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Tyrosine Mutations within the α Platelet-Derived Growth Factor Receptor Kinase Insert Domain Abrogate Receptor-Associated Phosphatidylinositol-3 Kinase Activity without Affecting Mitogenic or Chemotactic Signal Transduction

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A phosphatidylinositol-3 (PI-3) kinase activity of unknown biological function associates with tyrosine kinase-containing proteins, including a number of growth factor receptors after ligand stimulation. In the β platelet-derived growth factor (βPDGF) receptor, phosphorylation of a specific tyrosine residue within the kinase insert domain was required for its interaction with this enzyme. We show that substitutions of phenylalanine for tyrosine residue 731 or 742 within the kinase insert domain of the αPDGF receptor do not impair PDGF-induced tyrosine phosphorylation of the receptor or of an in vivo substrate, phospholipase C-γ. Moreover, phosphatidylinositol turnover in response to ligand stimulation is unaffected. However, both lesions markedly impair receptor association with PI-3 kinase. Antiphosphotyrosine antibody-recoverable PI-3 kinase was also dramatically reduced in PDGF-stimulated cells expressing either mutant receptor. Since neither mutation abolished PDGF-induced mitogenesis or chemotaxis, we conclude that αPDGF receptor-associated PI-3 kinase activity is not required for either of these major PDGF signalling functions.

The interaction of platelet-derived growth factor (PDGF) with its receptors causes activation of the receptor tyrosine kinase, which leads to a cascade of biochemical events, culminating in mitogenesis (38). Two PDGF receptor (PDGFR) genes, designated α and β, encode related proteins which can act independently to perform major PDGF signalling functions, including proliferation and chemotaxis (18). Certain enzymes that appear as tyrosine-phosphorylated substrates after PDGF stimulation are believed to participate in the intracellular signal transmission. In fibroblasts, phospholipase C-γ (PLCγ) and the GTPase-activating protein (GAP) have recently been shown to be tyrosine phosphorylated rapidly in response to PDGF triggering (20, 21) and to be coimmunoprecipitated by PDGFR antibodies (13, 17, 22). PLCγ hydrolyzes phosphatidylinositol 4,5-bisphosphate into two second messengers, 1,2-diacylglycerol and inositol 1,4,5-trisphosphate. The former activates protein kinase C, and the latter promotes the release of Ca^{2+} from intracellular stores (2). GAP enhances hydrolysis of *ras*-GTP to *ras*-GDP, which normally inactivates *ras* function (19, 30). Accumulating evidence indicates that the activities of both enzymes are affected by tyrosine phosphorylation (7, 21, 34).

Another enzyme that appears to be activated rapidly in PDGF-treated cells is a phosphatidylinositol-3 (PI-3) kinase (4, 14). This kinase was initially identified in immune complex with the v-*src* protein (28) and later found to be physically associated with activated PDGFRs as well as other tyrosine kinases (4, 5, 14, 26, 33). In cells transformed by v-*src* and v-*fms* and in fibroblasts stimulated with PDGF, PI-3 kinase is also immunoprecipitated by antiphosphotyrosine (anti-P-Tyr) antibody (1, 14, 27, 33, 37), implying that this enzyme is itself tyrosine phosphorylated or associated with other tyrosyl-phosphoproteins. PI-3 kinase phosphorylates the inositol ring of phosphatidylinositol (PI) at the D3 position instead of the regular D4 position, but there are as yet no clues as to the biological functions of such PI metabolites (1, 36).

Evidence that transforming $p60^{v\text{-}src}$ mutants invariably show associated PI-3 kinase activity suggested that this enzyme may be necessary for the growth-promoting action of the v-*src* protein (10). In the case of the βPDGFR, kinase insert (ki) deletion mutants which failed to associate with PI-3 kinase lacked the ability to induce DNA synthesis in response to PDGF (8). Thus, it has been proposed that association with PI-3 kinase is necessary for PDGFR-mediated mitogenesis (4). Recently, Kazlauskas and Cooper localized a specific site of tyrosine phosphorylation at residue 751 in the βPDGFR ki domain, which was required for PI-3 kinase association (15, 16). In the present report, we introduced substitutions for tyrosine residues within the αPDGFR ki domain in order to determine the effects on PDGF-induced biochemical and biological responses, with the goal of defining the role of receptor-associated PI-3 kinase in major PDGFR signalling functions.

