Phosphorylation Sites at the C-terminus of the Platelet-Derived Growth Factor Receptor Bind Phospholipase C γ 1

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We have identified two tyrosine phosphorylation sites, Tyr 1009 and Tyr 1021, in the C-terminal noncatalytic region of the human platelet-derived growth factor (PDGF) receptor β subunit. Mutant receptors with phenylalanine substitutions at either or both of these tyrosines were expressed in dog epithelial cells. Mutation of Tyr 1021 markedly reduced the PDGF-stimulated binding of phospholipase C (PLC) γ 1 but had no effect on binding of the GTPase activator protein of Ras or of phosphatidylinositol 3 kinase. Mutation of Tyr 1009 reduced binding of PLC γ 1 less severely. Mutation of Tyr 1021, or both Tyr 1009 and Tyr 1021, also reduced the PDGF-dependent binding of a transiently expressed fusion protein containing the two Src-homology 2 domains from PLC γ 1. Mutation of Tyr 1021, or both Tyr 1009 and Tyr 1021, greatly reduced PDGF-stimulated tyrosine phosphorylation of PLC γ 1 but did not prevent the tyrosine phosphorylation of other cell proteins, including mitogen-activated protein kinase. We conclude that Tyr 1021, and possibly Tyr 1009, is a binding site for PLC γ 1.

INTRODUCTION

The discovery that mitogens induce significant decreases in the phosphoinositide content of cell membranes focused attention on the regulation of the enzymes that synthesize and degrade these phospholipids (reviewed by Berridge and Irvine, 1989). Polyphosphoinositide (PI)-specific phospholipase C (PLC) activities have been purified from a variety of sources and have been found to belong to a family of isoenzymes (reviewed by Rhee *et al.*, 1989). Sequences conserved between the PLC isozymes are presumed to comprise the catalytic sites. The two PLC isozymes of the PLC γ type, PLC γ 1, found in fibroblasts, and PLC γ 2, found in hematopoietic cells, are atypical in that their catalytic regions are interrupted by heterologous sequences, known as src-homology (SH) domains (Stahl *et al.*, 1988; Suh *et al.*, 1988b; reviewed by Koch *et al.*, 1991). In PLC γ 1 and PLC γ 2, there are two ca. 100-residue SH2 domains followed by one ca. 50-residue SH3 domain. It now appears that PLC γ 1 and PLC γ 2 are regulated by tyrosine kinases and that PLC β is regulated, directly or indirectly, by G-proteins.

PLC activity was detected by Wahl *et al.* (1988) in antiphosphotyrosine immunoprecipitates prepared from epidermal growth factor (EGF)-stimulated, but not control, cells. They suggested that one or more PLCs, or

proteins to which PLC was bound, became phosphorylated on tyrosine in response to EGF. The specific PLC isozymes involved were identified by immunoprecipitation studies. Although murine fibroblasts contain PLC γ , PLC β 1, and PLC δ , stimulation with EGF or platelet-derived growth factor (PDGF) induces the tyrosine and serine phosphorylation of only PLC γ (Meisenhelder *et al.*, 1989; Wahl *et al.*, 1989b). PLC γ 1 is also present in human A431 cells and is tyrosine and serine phosphorylated in response to EGF (Margolis *et al.*, 1989; Wahl *et al.*, 1989a). Phosphorylation of PLC γ at tyrosine residues in vitro increases its enzymatic activity when assayed with polyphosphoinositide substrates that are either contained in detergent micelles or bound to profilin (Nishibe *et al.*, 1990; Goldschmidt-Clermont *et al.*, 1991). The residues at which PLC γ is phosphorylated by the EGF receptor tyrosine kinase in vitro and in vivo have been identified (Kim *et al.*, 1990; Wahl *et al.*, 1990). The same residues are phosphorylated in response to PDGF (Kim *et al.*, 1991). Two of the phosphorylation sites are needed for full activation of PLC γ 1 in the cell. Overexpression of PLC γ 1 increases the rate of phosphoinositide degradation elicited by suboptimal doses of PDGF (Cuadrado and Molloy, 1990; Margolis *et al.*, 1990b; Sultzman *et al.*, 1991). Mutant forms of PLC γ 1, in which the tyrosine phosphorylation sites

were substituted with phenylalanine, were less active in this assay (Kim *et al.*, 1991).

