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Tyrosines 1021 and 1009 Are Phosphorylation Sites in the Carboxy Terminus of the Platelet-Derived Growth Factor Receptor β Subunit and Are Required for Binding of Phospholipase Cγ and a 64-Kilodalton Protein, Respectively

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Binding of platelet-derived growth factor (PDGF) to the PDGF receptor (PDGFR) β subunit triggers receptor tyrosine phosphorylation and the stable association of a number of signal transduction molecules, including phospholipase Cγ (PLCγ), the GTPase activating protein of *ras* (GAP), and phosphatidylinositol-3 kinase (PI3K). Previous reports have identified three PDGFR tyrosine phosphorylation sites in the kinase insert domain that are important for stable association of GAP and PI3K. Two of them, tyrosine (Y) 740, and Y-751 are required for the stable association of PI3K, while Y-771 is required for binding of GAP. Here we present data for two additional tyrosine phosphorylation sites, Y-1009 and Y-1021, that are both in the carboxy-terminal region of the PDGFR. Characterization of PDGFR mutants in which these phosphorylation sites are substituted with phenylalanine (F) indicated that Y-1021 and Y-1009 were required for the stable association of PLCγ and a 64-kDa protein, respectively. An F-1009/F-1021 double mutant selectively failed to bind both PLCγ and the 64-kDa protein, whereas all of the carboxy-terminal mutants bound wild-type levels of GAP and PI3K. The carboxy terminus encodes the complete binding site for PLCγ, since a phosphorylated carboxy-terminal fusion protein selectively bound PLCγ. To determine the biological consequences of failure to associate with PLCγ, we measured PDGF-dependent inositol phosphate production and initiation of DNA synthesis. The PDGFR mutants that failed to associate with PLCγ were not able to mediate the PDGF-dependent production of inositol phosphates. Since tyrosine phosphorylation of PLCγ enhances its enzymatic activity, we speculated that PDGFR mutants that failed to activate PLCγ were unable to mediate its tyrosine phosphorylation. Surprisingly, the F-1021 receptor mediated readily detectable levels of PDGF-dependent PLCγ tyrosine phosphorylation. Thus, the production of inositol phosphates requires not only PLCγ tyrosine phosphorylation but also its association with the PDGFR. Comparison of the mutant PDGFRs' abilities to initiate PDGF-dependent DNA synthesis indicated that failure to associate with PLCγ and produce inositol phosphates diminished the mitogenic response by 30%. In contrast, preventing the PDGFR from binding the 64-kDa protein did not compromise PDGF-triggered DNA synthesis at saturating concentrations of PDGF. Thus, it appears that phosphorylation of the PDGFR at Y-1021 is required for the stable association of PLCγ to the receptor's carboxy terminus, the production of inositol phosphates, and initiation of the maximal mitogenic response.

Platelet-derived growth factor (PDGF) is a polypeptide growth factor able to initiate proliferation and chemotaxis in cells expressing PDGF receptors (PDGFRs) (9, 46). PDGF consists of a homo- or heterodimer of A and B chains, each of which recruits a PDGFR subunit, resulting in a dimeric PDGFR (9, 42, 46). There are two distinct PDGFR subunits, α and β, that differ in their affinity for the PDGF A and B chains (9, 42, 46). Thus, the type of PDGF that a cell encounters will at least in part determine the subunit composition of the PDGFR with which it interacts. The studies presented in this paper focus on the PDGFR consisting of ββ subunits, which is activated by the BB form of PDGF.

The extracellular domain of the PDGFR encodes a ligand binding portion, while the intracellular region of the PDGFR includes a protein tyrosine kinase. The kinase activity of the PDGFR is greatly stimulated by binding of PDGF, resulting in tyrosine phosphorylation of a number of cellular substrates including the receptor itself (9, 46). The tyrosine-phosphorylated PDGFR associates with several cellular proteins: phospholipase Cγ (PLCγ), the GTPase activating protein of *ras* (GAP), phosphatidylinositol-3 kinase (PI3K), c-*raf*, pp60$^{c\text{-}src}$, p62$^{c\text{-}yes}$, and p59^{fyn}, as well as 120- and 64-kDa species (3, 12, 16, 19, 24, 25, 33, 34, 45).

The proteins that associate with the activated PDGFR are in some instances known signal transduction enzymes. For instance, PLCγ, a substrate for the PDGFR (29, 34, 45), is activated by tyrosine phosphorylation (8, 21, 35), resulting in the hydrolysis of phosphatidylinositol-4,5-bisphosphate that produces two second messengers: inositoltrisphosphate and diacylglycerol. These two second messengers then trigger numerous intracellular changes that may contribute to the relay of PDGF's biological signals.

The interaction of signal transduction molecules with activated growth factor receptors requires (i) that the growth factor receptor is tyrosine phosphorylated and (ii) that the proteins that bind contain an appropriate *src* homology (SH2) domain (2). The specificity of the interaction is defined

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by both the amino acid context surrounding the phosphotyrosine and the amino acid sequence of the SH2 domain (1, 5, 6, 11, 22, 23, 28, 32). There appear to be additional ways for phosphorylated proteins to interact with SH2-containing partners, as illustrated by the finding that threonine-phosphorylated BCR associates with the *abl* SH2 domain in the apparent absence of phosphotyrosine (37).

For the human PDGFR β subunit, phosphorylation at tyrosine (Y) 740 and Y-751 is necessary for stable association of PI3K, whereas phosphorylation of Y-771 permits binding of GAP (6, 13, 20). PLCγ associates with the fibroblast growth factor receptor (FGFR) in a region surrounding phosphorylated Y-766 (31, 38), while binding of PLCγ to the epidermal growth factor receptor (EGFR) requires Y-992, which is also a phosphorylation site (41, 44).

Here we report the identification of two additional tyrosine phosphorylation sites (Y-1009 and Y-1021) in the carboxy terminus of the human PDGFR β subunit and demonstrate that Y-1021 is important for the stable association of PLCγ with the activated PDGFR, while Y-1009 is required for the binding of a 64-kDa protein. Receptor mutants that cannot associate with PLCγ fail to mediate PDGF-dependent inositol phosphate production and initiate DNA synthesis to a reduced level.

MATERIALS AND METHODS

Cell lines. TRMP cells, a line of canine kidney epithelial cells, were maintained as previously described (20). The wild-type and mutant human PDGFR β subunit was expressed in TRMP cells by using the pLXSN retroviral vector (30), as previously described (16). The level of PDGFR expression in mass populations of G418-selected cells used in these studies was approximately 1.6×10^6 receptors per cell, as determined by receptor Western blot (immunoblot) analysis and quantitation of the PDGFR immunoprecipitated from ^{35}S-labeled TRMP cells (data not shown).

Antibodies. The PDGFR antibody used in these studies was a rabbit polyclonal antiserum raised against a purified fusion protein including the carboxy terminus of the PDGFR (17, 18). A polyclonal antiserum to the kinase insert region or a monoclonal antibody (PR7212) to the extracellular domain of the receptor gave results indistinguishable from those presented here (not shown), indicating that at least a portion of the antiserum to the carboxy terminus recognized epitopes that do not interfere with binding of PLCγ or the 64-kDa protein. The PLCγ and PI3K antibodies were purchased from UBI. The antiphosphotyrosine antibodies were mouse monoclonals; PY20 was purchased from ICN and 2bk was purchased from UBI. The GAP antiserum was raised in a rabbit against a purified glutathione-S-transferase (GST) (43) fusion protein that included amino acids 171 to 448 of human GAP.

