Tethering of the Platelet-derived Growth Factor β Receptor to G-protein-coupled Receptors

A NOVEL PLATFORM FOR INTEGRATIVE SIGNALING BY THESE RECEPTOR CLASSES IN MAMMALIAN CELLS*

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Mitogenic stimuli initiate cell proliferation via different classes of cell surface receptors that include growth factor receptor tyrosine kinase receptors and G-protein-coupled receptor (GPCRs). This involves stimulation of the p42/p44 mitogen-activated protein kinase (MAPK) cascade. For many years it has been known that certain GPCR agonists can function as co-mitogens with growth factors to stimulate DNA synthesis. However, the molecular mechanism for this interaction has not been fully defined. It is known that both growth factors and GPCR agonists stimulate the tyrosine phosphorylation of Shc (SH2-containing protein) and the sequential activation of Grb-2-mSos (son of sevenless), Ras, Raf, MEK1, and p42/p44 MAPK. GPCR agonists also activate non-receptor tyrosine kinases (e.g. c-Src), which function as intermediates between Gβγ subunits and Ras-dependent p42/p44 MAPK activation (2–4).

A significant advance in our understanding of co-mitogenicity comes from recent studies showing that certain growth factors can use classical GPCR-mediated signaling pathways to stimulate p42/p44 MAPK in mammalian cells. For instance, the insulin like growth factor-1 (IGF-1) receptor utilizes the G-protein, Gi, to stimulate activation of p42/p44 MAPK in fibroblasts (3). This was established using pertussis toxin (which uncouples G-protein-coupled receptors from inhibitory G-proteins) reduced the platelet-derived growth factor (PDGF) dependent growth factor stimulation of p42/p44 mitogen-activated protein kinase.

Second, transfection of cells with inhibitory G-protein α subunits increased the activation of p42/p44 mitogen-activated protein kinase by platelet-derived growth factor. Third, platelet-derived growth factor stimulated the tyrosine phosphorylation of the inhibitory G-protein α subunit, which was blocked by the platelet-derived growth factor kinase inhibitor, tyrphostin AG 1296. We have also shown that the platelet-derived growth factor β receptor forms a tethered complex with Myc-tagged endothelial differentiation gene 1 (a G-protein-coupled receptor whose agonist is sphingosine 1-phosphate) in cells co-transfected with these receptors. This facilitates platelet-derived growth factor-stimulated tyrosine phosphorylation of the inhibitory G-protein α subunit and increases p42/p44 mitogen-activated protein kinase activation. In addition, we found that G-protein-coupled receptor kinase 2 and β-arrestin 1 can associate with the platelet-derived growth factor β receptor. These proteins play an important role in regulating endocytosis of G-protein-coupled receptor signal complexes, which is required for activation of p42/p44 mitogen-activated protein kinase. Thus, platelet-derived growth factor β receptor signaling may be initiated by G-protein-coupled receptor kinase 2β-arrestin 1 that has been recruited to the platelet-derived growth factor β receptor by its tethering to a G-protein-coupled receptor(s). These results provide a model that may account for the co-mitogenic effect of certain G-protein-coupled receptor agonists with platelet-derived growth factor on DNA synthesis.
tor-stimulated endocytic signaling and activation of the p42/p44 MAPK pathway (9–11). GRK2 is activated in an agonist- and Gβγ subunit-dependent manner. β-arrestin II is a clathrin adapter protein that is recruited to ligand-bound GPCRs that have been phosphorylated by GRK2, and this promotes dynamin II-mediated endocytosis of receptor signal complexes containing Raf-1-MEK1 for subsequent activation of p42/p44 MAPK. Recent studies by Dalle et al. (12) have shown that IGF-1 promotes the binding of β-arrestin I to the IGF-1 receptor in adipocytes. Rakhit et al. (13) have also shown that NGF stimulates β-arrestin I binding to a GRK2-Trk A receptor complex in PC 12 cells. In this case, GRK2/β-arrestin I appears to initiate endocytosis of Trk A signal complexes, which leads to the activation of p42/p44 MAPK in response to NGF.