It is now evident that PLC γ 1 and PLC γ 2 are not only substrates for tyrosine phosphorylation but also bind to other tyrosine-phosphorylated proteins. Complexes of PLC γ with the tyrosine-phosphorylated receptors for EGF, PDGF, nerve growth factor (NGF), and fibroblast growth factor (FGF), and with the human EGF receptor related protein, HER2, have been detected (Kumjian *et al.*, 1989; Margolis *et al.*, 1989; Wahl *et al.*, 1989a; Burgess *et al.*, 1990; Morrison *et al.*, 1990; Peles *et al.*, 1991; Vetter *et al.*, 1991). PLC γ is tyrosine phosphorylated when these receptors are activated and also when the antigen receptors of B and T cells are cross-linked (Mustelin *et al.*, 1990; Carter *et al.*, 1991; Park *et al.*, 1991; Weiss *et al.*, 1991). PLC γ probably binds via its SH2 domains (Anderson *et al.*, 1990; Koch *et al.*, 1991). There are several ways that the association of PLC γ with activated receptors could regulate PLC γ activity. First, binding of PLC γ to a receptor may bring it close to the tyrosine kinase domain of that receptor, promoting the phosphorylations that then activate the PLC γ . Second, the receptor could regulate PLC γ directly, e.g., by binding to an allosteric site. Third, PLC γ is normally soluble, and binding to the membrane, via the receptor, could situate it better to contact its substrates (Todderud *et al.*, 1990). Assessing the significance of PLC γ binding to an activated receptor requires the creation of specific mutations in both the PLC γ and the receptor that interfere with binding without having other effects.

Several proteins that have SH2 domains bind to activated PDGF β receptors (reviewed by Cantley *et al.*, 1991; Koch *et al.*, 1991). Some of these bind to the kinase insert region of the receptor, a region of ~ 100 residues that interrupts the sequence of the catalytic domain. However, results from Morrison *et al.* (1990) have indicated that PLC γ does not need the kinase insert regions of the PDGF receptor for binding. Here we report the identification of tyrosine phosphorylation sites in the C-terminal tail of PDGF receptor that are required for PLC γ binding.

MATERIALS AND METHODS

Site-Directed Mutagenesis

Site-directed mutagenesis was done as previously described (Kazlauskas and Cooper, 1989) on pRR8, a 1.44-kilobase *Bam*HI-*Xba*I fragment that contains the tail region of the human PDGF receptor β -subunit. Sequences of oligonucleotides are available on request. All mutations were confirmed by sequencing. Mutated *Bam*HI-*Xba*I fragments were moved from pRR8 into pRR6 (Kashishian *et al.*, 1992). 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) as described (Kashishian *et al.*, 1992). pLXSN derivatives were transfected into PE501 cells, and viral supernatants were passaged through PA317 cells before infection of TRMP cells and selection for resistance to G418 (Kazlauskas and Cooper, 1989).

Immunoprecipitation, Kinase Assays, and Peptide Mapping

Confluent cultures ($\sim 3 \times 10^6$ cells) of TRMP cells expressing the various mutant derivatives were placed in Dulbecco's modified Eagle's

medium (DMEM) containing 0.1% calf serum overnight and then stimulated for 5 min at 37°C with 0 or 40 ng/ml recombinant PDGF-BB (Zymogenetics, Seattle, WA). Cells were washed in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered saline, lysed in EB (Kazlauskas and Cooper, 1989), immunoprecipitated with monoclonal antibody PR7212 (Hart *et al.*, 1987), and subjected to *in vitro* kinase assays as described (Kazlauskas and Cooper, 1989). A glutathione-S-transferase (GST) fusion protein containing PDGF receptor C-terminal sequences was described previously (Kazlauskas *et al.*, 1991). Phosphopeptide mapping was performed using both trypsin and thermolysin, as described (Kashishian *et al.*, 1992). Before immunoprecipitating with anti-PLC γ , quiescent TRMP cells were incubated for 1 h at 37°C in DMEM containing 0.5 mM Na₃VO₄ and 10 mM HEPES, pH 7.2 and then lysed in WLB (20 mM tris(hydroxymethyl)aminomethane [Tris], pH 8.0, 137 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride [PMSF], 10 μ g/ml aprotinin, 20 μ M leupeptin, 1 mM Na₃VO₄, 1% NP40, 10% glycerol) (Morrison *et al.*, 1990). Insoluble material was removed by centrifugation at 4°C for 30 min at 15 000 $\times$ g. Immunoprecipitations were performed by incubating at 0°C for 1.5 h with a mixture of monoclonal antibodies to PLC γ (Upstate Biotechnology, Inc., Lake Placid, NY) (Suh *et al.*, 1988a). Immune complexes were collected on a suspension of formaldehyde-fixed *Staphylococcus aureus* that had been pretreated with goat anti-mouse IgG serum, spun through a 500- μ l cushion of WLB-10% sucrose, and then washed four times with 400 μ l WLB. Proteins were resolved by electrophoresis on 7.5% acrylamide, 0.193% bisacrylamide sodium dodecyl sulfate (SDS) gels.

