Platelet-derived-growth-factor stimulation of the p42/p44 mitogen-activated protein kinase pathway in airway smooth muscle: role of pertussis-toxin-sensitive G-proteins, c-Src tyrosine kinases and phosphoinositide 3-kinase

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The mechanism used by the platelet-derived growth factor receptor (PDGFR) to activate the mitogen-activated protein kinase (p42/p44 MAPK) pathway was investigated in cultured airway smooth muscle (ASM) cells. We have found that pertussis toxin (PTX, which was used to inactivate the heterotrimeric G-protein G_i) induced an approx. 40–50 % decrease in the activation of c-Src and p42/p44 MAPK by PDGF. An essential role for c-Src was confirmed using the c-Src inhibitor, PP1, which abolished p42/p44 MAPK activation (PP1 and PTX were without effect on PDGFR tyrosine phosphorylation). Furthermore, the PTX-dependent decrease in c-Src and p42/p44 MAPK activation appeared correlated. These findings suggest that the PDGFR can utilize the PTX-sensitive G-protein, G_i , to regulate c-Src and subsequent p42/p44 MAPK activation. Phosphoinositide 3-kinase (PI3K) has been shown by others to be involved in p42/p44 MAPK activation. This is confirmed here

by experiments which showed that PI3K inhibitors (wortmannin and LY294002) reduced the activation of p42/p44 MAPK by PDGF. PI3K activity was increased in Grb-2 immunoprecipitates from PDGF-stimulated cells and was decreased by pretreating these cells with PTX. These findings show that G_i might also promote Grb-2–PI3K complex formation and that Grb-2 may be a site at which PI3K is integrated into the p42/p44 MAPK cascade. In conclusion, our results demonstrate that G_i enables the PDGFR to signal more efficiently to p42/p44 MAPK, and this appears to be achieved through the regulation of c-Src and Grb-2/PI3K, which are intermediates in the p42/p44 MAPK cascade.

Key words: growth-factor receptor, lipid kinase, mitogenesis, protein kinase, Ras.

INTRODUCTION

Mitogenic stimuli that initiate smooth-muscle proliferation do so via different classes of cell-surface receptors, such as growth-factor tyrosine kinase and G-protein-coupled receptors. Growth-factor tyrosine kinase receptors activate the mitogen-activated protein kinase (p42/p44 MAPK) pathway and stimulate immediate early gene induction and cell-cycle progression from G_0 to G_1 . Activation of the p42/p44 MAPK pathway involves the tyrosine phosphorylation of Shc (SH-domain-containing protein) and the recruitment of the guanine-nucleotide-releasing binding protein Grb-2. Grb-2 localizes the guanine-nucleotide exchange factor mSos close to Ras (a low-molecular-mass G-protein). GTP $\leftrightarrow$ GDP exchange in Ras promotes activation of the serine/threonine protein kinase c-Raf, MAPK kinase (MEK-1) and p42/p44 MAPK. Duckworth and Cantley [1] have also demonstrated an essential role for phosphoinositide 3-kinase (PI3K) in platelet-derived growth-factor-receptor (PDGFR)-dependent regulation of this pathway in Swiss 3T3 fibroblasts.

G_i -coupled receptor agonists activate non-receptor tyrosine kinases such as c-Src (Btk, Syk, Pyk-2), which function as intermediates between $G_i/\beta\gamma$ and Ras-dependent p42/p44MAPK activation [2–4]. An essential role for a $G_i/\beta\gamma$ -regulated PI3K (γ isoform) was evinced by studies which showed that its over-expression in transfected Cos cells induced p42/p44 MAPK activation [5]. Furthermore, $G_i/\beta\gamma$ -dependent PI3K has been

shown to participate in the regulation of a novel tyrosine kinase responsible for the phosphorylation of the Grb-2 adaptor protein, p100 [6].

These studies and others suggest that growth-factor and G_i -coupled-receptor stimulation of the Ras-dependent p42/p44 MAPK pathway involves intermediate tyrosine kinase- and PI3K-regulated steps [5,7].

There is little evidence to support the idea that signal transmission from growth-factor tyrosine kinase receptors to p42/p44 MAPK involves $G_{i/o}$. There are two exceptions, namely insulin and insulin-like-growth-factor-1 receptor, which stimulate p42/p44 MAPK activation in a pertussis toxin (PTX)-sensitive manner in fibroblasts [8]. In contrast, there is a growing body of evidence to suggest that G_i -coupled receptor agonists can stimulate growth-factor-receptor transactivation [9–12].