In recent studies, we reported that the platelet-derived growth factor (PDGF) receptor stimulates c-Src and p42/p44 MAPK via a pertussis toxin-sensitive pathway in airway smooth muscle cells (14, 15). We suggested that G, might function to recruit c-Src close to the PDGF receptor tyrosine kinase for activation. PDGF also stimulates a pertussis toxin-sensitive tyrosine phosphorylation of the Grb-2-associated binding protein, Gab1. This promotes the binding of tyrosine-phosphorylated phosphoinositide 3-kinase (PI3K) to Gab1, which is required for dynamin II-mediated endocytic signaling to the p42/p44 MAPK pathway in response to PDGF (15).

The realization that growth factor receptors can use G-proteins to signal to p42/p44 MAPK provides a mechanism that begins to explain the co-mitogenic properties of some GPCR agonists. Thus, GPCR agonists might provide G-protein α and βγ subunits for use by growth factor receptors to produce stronger activation of the p42/p44 MAPK pathway and therefore DNA synthesis. Certain G-protein-coupled receptor agonists have also been shown to stimulate the tyrosine phosphorylation and transactivation of growth factor receptors. The subsequent phosphorylated sites on the receptor act as acceptors for the recruitment of signaling proteins, such as Grb-2, PLCγ (phospholipase C γ), and PI3K, and complex assembly to elicit mitogenic responses. For instance, lysophosphatidic acid has been shown to transactivate the EGF receptor and p185MET (16) to stimulate p42/p44 MAPK activation in Cos-7 cells (16), while angiotensin II promotes PDGF receptor transactivation in vascular smooth muscle (17). However, this model does not adequately explain the co-mitogenicity of certain GPCR agonists with growth factors, since the transactivation of the growth factor receptor by GPCR agonist appears to be growth factor-independent.

In the current paper we have used HEK 293 cells transfected with PDGFB receptor to investigate the molecular mechanism for the involvement of Gi in regulating the transmission of signals from this receptor. We have focused on the possibility that the PDGFB receptor might exist in a functional signaling complex with GPCRs. We have also tested this model by looking at the specific interaction between the PDGFB receptor and EDG1 (endothelial differentiation gene 1), whose natural agonist is sphingosine 1-phosphate (S1P). To date, five closely related GPCRs of the EDG family (EDG1, EDG3, EDG5/AGR16/H218, EDG6, and EDG5/arg-1) have been identified as high affinity S1P receptors (18–21). The rationale for looking at interaction between the PDGFB receptor and EDG1 was 2-fold. First, S1P is co-mitogenic with PDGF in airway smooth muscle cells, which predominantly express EDG1 (22), and second Hobson et al. (23) showed that PDGF-stimulated cell motility is EDG1-dependent. While these authors reported the existence of cross-talk regulation between these different receptor classes, they did not establish the molecular basis of this interaction.

Here we show that the PDGFB receptor is tethered to GPCR(s) in a complex. The tethered receptor complex provides a platform on which receptor tyrosine kinase and GPCR signals can be integrated to produce more efficient regulation of downstream effector pathways. This model provides a molecular dynamic that may explain the co-mitogenic effect of GPCR agonists on PDGF-stimulated DNA synthesis.

**EXPERIMENTAL PROCEDURES**

**Materials**—All biochemicals, including collagenase, elastase, soybean trypsin inhibitor, and PDGF were from Sigma. Enhanced chemiluminescence reagents were from Amersham Pharmacia Biotech. Cell culture supplies were from Life Technologies, Inc. Anti-phospho-p42/p44 MAPK, p42 MAPK, and PY20 HR-linked anti-phosphotyrosine antibodies were from Transduction Laboratories. Anti-Gα12 antibody was from Calbiochem. Anti-PDGFB receptor and anti-β-arrestin I antibodies were from Santa Cruz. Reporter HRP-anti rabbit/mouse antibodies were from the Scottish Antibody Production Unit (Carlake, Scotland).

**Cell Culture**—HEK 293 cells were maintained in minimum essential medium (MEM) containing 20% (v/v) fetal calf serum. These cells were placed in MEM for 24 h before experimentation. Cultured airway smooth muscle cells were prepared and maintained as described previously (14, 15).