Immunoblotting and PI3 Kinase Assays

Proteins precipitated with PR7212 or anti-PLC γ antibodies were resolved on 7.5% acrylamide, 0.193% bisacrylamide SDS gels, and blotted onto Immobilon (Millipore, Bedford, MA) or nitrocellulose (Schleicher and Schuell, Keene, NH), respectively. PDGF receptors were detected using the rabbit polyclonal antibody 2897 raised against the C-terminal domain of the PDGF receptor as described (Kazlauskas *et al.*, 1991). GTPase activator protein of Ras (GAP) was detected using anti-GAP B (Ellis *et al.*, 1990). PLC γ 1 was detected using the anti-PLC γ antibody mixture used for immunoprecipitation.

To analyze total cell proteins, TRMP cells were stimulated for 5 min at 37°C with 0 or 40 ng/ml PDGF BB, lysed in 1 $\times$ SB (2.5 mM EDTA, 2% SDS, 2.8 M β -mercaptoethanol, 10% glycerol, 100 mM Tris, pH 6.8, 0.03% bromophenol blue), resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotted. Mitogen-activated protein (MAP) kinase was detected in cell lysates with 1913.2 rabbit antiserum (Posada and Cooper, 1992). Phosphotyrosine-containing proteins were detected with anti-phosphotyrosine mouse monoclonal PY20 (ICN, Irvine, CA).

Two procedures were used for detection of proteins on immunoblots. For Figures 3, 5, and 7, immunoblots were blocked in BLOCK (1% bovine serum albumin, 1% ovalbumin, 0.05% Tween 20, 0.005% NaN₃ in 10 mM Tris HCl, pH 7.5, 154 mM NaCl), then probed with the appropriate antibodies followed by alkaline phosphatase-conjugated anti-rabbit or anti-mouse immunoglobulin and developed with 5-bromo-4-chloro-3-indolylphosphate (BCIP) and *p*-nitroblue tetrazolium (NBT) (Kashishian *et al.*, 1992). For Figures 4 and 6, immunoblots were blocked in 5% nonfat dry milk, 0.154 M NaCl, 10 mM Tris HCl, pH 7.5, 0.5% Tween-20, then probed with the appropriate antibodies followed by horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin, and developed using Enhanced Chemiluminescence (Amersham, Arlington Heights, IL).

PI3 kinase assays were performed on PR7212 immunoprecipitates as described (Kashishian *et al.*, 1992).

mt-PLC Fusion Protein

A fusion protein, mt-PLC, containing six copies of a 13-residue epitope for monoclonal antibody 9E10 (Evan *et al.*, 1985) fused to the SH2 domains of PLC γ 1, was expressed in TRMP cells using the vaccinia virus/T7 phage system of Fuerst *et al.* (1986). The vector, pJC1100,

was created by replacing the *Sma* I to *Sac* I portion of the polylinker of pTM-3 (Elroy-Stein and Moss, 1992) with a *Hinc*II to *Sac* I fragment from pBS/KS + MT6D (Roth *et al.*, 1991). This places the reiterated 9E10 epitope in frame with the initiator ATG contained in the encephalomyocarditis virus 5' untranslated region of pTM-3. The sequence encoding residues 514–769 of rat PLC γ 1 (Suh *et al.*, 1988b) was polymerase chain reaction (PCR) amplified using primers PLC5.1 (CTCTGGATCCCAGCAAGATCTACTAC) and PLC3.3 (CTCTGGATCCGGGTCAGCTGTCCC), which contain *Bam*HI sites (underlined). The PCR product (306 base pair) was cut with *Bam*HI and cloned into the *Bam*HI site of a pBS derivative and then moved into *Bam*HI-cut and phosphatase-treated pTM-3. The orientation of the insert was checked with *Xho* I.

