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Deletion or Substitution within the α Platelet-Derived Growth Factor Receptor Kinase Insert Domain: Effects on Functional Coupling with Intracellular Signaling Pathways

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The tyrosine kinase domains of the platelet-derived growth factor (PDGF) and colony-stimulating factor-1 (CSF-1)/c-*fms* receptors are interrupted by kinase inserts (ki) which vary in length and amino acid sequence. To define the role of the ki in the human αPDGF receptor (αPDGFR), we generated deletion mutants, designated αRΔki-1 and αRΔki-2, which lacked 80 (710 to 789) and 95 (695 to 789) amino acids of the 104-amino-acid ki region, respectively. Their functional characteristics were compared with those of the wild-type αPDGFR following introduction into a naive hematopoietic cell line, 32D. Biochemical responses, including PDGF-stimulated PDGFR tyrosine phosphorylation, phosphatidylinositol (PI) turnover, and receptor-associated PI-3 kinase activity, were differentially impaired by the deletions. Despite a lack of any detectable receptor-associated PI-3 kinase activity, 32D cells expressing αRΔki-1 showed only partially impaired chemotactic and mitogenic responses and were capable of sustained proliferation in vitro and in vivo under conditions of autocrine stimulation by the c-*sis* product. 32D transfectants expressing the larger ki deletion (αRΔki-2) showed markedly decreased or abolished biochemical and biological responses. However, insertion of the highly unrelated smaller c-*fms* (685 to 750) ki domain into αRΔki-2 restored each of these activities to wild-type αPDGFR levels. Since the CSF-1R does not normally induce PI turnover, the ability of the c-*fms* ki domain to reconstitute PI turnover in the αRΔki-2 transfectant provides evidence that the ki domain of the αPDGFR does not directly couple with this pathway. Taken together, all of these findings imply that their ki domains have evolved to play very similar roles in the known signaling functions of PDGF and CSF-1 receptors.

Platelet-derived growth factor (PDGF) is a potent mitogen for cells of mesenchymal origin. It exists as disulfide-linked dimers consisting of two related polypeptide chains which are encoded by distinct genes (1, 10). PDGF-A and PDGF-B chains can form homodimers as well as the AB heterodimer (22). These isoforms differentially bind and activate the products of two distinct genes encoding, respectively, the αPDGF and βPDGF receptors (αPDGFR and βPDGFR) (6, 28, 52). The αPDGFR binds to all three isoforms of PDGF, whereas the βPDGFR exhibits high-affinity binding only for PDGF-BB (5, 6, 16, 29). Both αPDGFR and βPDGFR gene products share structural and sequence similarities with the receptor for colony-stimulating factor-1 (CSF-1) (c-*fms*) (36, 40), a *fms*-like tyrosine kinase (*flt*) (41), and c-*kit* (53). Each possesses an extracellular ligand-binding segment with five immunoglobulinlike domains, a membrane-spanning segment, and an intracellular tyrosine kinase domain. Unlike the majority of protein tyrosine kinases, the catalytic domains of this small family are interrupted by intervening sequences, designated the kinase insert (ki) domains (52). Although tyrosine kinase domains of this family are very similar, their ki domains are highly unrelated in both predicted sequence and length (35).

The role of the ki domains in biochemical and biological functions of the βPDGFR and CSF-1 receptor (CSF-1R) has been the subject of investigation in several laboratories. According to one report, a deletion of 82 amino acids (residues 716 to 797) within the mouse βPDGFR ki resulted in a mutant receptor which induced apparently normal levels of phosphatidylinositol (PI) turnover and calcium mobilization, but was completely defective in transducing any detectable mitogenic signal in response to PDGF (13). Moreover, results published by the same laboratory indicated that this mutant exhibited normal kinase activity and phospholipase C-γ phosphorylation (13, 32). However, this mutant lacked the ability to associate with either PI-3 kinase (7) or GTPase activating protein (GAP) (23), putative targets of receptor kinase action (47). A somewhat larger deletion of 98 amino acids (701 to 798) in the human βPDGFR ki generated a mutant which was also incapable of inducing a mitogenic response (39). However, this mutant displayed substantially decreased autophosphorylation after ligand stimulation and had decreased ability to phosphorylate known exogenous substrates. In contrast, a deletion of 58 of 70 residues of the c-*fms* ki domain was reported not to inhibit its enzymatic or mitogenic activity in response to CSF-1 (45). Moreover, a human CSF-1R Δki mutant was also reported to be partially impaired in its mitogenicity and exhibited a significant although incomplete reduction in its associated PI-3 kinase activity (42). These findings have suggested that specific functions of the ki domains of these structurally similar receptor kinases may differ markedly.

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We have exploited a naïve hematopoietic cell, 32D (15), which is normally dependent upon interleukin-3 (IL-3) for proliferation, to investigate tyrosine kinase receptor signaling (21). Mitogenic and chemotactic signaling pathways inherently expressed by these cells could be efficiently coupled by introduction of either αPDGFR or βPDGFR and triggering by the appropriate PDGF ligand (29). This system provided the opportunity to systematically compare the effects of progressive deletions in the ki of the αPDGFR on distinct responses mediated by this receptor. We also investigated the effects of substituting αPDGFR ki with c-*fms* ki, which exhibits only 10% amino acid sequence identity with αPDGFR ki (28), on biological and biochemical responses to αPDGFR triggering.

