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Functions of the major tyrosine phosphorylation site of the PDGF receptor β subunit

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Two tyrosine phosphorylation sites in the human platelet-derived growth factor receptor (PDGFR) β subunit have been mapped previously to tyrosine (Y)751, in the kinase insert, and Y857, in the kinase domain. Y857 is the major site of tyrosine phosphorylation in PDGF-stimulated cells. To evaluate the importance of these phosphorylations, we have characterized the wild-type (WT) and mutant human PDGF receptor β subunits in dog kidney epithelial cells. Replacement of either Y751 or Y857 with phenylalanine (F) reduced PDGF-stimulated DNA synthesis to ~50% of the WT level. A mutant receptor with both tyrosines mutated was unable to initiate DNA synthesis, as was a kinase-inactive mutant receptor. Transmodulation of the epidermal growth factor receptor required Y857 but not Y751. We also tested the effects of phosphorylation site mutations on PDGF-stimulated receptor kinase activity. PDGF-induced tyrosine phosphorylation of two cellular proteins, phospholipase C γ1 (PLCγ1) and the GTPase activating protein of Ras (GAP), was assayed in epithelial cells expressing each of the mutant receptors. Tyrosine phosphorylation of GAP and PLCγ1 was reduced markedly by the F857 mutation but not significantly by the F751 mutation. Reduced kinase activity of F857 receptors was also evident in vitro. Immunoprecipitated WT receptors showed a two- to fourfold increase in specific kinase activity if immunoprecipitated from PDGF-stimulated cells. The F751 receptors showed a similar increase in activity, but F857 receptors did not. Our data suggest that phosphorylation of Y857 may be important for stimulation of kinase activity of the receptors and for downstream actions such as epidermal growth factor receptor transmodulation and mitogenesis.

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Introduction

Growth factors bind to cell surface receptors and initiate many changes, including DNA synthesis, mitosis, and cell division. The receptor for platelet-derived growth factor (PDGF) is a protein tyrosine kinase. Ligand binding activates its intrinsic enzymatic activity (reviews in Williams, 1989; Heldin and Westermark, 1990). This leads to the tyrosine phosphorylation of numerous intracellular proteins, including the receptor itself. The importance of these events in signal relay is best illustrated by the fact that mutant receptors lacking kinase activity fail to signal (Escobedo *et al.*, 1988a; Westermark *et al.*, 1990).

Some of the proteins that become tyrosine phosphorylated in a PDGF-stimulated cell are phospholipase C γ1 (PLCγ1), the GTPase activating protein of Ras (GAP), an 85-kDa subunit of phosphatidylinositol 3 kinase (PI3 kinase), and a number of less well-characterized proteins (reviewed in Cantley *et al.*, 1991). Some of the functional consequences of these phosphorylation events are starting to be determined, and it appears that they may contribute to the relay of a biological signal. For instance, tyrosine phosphorylation activates PLCγ1 (Nishibe *et al.*, 1990), leading to increased hydrolysis of phosphatidylinositol-4,5-bisphosphate, accumulation of diacylglycerol and inositol trisphosphate, and activation of protein kinase C. Tyrosine phosphorylation of GAP may be responsible for the two- to threefold increase in the GTP/GDP ratio of Ras observed in PDGF-stimulated cells (Gibbs *et al.*, 1990; Satoh *et al.*, 1990). These examples suggest that the activated receptor kinase mediates an intracellular signal by modifying the activities of cytoplasmic enzymes.

The PDGF receptor (PDGFR) is also tyrosine phosphorylated after activation by PDGF (Frackelton *et al.*, 1984). This may be important for activation, because some protein kinases are known to be regulated by tyrosine phosphorylation. Phosphorylation of a homologous tyrosine (Y) residue (equivalent to Y416 of Src) activates Src, the insulin receptor, Fps, and Lck (Rosen *et al.*, 1983; Weinmaster *et al.*, 1984;

Ellis *et al.*, 1986; Kmiecik *et al.*, 1988; Abraham and Veillette, 1990). In contrast, phosphorylation of Y15 of the *Schizosaccharomyces pombe* serine/threonine-specific cdc2 kinase inhibits kinase activity (Gould and Nurse, 1989). The β subunit of the human PDGFR is phosphorylated at Y751 and Y857 in response to binding PDGF (Kazlauskas and Cooper, 1989). One previous study has suggested that phosphorylation of the mouse PDGFR β subunit at the Y857 homologue is needed for receptor kinase activation (Fantl *et al.*, 1989). The contribution of phosphate at Y751 for receptor kinase activity has not yet been investigated.

In addition to the phosphorylation of numerous intracellular proteins, exposure of target cells to PDGF results in the stable association of the activated PDGFR with a number of intracellular proteins, including PLCγ1, GAP, a PI3 kinase activity, Src, Fyn, Yes, and several other less well-characterized proteins (reviewed in Ullrich and Schlessinger, 1990; Cantley *et al.*, 1991). These associations require receptor phosphorylation. Phosphorylation of Y751 is required for the stable association of PI3 kinase, GAP, and several other proteins, whereas phosphorylation of Y857 is not required for association of PI3 kinase, but is required for association of GAP (Kazlauskas and Cooper, 1989, 1990; Kazlauskas *et al.*, 1990).

Here we provide evidence that phosphorylation of the PDGFR at both of the phosphorylation sites is necessary to elicit a full mitogenic response to PDGF. In contrast, we found that phosphorylation of Y857, but not Y751, is required for PDGF-mediated receptor kinase activation and subsequent phosphorylation of a number of cellular substrates. Last, Y857 is necessary for transmodulation of the epidermal growth factor (EGF) receptor.

Results

Biological properties of epithelial cells expressing mutant PDGFRs

We have previously described certain properties of dog kidney epithelial TRMP cells expressing the wild-type (WT) and mutant PDGFR β subunits (Kazlauskas and Cooper, 1989). Mutant PDGFRs include kinase-inactive substitution R635 and phenlyalanine (F) substitutions F751, F857, and F751/F857. All of these mutants have the same binding affinity for pure yeast recombinant PDGF-BB as does the WT PDGFR. We have recently derived populations of TRMP cells expressing higher levels of the PDGFR, $\sim 10^5$ receptors/cell (Kazlauskas *et al.*, 1990), and we describe their biological and biochemical properties here.

Receptor abundance was assessed by ^{125}I-PDGF binding (data not shown) and by [^{35}S]methionine labeling (Figure 1). Receptor expression varied <10% between different cell lines. Mitogenic responsiveness was assayed by [^{3}H]thymidine uptake. As a positive control we stimulated cells with 10% fetal bovine serum (FBS), and incorporation is expressed as a percentage of the positive control. As with Balb/c 3T3 cells, for which PDGF is a poor mitogen in the absence of cooperating factors (Scher *et al.*, 1979), we found that the PDGF response of