MATERIALS AND METHODS

Site-directed mutagenesis and transfection of 32D cells. A 1.7-kb *Pst*I-*Bam*HI fragment containing the cytoplasmic region of the αPDGFR was subcloned into M13mp19 replicative form. Two oligonucleotides (5′-AATGGTGACTTC ATGGACATG-3′ and 5′-ACTACACAGTTTGTCCCCA T-3′), encompassing tyrosine 731 and tyrosine 742, were used to direct mutagenesis. The mutagenesis reaction was performed according to the manufacturer's protocol ("Mutagene M13 *in vitro* mutagenesis"; Bio-Rad Laboratories). Each mutated *Pst*I-*Bam*HI fragment was then cloned into

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pUC18 containing the extracellular domain of the αPDGFR. The entire αPDGFR cDNA was then subcloned into the LTR-2 eucaryotic expression vector (6).

DNA transfection of 32D cells was performed by the electroporation procedure (24). Mass populations of stably transfected cells were selected by their ability to survive in growth medium containing mycophenolic acid (80 mM).

Immunoblot and immunoprecipitation analysis. 32D cells were washed twice in Dulbecco's modified Eagle's medium and incubated at 37°C for 2 h in serum-free medium. The quiescent 32D cells were then stimulated with PDGF-BB (100 ng/ml; Upstate Biotechnology, Inc.) for 5 min at 37°C. After centrifugation, the cell pellets were lysed in 100 mM Tris buffer (pH 8) containing 1% sodium dodecyl sulfate (SDS), 1 mM Na_3VO_4, and 1 mM phenylmethylsulfonyl fluoride (PMSF) for 10 min at 90°C. Clarified lysates (100 μg per lane) were then resolved by electrophoresis on 7% polyacrylamide gels with SDS, followed by immunoblotting with polyclonal antipeptide serum against amino acids 1074 to 1089 of the human αPDGFR or monoclonal anti-P-Tyr (Upstate Biotechnology, Inc.) as described previously (12). For immunoprecipitation analysis, stimulated cells were treated with 5 mM diisopropyl fluorophosphate (DFP) at 4°C for 5 min and lysed in a P-Tyr buffer containing 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.5), 1% Triton X-100, 50 mM NaCl, 50 mM NaF, 10 mM sodium pp_i, 5 mM EDTA, 1 mM Na_3VO_4, 1 mM PMSF, 10 μg of aprotinin per ml, 10 μg of leupeptin per ml, and 5 mM DFP. Soluble lysates (2 mg) were immunoprecipitated with anti-P-Tyr antibody. Immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with monoclonal anti-PLCγ (29).

In vitro PDGFR autophosphorylation. Quiescent 32D transfectants were triggered with PDGF-BB (100 ng/ml) for 5 min at 37°C and lysed in P-Tyr buffer. Soluble lysates (2 mg) were immunoprecipitated with a monoclonal antibody directed against the extracellular domain of the human αPDGFR (Genzyme). Recovered immunoprecipitates were incubated with 50 μl of kinase buffer containing 50 mM Tris (pH 7.4), 10 mM $MgCl_2$, 20 μg of aprotinin per ml, 10 μM ATP, and 5 μCi of [γ-^{32}P]ATP for 20 min at room temperature. Reactions were terminated by addition of 50 μl of 2× sample buffer (100 mM Tris [pH 6.8], 4% SDS, 20% glycerol, 1.7 M β-mercaptoethanol, 0.02% bromophenol blue). Samples were heated to 90°C for 3 min, and proteins were resolved by SDS-PAGE.

