Platelet-Derived Growth Factor AB Heterodimer Interchain Interactions Influence Secretion as Well as Receptor Binding and Activation

Mary May, Stuart A. Aaronson, and William J. LaRochelle*

Laboratory of Cellular and Molecular Biology, National Cancer Institute, Building 37, Room 1E24, Bethesda, Maryland 20892

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ABSTRACT: Platelet-derived growth factor (PDGF) is a disulfide-linked dimer comprised of two related polypeptide chains. To investigate the effects of an inactivating lesion introduced into one chain of the nascent PDGF dimer, approaches were developed to optimize synthesis, assembly, secretion, and purification of heterodimers between normal PDGF A and wild-type or mutant PDGF B. PDGF AB heterodimers were released into culture fluids less efficiently than PDGF AA, but to a greater degree than the cell-associated PDGF BB. These results suggest that interactions between two chains influence PDGF secretion. Analysis of heterodimers between PDGF A and disabled PDGF B mutants on cells that express either α or β PDGFRs demonstrated that the impaired biologic activity of the mutant PDGF B chain was ameliorated with respect to binding and triggering of α PDGFRs. In cells that expressed both receptor types, heterodimers of mutant PDGF B and wild-type PDGF A gained substantially in their ability to recruit and trigger α, but not β, PDGFRs. Partial rescue of impaired PDGF B mutant chain function by dimer formation with a wild-type PDGF A chain implies that interchain interactions markedly affect PDGFR binding and activation.

Human platelet-derived growth factor (PDGF) is a prototype for dimeric ligands. PDGF exists either as homodimers of PDGF A or B chains or as the PDGF AB heterodimer (Heldin & Westermark, 1990; Aaronson, 1991). PDGF BB is the human homolog of the *v-sis* oncogene product (Devare et al., 1983; Doolittle et al., 1983; Waterfield et al., 1983). Each PDGF isoform has been purified from naturally occurring sources (Johnsson et al., 1982). These PDGF isoforms bind and interact with varying affinities to the products of two independent genes which encode the α and β PDGF receptors. PDGF BB binds both PDGFRs, while PDGF AA binds only the α PDGFR (Yarden et al., 1986; Hart et al., 1988; Heldin et al., 1988; Matsui et al., 1989b). PDGF AB, like either PDGF AA or PDGF BB, binds α PDGFRs with high affinity, while its affinity for β PDGFRs is substantially lower than that of PDGF BB. The complexity of the PDGF system and the potential involvement of aberrant PDGF receptor stimulation in a variety of diseases including arteriosclerosis (Ross et al., 1990; Ferns et al., 1991) and cancer (Heldin & Westermark, 1990; Nister et al., 1991; Fleming et al., 1992) have led to a variety of approaches aimed at elucidating ligand–receptor interactions.

The current model of PDGF ligand–receptor interaction suggests that each polypeptide chain of the nascent PDGF dimer binds an individual PDGF receptor and thus recruits and stabilizes PDGF receptor complexes (Heldin et al., 1989; Seifert et al., 1989). However, Andersson et al. (1992) have recently reported that monomeric PDGF can activate PDGF receptors as well. We reasoned that if a PDGF dimer were required for efficient receptor interactions leading to triggering, then a heterodimer involving a wild-type chain and one impaired in binding should influence the receptor signaling ability. Thus, we expressed heterodimers between PDGF A and wild-type or impaired PDGF B deletion mutants to investigate the role of interchain interactions in PDGF ligand function(s).

* Correspondence should be addressed to this author. FAX: 301-496-8479.

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EXPERIMENTAL PROCEDURES

PDGF Immunochemical Probes. Goat anti-PDGF serum was previously described (Robbins et al., 1982; LaRochelle et al., 1991). PDGF A chain specific anti-peptide serum was raised against amino acid residues 185–199. For analysis of PDGF A or PDGF B carboxy-terminal processing, anti-peptide sera 200–211 or 227–241, respectively, were utilized (LaRochelle et al., 1991). A monoclonal antibody directed against the PDGF A chain was obtained from Dr. Michael Pech (Hoffmann-La Roche, Basel, Switzerland). PDGF B chain specific monoclonal antibodies included anti-PDGF BB mab obtained from Upstate Biotechnology Inc. (Lake Placid, NY) and mab sis I (LaRochelle et al., 1989). All PDGF antibodies recognize the nondenatured biologically active PDGFs. Anti-peptide sera specific for the α (peptide 959–973) and β (peptide 967–981) PDGF receptors have been described (Matsui et al., 1989a,b; Jensen et al., 1992). MOPC21, used here as a negative control, was purchased from Sigma (St. Louis, MO).

PDGF Constructs. Human PDGF cDNAs were subcloned as *Bam*HI fragments into the *Bgl*II site of the mouse metallothionein expression vector MMTneo (LaRochelle et al., 1990) or MMTgpt. MMTgpt was constructed for these studies by substitution of the gpt gene for the neomycin resistance gene of MMTneo at common *Eco*RI sites. PDGF B stop 185 (LaRochelle et al., 1991) and PDGF B deletion mutants (Giese et al., 1990) were previously described. PDGF B Δ^{109} and PDGF B Δ^{112} are analogous to deletions of amino acid residues 139 and 142, respectively, within the nascent *v-sis* gene product. For analysis of PDGF expression and biologic activity, MMTneo or MMTgpt vectors containing PDGF cDNAs were transfected into NIH 3T3 fibroblasts. Transfectants were selected for growth in DMEM/10% calf serum containing either geneticin or mycophenolic acid. Transfectants expressing PDGF heterodimers were doubly selected with geneticin and hypoxanthine–aminopterin–thymidine (HAT) medium containing mycophenolic acid.