MATERIALS AND METHODS

Expression vectors and transfection of 32D cells. To generate αRΔki-1 and αRΔki-2, a plasmid containing a *Pst*I-*Bam*HI fragment of the αPDGFR cDNA (designated pUCTKαR) was digested either with *Eco*RV (nucleotide 2258) and *Stu*I (nucleotide 2506) or with *Hin*cII (nucleotide 2184) (partially) and *Stu*I (nucleotide 2506). The digested plasmids were then ligated in the presence of either oligonucleotides 5'-ATCTTTGG and 5'-CCAAAGAT or oligonucleotides 5'-AACTATTTGCATAAGAATAGGGATAGCTTC CT and 5'-AGGAAGCTATCCCTATTCTTATGCAAATA GTT, respectively. Correct mutants were identified by restriction mapping, and mutations were confirmed by DNA sequencing. To generate the αRΔki-*fms* chimera, the c-*fms* ki fragment was synthesized by the polymerase chain reaction (37) with oligonucleotides 5'-AACTATTTGCATAA GAATAGGGATAGCTTCC-TGGGACCCAGCCTGAG CCC and 5'-GGCCGTCCATCCTCCTTGTC. The c-*fms* ki fragment was then ligated with pUCTKαR, which had been digested with *Hin*cII (partially) and *Stu*I. The sequence of this clone was similarly confirmed by DNA sequencing. Following reconstruction of the entire coding regions for each mutant, each was then cloned into the LTR-2 eukaryotic expression vector (8). The pSV_2 (c-*sis*) neo vector is a long terminal repeat-driven construct which contains a geneticin selectable marker (13a).

The IL-3-dependent mouse hematopoietic cell line 32D has been described previously (15). DNA transfection of 32D cells was performed by the electroporation procedure (34). Mass populations of stably transfected cells were selected by their ability to survive in growth medium containing either mycophenolic acid (80 mM) (33) or geneticin (750 μg/ml).

RNA hybridization. Total cellular RNA was extracted as described previously (3). RNA samples were denatured and applied to nitrocellulose filters. Filters were then baked and hybridized at 42°C with ^{32}P-labeled cDNA probes in a buffer containing 40% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0]), 1× Denhardt solution (0.02% bovine serum albumin [BSA], 0.02% Ficoll, 0.02% polyvinylpyrrolidone), 5 mM NaH_2PO_4, 5 mM Na_2HPO_4, 0.1% sodium dodecyl sulfate (SDS), and salmon sperm DNA (100 μg/ml). After a 16-h hybridization, filters were washed twice for 20 min in 2× SSC at room temperature and then for 30 min in 0.1× SSC–0.1% SDS at 50°C and subjected to autoradiography at −70°C with Kodak XAR-5 film.

Immunoblotting and immunoprecipitation analysis. Quiescent cells were incubated in the presence of PDGF-BB (100 ng/ml; Upstate Biotechnology, Inc.) for 10 min at 37°C followed by 5 mM diisopropyl fluorophosphate at 4°C for 5 min. Cell pellets were lysed in a phosphotyrosine (P-Tyr) buffer containing 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid; pH 7.5), 1% Triton X-100, 50 mM NaCl, 50mM NaF, 10 mM sodium PP_i, 5 mM EDTA, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin and leupeptin per ml, and 5 mM diisopropyl fluorophosphate. Lysates were clarified, and the soluble fraction (2 mg) was then immunoprecipitated with monoclonal anti-P-Tyr antibody (PY 20, PY 69; ICN). The immune complex was recovered by using protein G-Sepharose (Gammabind G; Genex). Immunoprecipitates were then washed three times in P-Tyr lysis buffer lacking diisopropyl fluorophosphate and solubilized in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer. Immunoblotting was performed as described previously (9). For some experiments, cells were lysed for 10 min at 90°C in 100 mM Tris (pH 8.0) containing 1% SDS, 1 mM Na_3VO_4, and 1 mM phenylmethylsulfonyl fluoride. The clarified lysates (150 μg per lane) were then immunoblotted with anti-peptide serum (amino acids 959 to 973 of the human αPDGFR) or anti-P-Tyr. Immunoprecipitations were performed as previously described (30), except that lysates were diluted 10-fold in the presence of 50 mM HEPES (pH 7.5)–1% Triton X-100–1 mM Na_3VO_4–1 mM phenylmethylsulfonyl fluoride to bring the SDS concentration to 0.1%. For analysis of c-*sis* protein, cells were subjected to biosynthetic labeling and immunoprecipitation with a c-*sis*-specific goat anti-peptide serum as described previously (14).

Ligand binding, proliferation, and chemotactic assays. PDGF-BB was labeled by the method of Bolton and Hunter (specific activity, 2×10^4 cpm/ng) (2). ^{125}I-PDGF-BB binding to cells was performed as previously described (28). 32D cells and transfectants were plated in 24-well plates under serum-free conditions 1 h prior to assay. Adherent cells (5×10^5 cells per well) were washed with ice-cold HEPES binding buffer (HBB; 25 mM HEPES, 150 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 0.1% BSA [pH 7.5]), and incubated in the presence of increasing concentrations of ^{125}I-labeled ligand for 2 h at 4°C. Free ligand was removed by washing the wells three times with HBB. Cells were then lysed in 1% Triton, and radioactivity in the Triton extracts of triplicate samples was measured in a gamma counter. The extent of nonspecific binding was measured by incubating cells in the presence of a 100-fold excess of unlabeled ligand.

For mitogenic assays, 32D transfectants were washed twice with phosphate-buffered saline (PBS) and plated at 3×10^5 cells per ml into Costar 24-well plates in RPMI 1640 medium containing 15% fetal calf serum with or without various concentration of PDGF-BB or murine IL-3 (Genzyme) as previously described (29). For the soft-agar assay, cells were plated at 10-fold serial dilutions in semisolid agarose medium containing RPMI 1640 medium supplemented with 15% fetal calf serum and 0.45% SeaPlaque agarose (FMC Corp.). Colony formation was scored at 14 days. For determination of directed cell migration in response to PDGF-BB, modified Boyden chambers and Nuclepore filters (pore size, 5 μm) were used as described previously (17, 29).

