Type-Specific Antibodies to the Platelet-Derived Growth Factor Receptors: Role in Elucidating the Structural and Functional Characteristics of Receptor Types†

Sanjiv Raj,‡ Palanisamy Kanakaraj,‡ Shabbir A. Khan,§ and Subal Bishayee*,‡

Coriell Institute for Medical Research, 401 Haddon Avenue, Camden, New Jersey 08103, and The Wistar Institute, 36th and Spruce Streets, Philadelphia, Pennsylvania 19104

Received August 23, 1991; Revised Manuscript Received October 17, 1991

ABSTRACT: Two types of platelet-derived growth factor receptors have been cloned and sequenced. Both are glycoproteins with similar molecular weights. We have earlier established the ligand binding specificity, ligand-induced dimerization, and kinase activation of these two receptor types [Bishayee et al. (1989) *J. Biol. Chem. 264*, 11699–11705; Kanakaraj et al. (1991) *Biochemistry 30*, 1761–1767]. In the present studies, we have investigated the biosynthesis, processing, and glycosylation of the α-receptor and compared its structural and functional characteristics to those of the β-receptor. Unlike an anti-peptide antibody, AbP_2 (amino acid residues 964–979), to the human β-receptor which detects a phosphorylation-specific conformation of the receptor, an antibody, $AbP\alpha_2$ (amino acid residues 956–971), to the corresponding region of the human α-receptor failed to do so. However, our studies revealed that the stability of the α-receptor is comparable to that of the β-receptor. In addition, N-linked glycosylation of the α-receptor, like that of the β-receptor, is not important in kinase activation. We have exploited the lack of an effect of N-linked oligosaccharides on the functioning of the α-receptor to develop a simple and rapid method for direct demonstration of ligand-induced noncovalently linked α–β-receptor heterodimer formation. This method is based on the interaction between functionally active short and the long forms of two receptor types which can be resolved by denaturing gel electrophoresis.

Platelet-derived growth factor (PDGF)[1] is a family of disulfide-bonded dimeric proteins that consist of two highly homologous but nonidentical polypeptides, known as A- and B-chains. PDGF is a mitogen for cells of mesenchymal origin and is capable of transforming cells in culture [reviewed in Ross et al., (1986) and Heldin and Westermark (1990)]. The B-chain of PDGF is a product of the c–sis proto-oncogene (Waterfield et al., 1983; Doolittle et al., 1983). The mitogenic and transforming activities of PDGF are mediated through its interactions with high-affinity cell-surface receptors. Two types of PDGF receptor genes, an α-receptor (Matsui et al., 1989; Claesson-Welsh et al., 1989; Lee et al., 1990) and a β-receptor (Yarden et al., 1986; Gronwald et al., 1988; Claesson-Welsh et al., 1988), have been cloned and sequenced. These two receptors have similar structural features; e.g., in both receptor types, a single transmembrane domain separates the extracellular ligand binding domain from the intracellular tyrosine kinase domain. Interestingly, the kinase site in both receptor types is interrupted by a span of about 100 amino acids. This kinase insert domain of the α- and β-receptors is obligatory for mitogenesis [Heidaran et al., 1991; reviewed in Williams (1989)]. One of the early consequences of interaction between PDGF and its receptor is the formation of receptor dimers (Seifert et al., 1989; Heldin et al., 1989; Bishayee et al., 1989; Hammacher et al., 1989; Ueno et al., 1991). We have recently shown that, like the β-receptor, the α-type PDGF receptor also undergoes ligand-induced dimerization and such dimerization results in kinase activation leading to autophosphorylation (Kanakaraj et al., 1991). Apart from these similarities, α- and β-receptors differ considerably in a number of ways. For example, these receptors have only 43% overall sequence homology at the amino acid level with maximum sequence identity (80%) at two kinase domains and least identity (27%) at the kinase insert domain (Matsui et al., 1989). In addition, these receptors have different ligand binding characteristics; α-receptor binds to all three isoforms of PDGF whereas β-receptor binds only to PDGF BB (Hart et al., 1988; Nister et al., 1988; Heldin et al., 1988; Seifert et al., 1989; Kanakaraj et al., 1991). Moreover, actin reorganization is thought to be mediated by the β-receptor only (Hammacher et al., 1989).

Since the α-receptor has only recently been identified, it is relatively uncharacterized. In addition, its signal transduction pathway remains to be elucidated. For the β-type receptor, we and others have previously identified an epitope, P_2 (amino acid residues 964–979 of the human receptor), whose conformation is highly susceptible to the phosphorylation state of the receptor (Bishayee et al., 1988; Keating et al., 1988). It remains to be established whether the α-receptor also undergoes similar autophosphorylation-induced conformational changes.

In the present studies, we have characterized the α-receptor with respect to its biosynthesis, processing, and glycosylation, and compared its properties with those of the β-receptor. Our results indicate that the biological half-life of the α-receptor is comparable to that of the the β-receptor. In addition, using an anti-peptide antibody, we have identified an epitope in the

† This work was supported by National Institutes of Health Grants CA 44441 (to S.B.) and CA 47983 (to S.B. and S.A.K.) and by grants from the Council for Tobacco Research and the W. W. Smith Charitable Trust (both to S.B.).

