Biological Function of PDGF-induced PI-3 Kinase Activity: Its Role in αPDGF Receptor-mediated Mitogenic Signaling

Jin-Chen Yu,* J. Silvio Gutkind,‡ Daruka Mahadevan,* Weiqun Li,* Kimberly A. Meyers,* Jacalyn H. Pierce,* and Mohammad A. Heidaran*

*Laboratory of Cellular and Molecular Biology, National Cancer Institute (37-1E24), and ‡Laboratory of Cellular Development and Oncology, National Institute of Dental Research (30-211), National Institutes of Health, Bethesda, Maryland 20892

Abstract. The tyrosine phosphorylation sites in the human αPDGF receptor (αPDGFR) required for association with PI-3 kinase have been identified as tyrosines 731 and 742. Mutation of either tyrosine substantially reduced PDGF-induced PI-3 kinase activity but did not impair the receptor-mediated mitogenic response. We sought to determine whether PDGF-induced PI-3 kinase activity could be further ablated so as to exclude a low threshold requirement for PDGFR signal transduction. Thus, we mutated both tyrosine 731 and 742 and expressed the double mutant (Y731F/Y742F) in 32D hematopoietic cells. In such transfectants, PDGF induced no detectable receptor-associated or anti-P-Tyr recoverable PI-3 kinase activity. Under the same conditions, neither mobility shift of raf-1 nor tyrosine phosphorylation of either PLCγ or MAP kinase was impaired. 32D transfectants expressing the double mutant showed wild-type αPDGFR levels of mitogenic and chemotactic responses to PDGF. To examine the effect of the double mutation in cells that normally respond to PDGF, we generated chimeras in which the cytoplasmic domains of wild-type αPDGFR, Y731F, and Y731F/Y742F were linked to the extracellular domain of colony-stimulating factor-1 (CSF-1) receptor (fms). After introduction of the chimeric receptors into mouse NIH/3T3 fibroblasts, the ability of CSF-1 to stimulate growth of these transfectants was examined. Our data show that all these chimeric receptors exhibited similar abilities to mediate CSF-1-stimulated cell growth. These findings lead us to conclude that PDGF-induced PI-3 kinase activity is not required for PDGF-stimulated mitogenic pathway in both NIH/3T3 fibroblasts and 32D hematopoietic cells.

INTERACTION of PDGF with its receptors causes activation of the receptor tyrosine kinase, which leads to a cascade of biochemical events culminating in mitogenesis (Williams, 1989). Two PDGF receptor (PDGFR)[1] genes, designated α and β, encode related proteins that can act independently to perform major PDGF signaling functions, including proliferation and chemotaxis (Matsui et al., 1989). Several cellular proteins, including phospholipase Cγ (PLCγ), GTPase-activating protein (GAP), and phosphatidylinositol-3 (PI-3) kinase become associated with and tyrosine-phosphorylated by the activated PDGFR (Meisenhelder et al., 1989; Molloy et al., 1989; Kaplan et al., 1990; Morrison et al., 1990; Kumjian et al., 1989; Coughlin et al., 1989; Kavanaugh et al., 1992). These interactions are mediated by high affinity binding between the *src* homology 2 (SH2) domains of the substrates and specific phosphorylated tyrosine residues of receptor (Cantley et al., 1991). For example, in the human βPDGFR, phosphorylation of tyrosine 771 is necessary for association with GAP (Kashishian et al., 1992; Kazlauskas et al., 1992). In contrast, phosphorylation of tyrosine 1021 in the carboxy-terminal region is required for binding of PLCγ (Valius et al., 1993), while PI-3 kinase interaction sites are tyrosine 740 and tyrosine 751 (Kashishian et al., 1992; Kazlauskas et al., 1992).

PI-3 kinase phosphorylates the inositol ring of PI, PI-4P, and PI-4,5P2 at the D3 position (Auger et al., 1989; Whitman et al., 1988). Purification of PI-3 kinase revealed that it consists of an 85-kD (p85) regulatory and a 110-kD (p110) catalytic subunit (Skolnik et al., 1991; Escobedo et al., 1991; Ostu et al., 1991; Hiles et al., 1992). The p85 subunit lacks PI-3 kinase activity and functions as a regulator, which binds to the activated tyrosine kinase (Hu et al., 1992; McGlade et al., 1992). Although the precise role of PI-3 kinase in regulating cell growth remains unknown, evidence that transforming p60[v-src] and polyoma middle-T mutants invariably show associated PI-3 kinase activity has suggested that this enzyme may be necessary for mitogenic signaling (Fukui and Hanafusa, 1989).

1. *Abbreviations used in this paper*: CSF-1, colony-stimulating factor-1; DFP, diisopropyl fluorophosphate; MAP, mitogen-activated protein; PDGFR, PDGF receptor; PLCγ, phospholipase Cγ; GAP, GTPase-activating protein; PI-3, phosphatidylinositol-3; PIP, phosphatidylinositol phosphate; wt, wild type.

In the αPDGFR, mutation of either tyrosine 731 or tyrosine 742 substantially impairs PDGF-induced PI-3 kinase activity, but not PDGF-stimulated mitogenesis in 32D cells (Yu et al., 1991). Similar results have been observed using analogous mutants of the βPDGFR in epithelial cells (Fantl et al., 1992). These results have implied that PDGF-induced PI-3 kinase is not required for mitogenic signal transduction. However, we could not exclude the possibility that the low levels (~5–15%) of residual PDGF-induced PI-3 kinase activity observed with such mutants were sufficient to transduce the PDGF mitogenic signal. In addition, neither the hematopoietic nor epithelial cell lines used in these investigations represented a physiological system for PDGF signaling because they do not normally express PDGFRs. To better define the role of PI-3 kinase in αPDGFR signal transduction, we generated a double mutant (Y731F/Y742F) by substituting phenylalanines for tyrosines 731 and 742 within the αPDGFR. The present studies describe the effects of these mutations on PDGF-induced PI-3 kinase activity, other downstream signaling molecules, as well as biological functions of the αPDGFR in two different cell systems.

Materials and Methods

Site-directed Mutagenesis and Generation of fms/αPDGFR Chimeras

Construction of the single mutant Y731F clone has been described previously (Yu et al., 1991). To generate the double mutant, an M13 subclone containing the cytoplasmic region of the Y731F was used as a template, and an oligonucleotide (5′ACTACACAGTTTGTCCCCAT-3′) encompassing tyrosine 742 of human αPDGFR was used to direct mutagenesis. The mutagenesis reaction was performed according to the manufacturer's protocol ("Mutagene M13 in vitro mutagenesis"; Bio Rad Laboratories, Hercules, CA). The entire mutant αPDGFR cDNA was then subcloned into the LTR-2 eucaryotic expression vector (Di Fiore et al., 1987a).

To generate fms/αPDGFR chimeras, the SalI-PvuII fragment of human CSF-1R (fms) extracellular domain and PvuII-BamHI fragment of the cytoplasmic domain of wt αPDGFR, Y731F and Y731F/Y742F were ligated to the LTR-2 expression vector digested with XhoI and BamHI (Di Fiore et al., 1987a).

Transfection and Cotransfection Assays

DNA transfection of 32D cells was performed by the electroporation procedure (Pierce et al., 1988). Mass populations of stably transfected cells were selected by their ability to survive in growth medium containing mycophenolic acid (80 mM). Transfection of NIH/3T3 cells was performed by the calcium phosphate precipitation technique, as previously described (Heidaran et al., 1990). To analyze the effects of increased wild-type (wt) or mutant αPDGFR expression on PDGF-A-transforming efficiency, cotransfection of αPDGFR, and PDGF-A expression cDNAs was performed using similar amounts (~1 μg) of each construct. Since PDGF-A and αPDGFR construct each contain different drug-resistant markers (Beckmann et al., 1988; Di Fiore et al., 1987a), the number of cells expressing both cDNAs was determined by measuring the number of colonies that survived in the presence of both mycophenolic acid (80 mM) and geneticin (750 μg/ml). The number of transformed foci was determined 2–3 wk after transfection in plates that did not undergo any marker selection. For measuring the colony-stimulating factor-1 (CSF-1)-dependent transformation of NIH/3T3 expressing fms/αR chimeras, cells were transfected as described above. After 5 d, cell cultures were maintained in DME containing 2% calf serum supplemented with or without human CSF-1 (100 ng/ml).

