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Ligand-Induced Interaction between α- and β-Type Platelet-Derived Growth Factor (PDGF) Receptors: Role of Receptor Heterodimers in Kinase Activation†

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ABSTRACT: Two types of PDGF receptors have been cloned and sequenced. Both receptors are transmembrane glycoproteins with a ligand-stimulatable tyrosine kinase site. We have shown earlier that ligand-induced activation of the β-type PDGF receptor is due to the conversion of the monomeric form of the receptor to the dimeric form [Bishayee et al. (1989) *J. Biol. Chem. 264*, 11699-11705]. In the present studies, we have established the ligand-binding specificity of two receptor types and extended it further to investigate the ligand-induced association state of the α-receptor and the role of α-receptor in the activation of β-receptor. These studies were conducted with cells that express one or the other type of PDGF receptor as well as with cells that express both types of receptors. Moreover, ligand-binding characteristics of the receptor were confirmed by immunoprecipitation of the receptor-^{125}I-PDGF covalent complex with type-specific anti-PDGF receptor antibodies. These studies revealed that all three isoforms of PDGF bind to α-receptor, and such binding leads to dimerization as well as activation of the receptor. In contrast, β-receptor can be activated only by PDGF BB and not by PDGF AB or PDGF AA. However, by using antipeptide antibodies that are specific for α- or β-type PDGF receptor, we demonstrated that in the presence of α-receptor, β-receptor kinase can be activated by PDGF AB. We present here direct evidence that strongly suggests that such PDGF AB induced activation of β-receptor is due to the formation of a noncovalently linked α-β receptor heterodimer.

Platelet-derived growth factor (PDGF)[1] is a mitogen for cells of connective tissue origin. It is a disulfide-bonded dimeric protein composed of two nonidentical but highly homologous chains, known as A and B chains [for reviews, see Ross et al. (1986) and Heldin and Westermark (1989)]. All three possible isoforms of PDGF, namely, PDGF AA, PDGF BB, and PDGF AB, have been identified and isolated from different sources including tumor cells (Antoniades et al., 1979; Deuel et al., 1981; Raines & Ross, 1982; Stroobant & Waterfield, 1984; Heldin et al., 1986; Hammacher et al., 1988). They are biologically active in inducing mitogenesis. The B chain of PDGF has more than 90% sequence homology with the cellular homologue of the v-sis oncogene protein of simian sarcoma virus (Waterfield et al., 1983; Doolittle et al., 1983). The mitogenic and transforming activities of PDGF are mediated through its interaction with a high-affinity cell-surface receptor. PDGF receptor, a transmembrane glycoprotein of 180 kDa, is a member of the tyrosine kinase family of receptors. The intrinsic tyrosine kinase activity of the receptor is obligatory for its mitogenic function [reviewed in Williams (1989)]. Two types of PDGF receptors (α and β) have been cloned and sequenced (Yarden et al., 1986; Gronwald et al., 1988; Claesson-Welsh et al., 1988; Matsui et al., 1989). α- and β-type PDGF receptors have identical structural features. However, at the amino acid level, there is only 43% overall sequence homology between the two receptor types (Matsui et al., 1989). The ligand-binding specificity of these two receptors also differs considerably (Heldin et al., 1988; Hart et al., 1988).

We and others have investigated changes in the structure and association state of the PDGF receptor that are induced by ligand binding (Bishayee et al., 1989; Heldin et al., 1989). Such studies conducted either with a homogeneous preparation of β-type receptor (Heldin et al., 1989) or with β-receptor-specific antibodies (Bishayee et al., 1989) revealed that one

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[1] Abbreviations: BS^3, 3,3′-bis(sulfosuccinimido)suberate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NP-40, Nonidet P-40; PDGF, platelet-derived growth factor; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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of the earliest effects of the interaction of PDGF with its receptor is the complete conversion of the receptor monomer to a noncovalently linked dimer. On the basis of these studies, it was concluded that the ligand-induced dimerization of the β-receptor is linked to the process of the kinase activation leading to autophosphorylation and autophosphorylation-induced changes in the receptor conformation (Bishayee et al., 1988). In this respect, very little is known about the molecular basis for the activation of α-receptor kinase; this is mainly due to the fact that α-receptor has been identified only recently. Moreover, it is also not known whether there is any ligand-induced interaction between two types of PDGF receptors and, if so, whether such heterologous interaction plays any role in the activation of receptor kinase. The availability of type-specific anti-PDGF receptor antibodies and cell lines that express one or the other type of PDGF receptor as well as cell lines that contain both types of receptors allowed us to investigate the effect of different isoforms of PDGF on the activation of α- and β-type receptor kinases. Our studies presented here demonstrate that α-receptor can be activated by all three isoforms of PDGF whereas β-receptor can be activated only by PDGF BB and not by PDGF AA or AB. However, interestingly, β-receptor can be activated by PDGF AB provided α-receptor is also present. We also present evidence that indicates that the activation of β-receptor by PDGF AB is probably due to PDGF receptor heterodimer formation in which one α-receptor forms a noncovalent bond with a β-receptor.

Experimental Procedures

Materials. The cross-linking reagent 3,3′-bis(sulfosuccinimido)suberate (BS^3) was purchased from Pierce Chemical Co. (Rockford, IL). [^{32}P]P_i was obtained from ICN Radiochemicals Inc., whereas Na^{125}I was from Amersham Corp. Chemicals for electrophoresis were obtained from Bio-Rad.

