Assignment of Intrachain Disulfide Bonds in Platelet-derived Growth Factor B-chain*

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Platelet-derived growth factor (PDGF)-BB is a dimeric protein held together by two disulfide bonds involving the 2nd and 4th cysteine residues from the NH2 terminus. To localize the three intrachain disulfide bonds in PDGF, a method was devised that made it possible to cleave PDGF at specific sites. A set of PDGF derivatives in which specific amino acids were mutated to methionine residues was generated. The recombinant proteins, immunoprecipitated from metabolically labeled transfected COS cells, were then subjected to CNBr cleavage and analyzed by SDS-gel electrophoresis under nonreducing conditions. Based on whether the mutated proteins remained in one piece or fell apart after CNBr cleavage, it was possible to deduce the disulfide bond arrangement in the PDGF B-chain; one bond involves the 1st and 6th cysteine residues, another the 3rd and 7th, and the last the 5th and 8th. The latter disulfide bond was found to be dispensable for receptor binding, whereas the former two were found to be essential for the correct folding or stability of the PDGF B-chain.

Platelet-derived growth factor (PDGF)† is a potent mitogen for connective tissue cells and occurs as homo- or heterodimers of disulfide-bonded A- and B-polypeptide chains (reviewed in Refs. 1 and 2). The PDGF isoforms exert their biological effects via binding to two structurally similar protein-tyrosine kinase receptors. Ligand binding induces receptor dimerization; since the α-receptor binds both PDGF A- and B-chains, whereas the β-receptor only binds the B-chain, the different isoforms induce different dimeric receptor complexes (3–5). Receptor dimerization leads to autophosphorylation of the receptor molecules, which occurs in trans in the receptor complex (6). The autophosphorylation provides attachment sites for downstream components in the signaling pathway and allows their phosphorylation (reviewed in Ref. 7).

The PDGF A- and B-chains display 60% amino acid identity in their mature parts with a perfect conservation of the 8 cysteine residues (8). An identical spacing of the cysteine residues has recently also been found in two other dimeric protein-tyrosine kinase receptors. Ligand binding induces receptor dimerization; since the α-receptor binds both PDGF A- and B-chains, whereas the β-receptor only binds the B-chain, the different isoforms induce different dimeric receptor complexes (3–5). Receptor dimerization leads to autophosphorylation of the receptor molecules, which occurs in trans in the receptor complex (6). The autophosphorylation provides attachment sites for downstream components in the signaling pathway and allows their phosphorylation (reviewed in Ref. 7).

The PDGF A- and B-chains display 60% amino acid identity in their mature parts with a perfect conservation of the 8 cysteine residues (8). An identical spacing of the cysteine residues has recently also been found in two other dimeric growth factors, vascular endothelial growth factor/vascular permeability factor (9, 10) and placental growth factor (11). A detailed structural characterization of PDGF is important in order to understand its interaction with the receptors and for the design of specific antagonists. We recently identified the 2nd and 4th of the 8 cysteine residues in PDGF as being involved in interchain disulfide bonding; mutation of these residues to serine residues led to the production of monomeric PDGF with retained receptor binding activity (12).

The aim of this study was to determine the localization of the three intrachain disulfide bonds in each PDGF chain. Due to the high density of cysteine residues in PDGF, it is virtually impossible to use the conventional method involving proteolytic digestion, followed by the isolation of disulfide-bonded fragments. We have therefore produced a series of mutants of the monomeric PDGF B-chain in which we have replaced specific amino acid residues with methionine residues. Through an analysis of these mutants by SDS-gel electrophoresis under nonreducing conditions after incubation with CNBr, which induces specific cleavages at methionine residues, we have been able to deduce the localization of all three intrachain disulfide bonds in PDGF. One of these bonds, between the 5th and 8th cysteine residues, was found not to be essential for bioactivity.