* Address correspondence to this author.
‡ Coriell Institute for Medical Research.
§ The Wistar Institute.

[1] Abbreviations: DMEM, Dulbecco's modified Eagle's medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NP-40, Nonidet P-40; PAGE, polyacrylamide gel electrophoresis; PDGF, platelet-derived growth factor; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dedecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/92/0431-1774$03.00/0

α-receptor whose conformation appears to differ from the corresponding epitope (P_2) in the β-receptor. Furthermore, we show that, like the β-receptor, the α-receptor lacking N-linked oligosaccharides is functionally active. We have used the PDGF receptor lacking N-linked oligosaccharides to develop a simple and rapid method to study the heterologous interaction between two receptor types. Such a method will be useful in investigating the mechanism of kinase activation and receptor autophosphorylation.

Materials and Methods

Materials. Tunicamycin was obtained from Sigma Chemical Co. (St. Louis, MO). [^{32}P]P_i and Tran ^{35}S-label were purchased from ICN Pharmaceuticals Inc. Chemicals for electrophoresis were obtained from Bio-Rad.

PDGF. All three isoforms of PDGF were gifts of Drs. Marc Charette and Lynn Baird (Creative Biomolecules, Hopkinton, MA). Homodimeric PDGF AA or PDGF BB was not detected in PDGF AB preparation (Kanakaraj et al., 1991). PDGF BB was also obtained from Amgen Biologicals (Thousands Oak, CA).

[γ-^{32}P]ATP. Labeled ATP was prepared with [^{32}P]P_i and the 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 specific activity of 40–60 Ci/mmol (Bishayee et al., 1988).

Antibodies. Anti-peptide antibodies AbP_1 and AbP_2 are directed to amino acid residues 977–988 (peptide 1) and 932–947 (peptide 2) of the cytoplasmic domain of the murine β-type PDGF receptor (Yarden et al., 1986). In the 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 single amino acid substitution (serine to glycine at position 1017) (Gronwald et al., 1988; Claesson-Welsh et al., 1988). Antibodies $AbP\alpha_1$ and $AbP\alpha_2$ are directed respectively to amino acid residues 993–1009 (peptide α_1) and 956–971 (peptide α_2) of the cytoplasmic domain of the human α-type PDGF receptor (Matsui et al., 1989). All these antibodies recognize human and murine PDGF receptors in immunoprecipitation and Western blotting (Bishayee et al., 1988). These anti-peptide antibodies were generated as described earlier (Bishayee et al., 1988). The monoclonal anti-phosphotyrosine antibody, 2G8, was generated as described (Bishayee et al., 1986).

Cells. The human astrocytoma cell line (A1690) was a gift of Dr. Stuart Aaronson (NIH), whereas all other cell lines were obtained from the American Type Culture Collection (Rockville, MD). These cells were grown in either RPMI 1640 (for A1690) or Dulbecco's modified Eagles medium (DMEM) (for all other cell lines) supplemented with 10% fetal bovine serum. Plasma membranes from these cells were prepared as described elsewhere (Bishayee et al., 1986).

Isolation of Endoplasmic Reticulum from Tunicamycin-Treated Cells. Confluent cultures of cells grown in p150 plates were incubated at 37 °C for 18 h with 1 μg/mL tunicamycin in 2% fetal bovine serum supplemented medium. Under these conditions, the newly synthesized PDGF receptor lacking N-linked oligosaccharides is associated with the endoplasmic reticulum. After the cells were scraped off the plates, they were washed twice with 10 mM HEPES (pH 7.4)–0.15 M NaCl and then homogenized with 10 mM HEPES (pH 7.4)–0.25 M sucrose–1 mM PMSF–10 μg/mL leupeptin–0.15 unit/mL aprotinin in a Dounce homogenizer. Nuclear and mitochondrial fractions were removed by successive centrifugations at 1000 rpm for 10 min and at 10000 rpm for 30 min, respectively, in an SS34 rotor at 4 °C in Sorvall RC 5B centrifuge. Supernatant from the 10000 rpm spin was further centrifuged at 100000*g* for 1 h to isolate microsomal fraction. This fraction also contains endoplasmic reticulum. Microsomal fraction was suspended in 10 mM HEPES (pH 7.4)–0.15 M NaCl–1 mM PMSF and stored at −70 °C.

Metabolic Labeling of Cells. This was carried out as described (Bishayee et al., 1988) with some modifications. Briefly, MG-63 cells were seeded onto 2 cm^2 24-well plates in DMEM containing 10% fetal bovine serum. After 48 h when cells reached confluency, cultures were washed with methionine- and cysteine-free DMEM containing 2% dialyzed platelet-poor plasma. Cells were then incubated at 37 °C for the indicated time periods with Tran ^{35}S-label (100 μCi/mL, 1100 Ci/mmol) in the same medium. After cells were labeled, they either were solubilized with 1% NP-40 in 20 mM HEPES (pH 7.4)–0.15 M NaCl–protease inhibitors or were washed twice and incubated at 37 °C in fresh unlabeled methionine- and cysteine-supplemented DMEM containing 2% dialyzed platelet-poor plasma. The clarified soluble extracts after centrifugation were immunoprecipitated with the indicated antibody.