PDGF. All three isoforms of recombinant PDGF (PDGF AA, PDGF BB, and PDGF AB) were obtained as gifts from Dr. Lynn Baird and Dr. Mark Charette (Creative Biomolecules, Hopkinton, MA). Homodimeric PDGF AA or PDGF BB was not detected in a heterodimeric PDGF AB preparation (Lymm Baird, personal communication). PDGF BB was also obtained from Amgen Biologicals (Thousands Oak, CA).

Iodination of PDGF. PDGF AA and PDGF AB were iodinated by the Chloramine T procedure as described earlier (Bishayee et al., 1989). Both forms of ^{125}I-PDGF were homogeneous as judged by SDS–PAGE. ^{125}I-PDGF BB was obtained from Amersham Corp.

[γ-^{32}P]ATP. Labeled ATP was prepared with [^{32}P]P_i and gamma-Prep A kit (Promega Biotec, Madison, WI) according to the manufacturer's directions. The [γ-^{32}P]ATP was diluted with unlabeled ATP to a final specific activity of 40–60 Ci/mmol.

Antibodies. Antipeptide antibodies to the PDGF receptor were generated as described earlier (Bishayee et al., 1988). Antibodies AbP_1 and AbP_2 are directed to amino acid residues 977–988 (peptide 1) and 932–947 (peptide 2) of the murine β-type PDGF receptor (Yarden et al., 1986). In human β-type receptor, P_2 corresponds to amino acid residues 964–979 whereas P_1 corresponds to amino acid residues 1013–1024 with a substitution of serine to glycine at position 1017 (Gronwald et al., 1988; Claesson-Welsh et al., 1988). Both antibodies recognize human and murine PDGF receptors in immunoprecipitation and Western blotting (Bishayee et al., 1988). Antibody $AbP\alpha_1$ is directed to amino acid residues 993–1009 (peptide α_1) of the human α-type PDGF receptor (Matsui et al., 1989). The mouse monoclonal anti-phosphotyrosine antibody, 2G8, used for PDGF receptor purification was generated as described (Bishayee et al., 1986) and coupled to cyanogen bromide activated Sepharose before use.

Cells. Human osteogenic sarcoma (MG-63), glioblastoma (A172), and rhabdomyosarcoma (A204) cells were obtained from the American Type Culture Collection (Bethesda, MD) and were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Plasma membranes from these cells were prepared as described (Bishayee et al., 1986).

Autophosphorylation of the PDGF Receptor. This was done as described (Bishayee et al., 1989). Briefly, detergent-solubilized membrane extracts from cultured cells were incubated at 4 °C for 30 min with or without PDGF in 20 μL of 20 mM HEPES (pH 7.4), 0.15 M NaCl, 0.25% NP-40, 10% glycerol, 2.5 mM $MgCl_2$, 0.63 mM $MnCl_2$, 0.5 mM sodium vanadate, 1 mM PMSF, 10 μg/mL leupeptin, and 0.15 unit/mL aprotinin. Reaction was initiated by addition of 100 mM [γ-^{32}P]ATP to a final concentration of 20 μM. After 30 min at 4 °C, the reactions were terminated by the addition of EDTA to a final concentration of 10 mM, and the mixtures were processed as described in the Figure legends.

Purification of ^{32}P-Labeled PDGF Receptor Using Anti-Phosphotyrosine Antibody–Sepharose. This was carried out by using 2G8-Sepharose as described (Bishayee et al., 1989).

^{125}I-PDGF Binding to Cells. PDGF binding experiments with intact cells were performed on confluent cells in 1-cm^2 48-well plates. Cultures were incubated at 4 °C for 3 h with ^{125}I-PDGF with rocking in 100 μL of Earle's balanced salt solution containing 2.5 mg/mL bovine serum albumin (BSA) and 30 mM HEPES, pH 7.4. The cells were washed free of unbound radioactivity, solubilized with 0.5 mL of a solution of 1% Triton X-100 containing 1 mg/mL BSA, and then counted in a γ-counter. Nonspecific binding was measured by incubating the cells with ^{125}I-PDGF in the presence of 20 nM unlabeled PDGF.

Affinity Labeling and Chemical Cross-Linking of the PDGF Receptor in Intact Cells. These were done as described (Bishayee et al., 1988). Briefly, ^{125}I-PDGF was allowed to bind with confluent cultures of cells in 35-mm dishes according to the method described above. The cells were then incubated at 4 °C for 10 min with 0.5 mM BS^3. Under these conditions, ^{125}I-PDGF becomes covalently linked to the receptor. Cells were then washed, solubilized in 0.5% NP-40-containing buffer, and subjected to immunoprecipitation with anti-PDGF receptor antibodies and SDS–PAGE on nonreducing 3.5–10% gradient polyacrylamide gels. The labeled receptors were visualized by autoradiography.

Chemical Cross-Linking of ^{32}P-Labeled PDGF Receptor. This was carried out as described (Bishayee et al., 1989). Briefly, ^{32}P-labeled PDGF receptor purified by using anti-phosphotyrosine antibody–Sepharose was chemically cross-linked by incubation at 4 °C for 10 min with 50 μM BS^3. Excess cross-linker was inactivated by the addition of Tris (pH 7.5), and the samples were processed as described in the figure legends.

Immunoprecipitation of the PDGF Receptor. This was done as described (Bishayee et al., 1988) using AbP_1, AbP_2, and $AbP\alpha_1$.