Expression of PDGF Homodimers and Heterodimers. For PDGF homodimer expression, NIH 3T3 fibroblasts were transfected with 5 μg of MMTneo PDGF cDNA. PDGF

heterodimers were expressed after first determining the ratio of MMTgpt PDGF B to MMTneo PDGF A cDNA required for optimal heterodimer formation. Thus, MMTneo PDGF A was titered in cells transfected with 5 μg of MMTgpt PDGF B. After coselection, NIH 3T3 transfectants were metabolically labeled for 4 h with [^{35}S]cysteine and [^{35}S]methionine (125 μCi/mL, 1200 Ci/mmol each; Amersham) in cysteine- and methionine-free Dulbecco's modified Eagle's medium (DMEM) containing 25 μM zinc chloride (LaRochelle et al., 1990). The conditioned medium was collected and a crude cell membrane fraction prepared from the harvested cells (LaRochelle et al., 1991). Crude membrane fractions were boiled for 10 min in 10 mM potassium phosphate, pH 7.4, to release membrane-associated PDGF. Conditioned medium and crude membrane fractions were incubated separately with the appropriate antisera or monoclonal antibody for 2 h at 4 °C. The immunoprecipitates were recovered with *Staphylococcus aureus* protein A–Sepharose (Pharmacia, Piscataway, NJ) in 10 mM sodium phosphate, pH 7.5, 0.15 M NaCl, 1.0% TX-100, 0.1% SDS, 0.5% sodium deoxycholate, 0.02% sodium azide, and 1 mg/mL bovine serum albumin. After solubilization by boiling in SDS–PAGE sample buffer, proteins were analyzed by SDS–PAGE using 14% polyacrylamide gels. Transfectants containing 5 μg of MMTgpt PDGF B with 0.05 μg of MMTneo PDGF A were selected for optimal wt PDGF AB heterodimer formation.

To produce the PDGF AB heterodimer in quantities sufficient for purification from the conditioned medium of NIH 3T3 transfectants, a secretory form of PDGF B, PDGF B stop 185 (LaRochelle et al., 1991), was utilized. PDGF BΔ mutant (Giese et al., 1990) cDNAs were also expressed with a stop codon introduced at position 185. To determine the optimal conditions for PDGF AB stop 185 heterodimer formation, the MMTgpt PDGF A construct was titered in a cotransfection assay with 5 μg of the MMTneo PDGF B stop 185. Metabolic labeling demonstrated that culture fluids from cotransfectants containing 5 μg of MMTneo PDGF B stop 185 and 1 μg of MMTgpt PDGF A contained the greatest percentage of PDGF AB stop 185 heterodimers. For optimal expression of PDGF ABΔ, 5 μg of MMTneo PDGF BΔ was cotransfected with 0.5 μg of MMTgpt PDGF A.

Immunoaffinity Purification of PDGF Homodimers and Heterodimers. PDGF B homodimers were purified by elution from a PDGF B chain specific monoclonal antibody immunoaffinity column. The strategy for PDGF heterodimer purification relied on sequential immunoaffinity chromatography utilizing a PDGF B chain specific, followed by a PDGF A chain specific, monoclonal antibody (mab) immunoaffinity column. The PDGF B chain specific immunoaffinity column was prepared by conjugation of the PDGF B mab to Affigel 10 (Bio-Rad) according to the manufacturer's protocol. Briefly, 1.2 mg of mab was coupled to 2 mL of Affigel 10 in 100 mM HEPES, pH 7.4, for 16 h at 4 °C on a rotating wheel. Next, unconjugated *N*-hydroxysuccinimidyl groups were blocked with 100 mM ethanolamine, pH 8.0, for 4 h.

The PDGF A chain specific immunoaffinity column was prepared by conjugation of the PDGF A mab to CNBr-activated Sepharose 4B (Pharmacia) by the manufacturer's protocol. Briefly, 300 mg of resin was washed with 200 mL of 1 mM HCl followed by 100 mM sodium bicarbonate, pH 8.3, and 500 mM NaCl (coupling buffer); 5 mg of PDGF A mab was added to the activated resin in 4 mL of coupling buffer and incubated 16 h at 4 °C. Uncoupled resin was blocked for 2 h with 100 mM Tris-HCl, pH 8.0, and washed with three cycles of alternating pH using 100 mM sodium acetate (pH 4.0)/0.5 M NaCl followed by 100 mM Tris (pH 8.0)/0.5 M NaCl. Both PDGF B mab–Affigel and PDGF A mab–Sepharose were washed in PBS, mock-eluted with 200 mM glycine, pH 2.5, and stored at 4 °C in PBS/0.02% sodium azide. The PDGF B mab–Affigel column readily bound PDGF AB and PDGF BB, while the PDGF A mab–Sepharose column bound PDGF AA and PDGF AB. Analysis of PDGF B mab–Affigel with ^{125}I-PDGF AA showed that less than 1% of the loaded homodimer was retained and eluted with 200 mM glycine, pH 2.5. Similar results were obtained with PDGF A mab–Sepharose using ^{125}I-PDGF BB.