Autophosphorylation of the PDGF Receptor. Unless otherwise indicated, ligand-induced autophosphorylation was carried out using PDGF BB according to the method described earlier (Bishayee et al., 1989). The tyrosine-phosphorylated receptor was purified using anti-phosphotyrosine monoclonal antibody, 2G8, coupled to Sepharose.

Immunoprecipitation of the PDGF Receptor. This was done as described using α- and β-receptor-specific antibodies (Bishayee et al., 1988).

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

Results and Discussion

Biosynthesis of the α-Type PDGF Receptor. We have studied the biosynthesis of the α-type PDGF receptor in a human osteogenic sarcoma cell line, MG-63. We and others have shown earlier that MG-63 cells express both α- and β-type PDGF receptors in approximately equal proportions (Seifert et al., 1989; Kanakaraj et al., 1991).

To study the biosynthesis of the receptor, cells were labeled with Tran ^{35}S-label and chased with unlabeled methionine- and cysteine-supplemented medium, and then the receptor was immunoprecipitated with $AbP\alpha_1$, an anti-peptide antibody directed to amino acid residues 993–1009 of the human α-type PDGF receptor (Matsui et al., 1989). Immunoprecipitation was carried out in the presence of suramin which prevents interreceptor interaction (Bishayee et al., 1989; Kanakaraj et al., 1991). SDS–PAGE followed by autoradiography revealed that, after a 15-min pulse, all of the receptor was in the 155-kDa form (Figure 1, bottom arrow) and following the chase this form persisted up to 30 min. Between 30 and 60 min, most of the 155-kDa form was converted to the 170-kDa form, and by 2 h, almost all of the receptor was in the 170-kDa form. This suggests a precursor–product relationship between 155- and 170-kDa forms. It should be noted that like the α-receptor, the β-type PDGF receptor is synthesized as a 160-kDa form and between 30 and 60 min it is also converted to a high molecular mass form (180 kDa) (Keating & Williams, 1987).

In the experiment depicted in Figure 1, in addition to the 155- and 170-kDa forms of the α-type PDGF receptor, minor proteins mostly with molecular masses less than 150 kDa could also be detected. These bands were also seen when the ^{35}S-

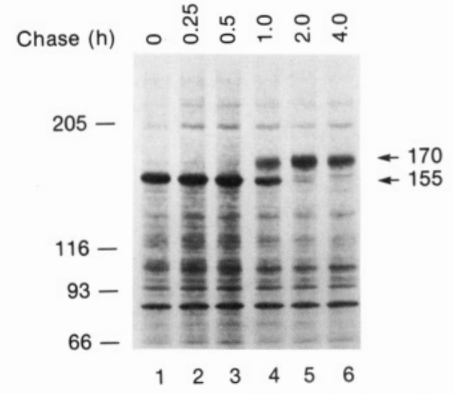

FIGURE 1: α-Type PDGF receptor biosynthesis. MG-63 cells in 2 cm^2 24-well plate were labeled with Tran ^{35}S-label for 15 min and then chased with methionine- and cysteine-supplemented DMEM containing 2% platelet-poor plasma for the indicated lengths of time. NP-40-clarified extracts were immunoprecipitated with the α-receptor-specific antibody Ab$P\alpha_1$ in the presence of suramin as described under Materials and Methods. 155- and 170-kDa forms of the α-receptor are indicated by arrows.

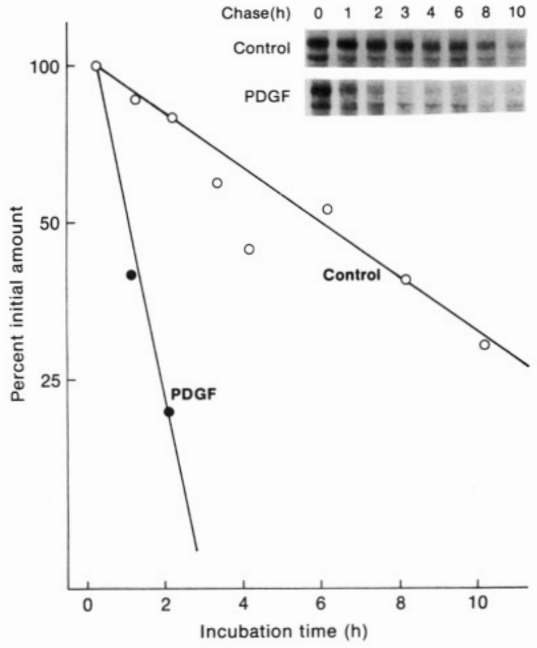

FIGURE 2: Effect of PDGF on the stability of the α-type receptor. MG-63 cells were labeled with Tran ^{35}S-label for 2 h and then chased with unlabeled methionine- and cysteine-supplemented DMEM containing 2% platelet-poor plasma in the absence (○) or presence (●) of 6 nM PDGF AB for the indicated times. The NP-40 cell lysates were subjected to immunoprecipitation with Ab$P\alpha_1$ in the presence of suramin and then SDS–PAGE and fluorography. The region containing the 170-kDa α-receptor was densitometrically scanned. Similar densitometric scans were performed for samples treated with nonimmune serum. The plot represents the results of the densitometric scan after subtraction of the nonimmune values.

labeled cell lysates were incubated with nonimmune serum instead of Ab$P\alpha_1$ (data not shown), indicating that these proteins are not related to the α-type PDGF receptor.