SDS Gel Electrophoresis. SDS–PAGE was performed as described (Bishayee et al., 1989). Unless otherwise mentioned, the samples were run under reducing conditions on 3.5–10% gradient polyacrylamide–SDS gels.

Results

Here we examined the ability of different isoforms of PDGF

Table I: Binding of ^{125}I-Labeled PDGF AA, PDGF AB, and PDGF BB with Different Cell Lines[a]

	specific binding (per 10^5 cells) with					
	A204		MG-63		A172	
labeled ligand	cpm	ng	cpm	ng	cpm	ng
^{125}I-PDGF AA	9275	0.15	16000	0.27	ND[b]	ND
^{125}I-PDGF BB	6250	0.15	20747	0.51	6150	0.15
^{125}I-PDGF AB	11900	0.14	42500	0.51	700	0.01

[a] For PDGF binding assays, 1.26 ng of ^{125}I-PDGF AA (60×10^3 cpm/ng), 1.2 ng of ^{125}I-PDGF AB (84×10^3 cpm/ng), or 1.34 ng of ^{125}I-PDGF BB (41×10^3 cpm/ng) in a total volume of 100 μL of Earle's balanced salt solution containing 30 mM HEPES, pH 7.5, and 2.5 mg/mL BSA was incubated at 4 °C for 3 h with cells grown in 1 cm^2 48-well plates. Cell numbers per well were 60×10^3, 57×10^3, and 30×10^3, respectively, for A204, MG-63, and A172. Nonspecific binding, which was 10–15% of the total ^{125}I-PDGF AA and ^{125}I-PDGF AB and 25–30% of the total ^{125}I-PDGF BB binding, was measured by incubating the cells with ^{125}I-PDGF in the presence of 20 nM respective unlabeled PDGF. [b] Not detectable.

to bind and activate α- and β-type PDGF receptors. Receptor activation was studied by ligand-induced dimerization and autophosphorylation of the receptor. Identity of the receptor was established by the use of type-specific anti-PDGF receptor antibodies. We have previously reported the generation and characterization of two antipeptide antibodies (AbP_1 and AbP_2) to the β-type PDGF receptor. AbP_1 and AbP_2 recognize both murine and human β-type PDGF receptors in immunoprecipitation and Western blot. In the present studies, in addition to AbP_1 and AbP_2, we have also used a newly developed antipeptide antibody, $AbP\alpha_1$. $AbP\alpha_1$ is directed to amino acid residues 993–1009 (peptide α_1) of human α-type PDGF receptor. $AbP\alpha_1$ recognizes only α-type (both human and murine) and not β-type PDGF receptor; similarly, AbP_1 and AbP_2 are specific for β-type receptor, and they do not cross-react with type-α receptor (see figures).

Binding of ^{125}I-Labeled PDGF with Different Cell Lines. We have tested various cell lines for their ability to bind to different isoforms of PDGF; these binding studies were carried out with saturating concentrations of labeled PDGF. As shown in Table I, all three cell lines that we have tested, namely, human rhabdomyosarcoma (A204), osteosarcoma (MG-63), and glioblastoma (A172), were capable of specific binding to ^{125}I-PDGF BB. However, among these cell lines, only A204 and MG-63 but not A172 bound to ^{125}I-PDGF AA (Table I); in addition, this binding was competitively inhibited by an excess of unlabeled PDGF (not shown). Since PDGF AA is known to bind only to α-type and not β-type PDGF receptor, these results suggest the presence of α-type receptor in A204 and MG-63 but not in A172 cells. Our binding studies also revealed that ^{125}I-PDGF AB binds very efficiently to A204 and MG-63 cells and it binds very poorly to A172 cells (Table I).

Comparison of binding of different isoforms of PDGF with these cell lines revealed that all three isoforms bind to the same extent with A204 cells. Since PDGF BB binds to both types of PDGF receptors whereas PDGF AA binds only to α-type receptor, these results suggest that A204 expresses only α-type PDGF receptor and it lacks type β-receptor. However, in MG-63 cells, binding of PDGF AA is nearly half of that of PDGF BB, suggesting that MG-63 cells express equal numbers of α- and β-type PDGF receptors. Failure of the binding of A172 cells with PDGF AA suggests that this cell line expresses only β-type PDGF receptor.

Immunoprecipitation of Receptor-^{125}I-PDGF Covalent Complex from A204 and MG-63 Cells. To demonstrate directly that A204 cells express only α-type receptors whereas