For purification of PDGF heterodimers, 2 L of NIH 3T3 conditioned medium was collected and cell debris removed by centrifugation. The medium was concentrated to 100 mL with a Minitan concentrator (Millipore) and adjusted to 1.0% TX-100. The supernatant was batch-loaded overnight at 4 °C on PDGF B mab–Affigel using a rotator. After extensive washing with 20 column volumes of PBS, bound PDGF was eluted with 200 mM glycine, pH 2.5. Western blot analysis detected PDGF BB and PDGF AB. PDGF AA was detected in the flow-through (data not shown). The PDGF eluate was neutralized with 1 N NaOH in the presence of DMEM/0.5% BSA, made 1.0% in Triton X-100, and applied to PDGF A mab–Sepharose. After extensive washing with PBS, the PDGF AB heterodimer was eluted with 200 mM glycine, pH 2.5. The eluate was added to SDS sample buffer, boiled, and resolved using 14% SDS–polyacrylamide gels. Silver staining of proteins was performed with a Bio-Rad silver stain kit. For Western blot analysis, PDGF heterodimers were transferred to Immobilon-P and immunoblotted with PDGF A chain specific anti-peptide serum 185–199. Recombinant PDGF BB used for quantitation and normalization was obtained from Upstate Biotechnology.

PDGF Mitogenic Assay. 32D cells transfected with either the α or the β PDGFR (Matsui et al., 1989a,b) were harvested by centrifugation, washed, and gently resuspended in RPMI 1640/10% serum. PDGF or PDGF deletion mutant homodimer or heterodimer was added to 50 μL of RPMI 1640/10% serum in a 96-well microtiter plate followed by 50 μL of cell suspension containing 4×10^4 cells. The plate was incubated at 37 °C for 24 h. [^{3}H]Thymidine (2 μCi) was then added for 3 h. Cells were harvested and washed, and [^{3}H]thymide uptake was quantitated usinbg a Beckman 5500 scintillation counter. In some cases, a neutralizing PDGF antibody was added simultaneously with ligand to further confirm the PDGF-specific nature of the response.

PDGF Binding Analysis. 32D cell transfectants expressing either α or β PDGFRs were harvested by centrifugation, washed in DMEM, gently resuspended in binding buffer [DMEM/25 mM HEPES (pH 7.4)/1 mg/mL BSA], and maintained at 37 °C. Next, saturating levels of ^{125}I-PDGF AA (1 ng) or ^{125}I-PDGF BB (2 ng) were added to increasing concentrations of unlabeled PDGF competitor in 50 μL of binding buffer at 4 °C. A total of 1.2×10^6 32D cells expressing either receptor were added in an equivalent volume of binding buffer and incubated at 16 °C. After 1 h, the cell suspension was layered onto 300 μL of a chilled oil mix [*n*-butyl phthalate (Fischer)/bis(2-ethylhexyl) phthalate (Kodak), 1.5:1]. Cells were centrifuged in an Eppendorf microfuge at 10 000 rpm for 10 min at 4 °C. The cell pellet was removed and counted in a Beckman 5500 γ counter.

PDGFR Phosphorylation. NIH 3T3 cells were starved overnight and triggered for 10 min with 50 ng/mL purified PDGF BB stop 185, PDGF BB Δ^{109}, PDGF BB Δ^{112}, PDGF AB stop 185, PDGF AB Δ^{109}, or PDGF AB Δ^{112}. Whole cell

