β PDGF receptor mutants defective for mitogenesis promote neurite outgrowth in PC12 cells

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Background: Platelet-derived growth factor (PDGF) promotes mitogenesis in fibroblast cell lines but stimulates neurite outgrowth in PC12 cells that ectopically express the β PDGF receptor. To determine which substrates must associate with this receptor protein-tyrosine kinase in order to promote neurite outgrowth, we introduced into PC12 pheochromocytoma cells three mutant forms of the β PDGF receptor that no longer associate with specific substrate proteins. We then assayed the ability of these receptor mutants to affect neurite extension.

Results: Receptors lacking the kinase-insert domain did not associate with either phosphatidylinositol 3-kinase (PI 3-kinase) or Ras GTPase-activating protein (Ras–GAP) in PC12 cells. A carboxy-terminal truncation of the β PDGF receptor eliminated the association of phospholipase C-γ1 (PLC-γ1) with the receptor and prevented phosphorylation of PLC-γ1 in PC12 cells. Finally, β PDGF receptors that have tyrosine-to-phenylalanine point mutations at positions 708, 719, 977 and 989 did not associate with either PI 3-kinase or PLC-γ1. All three mutant forms of the β PDGF receptor promoted PDGF-dependent neurite outgrowth in PC12 cells and elicited activation of mitogen-activated protein (MAP) kinases.

Conclusions: PC12 cells expressing the β PDGF receptor extend neurites in response to PDGF in the absence of signalling through PI 3-kinase, RasGAP, and PLC-γ1. This contrasts with the requirements for mitogenesis for epithelial and fibroblast cell lines, in which the association of PI 3-kinase with the β PDGF receptor is essential. This receptor protein-tyrosine kinase therefore phosphorylates and activates a similar set of intracellular signalling molecules in the context of both mitogenesis and differentiation, but the importance of particular pathways for each phenotypic response is distinct.

Current Biology 1995, **5**:168–178

Background

Many peptide growth factors exert their effects on a target cell by binding to and activating receptors that have intrinsic protein-tyrosine kinase activity [1]. Some receptor protein-tyrosine kinases promote proliferation and some support differentiation and survival, depending both on the type of receptor and the cell type that expresses it. Despite this diversity of cellular responses, most receptor tyrosine kinases have been shown to interact with and phosphorylate similar cytoplasmic signalling molecules and to activate similar downstream signalling pathways in response to binding ligand [2]. Several cytoplasmic signalling molecules have been shown to be critical mediators of the proliferative response following receptor tyrosine kinase activation, but much less is understood about how differentiation is regulated.

The PC12 cell line, derived from a rat pheochromocytoma, has served as a model system for investigating how certain growth factors regulate neuronal differentiation. Within several days of treatment with nerve growth factor (NGF) or fibroblast growth factor (FGF), the cells differentiate and acquire features characteristic of sympathetic neurons, such as the ability to extend neurites [3,4]. NGF binds to Trk, a member of the receptor tyrosine kinase family, and Trk is likely to mediate most of the biological responses to NGF [5–7]. Upon ligand binding, Trk associates with a number of intracellular signalling molecules, including phospholipase C-γ1 (PLC-γ1), phosphatidyl inositol 3-kinase (PI 3-kinase), the Erk1 kinase, and the adaptor protein Shc [8–10]. It has recently been shown that Trk's ability to regulate neurite outgrowth depends upon association of both Shc and PLC-γ1 with the receptor [11,12].

Expression in PC12 cells of the β subunit of the receptor for platelet-derived growth factor (PDGF) renders the cells able to respond to PDGF by differentiating and extending neurites [13]. A number of intracellular signalling proteins have been shown to interact with the PDGF receptor, and by deleting or mutating the phosphotyrosine residues on the PDGF receptor that mediate association with many of these signalling molecules, it has been possible to assess the significance of specific interactions for the ability of the receptor to promote cell proliferation in fibroblast and epithelial cell lines. We therefore reasoned that this receptor could provide a means for further exploring the signalling pathways that are required downstream of receptor tyrosine kinase activation in order to elicit PC12 cell differentiation.

The kinase domain of the PDGF receptor is interrupted by a stretch of approximately 100 amino acids, known as the kinase insert domain. Within this region, two phosphotyrosine residues at positions 708 and 719 mediate

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the binding of PI 3-kinase [14–18], and phosphotyrosine residue 719 also mediates interactions with the adaptor-like protein, Nck [19]. Phosphotyrosine residue 739 is required for association of the receptor with the Ras GTPase-activating protein (Ras–GAP) [16–18]. Deletion of 82 amino acids from the kinase insert region renders the PDGF receptor incapable of promoting a mitogenic response in most fibroblast and epithelial cell lines, even though kinase activity is retained [20]. Point mutations suggest that the integrity of the PI 3-kinase/Nck binding sites is most critical for PDGF-mediated mitogenicity [16,17].

Two phosphotyrosines, at positions 977 and 989, in the carboxy-terminal tail of the PDGF receptor mediate association with the protein tyrosine phosphatase Syp and PLC-γ1, respectively [21–24]. Mutation of tyrosine 989 or both tyrosines 977 and 989 of the receptor results in it eliciting a normal or slightly reduced mitogenic response to PDGF stimulation in most fibroblast and epithelial cell lines [22–24]. Two phosphotyrosines in the juxtamembrane region of the receptor, at positions 557 and 559, seem to mediate association with three members of the Src family of non-receptor tyrosine kinases, Src, Yes and Fyn [25]. When both tyrosines corresponding to residues 557 and 559 in the human β PDGF receptor are mutated to phenylalanine, however, the kinase activity of the receptor is compromised, hindering assessment of the contribution of this association to mitogenesis. Injection of NIH 3T3 fibroblast cells with either dominant-negative mutant forms of Src and Fyn, or antibodies specific for Src, Yes and Fyn, inhibits PDGF-stimulated DNA synthesis, suggesting that these tyrosine kinases play important roles in mediating the mitogenic signal following binding of PDGF to its receptor [26].

Although receptor protein-tyrosine kinases interact with similar cytoplasmic signalling molecules when regulating both cell proliferation and differentiation, it is not yet clear whether the requirements for both processes are the same or distinct. We have therefore expressed several mutant forms of the PDGF receptor in PC12 cells, in an attempt to determine whether the association of certain cytoplasmic signalling molecules with a receptor tyrosine kinase is required to promote a differentiation response, such as neurite outgrowth. We show that regulation of neurite extension in PC12 cells by the PDGF receptor does not depend upon the receptor's association with PI 3-kinase, Ras–GAP or PLC-γ1. These results suggests that the PDGF receptor uses distinct signalling pathways to promote two different phenotypic responses, namely mitogenesis and differentiation. The role of Syp in regulating neurite outgrowth is still in question, as elimination of the reported binding site for Syp did not completely disrupt its association with the PDGF receptor.