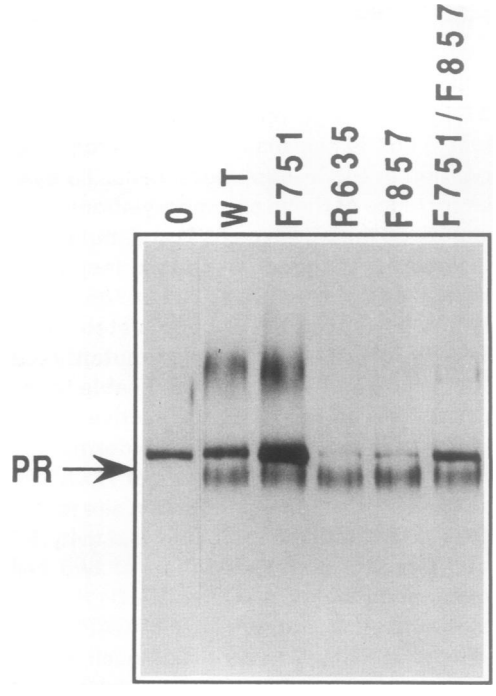

Figure 1. **Immunoprecipitation of the PDGFR from ^{35}S-labeled TRMP cells.** Confluent, quiescent cultures of TRMP cells were labeled overnight with 50 μCi/ml Tran-^{35}S-label, lysed, and immunoprecipitated with human-specific monoclonal PDGFR β subunit antibody PR7212 (see Materials and methods). The immunoprecipitates were resolved on a 7.5% acrylamide, 0.193% bisacrylamide SDS-PAGE gel, the gel was impregnated with 2,5-diphenyloxazole, and the radiolabeled proteins were detected by fluorography. The 180-kDa PDGFR (PR; marked with an arrow) was excised and the radioactivity quantitated and normalized relative to total incorporation. The receptor levels in all the cell lines were found to be within 10% of wild type. The amount of the 200-kDa species fluctuated in the various samples in replicate experiments and is probably unrelated to the PDGFR, because it is also recovered from the parental, PDGFR negative cell line. O: TRMP cells expressing an empty expression vector; WT: wild-type PDGFR; F751 and F857: phenylalanine substitutions for Y751 and Y857, respectively; R635: arginine substitution for K635; F751/F857: double phenylalanine substitution.

TRMP cells expressing WT PDGFRs was poor unless other growth factors were present. In serum-free medium, PDGF caused only ≤10% of the maximal response, but in the presence of 2% horse serum, PDGF initiated ~50% of the maximal response, with a half-maximal dose of 5–10 ng/ml of PDGF-BB (Figure 2). Two percent horse serum alone initiated 10–12% of the maximal response. Cells expressing an empty expression vector or the kinase-negative PDGFR were not stimulated by PDGF even in the presence of 2% horse serum. Mutation of either of the phosphorylation sites diminished the ability of the receptor to signal to ~50% of the WT level, whereas mutating both of the phosphorylation sites in the same PDGFR silenced the receptor. Whereas the response of the various cell lines to PDGF differed greatly, EGF (in the presence of 2% horse serum) stimulated DNA synthesis to 65–80% of maximal in all the cell lines. Thus it appears that mutation of either of the known PDGFR β subunit phosphorylation sites reduces the mitogenic response.

One effect of PDGF stimulation of cultured fibroblasts is to reduce the affinity of EGF binding to the EGF receptor (Wrann *et al.*, 1980). This is termed transmodulation. The mechanism of this effect is obscure, but it is rapid, occurring concomitantly with binding of PDGF to its receptor and even at 4°C (Bowen-Pope *et al.*, 1983). In TRMP cells expressing WT PDGFRs, we found a 30–39% reduction in specific binding of EGF after PDGF stimulation (Table 1). Stimulation with the phorbol diester 12-*O*-tetradecanoylphorbol-13-acetate resulted in a >98% reduction in EGF binding in all mutant cell lines (not shown). The F751 receptor could also mediate reduced EGF binding, although not as well as the WT receptor (Table 1). In contrast, the F857 PDGFR did not cause transmodulation. R635 and F751/F857 PDGFRs were also inactive. Hence Y857 of the PDGFR appears to be critical for transmodulation of the EGF receptor.

Tyrosine phosphorylation in intact cells

PLCγ1. In fibroblasts, PLCγ1 is tyrosine phosphorylated in response to activation of the PDGF or EGF receptors (Wahl *et al.*, 1988; Margolis *et al.*, 1989; Meisenhelder *et al.*, 1989). Anti-phosphotyrosine immunoprecipitates of EGF-

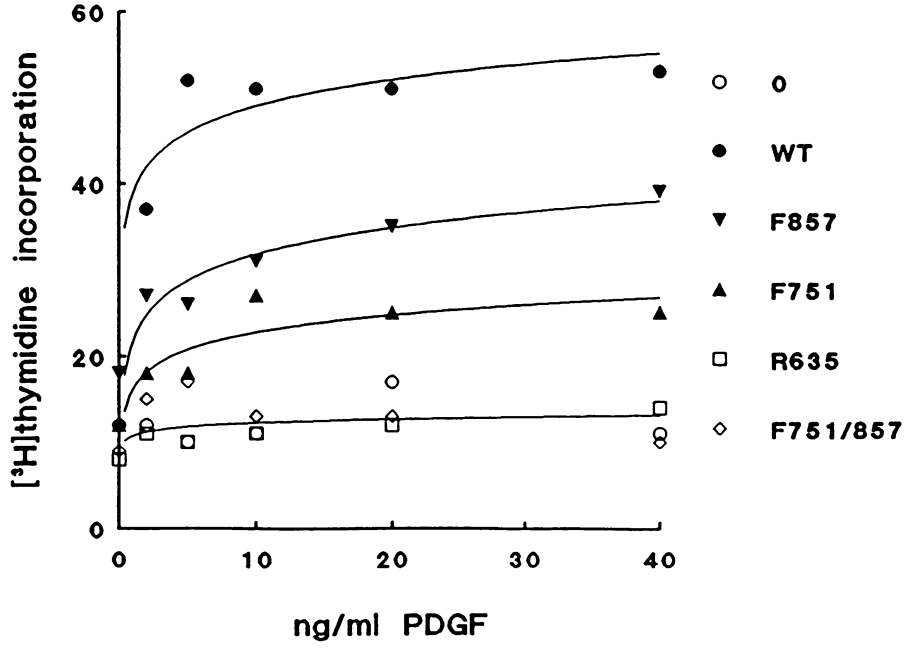

Figure 2. **Ability of PDGFR mutants to mediate DNA synthesis.** TRMP cells expressing the various PDGFR constructs (10^5/cell) were plated in triplicate at 4×10^4 cells/well in 24-well dishes and incubated for 2 d at 37°C, after which time one of the following additions was made: FBS to a final concentration of 10%, 2% horse serum, or 2% horse serum and the indicated concentration of PDGF-BB. After 18 h at 37°C, [³H]thymidine was added to 0.4 μCi/ml for 2 h at 37°C, after which time the TCA-precipitable material was harvested and counted in a scintillation counter. Replicate values were routinely within 1–2% of one another. The data are expressed as a fraction of the response to 10% FBS. Similar results were obtained in three other independent experiments. In this experiment, responses to EGF (50 ng/ml in the presence of 2% horse serum) were WT, 77%; F857, 80%; F751, 67%; R635, 64%; F751/F857, 70%; O, 43%. (The low response of TRMP-O cells to EGF was not reproducible).

Table 1. Transmodulation of EGF receptor by PDGF

Mutant	Reduction in specific EGF binding (%)	
	Experiment 1	Experiment 2
O	−4[a]	−13
WT	30	39
F751	23	28
R635	−3	−4
F857	10	−4
F751/F857	−3	−7

Confluent quiescent cultures of TRMP cells expressing similar amounts of the various PDGFR mutants were stimulated with 40 ng/ml PDGF for 30 min at 37°C, cooled to 4°C, and incubated with ^{125}I-EGF. Data are the percentages of reduction of specific ^{125}I-EGF binding to cells after PDGF stimulation, i.e., ratio of EGF binding in PDGF stimulated/unstimulated cells. Replicate values were routinely within 5% of each other. Specific binding was calculated by subtracting nonspecific binding from the total binding. 12-*O*-tetradecanoylphorbol-13-acetate (10 nM) reduced the binding of EGF by 98–100% in each of the cell lines tested in several different experiments.