**Transfection**—HEK 293 cells were transiently transfected with PDGFB receptor, Myc-tagged EDG1, EDG1, and/or β-arrestin 1 pcDNA3.1 plasmid constructs and pertussis toxin-resistant 22,000 P40, 10% (v/v) glycerol, 1 mg/ml bovine serum albumin, 0.5 mM sodium deoxycholate, 0.2 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, and 0.2 mM orthovanadate, 0.2 mM phenylmethylsulfonyl fluoride, leupeptin, antipain, pepstatin, and aprotonin (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 antibodies and 40 μl of one part immunoprecipitation buffer and one part protein A-Sepharose 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 150 mM NaCl, 10 mM sodium phosphate (pH 7.2), 2 mM sodium orthovanadate, 50 mM NaF, 2 mM EDTA, 1% (v/v) deoxycholate, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 20 μg/ml aprotinin, 0.1% (v/v) mercurcaptoethanol, 0.1% (v/v) Nonidet P-40, and 0.1% (v/v) SDS), twice in buffer B (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 solubilized with boiling sample buffer containing 62 mM-Tris HCl, pH 6.7, 1.25% (v/v) SDS, 10% (v/v) glycerol, 5.7% (v/v) mercaptoethanol and 0.05% (v/v) bromphenol blue. The samples were then subjected to SDS-polyacrylamide gel electrophoresis and Western blotting.

**Blotting**—Immunoblotting was performed as described previously (14, 15). Immunoreactive proteins were visualized using the enhanced chemiluminescence detection kit.

**Sphingosine 1-Phosphate Formation**—Cells (airway smooth muscle...
The presence of an equal volume of chloroform. [3H]S1P was isolated by thin well). After 3 min, the medium was removed, and the cells harvested the medium with that containing [3H]sphingosine (2.22

Overexpression of recombinant PDGF receptor

that were excised from the plate.

Western blot probed with anti-PDGF receptor antibody showing PDGF receptor expression in transected cells. These are representative results of an experiment performed three times.

cells or PDGF receptor-transfected HEK 293) were quiesced overnight before preincubation for 1 h in serum-free medium supplemented with fatty acid-free bovine serum albumin (2 mg/ml). Incubations, in the presence and absence of PDGF (10 ng/ml) were initiated by replacing the medium with that containing [3H]sphingosine (2.22 × 10^5 dpm/well). After 3 min, the medium was removed, and the cells harvested into 0.5 ml ice-cold methanol. Lipids were extracted by vortexing in the presence of an equal volume of chloroform. [3H]S1P was isolated by thin layer chromatography on silica gel G60 plates developed with chloroform:methanol:aetic acid:H2O (25:10:1:2, v/v/v). A [32P]S1P standard was run in parallel. [3H]S1P, which co-migrated with the S1P standard, was measured by scintillation counting of appropriate sections of silica that were excised from the plate.

RESULTS

PDGFβ Receptor Signaling Is G-protein-mediated in HEK 293 Cells—Vector transfected HEK 293 cells exhibit a PDGFβ receptor null background. Thus, endogenous PDGFβ receptor was not detected on Western blots probed with anti-PDGFβ receptor antibodies (Fig. 1a), and PDGF did not stimulate the p42/p44 MAPK pathway in vector-transfected HEK 293 cells (Fig. 1b). This provides an ideal cell model system for transfection with recombinant PDGFβ receptors to study the involvement of GPCR(s) in regulating PDGFβ receptor signaling. Overexpression of recombinant PDGFβ receptor (Rm = 180 kDa) in PDGFβ receptor-transfected cells was confirmed by Western blotting with anti-PDGFβ receptor antibody (Fig. 1a). Moreover, PDGF induced a robust activation of p42/p44 MAPK in these cells, a response that was abolished by the PDGF receptor kinase inhibitor, tyrophostin AG 1296 (Fig. 1b). In general we found that the extent to which p42 MAPK was phosphorylated in response to PDGF exceeded that of p44 MAPK, which on some Western blots was barely detectable.

