. Chem. 270, 16495–16498
29. Rakshit, S., Pyne, S., and Pyne, N. J. (2001) Mol. Pharmacol. 60, 63–70
30. Wattersson, K. R., Johnston, E., Claumiers, C., Prentin, A., Cook, S. J., Benovic, J. L., and Palmer, T. M. (1997) J. Biol. Chem. 272, 5767–5777
31. Rakshit, S., Pyne, S., and Pyne, N. J. (2000) Mol. Pharmacol. 58, 413–420
32. Madsen, S., Pire, K. L., Zanab, M., Miller, W. E., Ahn, S., Daaka, Y., Lefkowitz, R. J., and Luttrell, L. M. (2000) J. Biol. Chem. 275, 8572–8580
33. Hallak, H., Seiler, R. E. M., Green, J. S., Ross, B. N., and Rubin, R. (2000) J. Biol. Chem. 275, 2255–2258
34. Ahn, S., Madsen, S., Luttrell, L. M., Lefkowitz, R. J., and Daaka, Y. (1999) J. Biol. Chem. 274, 1185–1188
Sphingosine 1-Phosphate and Platelet-derived Growth Factor (PDGF) Act via PDGF β Receptor-Sphingosine 1-Phosphate Receptor Complexes in Airway Smooth Muscle Cells

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