Immunoblot and Immunoprecipitation Analyses

32D cells were washed twice in DME and incubated at 37°C for 2 h in serum-free medium. The quiescent 32D cells were then stimulated with PDGF-AA (Upstate Biotechnology, Inc., Lake Placid, NY) (100 ng/ml) for 5 min at 37°C followed by treatment with 5 mM diisopropyl fluorophosphate (DFP) at 4°C for 5 min. Cell lysis was performed using 1 ml of P-Tyr lysing buffer containing 50 mM Hepes (pH 7.5), 1% Triton X-100, 50 mM NaCl, 50 mM NaF, 10 mM sodium pyrophosphate, 5 mM EDTA, 1 mM Na₃VO₄, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 5 mM DFP/5 × 10⁷ cells. Clarified lysates (100 μg/lane) were resolved by 8% SDS-PAGE followed by immunoblotting with the appropriate antiserum. Polyclonal antipeptide serum against amino acids 959-973 of the human αPDGFR has been described (Heidaran et al., 1991; Yu et al., 1991). This antibody recognizes both the human and murine αPDGFR equally well. Monoclonal anti-P-Tyr was obtained from Upstate Biotechnology, Inc., and polyclonal anti-raf-1 serum was raised against a peptide CTLTTSPRLPVF encompassing the carboxy terminal domain of the human raf-1 (Gutkind, J. S., N. Xu, and K. C. Robbins, personal communication).

For immunoprecipitation, cells were lysed as described above. Around 2 mg of soluble lysate was immunoprecipitated with anti-P-Tyr antibody (Upstate Biotechnology, Inc.). Immunoprecipitates were resolved on SDS-PAGE and immunoblotted with monoclonal anti-PLCγ (Upstate Biotechnology, Inc.) or monoclonal anti-MAP kinase (Zymed Laboratories, Inc., South San Francisco, CA).

For lysing NIH/3T3 cells, the same lysing buffer lacking 5 mM DFP was used. Clarified lysates were subjected to immunoprecipitation using a monoclonal antibody against human αPDGFR that recognizes human, but not murine αPDGFR (Yu et al., 1994). After SDS-PAGE and electroblotting, the filter was immunoblotted with a polyclonal anti-αPDGFR antiserum, which reacts with both human and murine αPDGFR equally well (Yu et al., 1991).

PI-3 Kinase Assay

For measurement of in vivo αPDGFR-associated PI-3 kinase activity, quiescent 32D cells were exposed to PDGF-AA (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 MgCl₂, 1 mM CaCl₂, 1% Nonidet P-40, 10% glycerol, 1 mM Na₃VO₄, 5 mM DFP, 1 mM PMSF, and 10 μg/ml aprotinin and leupeptin. Soluble lysates (2 mg) were immunoprecipitated with monoclonal anti-αPDGFR antibody (Genzyme Corp., Boston, MA) or anti-P-Tyr antibody (Upstate Biotechnology, Inc.). Immunoprecipitates were recovered with protein G-Sepharose and assayed for PI-3 kinase activity as measured by their ability to phosphorylate PI to yield PIP (Gutkind et al., 1990).

Mitogenesis and Chemotaxis Assays

For measurement of DNA synthesis, cells were washed twice with PBS and plated at 3 × 10⁵ cells/ml into 24-well plates (Costar Corp., Cambridge, MA) in RPMI-1640 medium containing 15% fetal calf serum in the absence or presence of increasing concentrations of PDGF-AA or murine IL-3 (Genzyme Corp.) for 24 h followed by labeling with 5 μCi/ml of [³H]thymidine for 5 h. Cells were harvested and processed for measurement of [³H]thymidine incorporation, as previously described (Heidaran et al., 1991; Matsui et al., 1989). For determination of directed cell migration in response to PDGF-BB, modified Boyden chambers and Nucleopore filters (5-μm pore size; Costar Corp.) were used as described before (Heidaran et al., 1991; Matsui et al., 1989).

Soft Agar Assay

For analysis of proliferation in semisolid medium, 1 × 10⁵ of NIH/3T3 transfected cells were suspended in 0.4% agarose (SeaPlaque; FMC BioProducts, Rockland, ME) in DME containing 10% calf serum as described elsewhere (Di Fiore et al., 1987a). Cells were fed with DME containing 10% calf serum in the presence or absence of 100 ng/ml of human CSF-1 once per week. Colonies were stained with p-iodonitrotetrazolium violet (Sigma Immunochemicals, St. Louis, MO) and scored after 2 wk.

Results

Effects of Double Mutations on PDGF-induced PI-3 Kinase Activity

We have previously shown that mutation of tyrosine 731 or 742 within the kinase insert domain of the αPDGFR impairs

PDGFR-associated PI-3 kinase activity 95% or 80%, respectively (Yu et al., 1991). In an effort to further ablate αPDGFR-asssociated PI-3 kinase activity, we generated a double mutant αPDGFR (Y731F/Y742F), and cloned it into a long terminal repeat–driven expression vector (Di Fiore et al., 1987a). To analyze the double mutant, 32D cells were transfected by electroporation, and mass populations were marker selected. The level of expression of wt or mutant αPDGFR in independent 32D transfectants was found to be similar, ~5 × 10⁴ receptors/cell (Matsui et al., 1989, and data not shown). To assess the effects of the Y731F/Y742F mutant on PDGF-induced PI-3 kinase activity, cell lysates from PDGF-stimulated or unstimulated 32D transfectants were immunoprecipitated with a monoclonal anti-αPDGFR antibody. The immune complexes were subjected to an in vitro PI-3 kinase assay (Gutkind et al., 1990).

As shown in Fig. 1 A, addition of PDGF caused >50-fold increase in phosphatidylinositol phosphate (PIP) formation
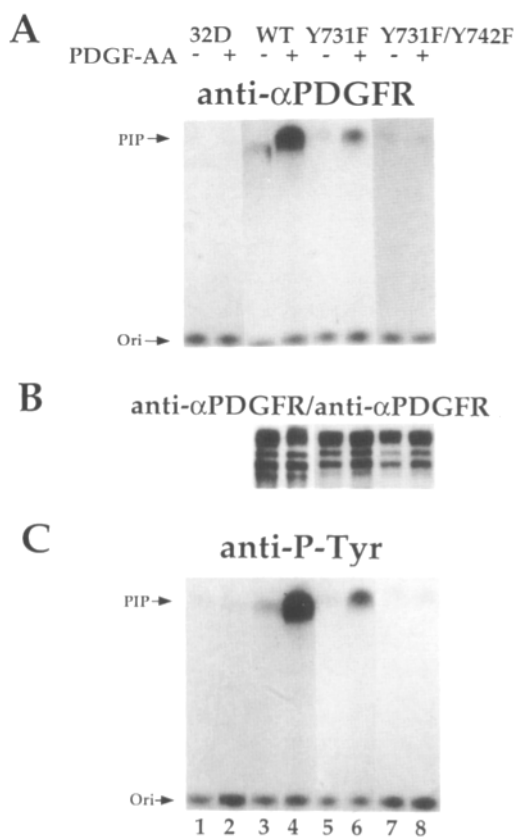

Figure 1. Comparison of PDGF-induced receptor-associated or anti-P-Tyr recoverable PI-3 kinase activities in PDGF-stimulated 32D transfectants. 32D cells (lanes 1 and 2) or 32D cells expressing wt αPDGFR (lanes 3 and 4), Y731F (lanes 5 and 6), or Y731F/Y742F (lanes 7 and 8) were either untreated (−) or treated (+) with PDGF-AA. Cell lysates were immunoprecipitated with a monoclonal antibody directed against αPDGFR (A) or anti-P-Tyr (C). Immune complexes were then subjected to the PI-3 kinase assay described before (Gutkind et al., 1990). 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. Fractions of anti-αPDGFR immune complex were immunoblotted with a polyclonal antiserum against carboxy terminus of human αPDGFR (B).

in PDGFR immunoprecipitates from 32D cells expressing the wt αPDGFR (Fig. 1 A, lanes 3 and 4). As previously reported (Yu et al., 1991), the PI-3 kinase activity associated with the Y731F upon PDGF triggering was ~5–10% of that associated with the wt receptor (Fig. 1 A, lanes 5 and 6). Under the same conditions, 32D cells expressing the Y731F/Y742F receptor showed no detectable increase in PIP level upon PDGF triggering (Fig. 1 A, lanes 7 and 8). As a control, a portion of each preparation of immunoprecipitated receptors was resolved by SDS-PAGE and immunoblotted with anti-αPDGFR antiserum. The levels of receptors among the preparations varied less than twofold (Fig. 1 B).