Using cultured airway smooth-muscle (ASM) cells, we have further investigated whether G_i is involved in growth-factor-receptor signalling and have focused on PDGFR and its regulation of p42/p44 MAPK.

MATERIALS AND METHODS

Materials

All biochemicals, including collagenase, elastase, soya-bean trypsin inhibitor, PTX and PDGF-AB (AB refers to ligand

Abbreviations used: ASM, airway smooth muscle; DTT, dithiothreitol; ERK, extracellular-signal-regulated kinase; p42/p44 MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; PTX, pertussis toxin; PDGF(R), platelet-derived growth factor (receptor); PI, phosphatidylinositol; PI3P, phosphatidylinositol 3-phosphate; PI3K, phosphoinositide 3-kinase; Shc, SH-domain-containing protein; DMEM, Dulbecco's modified Eagle's medium; FCS, foetal-calf serum; DHS, donor horse serum; NP-40, Nonidet P40; c-Raf, serine/threonine protein kinase; Ras, low-molecular-mass G-protein; G_i and G_o , heterotrimeric G-proteins.

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specificity at PDGF α and β receptors) were from Sigma Chemical Co. PPI and tyrphostin AG1296 were from Calbiochem. [γ - 32 P]ATP (3000 Ci/mol), [32 P]NAD⁺, MAPK Biotrak assay kits and Enhanced Chemiluminescence (ECL) reagents were from Amersham International. Cell-culture supplies were from Gibco. ERK-2 and PY20 anti-phosphotyrosine antibodies were from Affiniti (Mamhead, Exeter, Devon, U.K.). PDGFR antibody was from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Anti-c-Src and Grb-2 antibodies were from Santa Cruz (Santa Cruz, CA, U.S.A.). Reporter HRP-anti mouse/rabbit antibodies were from the Scottish Antibody Production Unit (Carlisle, Lanarkshire, Scotland, U.K.). Male Dunkin–Hartley guinea-pigs (200–400 g) were used for isolation of tracheal smooth muscle.

Cell culture

The preparation of the primary cultures of guinea-pig ASM cells was achieved as described previously [13]. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) foetal-calf serum (FCS) and 10% (v/v) donor horse serum (DHS) in 5% CO₂ at 37 °C and were passaged twice, using trypsin, prior to experimentation. Cells were grown to confluence on six-well plates and routinely used at 15–21 days after the initial preparation. Their identity was confirmed to be smooth muscle by the presence of α -actin using a smooth-muscle-specific mouse anti- α -actin monoclonal antibody.

p42/p44 MAPK assays

Confluent ASM cells were incubated in DMEM containing 1% (v/v) FCS and 1% (v/v) DHS for 24 h. Cells were then stimulated with PDGF in the presence and absence of inhibitors for the times indicated. In some cases, cells were pretreated with PTX (0.05–1 μ g/ml) for 24 h prior to stimulation with PDGF.

(i) p42 MAPK (ERK-2) phosphorylation assays

After stimulation with PDGF, the medium was removed and boiling sample buffer added [0.125 M Tris/HCl (pH 6.7)/0.5 mM Na₄P₂O₇/1.25 mM EDTA/2.5% (v/v) glycerol/0.5% (w/v) SDS/25 mM dithiothreitol (DTT)/1% (w/v) Bromophenol Blue]. Cell lysates were harvested, subjected to SDS/PAGE and transferred to nitrocellulose sheets. p42 MAPK activation was detected by a shift in its electrophoretic mobility on immunoblots probed with anti-ERK-2 antibody.

(ii) p42/p44 MAPK activity

Assays utilized a specific p42/p44 MAPK peptide substrate [EGFR-(661–680)-peptide, synthesized to contain one phosphorylation site]. Cell lysates were prepared by adding 0.5 ml of buffer containing 10 mM Tris/HCl, pH 7.4, 150 mM NaCl, 2 mM EGTA, 2 mM DTT, 1 mM orthovanadate, 1 mM PMSF, 10 μ g/ml aprotinin and 10 μ g/ml leupeptin. Cells were harvested in this buffer and homogenized by repeatedly (three times) passing through a 0.24-mm-gauge syringe. Each assay mixture contained 5–10 μ g of cell lysate protein and 0.5 μ Ci of [γ - 32 P]ATP (final concn. 0.2 mM). Assays were performed under conditions where less than 2% of the substrate was utilized and where product formation was linearly related to time. Assays were terminated by adding stopping solution. Samples were then spotted on to peptide-binding papers and washed three times in 1% (v/v) acetic acid and twice with distilled water. The amount

of [32 P]peptide on each disc was quantified using liquid-scintillation counting.