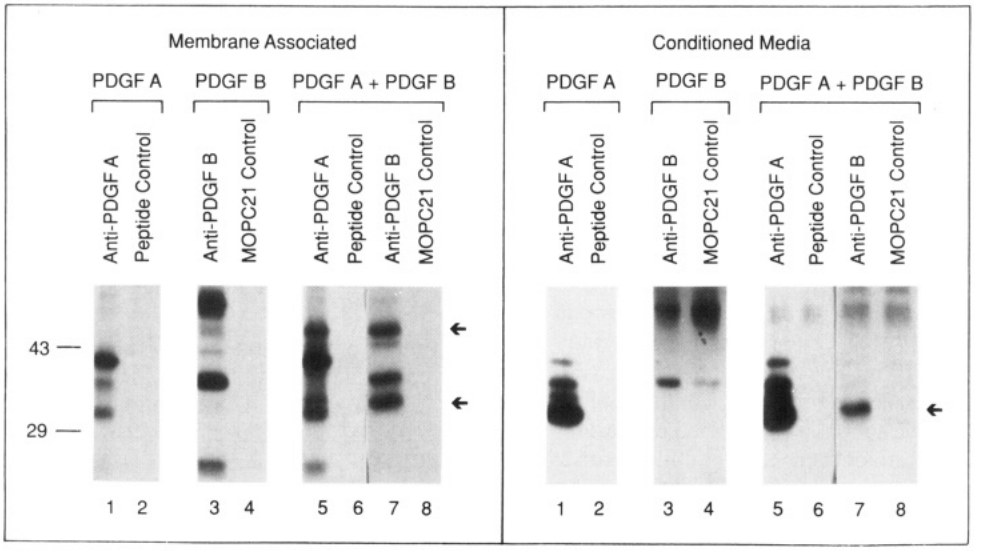

FIGURE 1: Cellular compartmentalization of PDGF homodimers and heterodimers. NIH 3T3 transfectants expressing PDGF A, PDGF B, or both PDGF A and PDGF B cDNAs were metabolically labeled as described under Experimental Procedures. The conditioned medium was collected, cells were harvested, and a crude cell membrane fraction was prepared. Conditioned medium or cell membranes from PDGF A transfectants were immunoprecipitated with a PDGF A chain specific anti-peptide serum 185–199 (lane 1). Similar cellular fractions from PDGF B transfectants were immunoprecipitated with a PDGF B chain specific monoclonal antibody (lane 3). Fractions were also immunoprecipitated from transfectants expressing both PDGF A and PDGF B cDNAs with either PDGF A anti-peptide serum 185–189 (lane 5) or PDGF B monoclonal antibody (lane 7). In some cases, PDGF A anti-peptide serum 185–199 was incubated with the homologous peptide (lanes 2 and 6). MOPC21 served as an additional specificity control (lanes 4 and 8). Immunoprecipitates were recovered with protein A–Sepharose. Protein species were resolved by SDS–PAGE and visualized by fluorography. Arrows designate the p50 and p33 PDGF AB heterodimeric species. Under the experimental conditions used here, no cross-reactivity between PDGF A or B chain specific immunochemical reagents was observed.

lysates were immunoprecipitated with α or β PDGFR-specific anti-peptide sera. Immunoprecipitates were subjected to SDS–PAGE using 7% gels, transferred to Immobilon-P membranes, and blocked with 3% dry milk. The filters were incubated with anti-phosphotyrosine mab (PY20) and washed with Tris-buffered saline/0.05% Tween 20, and bound primary antibody was detected with ^{125}I-protein A.

RESULTS

Assembly, Processing, and Secretion of Wild-Type PDGF Heterodimers. The secretory properties of PDGF A and B chains are determined by extracellular retention domains localized in the respective carboxyl termini of the PDGF A long isoform (residues 200–211) and PDGF B (residues 212–226) (LaRochelle et al., 1991; Ostman et al., 1991a). In fibroblasts, these domains are differentially cleaved, resulting in efficient release of PDGF A and cell-surface retention of PDGF B (LaRochelle et al., 1991) by cell-associated heparan–sulfate proteoglycans (Raines & Ross, 1992). To characterize the secretory properties of heterodimers of these two molecules, NIH 3T3 cells were cotransfected with varying ratios of expression vectors for each and marker selected mass cultures analyzed. Under optimal conditions, as much as 20–40% of immunodetectable PDGF A and B was assembled into heterodimers.

Figure 1 shows the results of a representative experiment in which PDGF A and B were measured in cell membranes or conditioned medium of transfected cultures. In cells transfected with either PDGF A or PDGF B, PDGF A was predominantly detected in conditioned medium, while PDGF B was retained in association with the cell membrane fraction (Figure 1). The characteristic mobilities of precursor and processed forms of the two molecules differed as well. PDGF A exhibited p42, p38, and p32 forms, while PDGF B was expressed as p54, p34, and p24 species (Figure 1). In PDGF A and B coexpressing cultures, species with the expected mobilities of wt PDGF A and B were observed (Figure 1). In addition, species with intermediate molecular weights, including p50 and p33, were recognized by both PDGF A and PDGF B specific antisera. Thus, it seemed likely that these new species reflected heterodimeric forms of the PDGF molecule.

Whereas >90% of the immunodetectable PDGF A was present in medium of PDGF A expressing cells, cultures coexpressing PDGF A and B showed higher levels of cell-associated PDGF A. The major secreted p33 heterodimer, although clearly detected within PDGF B specific immunoprecipitates of culture fluids, was partially masked within PDGF A specific immunoprecipitates by the larger amounts of PDGF A p32 present. Thus, to ensure that secreted p33 contained PDGF A as well as PDGF B determinants, conditioned medium was first purified by immunoaffinity chromatography using a PDGF B chain specific monoclonal antibody. Next, p33 was demonstrated to possess immunoreactivity with PDGF A chain specific anti-peptide serum 185–199 (Figure 2A). Thus, secreted p33 contained determinants of both the PDGF A and PDGF B chains, and unique to the PDGF AB heterodimer. These findings demonstrate that PDGF B determinants were released into culture fluids in greater amounts from PDGF A coexpressing cultures than from PDGF B transfectants. Furthermore, interactions between the two chains influenced their secretory properties, with greater cell retention of heterodimers relative to PDGF AA but more efficient secretion relative to PDGF BB.

The lack of p33 heterodimer retention at the cell surface suggested that either or both PDGF A and PDGF B carboxy-terminal retention sequences had been proteolytically removed. To investigate the mechanistic basis for heterodimer secretion and carboxy-terminal processing, anti-peptide sera to PDGF A carboxy-terminal amino acid residues 200–211 and PDGF B carboxy-terminal amino acid residues 227–241 were used to immunoprecipitate the partially purified p33. As shown