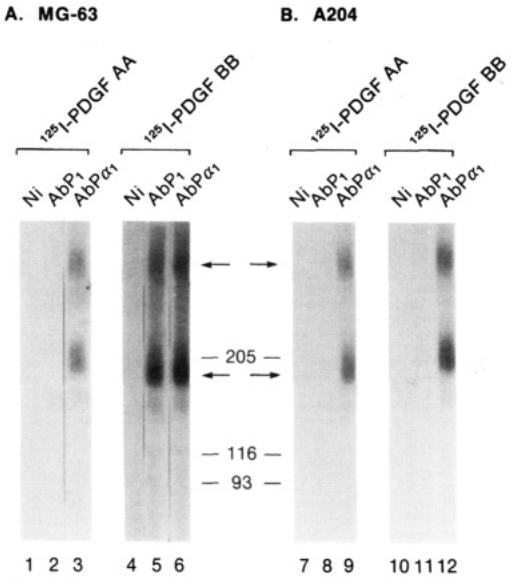

FIGURE 1: Identification of the PDGF receptor type by immunoprecipitation of receptor–^{125}I-PDGF covalent complexes. Human MG-63 and A204 cells grown in 35-mm dishes were incubated at 4 °C for 3 h with 0.2 nM ^{125}I-PDGF AA (60×10^3 cpm/ng) or 0.3 nM PDGF BB (41×10^3 cpm/ng) in Earle's balanced salt solution containing 30 mM HEPES, pH 7.5. ^{125}I-PDGF bound to the receptor was then covalently cross-linked by incubating the cells with BS^3 as described under Experimental Procedures. Aliquots of the Nonidet P-40 solubilized, clarified lysate were subjected to immunoprecipitation in the presence of 1 mM suramin with the antipeptide antibodies $AbP\alpha_1$ (specific for α-type receptor) or AbP_1 (specific for β-type receptor) or with nonimmune serum (Ni) and then subjected to SDS–PAGE under nonreducing conditions. Radioactive counts used for immunoprecipitation were as follows: 6500 and 11 000 cpm of ^{125}I-PDGF AA and ^{125}I-PDGF BB bound, respectively, to A204 cells; 5500 and 23 000 cpm of ^{125}I-PDGF AA and ^{125}I-PDGF BB bound, respectively, to MG-63 cells. The 180–200- and 370–390-kDa receptor–^{125}I-PDGF cross-linked complexes are indicated by arrows.

MG-63 cells express both α- and β-type receptors, we carried out chemical cross-linking experiments. In these experiments, cells in culture were incubated with ^{125}I-labeled PDGF, cross-linked with BS^3, and then subjected to immunoprecipitation with α- or β-receptor-specific antibodies. To prevent receptor coimmunoprecipitation, immunoprecipitations were carried out in the presence of suramin which is known to dissociate noncovalently linked PDGF receptor dimer (Bishayee et al., 1989). The immunoprecipitates were analyzed by nonreducing SDS–PAGE in 3.5–10% acrylamide gels. As shown in Figure 1, under nonreducing conditions, both ^{125}I-PDGF AA and ^{125}I-PDGF BB were found to be covalently linked with two components of 180–200 and 370–390 kDa. We have shown earlier that the slower and faster moving bands represent respectively the dimeric and monomeric forms of the PDGF receptor (Bishayee et al., 1989). When the cross-linking was carried out with ^{125}I-PDGF AA, both the bands in A204 and MG-63 were immunoprecipitated only by $AbP\alpha_1$ and not by AbP_1, suggesting that PDGF AA binds only to α-receptor.

In MG-63 cells, cross-linked complexes generated by incubation with ^{125}I-PDGF BB were recognized by $AbP\alpha_1$ as well as by AbP_1, confirming that PDGF BB interacts with both types of receptor and that MG-63 cells express α- and β-type PDGF receptors. However, in A204 cells, incubation of the cells with ^{125}I-PDGF BB resulted in the recognition of radiolabeled complexes only by $AbP\alpha_1$ and not by AbP_1, indicating that it lacks β-receptor. As expected, radiolabeled complexes generated by incubation of ^{125}I-PDGF BB with A172 cells were recognized only by AbP_1 and not by $AbP\alpha_1$

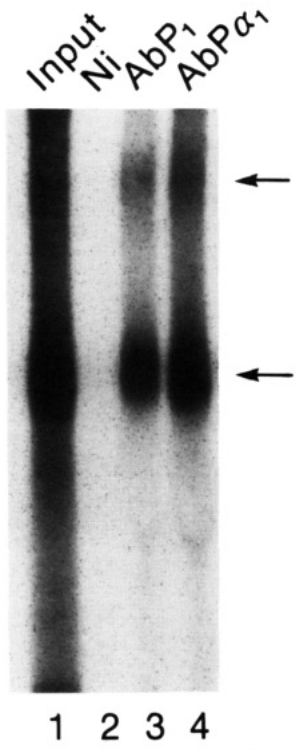

FIGURE 2: Direct evidence for the binding of ^{125}I-PDGF AB to β-type PDGF receptor in MG-63 cells. PDGF receptors in intact MG-63 cells were covalently cross-linked with ^{125}I-PDGF AB (0.4 nM; 80 $\times 10^3$ cpm/ng) and subjected to immunoprecipitation with antipeptide antibodies to AbPα_1 or AbP$_1$ or with nonimmune serum (Ni) as described in the legend to Figure 1. The immunoprecipitates were analyzed by nonreducing SDS–PAGE and autoradiography. Lane 1 (input) represents labeled sample that has not been subjected to immunoprecipitation. The 180–200- and 370–390-kDa receptor–^{125}I-PDGF AB cross-linked complexes are indicated by arrows.

(not shown). This confirms the absence of α-receptor in this cell line.

^{125}I-PDGF AB Binds to α- and β-Type Receptors in MG-63 Cells. Next we tested whether the interaction of ^{125}I-PDGF AB with MG-63 cells which express both types of PDGF receptors is due to its binding to α- as well as to β-type receptors. As described above (Table I), PDGF AB binds very poorly with A172, a cell line that lacks α-type receptor. However, the extent of binding of PDGF AB with MG-63 cells is the same as that of PDGF BB and is twice that of PDGF AA. Such results can be explained if PDGF AB binds with β-receptor only in the presence of α-receptor. As shown in Figure 2, in MG-63 cells, cross-linked complexes generated by incubation with ^{125}I-PDGF AB were recognized by AbPα_1 as well as by AbP$_1$, indicating that PDGF AB binds with both types of receptors. In A204 cells, both the higher and the lower molecular weight components generated by cross-linking with ^{125}I-PDGF AB were only recognized by AbPα_1 and not by AbP$_1$ (not shown). These results again confirm that A204 cells contain α-type and not β-type PDGF receptor.