Receptor-associated PI-3 kinase assays. For measurement of in vivo αPDGFR-associated PI-3 kinase activity, quiescent 32D cells were exposed to PDGF-BB (100 ng/ml) for 5 min at 37°C, incubated with 5 mM DFP at 4°C for 5 min, and lysed in a buffer containing 20 mM Tris (pH 8), 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% Nonidet P-40, 10% glycerol, 1 mM Na_3VO_4, 5 mM DFP, 1 mM PMSF, and 10 μg of aprotinin and of leupeptin per ml. Soluble lysates (2 mg) were immunoprecipitated with monoclonal anti-αPDGFR antibody (Genzyme) or anti-P-Tyr antibody. Immunoprecipitates were recovered with protein G-Sepharose and assayed for PI-3 kinase activity as measured by the ability of the coimmunoprecipitates to phosphorylate PI to yield phosphatidylinositol phospate (PIP) (11).

For measurement of in vitro PI-3 kinase binding to ATP-treated PDGFRs, wild-type (wt) or mutant αPDGFRs were recovered from unstimulated 32D transfectants by immunoprecipitation with monoclonal anti-αPDGFR. After incubation with 50 μl of 50 mM Tris (pH 7.4)–10 mM $MgCl_2$–20 μg of aprotinin per ml in the presence or absence of 10 μM ATP at room temperature for 20 min, the immunoprecipitates were washed twice with the same lysing buffer. The receptor immunoprecipitates were then incubated with soluble extracts of quiescent 32D cells prepared under the same lysing conditions. The in vitro association reaction was performed at 4°C for 1 h with tube rotation. After the reaction, half of the sample was assayed for receptor-associated PI-3 kinase activity as described previously (11). The other half was assayed by SDS-PAGE and then immunoblotting with polyclonal antipeptide serum against amino acids 1074 to 1089 of the human αPDGFR.

PI turnover. PI turnover was measured as described previously (18).

Mitogenesis and chemotaxis assays. For the mitogenesis assay, 32D transfectants were washed twice with RPMI-1640 medium (GIBCO), plated at 3×10^5 cells per ml in Costar 24-well plates in RPMI-1640 medium containing 15% fetal calf serum in the absence or presence of increasing concentrations of PDGF-BB or murine interleukin-3 (IL-3; Genzyme) for 24 h, and labeled with 5 μCi of [^{3}H]thymidine per ml for 3 h, as described previously (12, 18). For determination of directed cell migration in response to PDGF-BB, modified Boyden chambers and Nuclepore filters (5-μm pore size) were used as described before (12, 18).

RESULTS

Expression and receptor autophosphorylation of mutant αPDGFR in 32D cells. Tyrosine 742 of the αPDGFR corresponds to tyrosine 751 of the βPDGFR by sequence alignment (Fig. 1A). Moreover, based on consensus sequences of tyrosine kinase major autophosphorylation sites (23), tyrosine 731 was also a putative autophosphorylation site in the ki domain of the αPDGFR. Thus, we made phenylalanine substitutions for tyrosine 731 and tyrosine 742 by site-directed mutagenesis. Each mutant receptor cDNA (designated Y731F and Y742F) was then cloned into a murine leukemia virus long terminal repeat-driven expression vector (6) and transfected into naive 32D cells. Expression of PDGFRs in IL-3-dependent 32D cells has previously been shown to allow efficient coupling with intracellular pathways of mitogenic and chemotactic signalling (18).