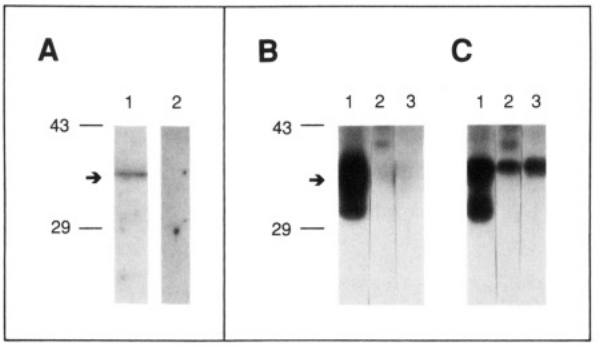

FIGURE 2: Immunochemical identification and analysis of wt PDGF AB heterodimer carboxy-terminal processing. The PDGF AB p33 heterodimer was partially purified by immmunoaffinity chromatography with a PDGF B chain specific monoclonal antibody. Panel A: PDGF B mab eluate was subjected to SDS–PAGE, transferred to Immobilon-P, and immunoblotted with PDGF A anti-peptide serum 185–199 (lane 1) or control serum (lane 2) followed by ^{125}I-protein A. Panels B and C: PDGF B mab eluate (panel B) or bacterial-expressed PDGF AB (panel C) was immunoprecipitated with goat anti-PDGF serum (lane 1), PDGF B anti-peptide serum 227–241 (lane 2), or PDGF A anti-peptide serum 200–211 (lane 3). Arrows designate the p33 heterodimer. Immunoprecipitates were subjected to SDS–PAGE and immunoblotted with goat anti-PDGF serum followed by ^{125}I-protein A.

in Figure 2B, PDGF antisera efficiently immunoprecipitated the p33 PDGF AB heterodimer, while neither PDGF A anti-peptide 200–211 nor PDGF B anti-peptide 227–241 sera recognized the p33 species. As a positive control, both anti-peptide sera immunoprecipitated bacterially expressed non-carboxy-terminal-processed PDGF AB p36 (Figure 2C). Thus, PDGF AB p33 released into culture fluids was carboxy-terminal-processed within both the PDGF A and the PDGF B chains.

Identification and Purification of Heterodimers Involving Wild-Type PDGF A and PDGF B Mutants Lacking Retention Properties. In order to determine the influence of defective PDGF B chains on the biologic properties of heterodimers with wt PDGF A, we sought an approach that would allow more efficient heterodimer secretion. We previously showed that truncation of the PDGF B carboxyl terminus at or beyond amino acid 185 results in efficient PDGF B secretion (LaRochelle et al., 1991). Thus we generated heterodimers by coexpressing PDGF A with wt PDGF B stop 185 or with either of two mutants, PDGF B Δ^{109} and PDGF B Δ^{112}, previously shown to exhibit substantially impaired transforming ability as homodimers (Giese et al., 1990). Furthermore, deletions of these single amino acid residues critically affected PDGFR recognition and functional activation (Giese et al., 1990).

As shown in Figure 3, the stop 185 forms of wild-type PDGF B as well as BΔ^{109} or BΔ^{112} mutants were efficiently secreted as p27 homodimers with greater than 90% in the culture medium. Under optimal conditions, cultures coexpressing any of these genes and wt PDGF A also efficiently secreted heterodimeric molecules, which accounted for as much as 30–50% of the immunodetectable PDGF released (Figure 3). The heterodimer in each case was around 29 kDa, intermediate in size between PDGF AA p32 and PDGF BB stop 185 p27. Unlike the wt homodimers, the p29 heterodimers were immunodetectable both with PDGF A anti-peptide serum 185–199 and with PDGF B specific monoclonal antibody (Figure 3). As expected, PDGF A anti-peptide serum 200–211 failed to recognize p29, consistent with the proteolytic removal of its PDGF A retention domain and efficient secretion (data not shown).

Purification of secreted heterodimers was achieved by sequential PDGF B and PDGF A immunoaffinity chromatography. The efficiency of each immunoaffinity step was greater than 50–60% in retaining homodimers containing the respective PDGF A or B chain (see Experimental Procedures). As revealed both by silver staining (Figure 4A) and by immunoblotting with PDGF A anti-peptide serum 185–199 (Figure 4B) or PDGF B specific monoclonal antibody (Figure 4C), these purification steps resulted in purification of the p29 heterodimer species. All of these results demonstrated the ability to generate, secrete, and purify PDGF AB heterodimers in sufficient quantity for analysis of biologic properties.

Effects of Heterodimer Formation with PDGF A on Mitogenic Signaling and PDGF Receptor Binding of PDGF B Δ^{109} and B Δ^{112} Mutants. To investigate the influence of a defective PDGF B chain on the biological properties of a PDGF AB heterodimer, we compared their mitogenic signaling abilities on 32D cell transfectants stably expressing either α or β PDGFRs (Matsui et al., 1989a,b). As shown in Table I, PDGF B Δ^{109} and PDGF B Δ^{112} mutant homodimers showed substantial impairment in their activities relative to wt PDGF BB, when tested on either 32D αR or 32D βR cell lines. The PDGF B Δ^{109} homodimer was more impaired, requiring a greater than 33-fold higher concentration than wt PDGF BB to induce a comparable increase in DNA synthesis in 32D αR cells and a greater than 200-fold increase over wt PDGF BB in 32D βR cells.

Heterodimers of a wt PDGF A chain with either mutant exhibited improved biological activity on 32D αR cells (Table I). For example, PDGF AB Δ^{112} was at least as active as wt PDGF AB, and PDGF AB Δ^{109} showed only 8-fold lower activity. Moreover, each of the heterodimers was more active than the respective mutant PDGF BB homodimer in stimulating DNA synthesis. As shown in Table I, PDGF AB was at least 10-fold less efficient than wt PDGF BB at signaling through the β PDGFR, and the activities of the mutant heterodimers were even more dramatically reduced. Thus, it was difficult to precisely quantitate mutant heterodimer signaling functions in 32D βR cells.

