Immunoglobulin-like Domain 4-mediated Receptor-Receptor Interactions Contribute to Platelet-derived Growth Factor-induced Receptor Dimerization*

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Platelet-derived growth factor (PDGF) is a dimeric growth factor that activates its tyrosine kinase receptor by inducing receptor dimerization. In this study, we investigated if receptor-receptor interactions, in addition to ligand-receptor interactions, contribute to the ligand-induced dimerization of the PDGF receptors. Analysis of two deletion mutants of the PDGF α-receptor indicated a role for Ig-like domain 4 in ligand-receptor or receptor-receptor interactions. When the fourth Ig-like domain of the PDGF α-receptor instead was replaced with the corresponding sequence of the stem cell factor receptor, the binding of PDGF-AA and -BB was not affected, nor was the ability to form homodimeric receptor complexes. This indicates that Ig-like domain 4 does not participate in ligand-receptor interactions. However, the chimeras did not form heterodimers with wild-type PDGF α- or β-receptors. Together, these findings suggest that Ig-like domain 4 mediates specific receptor-receptor interactions. This notion was also supported by the finding that a soluble form of Ig-like domain 4 of the PDGF α-receptor acted as a PDGF α-receptor antagonist. We conclude that specific receptor-receptor interactions contribute to PDGF receptor dimerization in vitro and that complementary epitopes in Ig-like domain 4 mediate these interactions. Our experiments also identify Ig-like domain 4 as a target for PDGF antagonists.

Platelet-derived growth factor (PDGF) constitutes a family of disulfide-bonded dimeric isoforms of A- and B-chains, which exert their effects on cells by binding to two structurally similar tyrosine kinase receptors, denoted α- and β-receptors (reviewed in Refs. 1 and 2). Each of the receptors consists of an extracellular membrane region, and an intracellular split tyrosine kinase domain (3–5). The receptors are activated by ligand-induced dimerization (6, 7). Since the A-chain binds only α-receptors, whereas the B-chain binds both α- and β-receptors with high affinity, the different isoforms will induce different dimeric receptor complexes.

Structurally, the PDGF B-chain consists of a tight cystine knot motif from which two loops (loops 1 and 3) point in one direction and one (loop 2) points in the other direction (8). The two subunits of the PDGF-BB molecule are arranged in an antiparallel manner so that loops 1 and 3 from one subunit will be close to loop 2 of the other. The receptor-binding epitopes reside mainly in loops 1 and 3, but loop 2 also contributes to some extent (9–12). It is likely that other PDGF isoforms are structurally similar. The dimeric PDGF molecule thus causes dimerization by simultaneously binding two receptor molecules. The ligand-binding region of the PDGF α-receptor has been mapped to Ig-like domains 1–3 (13, 14).

Several types of receptors are activated following ligand-induced dimerization (15, 16). In addition to the interactions between ligands and receptors, receptor-receptor interactions contribute to the dimerization of the growth hormone receptor (17) and possibly also to the ligand-induced dimerization of the SCF receptor, which is structurally related to the PDGF receptors, since deletion of Ig-like domain 4 of the SCF receptor was found to prevent dimerization, but not ligand binding (18). In this study, we have explored whether, in addition to ligand-receptor interactions, direct receptor-receptor interactions contribute to ligand-induced PDGF receptor dimerization.