Materials and Methods

Construction of cDNAs Encoding PDGF Mutants—cDNAs encoding the wild-type PDGF B-chain, PDGF-Bstop (13), and PDGF-monoB (12) have been described. Substitutions of codons corresponding to Ile-111, Ser-151, Val-139, Thr-144, and Lys-179 (numbering as described in Ref. 8) with methionine residues were done using the method of Kunkel et al. (14) on a uracil-containing template encoding PDGF-monoB, a mutant in which the 2nd and 4th cysteine residues (Cys-124 and Cys-133, respectively), which are involved in interchain disulfide bonding, were mutated to serine residues and in which a stop codon is present at position 191.

The expression vectors pSVmonoB-M131, pSVmonoB-M144, pSVmonoB-M111/M179, pSVmonoB-M139/M179, pSVmonoB-M111/M139/M144, and pSVmonoB-M139/M179 were generated by cloning the mutated fragments into the BglII/EcoRI sites of the expression vector pSV-PDGF-B (13), in which the corresponding fragments had been excised. Substitutions of the codons corresponding to the 1st and 6th (Cys-97 and Cys-133, respectively), 3rd and 7th (Cys-124 and Cys-133, respectively), 5th and 8th (Cys-134 and Cys-180, respectively) cysteine residues with serine residues were done using the Altered Sites™ in vitro mutagenesis system (Promega Biotec) on a template encoding PDGF-Bstop. Generation of the expression vectors pSVBstopSer16, pSVBstopSer37, and pSVBstopSer58 was done by cloning into the EcoRI site of pSV7d. All the plasmids were sequenced over the region encoding the mature parts of the proteins.

Expression and Immunoprecipitation of Recombinant Proteins—The pSV constructions encoding the PDGF chain mutants as well as pSVBstop and pSVmonoB were transfected into COS cells as described (13) using 15 μg of plasmid DNA and 0.5–1×10⁶ cells in 100-mm culture dishes. Two days after transfection, metabolic labeling was performed by growing the cells overnight in 1.5 ml of cysteine-free MCDB 104 medium supplemented with 0.1 μCi of [35S]cysteine/ml, 10% dialyzed fetal calf serum, and antibiotics. After labeling, the media were collected and cleared of cell debris by centrifugation. The cells were washed once in phosphate-buffered saline; collected by
scraping; and lysed in 0.5 ml of 0.5 M NaCl, 20 mM Tris-HCl, pH 7.5, 0.5% Triton X-100, 1% Trasylol (Sigma), and 1 mM phenethylsulfonyl fluoride. The cell lysates were centrifuged for 15 min at 10,000 × g, and the supernatants were preincubated by incubation with 15 μl of normal rabbit serum at 4 °C for 1 h, followed by the addition of 50% protein A-Sepharose slurry in phosphate-buffered saline. After incubation at 4 °C for 30 min, the beads were removed by centrifugation. The conditioned media and cell lysates were then subjected to immunoprecipitation; the samples were incubated for 2 h at 4 °C with 15 μl of rabbit antiserum against PDGF-BB (15). After incubation with protein A-Sepharose as described above, the beads were washed five times with 0.5 M NaCl, 20 mM Tris, pH 7.5, 5 mg/ml bovine serum albumin, 1% Triton X-100, and 0.1% SDS and once with 20 mM Tris-HCl, pH 7.5. The immunocomplexes were eluted by the addition of 200 μl of nonreducing sample buffer and incubation at 95 °C for 3 min. Half of the eluted material was reduced by the addition of dithiothreitol to a final concentration of 10 mM, followed by incubation at 95 °C for 2 min, and then alkylated by the addition of iodoacetamide to a final concentration of 50 mM. The samples were analyzed by SDS-gel electrophoresis using 12–18% polyacrylamide gels, followed by fluorography.