Receptor binding affinities of mutant PDGF BB homodimers and heterodimers with wt PDGF A were next analyzed by means of competition for labeled PDGF BB binding. Table I shows that the reduction in affinity generally corresponded with the degree to which mitogenic activity was impaired. Of note, the binding of heterodimers containing a wt PDGF A chain and either B Δ^{109} or B Δ^{112} chains to the α PDGFR was in each case improved relative to the respective mutant PDGF BB homodimer (Table I). A representative experiment in which binding of ^{125}I-PDGF BB to 32D αR cells was competed with either of the two mutant PDGF B homodimers or their respective heterodimer with wt PDGF A is shown in Figure 5. All of these results demonstrate that interactions between two chains of the PDGF ligand influence receptor binding and mitogenic signaling functions.

Triggering and Recruitment of PDGF Receptors by PDGF B Δ^{109} and B Δ^{112} Mutant Homodimers and Heterodimers. In 32D transfectants expressing only β PDGFRs, PDGF AB exhibited greatly diminished triggering ability relative to PDGF BB. In contrast, studies using cells that express both the α and β PDGFRs have indicated that PDGF AB interacts with β PDGFRs much more efficiently in the presence of α PDGFRs (Bishayee et al., 1989; Heidaran et al., 1991). Thus, we also investigated the ability of our mutant homodimers and heterodimers to recruit and activate β PDGFRs on NIH

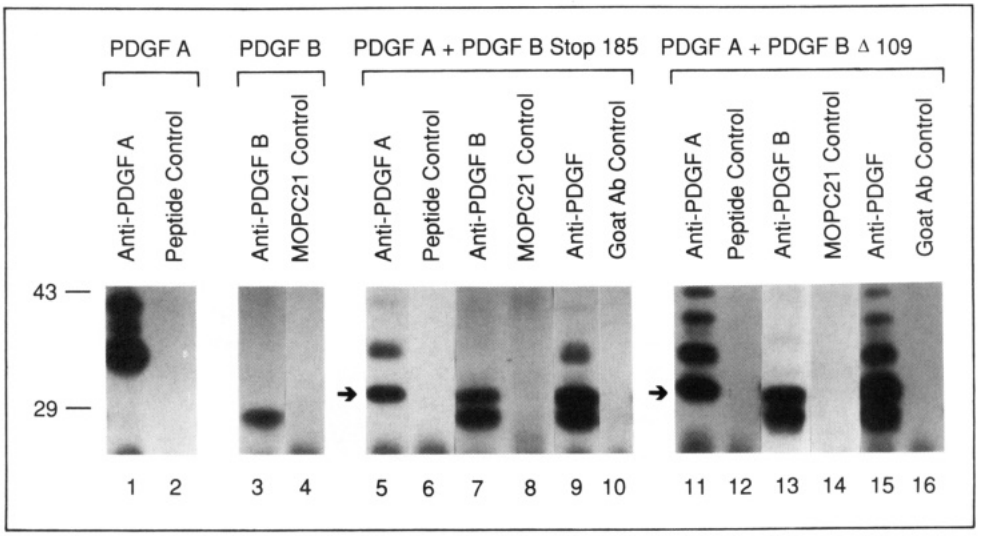

FIGURE 3: Assembly and secretion of wild-type or mutant PDGF B stop 185 homodimers or heterodimers with PDGF A. Metabolically labeled conditioned medium was harvested from NIH 3T3 transfectants expressing PDGF A, PDGF B stop 185, PDGF A and PDGF B stop 185, and PDGF A and PDGF B Δ^{109}. PDGF gene products were immunoprecipitated with a PDGF A chain specific anti-peptide serum 185–199 (lanes 1, 5, and 11), a PDGF B chain specific monoclonal antibody (lanes 3, 7, and 13), or goat anti-PDGF serum (lanes 9 and 15). In some cases (lanes 2, 6, and 12), the PDGF A anti-peptide serum was preincubated with the homologous peptide. MOPC21 (lanes 4, 8, and 14) and normal goat serum (lanes 10 and 16) were used as specificity controls. Arrows indicate PDGF AB heterodimers. Immunoprecipitated proteins were subjected to SDS–PAGE on 14% polyacrylamide gels and visualized by fluorography.

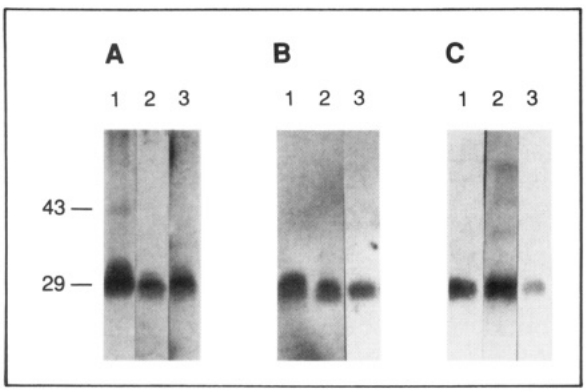

FIGURE 4: Silver stain and Western blot analysis of PDGF AB stop 185 and PDGF AB deletion mutant heterodimers. PDGF AB stop 185 (lane 1), PDGF AB Δ^{109} (lane 2), and PDGF AB Δ^{112} (lane 3) were purified by immunoaffinity chromatography as described under Experimental Procedures. Fifty nanograms of purified PDGF AB was subjected to SDS–PAGE followed by silver stain analysis (panel A) and immunoblotting with either PDGF A anti-peptide serum 185–199 (panel B) or a PDGF B specific monoclonal antibody (panel C).