[a] Negative values represent small increases in ^{125}I-EGF binding.

treated A431 cells or PDGF-treated Balb/c 3T3 cells, but not the corresponding unstimulated cells, have readily detectable PLC activity. Presumably this reflects tyrosine phosphorylation of either PLCγ1 or a protein with which it associates.

We compared the ability of the various PDGFRs to mediate a PDGF-dependent increase in the reactivity of PLC with anti-phosphotyrosine antibodies. Confluent quiescent cultures of TRMP cells expressing the various introduced human β subunits were stimulated with PDGF and lysed; the lysates were immunoprecipitated with 1G2 anti-phosphotyrosine antibodies. The immunoprecipitated proteins were eluted and then incubated with [^{3}H]phosphatidylinositol-4,5-bisphosphate to detect PLC activity. Immunoprecipitates from cells expressing the WT PDGFR had a 10- to 200-fold increase in PLC activity in response to PDGF (Table 2). Like the cells expressing an empty expression vector, those expressing the R635 (kinase inactive) PDGFR failed to show a PDGF-dependent increase in PLC activity. The F751 receptor was similar to the WT PDGFR, whereas the F857 and F751/F857 receptors were either unable or barely able to mediate a PDGF-dependent increase in the PLC activity detected in anti-phosphotyrosine immunoprecipitates (Table 2). The level of PDGF stimulation seemed to vary in replicate experiments because of different basal levels of activity, but the relative response of the mutant receptors within an experiment was highly reproducible.

The presence of PLCγ1 in anti-phosphotyrosine immunoprecipitates could be explained by its phosphorylation or its association with the tyrosine-phosphorylated proteins, including the PDGFR (Kumjian *et al.*, 1989; Kaplan *et al.*, 1990; Morrison *et al.*, 1990). To test directly whether PLCγ1 is phosphorylated on tyrosine in PDGF-stimulated TRMP cells, we labeled quiescent cells with ^{32}Pi, stimulated them with PDGF, lysed them, and immunoprecipitated them with a mixture of PLCγ1-specific monoclonal antibodies (Meisenhelder *et al.*, 1989). The proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the radiolabeled proteins were detected by autoradiography. The phosphorylation state of a 148-kDa phosphoprotein (the expected molecular mass of PLCγ1) increased modestly but reproducibly after PDGF stimulation of cells expressing the WT (1.8-fold increase) or F751 (1.6-fold) PDGFR (Figure 3A). In contrast, no increase in the 148-kDa band was detected after PDGF stimulation of cells expressing the F857 PDGFR. Phosphoaminoacid analysis of the 148-kDa phosphoprotein revealed primarily phosphoserine in unstimu-

Table 2. PLC activity in anti-phosphotyrosine immunoprecipitates

Mutant	Relative PLC activity (+PDGF/−PDGF)		
	Experiment 1	Experiment 2	Experiment 3
O	1.2	2.2	4
WT	9.7	207	105
R635	3.1	2.8	0
F751	11.7	139	79
F857	1.6	2.1	27
F751/F857	2.0	2.2	21

TRMP cells expressing equivalent numbers of PDGFR (10^4/cell) were grown to confluence, serum starved overnight, then left quiescent or stimulated with 50 ng/ml PDGF-BB, and lysed; the cell lysates were immunoprecipitated with the 1G2 phosphotyrosine monoclonal antibody. Proteins eluted from immunoprecipitates representing 10% of a 5-cm dish (2×10^9 receptors) were incubated with [^{3}H]phosphatidylinositol-4,5-bisphosphate for 15 min at 37°C; protein and uncleaved substrate were precipitated by adding TCA; and the amount of [^{3}H]inositol trisphosphate liberated was quantitated by counting the soluble fraction of the samples in a scintillation counter. The data are expressed as a ratio of PLC activity present in anti-phosphotyrosine immunoprecipitates from PDGF stimulated/unstimulated cells.

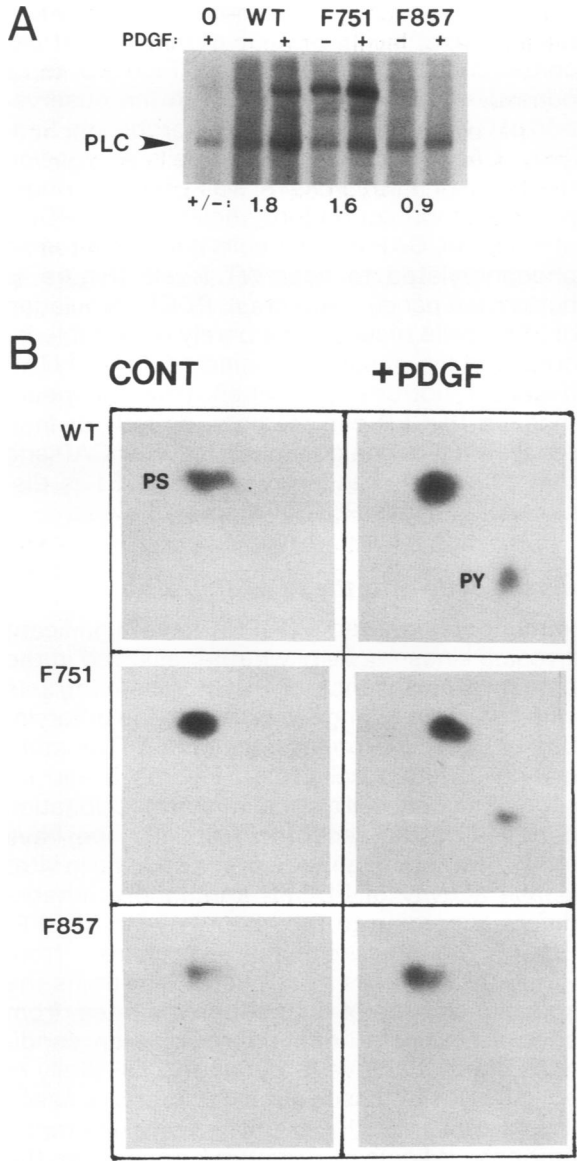

Figure 3. **Phosphorylation of PLCγ1.** Confluent, quiescent cultures of TRMP cells expressing the indicated PDGFR construct (10^4/cell) were metabolically labeled with 2.5 mCi/ml ^{32}Pi for 5 h, exposed to nothing (−) or 50 ng/ml PDGF-BB (+), and lysed; and PLCγ1 was immunoprecipitated with a mixture of PLCγ1-specific monoclonal antibodies. (A) Proteins were resolved on a 7.5% acrylamide, 0.193% bis-acrylamide SDS-PAGE gel, and the radiolabeled proteins were detected by autoradiography. The arrowhead points to a 148-kDa phosphoprotein that was excised and had its radioactivity quantitated by scintillation counting. The +/− at the bottom refers to the ratio of radioactivity in the 148-kDa PLCγ1 protein from PDGF treated/control samples. (B) Phosphoaminoacid analysis of the 148-kDa PLCγ1 protein. The 148-kDa protein from A was excised and hydrolyzed, and the resulting phosphoamino acids were resolved in two dimensions. Cont, unstimulated samples; +PDGF, PDGF-stimulated samples; PS, phosphoserine; PY, phosphotyrosine.

lated cells. PDGF treatment of cells expressing either the WT or F751 PDGFR, but not the F857 PDGFR, resulted in a detectable increase of the phosphotyrosine content of PLCγ1. Curiously, the WT and the F857 PDGFRs were also able to reproducibly enhance the phosphoserine content of PLCγ1, whereas the F751 PDGFR mediated an increase in tyrosine phosphorylation only. A 190-kDa phosphoprotein was occasionally detected in PLCγ1 immunoprecipitates, but phosphopeptide mapping of this protein revealed that it was not the PDGFR. Given that PLCγ1 and PDGFR can associate (Kumjian *et al.,* 1989; Kaplan *et al.,* 1990; Morrison *et al.,* 1990), it seems likely that this event is not detected by use of these immunoprecipitation conditions.

