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Phosphorylation sites in the PDGF receptor with different specificities for binding GAP and PI3 kinase *in vivo*

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Tyrosine residues have been identified in the human platelet-derived growth factor (PDGF) receptor β-subunit whose phosphorylation is stimulated by PDGF. These sites are also *in vitro* autophosphorylation sites. There are a total of three phosphorylation sites in the kinase insert region, tyrosines 740, 751 and 771. Mutagenesis studies show that Tyr740 and 751 are involved in the PDGF-stimulated binding of phosphatidylinositol (PI) 3 kinase, and Tyr771 is required for efficient binding of GAP, the GTPase activator of Ras. The requirement for Tyr751 is only detected at low PDGF receptor levels, suggesting that it increases the affinity of binding of PI3 kinase but is not absolutely required. Small deletions in the kinase insert only 10 residues from Tyr740 and Tyr771 do not significantly reduce binding of PI3 kinase or GAP, indicating that distant sequences are probably unimportant for recognition. The data suggest that the receptor signals to different pathways via different phosphorylated tyrosines, and that certain proteins, such as PI3 kinase, can recognize two phosphorylated tyrosines in a single receptor.

Key words: platelet-derived growth factor receptor/phosphatidylinositol kinase/Ras GTPase activator protein (GAP)

Introduction

One function of tyrosine phosphorylation appears to be to create binding sites for a set of proteins characterized by ~100 residue sequence motifs known as Src homology 2 (SH2) domains (reviewed in Koch *et al.*, 1991; Pawson, 1988). SH2-containing proteins, such as phospholipase Cγ (PLCγ), an 85 kDa polypeptide subunit (p85) of phosphatidylinositol (PI) 3 kinase, the GTPase activating protein of Ras (GAP), and members of the Src, Fps and Abl families of tyrosine kinases, are thought to be involved in intracellular signaling (Koch *et al.*, 1991). Many SH2-containing proteins are also substrates for tyrosine phosphorylation (reviewed in Ullrich and Schlessinger, 1990; Cantley *et al.*, 1991). Such phosphorylation could induce intramolecular binding of phosphotyrosine to the SH2 domain, and hence modify the conformation and function of the protein.

There appears to be some selectivity in the binding of SH2-containing proteins. Some bind to a wide spectrum of tyrosine phosphorylated proteins. PI3 kinase binds to the substrate IRS-1, the PDGF receptor, Fms, Kit, and the cellular Src-polyomavirus medium T antigen (mT) complex (Sun *et al.*, 1991; Kaplan *et al.*, 1987; Varticovski *et al.*, 1989; Reedijk *et al.*, 1990; Rottapel *et al.*, 1991; Lev *et al.*, 1991; Whitman *et al.*, 1985; Courtneidge and Heber, 1987). Other SH2-containing proteins are more restricted. PLCγ has not been found associated with Fms (Downing *et al.*, 1989), although it binds tightly to the PDGF, epidermal growth factor (EGF), fibroblast growth factor (FGF) and Kit receptors (Margolis *et al.*, 1989; Kumjian *et al.*, 1989; Morrison *et al.*, 1990; Mohammadi *et al.*, 1991; Rottapel *et al.*, 1991). GAP appears not to bind Kit or Fms (Rottapel *et al.*, 1991; Reedijk *et al.*, 1990), but does bind to the PDGF receptor (Kazlauskas *et al.*, 1990; Kaplan *et al.*, 1990; Margolis *et al.*, 1990). Therefore, it seems likely that the tertiary structure or primary sequences of different phosphotyrosine-containing proteins limit the SH2 domains to which they can bind.

Studies with purified components or crude cell extracts have been used to investigate the requirements for binding (Moran *et al.*, 1990; Matsuda *et al.*, 1990; Anderson *et al.*, 1990; Koch *et al.*, 1991). In these studies, isolated SH2 domains or full-length SH2-containing proteins bind to tyrosine-phosphorylated polypeptides and oligopeptides, with dissociation constants estimated in the range of 10^{-9} M and below for proteins and 10^{-6} M for peptides (Kazlauskas and Cooper, 1990; Escobedo *et al.*, 1991a). The lower affinity of peptides relative to proteins, and the even lower affinity of free phosphotyrosine (Mayer *et al.*, 1991), suggests that the peptide environment of the phosphorylated residue is important for high affinity binding.

Many of the SH2 proteins discovered so far bind to PDGF receptors. Two different PDGF receptor polypeptides, the α-and β-subunits, differ in their specificity for different isoforms of PDGF (reviewed in Heldin and Westermark, 1990). The β-receptor binds PI3 kinase, PLCγ, GAP, Src, Fyn, Yes, tensin and Raf (which lacks an SH2 domain) (Kaplan *et al.*, 1987; Kumjian *et al.*, 1989; Morrison *et al.*, 1990; Kazlauskas *et al.*, 1990; Kaplan *et al.*, 1990; Kypta *et al.*, 1990; Courtneidge *et al.*, 1991; Davis *et al.*, 1991; Morrison *et al.*, 1989). Site-directed mutagenesis has been used to define the features of the PDGF β-receptor required for binding. Tyrosine kinase activity is absolutely necessary, as expected if the binding site contains phosphotyrosine (Coughlin *et al.*, 1989; Kazlauskas and Cooper, 1989). Catalytic activity is reduced by substitution of a conserved tyrosine phosphorylation site in the catalytic domain (Tyr857 in the human β-receptor; Fantl *et al.*, 1989; Kazlauskas *et al.*, 1991). This mutation also reduces the binding of GAP (Kazlauskas *et al.*, 1990) but not PI3 kinase (Kazlauskas and Cooper, 1989). Binding of PI3 kinase, GAP and Raf is abolished by deletion of 70 amino acids from a non-catalytic segment within the kinase domain, the kinase insert, even though catalytic activity is normal (Coughlin *et al.*, 1989; Kaplan *et al.*, 1990; Morrison *et al.*, 1989). In contrast, PLCγ binding is not reduced by deletions in the kinase insert

(Morrison *et al.*, 1990). A tyrosine within the kinase insert (Tyr751) becomes phosphorylated in PDGF-stimulated cells and mutation of this residue to phenylalanine or glycine greatly reduces binding of GAP and PI3 kinase *in vivo* (Kazlauskas and Cooper, 1989; Kazlauskas *et al.*, 1990). Mutation of the Tyr751 homolog in the α-receptor also reduces PI3 kinase binding (Yu *et al.*, 1991). Together these studies suggest that PI3 kinase binds to a site including Tyr751, that GAP also binds to the kinase insert, and PLCγ binds elsewhere. On the other hand, GAP and PLCγ may have overlapping binding sites because PDGF receptors isolated from cells overexpressing PLCγ show reduced binding *in vitro* to SH2 domains from either PLCγ or GAP (Anderson *et al.*, 1990).