Labeling of the PDGFR and fusion proteins. To label the PDGFR in intact cells, TRMP cells were labeled with 3 mCi of $^{32}P_i$ per ml, and the PDGFR was immunoprecipitated as previously described (16, 18). To label the PDGFR in vitro, receptor immunoprecipitates prepared from resting or PDGF-stimulated TRMP cells were subjected to an in vitro kinase assay (16). To phosphorylate the GST-tail fusion protein (expressed in *Escherichia coli* and purified as described elsewhere [18]), receptor immunoprecipitates were added to 1 μg of fusion protein and subjected to a standard in vitro kinase assay (16). In the absence of added PDGFR immunoprecipitate, there was only a very low level of incorporation of phosphate (18). In addition, the GST portion of the fusion protein was a very poor substrate for the PDGFR in vitro (data not shown).

Phosphorylation of the synthetic peptide. The synthetic Y-1021 peptide (VQPNEGDNDY) was phosphorylated in vitro by using v-*src* immunoprecipitates and 1 mM peptide under conditions described above for labeling of the PDGFR. The peptide was purified by thin-layer electrophoresis (TLE) first at pH 3.5 and 1.2 kV for 20 min and then at pH 8.9 and 1.2 kV for 20 min.

Phosphopeptide maps. The labeled PDGFR or carboxy-terminal fusion proteins were resolved on a 7.5 or 15%, respectively, sodium dodecyl sulfate (SDS)-polyacrylamide gel, localized by autoradiography, recovered from the gel, trichloroacetic acid precipitated, performic acid oxidized, and digested exhaustively with trypsin and then with thermolysin. The resulting phosphopeptides were resolved by TLE at pH 8.9, and then by ascending chromatography in buffer I (isobutyric acid-pyridine-glacial acetic acid-water-butanol, 65:5:3:29:2) as described in detail elsewhere (13). Quantitation of individual phosphopeptide spots was done with a Molecular Dynamic PhosphorImager.

Site-directed mutagenesis. The *Bam*HI-*Hin*dIII 0.77-kb fragment of the human PDGFR β subunit was subcloned into the pEMBL8$^+$ vector and subjected to site-directed mutagenesis by using the Amersham oligonucleotide-directed in vitro mutagenesis system. To change Y-1009 to phenylalanine (F), the following oligonucleotide, which introduced a *Sca*I site without affecting the amino acid sequence, was used: 5′CACCGCAGTAAAGAGTACTGAGCTGGT3′. The following oligonucleotide was used to mutate Y-1021 to F: 5′GGGGATGATAAAGTCGTTGTCACCTTCATTAGGCT GCAC3′, which introduced a new *Eco*NI restriction site without affecting the amino acid sequence. Y-966 was mutated to phenylalanine by using 5′GTACTTCTTTTTAAA ACCTTCGCC3′. This oligonucleotide also introduced a *Dra*I site. The following oligonucleotide was used to mutate Y-970 to phenylalanine: 5′CACCTGCTGGAACTTCTTTTT GTA3′. The oligonucleotide removed an *Rsa*I site that facilitated identification of the desired DNA constructs. The F-1009/F-1021 mutant was constructed by introducing the F-1009 mutation into the F-1021 mutant. All mutants were verified by sequencing. The 0.77-kb *Bam*HI-*Hin*dIII fragment was then subcloned into RR7 (the *Eco*RI-*Sal*I 4.2-kb RR3 [16] insert subcloned into pBS$^+$ modified to lack the *Sph*I and *Hin*dIII sites in the polylinker). The *Eco*RI-*Sal*I 4.2-kb fragment of RR7, encoding the entire PDGFR coding region, was subcloned in *Eco*RI-*Xho*I-cut pLXSN retroviral vector.

Western blot analysis. The PDGFR or PLCγ was immunoprecipitated from resting or PDGF-stimulated (40 ng of PDGF-BB per ml for 5 min at 37°C) cells as previously described (20), except the two radioimmunoprecipitation assay (RIPA) buffer (14) washes of the immunoprecipitates were omitted. After resolving the samples by SDS-polyacrylamide gel electrophoresis (PAGE), the proteins were transferred to Immobilon membranes and subjected to Western blot analysis, as previously described (20). The GAP antibody used was diluted 1/5,000 in Blotto (20); the PDGFR antibody was diluted 1/1,000 in Blotto, and the PLCγ and PI3K antibodies were diluted in Blotto as recommended by the manufacturer. For antiphosphotyrosine Western blots of PLCγ immunoprecipitates, a mixture of antiphosphotyrosine antibodies (prepared in Block [10 mM Tris-Cl {pH = 7.5}, 154 mM NaCl, 10 mg of bovine serum albumin {BSA} per ml, 10 mg of ovalbumin per ml, 0.05% Tween 20, 0.005% NaN_3]) was used at a dilution of 1 μg/ml (PY20) and 2 μg/ml

(2bk). All other antiphosphotyrosine Western blots were performed with PY20 diluted in Block to 1 μg/ml.

In vitro binding to fusion proteins. GST fusion proteins were coexpressed with *lacZ-elk* as described elsewhere (39). Briefly, bacteria expressing the appropriate GST constructs were grown to mid-log phase in Luria broth containing 0.2% maltose, $MgCl_2$ was added to a final concentration of 10 mM, an equal volume of λB1 (phage expressing the *lacZ-elk* construct in a λgt11 vector) (26) (10^{10} PFU/ml) was added, and the samples were incubated at 30°C for 30 min. Bacteria were plated on replicate plates and grown at 30 or 42°C, and the infected colonies were isolated by their inability to grow at 42°C.

To express the fusion proteins, bacteria were grown to mid-log phase at 30°C and then isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.1 mM and incubated for 1 h at 30°C. The cultures were incubated at 42°C for 15 min and then at 37°C for 1 h. The bacteria were lysed, and the fusion proteins were immunoprecipitated by using polyclonal antisera raised against the GST portion of the fusion protein.

The in vitro binding assay was exactly as previously described (20) except that immunoprecipitates containing 0.04 μg of fusion protein were used in place of PDGFR immunoprecipitates. PI3K activity was determined as previously described (20).

Measurement of inositol phosphate production. To measure the production of inositol phosphates, 5-cm dishes of TRMP cells that were approximately 80 to 90% confluent were labeled in Dulbecco modified Eagle medium containing 0.1% calf serum and 1 μCi of [^{3}H]*myo*-inositol per ml for 72 h. Approximately 15 min prior to adding PDGF, the labeling medium was aspirated, the cells were rinsed three times with LMP (Dulbecco modified Eagle medium-Hams F-12 [1:1] supplemented with 0.1% BSA, 15 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES, pH = 7.5], and 20 mM $LiCl_2$), and the cells were incubated in 2 ml of LMP for 15 min at 37°C. The LMP medium was then replaced with 1.5 ml of LMP containing 200 ng of PDGF-BB (a generous gift of Charlie Hart, Zymogenetics) or buffer per ml, and the incubation at 37°C was continued for 30 min. A total of 1.5 ml of ice-cold methanol–concentrated HCl (100:1; acidified methanol) was added, the cells were scraped and placed into tubes containing 6 ml of acidified methanol-water-$CHCl_3$ (1.5:1.5:3), the samples were vortexed, and the organic and aqueous phases were separated by centrifugation. The aqueous phase was applied to an AG 1-X8 (Bio-Rad) column, washed sequentially with water and a solution of 5 mM disodium tetraborate–60 mM sodium formate, and then eluted with a solution of 0.1 M formic acid–1.0 M ammonium formate. The radioactivity in both the eluted material (total inositol phosphates) and the organic phase of the samples (phospholipids) was quantitated and used to express the data as a percentage of labeled phospholipids.

Phosphoamino acid analysis. Confluent quiescent TRMP cells expressing a similar number of the wild-type and mutant PDGFRs were labeled with 1 mCi of $^{32}P_i$ per ml stimulated with nothing or 200 ng of PDGF-BB per ml for 5 min at 37°C, and lysed in EB (15); GAP or PLCγ was immunoprecipitated by using 0.5 μl of the GAP antiserum or 1.25 μg of UBI PLCγ antibody and subjected to phosphoamino acid analysis (18, 19). The amount of signal in the three phosphoamino acids was quantitated by using a Molecular Dynamics PhosphorImager.

