Assignment of Interchain Disulfide Bonds in Platelet-derived Growth Factor (PDGF) and Evidence for Agonist Activity of Monomeric PDGF*

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Platelet-derived growth factor (PDGF) is a dimeric factor stabilized by disulfide bonds. Using an approach involving partial reduction of PDGF, we have identified the 2nd and 4th cysteine residues in the PDGF chains as the cysteine residues forming interchain disulfide bonds. Analysis of PDGF mutants in which the 2nd and 4th cysteine residues were mutated to serine residues revealed that the disulfide bonds are arranged in a cross-wise manner, with the 2nd cysteine residue in one chain being linked to the 4th cysteine residue in the other.

A PDGF B-chain mutant, in which both the 2nd and 4th cysteine residues were substituted with serine residues, migrated as a monomer in sodium dodecyl sulfate gel electrophoresis and retained receptor binding activity. When analyzed in receptor dimerization and autophosphorylation assays, this mutant showed agonistic activity. Thus, structural information has been obtained that will allow the large scale production of properly folded monomeric PDGF, as well as design of specific PDGF heterodimers.

Platelet-derived growth factor (PDGF) is a potent mitogen for connective tissue cells and certain other cell types (for reviews see Refs. 1 and 2). PDGF occurs as different isoforms of disulfide-bonded homo- or heterodimers of A- and B-polypeptide chains. The biological effects of the PDGF isoforms are exerted via binding with different affinities to two similar receptor types, denoted α- and β-receptors, both of which are protein tyrosine kinases (3-5). The A-chain of PDGF can bind to the α-receptor whereas the B-chain can bind to α- as well as β-receptors (6-10). Ligand binding induces receptor dimerization and autophosphorylation, followed by the phosphorylation of tyrosine residues on specific cytoplasmic substrates.

The two polypeptide chains of PDGF are structurally similar. Both are synthesized as precursor molecules with N-terminal hydrophobic leader sequences (11, 12). After dimerization, the molecules are proteolytically processed in the N-termini and, in the case of the B-chain, in the C-terminus (13). In the mature parts of the molecules of about 100 amino acids each, there is a 60% amino acid sequence similarity with a perfect conservation of the 8 cysteine residues in each chain. Despite the fact that the B-chain has a hydrophobic leader sequence, it is only inefficiently secreted from the producer cell. This is due to the presence of a stretch of basic amino acid residues in the C-terminal part of the molecule which serves as a retention sequence (14, 15). Interestingly, a similar retention sequence is found in a longer splice variant of the A-chain, but not in the more common shorter variant.

Some information is available regarding the epitopes in the PDGF isoforms that are involved in receptor binding. A systematic screening of peptides derived from the B-chain of PDGF for their abilities to inhibit the binding of [3H]labeled PDGF isoforms to α- and β-receptors revealed that a peptide containing B-chain amino acids 116-121 and 157-163 was a fairly efficient inhibitor of binding to both receptor types. This peptide furthermore had antagonistic activity in receptor dimerization and phosphorylation assays. These data suggest that the two regions in PDGF contained in the peptide are important for the interaction with determinants common for the two receptor types. Also, by substituting or deleting amino acids in the PDGF B-chain, the region between codons 106-118 was found to be of importance for receptor binding (16, 17). To specifically identify those regions mediating the B-chains unique ability to bind the β-receptor, amino acids in the B-chain of PDGF were systematically replaced with the corresponding amino acids in the A-chain; Asn-115, Arg-154, and Ile-158 were thus identified as important for the unique property of the B-chain to interact with the β-receptor (18). By using cell transformation as an assay, it was concluded that B-chain amino acids 105-140 inserted in the A-chain gave full B-chain activity (19). The reason for the discrepancy between the data in these two investigations is not known but may be related to the different assay methods used.

In order to further explore the mechanism for dimerization of receptors and its role in the activation of the receptor kinases, monomeric PDGF subunits with retained receptor binding activity would be very valuable. Attempts have been made to use the conventional method to identify interchain disulfide bonds by isolation of disulfide-containing peptides after proteolytic cleavage of non-reduced PDGF; however, due to the high density of cysteine residues in PDGF, this approach has been futile. In the present communication we have identified two interchain disulfide bonds in PDGF using...
an approach involving partial reduction of the molecule. After mutation of the two implicated cysteine residues to serine residues, monomeric PDGF was produced by transient expression in COS cells. We show that the obtained product has at least partial agonistic activity in receptor dimerization and autophosphorylation assays.