Table I: Mitogenic Activity and Receptor Binding Properties of PDGF B Wild-Type or Deletion Mutant Homodimers and Heterodimers with PDGF A

	PDGF homodimer		heterodimer with PDGF A	
	mitogenic equiv[a]	K_o[b]	mitogenic equiv[a]	K_o[b]
32D αR cells				
wt B	0.9	2.2	2	2.2
B Δ^{109}	33	50	8	10
B Δ^{112}	6	21	0.5	1.3
32D βR cells				
wt B	0.5	0.2	6	2.3
B Δ^{109}	100	11	50	77
B Δ^{112}	25	30	>100	>100

[a] Exponentially growing 32D αR or βR transfectants were incubated with the indicated PDGF, and [^{3}H]thymidine uptake was measured as indicated under Experimental Procedures. PDGF protein concentrations were measured by silver staining and immunoblotting in parallel with known quantities of recombinant PDGF B homodimer. PDGF B mitogenic equivalents were determined by dividing a known quantity of recombinant PDGF B by the amount of deletion mutant which produced an equivalent mitogenic signal. A representative experiment is shown. In two experiments, determinations varied by less than 20%. 32D cells not transfected exhibited <1% of the PDGF mitogenic response and binding ability of 32D PDGFR transfectants. [b] Binding assays were performed on 32D αR or βR cells as described under Experimental Procedures. Dissociation constants (nanomolar) were determined by Scatchard analysis using saturating concentrations of ^{125}I-PDGF BB. A representative experiment is shown. Determined K_ds differed by less than 20% between two experiments.

3T3 fibroblasts which express both receptor types. As shown in Figure 6, PDGF AB and BB triggered α PDGFRs or β PDGFRs to similar extents. Consistent with their dramatically reduced binding and mitogenic signaling in 32D PDGFR transfectants, PDGF B Δ^{109} and PDGF B Δ^{112} mutant homodimers demonstrated little activation of either α or β PDGFRs in NIH 3T3 cells. In contrast, PDGF B Δ^{109} and PDGF B Δ^{112} mutant heterodimers with wt PDGF A showed significant triggering of α PDGFRs while demonstrating little or no increase in β PDGFR activation. Thus, heterodimers between PDGF A and PDGF B Δ^{109} or PDGF B Δ^{112} gained substantially in their ability to trigger α PDGFRs, but not in recruiting and activating β PDGFRs.

DISCUSSION

In the present studies, we expressed heterodimers between PDGF A and wild-type or mutant PDGF B chains to investigate the influence of interactions between the two polypeptide chains on PDGF biologic functions. Interchain cooperation was demonstrated with respect to both secretory properties and receptor/ligand interactions. Whereas PDGF B remains tightly cell-associated due to a carboxy-terminal retention sequence, alternative PDGF A variants (Betsholtz et al., 1986) either lack such a domain or contain an analogous domain which is efficiently cleaved by proteolytic processes (LaRochelle et al., 1991; Ostman et al., 1991a). In either case, PDGF AA is actively secreted by fibroblasts, which retain PDGF BB at the cell surface. We were able to demonstrate that PDGF AB is intermediate in its secretory phenotype. Both PDGF carboxyl termini, including the PDGF A retention motif, were found to be cleaved from secreted PDGF AB, confirming previous evidence (LaRochelle et al., 1991) that these domains are responsible for PDGF cell-surface

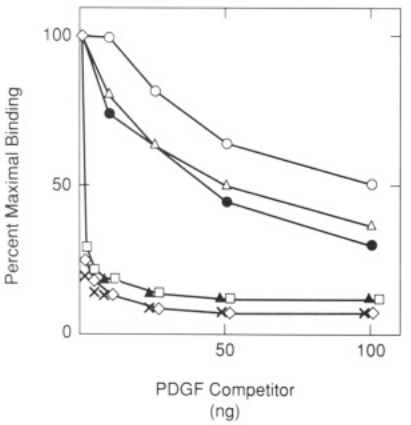

FIGURE 5: Inhibition of PDGF binding to the α PDGF receptor by PDGF homodimers and heterodimers. 32D αR were incubated with ^{125}I-PDGF BB in the presence of increasing concentrations of PDGF AA (□), PDGF BB (◇), PDGF AB stop 185 (×), PDGF BB Δ^{109} (○), PDGF BB Δ^{112}, (△), PDGF AB Δ^{109} (●), and PDGF AB Δ^{112} (▲) as described under Experimental Procedures. Cells were washed, and iodinated PDGF binding was measured with a Beckmann 5500 γ counter.