Results

Expression of PDGF receptor mutants in PC12 cells

To assess the importance of specific signalling molecules in a differentiation response, we expressed in PC12 cells

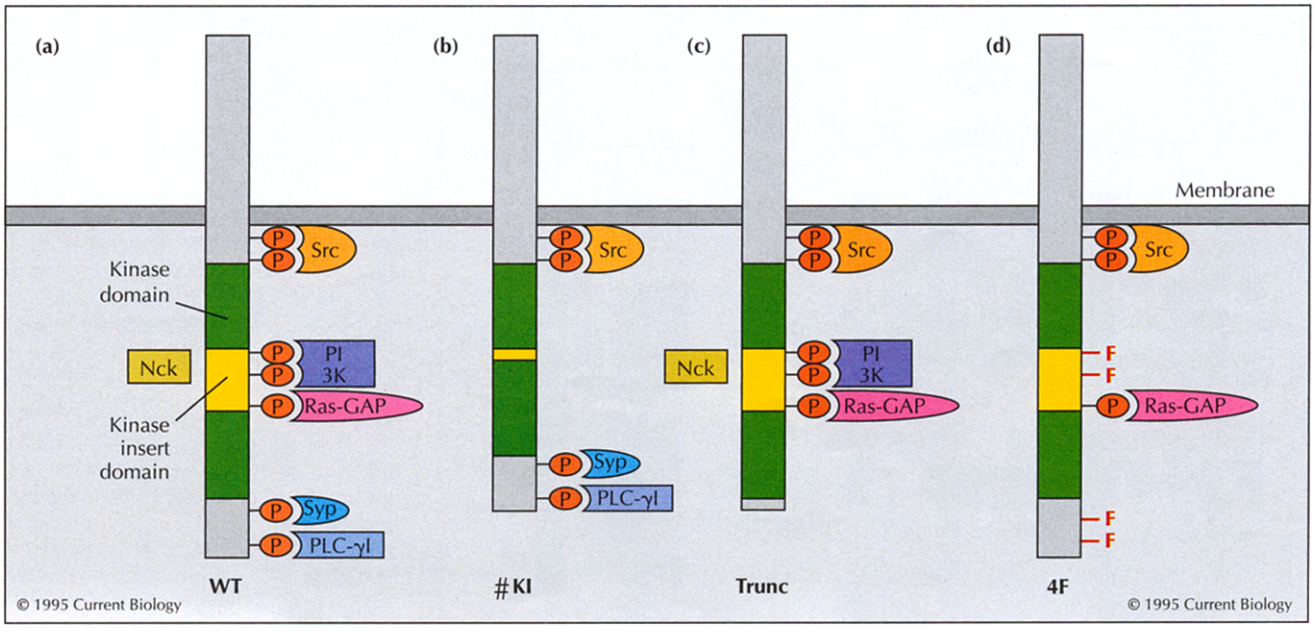

Fig. 1. Proteins that associate with wild-type and mutant forms of the PDGF β receptor. **(a)** Wild-type PDGF β receptor (WT) associates with Src family tyrosine kinases, PI 3-kinase (PI 3K), Nck, Ras–GAP, phospholipase C-γ1 (PLC-γ1), and the tyrosine phosphatase Syp [19,21,57,74–82]. **(b)** The ΔKI form of the PDGF β receptor has a deletion of residues 684–765 within the kinase insert region [20]. The ΔKI form lacks the binding sites for PI 3-kinase, Ras–GAP and Nck [16–19,80]. **(c)** The Trunc form of the PDGF β receptor has a truncation of the carboxyl terminus, caused by mutation of tyrosine 977 to a stop codon. The Trunc form lacks the reported binding sites for PLC-γ1 and Syp [21–24]. **(d)** The 4F PDGF β receptor has tyrosine-to-phenylalanine mutations at positions 708, 719, 977 and 989. These mutations alter the reported binding sites for PI 3-kinase, PLC-γ1, Syp and Nck [1–19,21–24,80].

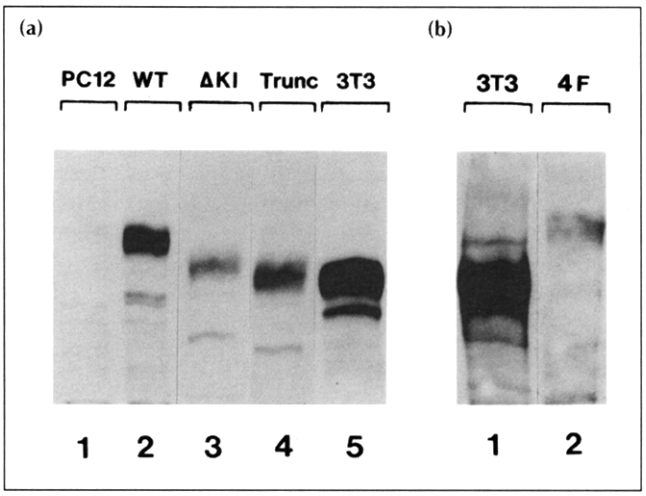

Fig. 2. Ectopic expression of PDGF receptor in PC12 cells. 50 μg of total protein were resolved on a 6 % SDS polyacrylamide gel. Following transfer to nitrocellulose, the blot was probed with an antibody that recognizes the mouse PDGF β receptor (Ab 65). **(a)** Cell lysates were prepared from untransfected PC12 cells (lane 1), PC12 cells transfected with cDNAs encoding either WT (lane 2), ΔKI (lane 3) or Trunc (lane 4) forms of the mouse PDGF β receptor, or from Balb/c 3T3 cells (lane 5). **(b)** Cell lysates were prepared from PC12 cells transfected with a cDNA encoding the 4F form of the PDGF receptor (lane 1). Lysate from Balb/c 3T3 cells (lane 2) is included as a reference.

either the full-length mouse PDGF receptor β subunit (WT in Fig. 1a), or one of three mutant versions of the receptor described in Figure 1. PC12 cells do not normally express the PDGF receptor (Fig. 2a, lane 1). PC12 cell clones expressing either the WT, ΔKI, Trunc or 4F forms of the mouse PDGF receptor β subunit were generated and their expression assessed by western immunoblotting (Fig. 2a, lanes 2–4, Fig. 2b, lane 2, one example of each shown). The electrophoretic mobility of the full-length PDGF receptor on SDS polyacrylamide gels was slower in lysates of PC12 cells (Fig. 2a, lane 2) than in lysates of mouse Balb/c 3T3 cells, in which the receptor is endogenously expressed (Fig. 2a, lane 5). This may be attributed to glycosylation differences between the two cell types, as the immature receptors (lower band in Fig. 2a, lanes 2 and 5) in each cell type were more similar to each other in electrophoretic mobility. The ΔKI and Trunc forms of the receptor both migrated on SDS gels more quickly than the full length PDGF receptor (WT), as expected. Figure 2b shows expression of the 4F form of the PDGF receptor in PC12 cells (lane 2) relative to expression of the PDGF receptor in Balb/c 3T3 cells (lane 1). When normalized to the levels of expression in Balb/c 3T3 cells, the 4F form of the PDGF receptor seems to be expressed at lower levels than the other forms of the PDGF receptor.

