Role of Immunoglobulin-like Domains 2–4 of the Platelet-derived Growth Factor α-Receptor in Ligand-Receptor Complex Assembly*

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Platelet-derived growth factor (PDGF) is a dimeric protein that exerts its effects through tyrosine kinase α- and β-receptors. The extracellular part of each receptor is composed of five Ig-like domains. Recombinant forms of α-receptor domains 1–4 (αRD1–4), 1–3 (αRD1–3), and 1 and 2 (αRD1–2) were prepared after expression in Chinese hamster ovary cells and were used to study the assembly of soluble ligand-receptor complexes. When incubated with micromolar concentrations of PDGF, both αRD1–3 and αRD1–4 formed complexes of 1:2 molar composition, i.e. one dimeric PDGF molecule bound two soluble receptors. αRD1–3, in contrast to αRD1–4, formed detectable 1:1 complexes under conditions of ligand excess. αRD1–4 displayed an increased ability to form 1:2 complexes as compared with αRD1–3 under conditions of limiting concentrations of ligand. We thus conclude that Ig-like domain 4-mediated receptor-receptor interactions contribute to 1:2 PDGF·αRD1–4 complex formation. Since αRD1–4 and αRD1–3 were equipotent in blocking binding of subnanomolar concentrations of PDGF to cell-surface receptors, we also conclude that this effect is predominantly achieved through formation of Ig-like domain 4-independent 1:1 ligand-receptor complexes. Finally, since αRD1–2 bound PDGF-BB with high affinity, whereas PDGF-AA was bound only with low affinity, we conclude that Ig-like domain 3 of the PDGF α-receptor contains epitopes of particular importance for PDGF-AA binding and that most of the PDGF-BB-binding epitopes reside in Ig-like domains 1 and 2.

Platelet-derived growth factors (PDGFs)† are a family of disulfide-bonded dimeric isoforms of A- and B-chains with potent mitogenic activity on connective tissue cells, glia cells, and endothelial cells (reviewed in Ref. 1). PDGF has been implicated in a number of diseases involving proliferation of PDGF-responsive cells, such as atherosclerosis, restenosis, glomerulonephritis, and certain malignancies (2).

PDGF A- and B-chains, which have ~60% identical amino acid sequences in their mature parts, form homo- and heterodimers that exert their cellular effects through two structurally related tyrosine kinase receptors, denoted α- and β-receptors (3). The A-chain binds only α-receptors, whereas the B-chain binds both α- and β-receptors.

Crystallographic analysis of PDGF-BB revealed that the two subunits are arranged in an antiparallel manner (4). Each subunit consists of a tight cystine knot motif from which two loops (loops 1 and 3) point in one direction and one loop (loop 2) points in the other direction. As a consequence of the antiparallel arrangement of the dimer, loops 1 and 3 of one subunit are juxtaposed to loop 2 of the other subunit. Mutational analysis has mapped the receptor-binding amino acid residues mainly to loops 1 and 3, but loop 2 also contributes to some extent (4–8). The dimeric PDGF molecule thus displays two receptor-binding regions, each one made up of epitopes derived from both subunits.

Both PDGF α- and β-receptors consist of an extracellular part composed of five Ig-like domains, a single transmembrane region, and an intracellular split tyrosine kinase domain (9, 10). The receptors are activated by ligand-induced dimerization (11, 12). Soluble forms of the extracellular parts of the PDGF α- and β-receptors undergo ligand-dependent dimerization, and functional bivalency of PDGF has been demonstrated, suggesting the formation of a 1:2 ligand-receptor complex (13–15). Ligand-binding regions of the α-receptor have been mapped to Ig-like domains 1–3 by analysis of deletion mutants, αβ-receptor chimeras, and soluble receptor fragments (16–18). More recently, evidence has been presented suggesting that PDGF-induced receptor dimerization not only involves ligand-receptor interactions, but also receptor-receptor interactions mediated by Ig-like domain 4 (18–20).