DNA synthesis assay. The ability of PDGF to initiate DNA synthesis was tested as previously described (18).

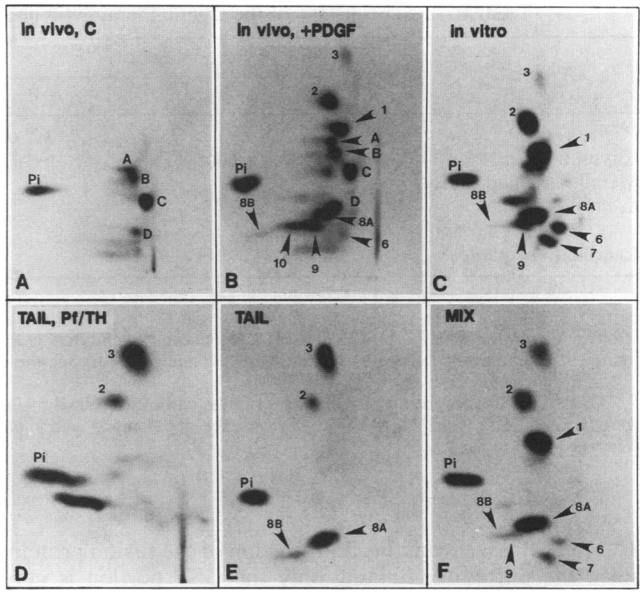

FIG. 1. Phosphopeptide maps of the PDGFR and a PDGFR carboxy-terminal fusion protein. The $^{32}P_i$ in vivo- or in vitro-labeled PDGFRs were subjected to two-dimensional tryptic-thermolytic maps as described in Materials and Methods. (A and B) In vivo-labeled PDGFRs from resting and PDGF-stimulated cells, respectively. Spots A to D are phosphoserine-containing peptides, whereas the numbered spots are phosphotyrosine-containing peptides. Pi indicates $^{32}P_i$. (C) Map of the PDGFR isolated from unstimulated cells and labeled in vitro; (E) map of the GST-tail fusion protein labeled in vitro; (D) the same GST-tail sample digested with *P. fragi*-thermolysin instead of trypsin-thermolysin; (F) mixture of the samples in panels C and E.

RESULTS

Identification of tyrosine phosphorylation sites in the carboxy terminus of the PDGFR. Initial attempts to map PDGFR tyrosine phosphorylation sites via tryptic phosphopeptide mapping identified Y-751 and Y-857 (16). Subsequent mapping studies, in which the receptor was cleaved into smaller peptides by the sequential digestion with trypsin and thermolysin, have identified two additional tyrosine phosphorylation sites as Y-740 and Y-771 (6, 13). These studies also indicated that there are still other, as yet unidentified, PDGFR tyrosine phosphorylation sites (13).

To test whether the carboxy-terminal region of the PDGFR contains any tyrosine phosphorylation sites, phosphopeptide maps of the intact PDGFR were compared with maps of the carboxy-terminal domain. The intact PDGFR, or a purified GST fusion protein that included the carboxy terminus of the PDGFR β subunit, were radiolabeled in an in vitro kinase assay, resolved on an SDS-polyacrylamide gel, and subjected to two-dimensional tryptic-thermolytic mapping. Maps of the carboxy terminus and intact PDGFR (Fig. 1C, E, and F) had four common spots (2, 3, 8A, and 8B). Phosphoamino acid analysis of these spots revealed exclusively phosphotyrosine (not shown). Two-dimensional maps of a radiolabeled fusion protein that included the kinase insert exhibited a distinct phosphopeptide map that did not include spots 3, 8A, or 8B; however, it did include spot 2 (13, and data not shown). Spot 2 is also present in tryptic-thermolytic maps of a number of unrelated proteins, so it appears not to be specific for the carboxy terminus of the PDGFR β subunit (not shown). It is unlikely that spots 3,

TABLE 1. Subcleavage of the tryptic-thermolytic phosphopeptides of the PDGFR carboxy-terminal fusion protein[a]

Protease	Specificity		Susceptibility		
	After	Before	Spot 3	Spot 8A	Spot 8B
P. fragi	–	C[b] or D	–	+	+
Chymotrypsin	P, W, Y (not PY)	–	–	–	–
Metalloendopeptidase	–	K	–	–	–
S. aureus V8	E	–	–	–	–
Candidate sequence[c]			(V)LYTA	VQPNEGDNDY(I)	

[a] Phosphopeptides 3, 8A, and 8B (resolved in Fig. 1E) were recovered from the TLE plate and subcleaved with a panel of site-specific protease as previously described (16). The amino acid and the site of cleavage (N or C terminus) are indicated for each of the proteases. After subcleavage, each sample was divided into two aliquots and resolved by TLE at pH 3.5 or ascending chromatography as described in Materials and Methods. A positive was scored when the protease treatment altered the mobility relative to that of the undigested tryptic-thermolytic phosphopeptide.

[b] C, cysteic acid.

[c] There are a total of four tyrosine residues in the carboxy-terminal region of the human PDGFR β subunit; the two tryptic-thermolytic peptides not chosen were (L)GEGYK(K) and (K)(K)YQQ. The presence of the amino acid in parenthesis in a peptide is variable and depends on which of two adjacent proteolytic cleavage sites are used.

8A, and 8B arise from the GST portion of the fusion protein, since the protein containing only the GST portion is very poorly phosphorylated (not shown). Importantly, spots 3, 8A, and 8B are also present in PDGFRs isolated from PDGF-stimulated cells labeled in vivo (Fig. 1B). These experiments indicated that the PDGFR carboxy terminus contains a number of tyrosine phosphorylation sites that are phosphorylated both in vitro and in an intact cell.

To sort out which of the four carboxy-terminal tyrosine-containing peptides correspond to spots 3, 8A, and 8B, each of these spots was recovered from the thin-layer plate and subcleaved with a panel of site-specific proteases. Spot 3 was not cleaved by any of the four proteases tested (Table 1), implicating the following sequences: either (K)(K)Y-970QQ, or (V)LY-1009T(A). To distinguish between these two possibilities, the radiolabeled carboxy-terminal fusion protein was digested with *Pseudomonas fragi* and then with thermolysin. This combination of proteases would yield the same 1009 peptide, but a very different 970 peptide [(L) LGFGY-966KKKY-970QQ(V)] that should have a different mobility than the tryptic-thermolytic 970 peptide. Figure 1D and E show that spot 3 has the same mobility in the tryptic-thermolytic and *P. fragi*-thermolytic phosphopeptide maps, strongly suggesting that spot 3 is the Y-1009 peptide. Spot 2 is also common to Fig. 1D and E; however, as stated above, it does not appear to be specific to the carboxy terminus of the PDGFR.

The subcleavage analysis predicted that spots 8A and 8B are related, since they behave identically. In addition, VQP-NEGDNDY-1021(I) is the best candidate for spots 8A or 8B, since it is the only peptide that has a *P. fragi* cleavage site. The candidate sequence also contains a *Staphylococcus aureus* V8 cleavage site, but neither spot 8A or 8B was susceptible to digestion with two different lots of this enzyme. A phosphorylated synthetic Y-1021 peptide (see below) was also not cleaved by *S. aureus* V8 (not shown), suggesting that either there was not a detectable shift in the peptide's mobility or that this particular cleavage site was not readily utilized.

To determine whether spots 8A or 8B corresponded to the Y-1021 candidate peptide, a synthetic Y-1021 peptide (VQP NEGNDY) was synthesized, phosphorylated in vitro, and purified by TLE. Figure 2 shows that the phosphorylated synthetic Y-1021 peptide comigrates with spot 8A, demonstrating that spot 8A corresponds to the phosphorylated Y-1021 peptide.