PD098059, which is universally used to inhibit MEK-1 activation, abolished PDGF-stimulated ERK activation seen in both shift and radioactive assays [14]. These data validate the p42/p44 MAPK assay.

Immunoprecipitation

After stimulation with PDGF, 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) Nonidet P40 (NP-40), 10% (v/v) glycerol, 1 mg/ml BSA, 0.5 mM sodium orthovanadate, 0.2 mM PMSF, 10 μ g/ml leupeptin, antipain, pepstatin and aprotinin, pH 8] for 10 min at 4 °C. The material was harvested, centrifuged at 14000 g for 5 min at 4 °C, and 200 μ l of cell lysate supernatant (equalized for protein, 0.5–1 mg/ml) taken for immunoprecipitation with antibodies (5 μ g of either anti-c-Src, anti-phosphotyrosine, anti-PDGFR or anti-Grb-2 antibodies and 40 μ l of 1:1 Protein A– or Protein G–Sepharose CL4B; 1:1 indicates equal parts of Protein A and immunoprecipitation buffer). After agitation for 2 h at 4 °C, the immune complex was collected by centrifugation at 14000 g for 15 s at 4 °C. For c-Src, the beads were washed twice with buffer A containing 150 mM NaCl, 10 mM sodium phosphate, pH 7, 2 mM EDTA, 1% (w/v) deoxycholate, 1% (v/v) NP-40, 0.1% SDS, 0.2 mM PMSF, 10 μ g/ml leupeptin, 20 μ g/ml aprotinin, 50 mM NaF, 2 mM Na₃VO₄, 0.1% (v/v) mercaptoethanol, and then twice with buffer containing 10 mM Hepes, pH 7, 100 mM NaCl, 0.2 mM PMSF, 10 μ g/ml leupeptin, 20 μ g/ml aprotinin and 0.5% (v/v) NP-40, and once in buffer without NP-40. For Grb-2 and PDGFR the beads were washed as described above, but with the omission of buffer A. For the anti-phosphotyrosine immunoprecipitates, the beads were washed three times with lysis buffer, twice with 0.5 M LiCl/100 mM Tris/HCl, pH 8, once with 0.15 M NaCl/10 mM Tris/HCl (pH 7.6)/1 mM EDTA, and once with 20 mM Hepes (pH 7.5)/1 mM DTT/5 mM MgCl₂.

c-Src immunoprecipitates were resuspended in buffer A for c-Src assays, while the anti-phosphotyrosine/Grb-2 immunoprecipitates were resuspended in 20 mM β -glycerophosphate/5 mM Na₄P₂O₇/30 mM NaCl/1 mM DTT, pH 7.2, for PI3K activity assays. In some cases, immunoprecipitates were resuspended in boiling sample buffer [0.125 M Tris/HCl (pH 6.7)/0.5 mM Na₄P₂O₇/1.25 mM EDTA/2.5% (v/v) glycerol/0.5% (w/v) SDS/25 mM DTT/1% (w/v) Bromophenol Blue]. Samples were then subjected to SDS/PAGE and transferred to nitrocellulose sheets for Western blotting.

Blotting

After transfer of proteins (immunoprecipitates and cell lysate samples) from SDS/PAGE to nitrocellulose, the sheets were blocked in 3% (w/v) BSA in 10 mM Tris/HCl, pH 8, and 0.15 M NaCl (TBS) for 1 h at 23 °C. These were then probed with anti-ERK-2, -Grb-2, -c-Src, -PDGFR or HRP-linked anti-phosphotyrosine antibodies in TBS containing 1% BSA (w/v) plus 0.05% (v/v) Tween-20 for 12 h at 23 °C. After this time, the nitrocellulose sheets were washed in TBS plus 0.05% (v/v) Tween-20. Detection of ERK-2, PDGFR or c-Src was by incubating nitrocellulose for 2 h at 23 °C with reporter HRP-linked anti-mouse/rabbit antibodies in TBS containing 1% BSA (w/v) plus 0.05% (v/v) Tween-20. After washing the blots as

described above (to remove excess reporter antibody) immuno-reactive bands were revealed using the ECL detection kit.

c-Src assay

c-Src immunoprecipitates were incubated with 1.6 μ g of acid-denatured rabbit muscle enolase (Sigma), 20 mM Hepes, pH 7, 10 mM MnCl_2 , 20 μ g/ml aprotinin and 5 μ Ci of [γ - 32 P]ATP (3 μ M) in a total reaction volume of 20 μ l. The reaction was stopped by adding 2 $\times$ sample buffer containing 10 mM EDTA, 4% (w/v) SDS, 5.6 M mercaptoethanol, 20% (v/v) glycerol, 200 mM Tris/HCl, pH 6.8, and 1% (w/v) Bromophenol Blue. Samples were then boiled for 2 min, cooled and subjected to SDS/PAGE.