TRMP cell lines stably expressing wild-type or mutant PDGF receptors, or the empty pLXSN vector, were plated into 24-well plates and allowed to reach confluence ($\sim 3 \times 10^5$ cells/well). Culture fluids were removed and replaced with 0.25 ml of DMEM containing 7.5×10^6 pfu vaccinia virus vTF7-3 (propagated in HeLa cells and titered in CV-1 cells) (Elroy-Stein and Moss, 1992). After 45–60 min, the virus was removed and replaced with 0.25 ml of DMEM containing 3.75 μ l of Lipofectin (GIBCO-BRL, Grand Island, NY) and 2.5 μ g of DNA. After a further 5–6 h at 37°C, PDGF-BB was added to 0 or 40 ng/ml final concentration, and incubation continued for 5 min. Monolayers were washed twice in ice-cold Tris-buffered saline, and lysed in 0.25 ml EB containing 1 mM PMSF and 0.2 mM Na₃VO₄. Lysates from duplicate wells were pooled, and 25 μ l was sampled for subsequent analysis. The remainder of the lysate was centrifuged at $10\,000 \times g$ for 10 min at 4°C, and the supernatant was precipitated with excess PR7212 antibody, rabbit anti-mouse antiserum, and fixed *S. aureus*. Immunoprecipitates were pelleted through EB-10% sucrose and then washed twice with EB and once with 0.1 M NaCl, 10 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid), pH 7.0, 20 μ g/ml aprotinin. A 10% polyacrylamide, 0.13% bisacrylamide SDS gel was loaded with samples corresponding to lysate from 1.2×10^4 cells and immunoprecipitate from 6×10^5 cells. After electrophoresis, the gel was blotted to Immobilon (Millipore) and probed with antibody 9E10 (2.6 μ g/ml), alkaline-phosphatase-conjugated anti-mouse Ig, and developed with BCIP/NBT (Kashishian *et al.*, 1992). The control fusion proteins, mt-GAP and mt-p85, contained residues 130–450 of human GAP and residues 303–724 of bovine p85 α , respectively.

RESULTS

Phosphorylation Sites in the C-terminal Noncatalytic Region of the PDGF Receptor β -Subunit

Our previous mapping studies have revealed a complex pattern of phosphopeptides that are phosphorylated on tyrosine *in vivo*, in response to PDGF, and *in vitro*. To test which of these phosphopeptides might correspond to tyrosines contained in the C-terminal noncatalytic tail, we used a bacterially synthesized GST fusion protein containing residues 939–1108 from the human PDGF β receptor as a substrate for tyrosine phosphorylation *in vitro* (GST is not phosphorylated by the PDGF receptor). After phosphorylation with [γ -³²P]ATP, the labeled fusion protein was analyzed by phosphopeptide mapping (Figure 1B). Comparison with phosphopeptide maps of PDGF receptors autophosphorylated *in vitro* (Figure 1A) showed that three phosphopeptides derived from the C-terminal region comigrated with phosphopeptides 2, 3, and 11 from the complete receptor (Figure 1C). In addition, phosphopeptide 8 appears to be a mixture of peptides, of which apparently one or two are contained in the C-terminal region of the receptor and

Figure 1. Tryptic/thermolytic phosphopeptide maps of the PDGF receptor. Wild-type PDGF receptors were immunoprecipitated from quiescent unstimulated TRMP cells and incubated with [γ -³²P]ATP in the presence of a GST fusion protein containing the C-terminal region of the PDGF receptor. After SDS-PAGE, the radioactive receptor and GST fusion protein were excised from the gel and analyzed by peptide mapping (MATERIALS AND METHODS). (A) PDGF receptor. (B) GST fusion protein. (C) Mixture of A and B.

one is in the kinase insert. Comparison with previous results (Kashishian *et al.*, 1992; our unpublished data) suggests that peptides 2, 3, 8, and 11 are also phosphorylated on tyrosine when intact cells are exposed to

Figure 2. Tryptic/thermolytic phosphopeptide maps of mutant PDGF receptors. PDGF receptors were immunoprecipitated from TRMP cells expressing wild-type (A) or phenylalanine mutant (B, F1009; C, F1021; D, F1009/1021) PDGF receptors, labeled in vitro by autophosphorylation with [γ^{32} P]ATP and analyzed by peptide mapping (MATERIALS AND METHODS). Phosphopeptides missing in each of the mutant receptors are arrowed.

PDGF. These four peptides are labeled to estimated 49, 15, 88, and 15% stoichiometries, normalized to a phosphoserine-containing peptide, 10 min after exposing cells to 13 nM PDGF. But, under our more usual conditions of stimulation with 1.3 nM PDGF for 5–10 min, phosphorylation of peptides 3 and 11 is almost undetectable (Kashishian *et al.*, 1992).