Several lines of evidence suggest that the PDGFβ receptor uses a classical GPCR signaling pathway to stimulate p42/p44 MAPK in PDGFβ receptor-transfected HEK 293 cells. Thus, pretreatment of cells with pertussis toxin, which uncouples GPCRs from Gαi, substantially reduced the PDGF-dependent activation of p42/p44 MAPK (Fig. 2a). The specificity of pertussis toxin was established by results showing that the stimulation of p42/p44 MAPK by the Gαi receptor agonist, thrombin, was also reduced by pretreating cells with this toxin (Fig. 2b), while the response to EGF, which does not use Gαi to signal, was unaffected (Fig. 2b). Pertussis toxin did not increase basal cAMP (data not shown), which in some cases can block (via protein kinase A) growth factor-stimulated p42/p44 MAPK activation. Because pertussis toxin is an inhibitor of Gα signaling, we tested the effect of transfecting cells with recombinant Gα for the PDGF-dependent stimulation of the p42/p44 MAPK pathway. Fig. 2c (upper panels) shows that the overexpression of recombinant Gα markedly increased the stimulation of p42/p44 MAPK by PDGF. This reflects more efficient downstream signaling from the PDGFβ receptor because recombinant Gα was without effect on PDGFβ receptor expression (Fig. 2d) and reduced PDGFβ receptor auto-phosphorylation (Fig. 2e). Studies to delineate the mechanism by which Gα potentiates the PDGF-dependent activation of p42/p44 MAPK were performed. These results show that both endogenous and recombinant Gα were basally tyrosine-phosphorylated in PDGFβ-transfected cells and that PDGF induced an increase in their tyrosine phosphorylation (Fig. 2c, lower panels). Tyrosine-phosphorylated recombinant Gα migrated on SDS-polyacrylamide gel electrophoresis as a 40-kDa protein. Two lines of evidence were used to identify Gα. First, endogenous Gα co-migrated with recombinant protein on SDS-polyacrylamide gel electrophoresis. Second, tyrosine-phosphorylated endogenous Gα was immunoprecipitated by anti-Gα antibodies (Fig. 2e). These results show that only a small fraction of the recombinant Gα is tyrosine-phosphorylated in response to PDGF but that this is sufficient to increase p42/p44 MAPK activation in response to this growth factor. The pretreatment of cells with the PDGF receptor kinase inhibitor, tyrophostin AG 1296, reduced the PDGF-stimulated tyrosine phosphorylation of endogenous Gα (Fig. 2f), suggesting that the PDGFβ receptor kinase might catalyze the phosphorylation of this G-protein. The concentration range of tyrophostin AG 1296 that was inhibitory (1–5 μM) was the same as that which inhibits PDGF receptor kinase activity and PDGF-stimulated p42/p44 MAPK activation (14). Interaction of the PDGFβ Receptor and EDG1 Receptors—These results are compatible with a model in which an endogenous ligand-bound GPCR(s) releases Gα from the αβγ heterotrimetric G-protein complex for subsequent tyrosine phosphorylation by the PDGFβ receptor kinase. The efficiency of this process could be greatly increased by proximity-induced effects if the PDGFβ receptor was tethered in a complex with the GPCR. To test the model, we investigated whether the recombinant forms of the PDGFβ receptor and the GPCR EDG1 (whose natural agonist is S1P) form a functional signaling complex in HEK 293 cells. The overexpression of recombinant EDG1 was demonstrated by a more robust stimulation of p42/p44 MAPK in response to S1P compared with that observed in vector-transfected cells (Fig. 3a). The evidence that the PDGFβ receptors and EDG1 form a complex was obtained from results showing that PDGFβ receptor and Myc-tagged EDG1 were co-immunoprecipitated from cell lysates using anti-Myc antibodies (Fig. 3b) or anti-PDGFβ receptor antibodies (Fig. 3c). Neither PDGF nor S1P stimulated an increase in the amount of each receptor in the complex. Controls were included to show that the PDGFβ receptor was not co-immunoprecipitated using anti-Myc tag antibodies when PDGFβ receptor plasmid construct was omitted from the co-transfection (Fig. 3b), while Myc-tagged EDG1 was not co-immunoprecipitated using anti-PDGFβ receptor antibodies when Myc-tagged EDG1 plasmid construct was omitted from the co-transfection (Fig. 3c).