Inositol phosphate and PI-3 kinase assays. Inositol phosphate formation induced in response to various concentrations of PDGF-BB was determined as described previously (29). For the PI-3 kinase assay, 32D cells were rendered quiescent, treated with PDGF-BB (100 ng/ml), and lysed at 4°C for 5 min in buffer containing 20 mM Tris (pH 8.0), 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM Na_3VO_4, 1% Nonidet P-40, 10% glycerol, 10 μg of aprotinin and leupeptin per ml, 5 mM diisopropyl fluorophosphate,

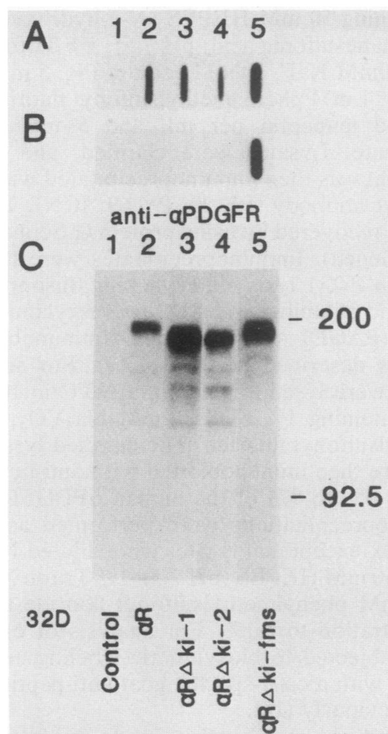

FIG. 1. Expression of ki mutants of the αPDGFR in 32D transfectants. For determination of αPDGFR RNA expression, total RNA (5 μg) extracted from 32D (lane 1), 32D-αR (lane 2), 32D-αRΔki-1 (lane 3), 32D-αRΔki-2 (lane 4), or 32D-αRΔki-*fms* (lane 5) was applied to nitrocellulose filters. Filters were hybridized with either ^{32}P-labeled αPDGFR cDNA (A) or c-*fms* cDNA (B). The presence of an equivalent amount of RNA was confirmed by hybridization of an identical filter with a β-actin probe (data not shown). For determination of αPDGFR protein expression (C), 100 μg of total lysate prepared from 32D (lane 1), 32D-αR (lane 2), 32D-αRΔki-1 (lane 3), 32D-αRΔki-2 (lane 4), or 32D-αRΔki-*fms* (lane 5) was subjected to immunoblot analysis with an anti-αPDGFR peptide serum as described in Materials and Methods.

and 1 mM phenylmethylsulfonyl fluoride. Soluble lysates (2 mg) were incubated with monoclonal antibody directed against the extracellular domain of the human αPDGFR (Genzyme). Immunoprecipitates were recovered with the aid of protein- G-Sepharose and assayed for PI-3 kinase activity as measured by ability of the coimmunoprecipitate to phosphorylate PI to yield phosphatidylinositol 3-phosphate (18).

RESULTS

Expression of αPDGFR ki mutants in IL-3-dependent 32D cells. Mutant or wild-type αPDGFR cDNAs were introduced into the LTR-2 expression vector (8), which contains a linked *Eco-gpt* selectable marker. 32D cells were then electroporated with the receptor constructs and selected for their ability to survive in the presence of mycophenolic acid and IL-3. To analyze the expression of different constructs, we prepared duplicate filters by slot blotting an equal amount of RNA (5 μg) isolated from the representative transfectants. Filters were then hybridized to either probes of the tyrosine kinase domains of αPDGFR or c-*fms*. αPDGFR-specific RNA transcripts were readily detectable in each transfectant but not in the parental 32D cell line (Fig. 1A). Under identical conditions, the c-*fms*-specific probe detected transcripts only in RNA isolated from the αRΔki-*fms* transfectant (Fig. 1B). The expression of receptors was further analyzed by subjecting total lysates of transfectants to immunoblot analysis. Anti-αPDGFR peptide serum specifically recognized proteins of approximately 190 kDa in 32D-αR, 180 kDa in 32D-αRΔki-1 and 32D-αRΔki-2, and 185 kDa in 32D-αRΔki-*fms* cells, consistent with the predicted sizes of these mutants (Fig. 1C).

To directly quantitate the level of receptors expressed at the cell surface, we subjected transfectants to Scatchard binding analysis (38). High-affinity binding with a single affinity constant was observed for each transfectant (Fig. 2). Moreover, in each case, the number of binding sites was similar to that of 32D cells expressing wild-type αPDGFR receptors. These results indicated that deletion or substitution of the ki domain did not significantly alter cell surface expression or PDGF-binding affinity of any of the mutant αPDGF receptors.

Effects of ki mutations on PDGF-induced receptor tyrosine phosphorylation. Earlier studies have shown that PDGF induces phosphorylation of its own receptor on tyrosine residues (12). Sites of receptor autophosphorylation have been mapped within the ki domains of both βPDGFR (25) and CSF-1R (44, 48). The αPDGFR ki domain contains analogous tyrosine residues, which are deleted in both αPDGFR ki mutants. To investigate the effects of each mutation on this biochemical parameter, we examined the ability of αPDGFR mutants to undergo PDGF-induced receptor tyrosine phosphorylation. PDGF-BB stimulated comparable levels of receptor phosphorylation in 32D cells which expressed either the wild-type receptor or αRΔki-*fms* (Fig. 3). In contrast, 32D cells expressing αRΔki-1 and αRΔki-2 showed a 90 and 99% reduction, respectively, in receptor tyrosine autophosphorylation when compared with the wild-type αR transfectant. Thus, progressive deletions of the αPDGFR ki reduced or essentially abolished receptor autophosphorylation, while substitution of the c-*fms* ki domain into the αRΔki-2 restored the level of P-Tyr incorporation of this chimeric molecule to that of wild-type αPDGFR.