PI3K assay

Resuspended anti-phosphotyrosine or -Grb-2 immunoprecipitates (40 μ l) were each combined with 20 μ l of phosphatidylinositol (3 mg/ml) in incubation buffer containing 1% cholate. To each, 40 μ l of [32 P]ATP (3 μ M Na_2ATP , 7.5 mM MgCl_2 and 0.25 mCi of [32 P]ATP/ml) was added. The reaction was performed at 37 °C for 15 min and was terminated by adding 450 μ l of chloroform/methanol (1:2, v/v). Organic and aqueous phases were resolved by adding 150 μ l of chloroform and 150 μ l of 0.1 M HCl. Samples were mixed and centrifuged (4200 *g* for 10 min). This was repeated and the lower phase harvested, dried, and [32 P]phosphatidylinositol 3-phosphate ([32 P]PI3P) resolved by TLC using chloroform/methanol/ammonia/water (42:60:9:15, by vol.) in parallel with a non-radioactive standard. Radioactive bands were revealed by autoradiography, and samples corresponding to [32 P]PI3P scraped from the plate and subjected to Čerenkov counting.

PTX ADP-ribosylation

Confluent ASM cells were pretreated with PTX (0.1 μ g/ml), or not pretreated, in DMEM containing 1% (v/v) FCS and 1% (v/v) DHS for 24 h. Cells were harvested in 0.25 M sucrose/10 mM Tris/HCl (pH 7.4)/1 mM EDTA/0.1 mM PMSF/2 mM benzamidine and homogenized by repeatedly (three times) passing through a 0.24-mm-gauge syringe. Membranes were collected by centrifugation at 22000 *g* for 10 min at 4 °C and subjected to PTX-catalysed ADP-ribosylation. This involved combining membranes (20 μ g of protein) with buffer containing (final concns.) 30 mM thymidine, 1 mM ATP, 80 mM potassium phosphate, pH 7.5, 20 mM arginine, 1 mM MgCl_2 and 7.5 μ Ci of 20 μ M [32 P]NAD $^{+}$. PTX (1.33 μ g/assay) was added to each assay mixture and incubated at 37 °C for 1 h (PTX was pre-activated by combining it with an equal volume of 50 mM DTT). PTX-catalysed ADP-ribosylation reactions were stopped by adding an equal volume of 2 $\times$ sample buffer containing 0.125 M Tris/HCl, pH 6.7, 0.5 mM $\text{Na}_4\text{P}_2\text{O}_7$, 1.25 mM EDTA, 2.5% (v/v) glycerol, 0.5% (w/v) SDS, 25 mM DTT, 1% (w/v) Bromophenol Blue, and then subjected to SDS/PAGE.

RESULTS

PDGFR tyrosine phosphorylation and c-Src/p42/p44 MAPK activation

Pretreating cells with PTX (0.1 μ g/ml) decreased the activation of p42/p44 MAPK by PDGF (Table 1 and Figure 1a). The maximal inhibitory effect (approx. 40–50%) was elicited by 0.1–1 μ g/ml PTX (Figure 1b). We have previously shown that PTX catalyses the ADP-ribosylation of G_i (the heterotrimeric G-

Table 1 Effect of PTX and inhibitors on the PDGF-dependent activation of p42/p44 MAPK

ASM cells were treated with and without PTX (0.1 μ g/ml) for 24 h. These cells were then treated with or without PP1 (10 μ M) or wortmannin (50 nM) or LY294002 (50 μ M) for 15 min prior to stimulation with PDGF (10 ng/ml, 10 min). p42/p44 MAPK activity was measured in cell lysates using a specific peptide assay kit. Basal p42/p44 MAPK activity in control cells was 115 ± 23 pmol/min per mg of protein. Results are expressed as the fold increase over basal p42/p44 MAPK activity in control cells (means $\pm$ S.D. for $n = 5$ –10 separate cell preparations). The inhibitor of MEK-1 activation, PD098059, abolished p42/p44 MAPK activation in response to PDGF [14]. $\dagger P < 0.001$ significance versus PDGF alone (Student's *t* test).