MATERIALS AND METHODS

Partial and Complete Reduction Followed by Alkylation of PDGF-AA—For partial reduction, 90 μg of recombinant PDGF-AA (long splice version) was treated with 3 mM DTT in 220 μl of 0.36 M Tris-HCl, pH 8.2, for 2 h at 30 °C. The exposed SH groups were allowed to react with 9 mM iodoacetic acid in the same solution for 15 min. The obtained monomers were isolated by gel chromatography on Superose 12 (1 × 50 cm) in 0.5 M NaCl, and 1 M acetic acid at a flow rate of 15 ml/h. The resulting fractions were analyzed by SDS-gel electrophoresis (20) followed by silver staining; the fractions containing monomeric PDGF were desalted by reversed-phase HPLC on a narrow bore Brownlee Aquapore C4 column (2.1 × 30 mm). An aliquot of the material was subjected to analysis by a receptor binding assay (see below); a major part of the material was then subjected to complete reduction with 20 mM DTT in 4 M guanidine HCl, 1 M Tris-HCl, pH 8.0, and 10 mM EDTA for 2 h at 37 °C. The freed SH groups (previously involved in the intrachain disulfide bonds) were alkylated by the addition of 2 μl of 4-vinylpyridine and incubation for 2 h at room temperature. After another desalting, as described above, the material was dried in a rotator evaporator and subjected to proteolytic cleavage.

Digestion with Staphylococcal Protexase V8 (Glu-C) and Fragment Separation—The completely reduced and pyridyl-ethylated PDGF-A chain was digested with Glu-C protease (Boehringer Mannheim) at an enzyme to substrate ratio of 1:50 (w/w) for 15 h at 37 °C in 200 μl of 2 M urea and 0.1 M ammonium bicarbonate. At the end of the reaction time the mixture was applied on a Brownlee Aquapore C4 (2.1 × 30 mm) narrow bore column, and the fragments were eluted by a linear gradient of propanol-1 (0–27% in 60 min) in 0.16 M trifluoroacetic acid at a flow rate of 100 μl/min. The effluent was monitored by a photodiode array detector (Waters 990), and spectral data between 200 and 300 nm were collected for later evaluation. Fractions were collected manually.

N-terminal Sequence Analysis of Proteolytic Fragments—The appropriate HPLC fractions were digested directly onto a Polybrene-treated glass fiber disc and subjected to Edman degradation in an Applied Biosystems 477A peptide sequencer equipped with an on-line phenylthiohydantoin-amino acid analyzer (120A). The instruments were operated according to the manufacturer with minor modifications as described.

Construction of cDNAs Encoding Monomeric PDGF A- and B-Chains—The corresponding cDNAs were ligated into the EcoRI/HindIII sites of the mammalian expression vector pSV-PDGF-A102 (pSVA) (22), where the expression cassettes were driven by the cytomegalovirus immediate-early promoter. The expression vector pSV-PDGF-A102 contains an appropriate HPLC column (2.1 × 30 mm) in 0.1 M ammonium bicarbonate. The effluent was monitored by a photodiode array detector (Waters 990), and spectral data between 200 and 300 nm were collected for later evaluation. Fractions were collected manually.