PI-3 kinase activity has also been observed in anti-P-Tyr immunoprecipitates after ligand stimulation (Auger et al., 1989; Kaplan et al., 1987). Moreover, the p85 subunit has been shown to be tyrosine-phosphorylated in PDGF-triggered cells (Kavanaugh et al., 1992). Thus, we also performed PI-3 kinase assays using anti-P-Tyr immune complexes after PDGF stimulation. As shown in Fig. 1 C, the Y731F mutant showed ~5% of wt αPDGFR levels of anti-P-Tyr recoverable PI-3 kinase activity (Fig. 1 C, lanes 5 and 6), while the double mutant resulted in complete loss of recoverable activity (Fig. 1 C, lanes 7 and 8). Anti-P-Tyr immune complexes from cells expressing wt or mutant αPDGFRs were resolved by SDS-PAGE and immunoblotted with polyclonal anti-p85 antibody. Consistently, cells expressing αPDGFR (Y731F/Y742F) showed no increase in anti-P-Tyr recovery of p85 upon PDGF stimulation, while cells expressing the Y731F mutant showed a low but detectable level of anti-P-Tyr recovery of p85 as compared to that of wt αPDGFR (data not shown). All of these results confirm that mutations affecting both tyrosines 731 and 742 resulted in complete loss of PDGF-stimulated receptor-associated or anti-P-Tyr recoverable PI-3 kinase activity.

Effects of Tyrosine Mutations on PDGF-stimulated Activation of Other Cellular Signaling Molecules

To study the effects of the mutations on receptor interactions with other signaling molecules, we analyzed PDGF-stimulated tyrosine phosphorylation of PLCγ and mitogen-activated protein (MAP) kinase as well as activation of raf-1. Accordingly, cell lysates of PDGF-treated or untreated 32D transfectants were immunoprecipitated with anti-P-Tyr followed by immunoblotting with PLCγ monoclonal antibody. The levels of expression and tyrosine phosphorylation of αPDGFRs upon triggering were similar among the transfectants analyzed (Fig. 2, A and B). Fig. 2 C shows that PLCγ was tyrosine-phosphorylated at comparable levels in PDGF-stimulated cells expressing wt or mutant receptors (Fig. 2 C, lanes 4, 6, and 8). These results imply that mutation of neither Y731 nor Y731/Y742 impaired in vivo kinase activity of the αPDGFR for this well-characterized substrate. Such results are consistent with recent findings that the tyrosine required for βPDGFR to associate with PLCγ is tyrosine 1021 within its carboxy-terminal domain (Valius et al., 1993).

Both raf-1 and MAP kinase are cytoplasmic serine/threonine protein kinases that are indirectly activated in response to PDGF (Morrison et al., 1989; L'Allemain et al., 1991; Pelech and Sanghera, 1992). The raf-1 protein kinase is re-
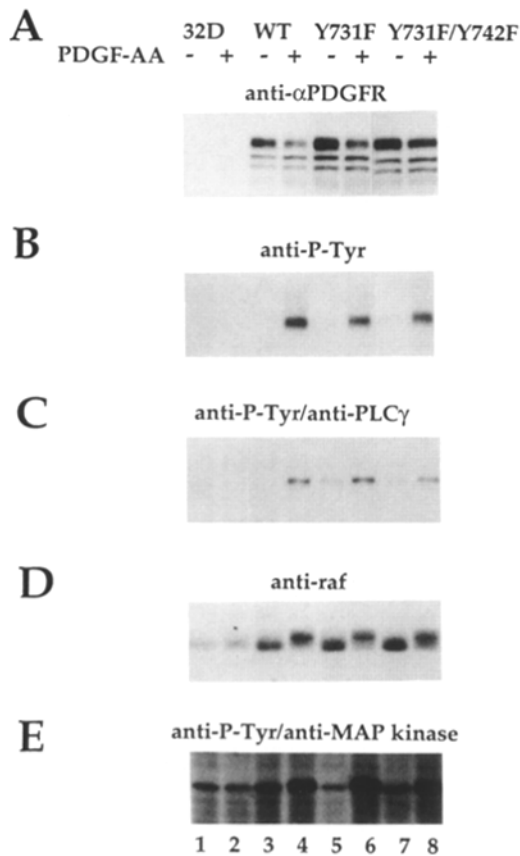
Figure 2. Effect of tyrosine-mutated αPDGFRs on activating downstream signal molecules in PDGF-stimulated 32D transfectants. 32D cells (lanes 1 and 2) or 32D cells transfected with wt αPDGFR (lanes 3 and 4), Y731F (lanes 5 and 6), or Y731F/Y742F (lanes 7 and 8) were either untreated (−) or treated (+) with 100 ng/ml of PDGF-AA. Clarified lysates (100 μg/lane) were resolved by SDS-PAGE and transferred to Immobilon-P (Millipore). The transferred blot was immunoprobed with anti-αPDGFR serum (A), monoclonal anti-P-Tyr antibody (B), or polyclonal anti-raf-1 antiserum (D). In C and E, 2 mg of clarified lysates were subjected to immunoprecipitation using anti-P-Tyr antibody followed by SDS-PAGE. The transferred blot was immunoblotted with anti-PLCγ antibody (C) or monoclonal anti-MAP kinase antibody (E).

quired for proliferation of NIH/3T3 cells (Kolch et al., 1991), and it has been reported to be upstream of MAP kinase (Kyriakis et al., 1992; Howe et al., 1992), which can activate c-*jun* by specific phosphorylation of two serine sites within c-*jun* (Pulverer et al., 1991). Therefore, *raf*-1 and MAP kinases seem to play important roles in PDGF-stimulated mitogenic signaling. To investigate the effects of Y731F and/or Y731F/Y742F receptor mutations on PDGF-stimulated activation of *raf*-1, we studied the mobility shift of this kinase as it was used as an index of its activation. Accordingly, total cell lysates from PDGF-triggered 32D transfectants were resolved by SDS-PAGE and immunoblotted with antiserum against *raf*-1. As shown in Fig. 2 *D*, wt and each of the mutant receptors caused a similar *raf*-1 mobility shift upon PDGF stimulation (lanes *3–8*).

We further tested the effects of the same mutations on PDGF-induced tyrosine phosphorylation of MAP kinase. Accordingly, anti-P-Tyr immune complexes from 32D transfectants were resolved by SDS-PAGE and then immunoblot-

ted with monoclonal anti-MAP kinase antibody. Fig. 2 *E* shows that neither Y731F nor Y731F/Y742F mutant receptors impaired PDGF-stimulated tyrosine phosphorylation of MAP kinase (Fig. 2 *E*, lanes *3–8*). Since tyrosine phosphorylation of MAP kinase correlates well with and is required for its activation (Anderson et al., 1990; Pelech and Sanghera, 1992), these data further suggest that neither Y731F nor Y731F/Y742F mutations impaired the ability of the αPDGFR to cause activation of MAP kinase in response to PDGF.

Mitogenic and Chemotactic Responses of Mutant αPDGFRs in 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 signaling (Matsui et al., 1989). To test the biological effects of the double mutant in 32D cells, we compared mitogenic activation by wt and mutant αPDGFRs. As shown in Fig. 3 *A*, PDGF induced a dose-dependent increase in DNA synthesis in 32D transfectants containing either wt or double-mutant αPDGFRs with ~10–20 ng/ml required for half maximal responses. Moreover, the maximal stimulation in DNA synthesis observed with 32D cells expressing the Y731F/Y742F receptor was similar to that of transfectants expressing the wt receptor (Fig. 3 *A*). Thus, despite complete abrogation of PDGF-induced receptor-associated or anti-P-Tyr recoverable PI-3 kinase activity, the double mutation was not associated with any significant reduction in mitogenic signaling in 32D cells.