GAP. Like PLCγ1, GAP becomes tyrosine phosphorylated in PDGF-treated cells (Molloy *et al.,* 1989; Kaplan *et al.,* 1990; Kazlauskas *et al.,* 1990). GAP is also readily phosphorylated by the PDGFR in vitro (unpublished data), suggesting that the PDGFR may directly phosphorylate GAP in an intact cell. We tested whether mutations in the phosphorylation sites of the PDGFR affect its ability to tyrosine phosphorylate GAP. TRMP cells expressing similar levels of the various PDGFR constructs (10^5/cell) were either stimulated with PDGF or left quiescent and lysed; the lysates were immunoprecipitated with the monoclonal anti-phosphotyrosine antibody 1G2. Immunoprecipitated proteins were resolved on an SDS-PAGE gel and transferred to Immobilon. The high-molecular-mass proteins (150–250 kDa) were probed with an anti-PDGFR antibody, whereas the proteins in the 150- to 100-kDa range were probed with an anti-GAP antibody. As expected, the WT and the F751 PDGFRs (which contain the major tyrosine phosphorylation site) are readily recovered with the anti-phosphotyrosine antibody in response to PDGF stimulation (Figure 4). No PDGFR was immunoprecipitated from cells expressing an empty vector or from the kinase-inactive PDGFR, even after PDGF treatment. The F857 PDGFR, lacking the major autophosphorylation site, and, to a lesser extent, the F751/F857 PDGFRs were also immunoprecipitated from PDGF-treated cells. These PDGFRs contain other minor sites of tyrosine phosphorylation (Kazlauskas and Cooper, 1989), which probably explains the ability to immunoprecipitate phenylalanine mutant PDGFRs with anti-phosphotyrosine antibody. About 4% of cellular GAP was detected in immunoprecipitates from PDGF-stimulated WT and F751 cells (Figure 4,

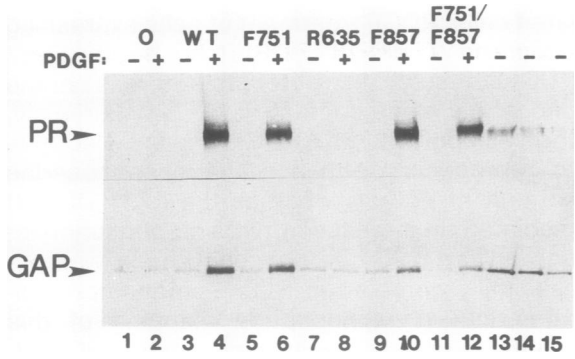

***Figure 4.* Detection of the PDGFR and GAP in anti-phosphotyrosine immunoprecipitates of cells expressing the various PDGFR mutants.** Confluent, quiescent 5-cm dishes of TRMP cells expressing similar levels (10^5/cell) of PDGFR mutants were stimulated with 40 ng/ml PDGF-BB (+) or left quiescent (−) and lysed, and the cell lysates were immunoprecipitated with monoclonal antiphosphotyrosine antibody 1G2. The proteins immunoprecipitated from the entire 5-cm dish (2×10^6 cells) were resolved on a 7.5% acrylamide, 0.193% bisacrylamide SDS-PAGE gel. After transfer to Immobilon, the top and bottom portions were probed with anti-GAP and anti-PDGFR antibodies, respectively. The top arrowhead points to the PDGFR (PR); the bottom arrowhead points to GAP. Lanes 13, 14, and 15 contain 2, 1, and 0.5%, respectively, of total cell lysate of TRMP cells expressing the WT PDGFR. The type of PDGFR expressed in the various cell lines is along the top of the figure and explained in the legend of Figure 1; R635 denotes the mutant in which lysine at position 635 is replaced with arginine.

lanes 3–6), 0.5–1.0% in anti-phosphotyrosine immunoprecipitates from PDGF-stimulated F857 and F751/F857 cells (Figure 4, lanes 9–12), and background levels (0.2% of cellular GAP) in immunoprecipitates from cells expressing the kinase negative PDGFR or no PDGFR (Figure 4, lanes 1, 2, 7, and 8).

GAP is known to associate with a number of tyrosine-phosphorylated proteins in transformed or growth factor-stimulated cells (Ellis *et al.,* 1990; Bouton *et al.,* 1991). We have found that only 20% of the GAP that binds to the activated WT PDGFR binds to activated F751, F857, or F751/F857 mutant PDGFRs (Kazlauskas *et al.,* 1990). Thus the equal GAP recovery from PDGF-treated WT and F751 cells suggests that GAP itself is tyrosine phosphorylated. Correspondingly, the decreased immunoprecipitation of GAP from F857 cells suggests that GAP is poorly tyrosine phosphorylated by this PDGFR. This was confirmed by examining the phosphoaminoacid content of GAP in the various cell lines after PDGF stimulation. Cells were metabolically labeled with ^{32}Pi, stimulated with PDGF, and lysed. GAP was immunoprecipitated, resolved by SDS-PAGE, and assayed for phosphoaminoacid content. GAP from resting cells expressing the WT PDGFR had primarily phosphoserine, and, after PDGF stimulation, it becomes tyrosine phosphorylated (Figure 5, top 2 panels), which is consistent with the observation of GAP phosphorylation in fibroblasts. Similarly, GAP from unstimulated cells expressing the F751 or F857 PDGFR was primarily phosphorylated on serine (not shown). After PDGF stimulation, GAP in F751 cells became tyrosine phosphorylated to near WT levels (Figure 5, bottom left panel). In contrast, PDGF stimulation of F857 cells resulted in a barely detectable increase in the phosphotyrosine content of GAP (Figure 5, bottom right panel). Thus it appears that the PDGFR can mediate, directly or indirectly, the tyrosine phosphorylation of GAP and that Y857 is necessary whereas Y751 is dispensable for this PDGFR-mediated event.

Kinase activity of the PDGFR in vitro

Immunoprecipitated PDGFRs have significant tyrosine kinase activity whether assayed in the presence or absence of PDGF. This contrasts with the large change in tyrosine phosphorylation of specific proteins caused by PDGF stimulation of intact cells (e.g., Figures 3 and 5), suggesting that cell lysis or immunoprecipitation deregulates the receptor. Recently, we have found that the magnitude of the specific in vitro kinase activity of PDGFR immunoprecipitates differs according to the history of the PDGFR. PDGFR immunoprecipitates prepared from PDGF-treated cells have two to four times the specific activity of immunoprecipitates from control cells. The immunoprecipitation conditions should remove the bound PDGF (Kelly *et al.,* 1991) and associated Src-family kinases (Kypta *et al.,* 1990), so this activity difference probably reflects a structural change in the PDGFR.