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cultures of COS cells transfected with pSVBstop, pSVmonoB, or from mock-transfected cells were desalted and concentrated as described above. A radioreceptor assay was carried out in order to determine the receptor binding activity. The media from the cells transfected with pSVmonoB or pSVBstop were then adjusted with mock-transfected medium to a receptor binding activity of 100 ng/ml. PAE cells expressing PDGF-B receptors (25), grown in 25-cm² dishes, were labeled in serum- and methionine-free MCDB 104 medium supplemented with 0.1 mg/ml BSA and 0.1 mCi of [35S]methionine/ml for 3 h at 37 °C. The cells were then stimulated with 1 ml of the different concentrated conditioned media for 30 min at 4 °C. As a positive control, 1 ml of mock-transfected medium with 100 ng/ml recombinant PDGF-BB was used. The cells were washed once with PBS, scraped into a lysis buffer of 20 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.5% deoxycholate, 0.5% Triton X-100, 30 mM pyrophosphate, 1% Trasylol, and 1 mM PMSF, and cleared by centrifugation for 15 min at 10,000 × g. Half of the cell lysate was incubated at 4 °C for 2 h with 5 μl of the antiserum PDGFR-3, raised against a peptide in the PDGF-B receptor sequence (26); the other half was incubated with 1 μl of antiserum against phosphotyrosine (27). The immunocomplexes were precipitated with 60 μl of a 50% slurry of Protein A-Sepharose in PBS. The beads were washed 3 times with lysis buffer, twice with 20 mM Tris-HCl, pH 7.5, 0.5 mM NaCl, 1% Trasylol, and 1 mM PMSF, and once in distilled water. Elution of the immunocomplexes was performed by adding 100 μl of sample buffer containing 4% SDS, 0.2 mM Tris-HCl, pH 8.8, 0.5 mM sucrose, 5 mM EDTA, 0.01% bromphenol blue, and 2% mercaptoethanol. Immunocomplexes were analyzed by SDS-gel electrophoresis, using a 7% acrylamide gel followed by fluorography.

**RESULTS**

Identification of Cysteine Residues in PDGF That Are Involved in Interchain Disulfide Bridges—Each PDGF chain contains 8 cysteine residues, and it has not been possible to demonstrate free SH groups in the molecule. Therefore, PDGF most likely contains an even number of interchain disulfide bonds; the most probable arrangement of disulfide bridges is thus two interchain bridges and, in addition, three intrachain bridges in each subunit. We hypothesized that the interchain disulfide bonds might be more susceptible to reduction than the intrachain disulfide bonds and therefore attempted to identify the interchain disulfide bonds using an approach involving partial reduction of the PDGF molecule.

In order to titrate the conditions for partial reduction, aliquots of PDGF-AA (the long splice variant) were incubated with different concentrations of DTT for 2 h at room temperature; samples were then alkylated and analyzed by SDS-gel electrophoresis under non-reducing conditions, followed by silver staining. As shown in Fig. 2A, PDGF-AA gradually shifted from a dimeric form of about 30 kDa to a monomeric form of about 17 kDa with increasing concentration of DTT. At 3 mM DTT almost all PDGF appeared as a monomer; however, this material migrated slower in SDS-gel electrophoresis than fully reduced PDGF and thus most likely still contained intrachain disulfide bonds. This finding was consistent with our hypothesis that the interchain disulfide bonds in PDGF are more susceptible to reduction than the intrachain bonds and encouraged to perform the partial reduction in a preparative scale to allow the identification of the cysteine residues involved.

Ninety μg of PDGF-AA was incubated in 3 mM DTT for 2 h at room temperature, then alkylated with iodoacetic acid and subjected to gel chromatography on a Superose 12 column.

**Fig. 2. Partial reduction of PDGF-AA.** A, PDGF-AA was incubated in different concentrations of DTT for 2 h at 20 °C in PBS or fully reduced in 10 mM DTT for 5 min at 95 °C in SDS sample buffer. The samples were then alkylated and analyzed by SDS-gel electrophoresis under non-reducing conditions, followed by silver staining. B, 90 μg of PDGF-AA was subjected to partial reduction with 3 mM DTT for 2 h at 20 °C, then alkylated and subjected to chromatography on a Superose 12 column, as described under “Materials and Methods.” Dimeric (pool I) and monomeric (pool II) forms of PDGF-AA are indicated. C, the ability of the monomeric form of PDGF-AA (pool II) (C3) to compete with [3H]-PDGF-AA for binding to PDGF-α-receptors was analyzed. Native (●) and fully reduced (■) PDGF-AA were analyzed in parallel.
The material eluted in two peaks, denoted I and II (Fig. 2B), which were shown by SDS-gel electrophoresis to represent dimeric and monomeric PDGF, respectively (data not shown). The monomeric PDGF-A was then subjected to a receptor binding assay; it was found to retain 3-10% of the ability to compete with 125I-PDGF-AA for binding to human fibroblasts, as compared with unreduced PDGF-AA (Fig. 2C). In contrast, fully reduced PDGF-A-chain had very low receptor competing activity. Partially reduced PDGF A-chain thus retains some ability to bind to the α-receptor.