Consistent with a model in which GPCR(s) release Gα for...
tyrosine phosphorylation by the PDGFβ receptor kinase, we found that PDGF induced a stronger tyrosine phosphorylation of G\textsubscript{\alpha} (Fig. 2c, lower panels) and a more robust activation of p42/p44 MAPK (Fig. 2, a and c, upper panels, Fig. 3d) in cells co-transfected with PDGFβ receptor and EDG1 versus PDGFβ receptor alone. This response remained sensitive to pertussis toxin (Fig. 2a). We have excluded the possibility that the improved effect of PDGF was due to an EDG1-induced increase in the expression (Fig. 1a) or tyrosine phosphorylation (data not shown) of recombinant PDGFβ receptor. Furthermore, PDGF did not stimulate p42/p44 MAPK in cells transfected with EDG1 alone (data not shown). Thus, our results suggest that the formation of the EDG1-PDGFβ receptor improves the efficiency of PDGFβ receptor signaling to p42/p44 MAPK. It is also important to note that PDGF did not increase S1P synthesis/release in these cells (Fig. 4), thereby excluding the possibility that S1P might function as an autocrine to stimulate p42/p44 MAPK activation by binding to recombinant EDG1 receptors. However, we do not exclude this as a possible mechanism in other cell types. As a positive control, we have shown that PDGF stimulated S1P formation in airway smooth muscle cells, measured after 3 min cell stimulation (Fig. 4). Furthermore, the pretreatment with DL-threo-dihydrosphingosine at a concentration (1 \( \mu \)M) that inhibits sphingosine kinase (23), an enzyme that catalyzes S1P formation, did not reduce PDGF-stimulated p42/p44 MAPK activation in PDGFβ receptor/EDG1-transfected cells or exogenous S1P stimulation of p42/p44 MAPK in cells overexpressing EDG1 alone (Fig. 5). We confirmed that the concentration of DL-threo-dihydrosphingosine was effective at inhibiting sphingosine kinase by showing that this inhibitor prevented PDGF-stimulated S1P formation in airway smooth muscle cells (Fig. 4).

**GRK2/β-Arrestin I Signaling**—Several growth factors can also use G-protein βγ subunits to initiate a GRK2- and β-arrestin-mediated stimulation of the p42/p44 MAPK pathway (9–11). The involvement of GRK2 and β-arrestin I might be explained by the binding of these proteins to a growth factor receptor-GPCR complex. Several lines of evidence support this model. Using cells transfected with recombinant forms of PDGFβ receptor and GRK2 (\( R_{m} = 85 \text{kDa} \)), we show that both proteins were co-immunoprecipitated from cell lysates using anti-PDGβ receptor antibodies (Fig. 6a), suggesting that these proteins form a complex. The stimulation of cells with PDGF or S1P did not increase the amount of GRK2 associated with the PDGFβ receptor (Fig. 6a). Less than 1% of the total GRK2 present in the anti-PDGβ receptor immunoprecipitates was endogenous GRK2. This was evidenced by residual binding of GRK2 in anti-PDGβ receptor immunoprecipitates from cells where recombinant GRK2 plasmid construct had been omitted from the co-transfection (Fig. 6b). As with recombinant GRK2, the stimulation of cells with PDGF did not increase the association between endogenous GRK2 and the PDGFβ receptor. Using cells transfected with recombinant forms of PDGFβ receptor and β-arrestin I (\( R_{m} = 55 \text{kDa} \)), we show that β-arrestin I can also be co-immunoprecipitated from cell lysates using anti-PDGβ receptor antibodies (Fig. 6c). In common with GRK2, we found that stimulation of cells with PDGF did not induce further association of recombinant β-arrestin I with the PDGFβ receptor (Fig. 6c). We also detected residual endogenous β-arrestin I (<1% of recombinant β-arrestin I) in the anti-PDGβ receptor immunoprecipitates. Treatment of cells with PDGF antibodies. 