**Digestion of Immunoprecipitated Proteins with CNBr**—Washed Sepharose beads containing material immunoprecipitated with PDGF-BB antiserum from [35S]cysteine-labeled transfected COS cells were divided into two parts that were incubated in the absence or presence of 10 mg/ml CNBr in 75 μl of 70% formic acid. After centrifugation to remove the Sepharose beads, the samples were kept at room temperature in the dark for 36 h. The samples were then dried under nitrogen, dissolved in 100 μl of distilled H2O, and dried in a rotor evaporator. Samples were dissolved in 200 μl of nonreducing sample buffer and analyzed as described above.

**Receptor Binding Analysis of Recombinant Proteins**—COS cells were transfected with DNA encoding PDGF mutants, and conditioned media were collected as described above. The amount of PDGF-BB receptor binding activity was determined by analyzing serial dilutions with regard to their ability to compete with 125I-PDGF-BB for binding to human foreskin fibroblasts (AG 1518, purchased from the Human Mutant Cell Repository, Camden, NJ). Cells were grown in Falcon 24-well plates to confluence, depleted of α-receptors by a 30-min preincubation with 50 ng/ml PDGF-AA at 37 °C, and then washed once in binding buffer (phosphate-buffered saline containing 1 mg/ml bovine serum albumin, 0.9 mM CaCl2, and 0.5 mM MgCl2). Cell cultures were then incubated at 0 °C for 2 h in 200 μl of binding buffer containing different dilutions of the conditioned media or known amounts of PDGF-BB for standardization of the assay. The cells were washed twice with binding buffer before radiolabeled PDGF-BB (~0.5–2 ng containing 15,000–30,000 cpm), in 200 μl of binding buffer, was added. After incubation at 0 °C for 1 h, the cells were washed five times with binding buffer and then lysed in 200 μl of 20 mM Tris-HCl, pH 7.5, 1% Triton X-100, and 10% glycerol at room temperature for 20 min. The amount of solubilized 125I radioactivity was measured in a γ-counter. Labeled PDGF-BB and unleabeled PDGF-BB were obtained as described (16).

**Results**

**Assignment of Intrachain Disulfide Bonds in PDGF**—PDGF does not contain any free SH groups. The assignment of the 2nd and 4th cysteine residues in PDGF as being involved in interchain disulfide bonding leaves 15 possibilities for the remaining 6 cysteine residues to form three intrachain disulfide bonds (summarized in Fig. 1A). To deduce which of these possibilities is the correct one, methionine residues were introduced into the PDGF-monoB sequence at specific sites (Fig. 1A). We reasoned that analysis of these mutants by SDS-gel electrophoresis under nonreducing conditions before and after CNBr cleavage would make it possible to exclude some of the possible combinations of disulfide bonds.

The type B chains of PDGF contains of PDGF contain a methionine residue, located 4 amino acids NH2-terminal of the 1st cysteine residue in the molecule. Transfection of a PDGF-monoB DNA into COS cells, followed by metabolic labeling with [35S] cysteine and immunoprecipitation, yielded a component of 14 kDa when analyzed by SDS-gel electrophoresis and fluorography; in the absence of dithiothreitol, this component migrated at 16 kDa, indicating that it contained disulfide bonds, as expected (Fig. 2B). After CNBr cleavage, ~90% of the radioactivity shifted to a faster migrating species (Fig. 2A), consistent with the expected effect of removal of 12 amino acids NH2-terminal of the single methionine residue in the PDGF B-chain. To determine whether the 1st and 3rd cysteine residues (Cys-97 and Cys-130, respectively) are connected with a disulfide bond, Ser-131 was changed to a methionine residue. If the 1st and 3rd cysteine residues are connected with a disulfide bond, CNBr cleavage of this mutant would be expected to create two fragments, an NH2-terminal part with one disulfide