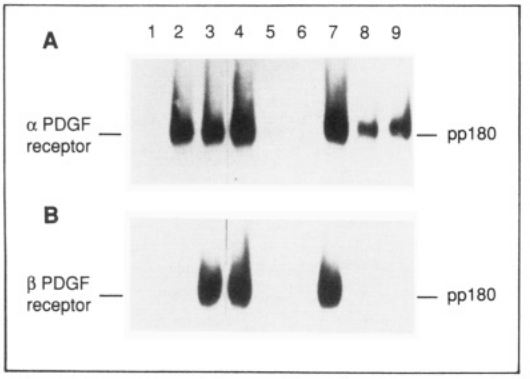

FIGURE 6: Tyrosine phosphorylation of α and β PDGF receptors by deletion mutant homodimers or heterodimers. NIH 3T3 fibroblasts were triggered for 10 min with immunoaffinity-purified PDGF BB stop 185 (lane 4), PDGF BB Δ^{109} (lane 5), PDGF BB Δ^{112} (lane 6), PDGF AB stop 185 (lane 7), PDGF AB Δ^{109} (lane 8), and PDGF AB Δ^{112} (lane 9) as described under Experimental Procedures. For comparison, untreated NIH 3T3 fibroblasts (lane 1) or those triggered with commercially available PDGF AA (lane 2) or PDGF BB (lane 3) are also shown. Cell lysates were immunoprecipitated with antisera specific for the α (panel A) or β (panel B) PDGFR. After SDS–PAGE, PDGFRs were immunoblotted with a monoclonal antibody specific for phosphotyrosine. Bound primary antibody was detected with ^{125}I-protein A.

retention. The fact that cell-surface-associated PDGF AB contained both PDGF A and B retention domains argues that cleavage processes are less efficient for the heterodimer than for PDGF AA. All of these findings establish that interactions between the PDGF A and B chains in the heterodimer influence ligand secretion in a codominant manner.

Previous studies have provided evidence that interactions between the two PDGF subunits can affect ligand binding and signaling properties. In an elegant dominant negative study, dimerization of an impaired PDGF A processing mutant with PDGF A or PDGF B suppressed PDGF A, but not PDGF B biologic activity (Mercola et al., 1990). In other studies, PDGF AB bound β PDGFRs but with significantly lower affinity than that of PDGF BB. In contrast, PDGF AA showed no detectable binding to the β PDGFR (Yarden et al., 1986; Hart et al., 1988; Heldin et al., 1988; Matsui et al., 1989b). Our present studies confirm these findings with respect to the β PDGFR and extend these observations to the α PDGFR. To investigate the effects of interactions between two PDGF subunits on α PDGFR binding, we examined PDGF B deletion mutants whose homodimeric forms showed progressively impaired binding to this receptor. In each case, the impaired activities of the mutant PDGF BB homodimers could be ameliorated by their forming heterodimers with a wild-type PDGF A chain. These findings establish cooperation between two chains of the PDGF heterodimer with respect to interactions with the α PDGFR.

There is increasing evidence for the role of growth factor receptor dimer formation in receptor activation (Ullrich & Schlessinger, 1990). Initial studies with EGF established that ligand addition results in chemically cross-linkable EGF receptor dimers (Schlessinger, 1988). Dimerization of these receptors correlates with activation of the receptor tyrosine kinase. Subsequent studies have extended these observations to other growth factor receptors as well (Bishayee et al., 1989; Heldin et al., 1989; Heidaran et al., 1991). Although evidence for PDGF-induced receptor aggregation has been indirect, the related colony-stimulating factor 1 (CSF-1) receptor has been shown to undergo noncovalent dimer formation *in vivo* in response to CSF-1 (Li & Stanley, 1991). There is convincing evidence that the ligand binding domains of these receptors aggregate (Bishayee et al., 1989; Margolis et al., 1989), but a direct causal role of receptor oligomerization in activation of membrane-spanning tyrosine kinase receptors has yet to be formally established.

Because of the dimeric nature of ligands like PDGF, it has been proposed that such molecules are bivalent, with each polypeptide chain of the dimer interacting with a single receptor (Heldin et al., 1989; Seifert et al., 1989). Recently, Andersson et al. reported that the PDGF A monomer was capable of binding and activating PDGF receptors, arguing against such a model (Andersson et al., 1992). However, these studies did not exclude the possibility of a noncovalent dimer that dissociated during SDS–PAGE. Our present findings imply that a PDGF monomer may not be sufficient to specify high-affinity receptor binding determinants because of influences demonstrated between the two chains on heterodimer interactions with either α or β PDGFRs.

The molecular interactions involved in ligand binding and recruitment of receptors into complexes have recently been elucidated in the case of human growth hormone, a monomeric ligand. Crystal structure analysis of the growth hormone receptor complex has demonstrated that a single growth hormone molecule forms a stable complex with two receptor external domains (de Vos et al., 1992). In the case of human growth hormone, two spatially distinct regions within the monomeric ligand appear to be required for recruitment and stabilization of the growth hormone receptor dimer (de Vos et al., 1992). Growth hormone receptor antagonists which exploit the sequential binding of receptors to these two sites, by eliminating or reducing affinity at site 2, have even been developed (Fuh et al., 1992). These act by preventing receptor dimer formation and concomitant signal transduction (Fuh et al., 1992).

There is evidence which supports the concept that PDGF interacts initially with one receptor and that such interactions affect subsequent recruitment of a second receptor (Bishayee et al., 1989; Heidaran et al., 1991). Molecular genetic analysis has already identified at least two and possibly three independent sites in the PDGF B molecule that specify high-affinity interaction with the β PDGFR (LaRochelle et al., 1990, 1992; Giese et al., 1990; Ostman et al., 1991b; Maher et al., 1993). PDGF A and B sites of interaction with the α PDGFR have yet to be elucidated. The dimeric nature of

PDGF and the cooperativity observed between its two chains suggest that ligand–receptor interactions leading to receptor activation are likely to be significantly more complex than those elegantly described for human growth hormone.

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