Treatment	Fold activation
No PTX treatment	
Control	1 $\pm$ 0.2
PDGF	9.3 $\pm$ 1.2
Wortmannin	0.8 $\pm$ 0.05
Wortmannin/PDGF	0.9 $\pm$ 0.1
LY294002	0.8 $\pm$ 0.02
LY294002/PDGF	5 $\pm$ 1.3 $\dagger$
PP1	0.9 $\pm$ 0.1
PP1/PDGF	1.2 $\pm$ 0.01
PTX-treated cells	
Control	0.8 $\pm$ 0.2
PDGF	5.4 $\pm$ 0.6 $\dagger$

protein G_o is not expressed in ASM cells [15]). The maximal inhibitory effect of PTX on p42/p44 MAPK activation was correlated with complete ADP-ribosylation of $G_{i\alpha}$ (molecular mass 41 kDa). Endogenous ADP-ribosylation of G_i was evidenced from experiments which showed that PTX failed to catalyse [32 P]ADP-ribosylation of $G_{i\alpha}$ in membranes prepared from PTX (0.1 μ g/ml, 24 h)-treated cells (Figure 1c). PTX can also increase intracellular cAMP levels in cells by disinhibiting tonic regulation of adenylate cyclase by G_i . This was an important consideration, because agents that increase intracellular cAMP block growth-factor-stimulated p42/p44 MAPK activation in aortic smooth-muscle cells [16]. Our experiments showed that PTX did not alter cAMP levels under these conditions; thus, cAMP-dependent inhibition of p42/p44 MAPK activation can be ruled out [cAMP levels (measured as pmol of cAMP/0.25 $\times$ 10 6 cells for $n = 3$ separate cell preparations; means $\pm$ S.D.): control, 1.5 ± 0.1 ; PTX (0.1 μ g/ml, 24 h), 1.3 ± 0.3 ; PDGF (10 ng/ml), 1.7 ± 0.05 ; PTX and PDGF, 1.3 ± 0.2]. These data provide strong evidence to support our contention that PTX blocks p42/p44 MAPK activation by inactivating G_i .

We also assessed the effect of PTX on p42/p44 MAPK activation kinetics. Figure 1(d) shows that PTX (0.1 μ g/ml) did not effect the initial burst (0–1 min), but decreased activation at later time points (5–60 min).

Figure 2(a) shows that PTX (0.1 μ g/ml) was without effect on PDGFR (molecular mass 180 kDa) tyrosine phosphorylation (measured between 0 and 60 min stimulation with PDGF; results not shown). These findings suggest that G_i does not modulate PDGFR tyrosine phosphorylation, but is instead involved in regulating signal transmission from the PDGFR to p42/p44 MAPK.

The 180 kDa protein was identified as PDGFR on the basis of several findings: (i) it was the major tyrosine-phosphorylated protein in cells stimulated with PDGF (50-fold increase in tyrosine phosphorylation compared with any other protein) (results not shown); (ii) it co-migrated with the PDGFR and was immunodepleted from cell lysates with a PDGFR antibody

Figure 1 Effect of PTX on p42/p44 MAPK activation

Cells were pretreated with and without PTX ('p-tox') for 24 h prior to stimulation with PDGF (10 ng/ml) for the indicated times. **(a)** Shift blot showing the effect of PTX (0.1 μ g/ml) on p42 MAPK/ERK-2 phosphorylation. ERK-2 and activated ERK-2 are denoted by arrows. **(b)** Concentration-dependent inhibition of p42/p44 MAPK activation by PTX (0.05–1 μ g/ml). Cells were stimulated with PDGF for 10 min. **(c)** Autoradiograph showing the PTX-catalysed [32 P]ADP-ribosylation of $G_i\alpha$ in cells pretreated with and without PTX (0.1 μ g/ml) for 24 h. **(d)** Time course of p42/p44 MAPK activation (○, control cells; ●, PTX-(0.1 μ g/ml) treated cells). **(a, c)** Representative results of experiments performed on three separate cell preparations. **(b, d)** Results expressed as the fold increase over basal p42/p44 MAPK activity in control cells (means $\pm$ S.D. for triplicate assays from a single experiment typical of three). Basal p42/p44 MAPK activities were 84 ± 5 and 93 ± 6 pmol/min per mg of protein for **(b)** and **(d)** respectively.

(results not shown); and (iii) its tyrosine phosphorylation was virtually abolished by the PDGFR kinase inhibitor AG1296 (Figure 2b).