In order to identify the cysteine residues involved in the interchain disulfide bonds, the partially reduced monomeric PDGF-A was subjected to full reduction, followed by labeling of the freed SH groups with 4-vinylpyridine; the pyridylethylcysteine residues, which were involved in intrachain disulfide bonds, were thereby rendered a specific absorption at 254 nm. The protein was thereafter subjected to proteolytic cleavage with Glu-C protease, and the peptides were fractionated on a Brownlee C4 HPLC column (Fig. 3A). The obtained peptides were then sequenced; the sequences of the peptides that gave information about the cysteine residues are shown in Fig. 3B. Cysteine residues involved in interchain disulfide bonds, which were alkylated with iodoacetic acid after partial reduction, would be expected to appear as carboxymethylcysteine, whereas the cysteine residues involved in intrachain disulfide bonds, which were alkylated with 4-vinylpyridine, would be expected to appear as pyridylethylcysteine. As shown in Fig. 3B, the 2nd and 4th cysteine residues (Cys-123 and Cys-132 in the PDGF A-chain) appeared as carboxymethylcysteine, whereas the other 6 appeared as pyridylethylcysteine. These results suggested that the 2nd and 4th cysteine residues in PDGF-AA form the interchain disulfide bonds.

**Mutation of Cysteine Residues Forming Interchain Disulfide Bonds in PDGF**—To verify the results obtained by the partial reduction approach and to allow further characterization of monomeric PDGF, codons for the 2nd and 4th cysteine residue (Cys-123 and Cys-132, respectively) in a PDGF-A cDNA were mutated to serine codons. The mutated fragment was cloned into an expression vector, and the obtained plasmid pSVmonoA, was transfected into COS cells; immunoprecipitation of conditioned medium from [35S]cysteine-labeled cells revealed only the monomeric form upon analysis on SDS-gel electrophoresis (Fig. 4A). The fact that PDGF-monoA shifted in migration when it was analyzed under reducing conditions suggests that it contains intrachain disulfide bonds. Furthermore, PDGF-monoA was well recognized by an antiserum raised against wild type PDGF-AA, which does not recognize denatured PDGF A-chain. These observations support the notion that the conformation of PDGF-monoA is similar to that of the two polypeptide chains in the corresponding dimer. A similar mutant of the PDGF B-chain, PDGF-monoB, in which the 2nd and 4th cysteine codons were mutated to serine codons, was also analyzed. In this mutant codon 191 was in

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**FIG. 3. Reversed-phase chromatography and sequence of staphylococcal protease V8 fragments of reduced and alkylated PDGF A-chain.** A, pool II of the Superose 12 chromatography (Fig. 2B) was fully reduced and alkylated with 4-vinylpyridine, de-salted, and then subjected to degradation with staphylococcal V8 protease. The fragments were separated on a narrow bore C4 column by a linear gradient of propanol-1 in 0.16% trifluoroacetic acid. SP1-SP4 denote fragments whose N-terminal sequences are marked in B. All other major peptides were also sequenced, but only those that contribute to the elucidation of the interchain disulfide bond configuration are indicated in the figure. B, N-terminal amino acid sequences of staphylococcal protease fragments Sp1-Sp4. The bars represent positive identification of the corresponding amino acid in the PDGF A-chain sequence. Carboxymethylcysteine and pyridylethylcysteine are represented by # and @, respectively.

**FIG. 4. Immunoprecipitations of transiently expressed monomeric PDGF chains.** A, conditioned media from [35S]cysteine-labeled COS cells transfected with pSVA (A) or pSVMonoA (mA), as well as media from mock-transfected COS cells (--), were immunoprecipitated with an antisera against PDGF-AA; samples were analyzed by SDS-gel electrophoresis in the absence or presence of DTT, followed by fluorography. B, conditioned media from [35S]cysteine-labeled COS cells transfected with pSVBstop (B) or pSVmonoB (mB), as well as medium from mock-transfected COS cells (--), were immunoprecipitated using an antisera against PDGF-BB; samples were analyzed by SDS-gel electrophoresis in the absence or presence of DTT, followed by fluorography.
addition changed to a stop codon to obtain a secreted product (14). This product showed the same pattern as the A-chain analog when expressed in COS cells and analyzed by immunoprecipitation and SDS-gel electrophoresis (Fig. 4B). In order to determine if PDGF-monoA and PDGF-monoB were able to bind to the PDGF receptors, concentrated conditioned media from COS cells transfected with pSVmonoA or pSVmonoB were analyzed for their abilities to compete with $^{125}$I-PDGF-AA and $^{125}$I-PDGF-BB for binding to $\alpha$- and $\beta$-receptors, respectively. In this assay, PDGF-monoA showed no detectable binding to the $\alpha$-receptor (data not shown), whereas PDGF-monoB competed relatively well for binding to the $\beta$-receptor (Fig. 5).