**Fig. 2. PDGFβ receptor and GPCR regulation of the p42/p44 MAPK pathway.** HEK 293 cells were transiently transfected with vector and/or plasmid constructs for EDG1, PDGFβ receptor, or G\textsubscript{\alpha} as indicated. Transfected cells were pretreated with and without pertussis toxin (0.1 \( \mu \)g/ml, 18 h) or tyrphostin AG 1296 (1, 5 \( \mu \)M, 20 min) prior to stimulation with PDGF (10 ng/ml, 10 min), thrombin (0.3 units/ml, 10 min), or EGF (100 ng/ml, 10 min). a, Western blot probed with anti-phospho-p42/p44 MAPK and anti-p42 MAPK antibodies showing the effect of pertussis toxin on the stimulation of p42/p44 MAPK by PDGF in PDGFβ receptor- versus EDG1/PDGFβ receptor-transfected cells. b, the effect of pertussis toxin on the stimulation of p42/p44 MAPK by thrombin or EGF in vector-transfected cells. c, upper panels are Western blots probed with anti-phospho-p42/p44 MAPK and anti-p42 MAPK antibodies showing that the effect of overexpressing recombinant G\textsubscript{\alpha} on the PDGF-dependent activation of p42/p44 MAPK in PDGFβ receptor- versus EDG1/PDGFβ receptor-transfected cells. d, Western blot probed with anti-phospho-p42/p44 MAPK and anti-p42 MAPK antibodies showing that the effect of overexpressing recombinant G\textsubscript{\alpha} on PDGFβ receptor tyrosine phosphorylation and expression in PDGFβ receptor-transfected cells. e, Western blot of anti-G\textsubscript{\alpha} immunoprecipitates from PDGFβ receptor-transfected cells that have been probed with HRP-linked anti-phosphotyrosine antibodies. f, Western blot probed with HRP-linked anti-phosphotyrosine antibodies showing the effect of pretreating cells with tyrphostin AG 1296 on the PDGF-stimulated tyrosine phosphorylation of G\textsubscript{\alpha}. These are representative results of an experiment performed three times. IP, immunoprecipitation; WB, Western blot.
did not increase β-arrestin I association with the PDGFβ receptor (Fig. 6c).

Using cells transfected with Myc-tagged EDG1 and recombinant GRK2, we show that both proteins can be co-immunoprecipitated from cell lysates using anti-Myc tag antibodies (Fig. 6d), suggesting that these proteins also form a complex. The stimulation of cells with S1P did not increase the amount of recombinant GRK2 associated with Myc-tagged EDG1 (Fig. 6d). However, the treatment of cells with S1P stimulated an increase in the association of recombinant β-arrestin I with Myc-tagged EDG1 (Fig. 6e). The presence of endogenous GRK2 and β-arrestin I in anti-Myc-tagged immunoprecipitates could not be detected, possibly as the amounts of these proteins may be below the level of detection (Fig. 6, d and e).

**DISCUSSION**

Certain growth factor receptor tyrosine kinases can use classical GPCR-mediated signaling pathways to stimulate p42/p44 MAPK activation.
MAPK in mammalian cells. It is therefore possible that these growth factor receptors might exist in functional signaling complexes with GPCRs. Here, we provide evidence to show that the PDGFβ receptor is tethered to an endogenous GPCR(s) and to recombinant EDG1 in HEK 293 cells. The tethered receptor complex provides a platform on which receptor tyrosine kinase and GPCR signals can be integrated to produce more efficient regulation of the p42/p44 MAPK pathway. This involves (i) tyrosine phosphorylation of Gαi, possibly catalyzed by the PDGFβ receptor kinase and (ii) the association of GRK2/i-arrestin I with the PDGFβ receptor. There are reports that show that Gαi is also a substrate for tyrosine phosphorylation by another growth factor receptor, the insulin receptor in vitro (6, 24).

Several lines of evidence suggest that the PDGFβ receptor can use Gαi to signal to the p42/p44 MAPK pathway in HEK 293 cells. First, pertussis toxin reduced the PDGF stimulation of p42/p44 MAPK. Second, transfection of cells with recombinant Gαi increased the activation of p42/p44 MAPK by PDGF. Third, PDGF stimulated the tyrosine phosphorylation of Gαi. These results are compatible with a model in which an unidentified ligand-bound GPCR(s) releases Gαi from the αβγ heterotrimeric G-protein complex for tyrosine phosphorylation by the PDGFβ receptor kinase. Indeed, the inhibitory effect of pertussis toxin on the PDGF stimulation of p42/p44 MAPK is consistent with the established effect of this toxin on GPCR signaling. This is to prevent ligand-bound GPCRs from activating inhibitory G-proteins. Therefore, the mode of action of pertussis toxin suggests involvement of a GPCR, particularly as there is no evidence that the PDGFβ receptor can directly activate G-protein αβγ complexes. The supply of Gαi to the PDGFβ receptor kinase suggests that the GPCR is either tethered to or is in close association with the PDGFβ receptor. The results showing the presence of Myc-tagged EDG1-PDGFβ receptor complexes in transfected HEK 293 cells suggest that the former possibility is more likely.