Both the subcleavage analysis and partial acid hydrolysis indicated that spot 8B is related to 8A (Table 1, and data not shown). Since there are two thermolytic cleavage sites at the C terminus of the Y-1021 sequence, perhaps 8A is a part of 8B; however, this possibility is inconsistent with the mobilities of spots 8A and 8B. We have previously observed two spots for the Y-740 peptide (spots 6 and 7 in Fig. 1C and reference 13) that are not readily explained by the expected peptide products. In strong support of the theory that spot 8B is related to spot 8A is the observation that the mutation of the Y-1021 phosphorylation site affects both spots 8A and 8B (Fig. 3).

The above-described studies convincingly implicated Y-1009 and Y-1021 as phosphorylation sites, so we mutated each of these tyrosines as well as the other two tyrosine residues in the PDGFR carboxy terminus to phenylalanine and expressed the mutant receptors in TRMP cells that lack endogenous PDGFRs (16). The mutant PDGFRs were immunoprecipitated from cells expressing similar levels of introduced PDGFR (see Fig. 9B), phosphorylated in vitro, and subjected to phosphopeptide mapping. As predicted from the experiments presented in the previous section, mutating Y-1009 ablated spot 3 but did not have any affect on any of the other phosphopeptides (Fig. 3). The F-1021 mutant lacked spot 8B but surprisingly only reduced the

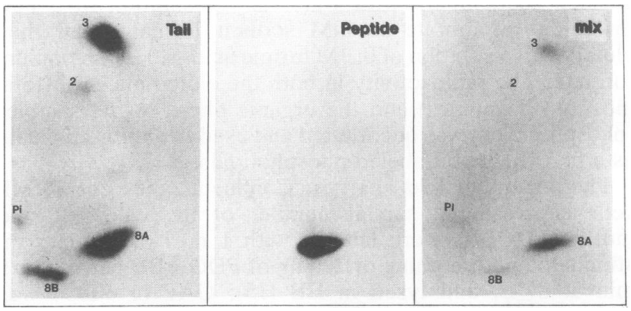

FIG. 2. Comparison of the mobility of the carboxy-terminal tail phosphopeptides and the phosphorylated Y-1021 peptide. The Y-1021 peptide [VQPNEGDNDY(I)] or the GST-carboxy-terminal tail fusion protein was phosphorylated in vitro, the fusion protein was digested with trypsin and thermolysin, and then the samples were resolved in two dimensions as in Fig. 1. mix, a 4:1 mixture of the fusion protein and peptide.

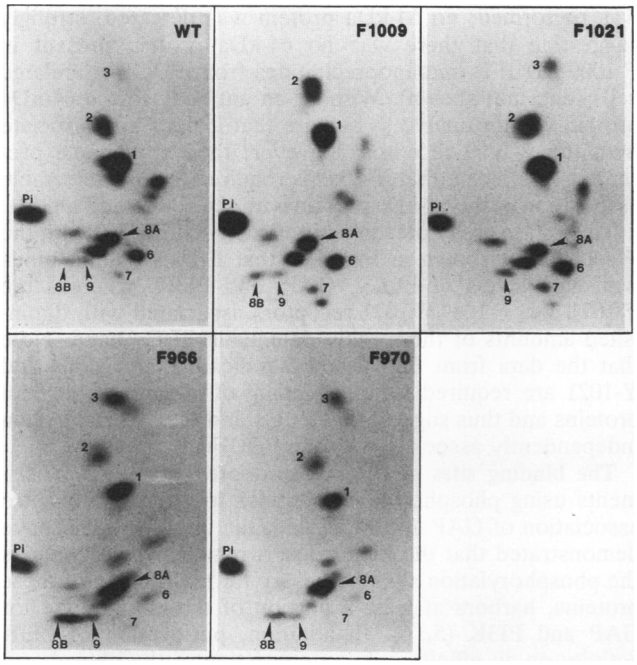

FIG. 3. Phosphopeptide maps of the wild-type and carboxy-terminal PDGFR mutants. The wild-type and mutant PDGFRs were immunoprecipitated from resting TRMP cells, labeled in vitro, and subjected to two-dimensional tryptic-thermolytic mapping as described in Materials and Methods. WT, wild type; F966, F970, F1009, and F1021 are the tyrosine-to-phenylalanine substitution mutants at positions 966, 970, 1009, and 1021, respectively.

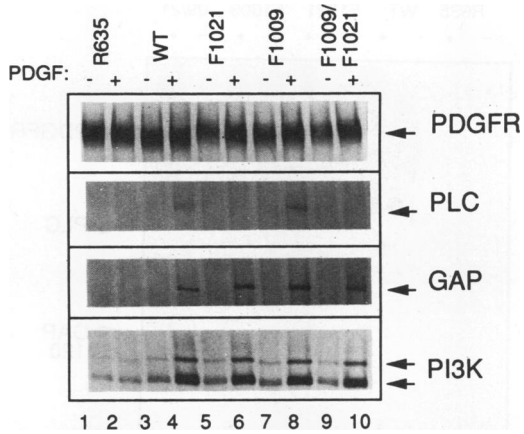

FIG. 4. Mutation of Y-1021 selectively blocks the association of PLCγ. Confluent, quiescent cultures of TRMP cells expressing similar numbers of the introduced human wild-type or mutant PDGFRs were exposed to nothing (−) or 40 ng of PDGF-BB per ml (+) for 5 min at 37°C, the PDGFR was immunoprecipitated, and the receptor-associated proteins were detected by Western blot analysis. The label to the right of each panel indicates which antibody was used in the Western blot, and the arrow points to the prominent immunoreactive species. R635, kinase inactive receptor; WT, wild-type receptor; F1021, F1009, and F1009/F1021, the tyrosine-to-phenylalanine mutants at the indicated residue.

intensity of spot 8A (Fig. 3). None of the other spots were affected by mutation of Y-1021. Importantly, mutating either of the two other tyrosine residues in the carboxy terminus (966 and 970) did not affect spots 3, 8A, 8B or any of the other phosphopeptides (Fig. 3).

While comigration of the phosphorylated synthetic Y-1021 peptide with spot 8A clearly demonstrated that spot 8A was the Y-1021 peptide, the persistence of spot 8A at a reduced level in the F-1021 mutant indicated that there may be more than one peptide that arises from the intact PDGFR that migrates in this region of the plate. For this reason carboxy-terminal fusion proteins harboring the various tyrosine-to-phenylalanine mutations were constructed and phosphorylated, and their phosphopeptide maps were compared. In these less complicated maps we found that mutation of Y-1021 selectively abolished both spot 8A and spot 8B (not shown). Thus, it seems likely that spot 8A in maps of the intact receptor is a mixture of the phosphorylated Y-1021 peptide and some other as yet undefined phosphopeptide.

In summary, we have used several independent approaches to identify Y-1009 and Y-1021 as phosphorylation sites in the carboxy terminus of the PDGFR.

The carboxy-terminal phosphorylation sites are needed for binding PLCγ and the 64-kDa protein. Studies to date have indicated that phosphorylation of the PDGFR can have two different types of consequences. Phosphorylation of the kinase insert tyrosine residues are important for stable association of PI3K and GAP but have no detectable influence on the receptor's kinase activity (4, 7, 18, and data not shown). Phosphate at Y-857 is important for both activation of the receptor's kinase activity and for the stable binding of numerous signaling molecules (4, 7, 18). We investigated whether any of the carboxy-terminal tail phosphorylation sites contribute to either the binding of the various receptor-associated proteins or the activation of the receptor's kinase activity.