EXPERIMENTAL PROCEDURES

Construction of Expression Vectors—Previously described cDNAs encoding the PDGF α-receptor (5) and the SCF receptor (19) were used to generate the mutants used in this study. Site-directed mutagenesis was performed with the Altered Sites™ system (Promega). To generate the deletion mutant αΔ5-R, Xhol sites (CTCGAG) were introduced into the α-receptor cDNA at positions corresponding to those encoding the ends of Ig-like domains 4 (amino acids 418–419) and 5 (amino acids 521–522), and the Xhol fragment was subsequently excised. In a similar manner, αΔ4.5-R was generated by introducing Xhol sites at positions encoding the ends of Ig-like domains 3 (amino acids 315–316) and 5, followed by excision of the Xhol fragment. In both deletion mutants, an NcoI site was also introduced at the position of the stop codon, enabling ligation to a vector encoding three tandem HA epitopes followed by a stop codon (a kind gift of Dr. Y. Xiong). To generate the chimeric receptors, a cDNA fragment encoding SCF receptor Ig-like domain 4 (amino acids 315–416) was obtained by polymerase chain reaction using primers introducing Xhol sites at the ends of the polypeptide chain reaction product. This fragment was ligated to a PDGF α-receptor cDNA in which an Xhol fragment encoding Ig-like domain 4 had been cut out. To make the truncated forms, a BglII site (AGATCT) was introduced into the sequence encoding the juxtamembrane portion (amino acids 560–561) of the α-receptor and used to ligate to the vector encoding three tandem HA epitopes followed by a stop codon. All constructs were inserted into the pSV7d vector (20) and used for transient expression in COS cells.

Immunoprecipitations of Metabolically Labeled Receptors Expressed in COS Cells—Plasmids were transfected into COS cells by the calcium phosphate method and metabolically labeled with [35S]methionine and [35S]cysteine for 3 h. After extraction with lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 0.5% deoxycholic acid, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1% Trasylol) and cen-
trifugation at 10,000 \times g for 15 min, the receptors were immunoprecipitated with rabbit antisera against the C-terminal part (amino acids 1066–1084) of the human PDGF \( \alpha \)-receptor (21) or the HA epitope (a kind gift of Dr. Lars Rönnstrand). After SDS-gel electrophoresis using 7% polyacrylamide gels, the labeled proteins were analyzed using a phosphoimager (FUJIX BAS 2000, Fuji).

Ligand Binding Analysis—PDGF-AA and -BB were \(^{125}\)I-labeled with the chloramine-T (22) and Bolton and Hunter (23) methods, respectively, to specific activities of \( \sim \)50,000 cpm/ng. Binding experiments were performed as described (24) using transfected COS cells in 12-well dishes.

Analysis of Receptor Homo- and Heterodimerization—Cross-linking and immunoprecipitation after binding of \(^{125}\)I-PDGF-AA in the absence or presence of excess unlabeled PDGF-AA were performed using transfected COS cells in 60-mm dishes. Cells were washed twice with PBS supplemented with 1 mg/ml bovine serum albumin and incubated for 90 min on ice with 10 ng/ml \(^{125}\)I-PDGF-AA in the absence or presence of 1 \( \mu \)g/ml unlabeled PDGF-AA. After three washes with PBS, ligand-receptor complexes were cross-linked by incubation in 1 mM bis(sulfosuccinimidyl) suberate for 30 min at room temperature. After incubation in 70 mM methylammonium chloride for 10 min, cell lysates were prepared as described above. Immunoprecipitations were performed with rabbit antisera against the PDGF \( \alpha \)-receptor or the HA epitope. After SDS-gel electrophoresis using 4–12% gradient gels, electrophoretic transfer was carried out onto a nitrocellulose membrane (Hybond-C Extra, Amersham Corp.). For immunoblotting, the filter was probed with 10 \( \mu \)g/ml monoclonal anti-HA epitope antibody (12CA5, Boehringer Mannheim) or a rabbit PDGF \( \beta \)-receptor antiserum (PR-4, a kind gift from Dr. Sara Courtneidge); precipitated