To further study the structural basis for PDGF-induced receptor dimerization, the properties of CHO cell-derived recombinant proteins consisting of PDGF α-receptor Ig-like domains 1–4 (αRD1–4), 1–3 (αRD1–3), and 1 and 2 (αRD1–2) were compared. Using these receptor fragments, we demonstrate that αRD1–4 at micromolar concentrations, in contrast to αRD1–3, forms a 1:2 ligand-receptor complex also under conditions of ligand excess. We show that this property is a consequence of Ig-like domain 4-mediated receptor-receptor interactions. We also show that these receptor-receptor interactions do not contribute significantly to the inhibitory effect of soluble receptors on binding of subnanomolar concentrations of PDGF to cell-surface receptors. Finally, characterization of the PDGF binding properties of αRD1–2 demonstrates that Ig-like domain 3 of the PDGF α-receptor contains epitopes of particular importance for PDGF-AA binding and that most of the PDGF-BB-binding epitopes are localized within the αRD1–2 fragment.

MATERIALS AND METHODS

Establishment of a CHO Cell Line Expressing αRD1–4·GST—A polynucleotide chain reaction product spanning amino acids 1–419 of the

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† The abbreviations used are: PDGFs, platelet-derived growth factors; CHO, Chinese hamster ovary; αRD1–4, αRD1–3, and αRD1–2; PDGF α-receptor Ig-like domains 1–4, 1–3, and 1 and 2, respectively; GST, glutathione S-transferase; PIPES, 1,4-piperazinediethanesulfonic acid.

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PDGF α-receptor flanked by XhoI sites was generated. The fragment was cloned into the pcScript SK+ vector (Stratagene) and sequenced by the dyeoxy chain termination method. The fragment was excised by XhoI and cloned into the SalI site of the pMT2SM-GST vector (21), a gift from Dr. M. F. Geibink (Netherlands Cancer Institute). CHO CHO Chinese hamster ovary cells were grown in exponential phase on a rotary shaker at 37 °C with 70% humidity. After 2 days and then cultured in serum-free RDF medium for 3 days. Serum-free conditioned medium (3 liters) was filtered through a mesh 50 μm filter, supplemented with 990 mg/liter glutamine, 10 mM HEPES, pH 7.4, and 200 mg/liter proline, 100,000 units/liter penicillin, 100 mg/liter streptomycin, and 50 μg/liter gentamycin in the presence of 10% fetal calf serum (FCS).

When cultured in roller bottles with 150 ml of medium, this clone secreted αPDGF at the thrombin cleavage site in the linker between the PDGF-encoding part by a thrombin cleavage recognition sequence (amino acids 347–351), and the third localized to the region between the part encoding the PDGF-α-receptor extracellular domain, the plasmid was transfected into CHO(dhfr−) cells, and after selection in increasing concentrations of methotrexate, a clone that secreted ~1 μg of αRD1–4-GST/ml/24 h was isolated. The second unique αRD1–4 peptide had a sequence of ILSWK (amino acids 347–351), and the third localized to the region between the two other peptides. Inspection of the amino acid sequence of the PDGF α-receptor revealed that the presence of PPPRIS at positions 343–347, which fits with the optimum sequence for thrombin cleavage (40).

During transfection, cells were grown in Ham’s F-12 medium containing 10% fetal calf serum and 0.4 mg/ml G418. G418-resistant clones were screened for secretion of αRD1–4-GST by an immunoprecipitation-based assay using 125I-labeled PDGF-BB and GST antiserum (22). After the first screening, the best cell line (clone 2–4) was maintained in Dulbecco’s modified Eagle’s medium containing 10% dialeyzed fetal calf serum (Hyclone Laboratories) and increasing concentrations of methotrexate (ICN Biomedicals Inc.) to obtain clones with higher expression of the recombinant protein. Clone C5–2, which was obtained after selection under 30 μM methotrexate, was used for large-scale expression.