Differential effects of αPDGFR ki deletions on PI metabolism and receptor-associated PI-3 kinase activities. PDGF stimulates several rapid cellular responses including PI hydrolysis (19) and receptor-associated PI-3 kinase activity (24). When the responses to stimulation with increasing amounts of PDGF-BB were analyzed, we observed a dose-dependent increase in PI breakdown by all the transfectants (Fig. 4). At saturating ligand concentration, 32D-αR cells demonstrated a 16- to 17-fold increase, whereas 32D-αRΔki-1 cells exhibited a 7- to 8-fold increase and 32D-αRΔki-2 cells showed only a small but reproducible increase over the unstimulated control. Thus, progressive deletions within the ki domain of the αPDGFR increasingly impaired its ability to induce PI turnover in response to PDGF-BB. Figure 4 further shows that the αRΔki-*fms* transfectant was indistinguishable from the wild type 32D-αR transfectant with respect to PI turnover. Since the c-*fms* product does not trigger PI turnover or tyrosine phosphorylation of phospholipase C-γ (11, 20, 50), the ki domain is unlikely to mediate interactions of the αPDGFR with this substrate.

PI-3 kinase, which phosphorylates the inositol ring of PI at the D3 position, has not been directly implicated in the regulation of PI turnover, and its biological function remains to be determined. However, this enzyme has been shown to be coimmunoprecipitated with activated βPDGFR (7) or

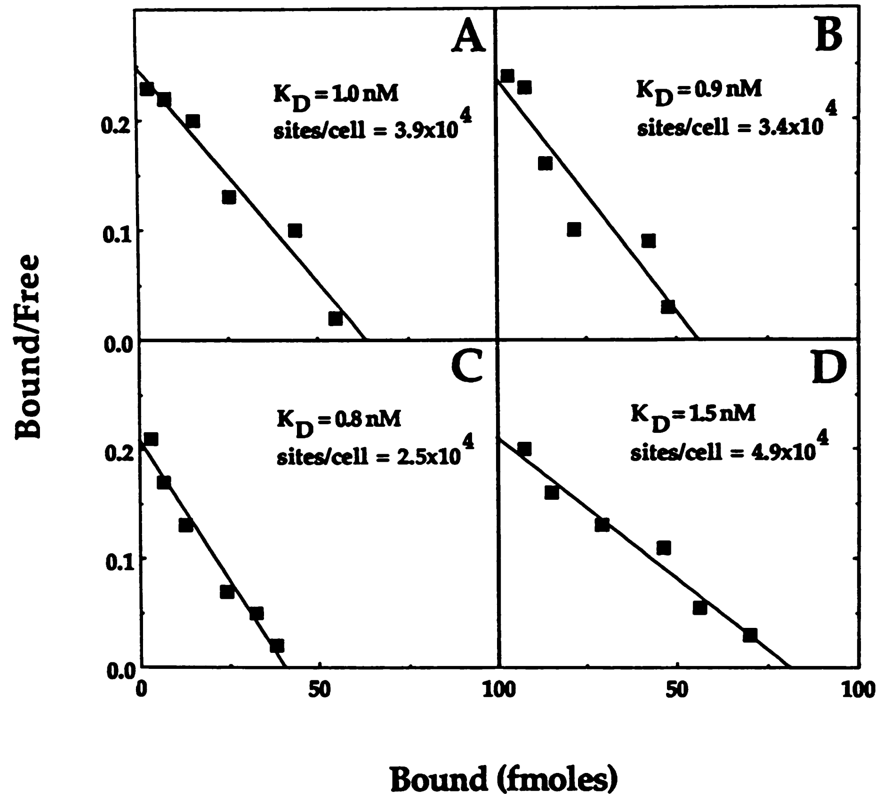

FIG. 2. Scatchard analysis of ^{125}I-labeled PDGF-BB binding to 32D transfectants. ^{125}I-PDGF-BB binding to 32D cells expressing 32D-αR (A), 32D-αRΔki-1 (B), 32D-αRΔki-2 (C), and 32D-αRΔki-*fms* (D) was determined as described in Materials and Methods. Binding studies were performed to saturation by using increasing concentrations of ^{125}I-PDGF-BB. The specific binding at each concentration was determined by subtraction of nonspecific binding from the mean value of total binding derived from triplicate samples. The binding data were then analyzed by the method of Scatchard (38).

CSF-1R (49). Moreover, mutation or loss of autophosphorylation sites located within the respective ki domains of the βPDGFR (25) and CSF-1R (42, 48) has been shown to diminish the ability of these mutant receptors to associate with PI-3 kinase. αPDGFR-associated PI-3 kinase activity was readily detectable and comparable in PDGF-BB-stimulated 32D-αR and αRΔki-*fms* cells (Fig. 5). In striking contrast, neither deletion mutant demonstrated detectable αPDGFR-associated PI-3 kinase activity. These findings implied that deletions within its ki domain more severely impaired this function than the ability to couple with PI metabolism.

Chemotactic and mitogenic responsiveness of wild-type and ki mutant αPDGFR transfectants. In view of the differential effects of ki deletions on biochemical activities of the αPDGFR, we sought to determine their influence on the major known PDGF-mediated biological responses, including chemotaxis and mitogenesis. PDGF-BB was highly chemotactic for 32D cells expressing either the wild-type αPDGFR or αRΔki-*fms* (Fig. 6). 32D-αRΔki-1 cells showed reduced but readily detectable directed migration in response to PDGF-BB, whereas neither 32D-αRΔki-2 nor control 32D cells showed any measurable chemotactic response under the same conditions.