To determine whether all four forms of the PDGF receptor were catalytically active when expressed in PC12 cells, we assayed for PDGF-stimulated changes in protein-tyrosine phosphorylation by western immunoblotting using an antibody (4G10) that is specific for phosphorylated tyrosine residues. Untransfected PC12 cells stimulated with NGF for 5 minutes showed typical changes in total cellular phosphotyrosine (Fig. 3a, lanes 1–2), but showed no changes in total cellular phosphotyrosine when stimulated with PDGF (Fig. 3a, lane 3). PC12 cells expressing all four versions of the β PDGF receptor responded to PDGF stimulation with an increase

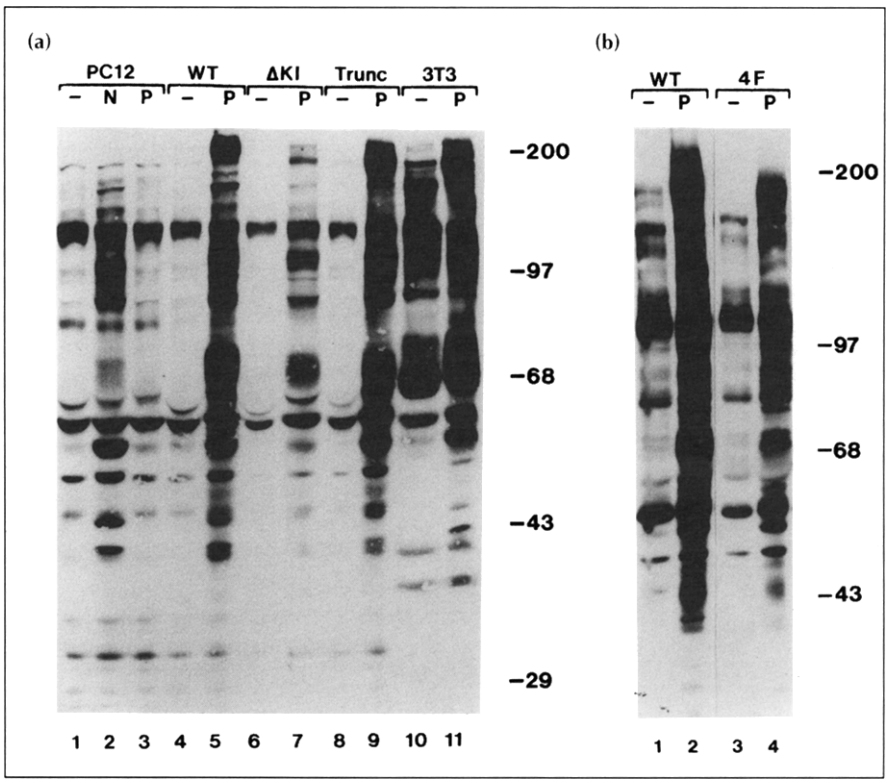

Fig. 3. PDGF induces rapid changes in protein-tyrosine phosphorylation in PC12 cells expressing each form of the PDGF receptor. Before lysis, cells were either left unstimulated (–) or stimulated with 50 ng ml^{-1} NGF (N) or 40 ng ml^{-1} PDGF BB (P) for 5 minutes at 37 °C. 50 μg of each cell lysate was resolved on a 7 % SDS polyacrylamide gel, transferred to nitrocellulose and the blot probed with 4G10, an anti-phosphotyrosine antibody.**(a)** Cell lysates were prepared from untransfected PC12 cells (lanes 1–3); PC12 cells expressing WT (lanes 4–5), ΔKI (lanes 6–7) or Trunc (lanes 8–9) forms of the PDGF receptor; and from Balb/c 3T3 cells (lanes 10–11). **(b)** Cell lysates were prepared from PC12 cells expressing the 4F form of the PDGF receptor (lanes 3–4). Lysates from PC12 cells expressing WT PDGF receptor (lanes 1–2) are included for reference.

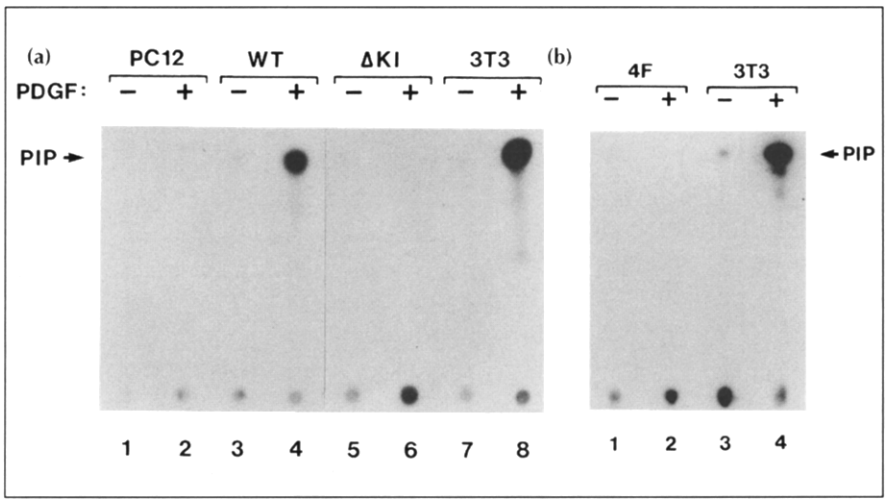

Fig. 4. PDGF receptor association with PI 3-kinase activity in PC12 cells is disrupted by deletion of the receptor's kinase insert region. Cells were either left unstimulated (–) or stimulated with 40 ng ml^{-1} PDGF BB (+) for 5 minutes at 37 °C before lysis. The PDGF receptor was immunoprecipitated and a PI 3-kinase assay performed on the immunoprecipitates. The products of the reaction were resolved from bottom to top on a silica gel plate by thin layer chromatography and the plate then exposed to Xomat AR film (Kodak). **(a)** Lysates were prepared from untransfected PC12 cells (lanes 1–2), PC12 cells expressing WT (lanes 3–4) or ΔKI (lanes 5–6) forms of the PDGF receptor, or Balb/c 3T3 cells (lanes 7–8). **(b)** Lysates were prepared from PC12 cells expressing the 4F PDGF receptor (lanes 1–2) or from Balb/c 3T3 cells (lanes 3–4).

in the number of tyrosine phosphorylated proteins (Fig. 3a, lanes 4–9; Fig. 3b, lanes 3–4)

Following NGF stimulation, all PC12 cell clones showed identical patterns of protein-tyrosine phosphorylation, demonstrating that Trk activation was normal in all the clones (data not shown). The pattern of tyrosine-phosphorylated proteins following PDGF stimulation of receptor-expressing PC12 cells (Fig. 3a, lanes 5, 7, 9; Fig. 3b, lane 4) was comparable, but not identical, to that seen with NGF stimulation (Fig. 3a, lane 2). In particular, a tyrosine-phosphorylated protein migrating in the size range of the autophosphorylated PDGF receptor (approximately 180 kD) was quite prominent following PDGF stimulation (Fig. 3a, lanes 5, 7, 9; Fig. 3b, lane 4). In addition, there were some subtle differences in the patterns of tyrosine-phosphorylated proteins following PDGF stimulation of PC12 cells expressing the different forms of the PDGF receptor. These differences may be attributable to loss of binding sites for specific substrate molecules on the mutated forms of the PDGF receptor.