To test whether phosphorylation of the carboxy terminus of the PDGFR is important for binding the various receptor-associated proteins, confluent, quiescent TRMP cells expressing similar numbers of introduced PDGFRs were exposed to nothing or 40 ng of PDGF-BB per ml for 5 min at 37°C, the PDGFR was immunoprecipitated with a PDGFR antibody, and the associated proteins were detected by Western blot (immunoblot) analysis (Fig. 4). The top panel of Fig. 4 (samples were probed with an PDGFR antiserum) demonstrates that all the immunoprecipitates contained similar amounts of PDGFR. In response to PDGF stimulation, the wild-type PDGFR associated with PLCγ, GAP, and PI3K (Fig. 4, lanes 3 and 4) (3, 12, 16, 19, 24, 34, 45), whereas the kinase activity receptor (R-635) did not bind any of these receptor-associated proteins (Fig. 4, lanes 1 and 2). Mutating Y-1009 did not markedly affect the association of PLCγ, GAP, or PI3K (Fig. 4, lanes 7 and 8). Like the F-1009 mutant, F-1021 bound wild-type levels of GAP and PI3K; however, the amount of PLCγ that associated with the F-1021 mutant was dramatically reduced (Fig. 4, lanes 5 and 6). The F-1009/F-1021 double mutant also selectively failed to associate with PLCγ (Fig. 4, lanes 9 and 10). Direct measurement of PI3K activity indicated that PDGFRs mutated at any of the carboxy-terminal phosphorylation sites complexed with wild-type levels of PI3K activity (data not shown). Note that there are two bands recognized by the antibody raised against the 85-kDa subunit of PI3K, an 84- and 72-kDa band, corresponding to the 84- and 72-kDa receptor-associated proteins detected in an in vitro kinase reaction (Fig. 5, and data not shown). Mutating Y-966 or Y-970 had no effect on binding of PLCγ, GAP, or PI3K (data not shown). These data indicate that stable association of PLCγ requires Y-1021 but not Y-1009.

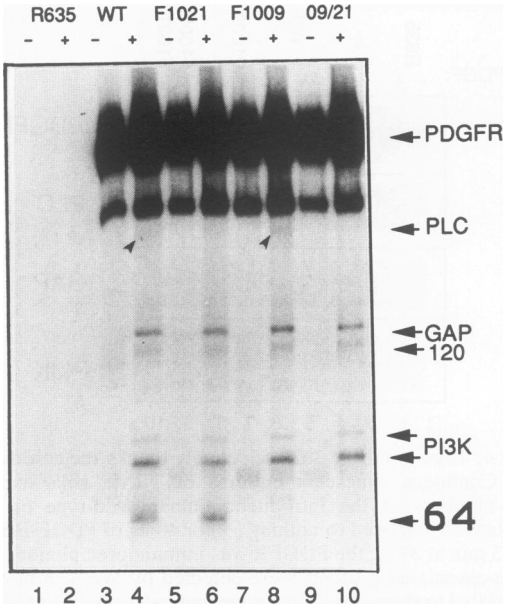

FIG. 5. Y-1009 is required for detecting the 64-kDa protein. The wild-type and mutant PDGFRs were immunoprecipitated from resting (−) or PDGF-stimulated (40 ng/ml for 5 min at 37°C) (+) TRMP cells, an in vitro kinase assay was performed, the samples were washed twice with radioimmunoassay buffer and resolved by SDS-PAGE (7.5%), and the resulting autoradiogram is presented. The arrows to the right point to the various PDGFR-associated proteins; the small arrowheads point to PLCγ. The abbreviations of the various mutants are as in the legend to Fig. 4, except that 09/21 indicates the F-1009/F-1021 double mutant.

In addition to PLCγ, GAP, and PI3K, there are several other proteins that associate with the activated PDGFR β subunit, including a 120- and a 64-kDa species that are readily detected in an in vitro kinase assay but for which antibodies are not yet available. Previous studies have shown that mutating Y-740 and Y-751 in the kinase insert ablates the association of any detectable 120-kDa protein (reference 20 and data not shown). To test whether the tail phosphorylation site mutants affect the binding of either of these proteins, PDGFR immunoprecipitates were isolated from resting or PDGFR-stimulated TRMP cells and subjected to an in vitro kinase assay. As illustrated in Fig. 5, lanes 3 and 4, the 64- and 120-kDa proteins, as well as PLCγ, GAP, and PI3K, were detected in PDGF-stimulated immunoprecipitates of the wild-type PDGFR. Wild-type levels of the 64-kDa protein associated with the F-1021 mutant (Fig. 5, lanes 4 and 6). In contrast, the F-1009 and the F-1009/F-1021 double mutants associated with a greatly diminished amount of 64-kDa protein (Fig. 5, lanes 8 and 10). The amount of 120-kDa protein that bound to the receptor was not affected by any of the carboxy-terminal mutations (Fig. 5, lanes 3 to 10). It is possible that the 64-kDa protein is present but that mutating Y-1009 alters the PDGFR's substrate specificity. This is inconsistent with the F-1009 mutant's ability to transmit PDGF-stimulated tyrosine phosphorylation of PLCγ and GAP, and the in vitro kinase activity of the F-1009 PDGFR towards an exogenous substrate is unchanged (Fig. 5, and data not shown). In addition, when wild-type PDGFRs isolated from resting cells (no associated proteins) were mixed with the F-1009 receptor isolated from PDGF-stimulated cells and a standard in vitro kinase assay was performed, no 64-kDa protein was detected, strongly suggesting that there was no 64-kDa protein present in F-1009 PDGFR immunoprecipitates from PDGF-stimulated cells (data not shown). Without an antibody to the 64-kDa protein we are unable to be sure that it does not associate with the F-1009 receptor; however, the experiments presented do indicate that Y-1009 is required for the detectable association of the 64-kDa protein with the activated PDGFR.

The kinase assay presented in Fig. 5 is consistent with the Western blot shown in Fig. 4 in that F-1009 mutant binds wild-type levels of PLCγ, GAP, and PI3K, whereas the F-1021 and F-1009/F-1021 receptors associated with diminished amounts of the weakly detectable PLCγ band. Note that the data from Fig. 4 and 5 indicate that Y-1009 and Y-1021 are required for the binding of apparently distinct proteins and thus suggest that PLCγ and the 64-kDa protein independently associate with the PDGFR.

The binding sites of PDGFR-associated proteins. Experiments using phosphorylated peptides to block the in vitro association of GAP and PI3K with the intact PDGFR have demonstrated that the kinase insert region, which contains the phosphorylation sites necessary for the binding of these proteins, harbors at least a portion of the binding site for GAP and PI3K (5, 6). In addition, purification of PI3K activity on an affinity column consisting of the phosphorylated peptide corresponding to Y-751 (36) indicated that the kinase insert region contains the complete binding site for PI3K. By analogy it seemed likely that the carboxy terminus of the PDGFR contains the complete binding site for PLCγ.

To test this possibility, GST fusion proteins including the kinase insert or the carboxy-terminal region of the PDGFR were expressed in bacteria alone or together with a promiscuous *lacZ-elk* tyrosine kinase fusion protein (26). The GST fusion proteins were immunopurified with antisera raised against GST, 0.04 μg of the immunopurified fusion proteins were mixed with TRMP cell lysates (prepared from TRMP cells expressing no PDGFRs), and the proteins that bound were detected by Western blot analysis or in a PI3K assay. The bottom panel of Fig. 6A demonstrates that only the fusion proteins coexpressed with the *lacZ-elk* fusion protein were detectably tyrosine phosphorylated. The phosphorylated kinase insert bound both GAP and PI3K but not PLCγ, whereas the phosphorylated tail associated with PLCγ but not GAP or PI3K activity (Fig. 6). Note that the unphosphorylated fusion proteins did not bind detectable levels of PLCγ, GAP, or PI3K activity (Fig. 6). Just as in the intact receptor, the F-1021 carboxy-terminal tail failed to associate with PI3K even when 10 times the amount of fusion protein was used (Fig. 6A, lanes 11 to 13). Although the antiphosphotyrosine immunoreactivity of the F-1021 fusion protein was significantly lower than those of the wild-type or F-1009 constructs (Fig. 6A), phosphopeptide maps of the F-1021 fusion proteins isolated from ^{32}P-labeled bacteria verified that Y-1009 was phosphorylated to apparently wild-type levels (not shown). These data demonstrate that the kinase insert region of the PDGFR contains the complete binding site for GAP and PI3K activity, whereas the carboxy terminus encodes the entire PLCγ binding site. Furthermore, the mutations of the carboxy terminus which prevent association of PLCγ with the intact receptor in vivo also prevent binding of PLCγ to a carboxy-terminal fusion protein in vitro.