Chemotaxis is another major biological response mediated by αPDGFR in 32D cells (Matsui et al., 1989). Thus, we also compared the chemotactic responsiveness of transfectants containing wt and mutant αPDGFRs. As shown in Fig. 3 *B*, cells expressing the wt αPDGFR exhibited an eightfold increase in chemotactic response to PDGF (100 ng/ml). 32D cells expressing the mutant αPDGFR (Y731F/Y742F) demonstrated a comparable fold increase under the same conditions. Thus, despite complete inhibition of PDGF-induced PI-3 kinase activity, the Y731/Y742 mutant showed no impairment in either mitogenic or chemotactic responses to PDGF in 32D cells.

Comparison of the Transforming Activity of wt and Mutant αPDGFR in NIH/3T3 Cells Cotransfected with PDGF-A Chain

We next sought to investigate the effect of the Y731/Y742 mutation in a cell type, NIH/3T3, which normally expresses PDGFRs. To do so, we used a PDGF-A chain transformation enhancement assay (Heidaran et al., unpublished results). PDGF-A chain is known to exhibit low transforming efficiency as compared to PDGF-B chain in NIH/3T3 cells, which express both α and βPDGFRs (Beckmann et al., 1988). αPDGFR levels have been shown to be limiting for PDGF-A chain transformation, since cotransfection of constructs expressing PDGF-A and the wt αPDGFR results in increased PDGF-A-transformed foci.

As shown in Fig. 4 *A*, transfection with PDGF-A resulted in ~20 transformed foci/μg (Fig. 4 *Aa*). In contrast, cotransfection of PDGF-A chain with the wt αPDGFR resulted in ~200 foci/μg of PDGF-A chain (Fig. 4 *Ab*). It can be ob-
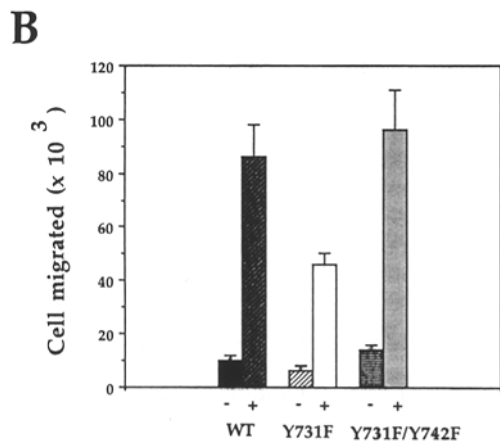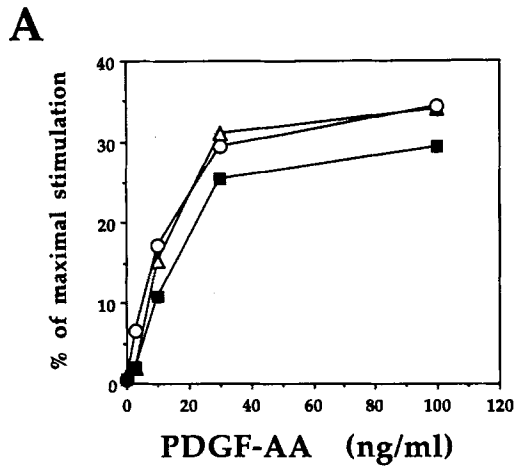
A

B

Figure 3. Comparison of PDGF-induced mitogenic and chemotactic response of 32D transfectants. DNA synthesis of 32D cell expressing wt αPDGFR (Δ), Y731F (O), or Y731F/Y742F (■) was measured by [³H]thymidine incorporation in IL-3-free medium as described in Materials and Methods (*A*). Each point represents the mean value of duplicate samples. Chemotaxis was determined by directed cell migration in response to 100 ng/ml of PDGF-BB (*B*). Modified Boyden chambers and Nucleopore filters (5-μm pore size) were used in this assay. Similar results were obtained in three independent experiments.

A

B
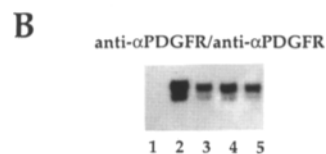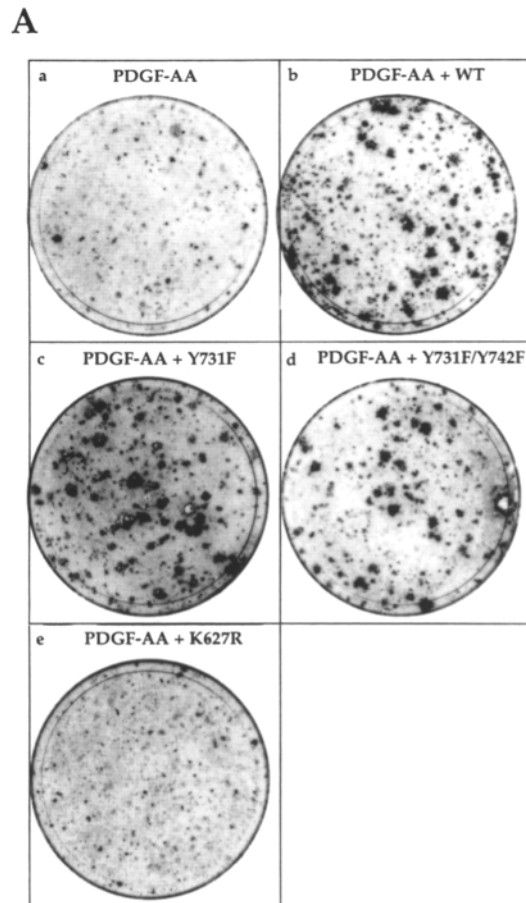
anti-αPDGFR/anti-αPDGFR

1 2 3 4 5

Figure 4. Comparison of the ability of wt and mutant αPDGFRs to enhance PDGF-A-transforming function in NIH3T3. NIH/3T3 cells were cotransfected with PDGF-A chain and LTR-gpt marker (*Aa*), wt αPDGFR (*Ab*), Y731F (*Ac*), Y731F/Y742F (*Ad*), or K627R (*Ae*). Transforming foci were detected 3 wk after transfection. Cells were fixed with 70% ethanol and stained with Giemsa stain. (*B*) The level of human αPDGFR expressed in each transfectant was analyzed. Cell lysates from marker-selected cultures were subjected to immunoprecipitation using monoclonal anti-αPDGFR antibody that recognizes human but not the murine αPDGFR. After SDS-PAGE and electroblotting, the filter was immunoblotted with a polyclonal anti-αPDGFR antiserum.

served that cotransfection with either Y731F or Y731F/Y742F caused a similar enhancement in PDGF-A focus induction to that observed with the wt receptor (Fig. 4 *A, c* and *d*). As a control, PDGF-A cotransfection with a mutant αPDGFR (K627R), which contains a mutation in the ATP-binding site, led to only ∿20 foci (Fig. 4 *Ae*). To ensure that the lack of increase in transforming activity of PDGF-A coexpressed with K627R receptor was not caused by lower level of expression of the mutant receptor, we examined the human αPDGFR levels expressed in each coselected culture. Accordingly, total cell lysates (2 mg) were subjected to immunoprecipitation using a monoclonal antibody that recognizes human but not the endogenous αPDGFR. The immune complexes were then subjected to immunoblot analysis using a polyclonal antibody to the αPDGFR. Results shown in Fig. 4 *B* indicate that the expression level of ectopically expressed αPDGFR among these cultures is comparable. Table I sum-

marizes the results of several independent experiments in which we measured enhancement of PDGF-A-transforming activity coexpressed with various αPDGFR constructs. Our results show that wt αPDGFR, Y731F and Y731F/Y742F all enhance PDGF-A-transforming activity to a similar level, as determined by the number of transformed foci/number of marker-selected colonies. Under the same conditions, transfection with the receptor constructs alone did not lead to any