In addition to the Tyr751 and Tyr857 phosphorylation sites identified previously, the PDGF β-receptor contains minor tyrosine phosphorylation sites (Kazlauskas and Cooper, 1989). We have now mapped two additional phosphorylation sites in the kinase insert region, Tyr740 and Tyr771, and show that GAP binding requires Tyr771 but not Tyr740, and, conversely, PI3 kinase binding requires Tyr740 but not Tyr771. Therefore these two SH2-containing proteins show complementary specificities *in vivo*. We also show that the locations of Tyr740 and Tyr771 within the kinase insert region can be altered without altering these specific binding interactions, indicating that local primary sequence probably contributes to the binding site. Finally, we suggest that PI3 kinase binds more tightly to doubly than to singly phosphorylated receptors.

Results

Additional phosphorylation sites in the PDGF receptor

Previously, we identified phosphorylation sites in the PDGF receptor by digesting immunoprecipitated, ^{32}P-labeled receptors with trypsin and resolving the phosphopeptides by two-dimensional thin-layer electrophoresis and chromatography. In addition to peptides containing Tyr751 and Tyr857, we detected a number of other peptides (Kazlauskas and Cooper, 1989). Attempts to characterize these additional peptides further were frustrated by poor recoveries. Therefore we tried other digestion protocols, and found that combined digestion with trypsin and thermolysin created a simple and reproducible pattern (Figure 1).

Wild-type human PDGF β-receptors were expressed at high level in dog epithelial TRMP cells, which have no detectable PDGF binding sites (Kazlauskas and Cooper, 1989). Duplicate dishes were labeled with $^{32}P_i$ and then

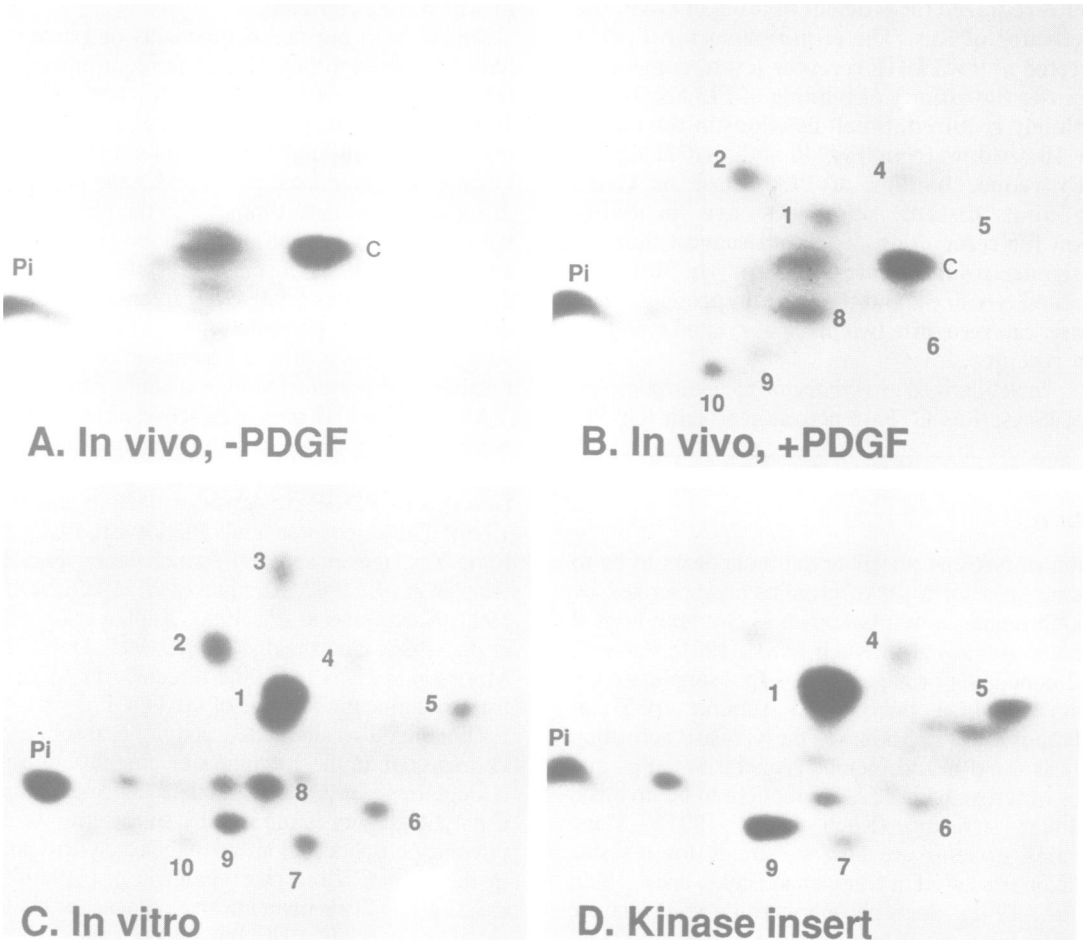

Fig. 1. Tryptic/thermolytic phosphopeptide maps of the PDGF receptor. PDGF receptors were immunoprecipitated from $^{32}P_i$-labeled (**A** and **B**) or non-radioactive (**C** and **D**) cells that had been exposed to 0 (A, C and D) or 1.3 (B) nM PDGF-BB for 5 min prior to lysis. Following washing, non-radioactive immunoprecipitates were incubated with [γ-^{32}P]ATP in the absence (C) or presence (D) of a GST fusion protein containing the kinase insert domain of the PDGF receptor (receptors from unstimulated cells have kinase activity that is not increased by PDGF). Following SDS–PAGE, radioactive receptors (A, B and C) and GST fusion protein (D) were excised from the gel and analyzed by peptide mapping (Materials and methods). Phosphopeptide C contains phosphoserine.

stimulated with 0 or 1.3 nM recombinant PDGF-BB for 5 min at 37°C. Receptors were recovered by immunoprecipitation and SDS−PAGE, and digested with trypsin and thermolysin. Phosphopeptide maps of unstimulated receptors showed one major phosphoserine peptide (c) and a smear of poorly resolved peptides (Figure 1A). Stimulated receptors contained additional phosphopeptides (Figure 1B). We tested which of these novel phosphopeptides were also *in vitro* phosphorylation sites by incubating non-radioactive, immunoprecipitated receptors with [γ-^{32}P]ATP, and subjecting the labeled receptors to the same digestion protocol (Figure 1C). All the peptides labeled in this reaction contained phosphotyrosine. Analysis of a mixture of *in vitro* and *in vivo* labeled samples showed that phosphopeptides 1, 2, 4, 5, 6, 8, 9 and 10 were phosphorylated both *in vivo* and *in vitro*. Peptides 1, 2, 8 and 10 were more heavily labeled *in vivo* than the others, but we do not know whether all phosphopeptides are recovered with equal efficiency.