The PDGFR β subunit was immunopurified from nonionic detergent lysates with an antibody raised to its carboxyterminal tail (Kazlauskas and Cooper, 1990), washed rigorously in ionic detergents, and incubated with exogenous substrates and [γ^{32}P]ATP. The labeled proteins were resolved by SDS-PAGE and detected by autoradiography. The kinase insert and the carboxyterminal tail of the human PDGFR β subunit expressed as bacterial fusion proteins are good in vitro substrates of the β subunit. In the absence of receptor these substrates are not phosphorylated (Figure 6, lane 11, and data not shown), and PDGFR immunoprecipitates prepared from TRMP cells expressing an empty PDGFR expression vector or the kinase-inactive

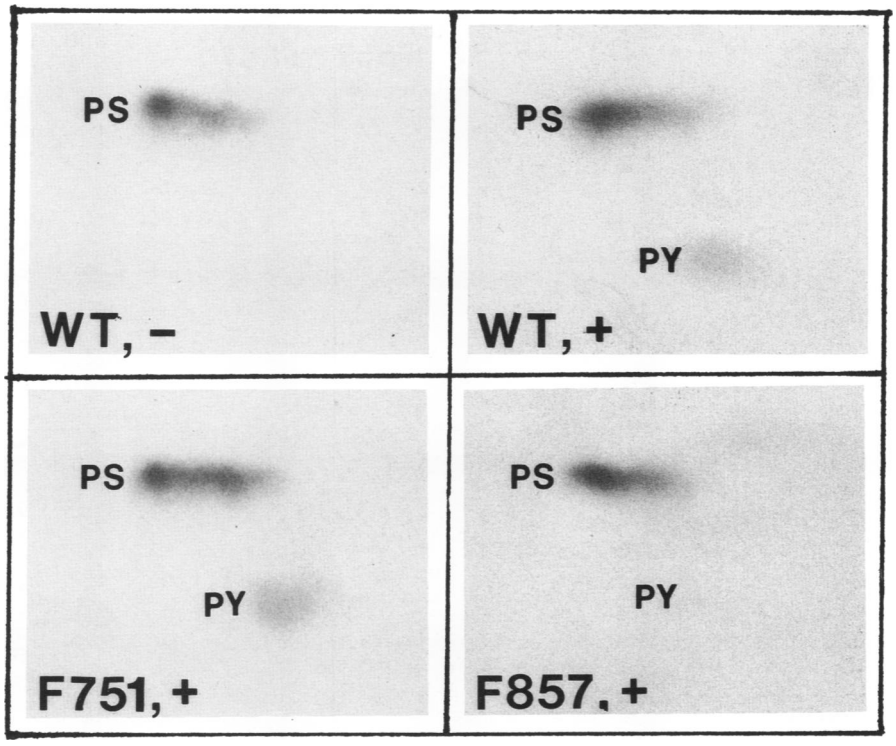

Figure 5. **Phosphoaminoacid analysis of GAP.** Confluent, quiescent 5-cm dishes of TRMP cells expressing similar (10^5/cell) levels of WT, F751, or F857 PDGFRs were labeled with 3 mCi/ml ^{32}Pi for 3 h, incubated with (+) or without (–) 40 ng/ml PDGF-BB for 10 min, and lysed; and the lysates were immunoprecipitated with an anti-GAP antibody. Immunoprecipitated proteins were resolved on a 7.5% acrylamide, 0.193% bisacrylamide SDS PAGE gel, and radiolabeled proteins were detected by autoradiography. The 124-kDa GAP phosphoprotein was excised, eluted from the gel, and acid hydrolyzed; and the resulting phosphoaminoacids were resolved electrophoretically in two dimensions. PS, phosphoserine; PY, phosphotyrosine.

β subunit phosphorylated the exogenous substrate to a very low level (Figure 6, lanes 1 and 2, and data not shown). The WT PDGFR β subunit isolated from PDGF-treated cells phosphorylated exogenous substrates to a 2.6 ± 1.0-fold (n = 4) greater extent than the WT PDGFR from unstimulated cells (Figure 6, lanes 3 and 4; Table 3). The F751 PDGFR β subunit mutant also had higher in vitro kinase activity toward exogenous substrates when isolated from PDGF-stimulated cells (2.3 ± 0.2, n = 4; Figure 6, lanes 5 and 6; Table 3). In contrast, we did not detect a reproducible PDGF-stimulated increase in the kinase activity of the F857 or the F751/F857 double mutant PDGFR (1.1 ± 0.2 and 1.0 ± 0.4, respectively, n = 4; Figure 6, lanes 7–10; Table 3). Note that, in contrast to substrate phosphorylation, receptor autophosphorylation tends to be diminished somewhat in PDGF-stimulated cells. Similar amounts of receptor were detected when these same immunoprecipitates were analyzed by PDGFR Western blot analysis (data not shown). The diminished receptor autophosphorylation of PDGF-stimulated samples may be due to the incorporation of nonradioactive phosphate before cell lysis. Thus it appears that tyrosine at position 857, but not 751, is required to mediate a PDGF-dependent increase in β subunit in vitro kinase activity.

Discussion

We have compared the ability of a number of PDGFR mutants to phosphorylate GAP and PLCγ1 in living dog epithelial cells and model substrates in vitro. From these studies we conclude that tyrosine at position 857 plays a role in the PDGF-dependent activation of PDGFR β subunit tyrosine kinase activity. In contrast, tyrosine at position 751 is not required for kinase activation, at least not for the substrates we have studied. Y857 is homologous to the major autophosphorylation sites in Src, Fps, Lck, and the insulin receptor. Phosphorylation of these homologous residues stimulates the activity of each of these tyrosine kinases (Weinmaster *et al.*, 1984; Ellis *et al.*, 1986; Kmiecik *et al.*, 1988; Abraham and Veillette, 1990). Given that Y857 is the major tyrosine phosphorylation site in the PDGFR in PDGF-stimulated cells (Kazlauskas and Cooper, 1989), we propose that phosphorylation of Y857 stimulates receptor tyrosine kinase activity.

Y857 is also necessary for a biological response to PDGF, namely, the heterologous down-regulation of the EGF receptor (Wrann *et al.*, 1980). PDGF stimulation causes a decrease in the affinity, not the number, of EGF receptors. This is a rapid effect of PDGF on cells, but the mechanism is unclear (Bowen-Pope *et al.*,