Association of the PDGF receptor with PI 3-kinase and Ras–GAP in PC12 cells

To confirm that the binding sites for PI 3-kinase on the PDGF receptor had been lost by deleting the kinase insert region or mutating specific tyrosine residues within this region, we performed a lipid kinase assay on PDGF receptor immunoprecipitates from the various cell lines. PI 3-kinase activity was detected in PDGF receptor immunoprecipitates from Balb/c 3T3 cells following PDGF stimulation (Fig. 4a, lanes 7–8; Fig. 4b, lanes 3–4), but not from untransfected PC12 cells (Fig. 4a, lanes 1–2). PI 3-kinase activity co-precipitated in a PDGF-dependent manner with the PDGF receptor from lysates of PC12 cells expressing the full length WT PDGF receptor (Fig. 4a, lanes 3–4), however, and this co-precipitation was disrupted in PC12 cells expressing either the ΔKI version of the receptor (Fig. 4a, lanes 5–6) or the 4F form of the receptor (Fig. 4b, lanes 1–2). Similar results were obtained when the lipid kinase assay was performed following immunoprecipitation with an anti-phosphotyrosine antibody (data not shown).

To demonstrate that the β PDGF receptor also associated with Ras–GAP in PC12 cells using sequences contained within the kinase insert region, we immunoprecipitated Ras–GAP from each of the cell lines and performed a western blot using the 4G10 anti-phosphotyrosine antibody. Ras–GAP was not phosphorylated in response to PDGF stimulation of untransfected PC12 cells (Fig. 5, lanes 1–2). Following PDGF treatment of Balb/c 3T3 cells (Fig. 5, lanes 7–8), or PC12 cells expressing the full length (WT) PDGF receptor (Fig. 5, lanes 3–4),

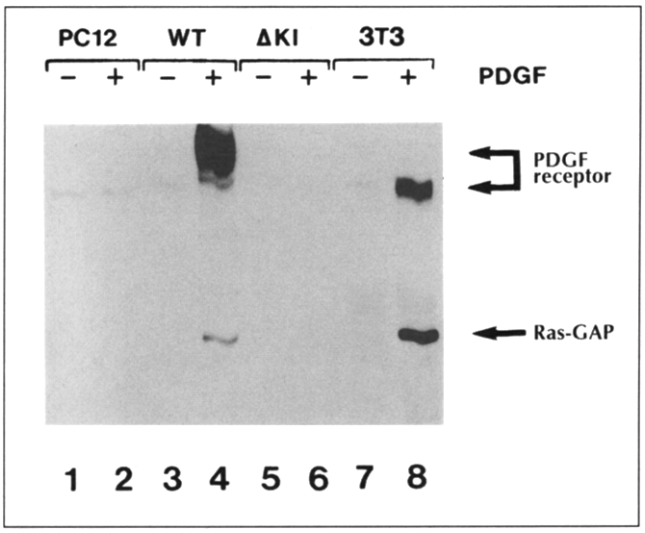

Fig. 5. Ras–GAP association with the PDGF receptor in PC12 cells is disrupted by deletion of the kinase insert region. Untransfected PC12 cells (lanes 1–2), PC12 cells expressing WT (lanes 3–4) or ΔKI (lanes 5, 6) PDGF receptor, or Balb/c 3T3 cells (lanes 7–8) were either left unstimulated (–) or stimulated with 40 ng ml^{-1} PDGF BB (+) for 5 minutes at 37 °C before lysis. Ras–GAP was immunoprecipitated and the immunoprecipitated proteins resolved on a 6 % SDS polyacrylamide gel, transferred to nitrocellulose and probed with an anti-phosphotyrosine antibody (4G10).

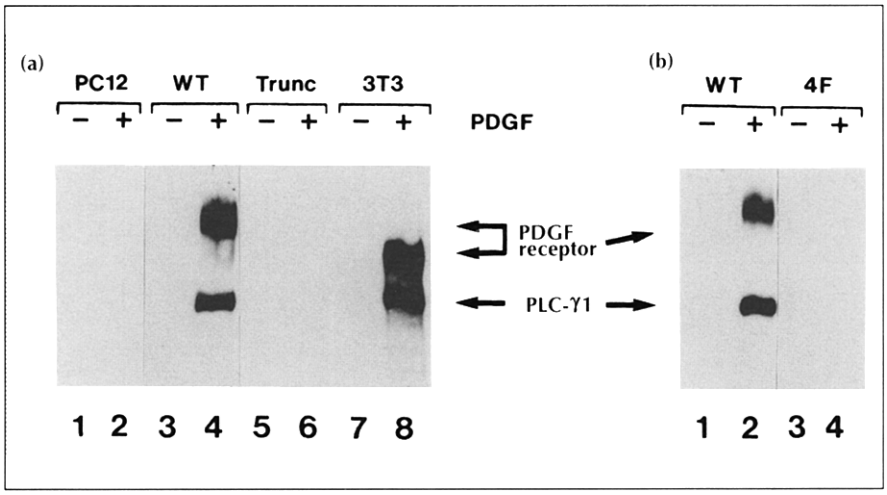

Fig. 6. PLC-γ1 association with the PDGF receptor in PC12 cells is disrupted by deletion of the receptor's carboxy-terminal tail. PLC-γ1 was immunoprecipitated from cell lysates and the immunoprecipitated proteins resolved on a 6 % SDS polyacrylamide gel, transferred to nitrocellulose and probed with an anti-phosphotyrosine antibody (4G10). **(a)** Untransfected PC12 cells (lanes 1–2), PC12 cells expressing WT (lanes 3–4) or Trunc (lanes 5–6) PDGF receptor, or Balb/c 3T3 cells (lanes 7–8) were either left unstimulated (–) or stimulated with 40 ng ml^{-1} PDGF BB (+) for 5 minutes at 37 °C before lysis. **(b)** PC12 cells expressing the 4F PDGF receptor (lanes 3–4) were either left unstimulated (–) or stimulated with 40 ng ml^{-1} PDGF BB (+), and then lysed. Lanes of lysates from unstimulated or PDGF-stimulated PC12 cells expressing the WT PDGF receptor (lanes 1–2) are included for reference.

Ras–GAP became phosphorylated on tyrosine and associated with tyrosine-phosphorylated PDGF receptor. This association was disrupted in PC12 cells expressing the ΔKI version of the PDGF receptor (Fig. 5, lanes 5–6).