Production and Purification of Recombinant Proteins—CHO cells expressing αRD1–4-GST (clone C5–2) were expanded in 18 roller bottles in the presence of 100 μM methotrexate. After the cells reached confluency, they were cultured in RDM medium (2:1:1 RPMI 1640 medium/Dulbecco’s modified essential medium/Ham’s F-12 medium) supplemented with 990 mg/liter glutamine, 10 mM HEPES, pH 7.4, 200 mg/liter proline, 100,000 units/liter penicillin, 100 mg/liter streptomycin, and 50 μg/liter gentamycin in the presence of 10% fetal calf serum for 2 days and then cultured in serum-free RDM medium for 3 days. Serum-free conditioned medium (3 liters) was filtered through a mesh 50 μm filter to remove cell debris and applied to a 2.5 × 6-cm Q-Sepharose column (Amersham Pharmacia Biotech). After washing the column with 600 ml of phosphate-buffered saline, the bound protein was eluted in 0.35 M NaCl and 10 mM sodium phosphate, pH 7.4. The crude protein preparation thus obtained was incubated with 750 μg of thioglycose-Sepharose (Amersham Pharmacia Biotech) for 16 h at 4 °C. The gel was then washed with 20 ml of binding buffer and further washed with 10 ml of thrombin cleavage buffer (50 mM Tris-HCl, pH 8.0, 0.15 μM NaCl, and 10 mM CaCl2). The protein bound on the gel was digested by 100 units of bovine thrombin (Sigma) in a total volume of 2.5 ml at 37 °C for 3 h. Cleaved protein was eluted by washing with cleavage buffer; concentrated with a Centricron 50 (Amicon, Inc.) to 800 μl, and applied to a Superdex 200 (16/60) column in 0.5 mM NaCl and 20 mM Tris-HCl, pH 7.5. The fraction eluted between 72 and 78 ml contained predominantly a 63-kDa protein (αRD1–3) and a small amount of the 80-kDa protein. A pure preparation of the 63-kDa protein was obtained by repeated gel permeation chromatography on the Superdex 200 column. To prepare the lysyl endopeptidase fragment, the 80-kDa protein (30 mg) was dialyzed against 0.1 mM Tris-HCl, pH 8.5, and concentrated with the Amicon 50 to 10 μM. The protein was then treated with Acrhomobacter lysii endopeptidase (100:1, w/w; Wako Bioproducts) at 37 °C for 16 h. The digestion was terminated by addition of 1 mM phenylmethylsulfonyl fluoride. The digest was applied to a Superdex 200 (16/60) column in 0.5 mM NaCl and 20 mM Tris-HCl, pH 7.5. The gel permeation chromatography resulted in a pure fraction of a 52–55-kDa protein (αRD1–4) eluting from 74 to 78 ml contained predominantly a 63-kDa protein (αRD1–3) and a small amount of the 80-kDa protein. A pure preparation of the 63-kDa protein was obtained by repeated gel permeation chromatography on the Superdex 200 column. To prepare the lysyl endopeptidase fragment, the 80-kDa protein (30 mg) was dialyzed against 0.1 mM Tris-HCl, pH 8.5, and concentrated with the Amicon 50 to 10 μM. The protein was then treated with Acrhomobacter lysii endopeptidase (100:1, w/w; Wako Bioproducts) at 37 °C for 16 h. The digestion was terminated by addition of 1 mM phenylmethylsulfonyl fluoride. The digest was applied to a Superdex 200 (16/60) column in 0.5 mM NaCl and 20 mM Tris-HCl, pH 7.5. The gel permeation chromatography resulted in a pure fraction of a 52–55-kDa protein (αRD1–4) eluting from 74 to 78 ml.

Purity of the proteins was assessed by SDS gel electrophoresis. The concentration of the purified proteins was determined by amino acid analysis.iodination of αRD1–3 and αRD1–4 was performed using the chloramine-T method (24).

Identification of the 80-, 63-, and 55-kDa Proteins as αRD1–4, αRD1–3, and αRD1–2, Respectively—The 80- and 63-kDa proteins were subjected to amino-terminal amino acid sequencing on an Applied Biosystems Model 49A peptide sequencer after gel electrophoresis and transfer to polyvinylidene difluoride membrane. In both cases, amino termini were blocked, suggesting that the amino terminus of each peptide is common, most likely located at Gln-24, which is the most conserved Gln residue of known PDGF α-receptors.