To examine the expression of the transfected cDNAs, cell lysates from mass populations of marker-selected 32D transfectants were subjected to immunoblot analysis with anti-αPDGFR peptide serum. As shown in Fig. 1B, anti-αPDGFR serum detected a major 190-kDa protein in 32D transfectants, consistent with our previous results (18). The 190-kDa protein levels of each mutant receptor were similar to that of the wt αPDGFR (Fig. 1B, lanes 3 to 8). Moreover, when the transfectants were exposed to PDGF, the wt and mutant receptors were tyrosine phosphorylated to comparable levels (Fig. 1C, lanes 4, 6, and 8), suggesting that they possessed similar in vivo kinase activities. As shown in Fig. 1C, we also observed a heterogeneous population of tyrosine-phosphorylated high-molecular-weight species in wt as well as Y731F and Y742F cells triggered with PDGF. The identity of these species is not known, but their relative level of phosphorylation was similar for the wt and each of the mutant receptors. As an independent measure of kinase function, in vitro kinase assays were performed with immunoprecipitates containing either wt or mutant αPDGFRs. The results revealed that neither mutation significantly impaired in vitro receptor autophosphorylation activity (data not shown).

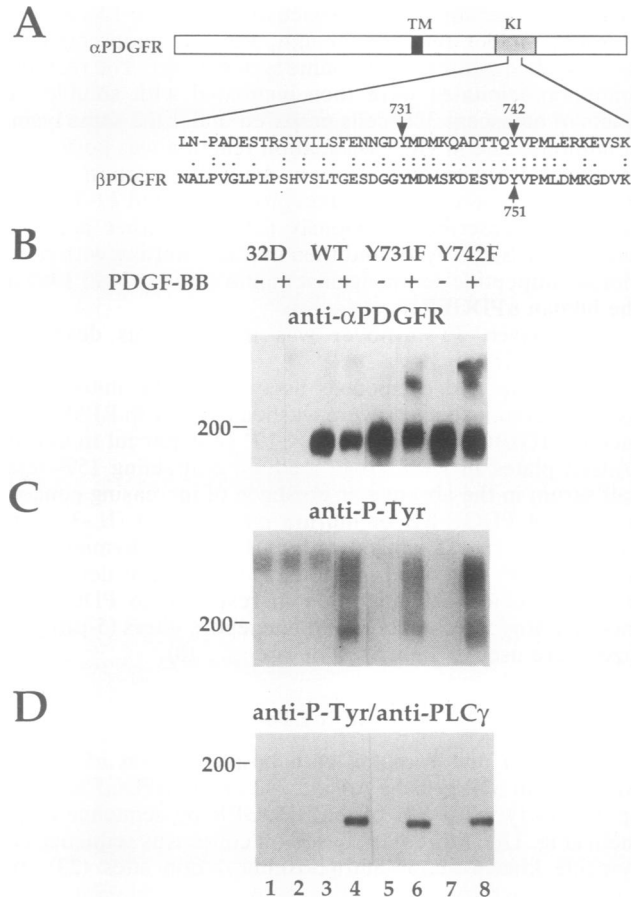

FIG. 1. Expression of Y731F and Y742F αPDGFR mutants. (A) Schematic diagram of the αPDGFR and amino acid sequence comparison of a portion of the ki domain of αPDGFR and βPDGFR. Black box, transmembrane (TM) domain; shaded box, kinase insert (KI) domain of αPDGFR. In the sequence alignment of ki domains of αPDGFR and βPDGFR, two dots show identities and a single dot shows conservative changes. (B and C) 32D cells (lanes 1 and 2) or 32D cells transfected with wt αPDGFR (lanes 3 and 4), Y731F (lanes 5 and 6), or Y742F (lanes 7 and 8) were either untreated (−) or treated (+) with PDGF-BB. Clarified lysates (100 μg per lane) were resolved by SDS-PAGE and transferred to Immobilon-P (Millipore). The transferred blot was immunoprobed with either anti-αPDGFR serum (B) or anti-P-Tyr (C). (D) Comparison of anti-P-Tyr recovery of PLCγ following PDGF stimulation. PLCγ immunoblots of anti-P-Tyr immunoprecipitates of cells treated as in panels B and C.