Association of the PDGF receptor with PLC-γ1 and Syp

To confirm that the binding sites for PLC-γ1 lie within the carboxy-terminal region of the β PDGF receptor and are lost by truncation, we immunoprecipitated PLC-γ1 and performed a western blot using the 4G10 anti-phosphotyrosine antibody. PDGF stimulation of untransfected PC12 cells did not promote phosphorylation of PLC-γ1 on tyrosine (Fig. 6a, lanes 1–2). Following PDGF stimulation of Balb/c 3T3 cells (Fig. 6a, lanes 7–8) or PC12 cells expressing the full-length (WT) PDGF receptor (Fig. 6a, lanes 3–4; Fig. 6b, lanes 1–2), however, PLC-γ1 was phosphorylated on tyrosine and co-precipitated with tyrosine-phosphorylated PDGF receptor. This association was disrupted in PC12 cells expressing either the Trunc (Fig. 6a, lanes 5–6) or the 4F PDGF receptor (Fig. 6B, lanes 3–4).

Syp also became phosphorylated and co-precipitated with tyrosine-phosphorylated PDGF receptor following PDGF stimulation of Balb/c 3T3 cells (Fig. 7a, lanes 8–9) and PC12 cells expressing the full-length WT PDGF receptor (Fig. 7a, lanes 4–5). This association between Syp and the PDGF receptor was not observed in untransfected PC12 cells stimulated with either PDGF or NGF (Fig. 7a, lanes 1–3). Syp was, however, also phosphorylated on tyrosine in response to PDGF in cells expressing either the Trunc (Fig. 7a, lanes 6–7) or the 4F PDGF receptor (Fig. 7b, lanes 3–4). The expression of the 4F PDGF receptor was lower than that of the WT receptor, perhaps contributing to the weaker observed Syp phosphorylation in response to PDGF in these cells. When the western blot was stripped and re-probed with an antibody specific for Syp, equal amounts of Syp were detected in all PC12 lanes, and the tyrosine-phosphorylated forms of the Syp protein were shifted in mobility (data not shown). Although the reported binding site for Syp, the tyrosine at residue 977, had been eliminated in

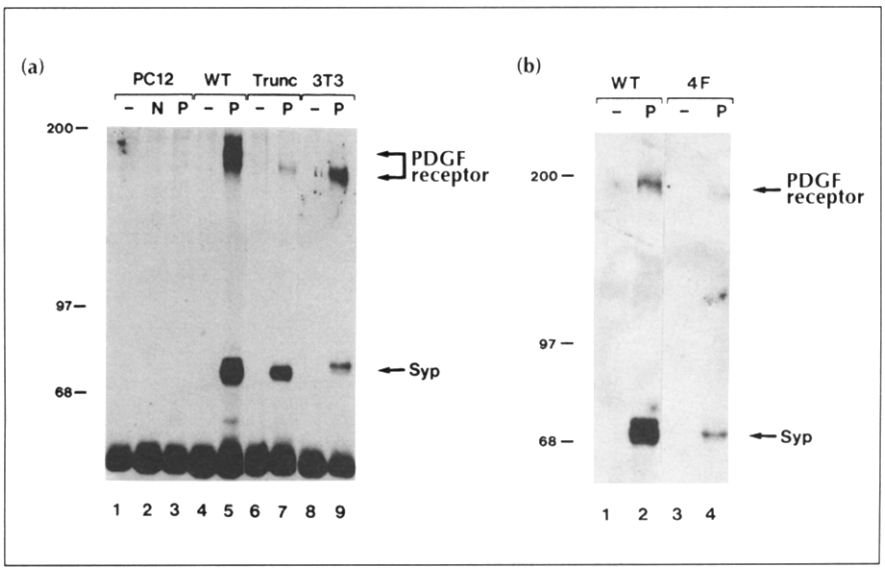

Fig. 7. Syp association with the PDGF receptor in PC12 cells is not disrupted by deletion of the receptor's carboxy-terminal tail. Syp was immunoprecipitated from cell lysates and the immunoprecipitated proteins resolved on a 6 % SDS polyacrylamide gel, transferred to nitrocellulose and probed with an anti-phosphotyrosine antibody (4G10). **(a)** Untransfected PC12 cells (lanes 1–3), PC12 cells expressing WT (lanes 4–5) or Trunc (lanes 6–7) PDGF receptor, or Balb/c 3T3 cells (lanes 8–9) were either left unstimulated (–) or stimulated with 50 ng ml^{-1} NGF (N) or 40 ng ml^{-1} PDGF BB (P) for 5 minutes at 37 °C before lysis. **(b)** PC12 cells expressing the 4F PDGF receptor (lanes 3–4) were either left unstimulated (–) or stimulated with 40 ng ml^{-1} PDGF BB (P). Lanes of lysates from unstimulated or PDGF-stimulated PC12 cells expressing the WT PDGF receptor (lanes 1–2) are included for reference.

both the Trunc and the 4F forms of the receptor, small amounts of tyrosine-phosphorylated receptor were still found to co-precipitate with Syp in each case (Fig. 7a, lanes 6–7; Fig. 7b, lanes 3–4), with the Trunc receptor migrating slightly faster than the full-length WT. These results suggest that, in PC12 cells, Syp may interact with alternative sites on the PDGF receptor when its primary binding sites are eliminated.

All forms of the PDGF receptor activate MAP kinase in PC12 cells stimulated with PDGF

MAP kinase activation is likely to be an important step in the signal transduction cascade leading to NGF-dependent differentiation of PC12 cells [27]. We wanted to determine whether PC12 cells expressing any forms of the PDGF receptor have lost the ability to activate the pp42/pp44 MAP kinases following PDGF stimulation. When an in-gel myelin basic protein (MBP) kinase assay was performed, we observed that stimulation of untransfected PC12 cells with NGF, but not with PDGF, caused pp42/pp44 MAP kinase activation (Fig. 8a, lanes 1–3). MAP kinase activation in response to PDGF was observed in Balb/c 3T3 cells (Fig. 8a, lanes 10–11) and in PC12 cells expressing the full-length (WT) PDGF receptor (Fig. 8a, lanes 4–5, Fig. 8b, lanes 3–4). MAP kinase activation was also apparent following PDGF stimulation of PC12 cells expressing both the ΔKI and the Trunc forms of the receptor (Fig. 8a, lanes 6–9). These results were confirmed by immune-complex kinase assays using soluble MBP as a substrate (M. McMahon, personal communication). In addition, PC12 cells expressing the 4F PDGF receptor responded to PDGF with activation of pp42/pp44 MAP kinases (Fig. 8b, lanes 1–2).

All forms of the PDGF receptor promote neurite outgrowth

Untransfected PC12 cells differentiated and extended neurites following treatment with NGF, but responded to neither PDGF BB nor PDGF AA (Fig. 9a–c; and data not shown). PC12 cells expressing the full-length β PDGF receptor (WT) responded to PDGF BB treatment by initiating a differentiation response and elaborating neurites (Fig. 9d–e) [13]. The neurite outgrowth response was maximal 3 days after addition of PDGF, but was not maintained, perhaps as a result of down-regulation of the PDGF receptor. PC12 cells expressing the ΔKI receptor extended neurites in response to PDGF (Fig. 9f–g), as did cells expressing the Trunc receptor (Fig. 9h–i) or the 4F receptor (Fig. 9j–k). But PC12 cells expressing the 4F receptor showed a reduced fraction of cells with neurites compared with the other clones. All clones had detectable neurite outgrowth with 0.5–1 ng ml^{-1} PDGF BB in the culture medium, with maximal responses obtained at 2.5–5 ng ml^{-1} PDGF (data not shown).