To locate the carboxyl terminus of the 80- and 63-kDa proteins, a tryptic peptide fragment was applied to a narrow-bore aRPC CPC C2/C18 SC 2.1/10 column was performed as described previously (23). After comparison of peptide maps, three peptides unique for the 80-kDa protein were selected for sequencing. One peptide was of the sequence YLVPR, corresponding to the expected carboxyl terminus of αRD1–4 and cleavage of αRD1–4-GST at the thrombin cleavage site in the linker between αRD1–4 and GST (see Fig. 1). No peptides corresponding to GST sequences were found. The second unique αRD1–4 peptide had a sequence of ILSWK (amino acids 347–351), and the third localized to the region between the two other peptides. Inspection of the amino acid sequence of the PDGF α-receptor revealed that the presence of PPPRIS at positions 343–347, which fits with the optimum sequence for thrombin cleavage (40).
with molecular masses of 80 and 63 kDa (data not shown). Pure fractions of the two forms were obtained by pooling the early and late eluting peak fractions separately and subjecting them to rechromatography (Fig. 2A). Sequencing of internal peptides and analysis of amino acid composition were used to structurally characterize the two proteins (see "Material and Methods" for details). From this analysis, it was concluded that the 80-kDa form was generated through cleavage at the linker sequence between the PDGF \( \alpha \)-receptor and GST and that the 63-kDa component was formed through thrombin cleavage at Arg-346 (Fig. 1). We thus conclude that the 80-kDa protein corresponds to Ig-like domains 1–4 of the PDGF \( \alpha \)-receptor and that the 63-kDa form is a carboxyl-terminally truncated form that is lacking most of Ig-like domain 4. The two proteins will hereafter be referred to as \( \alpha \)RD1–4 and \( \alpha \)RD1–3, respectively.

To obtain a fragment corresponding to Ig-like domains 1 and 2, we took advantage of the absence of any lysine residues between positions 37 and 194 in the PDGF \( \alpha \)-receptor. \( \alpha \)RD1–4 was subjected to cleavage by \( \text{Achromobacter} \) lysyl endopeptidase, and after gel permeation chromatography, a fraction of a 52–55-kDa protein was obtained (Fig. 2B). Since amino acid analysis indicated the presence of 1.5–2 mol of lysine/mol of protein, we concluded that the preparation was composed predominantly of a protein encompassing amino acid residues 24–194 with some contribution of a protein cleaved at Lys-36 and thus encompassing amino acid residues 37–194. Amino acid residues 24–194 correspond approximately to Ig-like domains 1 and 2 of the PDGF \( \alpha \)-receptor, and the preparation will hereafter be referred to as \( \alpha \)RD1–2 (Fig. 1).

\( \alpha \)RD1–4 Forms a 1:2 Complex with PDGF Also under Conditions of Ligand Excess—The complete extracellular region of the PDGF \( \alpha \)-receptor forms ligand-dependent dimers (14). To investigate if this was also a property of \( \alpha \)RD1–4, the protein was subjected to analyses, in the absence or presence of PDGF-BB, by dynamic light scattering, gel chromatography, and native gel electrophoresis.

As shown in Table I, addition of ligand to \( \alpha \)RD1–4 shifted the apparent molecular mass from 110 to 240 kDa, as analyzed by dynamic light scattering. When analyzed by gel permeation chromatography, \( \alpha \)RD1–4 eluted as a 130-kDa protein in the absence of PDGF-BB. In the presence of PDGF-BB, the peak shifted to an elution position corresponding to 260 kDa (Fig. 3A). SDS gel electrophoresis of the proteins of the 260-kDa peak confirmed the presence of PDGF-BB in this complex (Fig. 3B). Thus, we conclude that Ig-like domain 5 is not required for the formation of a stable ligand-receptor complex.
To determine the stoichiometry of the PDGF-BB-αRD1–4 complex, titration experiments were performed with a fixed receptor concentration of 50 μM and ligand/receptor ratios ranging between 0.06:1 and 4:1. Complex formation was assayed by a shift in mobility during electrophoresis under native conditions on agarose (Fig. 4A) and polyacrylamide (Fig. 4B) gels. At a 0.5:1 molar ratio of PDGF-BB and receptor, all ligand and receptor occurred in complex, confirming that the complex is composed of one PDGF dimer and two receptors. We also analyzed the complex formation under conditions of ligand excess. Interestingly, neither in the electrophoresis assays (Fig. 4) nor during gel chromatography (Fig. 3A) was there any evidence for 1:1 complexes under conditions of ligand excess, indicating that formation of 1:2 complexes is favored under these experimental conditions.