Effects of tyrosine mutations on PDGF-induced phosphorylation of PLCγ and PI turnover. We next investigated the effects of the αPDGFR ki mutations on PDGF-induced tyrosine phosphorylation of PLCγ, a known in vivo substrate of PDGFR kinases (20). Following stimulation with PDGF, cell lysates were first immunoprecipitated with anti-P-Tyr to enrich for P-Tyr-containing proteins and then immunoblotted and probed with anti-PLCγ monoclonal antibodies. Figure 1D shows that PLCγ was tyrosine phosphorylated in each of the 32D transfectants stimulated with PDGF. Moreover, the level of phosphorylation was similar in cells expressing wt or mutant receptors (Fig. 1D, lanes 4, 6, and 8). These results indicated that neither the tyrosine 731 nor 742 mutation impaired the in vivo kinase activity of the αPDGFR for this well-characterized substrate.

Since PLCγ hydrolyzes phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate and 1,2-diacylglycerol (2), we also analyzed PI turnover in the transfectants. As shown in Table 1, accumulation of inositol phosphates in 32D transfectants exhibited a dose-dependent increase in response to PDGF stimulation. At saturating ligand concentration, 32D cells expressing wt αPDGFR demonstrated an approximately eightfold increase in inositol phosphates, and cells expressing the Y731F or Y742F mutant showed comparable increases (Table 1). Thus, neither of these mutations caused any observable impairment in αPDGFR signalling through the PI hydrolysis pathway.

Effects of tyrosine mutations on αPDGFR-associated and anti-P-Tyr-recoverable PI-3 kinase activity. To analyze receptor-associated PI-3 kinase activity, we first measured the PDGF-induced increase in PIP with the anti-αPDGFR immune complex from wt αPDGFR transfectants. To conclusively identify the PIP product, it was scraped from the thin-layer chromatography plate, subjected to deacylation, and analyzed by high-pressure liquid chromatography. A $p60^{v\text{-}src}$ transformant was used as the source of phosphatidylinositol 3-phosphate standard (10), and [^{3}H]glyceroinositol 4-phosphate was used as another standard (11). The results revealed that most of the PIP generated by using the anti-αPDGFR immune complex from PDGF-stimulated 32D cells expressing wt αPDGFRs was phosphatidylinositol 3-phosphate, the product of the PI-3 kinase (data not shown). Furthermore, αPDGFR-associated PI-3 kinase activity following PDGF stimulation was at least 50-fold greater than in untreated cells (Fig. 2A, lanes 3 and 4). This increase was similar to that reported previously for cells expressing βPDGFRs (4).

In contrast to the wt αPDGFR transfectant, 32D transfectants expressing either Y731F or Y742F showed no detectable increase in PIP when analyzed under the same conditions for PDGF-stimulated receptor-associated PI-3 kinase activity (Fig. 2A, lanes 5 to 8). Levels of immunoprecipitated

TABLE 1. Comparison of PI turnover and chemotaxis of 32D transfectants in response to PDGF-BB

Transfectant	PI turnover[a] (cpm of inositol phosphates) at PDGF-BB concn:			Chemotaxis[b] (10^3 cells migrated) at PDGF-BB concn:		
	0	10 ng/ml	100 ng/ml	0	10 ng/ml	100 ng/ml
wt	654 ± 68	3,745 ± 728	5,231 ± 399	38 ± 10	78 ± 6	176 ± 12
Y731F	811 ± 79	5,484 ± 322	8,926 ± 459	50 ± 6	86 ± 26	156 ± 44
Y742F	882 ± 88	3,282 ± 183	7,801 ± 386	42 ± 12	104 ± 36	138 ± 30

[a] PI turnover was measured by accumulation of inositol phosphates in 32D transfectants stimulated with increasing concentrations of PDGF-BB. Each value represents the mean value of triplicate samples ± standard deviation.

[b] Chemotaxis was determined by directed cell migration in response to PDGF-BB as described in Materials and Methods. Each value represents the mean value ± standard deviation of triplicate samples. Similar results were obtained in three independent experiments.