To determine whether the PDGF receptor might promote PC12 cell differentiation indirectly by activating Trk, we tested the ability of PDGF to promote neurite outgrowth in the presence of K252a. This alkaloid blocks NGF-induced PC12 cell differentiation by inhibition of Trk tyrosine kinase activity, but does not inhibit differentiation in response to FGF [28–30]. Neurite extension in response to PDGF was not blocked by K252a at concentrations that completely blocked Trk activation and NGF-dependent differentiation (data not shown). In fact, in the presence of K252a PDGF-dependent neurite outgrowth in PC12 cells was enhanced and stabilized, with the neurites maintained for several weeks.

Many neurotrophic factors act not only as differentiation factors but also as survival factors. In PC12 cells, NGF not only promotes differentiation and neurite outgrowth but also supports cell survival in serum-free medium [31]. Although activation of the PDGF receptor was able to promote neurite outgrowth and differentiation, PDGF was not able to support PC12 cell survival in the absence of serum (data not shown).

Another hallmark of NGF-regulated PC12 cell differentiation is the enhanced expression pf the neuronal growth-associated protein, GAP-43. We observed an increase in GAP-43 expression in response to NGF but not PDGF in PC12 cells expressing the the WT PDGF receptor (data not shown). This suggests that the PDGF receptor may act primarily to regulate neurite outgrowth and not other aspects of neuronal differentiation in PC12 cells.

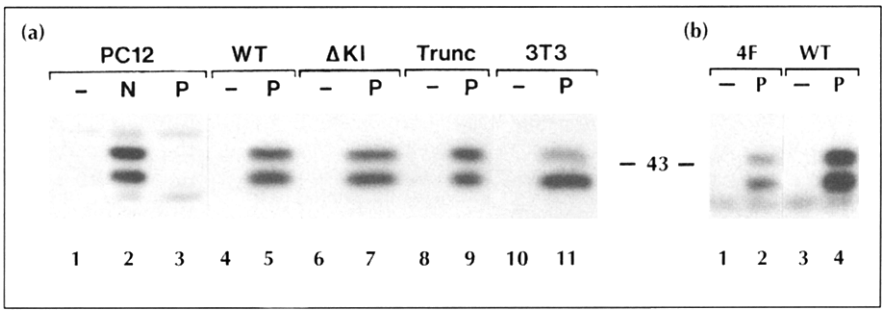

Fig. 8. PDGF receptor-mediated activation of pp42/pp44 MAP kinases in PC12 cells does not depend upon sequences in the kinase insert region or the carboxy-terminal tail. 50 μg of protein from each cell lysate was resolved on a 12 % SDS polyacrylamide gel containing 0.5 mg ml^{-1} myelin basic protein, and an in-gel myelin basic protein kinase assay was performed. The gel was then dried and exposed to Xomat AR film. **(a)** Lysates were prepared from untransfected PC12 cells (lanes 1–3); PC12 cells expressing WT (lanes 4–5), ΔKI (lanes 6–7) or Trunc (lanes 8–9) forms of the PDGF receptor; and from Balb/c 3T3 cells (lanes 10–11). **(b)** Lysates were prepared from PC12 cells expressing the 4F PDGF receptor (lanes 1–2) or from Balb/c 3T3 cells (lanes 3–4), for reference. Before lysis, cells were either left unstimulated (–) or stimulated with 50 ng ml^{-1} NGF (N) or 40 ng ml^{-1} PDGF BB (P) for 5 minutes at 37 °C.

Discussion and conclusions

Receptor protein-tyrosine kinases become activated in response to extracellular signals that regulate either cell proliferation or the initiation and maintenance of a differentiated phenotype. In both situations, similar intracellular signalling pathways seem to be activated. This raises the question of whether the signal transduction pathways involved in regulating mitogenesis are also important in controlling differentiation.

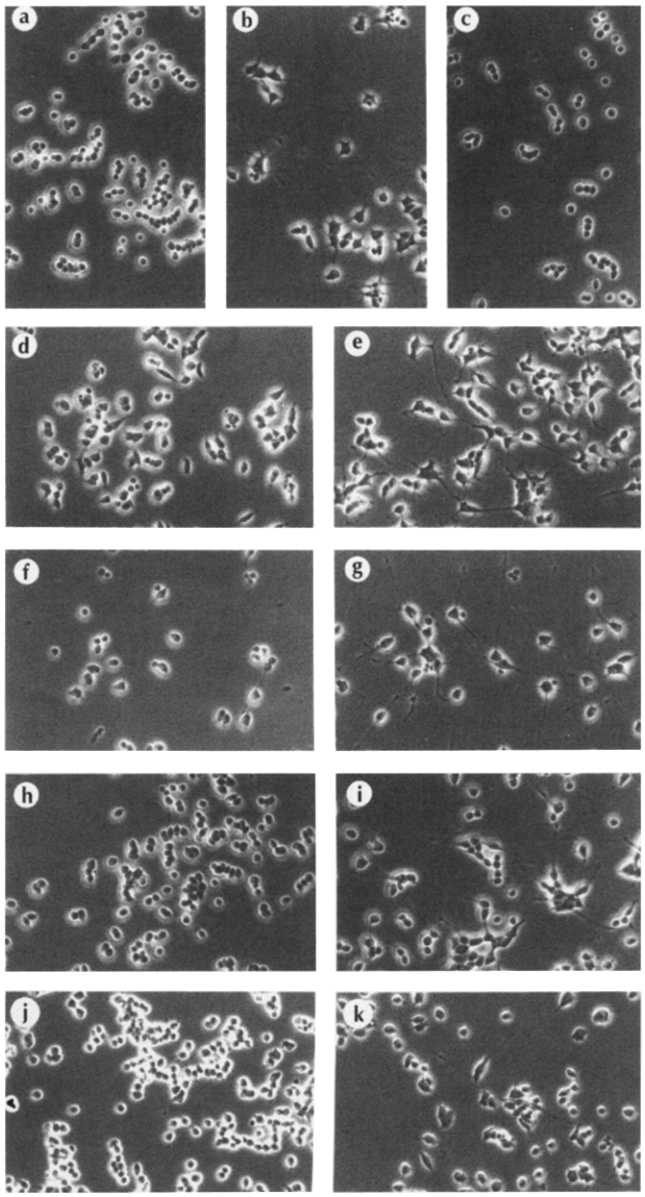

Fig. 9. PDGF promotes neurite outgrowth of PC12 cells expressing WT, ΔKI and Trunc forms of the PDGF receptor. PC12 cells were plated onto collagen IV-coated plates and grown in the presence or absence of growth factor for 3 days. **(a)** Untreated cells. **(b)** Cells treated with 50 ng ml^{-1} NGF. **(c)** Cells treated with 40 ng ml^{-1} PDGF BB. **(d)** Untreated cells expressing the WT PDGF receptor. **(e)** Cells expressing the WT PDGF receptor, treated with 40 ng ml^{-1} PDGF BB. **(f)** Untreated cells expressing the ΔKI PDGF receptor. **(g)** Cells expressing the ΔKI PDGF receptor, treated with 40 ng ml^{-1} PDGF BB. **(h)** Untreated cells expressing the Trunc PDGF receptor. **(i)** Cells expressing the Trunc PDGF receptor, treated with 40 ng ml^{-1} PDGF BB. **(j)** Untreated cells expressing the 4F PDGF receptor. **(k)** Cells expressing the 4F PDGF receptor, treated with 40 ng ml^{-1} PDGF BB.