Inositol phosphate production in cells expressing the carboxy-terminal phosphorylation site mutants. Activation of PLCγ results in the hydrolysis of phosphatidylinositol-4,5-bisphosphate, increasing the levels of cellular inositol phos-

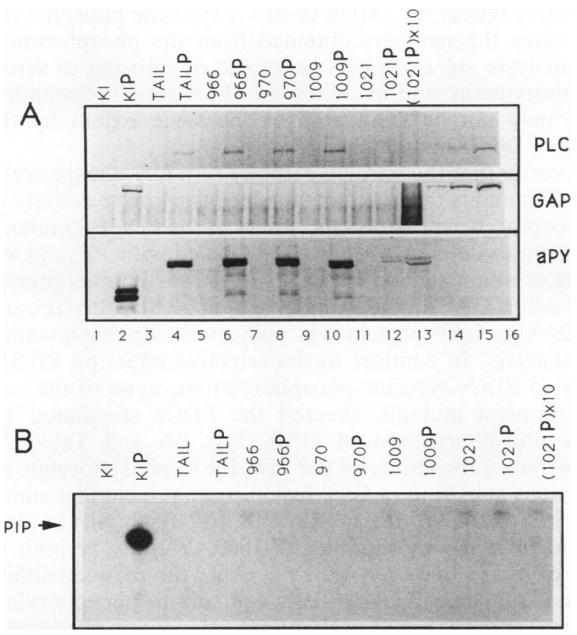

FIG. 6. In vitro binding of the various receptor-associated proteins to fusion proteins. Fusion proteins including the PDGFR kinase insert (KI), the carboxy terminus (TAIL), or carboxy-terminal phosphorylation site mutants (abbreviations are described in the legend to Fig. 3) were expressed in bacteria in the presence (indicated by P) or absence of a deregulated tyrosine kinase (*lacZ-elk*) and immunopurified, and 0.04 μg was used in an in vitro binding assay. Lane 13, 0.4 μg of fusion protein. Samples were subjected to Western blot analysis with PLCγ, GAP, or phosphotyrosine (aPY) antisera (A) or a PI3 kinase (B) assay. PIP, position of phosphatidylinositol phosphate. Lanes 14, 15, and 16, total cell lysates representing 1.2×10^4, 4.8×10^4, and 19.2×10^4 TRMP cells.

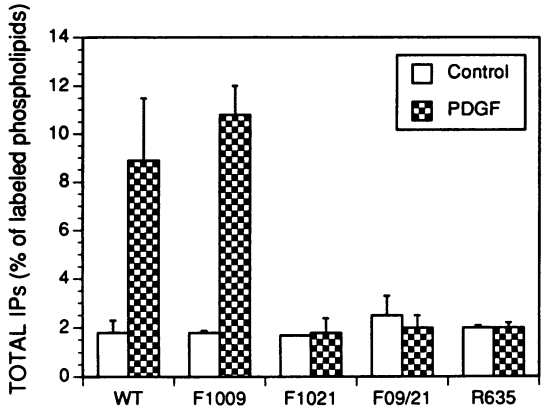

FIG. 7. PDGF-stimulated production of inositol phosphates. TRMP cells expressing similar levels of the wild-type (WT), carboxy-terminal phosphorylation site mutants, or the kinase-inactive (R635) PDGFR were labeled with [^{3}H]*myo*-inositol, exposed to buffer or 200 ng of PDGF-BB per ml, and then lysed, and the inositol phosphates were purified on an AG 1-X8 column. The data are expressed as percentages of labeled phospholipids calculated by dividing the radioactivity in the inositol phosphate (IP) pool (eluted from the column) by the radioactivity incorporated into the total phospholipid pool (the organic phase). Each sample was done in triplicate, and the error bars show the standard errors of the mean.

phates, and a number of groups have used the level of inositol phosphates as an indicator of PLCγ activation (10, 21, 31). To test whether detectable association of PLCγ with the PDGFR is necessary for increasing PLCγ activity, we measured PDGF-stimulated production of inositol phosphates in cells expressing equivalent levels of the various carboxy-terminal PDGFR mutants.

TRMP cells expressing similar levels of the carboxy-terminal PDGFR mutants or the kinase inactive PDGFR were labeled with [^{3}H]*myo*-inositol, stimulated with buffer or PDGF for 30 min at 37°C, and fixed in acidified methanol. The samples were extracted with H_2O-acidified methanol-chloroform 1.5:1.5:3, the aqueous phase was applied to an AG 1-X8 column, and the total inositol phosphates were eluted with a solution of 0.1 M formic acid–1.0 M ammonium formate. Figure 7 shows that the level of inositol phosphates increases 4.9-fold following PDGF stimulation of cells expressing the wild-type PDGFR, while the kinase inactive receptor (R-635) was unable to initiate any detectable increase. Mutation of Y-1009 has no effect on PDGF-stimulated inositol phosphate production, whereas mutating Y-1021 or both Y-1009 and Y-1021 completely eliminated the production of inositol phosphates (Fig. 7). Thus, mutants that abolish association of PLCγ also abolish the ability of the receptor to trigger PDGF-dependent inositol phosphate production.

Tyrosine phosphorylation of PLCγ. A possible explanation of why inositol phosphates are not produced in the F-1021-expressing cells is that the F-1021 receptor fails to tyrosine phosphorylate PLCγ, a prerequisite for activation of PLCγ (8, 21, 35). A precedent for this possibility is the observation that GAP is not tyrosine phosphorylated in cells that express PDGFR mutants that cannot associate with GAP (6, 13).

We measured PDGF-stimulated PLCγ tyrosine phosphorylation in the TRMP cells expressing the various carboxy-terminal phosphorylation site mutants. Confluent, quiescent TRMP cells were radiolabeled with $^{32}P_i$ and maintained quiescent or stimulated with PDGF, and then PLCγ was immunoprecipitated with PLCγ antibodies. The immunoprecipitates were resolved by SDS-PAGE, and the 148-kDa PLCγ band was excised and subjected to phosphoamino acid analysis. There was a small amount (4% of the total phosphoamino acid content) of phosphotyrosine in PLCγ in resting cells (Fig. 8A and Table 2). PDGF stimulation of cells bearing the wild-type or F-1009 PDGFR increased the phosphotyrosine content of PLCγ to 33 or 31% of the total phosphoamino acid content, respectively (Fig. 8A and Table 2), indicating that Y-1009 is dispensable for PLCγ phosphorylation. Mutation of Y-1021 reduced the PDGF-dependent tyrosine phosphorylation of PLCγ to 67% of the wild-type level, whereas combining the F-1009 and F-1021 mutations further reduced the levels of PLCγ tyrosine phosphorylation to 30% of the wild-type level (Fig. 8A and Table 2).