The size of αRD1–4 was estimated to 80 kDa by SDS gel electrophoresis. The predicted size of a complex composed of one PDGF molecule (30 kDa) and two receptors would thus be 190 kDa, which is somewhat lower than the sizes determined by both dynamic light scattering and gel chromatography. However, also the monomeric free receptor gave higher estimated values in the gel chromatography and dynamic light scattering assays than in SDS gel electrophoresis, most likely reflecting an extended structure of αRD1–4.

Ig-like Domain 4 Interactions Mediate Stability of 1:2 Complexes under Conditions of Ligand Excess—We have recently demonstrated that Ig-like domain 4-mediated receptor-receptor interactions contribute to PDGF-induced dimerization of receptors in intact cells (20). To investigate how Ig-like domain 4 is involved in this process, we compared αRD1–4 and αRD1–3 with regard to formation of ligand-receptor complexes.

In the complex forming assay using electrophoresis on agarose gels, αRD1–3 was found to form a complex of slower mobility after ligand addition (Fig. 5A, lanes 5–7). The slower migrating component was deduced to be a 1:2 PDGF-BB-αRD1–3 complex since all PDGF-BB and αRD1–3 were complexed at a 0.5:1 ligand/receptor molar ratio. Interestingly, in parallel to a decrease in the amount of the 1:2 complex, a novel slowly migrating component appeared with a mobility in between those of the 1:2 complex and the free receptor.
ligand, most likely representing a 1:1 ligand-receptor complex at conditions of ligand excess (Fig. 5A, lane 8). To confirm that the slowly migrating complex represented an αRD1–3-containing species rather than tailing PDGF, an experiment was performed using tracer amounts of 125I-labeled receptors. Free receptor and receptor complexes were detected by a phosphoimager (Fig. 5B). As in Fig. 5A, the addition of excess ligand did not lead to the appearance of any novel receptor-containing species in the case of αRD1–4 (Fig. 5B, lane 4); however, a more slowly migrating receptor-containing species was seen when PDGF and αRD1–3 were mixed at a 4:1 molar ratio (Fig. 5B, lane 8). We thus conclude that the preferential formation of 1:2 ligand-receptor complexes also under conditions of ligand excess is favored by the presence of Ig-like domain 4.

Ig-like Domain 4 Contributes to Complex-forming Ability at Micromolar Concentrations, but Not to PDGF-neutralizing Activity at Nanomolar Concentrations—To investigate if Ig-like domain 4 quantitatively contributes to the ability to form 1:2 ligand-receptor complexes, αRD1–4 and αRD1–3 were compared with regard to their abilities to form 1:2 ligand-receptor complexes under conditions of limiting amounts of ligand and using lower concentrations of receptor than in previous experiments (Fig. 6, A and B). In native acrylamide electrophoresis, the migratory positions of the 1:2 PDGF-BB-αRD1–4 complexes (Fig. 6A, lane 2) were well separated from the migratory positions of the 1:2 PDGF-BB-αRD1–3 complexes (lane 4). When αRD1–4 and αRD1–3, at concentrations of 12.5 μM, were mixed with limiting concentrations of PDGF-BB, complexes between PDGF and αRD1–4 were preferentially formed at the expense of PDGF-αRD1–3 complexes (Fig. 6A, lanes 6–8). Similar results were obtained when the formation of PDGF-AAreceptor complexes was analyzed (data not shown). Further evidence for an involvement of Ig-like domain 4 in the formation of soluble 1:2 ligand-receptor complexes was obtained when the fraction of free and complexed receptors was analyzed after incubation of 3 μM αRD1–3 and αRD1–4, with tracer amounts of 125I-labeled receptor, together with various concentrations of PDGF-BB (Fig. 6B). At 0.37 μM PDGF, 17% of αRD1–4 occurred as a ligand complex (Fig. 6B, lane 2), whereas <5% of αRD1–3 was present in complex with PDGF under the same conditions (lane 6). The reason that complete complex formation is not observed at a 1:2 ligand/receptor ratio in this experiment, in contrast to what was observed in Fig. 4, 5 and 6A, is most likely that lower receptor concentrations were used.