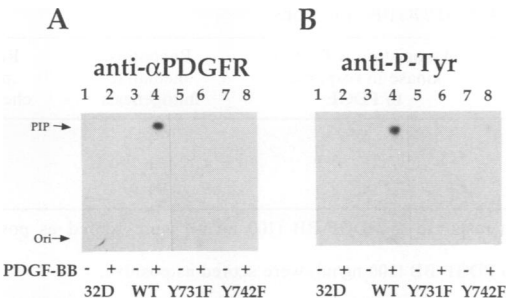

FIG. 2. Comparison of receptor-associated PI-3 kinase activities in PDGF-stimulated 32D transfectants. 32D cells (lanes 1 and 2) or 32D cells expressing wt αPDGFR (lanes 3 and 4), Y731F (lanes 5 and 6), or Y742F (lanes 7 and 8) were either untreated (−) or treated (+) with PDGF-BB. Cell lysates were immunoprecipitated with a monoclonal antibody directed against αPDGFR (A) or anti-P-Tyr (B). Immune complexes were then subjected to the PI-3 kinase assay described before (11). The PI-3 kinase reaction products were then analyzed by thin-layer chromatography. The positions of phosphatidylinositol phosphate (PIP) and the origin (Ori) are designated by arrows. Results are representative of at least three independent experiments.

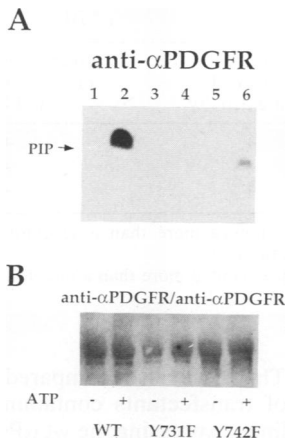

FIG. 3. Comparison of in vitro association of PI-3 kinase with wt and mutant αPDGFRs. PDGFRs from 32D cells expressing wt (lanes 1 and 2), Y731F (lanes 3 and 4), or Y742F (lanes 5 and 6) receptors were immunoprecipitated with monoclonal anti-αPDGFR antibody. Immunoprecipitates were incubated with kinase buffer containing 10 μM ATP (+) or no ATP (−) for 20 min at room temperature and then exposed to lysates of quiescent 32D cells as described in Materials and Methods. Immune complexes were then subjected to the PI-3 kinase assay (11) (A) or receptor analysis by immunoblotting with anti-αPDGFR serum (B). Similar results were obtained in two independent experiments.

wt and mutant receptors varied less than twofold (data not shown). As a specificity control, we engineered another point mutation, substituting phenylalanine for tyrosine 572, which corresponds to tyrosine 960 of the insulin receptor (35). This mutation exhibited no decrease in receptor-associated PI-3 kinase activity (data not shown). Thus, substitution of tyrosine 731 or tyrosine 742 within the ki domain appeared to specifically impair αPDGFR-associated PI-3 kinase activity in response to PDGF.

PI-3 kinase activity has also been observed following ligand stimulation in anti-P-Tyr immunoprecipitates (1, 14, 26, 27, 33, 37). Thus, we performed the PI-3 kinase assay under these conditions as well. As shown in Fig. 2B, PDGF induced more than a 50-fold increase in anti-P-Tyr-recoverable PI-3 kinase activity in 32D cells expressing wt αPDGFR (lanes 3 and 4). In contrast, Y731F and Y742F showed undetectable and about 15% of the wt αPDGFR level of anti-P-Tyr-recoverable PI-3 kinase activity, respectively, under the same conditions (Fig. 2B, lanes 5 to 8). These results strongly suggested that tyrosine 731 and tyrosine 742 were independently required for anti-P-Tyr recovery of PI-3 kinase.