Activation of α- and β-Type PDGF Receptors by Different Isoforms of PDGF. Availability of three different cell lines—one that contains both α- and β-type receptors (MG-63) and the other two that express either α-receptor (A204) or β-receptor (A172)—allowed us to investigate the effect of different isoforms of PDGF on the activation of receptor kinase. Receptor activation was studied by ligand-induced autophosphorylation of the receptor. For autophosphorylation assay, PDGF receptors in detergent-solubilized membrane extracts were phosphorylated with [γ-^{32}P]ATP in the presence or absence of PDGF, purified by anti-phosphotyrosine antibody, immunoprecipitated with type-specific anti-PDGF receptor antibody, and then subjected to SDS–PAGE and autoradiography. As shown in Figure 3, all three isoforms of PDGF stimulated the phosphorylation of α-type receptor in A204 cell membranes. However, only PDGF BB but not PDGF AA or AB stimulated the phosphorylation of β-receptor in A172 cell membranes.

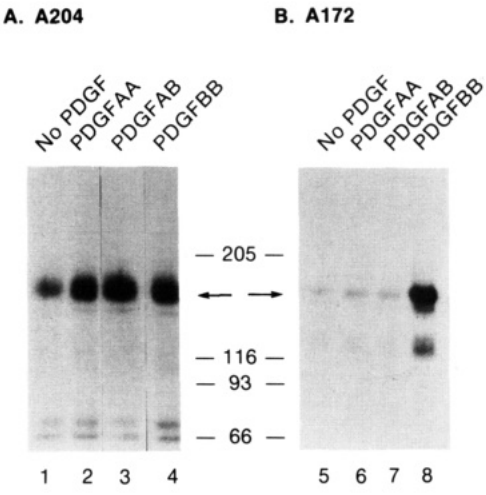

FIGURE 3: Effect of different isoforms of PDGF on the phosphorylation of its receptor in A204 and A172 cells. Detergent-solubilized A204 and A172 membrane preparations (pretreated with suramin; ~150 μg of protein) were phosphorylated with [γ-^{32}P]ATP in the presence of buffer alone (no PDGF), 30 nM PDGF AA, 10 nM PDGF AB, or 10 nM PDGF BB. After termination of the reaction with EDTA, the ^{32}P-labeled proteins were purified by using anti-phosphotyrosine antibody–Sepharose and then subjected to immunoprecipitation with AbPα_1 (for A204) or AbP$_1$ (for A172). The results of electrophoresis and autoradiography are depicted. The PDGF receptors are indicated by arrows.

We have previously shown that β-type PDGF receptor undergoes dimerization in response to PDGF. Therefore, we tested whether α-type receptor is also capable of forming dimer in a ligand-dependent fashion. To test this, PDGF receptor in detergent extracts of A204 cell membranes was phosphorylated with [γ-^{32}P]ATP in the presence of different isoforms of PDGF, and the ^{32}P-labeled receptor following purification with anti-phosphotyrosine antibody was chemically cross-linked with BS^3. Electrophoresis and autoradiography revealed the presence of a chemically cross-linked complex at 370–390 kDa. This band was absent when the receptor was not treated with BS^3 (Figure 4) or when PDGF bound to ^{32}P-labeled receptor was dissociated by incubation with suramin (not shown). This cross-linked complex of 370–390 kDa, in addition to the 180-kDa band, was recognized by AbPα_1, indicating that the 370–390-kDa complex represents α-type PDGF receptor. These results suggest that all three isoforms of PDGF that activate α-type PDGF receptor kinase are also capable of inducing dimerization of the α-type receptor. In parallel with the binding and activation of the β-receptor, only PDGF BB induces dimerization of β-type receptor in A172 cells (not shown).

In our previous studies, we reported the dimerization and activation of β-type PDGF receptor in MG-63 cells by PDGF AB (Bishayee et al., 1989). Those studies were conducted with human platelet-derived PDGF as the source of PDGF AB. Now it is known that platelet-derived PDGF is a mixture of about 70% PDGF AB and 25–30% PDGF BB (Hammecher et al., 1988). On the basis of the fact that β-type PDGF receptor in A172 cells is not activated by recombinant PDGF AB, it is possible that the observed activation of β-type receptor in MG-63 cells by human platelet-derived PDGF might be due