**FIG. 1.** Deletion of Ig-like domain 4 of the PDGF \( \alpha \)-receptor leads to loss of high affinity binding. A, schematic illustration of wild-type and altered PDGF receptors. The fifth or the fourth and fifth Ig-like domains were deleted to generate the deletion mutants \( \alpha \Delta 5-R \) and \( \alpha \Delta 4,5-R \), respectively. In the chimeras \( \alpha \)/kit-R and To/kit-R, Ig-like domain 4 of the PDGF \( \alpha \)-receptor was replaced with the corresponding sequence of the SCF receptor (c-Kit), as indicated by interrupted lines. The carboxyl-terminally truncated forms of the wild-type PDGF \( \alpha \)-receptor (To-R) and of the chimera (To/kit-R) as well as the deletion mutants have three tandem HA epitopes at their carboxyl termini. \( \alpha \)-R and \( \beta \)-R indicate the wild-type PDGF \( \alpha \)- and \( \beta \)-receptors, respectively. B, immunoprecipitations of transiently expressed receptors from metabolically labeled COS cells. Cells were transfected as indicated at the top of the figure and subjected to immunoprecipitations using antisera against the \( \alpha \)-receptor. The positions of \( \text{M}_r \) 200,000 and 97,000 marker proteins are indicated to the left, and the sizes of the immunoprecipitated components to the right. C, \(^{125}\)I-PDGF-AA binding to wild-type and mutant receptors. COS cells expressing the wild-type PDGF \( \alpha \)-receptor, \( \alpha \Delta 5-R \), or \( \alpha \Delta 4,5-R \) were tested for binding of \(^{125}\)I-PDGF-AA in the presence of various concentrations of unlabeled ligand. D, detection of \(^{125}\)I-PDGF-AA cross-linked to PDGF receptor complexes in transfected COS cells. Analysis was performed using COS cells transfected with the full-length wild-type PDGF \( \alpha \)-receptor, \( \alpha \Delta 5-R \), or \( \alpha \Delta 4,5-R \) as indicated at the top of the figure. Cross-linking and receptor immunoprecipitations were performed after binding of \(^{125}\)I-PDGF-AA in the absence (−) or presence (+) of competing unlabeled PDGF-AA. The positions of dimeric and monomeric receptors are indicated to the right. The positions of \( \text{M}_r \) 200,000 and 97,000 marker proteins are shown to the left. Ab, antibody.
chimeric receptors were tested for binding of $^{125}$I-PDGF-AA in the vector pGEX-3X (Pharmacia Biotech Inc.). Expression of GST-RIg4 was detected using antisera against the indicated at the top of the figure and subjected to immunoprecipitations from metabolically labeled COS cells. Cells were transfected as a ligand binding. The following experiments were designed to distinguish between these two possibilities.

The region of the PDGF-$\alpha$-receptor in which the fourth Ig-like domain of the SCF receptor ($\alpha$-Scf) was replaced with the corresponding domain of the SCF receptor (21) were used. Unstimulated cells were incubated in PBS supplemented with 1 mg/ml bovine serum albumin on ice for 60 min with or without 10 $\mu$g/ml GST control protein or GST-RIg4. Identical incubations were also performed in the presence of 3 ng/ml PDGF-AA and on cells that had been pretreated for 10 min at 37°C with a 50 mM concentration of the protein-tyrosine phosphatase inhibitor pervanadate. After incubation on ice, cells were lysed in lysis buffer, and PDGF-$\alpha$-receptor rabbit antisera was added. After SDS-gel electrophoresis, precipitated receptors were transferred to nitrocellulose filters, and the filters were probed with antiphosphotyrosine antibody PY20 (Transduction Laboratories). After incubation with horseradish peroxidase-conjugated anti-mouse antibodies, tyrosine-phosphorylated receptors were detected by enhanced chemiluminescence. The filters were subsequently stripped and reprobed with the PDGF-$\alpha$-receptor rabbit antisera, and the amount of receptors was determined by incubation with horseradish peroxidase-conjugated anti-rabbit antibodies followed by ECL. Control experiments performed on serum-starved PAE cells stably transfected with the SCF receptor (25) were performed in a similar fashion using a rabbit antiserum against the SCF receptor (25) for analysis by immunoprecipitation and immunoblotting.

RESULTS

Deletion of Ig-like Domain 4 in the PDGF-$\alpha$-Receptor Leads to Loss of High Affinity Binding—The region of the PDGF-$\alpha$-receptor primarily involved in direct PDGF binding has been mapped to Ig-like domains 1–3 (13, 14). In an initial attempt to determine if regions outside the ligand-binding domain are of any importance for high affinity binding of ligand, two deletion mutants were generated in which either Ig-like domain 5 ($\alpha$55-R) or Ig-like domain 4 and 5 ($\alpha$44,5-R) were deleted (Fig. 1A). Analysis by immunoprecipitations of the two deletion mutants after expression in metabolically labeled COS cells revealed precursor and mature forms of the expected sizes (Fig. 1B).