To determine the half-life of the α-receptor and the effect of PDGF on the stability of the receptor, MG-63 cells were pulsed with Tran ^{35}S-label for 2 h and then chased with unlabeled methionine/cysteine in the presence or absence of 6 nM PDGF AB for the indicated times up to 10 h (Figure 2). There was a gradual decrease in the amount of immunoprecipitable 170-kDa α-receptor with increasing chase time, and this process of receptor loss was accelerated by the addition of PDGF AB (Figure 2, insert). Similar results were also seen

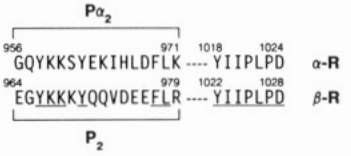

FIGURE 3: Amino acid sequences of Pα_2 and P_2 and their respective locations in the α- and β-receptors in relation to a seven amino acid long common sequence. Identical amino acids in both peptides are underlined.

when PDGF AA (50 nM) or PDGF BB (6 nM) was used during chase (data not shown). A semilog plot of the data obtained by scanning of the autoradiogram revealed that the α-type PDGF receptor has a half-life of 6–7 h and in the presence of PDGF it is reduced to about 1 h (Figure 2). An examination of the total protein ^{35}S radioactivity during the chase period indicated a loss of about 10% after 10 h of chase, and the overall loss was unaffected by PDGF (data not shown). Our studies indicate that the turnover rate of the α-type receptor is similar to that of the β-receptor which has a half-life of about 3 h in both murine and human cells (Keating & Williams, 1987; Bishayee et al., 1988).

Nonphosphorylated Form of α-Receptor Is Recognized by Ab$P\alpha_2$. In our earlier studies, we characterized an anti-peptide antibody, AbP_2, directed to amino acid residues 932–947 of the cytoplasmic domain of the murine β-type PDGF receptor; this corresponds to amino acid residues 964–979 of the human β-type PDGF receptor. AbP_2 is a novel conformation-specific antibody. This antibody specifically recognizes only the tyrosine-phosphorylated β-type PDGF receptor and not the unphosphorylated native receptor (Bishayee et al., 1988). A similar observation has also been reported by others (Keating et al., 1988).

To investigate whether an antibody corresponding to the homologous region in the α-type PDGF receptor can also detect a phosphorylation-specific conformation, we studied the characteristics of the antibody Ab$P\alpha_2$. This antibody is directed to amino acid residues 956–971 of the human α-type PDGF receptor. Although P_2 and Pα_2 have very little sequence homology, these two epitopes are probably located at the same site in the β- and α-type PDGF receptors. This is based on the following facts: (i) Both peptides are 16 amino acids long and contain 2 tyrosine residues at the third and seventh positions from the N-terminus end (Figure 3); (ii) in P_2, these 2 tyrosine residues are separated by 3 lysine residues whereas in Pα_2, only 1 (at position 6) out of 3 lysine residues is replaced by serine; (iii) the 2 penultimate amino acids at the C-terminus end in both peptides are same. In addition, there is a span of seven amino acids near the C-terminus end of both receptor types (amino acid residues 1018–1024 and 1022–1028 of the α- and β-type receptors, respectively) which are identical in sequence. These sequences are 48 and 44 amino acids downstream of Pα_2 and P_2, respectively.

Our studies revealed that, unlike AbP_2, Ab$P\alpha_2$ is capable of recognizing both the unphosphorylated and tyrosine-phosphorylated forms of the α-receptor in Mg-63 cells to the same extent (data not shown). This has been demonstrated by the immunoprecipitation of the ^{35}S-label α-type PDGF receptor from untreated control preparation as well as from the receptor preparation that had been incubated with PDGF and ATP. This suggests that the Pα_2 epitope in the native unphosphorylated α-receptor, unlike the corresponding P_2 epitope, is not cryptic and hence it is accessible to the antibody. This implies that the α- and β-type PDGF receptors probably differ in their conformation at least with respect to one epitope.