32D cells are normally strictly dependent on IL-3 for proliferation, and absence of this cytokine from the culture medium results in loss of cell viability within 24 h (15). Expression of wild-type αPDGFR or βPDGFR has been shown to allow efficient coupling by PDGF with mitogenic

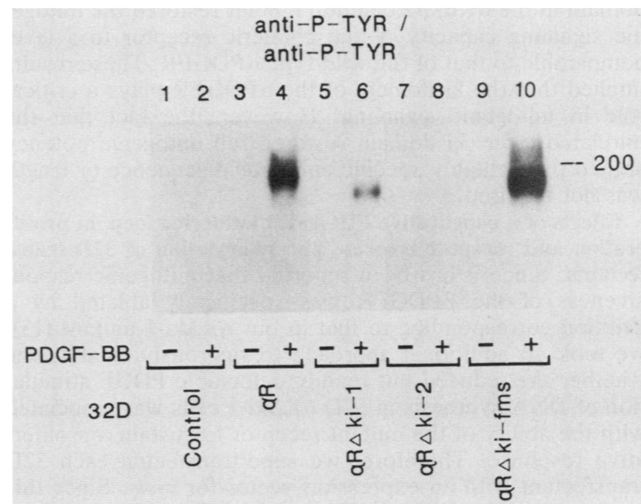

FIG. 3. In vivo αPDGFR tyrosine phosphorylation in 32D transfectants. Quiescent 32D cells were incubated with (+) or without (−) PDGF-BB (100 ng/ml) for 10 min at 37°C. Cells were then lysed, and soluble fractions (2 mg) were immunoprecipitated with anti-P-Tyr. Immunoprecipitates were electrophoretically separated, transferred to Immobilon-P (Millipore), and blotted with anti-P-Tyr antibody. Lanes: 1 and 2, 32D; 3 and 4, 32D-αR; 5 and 6, 32D-αRΔki-1; 7 and 8, 32D-αRΔki-2; 9 and 10, 32D-αRΔki-*fms*. The exposure time was 36 h.

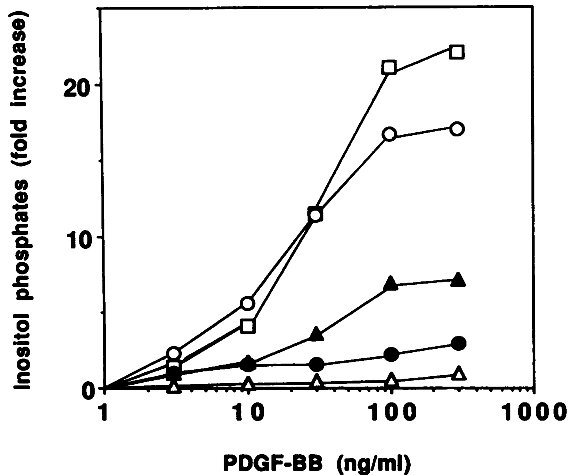

FIG. 4. Effect of PDGF-BB on inositol phosphate formation in 32D transfectants. 32D (△), 32D-αR (○), 32D-αRΔki-1 (▲), 32D-αRΔki-2 (●), and 32D-αRΔki-*fms* (□) cells were prelabeled with *myo*-[^{3}H]inositol for 48 h and then exposed to various concentrations of PDGF-BB. The reaction was stopped at 30 min, and total inositol phosphates were analyzed as described previously (29). Results are expressed as fold increase over unstimulated cells and represent mean values of triplicate samples.

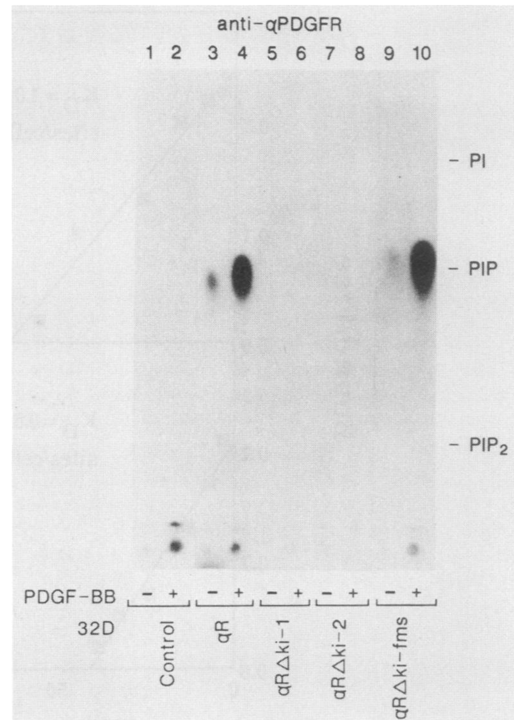

FIG. 5. Coimmunoprecipitation of PI-3 kinase activity with the human αPDGFR in 32D transfectants. Quiescent 32D cells (lanes 1 and 2), 32D-αR cells (lanes 3 and 4), 32D-αRΔki-1 cells (lanes 5 and 6), 32D-αRΔki-2 cells (lanes 7 and 8), and 32D-αRΔki-*fms* cells (lanes 9 and 10) were incubated with (+) or without (−) PDGF-BB (100 ng/ml) and immunoprecipitated with a monoclonal antibody against the human αPDGFR. Immune complexes were then subjected to a PI-3 kinase assay as described previously (18). A parallel Western immunoblot analysis of αPDGFR protein levels in immune complexes subjected to PI-3 kinase assay revealed the presence of at least equivalent amounts of each mutant compared with the wild-type αPDGFR (data not shown).

signaling pathways inherently expressed by the cells (29). Therefore, we analyzed the ability of increasing concentrations of PDGF-BB to induce DNA synthesis in all transfectants. PDGF-BB induced a dose-dependent increase in DNA synthesis with maximal levels of 50-fold over background observed at 300 ng/ml (Fig. 7). 32D-αRΔki-1 also demonstrated increased thymidine uptake, which plateaued at about 30% of the maximal response achieved with 32D-αR. In contrast, 32D-αRΔki-2 showed no detectable mitogenic response (Fig. 7). Of note, substitution of the c-*fms* ki domain in the αRΔki-2 deletion mutant restored the mitogenic signaling capacity of the chimeric receptor to a level comparable to that of the wild-type αPDGFR. These results implied that the ki domain of the αPDGFR plays a critical role in mitogenic signaling. However, the fact that the unrelated c-*fms* ki domain restored full mitogenic potency argued that a highly specific amino acid sequence or length was not required.