Effects of tyrosine mutations on in vitro αPDGFR binding to the PI-3 kinase. As an independent approach to confirm that the Y731F and Y742F mutants abrogated αPDGFR binding to the PI-3 kinase, we performed an in vitro PI-3 kinase association assay as described in Materials and Methods. As shown in Fig. 3A, the wt receptor showed at least a 50-fold increase in receptor-associated PI-3 kinase activity following in vitro phosphorylation and incubation with lysates of quiescent 32D cells (lanes 1 and 2). Under the same conditions, the Y731F and Y742F mutant receptors showed at least 95 and 80% reduction, respectively, relative to the wt receptor level (Fig. 3A, lanes 3 to 6). The amount of immunoprecipitated receptors utilized varied by less than twofold (Fig. 3B). Thus, the relative impairment of in vitro αPDGFR-associated PI-3 kinase activity in Y731F and Y742F correlated well with the impairment in anti-P-Tyr recovery of PI-3 kinase observed with each mutant.

Mitogenic and chemotactic responsiveness of mutant αPDGFR. To test the biological effects of tyrosine 731 and tyrosine 742 mutations, we compared mitogenic activation of wt and mutant αPDGFRs. As shown in Fig. 4, PDGF induced a dose-dependent increase in DNA synthesis in 32D transfectants containing either wt or mutant receptors, with about 10 to 20 ng/ml required for half-maximal responses. Moreover, the maximal stimulation in DNA synthesis observed with 32D cells expressing either Y731F or Y742F was similar to that of the wt receptor transfectant (Fig. 4). Thus, despite abrogation of receptor-associated or anti-P-Tyr-recoverable PI-3 kinase activity, neither the tyrosine 731 nor the tyrosine 742 mutation caused any significant reduction in mitogenic signalling.

Chemotaxis is another major PDGF signalling pathway which can be coupled in 32D cells transfected with the

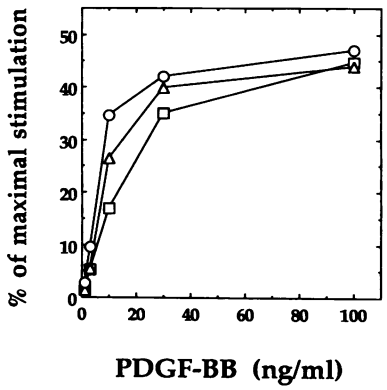

FIG. 4. Comparison of PDGF-BB-induced mitogenic response of 32D transfectants. DNA synthesis by 32D cells expressing wt αPDGFR (□), Y731F (△), or Y742F (○) was measured by [^{3}H]thymidine incorporation in IL-3-free medium as described before (12, 18). Each point represents the mean value for duplicate samples. Similar results were obtained in three independent experiments.

TABLE 2. Summary of wt, Y731F, and Y742F αPDGFR functions

αPDGFR	Ligand-activated kinase activity	Tyrosine phosphorylation of PLCγ in response to PDGF	Receptor-mediated PI turnover[a]	Association with PI-3 kinase in response to PDGF	Receptor-mediated mitogenesis	Receptor-mediated chemotaxis[b]
wt	+	+	+	+	+	+
Y731F	+	+	+	−	+	+
Y742F	+	+	+	−	+	+

[a] Cell lines which showed more than a sevenfold increase in inositol phosphates in response to PDGF-BB (100 ng/ml) were scored as positive for receptor-mediated PI turnover.

[b] 32D cell lines that exhibited more than a threefold increase in chemotactic response to PDGF-BB (100 ng/ml) were scored as positive.

αPDGFR (18). Thus, we also compared the chemotactic responsiveness of transfectants containing wt and mutant αPDGFRs. Cell lines expressing the wt αPDGFR exhibited a four- to fivefold increase in chemotactic response to PDGF (100 ng/ml), and cell lines expressing either mutant receptor demonstrated a comparable fold increase under the same conditions (Table 1). Thus, the severe inhibition in receptor-associated PI-3 kinase activity of either the tyrosine 731 or tyrosine 742 mutation was not responsible for any decrease in either of the major known biological responses to PDGF.