Several authors have reported that $G_i\beta\gamma$ subunits activate the tyrosine kinase c-Src, which has an important role in regulating p42/p44 MAPK activation [2–4]. We therefore tested the effect of PTX on the PDGF-dependent activation of c-Src. Our experiments showed that activation was maximal at 5 min, remained sustained for a further 15 min and then declined to a level above basal at 30 min (Figure 3a). Pretreatment with PTX was without effect on the initial burst (0–1 min), but decreased (did not abolish) activation at later time points (5–30 min). The effect on c-Src activation correlated with similar changes in p42/p44 MAPK activity in PTX-treated cells (see Figure 1d).

An essential role for c-Src was confirmed using the c-Src inhibitor PP1, which abolished p42/p44 MAPK activation (Table 1; used at a concentration that inhibits p42/p44 MAPK activation by epidermal-growth-factor receptor and lysophosphatidate in transfected Cos-7 cells [12]). PP1 specificity was tested against PDGFR tyrosine phosphorylation and was shown to have no effect (Figure 2c).

The PDGFR kinase inhibitor, tyrphostin AG 1296, was used to demonstrate that the activation of c-Src and p42/p44 MAPK was, as expected, dependent upon PDGFR kinase activity. Tyrphostin AG 1296 abolished PDGFR phosphorylation (Figure

2b) and c-Src (Figure 3b) and p42/p44 MAPK activation (Figures 4a and 4b).

Role of PI3K in regulating p42/p44 MAPK activation

Several authors have reported that PI3K inhibitors abrogate p42/p44 MAPK activation in response to growth-factor tyrosine kinase and G_i -coupled receptors [1,5–7]. We have shown here that PI3K inhibitors (LY294002, 50 μ M; wortmannin, 50 nM) markedly decreased the activation of p42/p44 MAPK by PDGF (Table 1, Figure 5).

We looked at intermediates in the p42/p44 MAPK cascade to identify at which level PI3K might act. We were able to demonstrate that PI3K activity was increased in Grb-2 immunoprecipitates from PDGF-stimulated cells. Interestingly, PI3K activity was decreased when these cells had been pretreated with either PTX (Figure 6a) or tyrphostin AG1296 (Figure 6b) (PI3K activity was not immunoprecipitated when antibody was omitted; results not shown). Figure 6(c) confirms that the Grb-2 antibody immunoprecipitated Grb-2 from cell lysates.

PTX was used to distinguish PI3K associated with Grb-2 from the PI3K present in anti-phosphotyrosine immunoprecipitates. Figure 7 shows the PI3K activity in anti-phosphotyrosine immunoprecipitates was stimulated up to 30-fold in

Figure 2 PDGFR tyrosine phosphorylation

Western blots showing PDGFR tyrosine phosphorylation in cells pretreated with and without (a) PTX ('p-tox') (0.1 μ g/ml) for 24 h, (b) tyrphostin AG1296 (20 μ M) for 15 min or (c) PP1 (10 μ M) for 15 min prior to stimulation with PDGF (10 ng/ml, 10 min). The phosphorylated PDGFR migrated with a molecular mass of 180 kDa (PDGFR was immunodepleted from detergent cell extract by anti-PDGFR antibody; results not shown). (a)–(c) are representative results of experiments performed on three separate cell preparations.

Figure 3 Effect of PTX and tyrphostin AG 1296 on c-Src activation

Autoradiographs show c-Src activation in cells pretreated with and without (a) PTX ('p-tox'; 0.1 μ g/ml) for 24 h or (b) tyrphostin AG 1296 (20 μ M) for 15 min prior to stimulation with PDGF (10 ng/ml). (a) PDGF for 0–30 min; (b) PDGF for 10 min. c-Src activity in anti-c-Src immunoprecipitates was measured with purified enolase and [γ - 32 P]ATP as substrates. [32 P]Enolase was then resolved on SDS/PAGE. Immunoprecipitates were also immunoblotted with anti-c-Src antibody to show that equal amounts of c-Src had been immunoprecipitated from each sample (results not shown). (a) and (b) are representative results of experiments performed on three to five separate cell preparations.