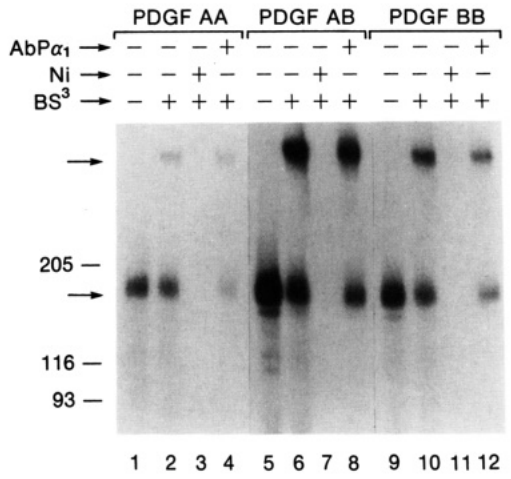

FIGURE 4: α-Type PDGF receptor undergoes dimerization in response to all three isoforms of PDGF. PDGF receptor in A204 membrane extracts was phosphorylated with [γ-^{32}P]ATP in the presence of PDGF AA (lanes 1–4), PDGF AB (lanes 5–8), or PDGF BB (lanes 9–12) under conditions as described in the legend to Figure 3. The ^{32}P-labeled PDGF receptor, purified by using anti-phosphotyrosine antibody–Sepharose, was subjected to chemical cross-linking by incubating with (lanes 2–4, 6–8, and 10–12) or without (lanes 1, 5, and 9) 50 μM BS^3 as described under Experimental Procedures. The cross-linked complexes either were directly subjected to SDS–PAGE and autoradiography (lanes 1, 2, 5, 6, 9, and 10) or were first immunoprecipitated with AbPα$_1$ (lanes 4, 8, and 12) or nonimmune serum (Ni) (lanes 3, 7, and 11) and then analyzed by SDS–PAGE and autoradiography. The 180–190- and 370–390-kDa forms of the PDGF receptor are indicated by arrows.

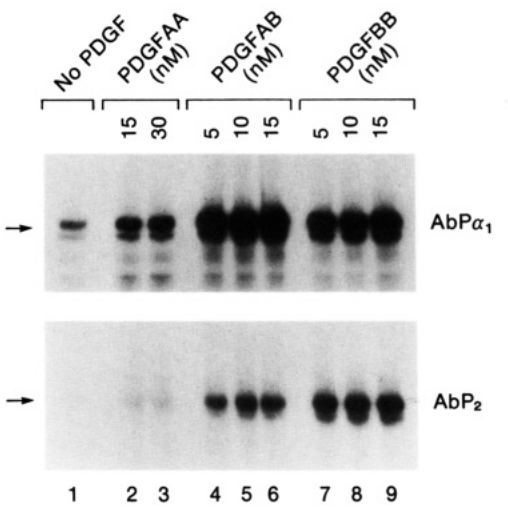

FIGURE 5: Autokinase activity of the β-type PDGF receptor in MG-63 cell membranes is stimulated by PDGF AB. Detergent-solubilized MG-63 membrane preparations (~100 μg of protein) were phosphorylated with [γ-^{32}P]ATP in the presence or absence of indicated concentrations of PDGF AA, PDGF AB, or PDGF BB. The ^{32}P-labeled PDGF receptors were purified by using antiphosphotyrosine antibody–Sepharose and then subjected to immunoprecipitation with AbPα$_1$ (upper panel) or AbP$_2$ (lower panel) in the presence of 1 mM suramin and SDS–PAGE/autoradiography. The autoradiographic exposure time for AbPα$_1$ immunoprecipitate is 2 days whereas for AbP$_2$ it is 1 day. The PDGF receptor bands are indicated by arrows.

to the presence of PDGF BB in our PDGF preparation. An alternative explanation may be that like the binding characteristics, the activation of the β-receptor in MG-63 cells by PDGF AB is influenced by the presence of α-receptor. In order to investigate this, we analyzed the phosphorylation of α- and β-type PDGF receptors in MG-63 cell membranes in response to different isoforms of PDGF. In this experiment, detergent extracts of MG-63 membranes were incubated with [γ-^{32}P]ATP in the presence or absence of PDGF, ^{32}P-labeled receptor was purified by anti-phosphotyrosine antibody, and then the type specificity of the phosphorylated receptor was identified by immunoprecipitation with AbPα$_1$ or AbP$_2$. Suramin was included during immunoprecipitation to prevent receptor–receptor interaction. The SDS–PAGE and autoradiography of the immunoprecipitates revealed that like α-receptor in A204 cells, phosphorylation of α-receptor in MG-63 cells could be stimulated by all three forms of PDGF (Figure 5, top panel). Similarly, β-receptor in MG-63 cells, like its counterpart in A172 cells, could be activated by PDGF BB but not by PDGF AA (Figure 5, bottom panel). However, there are considerable differences as to the effect of PDGF AB in inducing phosphorylation of β-receptor in MG-63 and A172 cells. PDGF AB is as effective as PDGF BB in inducing phosphorylation of β-receptor in MG-63 cells (Figure 5, bottom panel); in contrast, PDGF AB has no effect on the activation of β-receptor in A172 cells (see Figure 3). The activation of β-receptor by PDGF AB is not limited to MG-63 cells. In fact, β-receptor in any cell type that expresses both types of PDGF receptors (such as NIH 3T3, T 131) is activated by PDGF AB (not shown). This strengthens the possibility of direct involvement of α-receptor in the process of activation of β-receptor by PDGF AB.

Stimulation of Autokinase Activity of β-Type PDGF Receptor by PDGF AB Is Due to the Formation of Heterodimeric Receptor. To test whether phosphorylation of β-receptor by PDGF AB in MG-63 membranes is associated with dimerization of the receptor, we followed the association state of the receptor by chemical cross-linking analysis. In this experiment, PDGF receptors in detergent extracts of MG-63 membranes were phosphorylated with [γ-^{32}P]ATP in the presence of PDGF AB. Under these conditions, both receptor types are expected to be phosphorylated. Following purification with anti-phosphotyrosine antibody, phosphorylated receptors were cross-linked with BS^3 and then each receptor type was identified by immunoprecipitation with AbPα$_1$ and AbP$_2$ in the presence of suramin. Electrophoresis and autoradiography of the immunoprecipitates revealed that both AbPα$_1$ and AbP$_2$ immunoprecipitated 180–190- and 370–390-kDa bands in receptor preparations that were subjected to cross-linking with BS^3 (Figure 6). This suggests that 370–390-kDa bands in AbPα$_1$ and AbP$_2$ immunoprecipitates contain α- and β-type PDGF receptors, respectively.