Effects of a constitutive PDGF-BB autocrine loop on proliferation and receptor tyrosine phosphorylation of 32D transfectants. Since it has been reported that mitogenic responsiveness of the βPDGFR was specifically ablated by a deletion corresponding to that in our αRΔki-1 mutant (13), we took an additional approach to rigorously establishing whether the reduced but readily detectable PDGF stimulation of DNA synthesis in 32D-αRΔki-1 cells was associated with the ability of the mutant receptor to sustain a proliferative response. Therefore, we supertransfected each 32D transfectant with an expression vector for c-*sis*. Since this vector contained a linked pSV$_2$*neo* marker, it was possible to obtain double transfectants by selection of 32D transfectants in the presence of geneticin. We verified expression of the c-*sis* product, p27$^{c\text{-}sis}$, in each supertransfectant by radioimmunoprecipitation analysis with an anti-c-*sis* peptide serum (Fig. 8A).

To quantitate the steady-state level of αPDGFR phosphorylation relative to the level of receptor proteins under conditions of autocrine stimulation by c-*sis*, we probed immunoblots of cell lysates with anti-αPDGFR serum (Fig. 8B). Anti-P-Tyr immunoprecipitates of equal amounts of cell lysates were also subjected to immunoblotting with anti-P-Tyr to quantitate steady-state levels of receptor autophosphorylation. The results (Fig. 8C) demonstrate that wild-type 32D-αR(c-*sis*) and 32D-αRΔki-*fms*(c-*sis*) showed comparable levels of receptor autophosphorylation. In contrast, c-*sis* supertransfectants of 32D-αRΔki-1 and 32D-αRΔki-2 exhibited reduced receptor tyrosine phosphorylation at levels of around 10 and 1%, respectively, compared with 32D-αR(c-*sis*). Thus, steady-state receptor autophosphorylation observed in response to autocrine stimulation correlated well with the results of exogenous ligand stimulation of the same transfectants (Fig. 3).

To compare the effects of autocrine stimulation on the sustained proliferation of each cell line, we determined the ability of each to grow in liquid medium or form colonies in semisolid agar in the absence of IL-3. c-*sis*-supertransfected 32D-αR, 32D-αRΔki-1, and 32D-αRki-*fms* demonstrated IL-3-independent colony formation as well as growth in liquid culture, although 32D-αRΔki-1(c-*sis*) formed colonies at a somewhat lower efficiency (Table 1). Under the same conditions, c-*sis*-supertransfected 32D-αRΔki-2 cells failed to proliferate detectably.

Activation of a c-*sis* autocrine loop in NIH 3T3 cells

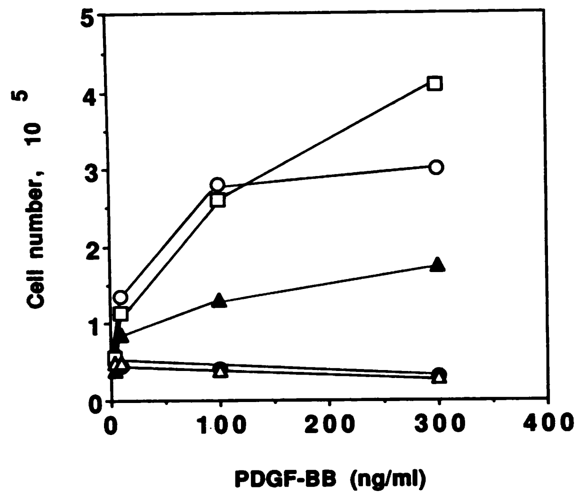

FIG. 6. Comparison of the chemotactic response of 32D transfectants. Cell migration of 32D (△), 32D-αR (○), 32D-αRΔki-1 (▲), 32D-αRΔki-2 (●), and 32D-αRΔki-*fms* (□) was assayed by means of a modified Boyden chamber technique with Nuclepore filters as described previously (17, 29). The results are from a representative experiment in which each point is the mean value of duplicate samples. Similar results were obtained in at least three independent experiments.

results in acquisition of the malignant phenotype (27). To test the effects of coexpression of c-*sis* and various αPDGFR constructs in 32D cells on their tumor-forming ability, athymic nude mice were inoculated subcutaneously with each cell line. 32D-αR and 32D-αRΔki-*fms* cells supertransfected with c-*sis* were rapidly tumorigenic in all mice tested (Table 1). The 32D-αRΔki-1 supertransfectant was also tumorigenic in the majority of the inoculated mice, although the average latency period for tumor development was increased. Finally, the 32D-αRΔki-2 and 32D lines expressing $p27^{c\text{-}sis}$ failed to form any tumors. Taken together, these results support the conclusion that a large deletion of the ki domain region inactivates mitogenic signaling, whereas the smaller ki deletion allows sustained signal transduction.