PDGF-treated cells. Activation reached a maximum at 5 min and then declined, although it remained sustained above basal even after 60 min. In contrast with Grb-2-associated PI3K,

Figure 4 Effect of tyrphostin AG1296 on p42/p44 MAPK activation

Cells with pretreated with and without various concentrations of tyrphostin AG1296 for 15 min prior to stimulation with PDGF (10 ng/ml, 10 min). (a) Shift blot showing p42 MAPK/ERK-2 phosphorylation. ERK-2 and activated ERK-2 are denoted by arrows. This is a representative result of an experiment performed on five separate cell preparations. (b) p42/p44 MAPK activity was measured in cell lysates using a specific peptide assay kit. Results are expressed as the percentage inhibition of PDGF-stimulated p42/p44 MAPK activity (100%) and are means $\pm$ S.D. for $n = 4$ separate cell preparations. PDGF-stimulated p42/p44 MAPK activity was calculated by subtracting the corresponding basal activity.

Figure 5 Effect of LY294002 on p42 MAPK phosphorylation

Shift blot showing p42 MAPK/ERK-2 phosphorylation in cells pretreated with and without LY294002 (50 μ M) for 15 min prior to stimulation with PDGF (10 ng/ml, 10 min). This is a representative result of an experiment performed on three separate cell preparations.

the PI3K activity in anti-phosphotyrosine immunoprecipitates was not affected by pretreating cells with PTX (Figure 7).

DISCUSSION

Role of c-Src

We have reported the surprising finding that PTX induced an approx. 40–50 % decrease in the activation of c-Src and p42/p44 MAPK by PDGF. This suggests that the PDGFR can utilize the PTX-sensitive G-protein, G_i , to regulate c-Src. In addition to this, the PDGFR can also directly bind to, and activate, c-Src via a PTX-insensitive mechanism ([17]; the present study). Both

Figure 6 PDGF-dependent Grb-2 associated PI3K activation

(a) Autoradiograph showing PI3K activity in Grb-2 immunoprecipitates from cells pretreated with and without PTX ('p-tox'; 0.1 μ g/ml) for 24 h prior to stimulation with PDGF (10 ng/ml) for 10 min or (b) Autoradiograph showing PI3K activity in Grb-2 immunoprecipitates from cells pretreated with or without tyrphostin AG1296 (20 μ M) for 15 min prior to stimulation with PDGF (10 ng/ml, 10 min). (c) Immunoblot showing the presence of Grb-2 in Grb-2 immunoprecipitates from cells treated with and without PDGF (10 ng/ml, 10 min). Immunoprecipitate proteins were resolved on SDS/PAGE, transferred to nitrocellulose and probed with anti-Grb-2 antibody. (a)–(c) are representative results of experiments performed on five separate cell preparations.

routes of regulation appear to have a mandatory requirement for the activated PDGFR kinase. On the basis of the findings presented here and elsewhere, we suggest that the PDGFR may promote the generation of free $G_i\beta\gamma$ subunits that directly stimulate c-Src. Alternatively, $G_i\beta\gamma$ subunits may act as adaptor proteins, locating c-Src close to the PDGFR kinase and/or intermediate activating proteins, e.g. other non-receptor tyrosine kinases. The adaptor model bears certain similarities with the mechanism employed by G-protein-coupled receptors to recruit GRKs to $\beta\gamma$ subunits [18]. A model for the adaptor hypothesis is proposed in Scheme 1(a).

The fact that PDGF elicits a larger c-Src and p42/p44 MAPK activation in the absence of PTX suggests that G_i enables PDGFR to produce more efficient stimulation of these kinases. The reason why the PDGFR activates c-Src via two distinct routes [one involving direct binding to the PDGFR (PTX-insensitive) and one involving G_i (PTX-sensitive)] is not known. One possibility is that G_i might increase the amount of c-Src that can otherwise be activated by direct binding to the PDGFR. This might represent a mechanism for producing stronger activation of the p42/p44 MAPK cascade by PDGF.

The mechanism promoting the generation of free $G_i\beta\gamma$ subunits is not known. One possibility might involve the phosphorylation

Figure 7 Tyrosine phosphorylated PI3K

The upper panel shows an autoradiograph showing PI3K activity in anti-phosphotyrosine immunoprecipitates from cells pretreated with and without PTX ('p-tox'; 0.1 μ g/ml) for 24 h prior to stimulation with PDGF (10 ng/ml, 10 min). The lower panel shows the time course of PDGF-stimulated PI3K (32 P]PI3P reaction product measured in anti-phosphotyrosine immunoprecipitates (10 ng/ml, 0–60 min). ○, Control; ●, PTX-treated cells. Each is a single experiment, typical of three.

of G_i by the PDGFR kinase, although there is no evidence to support this. Others have shown that peptides derived from another growth-factor receptor, insulin-receptor β subunit, activate G_i in lipid vesicles [19], suggesting direct protein–protein interaction. Direct interaction between growth-factor receptors and G-proteins has been demonstrated [20–22].