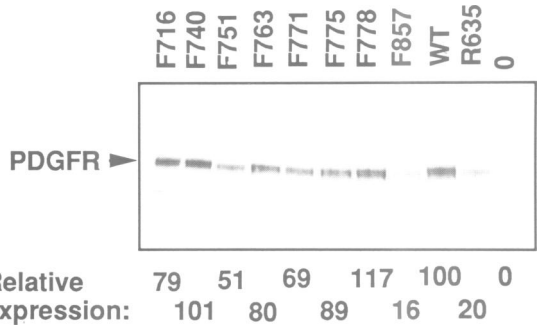

Fig. 2. Expression of mutant PDGF receptors in TRMP cells. TRMP cells were infected with pLXSN virus (0) or pLXSN derivatives containing wild-type (WT), kinase-inactive (R635) or phenylalanine (F716, F740, F751, F763, F771, F775, F778 and F857) mutant PDGF receptor cDNAs, and selected with G418. Drug-resistant mass cultures were analyzed for PDGF receptor expression by SDS−PAGE of samples from ~4×10^4 cells and immunoblotting with anti-receptor antibody 2897 (Materials and methods). The immunoblot was quantified and receptor expression level relative to WT is shown.

We have reported that Tyr857 is a major *in vivo* phosphorylation site, but a more minor *in vitro* phosphorylation site, relative to Tyr751 (Kazlauskas and Cooper, 1989). Phosphopeptide mapping of Phe857 mutant receptors labeled *in vivo* or *in vitro* showed that phosphopeptide 10 was missing in this mutant (data not shown). Phosphorylation of this peptide was enhanced *in vivo* relative to *in vitro* (Figure 1B and C). We therefore deduce that spot 10 probably contains phosphorylated Tyr857. To assign other phosphopeptides, we utilized a glutathione-*S*-transferase (GST) fusion protein that contains the kinase insert region

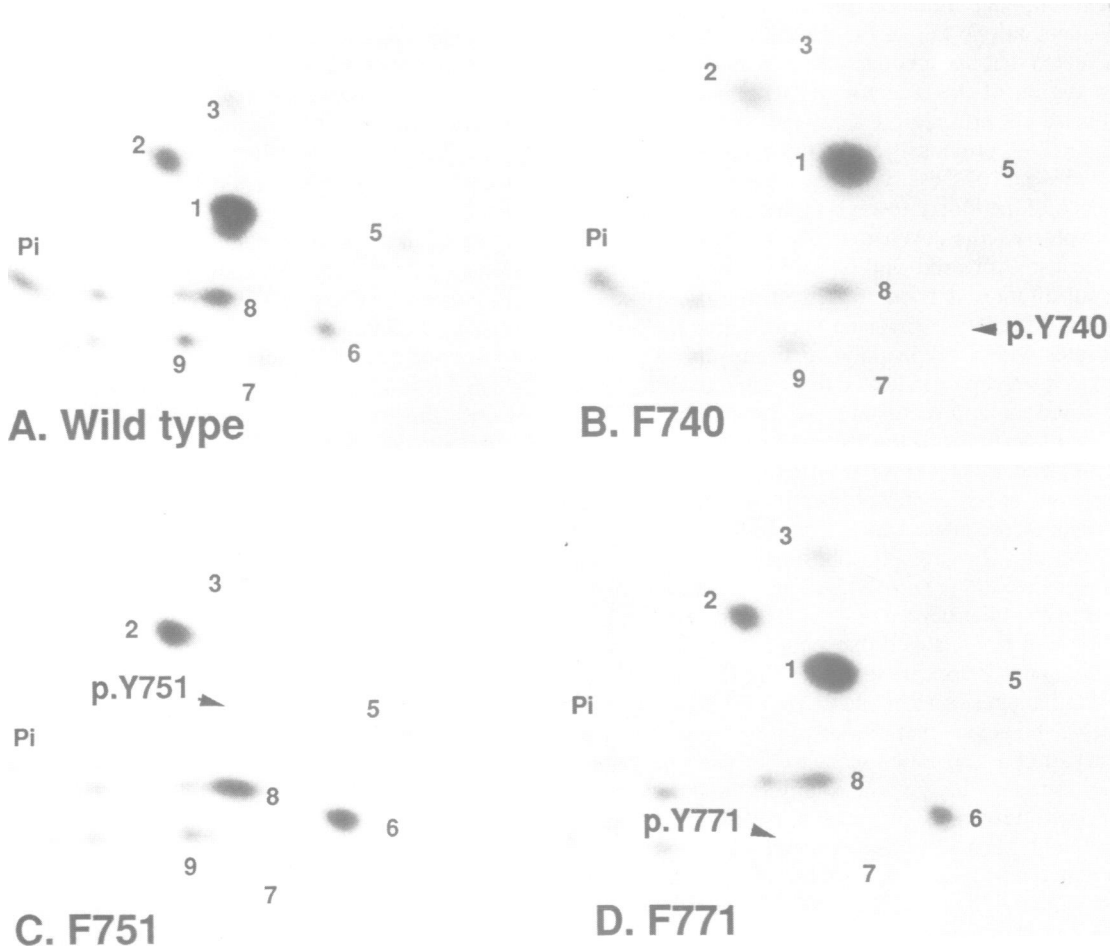

Fig. 3. Tryptic/thermolytic phosphopeptide maps of mutant PDGF receptors. PDGF receptors were immunoprecipitated from unstimulated TRMP cells expressing wild-type (**A**) or phenylalanine mutant (**B**, F740; **C**, F751; **D**, F771) PDGF receptors, labeled *in vitro* by autophosphorylation with [γ-^{32}P]ATP and analyzed by peptide mapping (Materials and methods). Phosphopeptides are labeled as in Figure 1. Phosphopeptides missing in each of the mutant receptors are arrowed.

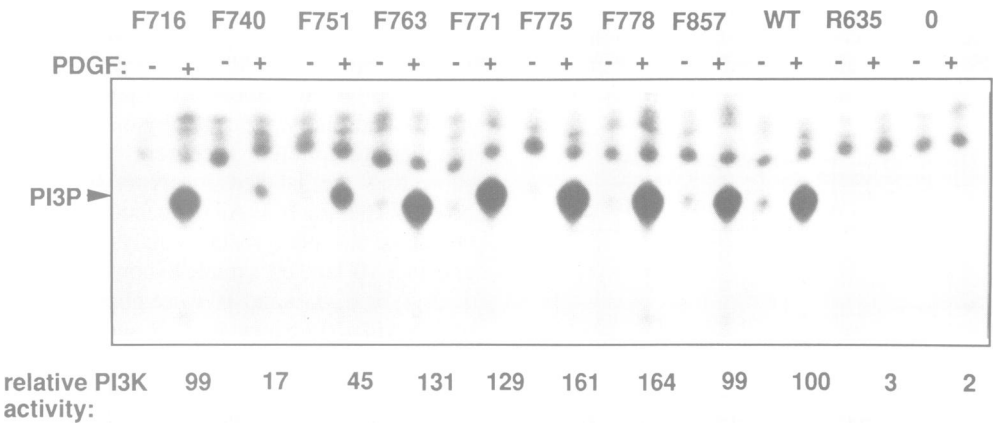

Fig. 4. PI3 kinase activity associated with wild-type and mutant PDGF receptors. TRMP cells infected with pLXSN (0) or expressing wild-type (WT), kinase-inactive (R635) or phenylalanine (F716, F740, F751, F763, F771, F775, F778 and F857) mutant PDGF receptors were treated with 0 or 1.3 nM PDGF-BB for 5 min at 37°C and lysed. PDGF receptor immunoprecipitates from 3×10^5 cells were incubated with PI and [γ-^{32}P]ATP and the labeled phospholipids analyzed (Materials and methods). The position of a non-radioactive PI phosphate marker, chromatographed in parallel, is indicated (PI3P). Radioactive PI3P was quantified using a Phosphorimager and expressed relative to the wild-type.

of the receptor (residues 699 to 798) as a substrate for *in vitro* tyrosine phosphorylation. GST itself was not a substrate for phosphorylation. Following trypsin and thermolysin digestion, the fusion protein was found to be phosphorylated at peptides 1, 4, 5, 6, 7 and 9, indicating that these tyrosines are contained in the kinase insert (Figure 1D). A similar experiment utilizing a fusion protein containing the C-terminal non-catalytic tail of the receptor (residues 939–1108) indicated that phosphopeptides 2, 3 and 8 are derived from that region of the receptor (data not shown).