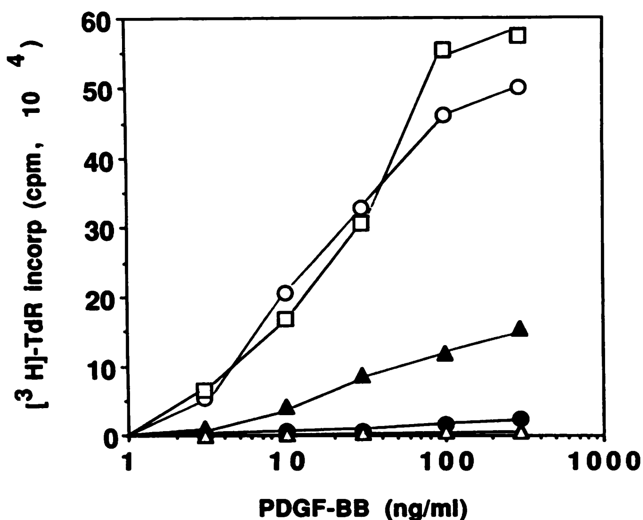

FIG. 7. Mitogenic response of 32D transfectants to PDGF-BB. DNA synthesis by 32D (△), 32D-αR (○), 32D-αRΔki-1 (▲), 32D-αRΔki-2 (●), and 32D-αRΔki-*fms* (□) cells was measured by [³H]thymidine incorporation in IL-3-free medium as described previously (29). The results are from a representative experiment in which each point is the mean value of duplicate samples. Similar results were obtained in three independent experiments.

DISCUSSION

The unusual structure of the split tyrosine kinase domains of members of the PDGF/CSF-1 receptor family has led to efforts to elucidate specific functions of their ki domains. In the present studies, we analyzed effects of progressive deletions and substitutions within the ki of the αPDGFR in efforts to clarify and, possibly, generalize the role of the ki domains of this group of structurally related receptors.

Analysis of major biological responses to PDGF signaling, including chemotaxis, mitogenesis, and sustained growth, established increasing impairment of these responses by the two progressive ki deletions. Although induction of DNA synthesis by the mutant with the smaller ki deletion was around 30% of the wild-type αPDGFR level, the mutant was still capable of inducing a sustained proliferative signal that led to colony formation in vitro and even to the formation of malignant tumors in vivo under conditions of autocrine stimulation by c-*sis*. These findings are consistent with recent studies using analogous c-*fms* ki deletion mutants, which were capable of transforming NIH 3T3 cells (45) and showed only a partial reduction of mitogenic signaling (42). All of these findings are at variance with those of Escobedo et al., who reported that an analogous βPDGFR ki deletion was associated with the specific loss of mitogenic signaling (13). The basis for the discrepancy of the effects of ki deletions on mitogenicity might potentially reflect intrinsic differences between the αPDGFR and CSF-1R in comparison with the βPDGFR. Among other possibilities, there may be differences in intracellular pathways inherent to the specific cell lines used in these experiments.

The larger deletion in the αPDGFR encompassed almost its entire ki domain and was analogous in size to a βPDGFR ki mutant generated by Severinsson et al. (39). The severe reduction or complete abolition of all measured responses in cells expressing these larger ki deletion mutations strongly suggests that this region must be important for maintaining the signaling capacity of both PDGFR molecules.

Tyrosine kinase activity is known to be essential for the function of a number of growth factor receptors. Receptor autophosphorylation, an indirect measure of tyrosine kinase activity, was progressively impaired by the deletion mutants. The αRΔki-1 mutant lacks tyrosine residues, which have been shown to be sites of tyrosine phosphorylation in βPDGF and CSF-1 receptors (25, 44, 48). Therefore, the 10-fold reduction in receptor phosphorylation of the αRΔki-1 mutant may be due in part to the loss of this putative autophosphorylation site. However, a further 10-fold reduction in receptor phosphorylation of the αRΔki-2 must be due to a decrease in receptor kinase activity, since this deletion mutant has not lost any additional tyrosine residues. Taken together, our results argue that structural alterations induced by deletions of the ki domain progressively impair receptor kinase function.

PI-3 kinase has previously been observed to be physically associated with ligand-stimulated PDGFR, epidermal growth factor receptor, and c-*fms* products, as well as with $p^{60v\text{-}src}$– and $p^{60c\text{-}src}$–polyomavirus middle T-antigen complexes (24, 43, 46, 49, 51). Recently, Coughlin et al. reported that the ki domain of the βPDGFR is essential for receptor-associated