Since α-receptor is obligatory for PDGF AB induced dimerization and activation of the β-receptor, we next tested whether 370–390-kDa cross-linked complex immunoprecipitated by AbP$_2$ represents α–β heterodimer. In order to investigate this, PDGF AB stimulated ^{32}P-labeled PDGF receptor from MG-63 membrane extracts was cross-linked with BS^3 and then incubated with AbPα$_1$; the receptor–AbPα$_1$ immune complex was isolated by the use of protein A–Sepharose. Under these conditions, in addition to α-receptor, β-receptor covalently cross-linked with α-receptor is also expected to be immunoprecipitated by AbPα$_1$. To test whether β-receptor is present in AbPα$_1$ immunoprecipitates, the PDGF receptor was dissociated from AbPα$_1$ by incubating the immunoprecipitate with excess α$_1$ peptide, and then the supernatant containing PDGF receptors was subjected to a second immunoprecipitation with AbP$_2$. As shown in Figure 7, 370–390-kDa cross-linked complex was precipitated by AbP$_2$, and the extent of immunoprecipitation was dependent on the amount of antibody. This cross-linked complex was absent when a second immunoprecipitation was carried out with nonimmune serum (Figure 7, lane 2) or with AbP$_2$ in the presence of excess peptide 2 (Figure 7, lane 3). It should be

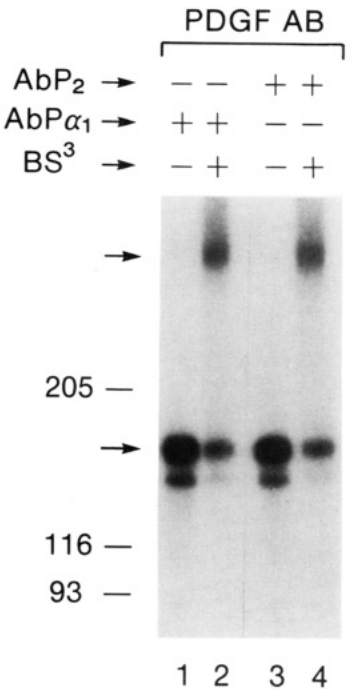

FIGURE 6: PDGF AB induces dimerization of both α- and β-type PDGF receptors in MG-63 cells. Detergent-solubilized membrane extracts from MG-63 cells ($\sim$100 μg of protein) were incubated with [γ-^{32}P]ATP in the presence of 10 nM PDGF AB. The ^{32}P-labeled PDGF receptors were purified by using anti-phosphotyrosine antibody–Sepharose and then incubated with (lanes 2 and 4) or without (lanes 1 and 3) 50 μM BS^3. The cross-linked complexes were then immunoprecipitated in the presence of 1 mM suramin with AbPα_1 (lanes 1 and 2) or AbP$_2$ (lanes 3 and 4) and subjected to SDS–PAGE/autoradiography.

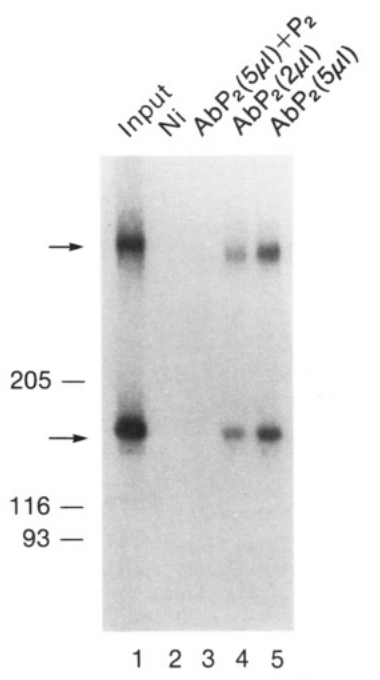

FIGURE 7: Evidence for the presence of β-receptor in PDGF AB induced dimeric forms of the α-receptor in MG-63 cells. The PDGF receptors in MG-63 membrane extracts were labeled with [γ-^{32}P]ATP in the presence of 10 nM PDGF AB, purified by anti-phosphotyrosine antibody–Sepharose, and then subjected to chemical cross-linking with BS^3. To separate the ^{32}P-labeled α-receptor from other proteins, the cross-linked complexes were incubated with AbPα_1, and the immune complexes were isolated by using protein A–Sepharose as described under Experimental Procedures. The α-receptors as well as other proteins that were associated with the receptor were then dissociated from the immune complex by incubating the protein A–Sepharose-bound radioactive complex at room temperature for 3 h with 50 μg/mL α_1 peptide. Equal aliquots of Pα_1-dissociated reaction mixture were immunoprecipitated with nonimmune serum (Ni, lane 2) or with β-receptor-specific antibody, AbP$_2$, either in the absence (lanes 4, 5) or in the presence (lane 3) of 25 μg/mL peptide 2 and subjected to SDS–PAGE/autoradiography. Lane 1 (input) represents α_1 peptide dissociated sample that has not been subjected to immunoprecipitation. Dimeric and monomeric forms of the PDGF receptors are indicated by arrows.