N-Linked Glycosylation Has No Effect on the α-Type

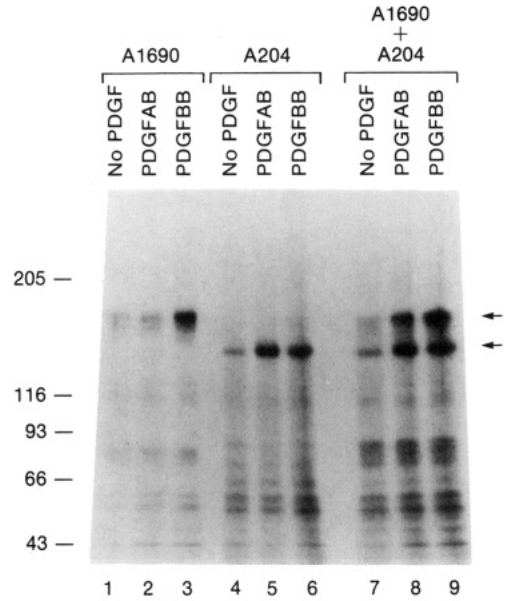

FIGURE 4: Short form of the α-receptor synthesized in the presence of tunicamycin retains its ligand-induced autokinase activity as well as its capability to activate β-receptor kinase in a PDGF AB dependent fashion. Detergent extracts of the plasma membrane fraction from A1690 (15 μg of protein) and the microsomal fraction from tunicamycin-treated A204 (7.5 μg of protein) were incubated alone (lanes 1–6) or in combination (lanes 7–9) at 4 °C for 15 min with [γ-^{32}P]ATP either in the absence (lanes 1, 4, and 7) or in the presence of PDGE AB (lanes 2, 5, and 8) or PDGF BB (lanes 3, 6, and 9). Following termination of the reaction with EDTA (10 mM), tyrosine-phosphorylated PDGF receptors were purified using anti-phosphotyrosine antibody and then subjected to SDS–PAGE/autoradiography. The short form of the α-receptor and the long form of the β-receptor are indicated by arrows.

PDGF Receptor. α-Type PDGF receptor binds to wheat germ agglutinin (unpublished observation), a lectin specific for *N*-acetylglucosamine. In addition, it also binds to Lens culinaris lectin (Claesson-Welsh et al., 1989), a mannose-specific lectin. These suggest the glycoprotein nature of the α-receptor. Since *N*-acetylglucosamine and mannose are present only in N-linked oligosaccharides [reviewed in Osawa and Tsuji (1987)], this implies that the α-receptor, like the β-receptor, contains N-linked sugar residues. To investigate whether the N-linked glycosylation is obligatory for the α-receptor activity, we isolated the microsomal fraction, which also contains the endoplasmic reticulum (site of N-linked glycosylation) from tunicamycin-treated A204 cells. We and others have shown earlier that A204 expresses only α-type PDGF receptor (Matsui et al., 1989; Kanakaraj et al., 1991). Since these cells secrete PDGF (unpublished observation), the α-type PDGF receptors in A204 will have a shorter half-life. Thus, after 18 h of tunicamycin treatment, there will be no fully glycosylated receptors in these cells. Western blot analysis using the α-receptor-specific antibody (AbPα_1) revealed the presence of only 140-kDa PDGF receptor in the microsomal fraction and not in the nuclear or mitochondrial fraction (data not shown). This 140-kDa receptor does not bind to wheat germ agglutinin (data not shown). To test whether the tunicamycin-treated α-type PDGF receptor has ligand-induced autokinase activity, a detergent-solubilized microsomal fraction was phosphorylated with [γ-^{32}P]ATP either in the absence or in the presence of PDGF; tyrosine-phosphorylated proteins were purified using anti-phosphotyrosine antibody and then subjected to SDS–polyacrylamide gel electrophoresis/autoradiography. As shown in Figure 4, lanes 4–6, like the 170–180-kDa long form, the phosphorylation of the 140-kDa receptor is stimulated by both PDGF AB and PDGF BB. In addition, phosphorylation was also stimulated by PDGF AA (data not shown). The identity of the 140-kDa phosphorylated protein as α-type receptor was confirmed by the immunoprecipitation of the protein by α-receptor-specific antibodies. This suggests that like the β-receptor (Keating et al., 1989), the ligand-induced autokinase activity of the α-receptor is independent of N-linked glycosylation.

Direct Demonstration of the Interaction between Two Types of PDGF Receptors. Studies from different laboratories including ours have suggested that the activation of the β-type receptor by PDGF AB is due to the heterologous interaction between α- and β-type PDGF receptors (Seifert et al., 1989; Hammacher et al., 1989; Kanakaraj et al., 1991; Kelly et al., 1991). In our studies, receptor heterodimer formation was demonstrated by isolating the chemically cross-linked α–β dimer using a complex multistep and hence time-consuming process that involved the use of α- and β-receptor-specific antibodies (Kanakaraj et al., 1991). Such a multistep process was necessitated since both the α- and β-type PDGF receptors have similar molecular weights. However, generation of the functionally active short 140-kDa form of the PDGF receptor by tunicamycin treatment allowed us to establish a simple and rapid method to investigate the heterologous interaction between two receptor types with two different molecular weights. In these experiments, we studied the interaction between the 140-kDa α-receptor from tunicamycin-treated A204 rhabdomyosarcoma cells and the 180-kDa β-receptor from A1690 astrocytoma cells. A1690 cells express mostly β-receptor and very little α-receptor messages (Matsui et al., 1989). In addition, receptor autokinase activity in A1690 membranes was stimulated only by PDGF BB and not by PDGF AB (Figure 4, lanes 2 and 3), suggesting that A1690 lacks the α-receptor. It should be mentioned that PDGF AB can activate β-type PDGF receptor only in the presence of the α-type receptor.