![Fig. 1. Schematic illustration of PDGF chain mutants used and possible arrangements of intrachain disulfide bonds in PDGF B-chain. A, the 8 conserved cysteine residues (C) in PDGF are indicated and numbered 1–8 from the NH2 terminus. S indicates replacement of a cysteine residue with a serine residue. M represents the naturally occurring methionine residue in the PDGF B-chain, and the arrows indicate the localization of the residues that have been mutated to methionine residues. B, the conclusions from the analysis of the CNBr sensitivity of the mutants illustrated in A are summarized. The data were obtained from the experiment described in the legend to Fig. 2. The filled circles indicate that analysis of the indicated mutant excludes the disulfide bond arrangement indicated to the left (see the text).](image-url)
FIG. 2. CNBr cleavage of PDGF B-chain mutants. A, conditioned media from [35S]cysteine-labeled COS cells transfected with the different PDGF B-chain methionine mutants as well as medium from mock-transfected cells were immunoprecipitated with an antiserum against PDGF-BB, cleaved with CNBr in formic acid, and then analyzed by SDS-gel electrophoresis in the absence or presence of dithiothreitol (DTT). B, conditioned media were collected as described for A, immunoprecipitated with a PDGF-BB antiserum, and analyzed by SDS-gel electrophoresis in the absence or presence of dithiothreitol.
bond and a COOH-terminal part containing the other two disulfide bonds (Fig. 1A). Analysis by SDS-gel electrophoresis of radiolabeled and immunoprecipitated material revealed a single component migrating slightly faster than the wild-type PDGF-monoB molecule (Fig. 2A). After reduction, two faster migrating components were obtained, indicating that the CNBr cleavage was effective. The faster migration of the unreduced component, compared to wild-type PDGF-monoB, is likely to be due to a conformational change upon cleavage of the chain into two fragments held together by a disulfide bond. Thus, the analysis of this mutant allowed us to exclude the possibility that the 1st and 3rd cysteine residues are connected with a disulfide bond.

We then asked whether the 1st and 8th cysteine residues are connected with a disulfide bond, and we constructed a mutant in which Ile-111 and Lys-179 were changed to methionine residues. Cleavage of this mutant with CNBr would lead to the formation of two components if these 2 cysteine residues were held together by a disulfide bond (Fig. 1A). Analysis by SDS-gel electrophoresis revealed that this was not the case (Fig. 2A); a single component was seen under nonreducing conditions, whereas two components appeared under reducing conditions, verifying that the cleavage with CNBr was effective. In conclusion, the 1st and 8th cysteine residues are not connected with a disulfide bond.

To determine whether the 7th and 8th cysteine residues are disulfide-bonded, a mutant with Thr-144 mutated to a methionine residue was constructed and analyzed. Only one band was seen after CNBr cleavage and analysis by SDS-gel electrophoresis in the absence of reducing agents (Fig. 2A), clearly showing that the 7th and 8th cysteine residues are not disulfide-bonded.

The possibility that the 6th and 7th cysteine residues were disulfide-bonded was explored by the construction of a mutant in which Val-139 and Lys-179 were mutated to methionine residues. Analysis by SDS-gel electrophoresis under nonreducing conditions revealed that two components were obtained, but these were both of a size similar to that of the wild-type PDGF-monoB molecule and thus probably represent incomplete cleavage by CNBr, a conclusion that is supported by the pattern of fragments seen after analysis by SDS-gel electrophoresis under reducing conditions (Fig. 2A). Both of these components thus most likely represent fragments held together by disulfide bonds; the faster migrating species has probably undergone a conformational change as a result of the CNBr cleavage, similar to mutant PDGF-monoB-M131. We conclude that the 6th and 7th cysteine residues are not connected with a disulfide bond.

The analysis of these four PDGF mutants thus allowed us to exclude 9 of the 15 possible combinations of disulfide bonds (Fig. 1B). To distinguish among the remaining six possibilities, it was necessary to construct two additional mutants, each with 3 disulfide bonds.

A mutant in which Ile-111, Val-139, and Thr-144 were mutated to methionine residues proved to be sensitive to CNBr cleavage; two faster migrating species were obtained (Fig. 2A). This result allowed the exclusion of three of the remaining six possibilities since, if the disulfide bonds had been arranged 1-5/3-7/6-8, 1-7/3-5/6-8, or 1-7/3-8/5-6, the four fragments obtained after CNBr cleavage of this mutant would have been held together by the disulfide bonds as one component.