The Interchain Disulfide Bonds in PDGF Are Arranged in a Cross-wise Manner—In order to determine the arrangement of the interchain disulfide bonds between the 2nd and 4th cysteine residues, two new mutants were constructed: PDGF-ASer2 with the 2nd cysteine residue mutated to a serine residue, and PDGF-ASer4 with the 4th cysteine residue mutated to a serine residue. If the 2nd and 4th cysteine residue are disulfide-bonded with the corresponding residue in the other chain, these mutants would be expected to form dimers. If on the other hand, the disulfide bonds are arranged in a cross-wise manner with the 2nd cysteine residue linked to the 4th, and vice versa, dimers can only be formed when PDGF-ASer2 and PDGF-ASer4 are expressed together. After transfection of pSVASer2, pSVASer4, or both plasmids together into COS cells, followed by immunoprecipitation of the conditioned media of $[^{35}]$S-methionine-labeled cells and analysis by SDS-gel electrophoresis, dimers were seen only when the two plasmids were cotransfected (Fig. 6A). Receptor binding analysis of concentrated conditioned media from COS cells transfected with pSVASer2, pSVASer4, or both plasmids together revealed a much more efficient competition for binding to the $\alpha$-receptor with medium from COS cells cotransfected with pSVASer2 and pSVASer4 compared with media from cells transfected with only one of the plasmids (Fig. 6B).

We conclude that the 2nd and 4th cysteine residues are disulfide-bonded cross-wise in the PDGF dimer.

Functional Properties of PDGF-monoB—An important question to answer was whether the binding of monomeric PDGF to PDGF receptors induced an agonistic or antagonistic effect. PDGF-monoB was therefore analyzed for its ability to activate the $\beta$-receptor in an autophosphorylation assay. $[^{35}]$S-Methionine-labeled $\beta$-receptor expressing PAE cells was therefore stimulated with concentrated conditioned media from COS cells transfected with pSVmonoB or pSVBstop (adjusted to a receptor binding activity of 100 ng/ml) or with medium from mock-transfected cells. The cells were lysed and subjected to immunoprecipitation with antiserum against the $\beta$-receptor and against phosphotyrosine. Analysis of the immunoprecipitates by SDS-gel electrophoresis revealed that both the products from pSVBstop and pSVmonoB stimulated autophosphorylation of the receptor (Fig. 7A). To determine if PDGF-monoB also caused dimerization of the receptor, $\beta$-receptor-expressing PAE cells were labeled with $[^{35}]$S-methionine and stimulated with concentrated conditioned media from COS cells transfected with pSVmonoB or pSVBstop. The cells were then lysed and the $\beta$-receptor cross-linked with BS$^3$. Immunoprecipitation, followed by SDS-gel electrophoresis and fluorography, showed that both PDGF-monoB and PDGF-BB caused dimerization of the receptor (Fig. 7B).
Control, 100 ng of recombinant PDGF-BB in mock-transfected medium were used or pSV monoB (mB) adjusted to a receptor binding activity of 100 ng/ml or with medium from mock-transfected cells (−). As a positive control, 100 ng of recombinant PDGF-BB in mock-transfected medium was used (rBB). The cell lysates were immunoprecipitated with antibodies against the PDGF receptor (R3) or with antibodies against phosphoryl tyrosine (α-P-Tyr). The immunoprecipitates were analyzed by SDS-gel electrophoresis in the presence of 2-mercaptoethanol, followed by fluorography. B, β-receptor expressing cells were labeled and stimulated as in A. The cells were then solubilized and the receptors cross-linked with BS3. Immunoprecipitations followed by SDS-gel electrophoresis and fluorography were performed as in A.