To investigate this issue, we have examined signalling by the PDGF receptor in PC12 cells. PDGF promotes neurite outgrowth when the PDGF receptor is expressed in PC12 cells, indicating that intracellular signalling pathways regulating neurite extension are activated by the PDGF receptor. PDGF-dependent neurites were maintained for less than a week, suggesting that the PDGF receptor might become down-regulated with prolonged exposure to PDGF.

The PDGF receptor in PC12 cells may activate a subset of the complete signalling cascade that is activated by endogenously expressed receptor tyrosine kinases, such as Trk and the FGF receptor, as the PDGF receptor promoted neurite outgrowth but did not promote PC12 cell survival in serum-free medium, nor did it induce GAP-43 expression. This would suggest that neurite outgrowth and these other manifestations of differentiation are functionally separable. Precedent exists for such functional segregation, as isoforms of the TrkC receptor promote neurite outgrowth but not survival in serum-free medium when expressed in PC12 cells [32].

The PDGF receptor promotes neurite outgrowth without signalling through PI 3-kinase, Ras–GAP or PLC-γ1

The neurite outgrowth response to PDGF did not depend upon sequences in the kinase insert region or the carboxy-terminal tail of the PDGF receptor, suggesting that association with the PDGF receptor of Ras-GAP, PI 3-kinase, or PLC-γ1 is not required for this differentiation response in PC12 cells. Association of Nck with the receptor may also be dispensable for neurite outgrowth, as Nck appears to share a binding site with PI 3-kinase [19]. It has recently been shown that the association of PI 3-kinase with Trk is not required for NGF-regulated PC12 cell differentiation, consistent with our observations using the PDGF receptor [12]. In addition, elimination of the PLC-γ1 binding site on Trk does not abolish neurite outgrowth in response to NGF, but does abrogate peripherin induction [33].

The fraction of cells bearing neurites following PDGF treatment was less for the cell lines expressing the 4F PDGF receptor than for the other PDGF receptor-expressing clones. The 4F receptor was expressed at lower levels than the other forms, perhaps accounting for the reduced fraction of cells bearing neurites. It is also possible that the effect of disrupting signalling through PLC-γ1 and PI 3-kinase in combination results in a reduced ability of these cells to extend neurites in response to PDGF.

We observed that association of both the Trunc and 4F forms of the PDGF receptor with the protein tyrosine phosphatase, Syp, was reduced but not eliminated, even though the reported binding site for Syp, tyrosine 977,

had been removed in these two receptor forms. It is possible that Syp binds to an alternative site in the PDGF receptor when its primary binding site is removed. Individual SH2 domains, such as that used by Syp to bind to the PDGF receptor, bind with high affinity to phosphotyrosine residues in the context of a specific peptide sequence, but may also interact with other phosphotyrosine-containing sequences with lower affinity [34]. Other mutants of the PDGF receptor may also retain some residual binding to associated proteins whose primary binding sites have been removed. Although not detectable in our assays, such residual associations might contribute functionally to signalling by the receptor. PC12 cells, in particular, may be more responsive to signalling by such residual associated proteins than other cell types.

MAP kinase activation by the PDGF receptor

We observed that the pp42/pp44 MAP kinases were activated in PC12 cells expressing the WT, ΔKI and Trunc forms of the PDGF receptor. In each case, therefore, some elements of the signal transduction cascade leading to activation of the MAP kinases must be intact. MAP kinase activation is central to the regulation of PC12 cell differentiation by NGF. If MAP kinase activation is blocked using interfering mutants of MAP kinase kinase, PC12 cells no longer extend neurites in response to NGF [35]. In addition, constitutively active forms of MAP kinase kinase activate MAP kinase and lead to neurite outgrowth when expressed in PC12 cells [35]. Additional isoforms of MAP kinase may also participate in signal transduction from the PDGF receptor but were not examined here. The activation of MAP kinases in response to NGF stimulation of PC12 cells is Ras-dependent, as dominant-negative alleles of *ras* block both PC12 cell differentiation and MAP kinase activation [36,37].

Receptor tyrosine kinases probably regulate MAP kinase activity in several ways. One pathway begins with Grb2, the SH2/SH3-domain-containing protein that is constitutively bound to the guanine nucleotide exchange factor mSOS [38–45]. When Grb2 associates with activated receptor tyrosine kinases, mSOS is brought into proximity with the plasma membrane, where it can regulate the amount of GTP-bound Ras. GTP-bound Ras forms a complex with Raf-1 [46–51], which becomes activated and phosphorylates MAP kinase kinase [52–54]. This kinase then phosphorylates and activates the MAP kinases (reviewed in [55]). In the case of the PDGF receptor, Grb2 has been shown to complex with the receptor indirectly, by binding to phosphotyrosine residues on the receptor-associated protein Syp [56]. As the Trunc PDGF receptor associated with Syp despite lacking the reported binding site for Syp, none of the PDGF receptor mutants that we expressed in PC12 cells completely disrupts this arm of the Ras activation pathway.

Receptor tyrosine kinases may also couple to Ras by other means. For example, the PDGF receptor associates with and activates the Src family tyrosine kinases, Src, Yes and Fyn [57]. Shc is phosphorylated on tyrosine in v-*src*-transformed cells and is potentially a direct substrate for the Src tyrosine kinase [58]. In addition, it has recently been shown that Shc interacts directly with the PDGF receptor [59]. As a result, following PDGF stimulation of cells, Shc becomes phosphorylated on tyrosine and associates with Grb2 [60,61], thus linking with the pathway described above. Alternatively, Ras may be activated as a consequence of protein kinase C activation, such as occurs following an increase in PI turnover [62,63]. In addition, there may be ways of regulating MAP kinase activity that are independent of Ras or independent of Raf-1, for example through MEKK [64], although it is not clear how these pathways might couple to receptor kinase activation.

The PDGF receptor elements required for neurite outgrowth are distinct from those required for mitogenesis

In PC12 cells, Ras–GAP, PI 3-kinase, and PLC-γ1 are not essential for PDGF-regulated neurite outgrowth. But the binding sites on the PDGF receptor for some of these signalling molecules are important for PDGF-mediated mitogenesis. Ras–GAP enhances the intrinsic GTPase activity of Ras and has been proposed to act as either a negative regulator of Ras or, in some cases, as an effector of Ras (reviewed in [65]). The phosphorylation of Ras–GAP on tyrosine and binding to the PDGF receptor is not important for PDGF-stimulated cell proliferation in epithelial and fibroblast cell lines, however [16–18]. In addition, association with Ras–GAP is not required for Ras activation following stimulation of either Chinese hamster ovary (CHO) cells or pro-B-cell BaF3 cells expressing the PDGF receptor [66]. Likewise, we have found that in PC12 cells, association of Ras–GAP with the PDGF receptor is not required for either differentiation or MAP kinase activation. In general, it is not clear what role Ras–GAP may play in mediating the biological effects of PDGF, as it has been shown that the PDGF receptor regulates Ras activity largely by modulating guanine nucleotide exchange activity [67]. As activation of Trk — the receptor for NGF — does not lead to phosphorylation of Ras–GAP or its association with Trk, it is perhaps not surprising that Ras–GAP does not seem to play a role in regulating PC12 cell differentiation.