noted that in addition to 370–390-kDa complex, a second immunoprecipitation by AbP$_2$ resulted also in the appearance of a band at 180–190 kDa (Figure 7). This band probably represents the noncovalently linked form of the α–β heterodimer in which BS^3 failed to create interreceptor cross-links (note that cross-linking is not quantitative). This possibility is supported by the fact that when the first immunoprecipitation by AbPα_1 was carried out in the presence of suramin, which prevents dimer formation, this 180–190-kDa band could not be seen when the subsequent immunoprecipitation was carried out with AbP$_2$ (not shown). All these results put together suggest that β-receptor forms a α–β heterodimer in the presence of PDGF AB. PDGF BB but not PDGF AA also induces heterodimer formation (not shown). However PDGF BB induced heterodimer formation is much less in comparison to that of PDGF AB.

Discussion

Two types of PDGF receptors have been cloned and sequenced (Yarden et al., 1986; Gronwald et al., 1988; Claesson-Welsh et al., 1988; Matsui et al., 1989). In certain respects, α- and β-type PDGF receptors have identical structural and functional characteristics, whereas with respect to other properties they differ considerably. For example, in both receptors, a single membrane-spanning segment separates the extracellular ligand-binding domain from the intracellular tyrosine kinase site. Moreover, the kinase domain of both receptors is interrupted by a span of about 100 amino acids (kinase insert domain). With the type-β receptor, it is known that the ligand-stimulated kinase activity and the kinase insert domain are required for mitogenic activity [reviewed in Williams (1989)]. With the α-receptor, it remains to be tested whether these sites are needed for activity. Aside from these similarities, α- and β-receptors have overall sequence identity at the amino acid level of only 43% with maximum sequence homology (80%) at two kinase domains and least homology (27%) at the kinase insert domain. It is not yet known whether these two receptor types differ in the signal transduction pathway. However, recent studies indicate that they may differ in substrate specificity (Welsh et al., 1990); in addition, actin reorganization is thought to be mediated by β-receptor only (Hammacher et al., 1989).

In the present studies, we have established the ligand-binding specificity of two receptor types and extended it further to investigate the ligand-induced association state of the α-receptor and the role of α-receptor in the activation of the β-receptor. There is considerable difference in our approach with those of others. Most of the studies conducted so far by others were done with cells that express both types of receptors in varying proportions, and conclusions on ligand-binding specificity were drawn based on cross-competition studies (Hart et al., 1988; Heldin et al., 1988). However, in our investigation, we have used cells that express one or the other PDGF receptor type. Moreover, ligand-binding characteristics of the receptors were established by immunoprecipitation of radiolabeled ligand–receptor covalent complex with type-specific PDGF receptor antibodies. In these studies, we have used rhabdomyosarcoma cells (A204) and glioblastoma cells (A172)

which are known to express only α- and β-type PDGF receptor messages, respectively (Matsui et al., 1989). In addition, we have also used human osteogenic sarcoma cells (MG-63). These cells are known to express β-type receptors (Bishayee et al., 1988, 1989). Our present studies reveal that MG-63 cells also express α-receptor (Table I, Figures 1 and 2). Our results can be summarized as follows: (i) α-receptor binds to all three isoforms of PDGF, and such binding leads to dimerization and activation of the α-receptor (Table I, Figures 1, 3, and 4); (ii) β-receptor binds with high affinity only to PDGF BB and not to PDGF AA or AB, and this leads to dimerization and activation of the β-receptor (Table I, Figure 3); (iii) in mixed population of receptors, PDGF AB binds to both receptor types with high affinity, and such binding leads to activation of α-receptor as well as β-receptor (Figure 5).

One of our significant findings is the direct demonstration of the influence of α-receptor in PDGF AB induced activation of β-receptor. Now the question is how α-receptor stimulates the β-receptor kinase. We have directly demonstrated that in the presence of α-receptor, the affinity of PDGF AB for β-receptor is increased several hundredfold; in addition, we have shown by the double immunoprecipitation technique using α- and β-receptor-specific antibodies the formation of α–β heterodimer in the presence of PDGF AB (Figure 7). On the basis of these findings, we propose the following model. In a mixed population of receptors, the A chain of PDGF AB binds with α-receptor whereas the B chain binds with either α- or β-receptor. Such binding results in the formation of PDGF AB induced α–α homodimer or α–β heterodimer with concomitant activation of receptor kinases. On the basis of indirect evidence, others have also proposed a similar model (Hammacher et al., 1989; Seifert et al., 1989). This PDGF AB induced activation of β-receptor kinase strongly supports our earlier conclusion that dimerization of the PDGF receptor is obligatory for the activation of the kinase.

PDGF AB has very low affinity for β-receptor; however, in the presence of α-receptor, it displays high-affinity binding that results in the formation of α–β heterodimer. This suggests that the ligand–receptor monovalent complex is easily dissociable but is stabilized by dimer formation. This opens up the question as to the significance of receptor dimerization in the activation of kinase, for example, whether the kinase activation is due to the stabilization of the ligand–receptor complex by dimer formation or due to a conformational change in the receptor by receptor–receptor interaction. Future studies with inhibitors of PDGF receptor kinase may elucidate the role of interreceptor interaction in kinase activation.

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Registry No. PDGF receptor kinase, 101463-26-7.

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