αRD1–4 and αRD1–3 were also analyzed for their abilities to neutralize the binding of subnanomolar concentrations of 125I-PDGF-AA to cell-surface receptors (Fig. 7). When mixed with 0.16 nM 125I-PDGF-AA, both proteins reduced, in a dose-dependent manner, the binding of 125I-PDGF-AA to cell-surface receptors. Half-maximal competition was observed at 30 and 100 nM for αRD1–3 and αRD1–4, respectively. Similar results were obtained with 125I-PDGF-BB (data not shown). Thus, Ig-like domain 4-mediated receptor-receptor interactions do not contribute significantly to the ability to sequester ligand in this concentration range.
aRD1–2 Binds PDGF-BB, but Not PDGF-AA—To further localize the region(s) within aRD1–3 that mediates ligand binding, we investigated the properties of aRD1–2 in complex forming assays and in cell-surface receptor binding inhibition assays (Figs. 8 and 9). The receptor fragment was mixed with PDGF-AA and PDGF-BB at a 1:2 ligand/receptor molar ratio, and the presence of complex was analyzed by native polyacrylamide gel electrophoresis (Fig. 8). When mixed with PDGF-BB, almost all of aRD1–2 appeared in complex with ligand (lane 6). In contrast, after mixing aRD1–2 with PDGF-AA, most of the receptor remained as free receptor (lane 5).

In cell-surface binding inhibition assays, inhibition of 125I-PDGF-AA binding to cell-surface PDGF-α-receptors was observed using 3–10-fold higher concentration of aRD1–2 as compared with aRD1–3 and aRD1–4 (Fig. 9, right panel). In contrast, when aRD1–2 was assayed for inhibition of 125I-PDGF-AA binding to PDGF-α-receptors, ~100-fold higher concentrations of aRD1–2 than of aRD1–3 or aRD1–4 were required for similar inhibitory effects (Fig. 9, left panel). Together, these findings demonstrate that aRD1–2 binds PDGF-BB, but not PDGF-AA, with rather high affinity and that Ig-like domain 3 of the PDGF-α-receptor contains epitopes of particular importance for PDGF-AA binding.

FIG. 6. Comparison of aRD1–4 and aRD1–3 in the formation of 1:2 ligand-receptor complexes. aRD1–4 and aRD1–3 at concentrations of 12.5 (A) and 3 (B) μM were incubated with PDGF-BB at various ligand/receptor ratios as indicated and analyzed by native electrophoresis on 7.5% polyacrylamide gels. Positions of PDGF-αRD1–4 and PDGF-αRD1–3 complexes are indicated by closed and open arrows, respectively. Proteins were visualized by Coomassie staining (A) or with a phosphoimager (B).

FIG. 7. Neutralization by aRD1–4 and aRD1–3 of 125I-PDGF-AA binding to cell-surface PDGF-α-receptors. Porcine aortic endothelial cells expressing the PDGF-α-receptor were incubated with 5 ng/ml 125I-PDGF-AA together with various concentrations of aRD1–4 (open circles) and aRD1–3 (closed circles). Unlabeled PDGF-BB at 160 ng/ml reduced the binding of 125I-PDGF-AA to 600 cpm.