	No. of transformed foci/ no. of marker-selected colonies		
DNA transfected	Trans-fection 1	Trans-fection 2	Trans-fection 3
PDGF-A + LTR-gpt	4/40	30/500	30/200
PDGF-A + WT	40/20	300/300	100/50
PDGF-A + Y731F	100/100	300/300	150/80
PDGF-A + Y731F/Y742F	20/10	150/100	50/30
PDGF-A + K627R	4/20	20/200	30/200

Transfection was performed by the calcium-phosphate precipitation method, using 1 μg of PDGF-A cDNA, 1 μg of indicated αPDGFR cDNA, and 40 μg of calf thymus DNA as carrier. Focus formation was scored 14–21 d after transfection in unselected cultures. To measure the number of cotransfected cells, marker selection was performed in paralleled cultures using medium containing both mycophenolic acid (80 mM) and geneticin (750 μg/ml). Colonies surviving double-marker selection were scored 14–21 d after transfection.

cellular transformation (data not shown). Thus, all of these results indicate that tyrosine mutations that specifically abrogate PDGF-induced PI-3 kinase activity do not impair the ability of the αPDGFR to enhance PDGF-A chain transformation of fibroblasts. In this cotransfection assay, we observed that the number of transformed foci exceeded the number of marker-selected colonies when PDGF-A-transforming activity was greatly enhanced. This may be caused by the differences in threshold levels of proteins required for forming foci and for surviving double-marker selection. Alternatively, it may result from the different levels of expression of transfected ligand and receptor vs that of marker-resistant genes, since they are driven by different promoters (Beckmann et al., 1988; Di Fiore et al., 1987a).

CSF-1-stimulated Cell Growth of NIH/3T3 Cells Transfected with Chimera Containing the Extracellular Domain of fms and the Cytoplasmic Domain of wt and Mutant αPDGFR

Since endogenous αPDGFR in NIH/3T3 cells may be activated in the PDGF-A cotransfection assay, we generated chimeric receptors between the extracellular domain of fms and the cytoplasmic domains of wt αPDGFR, Y731F, and Y731F/742F (Fig. 5 A). The chimeric receptors (designated as fms/αRWT, fms/αR731, and fms/αR731+742, respectively) were transfected into NIH/3T3 cells, which do not normally express fms. Cell lysates of NIH/3T3 transfectants treated with or without CSF-1 were resolved by SDS-PAGE and immunoblotted with either anti-αPDGFR serum against carboxy-terminal domain of αPDGFR (Yu et al., 1991) or anti-P-Tyr. As shown in Fig. 5 B, anti-αPDGFR serum detected 170-kD chimeric receptors (lanes 2–4), as well as the endogenous 180 kD αPDGFR (lanes 1–4). The intensity of the receptor signal derived from chimeras was comparable to that from endogenous αPDGFR, which is expressed at 6.5 × 10⁴/cell (Heidaran et al., 1993). CSF-1 stimulation induced tyrosine phosphorylation of the 170-kD protein species, but not the 180-kD species (Fig. 5 C, lanes 4, 6, and 8), suggesting endogenous αPDGFRs are not cross-phosphorylated by activated chimeric receptors. Since the expression level of fms/αR731 and fms/αR731+742 were approximately three- and twofold that of fms/αR, respectively, the tyrosine phosphorylation level among each chimera was
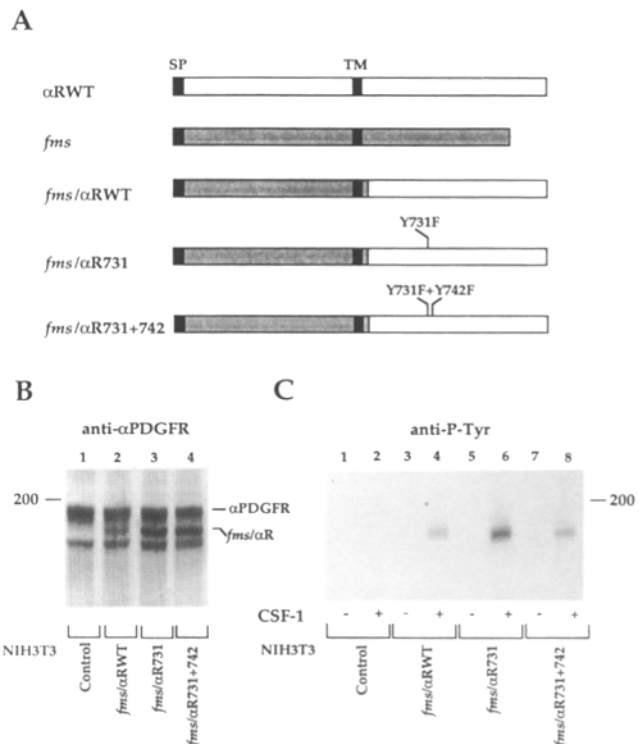

Figure 5. Expression and tyrosine-phosphorylation of fms/αPDGFR chimeric receptors. (A) Schematic diagram of wt αPDGFR, fms, and chimeric receptors between fms and αPDGFRs. Coding regions of the αPDGFR are represented by open boxes. Coding regions of fms are represented by shaded boxes. Black boxes correspond to signal peptide (SP) and transmembrane (TM) domains. The number indicates the site of mutated tyrosines of αPDGFR. (B) Total cell lysates of NIH/3T3 cells transfected with vector alone (lane 1), fms/αRWT (lane 2), fms/αR731 (lane 3), and fms/αR731+742 (lane 4) were resolved by SDS-PAGE and transferred to immobilon-P. The membrane was immunoblotted with anti-αPDGFR serum against the carboxy-terminal domain of the αPDGFR that recognizes both the human and murine αPDGFR equally well (C). Total cell lysates of NIH/3T3 stimulated or not with CSF-1 were resolved by SDS-PAGE, and transferred to immobilon-P. The membrane was then immunoblotted with anti-P-Tyr.

comparable after normalizing to their receptor level (Fig. 5, B and C). Under these conditions, NIH/3T3 cells expressing fms/αR731+742 showed no increase in anti-P-Tyr recovery of p85 upon CSF-1 stimulation, while cells expressing the fms/αR731 mutant showed a low but detectable level of anti-P-Tyr recovery of p85 as compared to that of fms/αRWT (data not shown).

We next compared the ability of the chimeric receptors to mediate CSF-1-dependent growth of NIH/3T3 transfectants by soft agar assay (Pierce et al., 1990). Thus, the NIH/3T3 transfectants were plated in a semisolid medium containing 10% calf serum supplemented with or without CSF-1. As shown in Fig. 6, NIH/3T3 transfectants failed to grow without CSF-1 stimulation (Fig. 6, A, C, E, and G). In contrast, NIH/3T3 transfected with fms/αPDGFR chimeras exhibited similar ability to form progressively growing colonies in the presence of CSF-1 (Fig. 6, D, F, and H). Under these conditions, CSF-1 stimulation did not induce proliferation of NIH/3T3 cells transfected with vector alone (Fig. 6, A and
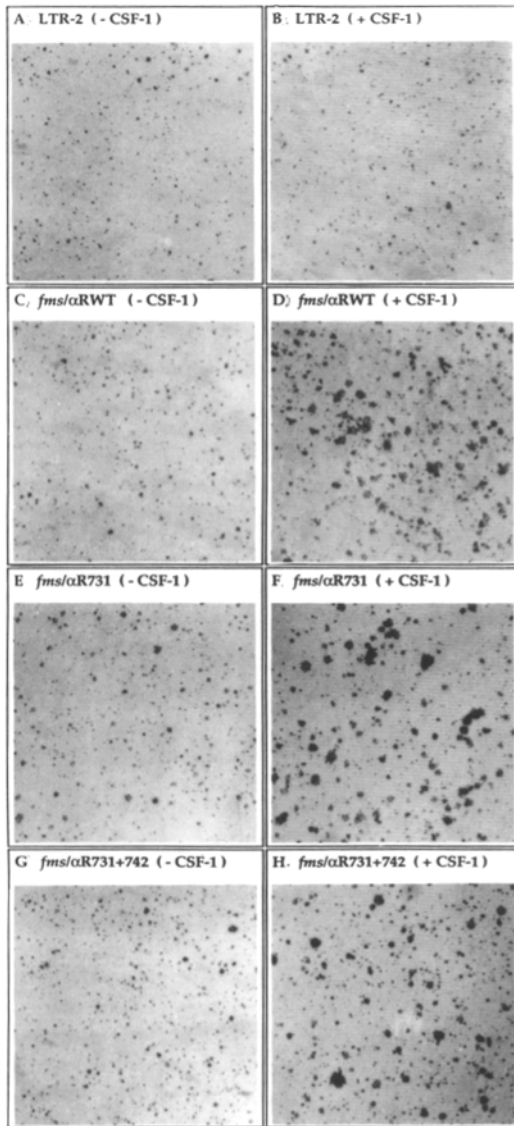
Figure 6. Comparison of the CSF-1-stimulated cell growth of NIH/3T3 cells transfected with *fms/αPDGFR* chimeric receptors. NIH/3T3 cells transfected with vector alone (*A* and *B*), *fms/αRWT* (*C* and *D*), *fms/αR731* (*E* and *F*), and *fms/αR731+742* (*G* and *H*) were suspended in 0.4% agarose diluted in DME supplemented with 10% calf serum as described in Materials and Methods. Cells were then fed with DME containing 10% calf serum in the presence or absence of CSF-1 (100 ng/ml) once per week. Photographs were taken by using light microscope after 2 wk.