Evidence that Gαi is involved in the PDGF-dependent activation of p42/p44 MAPK was based upon the fact that transfection of cells with recombinant Gαi potentiated p42/p44 MAPK activation by this growth factor. Moreover, this could be directly correlated with increased tyrosine phosphorylation of Gαi. We also noted that recombinant and endogenous Gαi were basally tyrosine-phosphorylated in PDGFβ receptor-transfected HEK 293 cells. This is not unexpected, as there is substantial PDGFβ receptor kinase activity in unstimulated cells (indicated by the basal tyrosine phosphorylation of the PDGFβ receptor) to support basal tyrosine phosphorylation of Gαi. However, it is important to note that the basally tyrosine-phosphorylated recombinant Gαi does not induce activation of p42/p44 MAPK on its own. Therefore, the potentiating effect of recombinant Gαi on the PDGF-stimulated p42/p44 MAPK activation may require the PDGF-induced recruitment to tyrosine-phosphorylated Gαi of other intermediates involved in the p42/p44 MAPK pathway. We also found that overexpression of recombinant Gαi reduced PDGF receptor auto-phosphorylation consistent with substrate competition between specific phosphotyrosine sites on the PDGFβ receptor and Gαi for the kinase. The fact that overexpression of recombinant Gαi reduced PDGFβ receptor tyrosine phosphorylation provides further evidence to support our proposal that Gαi interacts with the PDGFβ receptor kinase. It also is significant that the stimulatory effect of overexpressing recombinant Gαi on the PDGF-dependent activation of p42/p44 MAPK occurs in the face of reduced PDGFβ receptor autophosphorylation. This suggests that the PDGFβ receptor uses the Gαi signaling pathway as a major route for regulating p42/p44 MAPK in HEK 293 cells. Indeed, this is supported by other results reported here, which show that inactivation of endogenous Gαi signaling by pertus-
sis toxin reduced the PDGF stimulation of p42/p44 MAPK by ~90% in these cells.

A major finding confirming our proposal was obtained by data showing that the PDGFβ receptor can exist in a tethered complex with Myc-tagged EDG1 in cells co-transfected with these receptors. There is one other example of growth factor receptor tethering to a GPCR. This was shown for the insulin and β-adrenergic receptors in adipocytes (25, 26). These authors showed that insulin stimulates the phosphorylation of the β-adrenergic receptor on Tyr-350 and this promotes the binding of the insulin receptor via Grb-2, which serves to tether the two receptors. Moreover, the integrity of the insulin receptor-Grb-2-β-adrenergic receptor complex is critical for β-adrenergic agonist amplification of insulin-dependent activation of p42/p44 MAPK. Therefore, we suggest that the purpose of tethering the PDGFβ receptor with a GPCR is to improve the efficiency of PDGFβ receptor signaling, possibly via proximity-induced effects. This is borne out experimentally where we show that PDGF induced a stronger tyrosine phosphorylation of Gα and a more robust activation of p42/p44 MAPK in cells transfected with both PDGFβ receptor and EDG1 compared with PDGFβ receptor alone. We consider these studies to be very important in identifying novel signaling platforms. It is also possible that endogenous PDGF receptors form complexes with endogenous GPCRs in other cell types, such as airway smooth muscle cells. This is based upon data showing that pertussis toxin abolished PDGF-induced Gab1 tyrosine phosphorylation, PI3K-Gab1 association, and dynamin II binding to Grb-2 (15) and reduced the PDGF-dependent activation of p42/p44 MAPK by ~50% (14, 15).

The effect of recombinant EDG1 on PDGFβ receptor signaling was not a consequence of its binding S1P released from cells in response to PDGF but is instead probably due to constitutive activation of EDG1. Indeed, it is a common feature of recombinant GPCRs that they are often constitutively activated when expressed in cells. Moreover, others have shown this to be the case for EDG1, EDG3, and EDG5. These authors showed that when these receptors are overexpressed they form active complexes with G-proteins α subunits (27, 28). We have excluded a role for released S1P based on the following. First, we were unable to detect S1P formation in response to PDGF in PDGFβ receptor-transfected HEK 293 cells. Second, the sphingosine kinase inhibitor, ω-threo-dihydrosphingosine, had no effect on PDGF-stimulated p42/p44 MAPK activation in PDGFβ receptor/EDG1 co-transfected cells. However, we do not exclude the possibility that released S1P could act via tethered EDG1-PDGFβ receptors in other cell types.