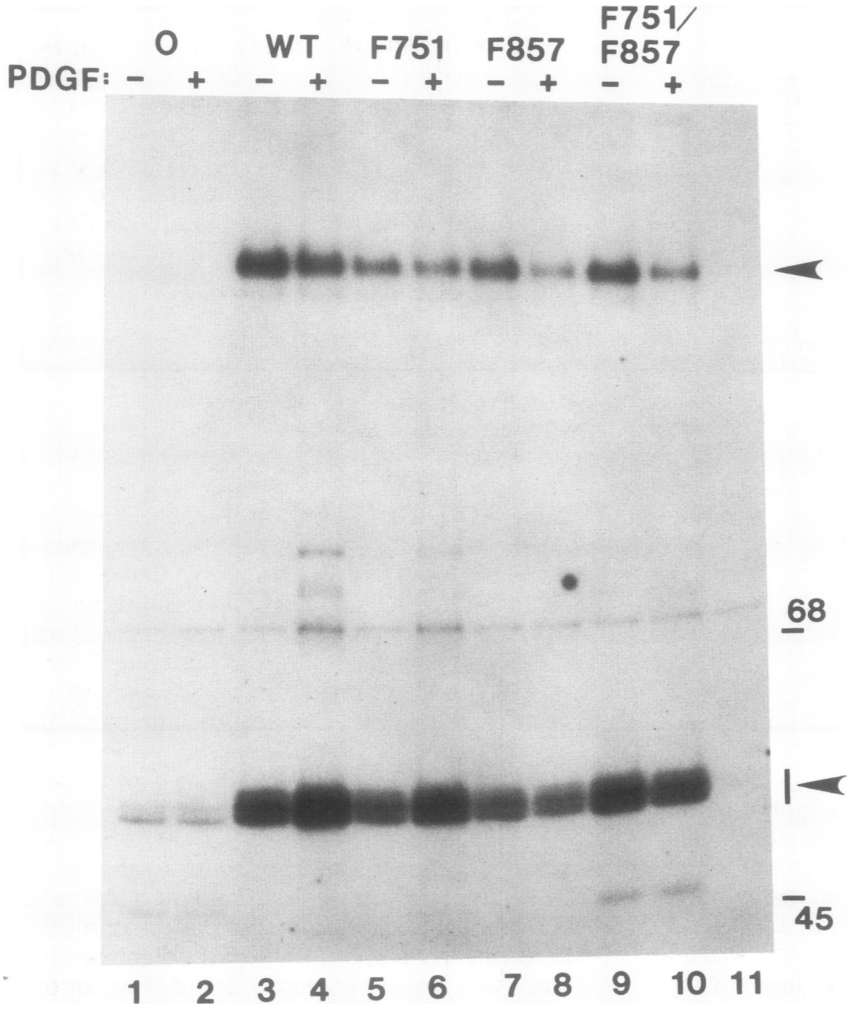

Figure 6. **In vitro kinase activity of PDGFR mutants.** Confluent, quiescent 5-cm dishes of TRMP cells expressing similar levels (10^5/cell) of introduced human PDGFR β subunit were stimulated with 40 ng/ml PDGF-BB (+) for 5 min at 37°C or left quiescent (−) and lysed; the PDGFR was immunoprecipitated with antibody 2678. Receptor immunoprecipitates representing 10% of a 5-cm dish ($\sim 2 \times 10^{10}$ receptors) were incubated with [γ^{32}P]ATP and 1 μg TAIL (the purified glutathione-S-transferase-PDGFR β subunit fusion protein) for 10 min at 30°C, the proteins were resolved on a 12.5% acrylamide, 0.10% bisacrylamide SDS-PAGE gel, and radiolabeled proteins were detected by autoradiography. Lane 11 contained all reaction ingredients with the exception of receptor immunoprecipitate. The upper arrowhead marks the position of the PDGFR; the lower arrowhead denotes the position of the fusion protein substrate, which is a doublet. The positions of molecular weight standards are indicated on the right. The designations above the lanes refer to the type of PDGFR expressed and are described in the legend of Figure 1.

1983). In human diploid fibroblasts, PDGF induces EGF receptor phosphorylation at threonine 654, a known site of protein kinase C phosphorylation (Davis and Czech, 1985). This suggests a mechanism in which PDGF activates protein kinase C, which then downregulates the EGF receptor. However, one report indicates that protein kinase C is not necessary for PDGF-induced phosphorylation of threonine 654, because the phosphorylation occurs normally in cells deficient in protein kinase C (Davis and Czech, 1987). Significantly, the phosphorylation may be unrelated to the reduction in EGF binding, because alanine 654 mutant receptors, expressed in Chinese hamster ovary (CHO) cells, are susceptible to downregulation by PDGF (Countaway *et al.,* 1989). Therefore, PDGF-induced downregulation may involve neither protein kinase C nor phosphorylation of the EGF receptor at threonine 654. An alternative explanation is that there exist two or more signal pathways with the potential to transmodulate the EGF receptor. The requirement for Y857 in the PDGFR may indicate that stimulation of PDGFR tyrosine kinase activity is required for both pathways. The particular substrates involved are not known, but the reduced phosphorylation of PLCγ1 by F857 mutant PDGFRs may lead to a reduced activation of protein kinase C. PDGF-induced tyrosine phosphorylation of the EGF receptor has not been reported. Possibly an uncharacterized substrate for the PDGF receptor is involved in EGF receptor downregulation.

Expression of the human PDGFR β subunit in dog kidney epithelial cells (which express no detectable PDGFRs) enabled these cells to synthesize DNA in response to PDGF. Thus, existing second-messenger pathways must be able to interface with the mitogenic signals initiated by the PDGFR. Others have observed mitogenic signaling by PDGFRs introduced into CHO cells

Table 3. Quantitation of in vitro substrate phosphorylation

	Relative phosphorylation (+PDGF/−PDGF)			
	Experiment 1		Experiment 2	
Mutant	KI	TAIL	KI	TAIL
WT	1.5	2.0	2.8	4.1
F751	2.4	2.2	2.1	2.5
F857	1.3	0.9	1.2	1.1
F751/F857	0.9	0.8	0.8	1.6

Two different substrates were used to measure the in vitro kinase activity of PDGFR mutants, as described in Figure 6. KI, fusion protein with the entire kinase insert domain of the β PDGFR (residues 699–798) M_r 45; TAIL, fusion protein with the carboxy terminus of the β PDGFR (residues 939–1108) M_r 53. Extent of phosphorylation was determined by excising the fusion proteins and counting in a scintillation counter. Fold increase was calculated as the ratio of substrate phosphorylation (radioactivity incorporated) by PDGFRs from PDGF stimulated vs. unstimulated cells.

(Escobedo *et al.*, 1988b) or myeloid-progenitor 32D cells (Matsui *et al.*, 1989), and there are many other examples of receptor tyrosine kinases that can function in heterologous cells. Either the PDGFR can engage many different sorts of signal relay cascades, or different membrane receptors use a common intracellular pathway to transmit extracellular signals to the nucleus, as suggested by the finding of common substrates. We found that expression of 10^5 receptors/cell, which is similar to the receptor levels on fibroblasts and smooth muscle cells that naturally express the PDGFR, was sufficient to mediate DNA synthesis in TRMP cells, but at 10^4 receptors/cell only a very weak mitogenic response was detected (data not shown). Perhaps a certain threshold of stimulation is required to overcome negative growth signals or to activate the necessary subsequent signaling events.

A full mitogenic response requires tyrosine at both of the identified receptor autophosphorylation sites. A double mutant receptor was mitogenically inactive, but single mutants each gave a partial response, suggesting that both tyrosine residues function independently in mitogenesis. Previously we have shown that Y751 is needed for PDGF-induced binding of PI3 kinase, GAP, and several unidentified polypeptides (Kazlauskas and Cooper, 1989; Kazlauskas *et al.*, 1990). In vitro studies demonstrated that phosphorylation of Y751 creates a binding site for these proteins (Kazlauskas and Cooper, 1990). Y857 is also needed for binding of GAP, but not for binding PI3 kinase. Our present results suggest that Y857 is needed for full kinase activation and for phosphorylation of GAP and PLCγ1. Thus there may be two pathways leading from the activated PDGF receptor: one involving binding of PI3 kinase and other proteins to a site created by phosphorylation of Y751, and another involving substrate tyrosine phosphorylation (requiring Y857). Blocking both these pathways by the double mutant blocks mitogenesis, but blocking either pathway alone allows a partial mitogenic response. Of course, the actual situation is undoubtedly more complicated, with additional pathways and cross talk between pathways. Even so, some of the phenotypes of the PDGFR mutants are surprisingly distinct. For example, F751 mutant PDGFRs fail to bind GAP, yet cause GAP tyrosine phosphorylation at almost WT levels. This suggests that binding of GAP may be functionally distinct from phosphorylation of GAP. F857 receptors bind PI3 kinase but not GAP, suggesting different binding sites. Furthermore, F751 receptors phosphorylate (Figures 3 and 4) and bind (S. Courtneidge, personal communication) PLCγ1. This suggests three classes of binding proteins: those requiring Y751 (PI3 kinase), those independent of Y751 (PLCγ1), and those requiring Y751 and Y857 (GAP).