The kinase insert region contains seven tyrosines, including Tyr751, previously found to be a phosphorylation site. To find which of these tyrosines is phosphorylated, we mutated each of the kinase insert tyrosines to phenylalanine (F) and expressed the seven mutants in TRMP cells. As controls we also expressed wild-type (WT), F857, R635 (an arginine substitution at lysine 635, causing loss of kinase activity) and an empty expression vector. Immunoblotting of cell lysates with a rabbit polyclonal anti-fusion protein antiserum to the receptor indicated that each mutant receptor was expressed at approximately the same level as WT (69–117% of WT), with the exceptions of F857 (16%), R635 (20%) and F751 (51%) (Figure 2).

To correlate specific phosphopeptides with individual tyrosine residues, we labeled each of the immunoprecipitated mutant receptors by autophosphorylation *in vitro* and prepared peptide maps (Figure 3). All the mutants autophosphorylated to a similar degree *in vitro*. The maps of mutants F740, F751 and F771 lacked phosphopeptides 6, 1 and 9, respectively. Thus it appears that Tyr740, 751 and 771 are phosphorylation sites *in vitro* and *in vivo* (Figure 1B). It is also possible, however, that one or more of these tyrosine residues is part of a recognition sequence for phosphorylation of another tyrosine in the kinase insert, and this other phosphorylated tyrosine is contained in the phosphopeptide. This is unlikely, because phosphopeptides 1, 6 and 9 were phosphorylated normally in each of the other mutants (data not shown). Mutation of Tyr716, 763, 775 and 778 did not lead to the loss of any phosphopeptides, suggesting they are not phosphoryated at high level. The minor kinase insert phosphopeptides, 4, 5 and 7, are difficult to detect, and their presence or absence in each of the mutants could not be confirmed. They could be partials containing one or more phosphorylation sites.

Effects of phosphorylation site mutations on protein binding

We assayed for the association of cell proteins with PDGF-stimulated wild-type and mutant receptors by immunoprecipitation of receptors from PDGF-treated and control cells, using a monoclonal antibody to the extracellular domain of the receptor. Kinase-inactive (R635) and kinase-reduced (F857) mutants were included as controls. Receptor immunoprecipitates were analyzed three ways.

First, immunoprecipitates were assayed for associated PI3 kinase activity by incubation with [γ-^{32}P]ATP and phosphatidylinositol (Figure 4). PI3 kinase activity was significantly reduced in the F740 and kinase-inactivating R635 mutants (Figure 4). PI3 kinase activity was also reduced in the F751 mutant, but this may be partly due to reduced receptor expression (Figure 2). Normal levels of PI3 kinase activity were associated with the other kinase insert mutants.

Second, immunoprecipitates were subjected to SDS–PAGE and immunoblotted for associated GAP. The same samples were immunoblotted with anti-receptor antibody, and the quantity of PDGF receptor determined. Bound GAP was normalized to the amount of receptor, and the ratio expressed relative to the wild-type. GAP association was reduced dramatically by the F771 and R635 mutations (Figure 5; the decreased binding of GAP to the F778 mutant in this experiment was not reproducible). GAP also bound poorly to the F857 mutant (Figure 5), as found previously (Kazlauskas *et al.*, 1990).

Third, immunoprecipitates were incubated with [γ-^{32}P]ATP and analyzed by SDS–PAGE. Receptor-associated proteins were detected in immunoprecipitates from PDGF-stimulated, but not control, cells. Using wild-type receptor immunoprecipitates, this procedure labels GAP (124 kDa), two forms of PI3 kinase p85 (74 and 85 kDa, unpublished results) and unidentified polypeptides of 120 and 64 kDa (Kazlauskas and Cooper, 1990). Conspicuously, the 120 kDa protein and p85 variants were phosphorylated much less, relative to the GAP and 64 kDa bands, in the F740 mutant, consistent with reduced PI3 kinase binding. The GAP band was phosphorylated much less, relative to the other bands, in the F771 mutant, consistent with reduced GAP binding (data not shown). All bands were phosphorylated poorly in the F857 mutant, consistent with

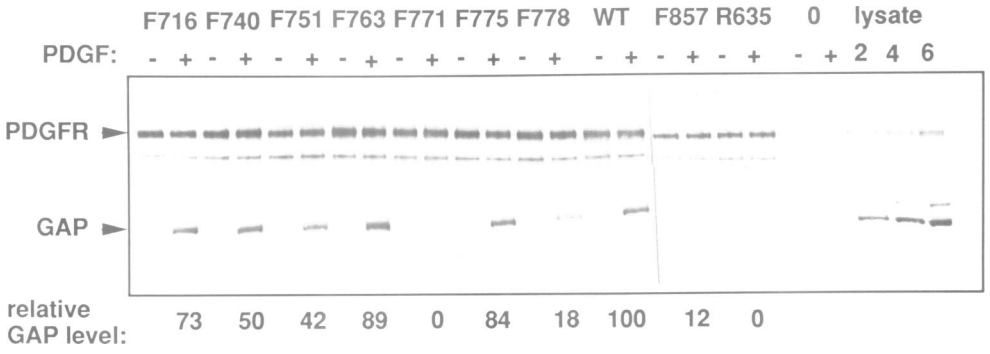

Fig. 5. GAP associated with wild-type and mutant PDGF receptors. PDGF receptor immunoprecipitates from 1.5×10^6 cells were resolved by SDS−PAGE and immunoblotted. The right hand three lanes (2, 4 and 6) were loaded with cell lysate from 3, 6 and 9×10^4 cells, respectively, and thus correspond to 2, 4 and 6% of the cell number used for immunoprecipitation. The upper part of the immunoblot was probed with antibody 2897, to detect the PDGF receptor, and the lower part with anti-GAP B to detect GAP (Materials and methods). GAP was quantified, normalized for the amount of PDGF receptor present in the immunoprecipitate, and expressed relative to the wild-type.

lower kinase activity (Fantl *et al.*, 1989; Kazlauskas *et al.*, 1991), or decreased binding (data not shown).