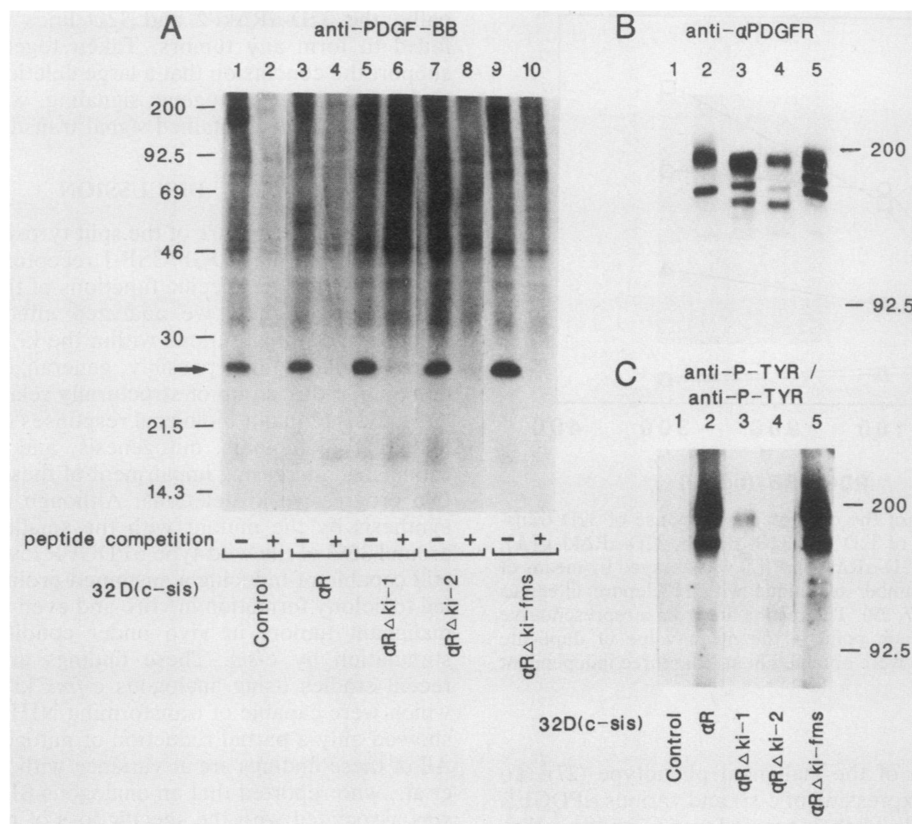

FIG. 8. Steady-state levels of αPDGFR tyrosine phosphorylation in 32D transfectants expressing both the receptor and PDGF-BB. (A) To determine the levels of PDGF-BB in 32D transfectants, 32D(c-*sis*) (lanes 1 and 2), 32D-αR(c-*sis*) (lanes 3 and 4), 32D-αRΔki-1(c-*sis*) (lanes 5 and 6), 32D-αRΔki-2(c-*sis*) (lanes 7 and 8), and 32D-αRΔki-*fms*(c-*sis*) (lanes 9 and 10) cell lysates were immunoprecipitated with anti-c-*sis* serum in the presence (+) or absence (−) of competing peptide. The arrow indicates the position of $p27^{c\text{-}sis}$. (B) For analysis of αPDGFR protein, 100 μg of total cell lysate prepared from 32D(c-*sis*) (lane 1), 32D-αR(c-*sis*) (lane 2), 32D-αRΔki-1(c-*sis*) (lane 3), 32D-αRΔki-2(c-*sis*) (lane 4), and 32D-αRΔki-*fms*(c-*sis*) (lane 5) was subjected to immunoblot analysis with anti-αPDGFR peptide serum. (C) For determination of the level of αPDGFR tyrosine phosphorylation, anti-P-Tyr immunoprecipitates from total cell lysates (2 mg) were subjected to immunoblot analysis with the same anti-P-Tyr as described in Materials and Methods. 32D(c-*sis*) (lane 1), 32D-αR(c-*sis*) (lane 2), 32D-αRΔki-1(c-*sis*) (lane 3), 32D-αRΔki-2(c-*sis*) (lane 4), 32D-αRΔki-*fms*(c-*sis*) (lane 5) were used.

PI kinase activity (7). Mutational analysis has revealed that tyrosine 751 in the βPDGFR ki domain is specifically required for this function (25). Our present studies demonstrate that the αPDGFR, like the βPDGFR, associates with PI kinase following PDGF stimulation. Moreover, our findings that ki deletion mutants abolished receptor-associated PI-3 kinase activity argue that the αPDGFR ki domain is either directly or indirectly responsible for this interaction. However, our evidence that the αRΔki-1 mutant was capable of mitogenic and chemotactic signaling in the absence of detectable receptor-associated PI-3 kinase activity indicates that PI-3 kinase interaction is not essential for these major αPDGFR biological functions.

Another approach toward assessing ki function was derived from generation of a chimera in which the c-*fms* ki domain was inserted into the αRΔki-2 deletion mutant. This chimera restored each of the biochemical and biological activities abolished in the αRΔki-2 deletion mutant to those of wild-type αPDGFR. The αPDGFR and c-*fms* ki domains differ in size by 34 amino acids and possess only 10% sequence identity. Thus, a highly specific amino acid sequence or length of the ki domain is not required for major biological and biochemical responses of the αPDGFR. However, they do exhibit similar predicted secondary structure

TABLE 1. Growth properties of αPDGFR mutants supertransfected with c-*sis*

Cell line	Growth in medium lacking IL-3[a]	% Colony-forming efficiency[b]	Tumorigenicity[c] (no. positive/no. tested)	Latency period (days)
32D(c-*sis*)	−	<0.1	0/10	
32D-αR(c-*sis*)	+	25.0	10/10	17
32D-αRΔki-1(c-*sis*)	+	5.5	9/10	45
32D-αRΔki-2(c-*sis*)	−	<0.1	0/10	
32D-αRΔki-*fms*(c-*sis*)	+	12.0	10/10	20

[a] Transfected cell lines growing in RPMI 1640 medium containing 15% fetal calf serum and 500 U of IL-3 per ml were shifted to growth medium lacking IL-3. Lines which could be continually propagated under these conditions were scored as positive. Lines which were scored as negative died within one or two passages in growth medium without IL-3. Similar results were obtained in three independent experiments.

[b] Colony-forming efficiency was determined as described in Materials and Methods. Visible colonies were scored at 14 days after plating, and results represent the mean values of duplicate plates.

[c] Tumorigenicity was determined by subcutaneous inoculation of adult 129 NFR nude mice with 10^6 cells per mouse. Inoculated mice were observed for 4 months. Pathological analysis of explanted tumors revealed cells with an immature myeloid phenotype (data not shown).

(4, 20a). Although this may explain the restoration of many biological and biochemical activities of this chimeric molecule, including tyrosine kinase function and PI-3 kinase interaction, it cannot account for the ability of 32D-αRΔki-*fms* to couple with PI turnover. The c-*fms* product has been shown not to induce tyrosine phosphorylation of phospholipase C-γ or PI turnover (11, 20, 50). Thus, the ability of the c-*fms* ki domain to restore αPDGFR-associated PI turnover establishes that the ki domain does not directly mediate this response. Although the exact mechanism by which the c-*fms* ki domain completely restores the biological activity of mutant αPDGFR remains to be elucidated, our findings argue that their respective ki domains have evolved to play similar roles in known signaling activities of both PDGF and CSF-1 receptors.

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