B). Table II summarizes the percentage of the cells forming progressively growing colonies after 2 wk of CSF-1 stimulation. These results strongly suggest that the ability of *fms/αRWT* to mediate CSF-1-dependent growth of NIH/3T3 cell is comparable to that of *fms/αR731* or *fms/αR731+742*.

It has been reported that overexpression of EGFR in NIH/3T3 fibroblasts is sufficient for ligand-dependent transformation of these cells (Di Fiore et al., 1987*a*). Using a similar strategy, we sought to compare the ability of each *fms/αPDGFR* chimeras to mediate CSF-1-induced transformation of NIH/3T3 fibroblasts. Accordingly, NIH/3T3 cells were transfected with the chimeric receptors or vector alone,

Table II. The Activity of CSF-1-induced Cell Growth of NIH/3T3 Cells Expressing fms/αPDGFR Chimeric Receptors

Plasmid	Soft agar growth (%)	
	− CSF-1	+ CSF-1
LTR-gpt	1	1
fms/αRWT	6	48
fms/αR731	6	53
fms/αR731 + 742	6	38

Marker-selected NIH/3T3 transfectants (1 × 10⁵) were suspended in 0.4% semisolid agarose in the presence of DME supplemented with 10% calf serum. Cells were then fed with this media lacking or containing CSF-1 (100 ng/ml) once per week. The number represents the percentage of the cells forming colonies of size >30 μm in diameter after 2 wk. Results represent mean values of duplicate plates.

and the cell cultures were maintained in DME containing 2% calf serum supplemented with or without human CSF-1 (100 ng/ml). As shown in Table III, transfection with vector alone did not result in any detectable focus formation. However, transfection with *fms/αRWT*, *fms/αR731*, and *fms/αR731+742* resulted in significant and similar level of focus formation in the presence of CSF-1. Together, our findings clearly suggest that ligand-induced association of PI-3 kinase activity with receptor is not necessary for αPDGFR-mediated cell proliferation and transformation in a physiologically relevant cell system.

Discussion

Our present studies demonstrate that mutations of tyrosines 731 and 742 within the kinase insert domain of the αPDGFR (Y731F/Y742F) completely impaired PDGF-induced receptor-associated or anti-P-Tyr recoverable PI-3 kinase activity. Yet the mutation of these tyrosines did not affect the ligand-dependent receptor autophosphorylation, tyrosine phosphorylation of PLC-γ, and MAP kinase or mobility shift of *raf*-1. In 32D cells, the ectopic expression of the mutant αPDGFR led to ligand-dependent mitogenic and chemotactic responses that were comparable to that induced by the

Table III. Comparison of the Ability of fms/αPDGFR Chimeric Receptors to Mediate CSF-1-induced Transformation of NIH/3T3 Cells

Plasmid	CSF-1	No. of transformed foci/ no. of marker-selected colonies	
		Transfection 1	Transfection 2
LTR-gpt	−CSF-1	0/500	0/500
	+CSF-1	0/500	0/500
fms/αRWT	−CSF-1	0/500	0/500
	+CSF-1	150/500	100/500
fms/αR731	−CSF-1	0/500	1/500
	+CSF-1	100/500	100/500
fms/αR731 + 742	−CSF-1	0/500	0/500
	+CSF-1	100/500	100/500

NIH/3T3 cells transfection was performed by the calcium-phosphate precipitation method, using 1 μg of indicated *fms/αPDGFR* chimeric receptor cDNA and 40 μg of calf thymus DNA as carrier. Cells were grown in medium containing 2% calf serum with or without 100 ng/ml of human CSF-1 after 5 d after transfection. Focus formation was scored 2–3 wk after transfection. Marker selection was performed in medium containing mycophenolic acid (80 mM).

wt αPDGFR. In addition, the mutant receptor demonstrated the same ability as the wt αPDGFR to enhance transforming activity of PDGF-A chain in NIH/3T3 cells. Furthermore, CSF-1 induced similar levels of cell growth and transformation of NIH/3T3 cells transfected with chimeric receptor containing the extracellular domain of *fms* and the cytoplasmic domain of wt or mutant αPDGFRs. Therefore, these results suggest that PDGF-induced PI-3 kinase activity is not necessary in αPDGFR-mediated mitogenic, proliferative, or transforming signaling pathways.

Previously, we have shown that mutation of tyrosine 731 or 742 of the αPDGFR impaired PDGF-induced PI-3 kinase activity 95% or 80%, respectively, but not PDGF-stimulated mitogenesis in 32D cells (Yu et al., 1991). Fantl et al. reported that similar mitogenic effects were observed using analogous single mutants of mouse βPDGFR in normal murine mammary gland (NMuMG) epithelial cells (Fantl et al., 1992). However, they found that mutation of both tyrosines of βPDGFR completely abrogated PDGF-induced PI-3 kinase activity and mitogenic response in that cell line (Fantl et al., 1992). These results suggest that a low level of PDGF-induced PI-3 kinase activity is required in PDGF mitogenic signaling. This is in contrast to our findings showing that complete abrogation of PDGF-induced PI-3 kinase activity does not impair PDGF-induced mitogenic response in 32D cells. To address this discrepancy, we used NIH/3T3 cell line since it provides a biologically relevant system to study αPDGFR-mediated signaling. Using chimeric receptors containing the extracellular domain of *fms* and the cytoplasmic domains of wt αPDGFR, Y731F, and Y731F/Y742F, we showed that the ability of these chimeric receptors to mediate cell growth and transformation of NIH/3T3 cells induced by CSF-1 is similar. Since *fms* is not expressed in NIH/3T3 fibroblasts, and endogenous αPDGFR is not cross-phosphorylated by the chimeric receptor, our data strongly argue that PDGF-induced association of PI-3 kinase activity with receptor is not required for αPDGFR-mediated cell growth and transformation in a physiologically relevant cell system. However, the exact role of receptor-associated PI-3 kinase activity in mediating migration of NIH/3T3 cells remains to be established.

Recently, Kazlauskas et al. described that an analogous double mutant of human βPDGFR in dog kidney TRMP epithelial cell can mediate PDGF-induced mitogenic response when expressed at high level (6 × 10⁶ receptors/cell) (Kazlauskas et al., 1992). Since the level of receptor expression has been shown to affect the biological property of *erb*B-2 receptor expressed in NIH/3T3 cells (Di Fiore et al., 1987b), it is possible that the mitogenic response mediated by double mutant of βPDGFR was caused by overexpression and a physiological level of mutant receptor expression may not be sufficient for efficient coupling of receptor to mitogenic signaling pathway. However, our data show that a double mutant of αPDGFR can transduce mitogenic and proliferation signaling, even when expressed at ∿5 × 10⁴ receptors/cell in both 32D cells and NIH/3T3 cells. Since the endogenous αPDGFR is expressed at similar level in fibroblast (see Fig. 5 B and Heidaran et al., 1993), our results suggest that PDGF-induced PI-3 kinase activity is not required for mitogenic signaling, even when the mutant receptor is expressed at a physiologically relevant level.