Effect of receptor expression level

We had previously found that mutation of Tyr751 to glycine or phenylalanine reduced binding of both PI3 kinase and GAP (Kazlauskas and Cooper, 1989; Kazlauskas *et al.*, 1990). However, in the present experiments, binding of PI3 kinase and GAP was reduced no more than 2-fold by the F751 mutation. One possible reason for this discrepancy is the level of expression of the receptor. Our previous experiments were done with a retroviral vector, pLJ (Korman *et al.*, 1987), which caused TRMP cells to express $\sim1\times10^5$ receptors per cell (Kazlauskas and Cooper, 1989). We have now utilized an alternative retroviral expression vector, pLXSN (Miller and Rosman, 1989). Immunoblotting of serial dilutions of cell lysates indicated that the pLXSN vector wild-type line expressed 60-fold more receptors than the pLJ vector line, i.e. 6×10^6 receptors per cell (Figure 6A). Direct binding assays with [^{125}I]PDGF-BB indicated a dissociation constant of $\sim2\times10^{-10}$ M and 5×10^6 receptors per cell (data not shown), in agreement with the immunoblotting estimate.

To test whether the level of expression affected the binding of PI3 kinase, immunoprecipitates of receptors from TRMP cells expressing 1×10^5 or 6×10^6 wild-type or F751 mutant receptors were compared (Figure 6B). PI3 kinase activity was reduced significantly at the low level of receptor expression, but only slightly at the high level of expression. This result was confirmed by examining the *in vitro* phosphorylation of receptor-associated 85 and 74 kDa forms of p85 (data not shown). Note that the absolute levels of PI3 kinase activity associated with wild-type receptors was decreased slightly by higher expression. We have shown before that phosphorylated wild-type receptors are in excess over PI3 kinase in TRMP cells expressing 1×10^5 receptors (Kazlauskas and Cooper, 1990). The decrease is probably due to antibody limitation when using cells overexpressing receptors.

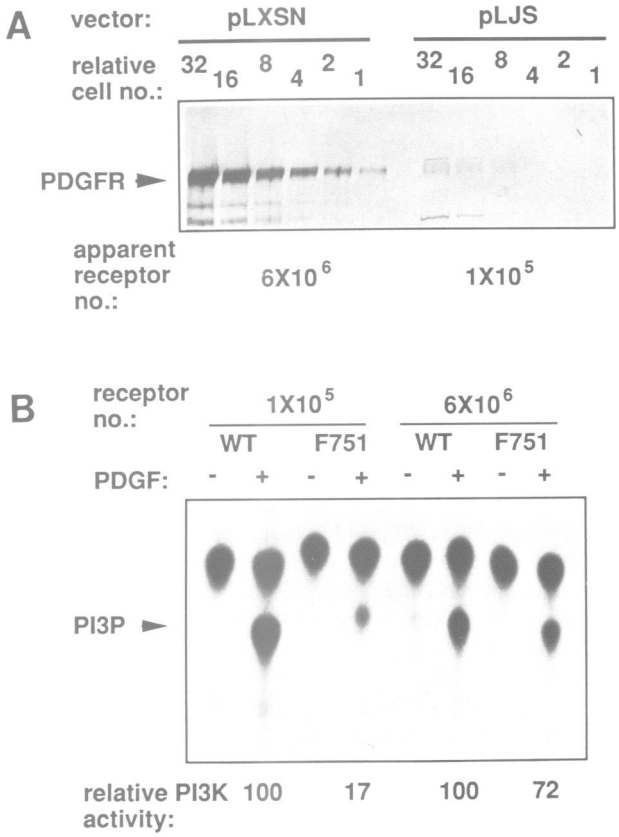

Fig. 6. Effect of level of PDGF expression on association of PI3 kinase activity. TRMP cells were infected with wild-type or F751 mutant PDGF receptor cDNA derivatives of the retroviral vectors pLJ or pLXSN. (**A**) Cells expressing wild-type receptors were analyzed for the level of PDGF receptor expression by immunoblotting (Figure 2). Serial 2-fold dilutions of a sample containing 7.5×10^4 cells were analyzed. Quantitation of the immunoblot indicated that the level of receptor expression was 60-fold higher in pLXSN- than pLJ-infected cultures. (**B**) Similar cultures were treated with 0 or 1.3 nM PDGF-BB for 5 min at 37°C and lysed. PDGF receptors were immunoprecipitated and incubated with PI and [γ-^{32}P]ATP and the labeled phospholipids analyzed (Materials and methods). The position of a non-radioactive PIP marker, chromatographed in parallel, is indicated. Radioactive PI3P was quantified and expressed relative to the wild-type.

Effects of deletions within the kinase insert

The preceding results indicate differences in the binding sites present on PI3 kinase and GAP, which must recognize phosphotyrosine in the context of distinct parts of the overall receptor structure. Depending on the folding of the kinase insert, and of the entire cytoplasmic domain of the receptor, the features of the receptor structure that are recognized by PI3 kinase and GAP could be local to the phosphotyrosine

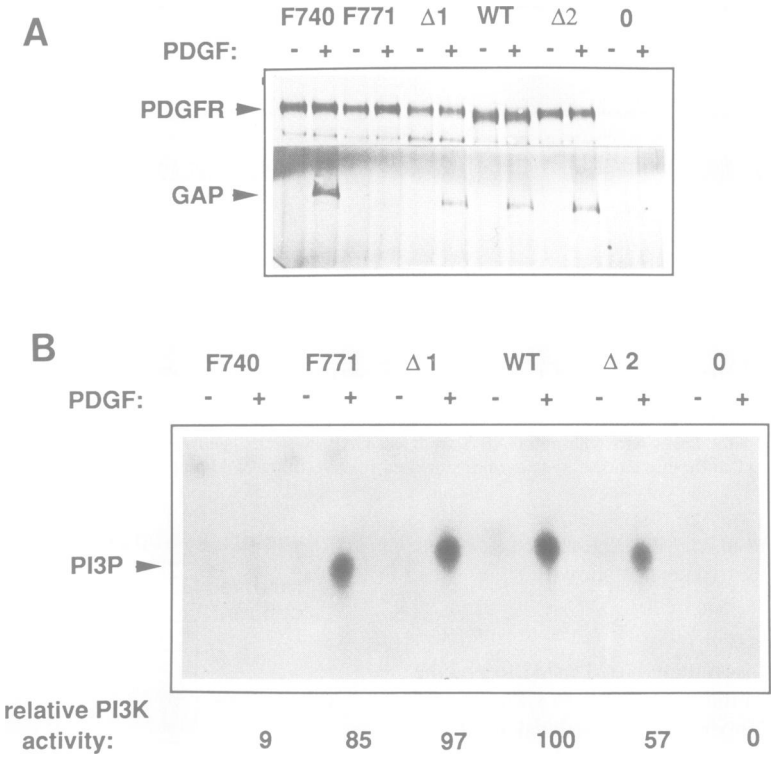

Fig. 7. Effects of deletions in the kinase insert on association of the PDGF receptor with GAP and PI3 kinase. TRMP cells were infected with pLXSN virus (0) or pLXSN derivatives containing wild-type (WT), phenylalanine (F740 and F771) and deletion mutant (Δ1 and Δ2) PDGF receptor cDNAs, and selected with G418. Drug-resistant mass cultures were treated with 0 or 1.3 nM PDGF-BB for 5 min at 37°C and lysed. PDGF receptors were immunoprecipitated and analyzed for associated GAP (**A**) and PI3 kinase activity (**B**) (Materials and methods).

or more distant in the primary sequence. To test whether distant features in the primary sequence might be important, we made two deletions in the kinase insert; Δ1 is a deletion of 12 residues C-terminal to Tyr771 (residues 782−793, inclusive) and Δ2 is a deletion of 22 residues N-terminal to Tyr740 (residues 708−729, inclusive). Both mutations also introduced two residues (LysLeu) at the site of deletion. These deletions have unknown effects on the folding of the kinase insert, but alter the spacing between the key tyrosine residues and the N- and C-terminal portions, respectively, of the kinase domain.