To determine the functional effects of the deletions, COS cells expressing the wild-type PDGF-$\alpha$-receptor or the deletion mutants were subjected to a $^{125}$I-PDGF-AA binding assay (Fig. 1C). Whereas deletion of Ig-like domain 5 had only minor effects on PDGF-AA binding, deletion of both Ig-like domains 4 and 5 decreased binding dramatically (Fig. 1C). When the results from the binding data were subjected to Scatchard analysis (26), a $K_d$ value of 0.5 nM was obtained for both the wild-type PDGF-$\alpha$-receptor and $\alpha$55-R. The two deletion mutants were also compared with the wild-type PDGF-$\alpha$-receptor using cross-linking of $^{125}$I-PDGF-AA to the receptor by bis(sulfosuccinimidyl) suberate, followed by immunoprecipitations with PDGF-$\alpha$-receptor antisera. As shown in Fig. 1D, deletion of Ig-like domain 5 affected neither the total amount of recovered ligand-receptor complex nor the ratio of monomeric and dimeric receptor forms, as compared with the wild-type $\alpha$-receptor. In contrast, deletion of both Ig-like domains 4 and 5 reduced dramatically the formation of both monomeric and dimeric receptor complexes.

Together, these experiments suggest that Ig-like domain 4 is required for high affinity binding of PDGF-AA to its receptor, either by mediating ligand-receptor interactions or by mediating receptor-receptor interactions required for high affinity ligand binding. The following experiments were designed to distinguish between these two possibilities.

The Fourth Ig-like Domain of the PDGF-$\alpha$-Receptor Can Be Replaced with the Corresponding Domain of the SCF Receptor without Affecting PDGF-AA Binding—A cDNA encoding a chimeric PDGF-$\alpha$-receptor in which the fourth Ig-like domain of the receptor was replaced with the corresponding sequence of the SCF receptor ($\alpha$-kitR) was generated (Fig. 1A). In addition, cDNAs encoding carboxyl-terminally truncated forms of the chimera (TcR or TcR) as well as of the wild-type PDGF-$\alpha$-receptor (TcR) were generated (Fig. 1A). Both truncated forms contain three tandem HA epitopes at their carboxy termini for immunodetection. When expressed transiently in COS cells, all four types of receptors were detected as precursor and mature forms of the expected sizes after immunoprecipitations from lysates of metabolically labeled cells (Fig. 2A).

The four receptors were expressed in COS cells and analyzed with regard to binding of PDGF-AA. As shown in Fig. 2B, no
major differences were observed between the full-length and truncated receptors on one hand and between the wild-type and chimeric receptors on the other (Fig. 2B). Scatchard analysis of the results from the binding experiments yielded $K_d$ values between 0.3 and 0.5 nM. Furthermore, when analyzed in cross-linking experiments, the full-length chimeric receptor formed cross-linked dimers in a manner indistinguishable from that of the wild-type PDGF $\alpha$-receptor (Fig. 3, lanes 1–4). The finding that Ig-like domain 4 of the PDGF $\alpha$-receptor can be substituted with the corresponding domain of the SCF receptor, which does not bind PDGF, suggests that Ig-like domain 4 is not involved in ligand-receptor interactions.

$a$-kit-R and $\alpha$-kit-R Do Not Form Heterodimers with the Wild-type PDGF $\alpha$-Receptor—To explore if Ig-like domain 4 is involved in direct and specific receptor-receptor interactions, we designed cross-linking experiments in which the ability of the receptor chimera to form homodimers could be compared with the ability to form heterodimers with wild-type PDGF $\alpha$-receptors. The full-length $\alpha$-receptor, either alone or together with the truncated wild-type or truncated chimeric receptor, was transiently expressed in COS cells. After binding of $^{125}$I-PDGF-AA, with or without an excess of unlabeled PDGF-AA, ligand-receptor complexes were cross-linked with bis(sulfosuccinimidyl) suberate, immunoprecipitated, and subjected to analyses by SDS-gel electrophoresis, followed by exposure using a phosphoimager (Fig. 3A). In the immunoprecipitations with PDGF $\alpha$-receptor antiserum, dimers of the full-length wild-type $\alpha$-receptor were recovered (lanes 3, 5, and 7). In addition, cross-linked dimers of full-length and truncated wild-type receptors could be demonstrated (lane 5). However, no heterodimers composed of the full-length wild-type PDGF $\alpha$-receptor and truncated chimeric receptors were detected (lane 7). In the HA tag precipitations, homodimeric forms of both truncated versions were easily identified as broad tailing components at positions that partially overlap with the positions of the heterodimeric complexes (lanes 9–14).
In an analogous experiment in which the full-length chimeric receptor was expressed alone or together with either of the two truncated forms, similar findings were obtained; all possible homodimeric receptor forms were identified (Fig. 3, lanes 3, 5, 7, 11, and 13). Also, dimers of full-length and truncated chimeric receptors were detected (lane 7). In contrast, no heterodimers between full-length chimeric and truncated wild-type receptors were observed (lane 5).