In some cell types, the PI 3-kinase/Nck binding sites on the PDGF receptor must be intact for PDGF to be able to promote DNA synthesis. When these residues are deleted or mutated and the PDGF receptor is expressed in epithelial cells, for example, PDGF no longer activates PI 3-kinase, no longer elicits an increase in the amount of GTP-bound Ras, does not stimulate the activities of Raf-1 and MAP kinases, and does not promote DNA synthesis [16,17,66]. Recently it has been shown that PI 3-kinase interacts physically with the GTP-bound form of Ras and is likely to serve as an effector for Ras [68]. Association of PI 3-kinase with the PDGF receptor is also required for normal endocytic trafficking of the receptor, although how this influences signalling by the receptor is not known [69].

In contrast, we have shown that in PC12 cells, the PI 3-kinase binding sites on the PDGF receptor are not required for neurite outgrowth nor for pp42/pp44 MAP kinase activation. In this respect, the requirements for PDGF-mediated mitogenesis are distinct from those for PDGF-dependent neurite outgrowth. In addition, there may be some cell-type specificity with respect to how the same receptor communicates with downstream signalling pathways. For example, in contrast to the situation in epithelial cells, when the PDGF receptor is expressed in the pro-B-cell line BaF3, the integrity of the PI 3-kinase binding sites on the PDGF receptor is not important for the observed increase in the amount of GTP-bound Ras [66].

When the phosphotyrosine residues mediating the association of PLC-γ1 with the PDGF receptor are mutated, there is a reduction in the amount of DNA synthesis detected following PDGF stimulation in some [23], but not all [24] cell types. In PC12 cells, association of PLC-γ1 with the PDGF receptor is not essential for the ability of the receptor to promote MAP kinase activation and neurite outgrowth, providing another example of a case in which the requirements for PDGF-stimulated mitogenesis and differentiation are distinct.

Our findings with PC12 cells suggest that the importance of individual signalling pathways may vary depending on the cell type in which the PDGF receptor is expressed, or on the ultimate phenotypic outcome of signalling. In addition, the PDGF receptor may use multiple redundant pathways to exert a biological effect, and there may be compensation between these pathways when any one pathway is compromised. PC12 cell neurite outgrowth may depend upon the activation of known signal transduction components, such as the MAP kinases, or may require the activity of other signalling molecules. These might include either identified signalling molecules, such as the Stat proteins [70–72], or novel proteins in growth factor receptor signal transduction. At this time, the identity of the critical signal transduction pathway(s) mediating PDGF-dependent neurite outgrowth in PC12 cells is unknown.

Materials and methods

Materials

Polyclonal antibodies specific for the platelet-derived growth factor receptor β subunit were obtained from L.T. Williams (Ab65) or from Upstate Biotechnology Inc. (catalog #06-131). The following antisera were also obtained from Upstate Biotechnology Inc.: polyclonal antisera specific for Ras–GAP (catalog #06-153), mixed monoclonal antibodies recognizing PLC-γ1 (catalog #05-163), and polyclonal antisera specific for Syp (catalog #06-118). A monoclonal antibody recognizing Syp was obtained from Transduction Laboratories (catalog #P17420). The anti-phosphotyrosine monoclonal antibody 4G10 was provided by D. Morrison, B. Drucker and T. Roberts. Nerve growth factor (Boehringer Mannheim) was used at a concentration of 50 ng ml^{-1} and human recombinant platelet derived growth factor BB (Upstate Biotechnology Inc.) at a concentration of 40 ng ml^{-1}.

Cell culture and neurite outgrowth assays

PC12 cells were maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 5% horse serum derived from platelet-poor plasma (Sigma). For analysis of neurite outgrowth, cells were seeded into 24-well collagen IV biocoat plates (Collaborative Biomedical Products), and cultured in the presence of NGF or PDGF BB for several days.

PDGF receptor cDNA constructs and expression

All mouse β PDGF receptor cDNAs (wild-type and mutant) were a generous gift from J. Escobedo and L.T. Williams. The ΔKI, Trunc and the 4F forms of the PDGF receptor are described in the Results section and in Fig. 1. The cDNAs encoding all forms of the receptor were cloned into the CMV expression vector pCEN (a generous gift from J. Majors). Plasmid was introduced into PC12 cells by electroporation and the cells selected in G418 (300 μg ml^{-1}). G418-resistant clones were screened for PDGF receptor expression by western blot analysis of cell lysates using an antibody specific for the β PDGF receptor (Ab 65; a gift from L.T. Williams). Two independent cell lines expressing each PDGF receptor construct were used in all experiments. In each case, all results were identical for both cell lines.

Preparation of cell extracts, immunoprecipitation and immunoblotting

For preparation of cell extracts, PC12 cells (1×10^7) or confluent Balb/c 3T3 cells (3×10^6) were stimulated with growth factor and the cells were lysed as described previously [10] in 1 % Nonidet P-40 lysis buffer (50 mM Hepes buffer, pH 7.4, 150 mM NaCl, 10 % glycerol, 1.5 mM $MgCl_2$, 1 mM EGTA, 100 mM NaF, 1 % Nonidet P-40, 1 mM Pefabloc, 10 μg ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin, 1 mM sodium orthovanadate). Immunoprecipitations were carried out and resolved by SDS polyacrylamide gel electrophoresis (SDS–PAGE) as described [10]. Western immunoblots were performed using the Enhanced Chemiluminescence (ECL) detection system (Amersham).

In-gel myelin basic protein kinase and PI 3-kinase assays

For the in-gel myelin basic protein kinase assay, 50–100 μg total cell protein was electrophoresed through a 12.5 % SDS polyacrylamide gel containing 500 μg ml^{-1} myelin basic protein, and the assay was carried out as described previously [73]. For the PI 3-kinase assay, the PDGF receptor was immunoprecipitated from PC12 or Balb/c 3T3 cell lysates and the PI 3-kinase assay carried out as described previously [16].

Acknowledgements: This work was supported by the National Institutes of Health Grant No. CA 44338 and by funds from the G.W. Hooper Research Foundation and the Lucille Markey Charitable Trust. M.L.V. was a Howard Hughes Medical Institute Predoctoral Fellow. We thank L.T. Williams, J. Escobedo and W. Fantl for many helpful discussions and the generous use of reagents, and D. Kalman for comments on the manuscript.

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Received: **14 September 1994**; revised: **4 November 1994**.
Accepted: **30 November 1994**.