As shown in Figure 7, both deleted receptors bound PI3 kinase and GAP at or close to wild-type levels, in contrast to the F740 and F771 mutants which served as controls. This implies that Tyr740 and 771 were both phosphorylated normally and that these phosphotyrosines constituted binding sites despite the removal of nearby sequences in the kinase insert. It is possible that the hydrophilic kinase insert is a poorly structured part of the receptor, and may be sufficiently flexible to adopt the required conformations for phosphorylation and binding even when certain regions are deleted.

Discussion

Phosphorylation sites in the β-receptor

We have now identified a total of three tyrosine phosphorylation sites in the kinase insert region of the PDGF receptor β-subunit and have evidence for at least one site in the C-terminal, non-catalytic tail. Thus, the PDGF receptor resembles the insulin and EGF receptors in having several tyrosine phosphorylation sites in non-catalytic regions of the receptor (Downward *et al.*, 1984; Tornquist and Avruch, 1988; White *et al.*, 1988). Moreover, we have found that each of the tyrosine phosphorylation sites in the kinase insert makes a distinct contribution, binding different SH2-containing proteins. Mutation of Tyr740 decreases the binding of PI3 kinase activity, PI3 kinase subunits of 85 and 74 kDa, and a polypeptide of unknown function, p120. Mutation of Tyr771 reduces the binding of GAP. Substituting Tyr751 also decreases the binding of PI3 kinase and p120 if receptors are expressed at low levels, but not if receptors are expressed at high levels. Another phosphorylation site, Tyr857, outside the kinase insert, also plays a role in binding GAP. Because Tyr857 is also involved in kinase activation (Fantl *et al.*, 1989; Kazlauskas *et al.*, 1991), we do not know whether Tyr857 is part of a GAP binding site or is required for phosphorylation of other tyrosines.

There are seven tyrosines in the kinase insert of which 740, 751, 763 and 771 are conserved between the α- and β-subunits (Yarden *et al.*, 1986; Matsui *et al.*, 1989). Our data indicate that Tyr740, 751 and 771 are phosphorylated, but Tyr763 appears not to be. Three other *in vitro* phosphopeptides, of which two are major *in vivo* sites, appear to lie in the C-terminal non-catalytic tail. The sequence of the tail shows four tyrosine residues, and additional mutants will be required to identify which of these are phosphorylated. We have no evidence on the possible functions of the C-terminal phosphotyrosines, but we note that PLCγ binding to the PDGF receptor is not reduced by deletion of the kinase insert (Morrison *et al.*, 1990), and that PLCγ binds to the C-terminal regions of the EGF and FGF

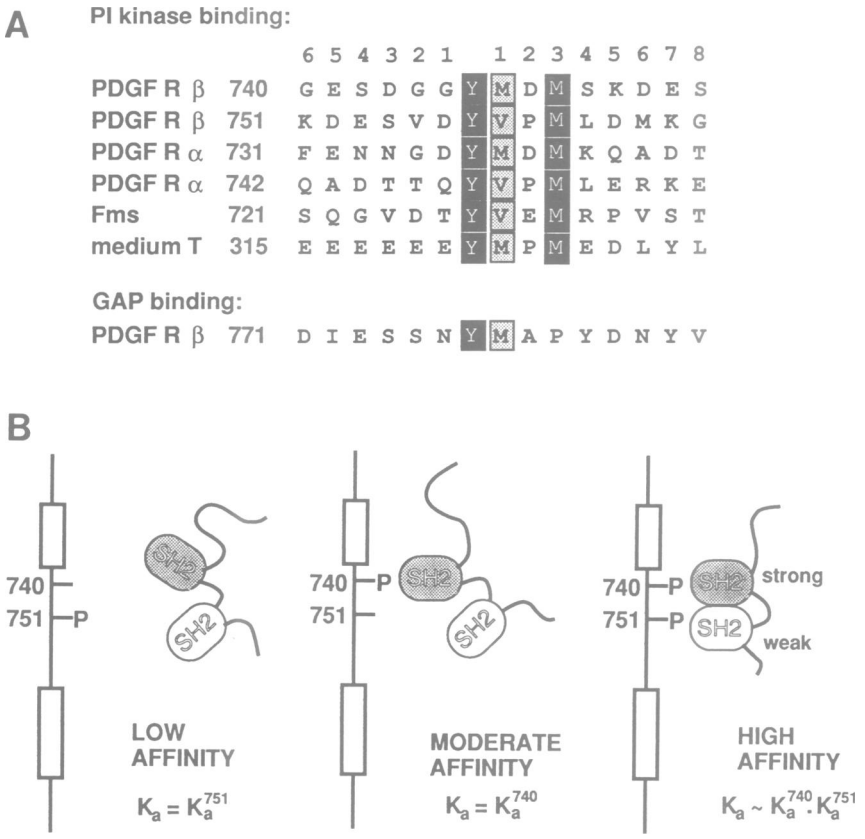

Fig. 8. (**A**) Sequences surrounding tyrosine residues implicated in binding of PI3 kinase and GAP. Only those sequences identified by site-directed mutagenesis as being required for binding are shown. The phosphorylated tyrosine, conserved methionine/valine and identical methionine are highlighted. For references, see text. (**B**) Model for the differential effects of mutations at Tyr740 and 751 on the binding of PI3 kinase. The p85 subunit of PI3 kinase is shown with two SH2 domains, one binding to phosphorylated Tyr740 and the other to phosphorylated Tyr751. The association constants (K_a) for binding to phosphotyrosine 740 (K_a^{740}) appears to be stronger than the K_a for binding to phosphotyrosine 751 (K_a^{751}), because Tyr740 is required for binding at high concentrations of receptor, whereas Tyr751 is only needed for binding at low concentrations of receptor. Assuming independent binding sites, the overall K_a for binding of PI3 kinase to receptors that are phosphorylated at both Tyr740 and 751 is approximately the product of K_a^{740} and K_a^{751}.

receptors (Margolis *et al.*, 1990; Mohammadi *et al.*, 1991). It is possible that PLCγ binds to a C-terminal phosphorylated tyrosine in the PDGF receptor.