The C-terminal region of the PDGF β receptor contains four tyrosine residues, tyrosines 966, 970, 1009, and 1021, of which only 1009 is not conserved in the PDGF α receptor (Gronwald *et al.*, 1988; Matsui *et al.*, 1989). To test which phosphopeptides correspond to which tyrosine residues, we used site-directed mutagenesis to mutate the codon for each tyrosine residue to encode phenylalanine, creating four mutants, designated F966, F970, F1009, and F1021. We also made a double mutant, F1009/1021. Each mutant cDNA was expressed, using a retroviral vector, in the dog epithelial cell line, TRMP, which has no detectable PDGF receptors. Wild-type and mutant PDGF receptors were expressed at equal levels, as detected by immunoblotting (our unpublished results and Figure 3B). Receptors were immunoprecipitated, labeled by autophosphorylation with [γ^{32} P]ATP in vitro, and analyzed by phosphopeptide mapping. Maps of mutants F966 and F970 were indistinguishable from wild-type. Mutant F1009 recep-

tors lacked phosphopeptide 3 (Figure 2B), whereas mutant F1021 receptor lacked phosphopeptide 11 (Figure 2C). Double mutant F1009/1021 lacked both peptides 3 and 11 (Figure 2D). Labeling of phosphopeptides in the vicinity of peptide 8 was also reduced in mutants F1021 and F1009/1021. Curiously, none of the mutations blocked the labeling of phosphopeptide 2, despite our evidence that this peptide is contained in the C-terminal region. Perhaps this phosphopeptide is actually a mixture of 2 peptides, a minor one derived from the C-terminus and a major one from outside the C-terminus. We conclude that Tyr 1009 and 1021 are both phosphorylated, corresponding, respectively, to peptides 3 and to peptides 8 and 11. Peptides 8 and 11 could be related by partial digestion.

Binding Properties of Mutant PDGF Receptors

TRMP cells expressing wild-type or mutant PDGF receptors were treated with PDGF, and the receptors and their associated proteins were isolated by immunoprecipitation. Levels of the SH2-containing proteins GAP and PI3 kinase were unchanged by any of the C-terminal mutations (Figure 3). However, binding of PLC γ was greatly reduced by the F1021 mutation, reduced less by the F1009 mutation, and almost completely

Figure 3. (A) PI3 kinase activity associated with wild-type and mutant PDGF receptors. TRMP cells infected with pLXSN vector (0) or expressing wild-type (WT) or phenylalanine (F966, F970, F1009, and F1021) mutant PDGF receptors, were treated with 0 or 1.3 nM PDGF-BB for 5 min at 37°C. PDGF receptor immunoprecipitates were incubated with PI and [γ - 32 P]ATP and the labeled phospholipids analyzed as described in MATERIALS AND METHODS. (B) GAP associated with PDGF receptors. PDGF receptor immunoprecipitates were resolved by SDS-PAGE and immunoblotted. The upper part of the immunoblot was probed with antiserum 2897 to detect the PDGF receptors and the lower part with anti-GAP B to detect GAP (MATERIALS AND METHODS).

blocked by mutation of both residues (Figure 4). It should be noted that PLC γ binding to the PDGF receptor was difficult to detect, whether by immunoblotting PDGF receptor immunoprecipitates with antibody to PLC γ or by immunoblotting PLC γ immunoprecipitates with antibody to PDGF receptors (Figure 4), so we pretreated the cells for 1 h before PDGF stimulation with 0.5 mM Na₃VO₄ to stabilize receptor tyrosine phosphorylation. Under these conditions, the F1021 mutation routinely reduced binding of PLC γ 1, and the F966 and F970 mutations had no effect. The F1009 mutation also reduced binding but less dramatically.

To increase the sensitivity of the assay for PLC γ binding, we made use of a DNA construct encoding a fusion protein, mt-PLC, containing the 2 SH2 domains of rat PLC γ 1 (previously known as PLC-II) (Suh *et al.*, 1988b) fused to a sequence containing a sixfold tandem repeat of a 13-residue epitope that is recognized by the monoclonal antibody 9E10 (Roth *et al.*, 1991). This DNA was introduced into TRMP cells by transfection, and mt-PLC was expressed from a phage T7 polymerase promoter by infecting the cells with a recombinant vaccinia virus encoding T7 polymerase (Fuerst *et al.*, 1986). Five to 6 h after transfection, the cells were treated with PDGF, and the receptor was isolated by immunoprecipitation and analyzed by SDS-PAGE. The bound