Thus, in the immunoprecipitations with PDGF a-receptor antiserum, dimers composed of one truncated and one full-length form of the PDGF a-receptor could be demonstrated as well as dimers composed of one truncated and one full-length SCF receptor/PDGF a-receptor chimera. However, no heterodimers between wild-type and chimeric receptors were seen.
These experiments thus suggest that PDGF α-receptor dimerization is not exclusively a consequence of ligand-receptor interactions, but also requires specific receptor-receptor interactions. Since the chimera’s ability to form homodimers was unaffected, we also conclude that the receptor-receptor interactions occur between complementary epitopes in Ig-like domain 4.

**Tαkit-R Does Not Form Heterodimers with the PDGF β-Receptor—**PDGF-AA binds exclusively to PDGF α-receptors. In contrast, PDGF-BB binds with equal affinity to α- and β-receptors and will thus, on cells expressing both receptor types, induce the formation of heterodimeric α- and β-receptor complexes in addition to homodimeric complexes. We therefore investigated whether Ig-like domain 4 of the α-receptor also is involved in interactions with the β-receptor. The truncated wild-type and chimeric α-receptors expressed in COS cells showed similar affinities for binding of PDGF-BB (Fig. 4A). A $K_d$ value of 0.7 nM was obtained when binding data were subjected to Scatchard analysis. The PDGF β-receptor was coexpressed with either the truncated wild-type or the truncated chimeric receptor; after ligand binding, cells were incubated with or without cross-linker, and lysates were subjected to immunoprecipitations with a PDGF α-receptor antiserum. A dimeric complex between the PDGF α-receptor and the truncated α-receptor complex could be demonstrated, but no heterodimeric complexes between the truncated chimera and the β-receptor (Fig. 4B, upper panel). Control experiments showed that equal amounts of the different receptor forms were expressed (Fig. 4B, middle and lower panels). The finding that substitution of Ig-like domain 4 altered the ability of the chimera to dimerize with wild-type PDGF β-receptors, without affecting the ability to bind PDGF-BB, extends the role for Ig-like domain 4 in homodimerization of PDGF α-receptors to include also heterodimerization with PDGF β-receptors.

**A Soluble Form of the Fourth Ig-like Domain of the PDGF α-Receptor Blocks PDGF α-Receptor Tyrosine Phosphorylation—**We next used a receptor autophosphorylation assay to investigate whether a soluble Ig-like domain 4 could interfere with receptor activation. PDGF α-receptor Ig-like domain 4 was expressed as a GST fusion protein (GST-αRIg4) in bacteria and purified through glutathione-Sepharose affinity purification. To investigate its ability to interfere with PDGF α-receptor activation, PAE cells stably expressing the PDGF α-receptor were used (Fig. 5). The potential inhibitory effect of GST-αRIg4 on PDGF α-receptor tyrosine phosphorylation was analyzed under three different conditions: using unstimulated cells, cells stimulated with 3 ng/ml PDGF-AA, and cells pre-treated with pervanadate, a potent inhibitor of protein-tyrosine phosphatases. After cell lysis, the extent of tyrosine phosphorylation of PDGF α-receptors was determined by immunoprecipitations of the PDGF α-receptor followed by immunoblotting with anti-phosphotyrosine antibodies. As shown in Fig. 5A (upper panel), the presence of 10 μg/ml GST-αRIg4 led to a reduction in background as well as ligand-stimulated tyrosine phosphorylation of the PDGF α-receptor. No such effect was seen when cells were incubated with GST (Fig. 5A, lower panel). The total amount of receptors was the same under the different experimental conditions (Fig. 5A, lower panel). To investigate the specificity of the antagonistic action of GST-αRIg4, PAE cells stably expressing the SCF receptor were subjected to similar experiments. As shown in Fig. 5B, GST-αRIg4 did not show any effect on ligand-independent tyrosine phosphorylation of the SCF receptor, nor was any effect seen on SCF-stimulated tyrosine phosphorylation of the SCF receptor (data not shown). The finding that GST-αRIg4 functions as an antagonist to PDGF α-receptor tyrosine phosphorylation supports the notion that Ig-like domain 4 is involved in direct receptor-receptor interactions.