A similar result was obtained when PDGF-stimulated tyrosine phosphorylation of PLCγ was measured by antiphosphotyrosine Western blotting of PLCγ immunoprecipitates. PLCγ immunoprecipitated from PDGF-stimulated cells expressing either the wild-type of F-1009 PDGFR was readily detected with antiphosphotyrosine antibodies (Fig. 8B, lanes 4 and 8), whereas F-1021 and to a greater extent the F-1009/F-1021 double mutant were less able to increase the antiphosphotyrosine immunoreactivity of PLCγ (Fig. 8B, lanes 6 and 10). The lower panel of Fig. 8B is a PLCγ Western blot of the same samples, demonstrating that there were similar levels of PLCγ immunoprecipitated and that more slowly migrating forms of PLCγ were present only in samples in which PLCγ was tyrosine phosphorylated. Quan-

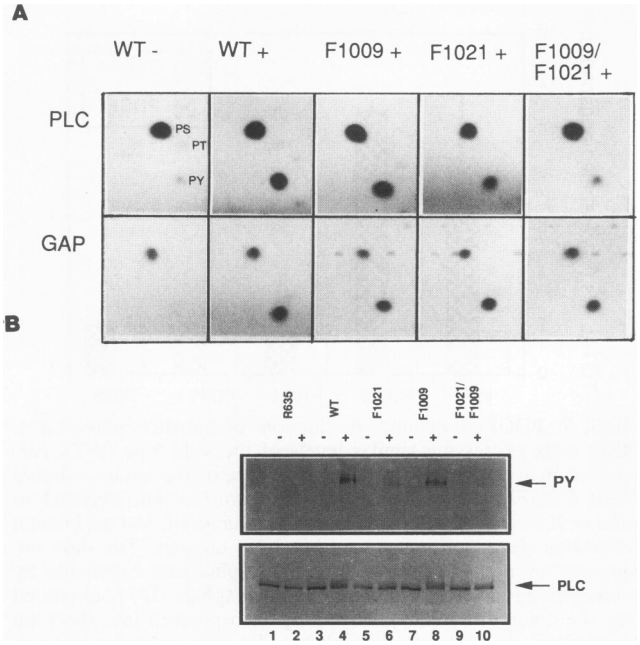

FIG. 8. Quantitation of the extent of PLCγ tyrosine phosphorylation. (A) TRMP cells expressing similar levels of the WT or mutant PDGFRs were made quiescent, labeled with 1 mCi of $^{32}P_i$ per ml, and exposed to nothing (−) or 200 ng of PDGF-BB per ml (+) for 5 min at 37°C, and then PLCγ or GAP was immunoprecipitated. The immunoprecipitates were resolved on an SDS–7.5% polyacrylamide gel, and the 148-kDa PLCγ band or the 124-kDa GAP band was excised and subjected to phosphoamino acid analysis. The top and bottom rows are the PLCγ and GAP data, respectively. PS, phosphoserine; PT, phosphothreonine; PY, phosphotyrosine. (B) PLCγ was immunoprecipitated from resting (−) or PDGF-stimulated (+) cells and subjected to a phosphotyrosine (PY; top panel) or PLCγ (bottom panel) Western blot analysis. Mutant abbreviations are outlined in the legend to Fig. 4.

titation of the extent of the antiphosphotyrosine signal indicated that PLCγ was phosphorylated to 74, 32.5, and 7.1% of the wild-type levels in the F-1009, F-1021 and F-1009/F-1021 cells, respectively. It is probable that the numbers obtained from the Western blot analysis more accurately reflect the extent of PLCγ tyrosine phosphorylation, since the numbers obtained from the phosphoamino acid analysis are expressed as a ratio of tyrosine to serine phosphorylation, and the extent of PLCγ serine phosphorylation may not be stimulated to the same extent by the various PDGFR mutants.

TABLE 2. Quantitation of the phosphoamino acid content of PLCγ and GAP phosphorylated in intact cells

Protein and phosphoamino acid	% Total phosphoamino acid content[a] in the following cells:				
	WT−	WT+	F-1009+	F-1021+	09/21+
PLCγ					
PS	94.4	66.7	69.0	77.3	88.1
PT	1.4	0.5	0.5	1.1	1.7
PY	4.1	32.7	30.7	21.7	10.2
GAP					
PS	92.6	38.0	34.1	30.5	44.6
PT	2.8	2.4	2.6	3.3	1.9
PY	4.6	59.6	62.5	66.2	53.5

[a] The data presented in Fig. 8 were quantitated by using a Molecular Dynamics PhosphorImager and are expressed as percentages of the total phosphoamino acid content. Samples from resting and PDGF-stimulated cells are denoted as − and +, respectively. WT, wild-type PDGFR; F-1009 and F-1021, the Y to F substitutions; 09/21, the double phenylalanine mutant; PS, phosphoserine; PT, phosphothreonine; PY, phosphotyrosine.

To verify that the various carboxy-terminal phosphorylation site mutants did not affect the intrinsic kinase activity of the receptor, we measured the ability of the PDGFR mutants to phosphorylate GAP. Cells were labeled with $^{32}P_i$ and left resting or stimulated with PDGF. GAP was immunoprecipitated with a GAP antibody and resolved by SDS-PAGE, and the 124-kDa GAP protein was subjected to phosphoamino acid analysis. In contrast to the selective effect on PDGF-mediated PLCγ tyrosine phosphorylation, none of the carboxy-terminal mutants affected the PDGF-stimulated tyrosine phosphorylation of GAP (Fig. 8A and Table 2). Furthermore, the ability of the PDGFR to phosphorylate an exogenous substrate (a GST fusion protein including amino acids 951 to 1089 of the human PDGFR α subunit) in vitro was not affected by mutating Y-1009, Y-1021, or both of these tyrosines (data not shown). Thus, the reduced ability of the F-1021 and F-1009/F-1021 mutants to phosphorylate PLCγ was not the result of an overall attenuation of receptor kinase activity. These experiments show, first, that the carboxy-terminal tyrosine phosphorylation sites are not required for PDGF-mediated activation of receptor kinase activity and, second, that stable association of PLCγ is not an absolute requirement for PDGF-stimulated PLCγ tyrosine phosphorylation.

The ability of the carboxy-terminal phosphorylation site mutants to stimulate DNA synthesis. We also tested the ability of the various carboxy-terminal phosphorylation site mutants to mediate a biological effect. TRMP cells expressing similar levels of wild-type or mutant PDGFRs (Fig. 9B) were tested for their abilities to stimulate DNA synthesis in response to increasing concentrations of PDGF. The F-1021 mutant triggered PDGF-dependent DNA synthesis to 70% of the wild-type levels (Fig. 9A). Mutating Y-1009 had little effect on the ability of the PDGFR to trigger DNA synthesis at saturating concentrations of PDGF, but at low doses, this mutant was slightly less able to relay the mitogenic response (Fig. 9A). The PDGFR that could not phosphorylate either Y-1009 or Y-1021 behaved as the F-1021 single mutant (Fig. 9A). The kinase-inactive (R-635) PDGFR failed to respond to PDGF at any of the doses tested (Fig. 9A). These experiments show that at saturating concentrations of PDGF, DNA synthesis in TRMP cells does not require the association of the 64-kDa protein, but that stable binding of PLCγ is necessary for the PDGFR to trigger a maximum mitogenic response.

DISCUSSION

We have identified two additional tyrosine phosphorylation sites in the carboxy-terminal tail of the human PDGFR β subunit. These tyrosines are phosphorylated both in vivo and in vitro, and Y-1021 is important for binding PLCγ, while Y-1009 is required for the stable association of an as yet uncharacterized 64-kDa protein. Binding of the 64-kDa protein appears dispensable for mediation of a biological signal, whereas PDGFRs that do not associate with PLCγ evoke a submaximal DNA synthesis response following PDGF stimulation.