Next we investigated whether the short form of the α-receptor from tunicamycin-treated cells, like the fully glycosylated receptor, is capable of activating the β-receptor in a ligand-dependent fashion and also whether these two receptor types could be resolved by SDS–PAGE. In these experiments, the detergent-solubilized plasma membrane fraction from A1690 and the microsomal fraction from A204 were mixed together and incubated with [γ-^{32}P]ATP either in the absence or in the presence of PDGF, and then the ^{32}P-labeled receptors were purified using immobilized anti-phosphotyrosine antibody. SDS–PAGE and autoradiography revealed that in mixed population of receptors, the phosphorylation of the 140-kDa α-receptor and the 180-kDa β-receptor was stimulated in response to both PDGF AB and PDGF BB (Figure 4, lanes 7–9). Stimulation of autokinase activity of the β-receptor by PDGF AB in the presence of the short form of the α-receptor suggests that aglyco-α-receptor is as effective as the fully glycosylated receptor in inducing β-receptor kinase.

Next we attempted to isolate the noncovalently linked α–β-receptor heterodimer by subjecting the ^{32}P-labeled receptors, generated as above, to immunoprecipitation with AbPα_1, an α-receptor-specific antibody. Under these conditions, in addition to the 140-kDa α-receptor, the 180-kDa β-receptor noncovalently linked to the α-receptor is also expected to be immunoprecipitated by AbPα_1 provided the α–β-receptor complex is stable. As shown in Figure 5, lane 1, AbPα_1 immunoprecipitated both the 140-kDa α-receptor and also the 180-kDa β-receptor when receptor phosphorylation was stimulated by PDGF AB. When suramin, which prevents receptor dimer formation, was present during immunoprecipitation, the 180-kDa β-receptor could not be seen (Figure 5, lane 2).

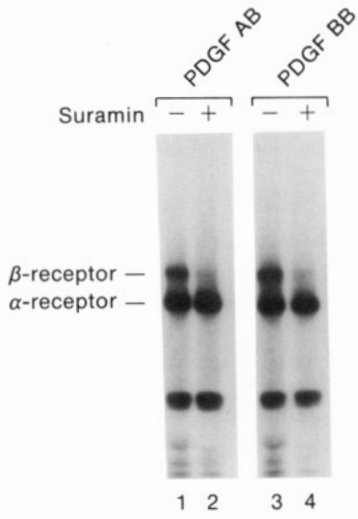

FIGURE 5: Effect of suramin on the co-immunoprecipitation of ^{32}P-labeled β-receptor formed in the presence of the short form of the α-receptor by α-receptor-specific antibody. Tyrosine-phosphorylated ^{32}P-labeled PDGF receptors formed in the presence of PDGF AB or PDGF BB (lanes 8 and 9 of Figure 4) were immunoprecipitated with Ab$P\alpha_1$ either in the absence (lanes 1 and 3) or in the presence (lanes 2 and 4) of 1 mM suramin, and subjected to SDS–PAGE. α- and β-receptor bands are indicated.

Similar results were also obtained when receptor phosphorylation was stimulated by PDGF BB, indicating that under these conditions, in addition to α–α and β–β homodimers, a fraction of the receptors was present as α–β heterodimer (Figure 5, lanes 3 and 4). Both these receptor bands were also seen when immunoprecipitation was carried out with the β-receptor-specific antibody AbP_1; furthermore, addition of suramin during immunoprecipitation resulted in the disappearance of the 140-kDa α-receptor band (data not shown). All these results put together not only confirm the formation of the α–β-receptor heterodimer under appropriate conditions but also point to the fact that the present method is simple and rapid in studying interreceptor heterologous interaction. This method will be useful in investigating how receptor dimerization is associated with kinase activation and whether autophosphorylation is an inter- or intrapeptide event.

Detection of the noncovalently linked receptor heterodimer by co-immunoprecipitation suggests that the α–β-receptor complex is stable. In some experiments, ^{32}P-labeled PDGF receptors were kept at 4 °C up to 8 h before immunoprecipitation, and this did not result in any loss of the relative intensities of the two receptor bands. In earlier studies, the noncovalently linked β–β homodimeric receptors were isolated using either high-pressure liquid chromatography or sucrose density gradient centrifugation which took 4–8 h to complete (Heldin et al., 1989; Bishayee et al., 1989; Ueno et al., 1991). This suggests that the α–β-receptor dimer is as stable as the β–β homodimer. However, this conclusion differs from that of Kelly et al. (1991). Using a monoclonal antibody to the β-receptor (PR7212), this group failed to detect the α–β-receptor heterodimer and concluded that the α–β heterodimer is less stable compared to β–β homodimer. At present we do not know the apparent reasons for this discrepancy; however, it is possible that the affinity of the monoclonal antibody PR7212, which is directed to an extracellular domain of the β-receptor (Hart et al., 1987), is reduced when the β-receptor is complexed with the α-receptor and this may result in the decreased immunoprecipitation of the α–β-receptor heterodimer.