DISCUSSION

We show in the present communication that the 2nd cysteine residue from the N terminus in the PDGF chains forms an interchain disulfide bond with the 4th cysteine residue of the other chain in the dimer. Since PDGF seems not to contain any free SH groups, it is likely that a second interchain disulfide connects the 4th cysteine residue in the first chain with the 2nd cysteine residue in the second chain. This suggests that the two subunits in the PDGF dimer are arranged in an antiparallel manner. The remaining 6 cysteine residues in each PDGF chain are thus most likely involved in intrachain disulfide bonds, the exact localization of which remains to be established. Two additional members in the PDGF family were recently identified, vascular endothelial growth factor (also called vascular permeability factor) (28, 29) and placenta growth factor (30). These factors are dimeric molecules which show a perfect conservation of the cysteine residues of PDGF; it will be interesting to see whether the analogous cysteine residues in these factors are involved in interchain disulfide bonds.

An initial characterization of the functional properties of the monomeric PDGF B-chain, in which the 2nd and 4th cysteine residues were mutated to serine residues, revealed agonist activity. Thus, PDGF-monoB induced PDGF β-receptor dimerization and autophosphorylation (Fig. 7). The mechanism whereby PDGF-monoB causes receptor dimerization is not known. It is possible that the binding of monomeric PDGF induces a conformational change in the extracellular domain of the receptor, which promotes receptor dimerization. The small monomeric ligand epidermal growth factor seems to induce receptor dimerization via such a mechanism (31). Alternatively, PDGF-monoB may form dimers, which are not stabilized by disulfide bonds. The ligand for the PDGF receptor-like molecule c-Kit has been shown to appear as a non-disulfide-bonded dimer (32, 33) and to induce dimerization of its receptor (34). Preliminary attempts to determine whether PDGF-monoB appears as a monomer or a dimer under physiological conditions have been inconclusive due to the poor recovery of PDGF-monoB in most chromatographic systems. Activation of the receptor kinase and autophosphorylation are intimately correlated to receptor dimerization (6–10). Therefore, regardless of the mechanism involved, it is possible that PDGF-monoB-induced receptor dimerization is sufficient for receptor activation.

Analysis of PDGF-monoA and PDGF-monoB after transient expression in COS cells revealed that PDGF-monoA, in contrast to PDGF-monoB, had a very low or no receptor binding activity. The reason for this difference remains to be elucidated. It is possible that the ability of PDGF-monoB to form noncovalently stabilized dimers is higher than that of PDGF monoA. There may also be a difference in stability or recovery of the products. Monomers formed after partial reduction of PDGF-AA did show receptor binding activity (Fig. 2C), suggesting that monomeric PDGF A-chain with the 2nd and 4th cysteine residues alkylated has different properties compared with one in which these residues were mutated to serine residues.

The assignment of the 2nd and 4th cysteine residues as being involved in interchain disulfide bonds is consistent with observations made on PDGF mutants expressed in bacteria (35). Mixing of a PDGF mutant with the 2nd cysteine residue mutated to a serine residue and one with the 4th cysteine residue mutated to a serine residue was found to yield disulfide-bonded dimers, whereas either one of the mutants alone did not (35). Previous studies, in which the transforming properties of PDGF B-chain mutants with individual cysteine residues were changed to serine residues were determined, revealed that mutation of cysteine residues 1, 3, 6, and 7 abolished the activity (36, 37). Apparently, perturbation of intrachain disulfide bonds, which involves these residues, has a severe effect on the ability of the product to activate its receptor. The finding that mutation of the 2nd and 4th cysteine residues has less effect on the transforming activity of the PDGF B-chain is consistent with our finding that PDGF-monoB has receptor agonist activity.

The fact that the interchain bonds in PDGF are arranged in a cross-wise manner gives a possibility to specifically assemble heterodimers, which could potentially be used as receptor antagonists. It would for instance be possible to co-express a PDGF chain with only the 2nd cysteine residue mutated, with a PDGF chain with the 4th cysteine residue mutated and which contains additional mutations that prevent receptor binding. Such a heterodimer would bind to receptors in a monovalent manner and might thus prevent receptor dimerization and activation. Specific PDGF antagonists could be clinically useful to inhibit PDGF in conditions involving excess PDGF stimulation, e.g. certain fibrotic conditions.

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