Given that F857 receptors have reduced kinase activity, reduced phosphorylation of Y751 and reduced binding of PI3 kinase might be expected, but this was not observed. Phosphopeptide mapping shows that Y751 phosphorylation in the F857 mutant appears to occur to WT levels in vivo (data not shown). Thus, unlike GAP, PLCγ1, and certain in vitro substrates, phosphorylation of Y751 in vivo may not require phosphorylation of Y857. Together with the observation that the stoichiometry of Y751 to Y857 phosphorylation in vivo is ~1:5, whereas in vitro Y751 is the favored site (Kazlauskas and Cooper, 1989), it appears that there are additional components regulating receptor phosphorylation.

Our results with the F857 receptor may be compared with those from the laboratory of Williams (1989), where a corresponding mutation has been made in the murine PDGFR β subunit and expressed in CHO cells. Researchers found that the phenylalanine mutant was essentially inactive in signaling cell proliferation (Fantl *et al.*, 1989), whereas our mutant had considerable residual ability to stimulate DNA synthesis (Figure 2). This may not be a serious difference, because different cell types may

have different requirements for activation of specific signaling pathways. Alternatively, cell proliferation in CHO cells may require more PDGFR functions than are needed for DNA synthesis. Like us, they found that mutation of the Y857 homologue did not affect binding of PI3 kinase (Coughlin *et al.,* 1989). In addition, they detected tyrosine phosphorylation of PLCγ1, although the phosphorylation level appears to be <50% of WT (Morrison *et al.,* 1990). Binding of PLCγ1 to the mutant receptor was also reduced. In vitro, they found that adding PDGF to cell lysates, in the presence of Mg^{2+} and ATP, resulted in increased kinase activity of the WT PDGFR and not the phenylalanine mutant, assayed after subsequent immunoprecipitation (Fantl *et al.,* 1989). This result resembles ours, obtained by pretreating cells with PDGF before lysis and immunoprecipitation. Presumably, in both assays, PDGF induces PDGFR phosphorylation at Y857, which survives immunoprecipitation and stimulates in vitro phosphorylation.

The emerging picture is that PDGF binding induces receptor tyrosine phosphorylation, which activates the receptor kinase toward exogenous substrates and creates binding sites for cellular signaling enzymes (Ullrich and Schlessinger 1990; Cantley *et al.,* 1991). The precise mechanisms that induce receptor phosphorylation and kinase activation require further study, but the evidence for ligand-induced receptor dimerization suggests a model (Bishayee *et al.,* 1989; Hammacher *et al.,* 1989; Seifert *et al.,* 1989). Phosphorylation of Y857 in the PDGFR β subunit, and of the homologous residue in Src, can occur in trans, with one kinase phosphorylating another (Cooper and MacAuley, 1988; Kelly *et al.,* 1991). Therefore PDGFR dimerization may promote "autophosphorylation" in trans by bringing two kinase domains together. Because both subunits of a dimer probably are bound to PDGF (which is itself dimeric), both subunits could become tyrosine phosphorylated by this mechanism. Phosphorylation may induce a conformation change (or stabilize an otherwise unstable conformation), causing enzymatic activation. A PDGF-induced conformation change in the PDGFR has been detected with an antipeptide antibody (Bishayee *et al.,* 1988; Keating *et al.,* 1988). Whether the molecules detected by this antibody represent enzymatically activated PDGFRs or functional dimers is not known. Once phosphorylated at Y857, the receptor may remain active even when PDGF dissociates, as shown for the insulin receptor (Rosen *et al.,* 1983). This result is indicated by our in vitro experiments, although we do not know what fraction of the ligand-stimulated activity survives after ligand is removed and receptors isolated. If PDGFR subunits remain active after PDGF dissociates, then it is possible that dimers may separate into active monomers, possibly with associated proteins remaining complexed. Activated monomeric or dimeric PDGFRs then phosphorylate cellular substrate proteins, leading ultimately to other downstream effects.

Materials and methods

Cell culture

The expression of WT and mutant forms of the human PDGFR β subunit in TRMP cells (Turker *et al.,* 1988) has been previously described (Kazlauskas and Cooper, 1989; Kazlauskas *et al.,* 1990). For most experiments TRMP cells expressing $\sim 10^5$ PDGFRs/cell were used, whereas the PLCγ1 studies were performed on cells expressing $\sim 10^4$ PDGFRs/cell. Infected TRMP cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 0.25 mg/ml G418.

Antibodies

A polyclonal antibody to the PDGFR was raised in rabbits against the carboxy terminus (amino acids 939–1108) of the human β PDGFR subunit fused to glutathione S transferase (see below). Two rabbits were immunized, resulting in antiserum 2678, which was used to immunoprecipitate the PR, and antiserum 2897, which was used to detect the PDGFR in a Western blot analysis. The antisera appear specific, inasmuch as they fail to detect any PDGFR in TRMP cells that express an empty expression vector. The mouse monoclonal antibody to PLCγ1 was a mixture of PLCγ1-specific reagents raised against purified bovine PLCγ1 and has been previously described (Suh *et al.,* 1988; Meisenhelder *et al.,* 1989). The monoclonal anti-phosphotyrosine 1G2 antibody, monoclonal anti-human β PDGFR PR7212 antibody, and a rabbit polyclonal antiserum to GAP have been previously described (Hart *et al.,* 1987; Huhn *et al.,* 1987; Ellis *et al.,* 1990).

Fusion proteins

The kinase insert (amino acids 699–798) and carboxy terminus (amino acids 939–1108) of the human PDGFR β subunit were amplified by polymerase chain reaction. In doing so, *Eco*RI and *Bam*HI restriction sites were introduced, permitting the inframe subcloning of these fragments into the pGEX-3X (Smith and Johnson, 1988) expression vector. Fusion proteins were purified to near homogeneity from bacterial cell lysates on a glutathione agarose affinity column and were specifically eluted with glutathione.

DNA synthesis assay

TRMP cells were grown to confluence in DMEM containing 10% FBS, trypsinized, diluted into DMEM with no serum, and seeded in triplicate at 4×10^4 cells/well in a 24-well dish in DMEM with no serum. After 2 d, horse serum was added to a final concentration of 2% together with various concentrations of PDGF or EGF. Alternatively, cells were stimulated with 10% FBS. [^{3}H]thymidine (0.4 μCi/ml) was added after 18 h, and 2 h later the cells were washed, the trichloroacetic acid (TCA)-precipitable material was har-

vested and quantitated as previously described (Kazlauskas *et al.*, 1988). Replicate samples routinely lay within a 10% range.