Acknowledgments

S.B. dedicates this paper to the memory of his long-standing collaborator, friend, and wife Dr. Manjusri Das. We thank Drs. Marc Charette and Lynn Baird for providing us with all three isoforms of PDGF.

Registry No. PDGF, 101463-26-7.

References

Bishayee, S., Ross, A. H., Womer, R., & Scher, C. D. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 6756–6760.

Bishayee, S., Majumdar, S., Scher, C. D., & Khan, S. (1988) *Mol. Cell. Biol. 8*, 3696–3702.

Bishayee, S., Majumdar, S., Khire J., & Das, M. (1989) *J. Biol. Chem. 264*, 11699–11705.

Claesson-Welsh, L., Eriksson, A., Moren, A., Severinsson, L., EK, B., Betsholtz, C., & Heldin, C. H. (1988) *Mol. Cell. Biol. 8*, 3476–3486.

Claesson-Welsh, L., Eriksson, A., Westermark, B., & Heldin, C. H. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 4917–4921.

Doolittle, R. F., Hunkapiller, M. W., Hood, L. E., Devare, S. G., Robbins, K. C., Aaronson, S. A., & Antoniades, H. N. (1983) *Science 221*, 275–277.

Gronwald, R. G. K., Grant, F. J., Haldeman, B. A., Hart, C. E., O'Hara, P. J., Hagen, F. S., Ross, R., Bowen-Pope, D. F., & Murry, M. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 3435–3439.

Hammacher, A., Mellstrom, K., Heldin, C. H., & Westermark, B. (1989) *EMBO J. 8*, 2489–2495.

Hart, C. E., Seifert, R. A., Ross, R., & Bowen-Pope, D. F. (1987) *J. Biol. Chem. 262*, 10780–10785.

Hart, C. E., Forstrom, J. W., Kelly, J. D., Seifert, R. A., Smith, R. A., Ross, R., Murry, M. J., & Bowen-Pope, D. F. (1988) *Science 140*, 1529–1531.

Heidaran, M. A., Pierce, J. H., Lombardi, D., Ruggiero, M., Gutkind, J. S., Matsui, T., & Aaronson, S. A. (1991) *Mol. Cell. Biol. 11*, 134–142.

Heldin, C. H., & Westermark, B. (1990) *Cell Regul. 1*, 555–566.

Heldin, C. H., Backstrom, G., Ostman, A., Hammacher, A., Ronnstrand, L., Rubin, K., Nister, M., & Westermark, B. (1988) *EMBO J. 7*, 1387–1393.

Heldin, C. H., Ernlund, A., Rorsman, C., & Ronnstrand, L. (1989) *J. Biol. Chem. 264*, 8905–8912.

Kanakaraj, P., Raj, S., Khan, S. A., & Bishayee, S. (1991) *Biochemistry 30*, 1761–1767.

Keating, M. T., & Williams, L. T. (1987) *J. Biol. Chem. 262*, 7932–7937.

Keating, M. T., Escobedo, J. A., & Williams, L. T. (1988) *J. Biol. Chem. 163*, 12805–12808.

Keating, M. T., Harryman, C. C., & Williams, L. T. (1989) *J. Biol. Chem. 264*, 9129–9132.

Kelly, J. D., Haldeman, B. A., Grant, F. J., Murry, M. J., Seifert, R. A., Bowen-Pope, D. F., Cooper, J. A., & Kazlauskas, A. (1991) *J. Biol. Chem. 266*, 8987–8992.

Lee, K. H., Bowen-Pope, D. F., & Reed, R. R. (1990) *Mol. Cell. Biol. 10*, 2237–2246.

Matsui, T., Heidaran, M., Miki, T., Popescu, N., LaRochelle, W., Kraus, M., Pierce, J., & Aaronson, S. A. (1989) *Science 243*, 800–804.

Nister, M., Hammacher, A., Mellstrom, K., Siegbahn, A., Ronnstrand, L., Westermark, B., & Heldin, C. H. (1988) *Cell 52*, 791–799.

Osawa, T., & Tsuji, T. (1987) *Annu. Rev. Biochem. 56*, 21–42.

Ross, R., Raines, E. W., & Bowen-Pope, D. F. (1986) *Cell 46*, 155–169.

Seifert, R. A., Hart, C. E., Phillips, P. E., Forstrom, J. W., Ross, R., Murry, M. J., & Bowen-Pope, D. F. (1989) *J. Biol. Chem. 64*, 8771–8778.
Ueno, H., Colbert, H., Escobedo, J. A., & Williams, L. T. (1991) *Science 151*, 844–848.
Waterfield, M. D., Scrace, G. F., Whittle, N., Stroobant, P., Johnsson, A., Wasteson, A., Westermark, B., Heldin, C. H., Huang, J. S., & Deuel, T. F. (1983) *Nature (London) 304*, 35–39.
Williams, L. T. (1989) *Science 243*, 1564–1570.
Yarden, Y., Escobedo, J. A., Kuang, W. J., Yang-Feng, T. L., Daniel, T. O., Tremble, P. M., Chen, E. Y., Ando, M. E., Harkins, R. N., Francke, U., Fried, V. A., Ullrich, A., & Williams, L. T. (1986) *Nature (London) 323*, 226–232.