DISCUSSION

Our present studies demonstrate that a point mutation in tyrosine 731 or tyrosine 742 within the ki domain of the αPDGFR markedly impairs receptor-associated PI-3 kinase activity, yet neither of these lesions adversely affected receptor kinase activity, as measured by in vivo or in vitro receptor tyrosine phosphorylation. These findings are consistent with the results of Kazlauskas and Cooper, who used an analogous βPDGFR mutant, which showed no decrease in its in vitro autophosphorylation, presumably due to vicarious phosphorylation (15). Neither of the mutants showed detectable in vivo impairment in its ability to tyrosine-phosphorylate PLCγ, a known PDGFR substrate (20). The absence of any inhibition in downstream signalling mediated by PLCγ was confirmed by evidence that PDGF stimulation of mutant receptors led to rapid PI turnover, comparable in magnitude to that associated with the wt receptor. The specific abrogation of receptor-associated PI-3 kinase activity was not associated with any detectable impairment in PDGFR-mediated mitogenesis or chemotaxis.

Our findings are consistent with previous studies localizing tyrosine residues within the ki domain of the βPDGFR as being essential for receptor interaction with PI-3 kinase (15, 16). PI-3 kinase has been purified and shown to be a heterodimer of 110- and 85-kDa subunits (3). Moreover, this 85-kDa subunit has been reported to be identical to an 85-kDa protein which is tyrosine phosphorylated and associates with activated PDGFRs in PDGF-stimulated cells (14). These findings argue that PI-3 kinase is tyrosine phosphorylated in response to PDGF triggering, although the effects of tyrosine phosphorylation on its function are not yet known. We showed that following PDGF stimulation, there was little if any anti-P-Tyr-recoverable PI-3 kinase activity in Y731F or Y742F cells. Although it is possible that tyrosine-phosphorylated PI-3 kinase is specifically not detected by the anti-P-Tyr used, the same antibody has been shown to widely recognize many known tyrosine-phosphorylated receptor kinases and their substrates. Thus, it seems more likely that the marked decrease in anti-P-Tyr-recoverable PI-3 kinase in PDGF-triggered Y731F and Y742F cells reflects the impaired ability of the mutant receptors to tyrosine phosphorylate the enzyme.

Recently, Roussel et al. reported that a point mutation in tyrosine 809 of the colony-stimulating factor 1 receptor did not affect receptor association with PI-3 kinase or induction of the c-*fos* and *junB* genes. However, these responses were not sufficient to induce a mitogenic response to colony-stimulating factor 1 (25). Similarly, a mutation of the major in vivo tyrosine autophosphorylation site of the βPDGFR did not impair its ability to associate with PI-3 kinase (15), yet this mutant lacked the ability to induce DNA synthesis in response to PDGF (9). All of these findings have indicated that receptor-associated PI-3 kinase activity is not sufficient to elicit a mitogenic response.

Our present studies indicate that despite markedly reduced receptor-associated or anti-P-Tyr-recoverable PI-3 kinase activity, our PDGFR mutants showed no impairment in PDGFR-mediated mitogenic or chemotactic signal transduction (Table 2). It is possible that the low residual receptor-associated PI-3 kinase activity within these mutants is sufficient to provide a threshold level required for an essential function in mitogenic signalling. It is also possible that there is sufficient redundancy in the mitogenic signalling cascade that any one target molecule is not required for an efficient response. Alternatively, it is possible that PI-3 kinase may be required for some signalling function yet to be elucidated. There is accumulating evidence that the level of in vivo phospholipid products of the PI-3 kinase correlates better with the status of proliferation or transformation than antibody-recoverable PI-3 kinase activity does (31, 32). Studies to characterize in vivo levels of PI-3 kinase metabolites in cells expressing wt or mutant receptors as well as the effects of combined Y731F and Y742F mutants on αPDGFR functions should help to resolve these possibilities.

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The first two authors made major contributions to the experiments presented in this article.

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