EGF receptor transmodulation

TRMP cells were plated in triplicate in 24-well dishes at 1 $\times 10^5$ cells per dish in DMEM containing 10% FBS and grown to confluency. Cells were then incubated overnight in DMEM containing 0.1% FBS. This medium was aspirated and replaced with 0.25 ml of DMEM containing 2 mg/ml bovine serum albumin (BSA) and 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.2 (BM), to which was added PDGF-BB (40 ng/ml final concentration) or phorbol myristate acetate (final concentration 10 nM). Cells were incubated for 30 min at 37°C and placed on ice; the medium was replaced with 0.25 ml of ice-cold BM. Excess unlabeled EGF (final concentration 100 nM) was then added to certain wells for the determination of nonspecific binding of ^{125}I-EGF. To all wells 120 pM ^{125}I-EGF (specific activity 107 μCi/μg; Collaborative Research, Bedford, MA) was added, and the plate was incubated at 4°C with continuous agitation for 2 h. The cells were then washed three times on ice with 1.0 ml of 1 mg/ml BSA in phosphate-buffered saline (RB), followed by the addition of 0.5 ml of a solubilization solution containing 1% Triton X-100 and 1 mg/ml BSA. Plates were agitated vigorously at room temperature for 15 min, after which time samples were removed and counted in a gamma counter. Specific binding of ^{125}I-EGF was calculated by subtracting the nonspecific binding (determined for each cell line by measuring the ^{125}I-EGF binding in the presence of 840-fold excess of unlabeled EGF) from the total binding. The nonspecific binding was similar for all the cell lines and ranged between 5 and 10% of the total EGF bound (data not shown).

Immunoprecipitation

TRMP cells were grown to confluence in DMEM containing 10% FBS, and switched to 0.1% calf serum in DMEM overnight. For phosphate labeling, DMEM containing 50 μM phosphate was used, and ^{32}Pi was added as indicated in the figure legends. For ^{35}S labeling, methionine-free DMEM was used and Tran-^{35}S-label (ICN Irvine, CA) was added at 50 μCi/ml overnight. Cells were then stimulated with PDGF-BB for 5 or 10 min (as indicated in specific experiments) at 37°C and rinsed twice with ice-cold H/S, lysed in EB (Kazlauskas and Cooper 1988); the lysate was cleared by centrifugation at 15 000 $\times$ *g* for 30 min at 4°C. Subsequent steps for immunoprecipitation with GAP and 1G2 antibodies have been described in detail elsewhere (Kazlauskas and Cooper, 1988; Kazlauskas *et al.*, 1990).

To precipitate PDGFRs for kinase assays, we added a 2-μl aliquot of 2678 antibody to clarified cell lysates; after 1.5 h at 0°C, 30 μl of a 10% solution of Formalin-fixed *Staphylococcus aureus* was added and incubated for an additional 30 min at 0°C. Immune complexes were spun through a cushion of 10% sucrose in EB, then washed twice with RIPA (Kazlauskas and Cooper, 1988), twice with EB, and twice with a solution of 20 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES) pH 7.0, 100 mM NaCl, and 20 μg/ml aprotinin (PAN). The final pellet was resuspended in 50 μl PAN and stored at −70°C.

Immunoprecipitation of ^{32}P-labeled cell lysates with PLCγ1 antibody and of [^{35}S]methionine-labeled cell lysates with PR7212 antibody was performed by incubating clarified RIPA lysates with 1–1.5 μg of antibody for 2 h, followed by 15 min with 6 μg of rabbit anti-mouse antibody, followed by 30 min with 25 μl of a 10% solution of Formalin-fixed *S. aureus*. Immune complexes were spun through a 10% sucrose solution in RIPA and washed twice with RIPA, twice with 0.5% Nonident P-40 in PAN, and twice with PAN. The washed pellets were resuspended in sample buffer (Kazlauskas and Cooper, 1988), heated at 98°C for 5 min, and resolved on a 7.5% acrylamide, 0.193% bisacrylamide SDS-PAGE gel.

Phospholipase C assay

Antiphosphotyrosine immunoprecipitates were recovered from the sepharose-immobilized 1G2 antibody by incubating in 40 μl of a solution containing 10 mM tris(hydroxymethyl)aminomethane (Tris)-HCl pH 7.5, 5 mM EDTA, 50 mM NaF, 10 mM NaCl, 0.1 mg/ml ovalbumin, 0.01% Triton X-100, 20 μg/ml aprotinin, and 1 mM phenylphosphate. The PLCγ1 activity of the liberated material was assayed as previously described (Wahl *et al.*, 1988). Briefly, a 4-μl aliquot was incubated in a final volume of 40 μl containing the following ingredients: 70 mM KCl, 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) pH 6.0, 4 μl of 5 mM $CaCl_2$, 50 μM [^{3}H]phosphatidylinositol-4,5-bisphosphate (5 mCi/mmole). The samples were incubated for 15 min at 37°C, and 100 μl of a 10 mg/ml solution of BSA was added, followed by 200 μl of ice-cold 20% TCA. Samples were incubated at 0°C for 5 min and spun at 8000 $\times$ *g* for 5 min, and the TCA soluble fraction was combined with scintillation fluid and counted. For preparation of the substrate, $\sim 2 \times 10^6$ cpm of phosphatidylinositol[2-^{3}H]-4,5-bisphosphate and 100 pmol unlabeled phosphatidylinositol-4,5-bisphosphate were evaporated to dryness, resuspended in 80 μl of 2.5% octylglucoside in water, and sonicated in a bath sonicator for 15 min at 4°C. The octylglucoside was diluted to 1% by adding 120 μl of H_2O and then the solution was resonicated for an additional 15 min at 4°C. A 1/10th volume of this solution was added to the reaction so that the final detergent concentration was 0.1% octylglucoside (from the substrate) and ~0.001% Triton X-100 (from the immunoprecipitate).

Phosphoaminoacid analysis

Phosphoproteins were detected by autoradiography; the protein of interest was excised from the SDS-PAGE gel, eluted, and hydrolyzed in 5.7 M HCl for 1 h at 110°C; and the resulting phosphoaminoacids were resolved electrophoretically in two dimensions, exactly as described previously (Cooper *et al.*, 1983).

Western blot analysis

After the resolution of samples on a 7.5% acrylamide, 0.193% bisacrylamide SDS-PAGE gel, they were transferred (5 mA-h/cm^2) to Immobilon, incubated in Blotto (1% dry nonfat milk in phosphate-buffered saline plus 0.05% Tween 20 and 0.005% NaN_3) for 1 h at room temperature, and then incubated overnight with either 2897 diluted 1/1000 or anti-GAP B diluted 1/200. Immobilon was washed four times, 5 min each wash, in Rinse buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl), then incubated for 4 h at room temperature with an alkaline phosphatase-conjugated goat anti-rabbit antibody (Southern Biotechnology Associates, Birmingham, AL). The Immobilon was washed twice with Rinse buffer and twice with alkaline phosphatase buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) and then developed by incubating with 0.33 mg/ml p-nitro blue tetrazolium chloride and 0.165 mg/ml 5-bromo 4-chloro 3-indolylphosphate toluidine salt in alkaline phosphatase buffer for 5–20 min at room temperature.

In vitro kinase assay

An aliquot of 2678 PDGFR immunoprecipitate, representing 10% of a 5-cm dish of cells (2×10^{10} receptors), was com-

bined with 1 μg of substrate (bacterially expressed fusion proteins consisting of glutathione S-transferase fused with either the kinase insert [amino acids 699–798] or the carboxy terminus [residues 939–1108] of the human PDGFR β subunit), 20 mM PIPES pH 7.0, 10 mM $MnCl_2$, 5 μCi [γ^{32}P]ATP (3000 Ci/mmole), and 20 μg/ml aprotinin in a final volume of 10 μl. After 10 min at 30°C, the reactions were terminated by adding 10 μl of twofold concentrated sample buffer (Kazlauskas *et al.*, 1988) and heated at 98°C for 5 min. Phosphoproteins were resolved in a 7.5% acrylamide, 0.193% bisacrylamide SDS-PAGE gel and visualized by autoradiography. Under these conditions, excess ATP remains at the end of kinase assay (not shown).

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