Distances between the Antigen-Binding Sites of Three Murine Antibody Subclasses Measured Using Neutron and X-ray Scattering†

T. R. Sosnick, D. C. Benjamin,‡ J. Novotny,§ P. A. Seeger, and J. Trewhella*

Life Sciences Division and LANSCE, Los Alamos National Laboratory, Los Alamos, New Mexico 87545, Department of Microbiology, University of Virginia, Charlottesville, Virginia 22908, and Department of Macromolecular Modeling, Bristol-Myers Squibb Research Institute, Princeton, New Jersey 08543

Received August 29, 1991; Revised Manuscript Received November 13, 1991

ABSTRACT: For three different murine immunoglobulins (IgG subclasses 1, 2a, and 2b), the distances between their antigen-binding sites have been measured using neutron scattering from deuterated antigens complexed with proteated IgG. Neutron-scattering data were measured for each antibody–antigen complex in a 41% D_2O solvent. Unlike the proteated antibody molecule, the deuterated antigens are strongly contrasted against the 41% D_2O solvent and give rise to a scattering profile that contains an interference term related to the distance between the deuterated antigens. For all three subclasses, the damping of this interference term, which gives information on the relative flexibility of the antigen-binding sites, indicates that a single distance is inadequate to describe the observed scattering and a distribution of distances is needed. The scattering profile has been modeled for each subclass to give the mean distance between the antigens and the variance of this distance. For all three IgG subclasses, the mean distance is between 117 and 134 Å, and the variance is large (≈40 Å), indicating a high degree of flexibility of the F_{ab} arms. Small-angle X-ray scattering measurements on the same samples are consistent with the neutron-scattering results.

Antibodies are protein products of the immune response which specifically recognize and bind to the foreign substance (antigen) which induced their synthesis. The antibody molecule is divided into two functional regions: the F_{ab} portion which contains the antigen-binding site and the non-antigen-binding F_c region. The totality of antigenic substances presents an almost infinite surface variability. Therefore, the immune system must be capable of responding by producing an almost limitless variety of antigen-binding sites. In contrast, a very limited number of classes and subclasses of antibody molecules have evolved, each of which is capable of performing one or more F_c region mediated biological functions such as the fixation of complement leading to lysis and/or phagocytosis of the offending foreign substance; interaction with F_c receptors on mast or phagocytic cells, leading to the release of pharmacologically active agents or phagocytosis, respectively; and transport across epithelial surfaces, including the intestine or the placenta providing maternal protection against infection.

Over 70% of the antibody in serum is of the IgG class. This class can be visualized as a "Y" or "T" shaped molecule having 2-fold symmetry with two identical antigen-binding sites, one at each end of two identical arms, or F_{ab} regions. The two F_{ab} regions are joined to an F_c region at a flexible hinge region (Noelken et al., 1965; Valentine & Green, 1967; Alpert et al., 1985; Reidler et al., 1982; Oi et al., 1984; Dangl et al., 1988). High-resolution crystallographic data exist on portions and fragments of IgG as well as hinge deleted IgG's [reviewed by Davies et al. (1990), Alzari et al. (1988), Davies and Metzger (1983), and Marquart and Deisenhofer (1982)]. However, information about the complete structure of functional IgG's is lacking. Detailed computer models of entire human IgG molecules incorporating the amino acid sequence of the hinge region of each subclass have been presented (Pumphrey, 1986), but there is little empirical evidence to support or refute these composite models. Small-angle X-ray and neutron solution scattering studies have been performed on human (Cser et al., 1976; Pilz et al., 1977; Kilar et al., 1985) and pig (Cser et al., 1978, 1981a) antibodies, but, due to the inherent problems of spherical averaging, it has not been possible to determine unique structures. Cser et al. (1978) used neutron solvent matching techniques to measure explicitly the distance between two antibody-bound antigens. The scattered neutron intensity from a molecule is proportional to the difference or contrast between the mean neutron-scattering length density of the molecule and that of the solvent. In the experiment of Cser et al. (1978), polyclonal pig serum IgG complexed with a

† This work was performed under the auspices of the DOE (Contract W-7405-ENG-36) and is supported by DOE/Office of Health and Environmental Research project KP-04-01-00-0 (J.T.) and National Institutes of Health Grant AI20745 (D.C.B.). This work has benefited from the use of facilities at the Manuel Lujan Jr. Neutron Scattering Center, a national user facility funded as such by DOE/Office of Basic Energy Sciences.

* Address correspondence to this author at Life Sciences Division, Los Alamos National Laboratory.
‡ University of Virginia.
§ Bristol-Myers Squibb Research Institute.

0006-2960/92/0431-1779$03.00/0