Like the kinase insert region, tyrosine phosphorylation of the PDGFR carboxy-terminal tail appears to be important

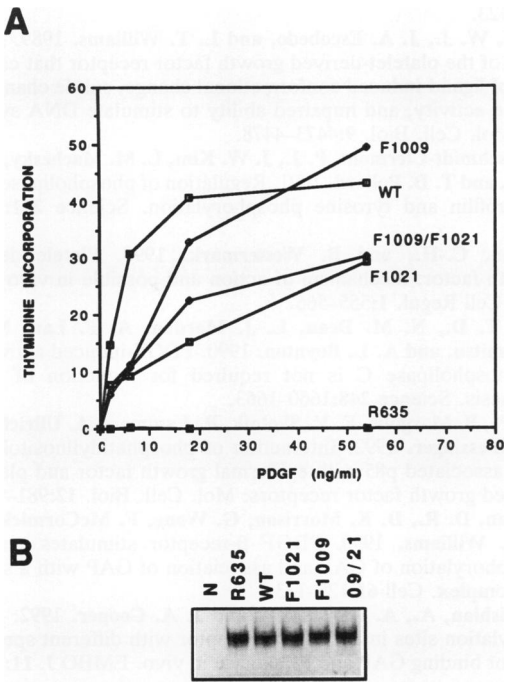

FIG. 9. Biological response of TRMP cells expressing the various PDGFR mutants. (A) TRMP cells expressing the indicated mutant receptors were plated at a low cell density and allowed to become quiescent, and the ability to incorporate [^{3}H]thymidine was measured 22 h following stimulation with either 10% fetal bovine serum or the indicated concentration of PDGF-BB in the presence of 2% horse serum. The data are expressed as percentage of the maximal response (i.e., response to 10% fetal bovine serum). The standard error of the mean was routinely less than 15%, and two other independent experiments gave similar results. (B) Total cell lysates representing 4 × 10^4 cells were resolved on an SDS–7.5% polyacrylamide gel and subjected to PDGFR Western blot analysis. N is the parental cell line expressing an empty expression vector; abbreviations for the other cell types are as in the legend to Fig. 4.

for the stable association of cellular proteins and not for activation of the receptor's kinase activity. Thus, the intracellular domain of the PDGFR contains a number of regions with distinct functions but a common theme of regulation by tyrosine phosphorylation. The kinase domain performs the phosphotransferase functions of the receptor and is activated by phosphorylation at Y-857 (7, 18). The F-857 PDGFR mutant is less able to associate with a number of the receptor-associated proteins (4, 17, 19), which at least in part reflects the decreased ability to phosphorylate the tyrosine residues in the PDGFR important for binding. In contrast, the kinase insert and carboxy-terminal regions contain a number of phosphorylation sites that are dispensable for PDGF-stimulated activation of the kinase activity but are pivotal for the stable binding of the receptor-associated proteins. In addition, the kinase insert and carboxy-terminal encode the complete binding sites for a number of the receptor-associated molecules (Fig. 6) (5, 6). Thus, the kinase insert and tail regions of the PDGFR appear to function in recruiting signal transduction molecules, and these same receptor domains serve a similar function in a number of related growth factor receptors (32, 39, 44, 47).

The identification of a group of proteins that bind PI3K has enabled the definition of a consensus PI3K binding sequence (2, 6). The tyrosine phosphorylation site necessary for binding of PLCγ had been identified for three different growth factor receptors. The important tyrosine in the EGFR is Y-992 (41, 44), and the surrounding amino acid sequence is EYLIPQ, with Y-992 underlined. In contrast, the region surrounding Y-966, which has been shown to be the phosphorylation site needed for PLCγ binding to the FGFR, EYLDLS, with Y-966 underlined (32), shares only a portion of the amino acid similarity. This region of the FGFR is conserved throughout evolution and never becomes more like the EGFR or PDGFR sequences (32). Given that SH2 domains have distinct affinities for phosphotyrosine-containing proteins (1, 22, 23, 28, 32), it is possible that the PLCγ SH2 domain that binds to the FGFR is not the same one that associates with the PDGF and EGFR. Of the two PLCγ SH2 domains, the N-terminal one has been shown to have a higher affinity for the tyrosine-phosphorylated PDGFRs and EGFRs (1). For the FGFR, a construct that included the PLCγ C-terminal SH2 and the SH3 domain was able to bind the FGFR equally as well as a construct that included both of the SH2 domains and the SH3 domain (32). This observation is consistent with the possibility that the C-terminal SH2 domain is mediating the binding of PLCγ to the phosphorylated FGFR. Thus, while PLCγ associates with a number of tyrosine-phosphorylated growth factor receptors, it may use different SH2 domains for binding to different growth factor receptors.

The kinetics of interaction of the proteins that associate with the PDGFR may not be the same for all of the receptor-associated proteins. Comparison of the amount of PDGFR that coprecipitates with PLCγ and GAP from ^{32}P-labeled TRMP cells indicated that there is at least 10× more PDGFR coprecipitating with GAP (data not shown). This suggests that, once bound to the PDGFR, GAP remains bound for a longer period, whereas PLCγ associates with the PDGFR transiently, as it does with the EGFR (23, 27). If indeed the interaction of PLCγ with the PDGFR is ephemeral, then it would explain why only relatively small amounts of PLCγ are detected complexed with the PDGFR, making it difficult to quantitate the fraction of PLCγ that associates with the PDGFR.

We have found that phosphorylation of Y-1009 is required for the stable association of a complex of proteins that we term 64 kDa. The molecular mass of the 64-kDa complex is distinct from that of p62 that associates with GAP or from the *src* family members *src*, *yes*, and *fyn* that have been shown to associate with the PDGFR (4, 25). The observation that PLCγ is tyrosine phosphorylated to a greater extent in cells expressing the F-1021 mutant (where 64-kDa is associated with the receptor) than in cells expressing the F-1009/F-1021 PDGFR (where 64 kDa is not associated) (Fig. 8), raises the possibility that the 64-kDa protein is itself a protein tyrosine kinase or an activator of protein tyrosine kinases. We are currently attempting to identify this receptor-associated protein.

The F-1021 PDGFR mutant that does not bind PLCγ is unable to initiate PDGF-dependent inositol phosphate production (Fig. 7). Surprisingly, PLCγ is detectably tyrosine phosphorylated in cells expressing the F-1021 mutant (Fig. 8). Perhaps in the F-1021 cells tyrosine phosphorylation of PLCγ is not on the tyrosine residues that enhance PLCγ activity. Alternatively, PLCγ is activated, but since it does not associate with the PDGFR it does not gain access to its substrate, phosphatidylinositol-4,5-bisphosphate, which is in the plasma membrane. If this is the case, then formation of a complex between PLCγ and the PDGFR is important not

only for the tyrosine phosphorylation of PLCγ but also to position PLCγ such that it can interact with its substrate.

The observations that phorbol esters that activate protein kinase C (PKC) are mitogens and that inhibitors of protein kinase C can block the initiation of DNA synthesis support the possibility that activation of PLCγ, and the subsequent activation of protein kinase C, is important for the relay of a biological signal. In contrast, several groups have recently found that fibroblast growth factor activates PLCγ but that this event is dispensable for mitogenesis (31, 38). Similar results have been reported for PDGFR; in mouse 10T1/2 cells, PDGF can initiate DNA synthesis in the apparent absence of PLCγ activation (10), and PDGFRs that do not associate with PLCγ are still fully able to initiate DNA synthesis in pig aortic endothelial cells (40). Surprisingly, we find that association with PLCγ and production of inositol phosphates are required for a maximal mitogenic response of TRMP cells (Fig. 9). Given that activation of growth factor receptors appears to trigger multiple, and perhaps redundant, mitogenic cascades, it is possible that PLCγ does play a role in mitogenic signaling, but its contribution is not readily detected when other signaling pathways are engaged.

A likely explanation of the discrepancy of PLCγ's relative contribution to mitogenesis may reflect that PLCγ plays a bigger role in relaying mitogenic signals in TRMP cells than in other cell types. Additional cell type-specific differences have been reported. When expressed in normal murine mammary gland epithelial cells, PDGFRs that do not bind PI3K are virtually unable to trigger DNA synthesis (6), whereas in TRMP cells, the same PDGFR mutant triggers approximately 60% of the wild-type response (20). Thus, while the tyrosine-phosphorylated PDGFR associates with the same signal transduction proteins when expressed in a variety of different cell types, the relative role of these proteins in the relay of a biological signal may be cell type specific.

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