Figure 4. PDGF receptors associated with PLC γ 1. TRMP cells infected with pLXSN vector (0) or expressing wild-type (WT) or mutant PDGF receptors were treated for 1 h at 37°C with 0.5 mM Na₃VO₄ and then treated with 0 or 1.3 nM PDGF for 5 min at 37°C. PLC γ 1 was immunoprecipitated and resolved by SDS-PAGE and immunoblotted. Samples containing 4% of the volume of cell lysate used for immunoprecipitation were also run as controls. The upper part of the immunoblot was probed with 2897 to detect PDGF receptors and the lower part with anti-PLC γ to detect PLC γ 1 (MATERIALS AND METHODS).

mt-PLC fusion protein was detected in the cell lysates (Figure 5, lanes a) and in anti-PDGF receptor immunoprecipitates (Figure 5, lanes b) by immunoblotting with the 9E10 antibody. The results show that binding of mt-PLC to wild-type receptors was PDGF dependent. About 6% of the mt-PLC in the cell lysate (estimated at 3×10^6 molecules per cell by comparison with other proteins containing the epitope tag) bound to wild-type PDGF receptors (these cells express $\sim 6 \times 10^6$ receptors per cell), suggesting that 3% of the receptors had mt-

Figure 5. Association of epitope-tagged SH2 domains of PLC γ with wild-type and mutant PDGF receptors. TRMP cells expressing wild-type (WT), mutant (F966, F970, F1009, F1021, F1009/F1021), or no PDGF receptors (0) were infected for 45–60 min at 37°C with vaccinia virus vTF7-3 and then transfected with mt-PLC DNA. After 5 h at 37°C, the cells were stimulated with 0 or 1.3 nM PDGF-BB for 5 min at 37°C. Monolayers were then lysed in EB, a sample of the lysate was taken, and the remainder was immunoprecipitated with PR7212. Lysates (lanes a) and immunoprecipitates (lanes b) were resolved by SDS-PAGE and immunoblotted. The immunoblot was probed with 9E10 antibody, to detect mt-PLC, as described in MATERIALS AND METHODS. Note that two forms of the mt-PLC protein were detected, one migrating with an apparent molecular size of 65 kDa and the other comigrating with immunoglobulin heavy chains. We do not know the relationship between these two forms but have found that similar constructs containing SH2 domains from GAP and PI3 kinase also give rise to two proteins. In those cases, the effects of receptor mutations on binding to the PDGF receptor are concordant for the two forms of each protein (Cooper and Kashishian, 1993).

Figure 6. PLC γ tyrosine phosphorylation and association with the PDGF receptor. TRMP cells expressing wild-type (WT), mutant receptors (F966, F970, F1009, F1021, F1009/F1021), or no receptors (0) were treated for 1 h at 37°C with 0.5 mM Na₃VO₄ and then stimulated with 0 or 1.3 nM PDGF-BB. PLC γ immunoprecipitates were resolved by SDS-PAGE and immunoblotted. Samples containing 4% of the volume of cell lysate used for immunoprecipitation were also run as controls. The immunoblot was probed with PY20 to detect phosphotyrosine-containing proteins (MATERIALS AND METHODS).

PLC bound. Each of the C-terminal mutant receptors tested also bound mt-PLC, with the exception of F1021 and F1009/1021 receptors, which bound little if any of the fusion protein. As controls, we also expressed comparable constructs encoding epitope-tagged SH2 domains derived from GAP (mt-GAP) and the p85 subunit of PI3 kinase (mt-p85) (Cooper and Kashishian, 1993). Binding of these fusion proteins was not reduced by any of the C-terminal mutations.

Signal Transduction by Mutant PDGF Receptors

We first tested whether mutations that reduced PLC γ binding also reduced PDGF-induced PLC γ tyrosine phosphorylation. Immunoprecipitates of PLC γ were immunoblotted with antibody to phosphotyrosine (Figure 6). Wild-type and mutant receptors were all capable of signaling tyrosine phosphorylation of PLC γ . Tyrosine phosphorylation was decreased by the F1021 mutation and reduced further by the F1009/1021 double mutation. Each mutant also induced a PDGF-dependent mobility shift in PLC γ (Figure 4, lower). This mobility shift may be due to phosphorylation at tyrosine or serine, or both. These results suggest that PDGF receptor mutations that decrease binding of PLC γ also reduce PDGF-dependent tyrosine phosphorylation of PLC γ .

Immunoblotting of immunoprecipitates of PLC γ with antibody to phosphotyrosine also detected PDGF receptors coimmunoprecipitated with PLC γ . The apparent decrease in tyrosine-phosphorylated F1021, and absence of F1009/1021, mutant receptors from the PLC γ immunoprecipitates provides further evidence for a role for Tyr 1021 in binding PLC γ .