An essential role for c-Src was confirmed here with the c-Src inhibitor, PP1, which abolished p42/p44 MAPK activation. Furthermore, the PTX-dependent decrease in c-Src and p42/p44 MAPK activation appear correlated. Others have also shown that c-Src has a mandatory role in regulating PDGFR signalling in fibroblasts. In these studies, mutant α PDGFRs, impaired in their ability to signal to c-Src (c-Src binding Tyr⁵⁷² and Tyr⁵⁷⁴ had been mutated to phenylalanine) failed to stimulate Shc phosphorylation, which is upstream of p42/p44 MAPK [17].

The involvement of G_i in regulating growth-factor-receptor signalling is supported by studies from Luttrell and colleagues [8]. These authors showed that PTX and an inhibitor of $G_i\beta\gamma$ -mediated signalling (C-terminal domain of β -adrenergic receptor kinase) blocked the activation of p42/p44 MAPK by insulin and insulin-like-growth-factor-1 receptors in rat 1A fibroblasts. Interestingly, in transfected rat 1A cells overexpressing the human insulin receptor at high density, insulin treatment stimulated a more robust and sustained activation of p42/p44 MAPK that was PTX-insensitive. The authors proposed that insulin receptors expressed at high density might utilize an additional $G_i\beta\gamma$ -independent pathway. Alternatively, $G_i\beta\gamma$ may be required for efficient signal transduction at low endogenous levels of insulin-receptor expression, but that at high receptor density the requirement for $G_i\beta\gamma$ is surmounted.

Scheme 1 Model describing the possible role of G_i in the regulation of the p42/p44 MAPK cascade by the PDGFR: proposed mechanism of c-Src activation (a) and PI3K activation (b)

The model in (a) shows the dependence upon PDGFR kinase activity, G-protein interaction with growth-factor receptors, recruitment of c-Src by $G_i/\beta\gamma$ adaptor proteins and modulation of intermediate kinases by the PDGFR kinase. Alternatively the PDGFR kinase might regulate c-Src without the requirement of an intermediate. The model also shows that the PDGFR can bind c-Src via tyrosine sites phosphorylated by the PDGFR kinase. Activated c-Src subsequently regulates SHC and the p42/p44 MAPK cascade. (b) shows that Grb-2/PI3K complex formation is regulated by G_i and that Grb-2 is a possible site for integration of PI3K into the cascade.

Role of PI3K

An essential role for PI3K was confirmed here by experiments which showed that two structurally dissimilar PI3K inhibitors decreased p42/p44 MAPK activation. We have provided support for these findings by showing that PI3K associates with Grb-2, an intermediate in the p42/p44 MAPK cascade, in response to PDGF. This suggests that Grb-2 might be a site at which PI3K is integrated into the p42/p44 MAPK cascade. Furthermore, G_i appears to have an essential role in promoting Grb-2-PI3K complex formation (Scheme 1b). Others have reported that certain PI3K isoforms (e.g. PI3K γ) are directly activated by $G_i/\beta\gamma$ [5], and this might be an important step regulating Grb-2-PI3K association. Further characterization is required to establish whether the interaction occurs through Grb-2 SH2 or SH3 domains, phosphotyrosine or PH domains in PI3K, or through other proteins in a complex.

Grb-2-associated PI3K was distinguished from PI3K activity present in anti-phosphotyrosine immunoprecipitates because the latter, although stimulated up to 30-fold in PDGF-treated cells, was insensitive to PTX. There is good evidence that the tyrosine-

phosphorylated PI3K isoform (found in anti-phosphotyrosine immunoprecipitates), associated with the PDGFR, is linked with regulation of the S6 ribosomal kinase p70^{S6K} [5]. In contrast, we suggest that the Grb-2 associated PI3K is probably a distinct isoform that appears to be involved in the regulation of the p42/p44 MAPK cascade.

Conclusion

The regulation of the p42/p44 MAPK cascade by certain growth factors (PDGF, insulin-like growth factor-1 and insulin) appears to involve both G_i -dependent and -independent pathways. The G_i -dependent route appears to provide a mechanism for stronger activation of this signal-transduction cascade by PDGF.

Understanding how these mitogenic signals interact is of vital importance to the development of novel therapeutic strategies designed to reverse proliferative smooth-muscle disease states, such as chronic obstructive airway disease, pulmonary hypertension and restenosis.

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