**DISCUSSION**

In this study, we provide evidence that PDGF receptor dimerization involves, in addition to the interaction between ligand and receptor, a direct interaction between the receptors. Whereas the ligand binds to epitopes in Ig-like domains 1–3 (13, 14), Ig-like domain 4 mediates direct receptor-receptor interactions (Fig. 6).

PDGF receptor dimerization can thus occur via two different routes: either the ligand binds first to one receptor and subsequently to another, or alternatively, the ligand binds to and stabilizes preformed transient receptor dimers (Fig. 6). It is noteworthy that in PAE cells stably expressing the PDGF α-receptor, which expresses receptor levels comparable to those in cells expressing endogenous receptors, the antagonistic effect of GST-αRIg4 was observed not only on ligand-stimulated cells, but also on the background phosphorylation of the receptor in unstimulated cells (Fig. 5, A and B). These findings suggest that the background receptor phosphorylation is due to the formation of transient ligand-independent receptor dimers, rather than to receptor monomers displaying a basal kinase activity. Thus, an equilibrium between monomeric and dimeric receptors appears to exist in the absence of ligand. It follows that the receptor density on the target cell is likely to influence which of the two routes in Fig. 6 are preferentially used.

The structurally best characterized example of ligand-receptor interaction is the binding of growth hormone (GH) to the
GH receptor (17), for which the crystal structure is known. In this case GH, binds with high affinity to one receptor and, through a different epitope, with lower affinity to another receptor, thus forming a 1:2 complex. The binding of the second receptor is stabilized by direct interactions between the receptors. Thus, the the PDGF ligand-receptor complex and the GH ligand-receptor complex are similar in that they both are stabilized by a combination of ligand-receptor and receptor-receptor interactions, but differ in that the two ligand-receptor interactions in the case of PDGF are of similar strength, but differ in the case of GH.

The PDGF receptors are structurally most related to the SCF and colony-stimulating factor-1 receptors (19,27). These receptors also bind dimeric ligands. In the case of the SCF receptor, an involvement of Ig-like domain 4-mediated receptor-receptor interactions in dimerization was recently postulated based on the finding that deletion of Ig-like domain 4 prevents dimerization and, to a lesser extent, ligand binding (18). Interestingly, mutations in the colony-stimulating factor-1 receptor that lead to its ligand-independent activation are localized to Ig-like domain 4 (27). It is possible that the activating effects of these mutations are mediated by causing increases in the affinity of receptor-receptor interactions. Together with the findings presented in this paper, these observations suggest that direct interactions between receptors may be common in ligand-induced dimeric receptor complexes.

PDGF antagonists are highly warranted given the overactivity of PDGF receptors in certain pathological situations, including tumorigenesis and atherosclerosis (28–31). Previous attempts to design antagonists acting on PDGF receptors have focused mainly on blocking direct ligand-receptor interactions or on inhibiting the tyrosine kinase activity of the receptors (13, 28, 32–34). In addition, dominant-negative forms of both PDGF itself and the receptors have been described (35–37). Our results suggest that blocking receptor-receptor interaction by targeting Ig-like domain 4 may serve as an additional strategy to interfere with signaling through PDGF receptors.

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