We also tested whether mutations that decrease binding of PLC γ interfere with other phosphorylation events in PDGF-stimulated cells. We have detected a number of proteins whose phosphotyrosine content is increased by PDGF, using antibodies to phosphotyrosine to probe immunoblots of total cell lysates (Figure 7, upper). One phosphoprotein (band a) comigrates with, and is probably identical to, PLC γ . The other phosphoproteins were detected in cells expressing each

of the mutant receptors (Figure 7), including the F1009/1021 mutant. One protein (band d), at ~42 kDa, is almost certainly a MAP kinase (early response kinase [ERK] 2), based on comigration with phosphorylated MAP kinase present in *Xenopus* eggs. Direct analysis of MAP kinase phosphorylation, by immunoblotting cell lysates with antibodies to MAP kinase and assaying for the characteristic phosphorylation-dependent mobility shift, showed that both the 42-kDa (ERK2) and 45-kDa (ERK1) MAP kinases were phosphorylated in response to PDGF stimulation in cells expressing each of the mutant receptors (Figure 7, lower). This suggests that MAP kinase phosphorylation, and hence activation, is not dependent on PLC γ binding to the PDGF receptor.

DISCUSSION

Our results implicate Tyr 1009 and 1021 as PDGF-dependent phosphorylation sites in the PDGF receptor

Figure 7. Protein tyrosine phosphorylation in response to PDGF. TRMP cells expressing wild-type (WT), mutant receptors (F966, F970, F1009, F1021), or no receptors (0) were stimulated with 0 or 1.3 nM PDGF-BB for 5 min at 37°C. Cells were lysed, and total cell proteins were resolved by SDS-PAGE, immunoblotted, and probed with anti-phosphotyrosine antibody PY20 (upper) or anti-MAP kinase antiserum 1913.2 (lower) as described in MATERIALS AND METHODS. (Upper) Bands a-f correspond to proteins that were phosphorylated in response to PDGF stimulation. (Lower) Phosphorylation-dependent mobility shifts of the 42-kDa (ERK2) and 45-kDa (ERK1) MAP kinases were detected in response to PDGF stimulation. Marker lanes were loaded with proteins from *Xenopus* oocytes and eggs (in which the 42-kDa MAP kinase is in the unphosphorylated and phosphorylated state, respectively) (Posada and Cooper, 1992).

and hence as candidate binding sites for cell proteins that contain SH2 domains. Studies with mutant receptors suggested that Tyr 1009 and 1021 both participate in binding of PLC γ 1, with Tyr 1021 playing a more important role. Tyr 966 and 970 are in a region of the noncatalytic tail of the receptor that contains a PDGF-dependent epitope and may undergo a PDGF-induced conformation change (Bishayee *et al.*, 1988; Keating *et al.*, 1988). However, we found no evidence for phosphorylation of either Tyr 966 or 970, and mutation of these residues did not decrease binding of PLC γ 1, GAP, or PI3 kinase.

The sequence around Tyr 1021 is highly conserved between the human and mouse PDGF β receptors and the human α receptor: DxxYIIPLDxxP, where the underlined Y is the phosphorylated tyrosine. The stretch of seven identical residues is the largest region of identity between α and β receptors in the C-tail and suggests a conserved function. A similar sequence, YLIP, is found around Tyr 992 of the EGF receptor and is implicated in PLC γ binding (Rotin *et al.*, 1992; Vega *et al.*, 1992). Comparison of the PDGF- and EGF-receptor sequences suggests a consensus, Y(L/I)IP, for binding PLC γ . However, the sequences around Tyr 766 of the FGF receptor (*flk*), YLDL, and Tyr 785 of the NGF receptor (*trk*), YLDV, both implicated in PLC γ binding (Mohammadi *et al.*, 1991, 1992; Peters *et al.*, 1992; Loeb, Stephens, Greene, and Kaplan, personal communication), share only the first leucine or isoleucine with the speculative consensus. Possibly the confusion arises from the presence of 2 SH2 domains in PLC γ . One SH2 could bind specifically to the Y(L/I)IP motif and the other to the YLD(L/V) motif.