Specificity of SH2 domain binding

What is the basis for the different binding properties of mutant receptors with substitutions at Tyr740, 751 and 771? The simplest hypothesis is that phosphorylation of Tyr740, 751 and 771 creates binding sites with different affinities for PI3 kinase and GAP. An alternative hypothesis is that the mutations reduce phosphorylation of distant tyrosines, thereby blocking binding. For Tyr740 and 751, the first hypothesis is supported by the *in vitro* competition experiments of Escobedo *et al.* (1991a). They found that phosphorylated synthetic peptides based on the Tyr740 or Tyr751 sequences competed for binding of PI3 kinase to the PDGF receptor *in vitro*. A scrambled phosphopeptide of the same amino acid composition but different sequence did not compete. Furthermore, the phosphopeptide that competed for PI3 kinase did not compete for GAP binding, indicating the sequence specificity for interaction. Reduced binding of GAP to the Phe771 mutant is consistent with a similar model in which phosphorylated Tyr771 is a binding site for GAP. However, it is possible that mutation of Tyr771 reduces phosphorylation of another tyrosine, thereby preventing GAP binding. This seems unlikely given that most other phosphopeptides are phosphorylated normally in the Phe771 mutant *in vitro* (Figure 3) and that GAP does not bind to the Phe771 mutant *in vitro* (A.Kazlauskas, unpublished results), but phosphopeptide competition studies would be required to exclude this possibility.

Deletion of 22 residues close to Tyr740, or of 12 residues close to Tyr771, had little effect on the binding of GAP, PI3 kinase or p120, suggesting that the specificity for different SH2 domains is not determined by the positions of phosphorylated Tyr740 and Tyr771 in the overall 3-dimensional structure of the receptor. This result is consistent with the primary determinant of binding specificity being the local sequence around the phosphorylated tyrosine (Escobedo *et al.*, 1991a).

The tyrosine residues required for binding of PI3 kinase to a number of phosphoproteins have now been identified by mutagenesis. These residues are Tyr740 and 751 in the β-subunit (Figures 3 and 6B), Tyr731 and Tyr742 in the α-subunit (Yu *et al.*, 1991), Tyr721 in Fms (Reedijk *et al.*, 1992; Downward *et al.*, 1991), and Tyr315 in mT (Talmage *et al.*, 1989). Alignment of these sequences indicates significant similarity (Figure 8A; Cantley *et al.*, 1991). Numbering residues from the phosphotyrosine, residue +1 is methionine or valine, +2 is proline or an acidic residue,

and +3 is invariably methionine. Except for methionine at +1, the Tyr771 sequence of the β-subunit required for GAP binding does not share these features. It has proline at position +3, which is conserved in the α-subunit sequence. The sequence conservation of PI3 kinase binding sites strongly suggests an important role for the methionine residue at position +3. The more common hydrophobic residues—leucine, valine and isoleucine—are not found. Methionine is more flexible than the other aliphatic residues, which may allow it to insert into hydrophobic pockets (Bernstein *et al.*, 1989), is often found on dimerization interfaces in oligomeric proteins (Argos, 1988). Methionine can also interact with metals via its sulfur atom (Chakrabarti, 1989): metal ions have not been detected in SH2 domains, but could provide additional stability to the phosphotyrosine−PI3 kinase interaction.

Stoichiometry of phosphorylation and binding

The amounts of GAP and PI3 kinase that bind to the β-subunit may be regulated by the levels of phosphorylation of their binding sites. *In vivo*, the percentage of receptors phosphorylated at each tyrosine can be estimated from the labeling of each phosphotyrosine peptide (this assumes that this phosphopeptide contains a single phosphate and that all receptors are phosphorylated at this site). By this method, we estimate the relative phosphorylation at Tyr751, 740, 771 and 857 is 33, 2, 10 and 19%, respectively (data not shown). These numbers may, however, be affected by differential dephosphorylation after lysis, differential recovery by immunoprecipitation, incomplete digestion, losses during peptide mapping and contributions from background radiation during quantitation.

A low level of phosphorylation of Tyr771 may explain the low extent of GAP binding in these cells. Calibration of the immunoblot analysis with reference to a recombinant human GAP standard indicates that a TRMP cell contains $\sim 3\times10^6$ molecules of GAP, of which only 8% (2.4×10^5 molecules) bind to the receptor, even though there are 6×10^6 receptors. This calculation suggests that 4% of the receptors have GAP bound, but could be an underestimate if the antibody used, raised to human GAP, poorly recognizes the dog GAP present in TRMP cells. The estimated value of 10% phosphorylation at Tyr771 is in the range to explain the binding of GAP to only 4% of receptors.

Only a small percentage of receptors are phosphorylated at Tyr740. Using TRMP cells expressing 10^5 receptors, we found previously that the PI3 kinase activity bound *in vivo* approximately equalled the activity that could bind *in vitro* under conditions of receptor excess, indicating that receptors were also in excess for complex formation *in vivo* (Kazlauskas and Cooper, 1990). Consistent with this, increasing receptor expression to 6×10^6 per cell did not increase PI3 kinase binding (Figure 6). Therefore, the low stoichiometry of phosphorylation of Tyr740 probably does not limit the binding of PI3 kinase.

Single versus multiple phosphorylation

The low levels of phosphorylation of individual kinase insert tyrosines raises the question of whether GAP and PI3 kinase bind to the same or different receptors. If the phosphorylations are independent, or mutually exclusive, then only a very small proportion of receptors would be phosphorylated simultaneously at both Tyr740 and 771, and PI3 kinase and GAP would bind to different receptors. On the other hand, if the phosphorylations are cooperative, then PI3 kinase and GAP could bind to the same receptor, provided there was no steric hindrance. PI3 kinase and GAP do not compete for binding to receptors *in vitro*, implying either that they bind to different receptors or have non-overlapping binding sites. The detection of PDGF-induced complexes of GAP with PLCγ and PI3 kinase (Kaplan *et al.*, 1990) has been cited as evidence for the possibility of simultaneous binding of more than one protein to a single receptor subunit, but could be explained by the dimerization of receptor subunits (Bishayee *et al.*, 1989; Hammacher *et al.*, 1989; Seifert *et al.*, 1989; Li and Schlessinger, 1991), each with a different associated protein. Direct measurements of the number of phosphates on a single receptor polypeptide have not been reported.

Double phosphorylation would provide a possible explanation for the effects of mutating Tyr751. Previously, using TRMP cells expressing 10^5 receptors, we found that mutating Tyr751 reduced binding of both PI3 kinase and GAP (Kazlauskas and Cooper, 1989; Kazlauskas *et al.*, 1990). Presently, using the same cell type expressing $\sim 6\times10^6$ receptors, Tyr751 was not required for binding either protein. Direct comparison of PI3 kinase binding by F751 mutant receptors at 10^5 and 6×10^6 per cell confirmed that Tyr751 is only needed for PI3 kinase binding when receptors are expressed at low level (Figure 6B). Thus Tyr751 may raise the affinity of binding. With F751 mutant receptors, the affinity may be reduced so that PI3 kinase binding is detected only if high levels of receptors are available to drive the equilibrium in the direction of binding. Tyr740 is required for binding even at high receptor levels, suggesting a strict requirement for this phosphorylation site. A physical model for this effect on binding affinity is shown in Figure 8B. Because PI3 kinase p85 has two SH2 domains (Escobedo *et al.*, 1991b; Otsu *et al.*, 1991; Skolnik *et al.*, 1991), one could bind tightly to phosphotyrosine 740 and the other less strongly to phosphotyrosine 751. (Which SH2 domain binds to which phosphotyrosine is not known.) Mutation of Tyr751 would allow tight interaction with phosphotyrosine 740 and complexes would form, provided the concentration of phosphorylated receptor was high (6×10^6 receptors/cell). Mutation of Tyr740 would leave only the potential for weak interaction with phosphotyrosine 751, so little PI3 kinase would bind, even at high receptor levels. If both phosphotyrosines are available, interactions with both SH2 domains would be possible, and the overall affinity of interaction would be increased (approximately multiplicatively) allowing detection of binding at either high or low levels of receptors (1×10^5/cell)(Figure 8B). This model implicitly acknowledges that doubly phosphorylated receptors exist and that both SH2 domains on a single p85 molecule participate. If this were not so, the effects of the mutations of Tyr751 and 740 on the quantity of PI3 kinase bound would be simply additive, and probably not detected by the crude methods used here.