FIG. 8. Comparison of aRD1–4 and aRD1–2 in complex forming assay. aRD1–4 or aRD1–2 at a concentration of 25 μM was incubated with or without 12.5 μM PDGF-AA or PDGF-BB, as indicated. Complex formation was analyzed by native electrophoresis on 7.5% polyacrylamide gels. Proteins were visualized by Coomassie staining.

**DISCUSSION**

In this study, we have used three forms of the PDGF-α-receptor extracellular part, aRD1–4, aRD1–3, and aRD1–2, to explore the mechanism and structural basis of ligand binding and receptor dimerization. Through the use of gel permeation chromatography and two different types of native gel electrophoresis, we confirmed, in agreement with previous studies (13, 14), that soluble aRD1–4 forms a complex at a 1:2 ligand/receptor ratio (Figs. 3 and 4). Using the same assay system, we further demonstrated that a 1:2 complex also forms under conditions of ligand excess, suggesting that the complex is stabilized by additional types of interactions.

The finding that the 1:2 PDGF-αRD1–3 complex, in contrast to the 1:2 PDGF-αRD1–4 complex, was partially disrupted under conditions of ligand excess (Fig. 5, A and B) localizes the interactions mediating stability under conditions of ligand excess to Ig-like domain 4. Receptor-receptor interactions involving Ig-like domain 4 of the PDGF-α-receptor have recently been shown to occur and also to be required for activation of PDGF.
various concentrations of Flt-4 (33).

(31), as well as in Flt-1-related receptors, such as KDR (32) and such as the colony-stimulating factor-1 receptor (30) and Flk2 in other receptors structurally related to the PDGF receptors, residues is involved. This structural feature is also conserved an anomalous Ig-like domain lacking two conserved cysteine receptor-receptor interactions. Interestingly, in all these cases, domain 4 (29). Together, these findings suggest that Ig-like domain 4 is important for receptor-receptor interactions. Thus, Ig-like domain 4 is of great importance for PDGF-AA binding, has been provided through the recent characterization of monoclonal antibodies against Ig-like domain 4 of the PDGF β-receptor that block receptor signaling without affecting ligand binding (18, 19). Ig-like domain 4 of the stem cell factor receptor has also been shown to be involved in receptor-receptor interactions (28). Moreover, a recent study comparing the properties of soluble forms of the vascular endothelial cell growth factor receptor Flt-1 indicated that at micromolar concentrations of soluble receptor domains, cross-linked 1:2 ligand-receptor complexes were detected only in receptors containing both the ligand-binding Ig-like domains 1–3 and Ig-like domain 4 (29). Together, these findings suggest that Ig-like domain 4 in several tyrosine kinase receptors is involved in receptor-receptor interactions. Interestingly, in all these cases, an anomalous Ig-like domain lacking two conserved cysteine residues is involved. This structural feature is also conserved in other receptors structurally related to the PDGF receptors, such as the colony-stimulating factor-1 receptor (30) and Flk2 (31), as well as in Flt-1-related receptors, such as KDR (32) and Flt-4 (33).

aRD1–4 and aRD1–3 were further compared with regard to their relative affinity for PDGF (Figs. 6 and 7). Whereas a difference between the two forms was detected in the assay performed at micromolar concentrations of receptor (Fig. 6), no difference was observed in the binding inhibition assay that scored for neutralization of subnanomolar concentrations of PDGF (Fig. 7). We therefore conclude that the neutralizing effect is achieved predominantly through the formation of 1:1 ligand-receptor complexes without involvement of receptor-receptor interactions. Thus, Ig-like domain 4 is important for ligand binding and complex formation on cell-surface receptors at subnanomolar concentrations of ligand, but not when soluble receptor domains are used; this difference is most likely due to the high local receptor concentration at the cell surface, which promotes receptor-receptor interactions.