The ligand-dependent activation of c-*raf* and *ras* have been

shown to be essential for PDGFR-mediated biological response in NIH/3T3 cells (Kolch et al., 1991; Cantley et al., 1991). Moreover, MAP kinase has also been implicated in mitogenic signaling pathways since it has been shown to be downstream of *raf*-1 and to activate c-*jun* in the nucleus (Kyriakis et al., 1992; Howe et al., 1992; Pulverer et al., 1991). Our data showing that mutations of tyrosines 731 and 742 of αPDGFR did not affect the mobility shift of c-*raf* and tyrosine phosphorylation of MAP kinase suggest this important mitogenic signal pathway to the nucleus is not impaired. We also found that activation of the wt αPDGFR and Y731F/Y742F led to a similar level of tyrosine phosphorylation of Shc molecule in 32D cells (data not shown). Shc has recently been shown to be involved in mediating *ras* activation in v-*src* transformed cells (Rozakis-Adcock et al., 1993; Egan et al., 1993). Thus, our findings also imply that the double mutation may not impair the ability of PDGFR to activate *ras* protein in vivo. These findings are consistent with results published recently by Valius and Kazlauskas, who reported characterization of a mutant βPDGFR that did not associate with PI-3 kinase, but was still capable of activating *ras* upon PDGF triggering (Valius and Kazlauskas, 1993). Together, all of these data suggest that abrogation of PI-3 kinase association does not block the critical mitogenic signaling pathways in vivo.

The ligand-dependent receptor association and/or tyrosine phosphorylation of GAP (Kazlauskas et al., 1992) or PLCγ (Valius et al., 1993) have recently been shown to be dispensible for PDGF mitogenic signaling in vivo. Our present report also suggests that PI-3 kinase association is not required for PDGF-stimulated mitogenic signaling. Thus, all of these findings suggest that it is possible that any one signal molecule can be dispensible for mitogenic signaling due to sufficient redundancy. Alternatively, these results suggest that none of these molecules are directly involved in mitogenic pathways, and they may have functions yet to be identified. In this regard, the sequence homology between the p110 catalytic subunit of PI-3 kinase and a yeast protein VPS34 suggest PI-3 kinase may play a protein-sorting function in mammalian cells as in yeast (Hiles et al., 1992; Herman and Emr, 1990). Future investigation of downstream signal molecules of PI-3 kinase and the biological function of its products should allow us to further dissect the role of this enzyme in vivo.

References

Anderson, N. G., J. L. Maller, N. K. Tonks, and T. W. Sturgill. 1990. Requirement for integration of signals from two distinct phosphorylation pathways for activation of MAP kinase. *Nature (Lond.)*. 343:651–653.

Auger, K. R., L. A. Serunian, S. P. Soltoff, P. Libby, and L. C. Cantley. 1989. PDGF-dependent tyrosine phosphorylation stimulates production of novel polyphosphoinositides in intact cells. *Cell*. 57:167–175.

Beckmann, M. P., C. Betsholtz, C.-H. Heldin, B. Westermark, E. Di Marco, P. P. Di Fiore, K. C. Robbins, and S. A. Aaronson. 1988. Comparison of biological properties and transforming potential of human PDGF-A and PDGF-B chains. *Science (Wash. DC)*. 241:1346–1349.

Cantley, L. C., K. R. Auger, C. Carpenter, B. Duckworth, A. Graziani, R.

Kapeller, and S. Soltoff. 1991. Oncogenes and signal transduction. *Cell.* 64:281–302.

Coughlin, S. R., J. A. Escobedo, and L. T. Williams. 1989. Role of phosphatidylinositol kinase in PDGF receptor signal transduction. *Science (Wash. DC).* 243:1191–1194.

Di Fiore, P. P., J. H. Pierce, T. P. Fleming, R. Hazan, A. Ullrich, C. R. King, J. Schlessinger, and S. A. Aaronson. 1987a. Overexpression of the human EGF receptor confers an EGF-dependent transformed phenotype to NIH/3T3 cells. *Cell.* 51:1063–1070.

Di Fiore, P. P., J. H. Pierce, M. H. Kraus, O. Segatto, C. R. King, and S. A. Aaronson. 1987b. erbB-2 is a potent oncogene when overexpressed in NIH/3T3 cells. *Science (Wash. DC).* 237:178–182.

Egan, S. E., B. W. Giddings, M. W. Brooks, L. Buday, A. M. Sizeland, and R. A. Weinberg. 1993. Association of Sos ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. *Nature (Lond.).* 363:45–51.

Escobedo, J. A., S. Navankasattusas, W. M. Kavanaugh, D. Milfay, V. A. Fried, and L. T. Williams. 1991. cDNA cloning of a novel 85 kd protein that has SH2 domains and regulates binding of PI3-kinase to the PDGF β-receptor. *Cell.* 65:75–82.

Fantl, W. J., J. A. Escobedo, G. A. Martin, C. W. Turck, M. del Rosario, F. McCormic, and L. T. Williams. 1992. Distinct phosphotyrosines on a growth factor receptor bind to specific molecules that mediate different signaling pathways. *Cell.* 69:413–423.

Fukui, Y., and H. Hanafusa. 1989. Phosphatidylinositol kinase activity associates with viral p60^{src} protein. *Mol. Cell. Biol.* 9:1651–1658.

Gutkind, J. S., P. M. Lacal, and K. C. Robbins. 1990. Thrombin-dependent association of phosphatidylinositol-3 kinase with p60^{c-src} and p59^{fyn} in human platelets. *Mol. Cell. Biol.* 10:3806–3809.

Heidaran, M. A., T. P. Fleming, D. P. Bottaro, G. I. Bell., P. P. Di Fiore, and S. A. Aaronson. 1990. Transformation of NIH3T3 fibroblasts by an expression vector for the human epidermal growth factor precursor. *Oncogene.* 5:1265–1270.

Heidaran, M. A., J. H. Pierce, D. Lombardi, M. Ruggiero, J. S. Gutkind, T. Matsui, and S. A. Aaronson. 1991. Deletion or substitution within the αPDGF receptor kinase insert domain: effects on functional coupling with intracellular signalling pathways. *Mol. Cell. Biol.* 11:134–142.

Heidaran, M. A., J. F. Beeler, J.-C. Yu, T. Ishibashi, W. J. LaRochelle, J. H. Pierce, and S. A. Aaronson. 1993. Differences in substrate specificities of α and β PDGF receptors. *J. Biol. Chem.* 268:9287–9295.

Herman, P. K., and S. D. Emr. 1990. Characterization of VPS34, a gene required for vacuolar protein sorting and vacuole segragation in *Saccharomyces cerevisiae. Mol. Cell. Biol.* 10:6742–6754.

Hiles, I. D., M. Ostu, S. Volinia, M. J. Fry, I. Gout, R. Dhand, G. Panayotou, F. Ruiz-Larea, A. Thompson, N. F. Totty, et al. 1992. Phosphatidylinositol 3-kinase: structure and expression of the 110 kd catalytic subunit. *Cell.* 70:419–429.

Howe, L. R., S. J. Leevers, N. Gomez, S. Nakielny, P. Cohen, and C. J. Marshall. 1992. Activation of the MAP kinase pathway by the protein kinase raf. *Cell.* 71:335–342.

Hu, P., B. Margolis, E. Y. Skolnik, R. Lammers, A. Ullrich, and J. Schlessinger. 1992. Interaction of phosphatidylinositol 3-kinase-associated p85 with epidermal growth factor and platelet-derived growth factor receptors. *Mol. Cell. Biol.* 12:981–990.