We also found that GRK2/β-arrestin I can associate with the PDGFβ receptor. The stimulation of cells with PDGF did not increase the amount of either protein in the receptor complex, suggesting that the PDGFβ receptor-GRK2-β-arrestin I complex is preformed. These data again support the possibility that GRK2 and β-arrestin I are bound to an endogenous ligand-GPCR(s) that is tethered with the PDGFβ receptor. This is a plausible explanation given that the PDGFβ receptor does not have binding sites for GRK2 and β-arrestin I. We surmise that the putative GPCR is ligand-bound based upon the fact that GRK2 and β-arrestin I association with GPCRs is dependent upon free Gβγ activation released upon ligand binding to the GPCR. In addition, the finding that PDGF did not stimulate further association of these proteins with the PDGFβ receptor suggest to us that this growth factor receptor cannot itself induce Gβγ dissociation and that PDGF does not stimulate release of the putative GPCR ligand from these cells. GRK2 and β-arrestin I play an important role in regulating endocytosis of GPCR signal complexes, which is required for activation of p42/p44 MAPK. In this regard, we have previously shown that endocytosis of PDGF receptor signal complexes is also required for p42/p44 MAPK activation and that this is G-protein-regulated in airway smooth muscle cells (14). It is therefore possible that endocytosis of PDGFβ receptor-signal complexes may be initiated by GRK2/β-arrestin I that have been recruited to the PDGFβ receptor by its tethering to GPCR(s). This possibility is currently under investigation in our laboratory.

The model proposed here for the interaction of the PDGFβ receptor with GRK2/β-arrestin I appears to be slightly different from that reported for two other growth factor receptors where binding of β-arrestin I is apparently growth factor-dependent. First, Dalle et al. (12) showed that IGF-1 uses Gbγ subunits to stimulate the binding of β-arrestin I to the IGF-1 receptor in adipocytes. These authors also showed that IGF-1 induced Gbγ subunits dissociation and stimulated binding of Gα to the IGF-1 receptor. These workers therefore proposed that the IGF-1 receptor uses both G-protein subunits to promote signal transduction. Second, we have shown that the NGF-dependent activation of p42/p44 MAPK can be potentiated in PC 12 cells transfected with GRK2 or β-arrestin I (13). GRK2 is preassociated with the Trk A receptor, while NGF stimulates the pertussis toxin-sensitive binding of β-arrestin I to the Trk A receptor-GRK2 complex. However, in the light of the current findings in this article, there is a need to establish whether these growth factor receptors are also tethered to GPCRs and whether they elicit release of the corresponding GPCR ligand, which may in turn trigger binding of β-arrestin I to the GPCR within the putative complex with growth factor receptor.

We also found that GRK2 and β-arrestin I are associated with recombinant EDG1, thereby providing further evidence that this receptor may be constitutively active when overexpressed. However, recombinant EDG1 appears to be capable of further activation based upon the fact that S1P induces an increase in the binding of β-arrestin I and can stimulate activation of p42/p44 MAPK. This is in line with current thinking where it is proposed that receptors can exist in different receptor-G-protein conformations and that ligands induce a more productive conformation in terms of efficacy. Thus, S1P may convert recombinant EDG1 from a partially constitutively activated conformation to one that is capable of stimulating further Gbγ release and binding of β-arrestin I.

In conclusion, growth factor receptor-GPCR complexes provide a platform for integrating signals from these different receptor classes. This represents a mechanistic model that may account for the co-mitogenic effect of GPCR agonists with growth factors. More specifically, our proposed model provides a mechanism that may account for the recent findings of Hobson et al. (23) who showed that PDGF-stimulated cell motility is EDG1-dependent. Thus, S1P released from cells (not HEK 293 cells) in response to PDGF could act back on EDG1-PDGFβ receptor complexes to induce more efficient downstream stimulation of effector pathways in response to PDGF. This might be specific to certain cell types where S1P functions as an autocrine with PDGF.

The findings in the current study break the conventional paradigm for growth factor receptor signaling and strengthen an emerging model that such receptors can use GPCR-mediated pathways to stimulate p42/p44 MAPK. Future goals will be to identify the structural determinants that govern the interaction between the PDGFβ receptor and GPCRs.

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Tethering of the Platelet-derived Growth Factor β Receptor to G-protein-coupled Receptors: A NOVEL PLATFORM FOR INTEGRATIVE SIGNALING BY THESE RECEPTOR CLASSES IN MAMMALIAN CELLS
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