The requirement for Tyr 1021 for binding of PLC γ 1 was confirmed by analysis of a fusion protein, mt-PLC, transiently expressed in TRMP cells that possess wild-type or mutant PDGF receptors. mt-PLC contained the two SH2 domains, and their immediate flanking sequences, derived from PLC γ 1. The mt-PLC was detected in PDGF receptor immunoprecipitates by virtue of an epitope tag that distinguished the fusion protein from endogenous cell proteins. Demonstration that the SH2 domains, not the epitope tag, conferred the PDGF- and Tyr 1021-dependent binding of mt-PLC to the PDGF receptor was provided by the control fusion proteins, mt-GAP and mt-p85, that bound to the PDGF receptor in a PDGF-dependent Tyr 1021-independent manner. Other studies show that binding of PDGF receptors to mt-GAP depends on Tyr 771 and mt-p85 on Tyr 740 and Tyr 751 (Cooper and Kashishian, 1992). Thus, all three fusion proteins recapitulate the binding specificities of their cognate full-length proteins. These results extend the phosphorylation-dependence of SH2 binding deduced from *in vitro* binding studies (Koch *et al.*, 1991) by showing that the SH2 domains confer sequence-specific binding to phosphotyrosine residues in the PDGF receptor in living cells. Also, since the known PLC γ tyrosine phosphorylation sites (Kim *et al.*, 1990)

were excluded from mt-PLC, we conclude that tyrosine phosphorylation of PLC γ is probably not needed for binding. Indeed, work from Schlessinger's laboratory has suggested that tyrosine phosphorylation of PLC γ may actually decrease its binding to the EGF receptor, perhaps by competition between the phosphotyrosines in PLC γ and the receptor for the PLC γ SH2 domains (Margolis *et al.*, 1990a). Tyrosine phosphorylation of PLC γ might actually provide a mechanism for the release of PLC γ from the receptor.

Receptor mutants with reduced binding of PLC γ also were less active in tyrosine phosphorylation of PLC γ . Therefore, stable binding of PLC γ through its SH2 domains to the PDGF receptor may be needed for phosphorylation of PLC γ by the receptor. Similarly, F771 mutant PDGF receptors that do not bind stably to GAP do not signal GAP tyrosine phosphorylation (Fantl *et al.*, 1992; Cooper and Kashishian, unpublished results). Binding of cellular proteins to phosphorylation sites on the PDGF receptor could increase their access to the receptor catalytic domain and increase their rate of phosphorylation.

PLC γ tyrosine phosphorylation is required for PLC γ activation *in vivo* (Kim *et al.*, 1991). This activation stimulates PI turnover, and the products of PI turnover increase cellular Ca²⁺ levels (Berridge and Irvine, 1989). These events activate Ca²⁺-dependent enzymes and protein kinase C (PKC). PKC activation by phorbol esters is sufficient to induce mitogenesis, but recent experiments suggest that neither PI turnover nor PKC activation is needed for PDGF-stimulated DNA synthesis (Hill *et al.*, 1990). Moreover, FGF receptor mutants unable to stimulate PI turnover are nonetheless mitogenic (Mohammadi *et al.*, 1992; Peters *et al.*, 1992). PKC activation also stimulates MAP kinase via a cascade of kinases. In some cell types, PKC appears to be necessary for MAP kinase activation by PDGF (Kazlauskas and Cooper, 1988). In TRMP cells expressing human PDGF β receptors, mutant receptors with reduced binding of PLC γ , PI3 kinase, and GAP were each able to activate MAP kinase (Fig. 7 and Kashishian and Cooper, unpublished results). However, we have been unable to establish assays for either PI turnover or Ca²⁺ flux in these cells and do not know whether PLC γ is activated in response to PDGF. Further analysis will require expression of the mutant receptors in cells which show significant PI turnover, Ca²⁺ flux, and mitogenic responses to PDGF.

While this work was in progress we learned of similar results from the laboratory of Dr. C.-H. Heldin. They also found that Tyr 1009 and Tyr 1021 are phosphorylation sites and that mutation of either Tyr 1021 or both tyrosines greatly reduced tyrosine phosphorylation and binding of PLC γ . Mutation of Tyr 1009 slightly reduced PLC γ phosphorylation. The single and double mutant receptors were mitogenic when assayed in pig aortic endothelial cells. This work was published recently (Ronnstrand *et al.*, 1991). Also, Seedorf *et al.*

(1992) have reported that deletion of C-terminal residues from the PDGF receptor inhibits PLC γ binding.

Note added in proof. Substantially similar results have been obtained by M. Valius, C. Bazenet, and A. Kazlauskas: Tyrosines 1021 and 1009 are phosphorylation sites in the carboxyterminus of the platelet-derived growth factor receptor β subunit and are required for binding of phospholipase C γ and a 64-kilodalton protein, respectively. *Mol. Cell. Biol.* (in press).

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