Kaplan, D. R., D. K. Morrison, G. Wong, F. McCormick, and L. T. Williams. 1990. PDGF β-receptor stimulates tyrosine phosphorylation of GAP and association of GAP with a signalling complex. *Cell.* 61:125–133.

Kaplan, D. R., M. Whitman, B. Schaffhausen, D. C. Pallas, M. White, L. Cantley, and T. M. Roberts. 1987. Common elements in growth factor stimulation and oncogenic transformation: 85 kd phosphoprotein and phosphatidylinositol kinase activity. *Cell.* 50:1021–1029.

Kashishian, A., A. Kazlauskas, and J. A. Cooper. 1992. Phosphorylation sites in the PDGF receptor with different specificities for binding GAP and PI3 kinase in vivo. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:1373–1382.

Kavanaugh, W. M., A. Klippel, J. A. Escobedo, and L. T. Williams. 1992. Modification of the 85-kilodalton subunit of phosphatidylinositol-3 kinase in PDGF-stimulated cells. *Mol. Cell. Biol.* 12:3415–3424.

Kazlauskas, A., A. Kashishian, J. A. Cooper, and M. Valius. 1992. GTPase-activating protein and phosphatidylinositol 3-kinase bind to distinct regions of the PDGF receptor β subunit. *Mol. Cell. Biol.* 12:2534–2544.

Kolch, W., G. Heidecker, P. Lloyd, and U. R. Rapp. 1991. Raf-1 protein kinase is required for growth of induced NIH/3T3 cells. *Nature (Lond.).* 349:426–428.

Kumjian, D. A., M. I. Wahl, S. G. Rhee, and T. O. Daniel. 1989. Platelet-derived growth factor (PDGF) binding promotes physical association of PDGF receptor with phospholipase C. *Proc. Natl. Acad. Sci. USA.* 86:8232–8236.

Kyriakis, J. M., H. App, X. Zhang, P. Banerjee, D. L. Brautigan, U. R. Rapp, and J. Avruch. 1992. Raf-1 activates MAP kinase-kinase. *Nature (Lond.).* 358:417–421.

L'Allemain, G., T. W. Sturgill, and M. J. Weber. 1991. Defective regulation of mitogen-activated protein kinase activity in a 3T3 cell variant mitogenically nonresponsive to tetradecanoyl phorbol acetate. *Mol. Cell. Biol.* 11:1002–1008.

Matsui, T., J. H. Pierce, T. P. Fleming, J. S. Greenberger, W. J. LaRochelle, M. Ruggiero, and S. A. Aaronson. 1989. Independent expression of human α or β PDGF receptor cDNAs in a naive hematopoietic cell leads to functional coupling with mitogenic and chemotactic signaling pathways. *Proc. Natl. Acad. Sci. USA.* 86:8314–8318.

McGlade, C. J., C. Ellis, M. Reedjik, D. Anderson, G. Mbamalu, A. D. Reith, G. Panayotou, P. End, A. Bernstein, A. Kazlauskas, M. D. Waterfield, and T. Pawson. 1992. SH2 domains of the p85 subunit of phosphatidylinositol 3-kinase regulate binding to growth factor receptors. *Mol. Cell. Biol.* 12:991–997.

Meisenhelder, J., P.-G. Suh, S. G. Rhee, and T. Hunter. 1989. Phospholipase C-γ is a substrate for the PDGF and EGF receptor protein-tyrosine kinases in vivo and in vitro. *Cell.* 57:1109–1122.

Molloy, C. J., D. P. Bottaro, T. P. Fleming, M. S. Marshall, J. B. Gibbs, and S. A. Aaronson. 1989. PDGF induction of tyrosine phosphorylation of GTPase activating protein. *Nature (Lond.).* 342:711–714.

Morrison, D. K., D. R. Kaplan, J. A. Escobedo, U. R. Rapp, T. M. Roberts, and L. T. Williams. 1989. Direct activation of the serine/threonine kinase activity of raf-1 through tyrosine phosphorylation by the PDGF β-receptor. *Cell.* 58:649–657.

Morrison, D. K., D. R. Kaplan, S. G. Rhee, and L. T. Williams. 1990. Platelet-derived growth factor (PDGF)-dependent association of phospholipase C-γ with the PDGF receptor signalling complex. *Mol. Cell. Biol.* 10:2359–2366.

Ostu, M., I. Hiles, I. Gout, M. J. Fry, F. Ruiz-Larrea, G. Panayotou, A. Thompson, R. Dhand, J. Hsuan, N. Totty, et al. 1991. Characterization of two 85 kd proteins that associate with receptor tyrosine kinases, middle-T/pp60c-src complexes, and PI3-kinase. *Cell.* 65:91–104.

Pelech, S. L., and J. S. Sanghera. 1992. MAP kinases: charting the regulatory pathways. *Science (Wash. DC).* 257:1355–1356.

Pierce, J. H., E. Di Marco, G. W. Cox, D. Lombardi, M. Ruggiero, L. Varesio, L. M. Wang, G. G. Choudhury, A. Y. Sakaguchi, P. P. Di Fiore, and S. A. Aaronson. 1990. Macrophage-colony-stimulating factor (CSF-1) induces proliferation, chemotaxis, and reversible monocytic differentiation in myeloid progenitor cells transfected with the human c-fms/CSF-1 receptor cDNA. *Proc. Natl. Acad. Sci. USA.* 87:5613–5617.

Pierce, J. H., M. Ruggiero, T. P. Fleming, P. P. Di Fiore, J. S. Greenberger, L. Varticovski, J. Schlessinger, G. Rovera, and S. A. Aaronson. 1988. Signal transduction through the EGF receptor transfected in IL-3 dependent cells. *Science (Wash. DC).* 239:628–631.

Pulverer, B. J., J. M. Kyriakis, J. Avruch, E. Nikolakaki, and J. R. Woodgett. 1991. Phosphorylation of c-jun mediated by MAP kinases. *Nature (Lond.).* 353:670–674.

Rozakis-Adcock, M., R. Fernley, J. Wade, T. Pawson, and D. Bowtell. 1993. The SH2 and SH3 domains of mammalian Grb2 couple the EGF receptor to the ras activator mSos1. *Nature (Lond.).* 363:83–85.

Skolnik, E. Y., B. Margolis, M. Mohammadi, E. Lowenstein, R. Fischer, A. Drepps, A. Ullrich, and J. Schlessinger. 1991. Cloning of PI3 kinase-associated p85 utilizing a novel method for expression/cloning of target proteins for receptor tyrosine kinases. *Cell.* 65:83–90.

Valius, M., C. Bazenet, and A. Kazlauskas. 1993. Tyrosines 1021 and 1009 are phosphorylation sites in the carboxy terminus of the PDGF receptor β subunit and are required for binding of phospholipase Cγ and a 64-kilodalton protein, respectively. *Mol. Cell. Biol.* 13:133–143.

Valius, M., and A. Kazlauskas. 1993. Phospholipase C-γ1 and phosphatidylinositol 3 kinase are the downstream mediators of the PDGF receptor's mitogenic signal. *Cell.* 73:321–334.

Whitman, M., C. P. Downes, M. Keeler, T. Keller, and L. Cantley. 1988. Type I phosphatidylinositol kinase makes a novel inositol phospholipid, phosphatidylinositol-3-phosphate. *Nature (Lond.).* 332:644–646.

Williams, L. T. 1989. Signal transduction by the platelet-derived growth factor receptor. *Science (Wash. DC).* 243:1564–1570.

Yu, J. C., M. A. Heidaran, J. H. Pierce, J. S. Gutkind, D. Lombardi, M. Ruggiero, and S. A. Aaronson. 1991. Tyrosine mutations within the αPDGFR kinase insert domain abrogate receptor-associated phosphatidylinositol-3 kinase activity without affecting mitogenic or chemotactic signal transduction. *Mol. Cell. Biol.* 11:3780–3785.

Yu, J. C., D. Mahadevan, W. J. LaRochelle, J. H. Pierce, and M. A. Heidaran. 1994. Structural coincidence of αPDGFR epitopes binding to PDGF-AA and a potent neutralizing monoclonal antibody. *J. Biol. Chem.* 269:10668–10674.