Soluble forms of the intact extracellular domain of the PDGF β-receptors have previously been shown to inhibit PDGF action through sequestration of the ligand (15). Similarly, β-receptor Ig-like domains 1–4 have also been shown to block PDGF action (34). In both cases, IC50 values between 20 and 100 nM were reported, which are similar to the values found in our study. Our data predict that one way to convert soluble extra-cellular domains of receptors to more potent antagonists would be to engineer them to adopt dimeric forms. In agreement with this prediction, a disulfide-linked dimeric form of Ig-like domains 1–3 of the PDGF β-receptor fused to the second and third constant Ig-like domains of the heavy chain of IgG was reported to block PDGF at significantly lower concentrations than the monomeric forms (35).

To further map the ligand-binding region(s) within Ig-like domains 1–3, we compared aRD1–3 and aRD1–2 with regard to PDGF-AA and PDGF-BB binding (Figs. 8 and 9). We found that Ig-like domain 3 is of great importance for PDGF-AA binding, but contributes less to PDGF-BB binding. The observation that Ig-like domain 3 is of great importance for PDGF-AA binding to PDGF α-receptors is in good agreement with a recent study assaying PDGF-AA binding to immobilized PDGF α-receptor fragments (18). However, our demonstration that Ig-like domain 3 of the PDGF α-receptor is of lesser importance for binding of PDGF-BB than PDGF-AA represents a novel finding.

The observation that the binding regions of PDGF-AA and PDGF-BB are not structurally coincident is also in agreement with previous studies; PDGF-AA binds with higher affinity than PDGF-BB to Ig-like domains 2 and 3 (36), and neomycin selectively inhibits PDGF-AA binding to PDGF α-receptors (37). Studies using a PDGF α-receptor mutant lacking the major part of Ig-like domain 2 (amino acid residues 150–189) suggested the presence of a PDGF-AA-specific epitope within that region (38). This is to some extent in contrast to our results, and the reason remains unclear at present. However, it is possible that the PDGF-BB epitope(s) in Ig-like domain 2 lies outside that region and that the loss of PDGF-AA binding of the deletion mutant is caused by indirect effects on the structure of Ig-like domain 3.

The demonstration that PDGF-BB binds aRD1–2, together with previous observations demonstrating the lack of importance of Ig-like domain 1 for PDGF binding (18, 36), identifies Ig-like domain 2 of the PDGF α-receptor as the structure containing the major binding epitope(s) for PDGF-BB binding. Interestingly, it was recently demonstrated by crystallographic

**FIG. 9.** Neutralization by aRD1–2 of 125I-PDGF-AA and 125I-PDGF-BB binding to cell-surface PDGF α-receptors. Porcine aortic endothelial cells expressing the PDGF α-receptor were incubated with 5 ng/ml 125I-PDGF-AA (left panel) or 125I-PDGF-BB (right panel) with various concentrations of aRD1–4 (open squares), aRD1–3 (closed circles), and aRD1–2 (open circles). Unlabeled PDGF-BB at 160 ng/ml reduced the binding of 125I-PDGF-AA and 125I-PDGF-BB to 50 and 18%, respectively.

Soluble PDGF Ligand-Receptor Complex
analysis that the structurally related vascular endothelial cell growth factor binds predominantly to Ig-like domain 2 of the vascular endothelial cell growth factor receptor Flt-1 (39).

Our present investigation thus confirms and extends previous studies characterizing the structural basis and mechanism of PDGF-induced receptor activation. We conclude that PDGF-AA binding occurs predominantly through interactions with Ig-like domains 2 and 3, whereas PDGF-BB binding occurs predominantly via Ig-like domain 2. Furthermore, Ig-like domain 4-mediated receptor-receptor interactions contribute to complex formation and promote the formation of 1:2 ligand-receptor complexes at micromolar concentrations of soluble receptor and at physiologic levels of cell-surface receptors. However, these interactions are not significantly involved in the neutralizing activity of subnanomolar concentrations of PDGF by soluble receptors. The availability of large amounts of well characterized soluble PDGF α-receptor, as described in this paper, will allow future studies aiming at an improved understanding of the structural basis for ligand-receptor interaction, such as crystallographic analysis, as well as the setup of solid-phase assays to be used in screens for PDGF receptor antagonists interfering with ligand-receptor or receptor-receptor interactions.

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