This model permits resolution of some seemingly contradictory results. Micromolar concentrations of phosphopeptides, containing the murine β-receptor sequence corresponding to residues 737−757 of the human receptor, compete for *in vitro* binding of PI3 kinase to the murine β-receptor, irrespective of whether the peptide is

phosphorylated at Tyr740 or Tyr751 (Escobedo *et al.*, 1991a). This suggests that the binding sites on PI3 kinase, which do show considerable sequence similarity, can be occupied by either phosphopeptide (Figure 8A). In that case, PI3 kinase would be expected to bind to receptors in which either Tyr740 or Tyr751 was phosphorylated, and mutation of either residue would not reduce PI3 kinase binding. However, mutation of either residue in the β-receptor (this work) or either homologous residue in the α-receptor (Tyr731 and 742; Yu *et al.*, 1991), affects binding *in vivo*, suggesting that each tyrosine has a distinct role in binding. We can now offer two alternate hypotheses to explain these results. (i) Micromolar concentrations of phosphopeptide may be sufficient to bind with low sequence specificity to both SH2 domains on PI3 kinase, irrespective of which tyrosine is phosphorylated. Thus, both binding sites on PI3 kinase would be blocked. (ii) Each phosphopeptide may bind only to its cognate SH2 domain in PI3 kinase, but the remaining, unblocked SH2 domain is available to bind to the PDGF receptor at reduced affinity, a situation analogous to a singly phosphorylated receptor (Figure 8B). This would predict that PI3 kinase blocked with a Tyr751 phosphopeptide would bind with moderate affinity to PDGF receptors, but PI3 kinase blocked with a Tyr740 phosphopeptide would only bind with very low affinity.

PLCγ and GAP resemble PI3 kinase p85 in having two SH2 domains, so each of these proteins may also bind to the PDGF receptor via two different phosphotyrosines. In that case, there may be a second tyrosine residue involved in binding of GAP. Tyr857 is a good candidate to be a second binding site for GAP (Figure 5, Kazlauskas *et al.*, 1990). However, since mutation of Tyr857 reduces kinase activity, phosphorylation of other tyrosines may be reduced in this mutant.

Our results beg the question of the biological function of binding proteins like PI3 kinase and GAP to the activated PDGF receptor. None of the tyrosine to phenylalanine substitution mutants described here is significantly impaired in mitogenic signaling in TRMP cells (data not shown). However, it is possible that receptor overexpression masked subtle signaling defects that might have been evident at lower expression levels. One question is whether receptors that fail to bind to PI3 kinase or GAP also fail to regulate those enzymes. This may be the case. By probing immunoblots of GAP immunoprecipitates with antibodies to phosphotyrosine, we have found that PDGF stimulates tyrosine phosphorylation of GAP in cells expressing wild-type and Phe740 mutant receptors, but not in cells expressing Phe771 mutant receptors (unpublished results). Thus one consequence of the binding of GAP to phosphorylated PDGF receptors may be to bring GAP to the receptor so that it can be phosphorylated by the receptor or an associated tyrosine kinase.

Materials and methods

Site-directed mutagenesis

Site-directed mutagenesis was done as described previously (Kazlauskas and Cooper, 1989) on pRR4, a 1.35 kb *Sph*I–*Bam*HI fragment that contains the kinase insert region of the human PDGF receptor β-subunit (Gronwald *et al.*, 1988). Sequences of oligonucleotides are available upon request. All mutations were confirmed by sequencing. R635, F751 and F857 mutants of pRR4 were described previously (Kazlauskas and Cooper, 1989). Mutated *Sph*I–*Bam*HI fragments were moved from pRR4 into pRR6, which is the same as pRR3 (Kazlauskas and Cooper, 1989) except that the *Sph*I site in the polylinker had been destroyed. The entire PDGF receptor open reading frame was excised from pRR6 and its mutant derivatives using *Eco*RI and *Sal*I and cloned into pLXSN (Miller and Rosman, 1989) that had been cleaved with *Eco*RI and *Xho*I. pLXSN derivatives were transfected into ψ2 cells and viral supernatants were passaged through PA317 cells before infection of TRMP cells and selection for resistance to G418 (Kazlauskas and Cooper, 1989).

Cell labeling, immunoprecipitation and peptide mapping

TRMP cells were labeled if required for 4 h with 1 mCi/ml $^{32}P_i$, stimulated for 5 min at 37°C with 0 or 40 ng/ml recombinant PDGF-BB, lysed in EB, immunoprecipitated with monoclonal antibody PR7212, and subjected to *in vitro* kinase assays as described (Kazlauskas and Cooper, 1989). GST fusion proteins containing residues 699–798 (kinase insert) or 939–1108 (C-terminus) were prepared and phosphorylated as described (Kazlauskas *et al.*, 1991). Proteins were resolved by electrophoresis on 7.5% acrylamide, 0.193% bisacrylamide gels. Phosphopeptide mapping was performed exactly as described before (Kazlauskas and Cooper, 1989), except that following two digestions with trypsin, 100 μl of 10% 2-mercaptoethanol was added, the sample was heated at 100°C for 5 min and lyophilized. The sample was then digested twice, for 8–12 h each, with 10 μg thermolysin in 50 μl of 50 mM NH_4HCO_4, 1 mM $CaCl_2$ at 55°C. A further 5 μg of thermolysin was added and digestion continued for 8–12 h. The sample was then relyophilized repeatedly as described before, and analyzed by electrophoresis at pH 8.9 and chromatography in buffer I (Kazlauskas and Cooper, 1989). Phosphopeptides were quantified using a Molecular Dynamics Phosphorimager.

Immunoblotting and PI3 kinase assays

Immunoblotting for the PDGF receptor (using antiserum 2897 to the C-terminal domain of the receptor) and GAP (using anti-GAP B) has been described in detail before (Kazlauskas *et al.*, 1991). PI3 kinase assays were done as described (Kazlauskas and Cooper, 1989), except that the PI stock was dissolved in DMSO. Data were analyzed using a Phosphorimager (PI kinase assays) and a BioImage Visage system (immunoblots).

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