Finally, a mutant in which Ser-131, Val-139, and Lys-179 were mutated to methionine residues was found to be sensitive to CNBr cleavage; part of the material showed a slow migration, but two fragments were liberated (Fig. 2A). This excludes two of the remaining three possibilities; had the disulfide bonds been arranged 1-6/3-8/5-7 or 1-7/3-5/6-8, small fragments would not have been released upon SDS-gel electrophoresis under nonreducing conditions.

Thus, through excluding 14 of the 15 possible arrangements of the intrachain disulfide bonds, the analysis of these six PDGF mutants indicates that the disulfide bonds are arranged 1-6/3-7/5-8. As outlined below, this configuration of the disulfide bonds is also compatible with the finding that the two triple mutants (but none of the other) yield more than one fragment after CNBr cleavage. Regarding the triple mutant PDGF-monoB-M111/M139/M144, which was found to be susceptible to CNBr cleavage, this arrangement of the disulfide bonds would be expected to lead to the formation of two components upon analysis by SDS-gel electrophoresis under nonreducing conditions, one component containing two fragments with the 1st and 6th cysteine residues held together by a disulfide bond and another with two fragments held together by the other two disulfide bonds. The result obtained is compatible with this prediction (Fig. 2A). The other triple mutant, PDGF-monoB-M131/M139/M179, which also was susceptible to CNBr cleavage, was expected to yield a small component that contained two fragments connected with a disulfide bond between the 5th and 8th cysteine residues and a larger component with two fragments held together by the other two disulfide bonds. Again, the result obtained is compatible with this prediction (Fig. 2A). We conclude that the disulfide bonds in the PDGF molecule are arranged 1-6/3-7/5-8.

**Effect of Removal of Individual Intrachain Disulfide Bonds on Receptor Binding Activity of PDGF**—To determine the importance of the individual disulfide bonds in wild-type PDGF-BB, the codons encoding the cysteine residues forming the intrachain bonds were mutated pairwise to encode serine residues. The DNA constructs were then transfected into COS cells metabolically labeled with [35S]cysteine, and the protein products were analyzed by immunoprecipitation and for receptor binding. Constructs in which the 1st and 6th or the 3rd and 7th cysteine residues were mutated did not yield any immunoprecipitable product (data not shown). This suggests that the corresponding disulfide bonds are essential for the correct folding or stability of the product. In contrast, the mutant in which the 5th and 8th cysteine residues were mutated was recognized by the PDGF-BB antiserum and yielded components of the expected size, i.e. 30 and 15 kDa before and after reduction, respectively (Fig. 3A). Furthermore, this mutant had the same ability to compete with I-125I-PDGF-BB for binding to the PDGF β-receptor compared to wild-type PDGF-BB (Fig. 3B).

**DISCUSSION**

In this study, we present evidence that the intrachain disulfide bonds in the PDGF B-chain are localized between the 1st and 6th, 3rd and 7th, and 5th and 8th cysteine residues (Fig. 4). Earlier work has shown that the 2nd cysteine residue forms a disulfide bond with the 4th cysteine residue in the other chain in the dimer and vice versa (12, 17). During the preparation of this manuscript, the three-dimensional structure of PDGF-BB at 3.0-Å resolution was published (18). The predictions regarding the localization of the disulfide bonds in the work by Oefner et al. (18) are supported by the results obtained by our mutational approach.

We present evidence that the disulfide bond between the 5th and 8th cysteine residues is not important for bioactivity; in fact, a mutant lacking this disulfide bond had receptor binding activity similar to that of wild-type PDGF-BB (Fig.
Intrachain Disulfide Bonds in PDGF

A. conditioned media from [35S]cysteine-labeled COS cells transfected with pSVBstop (lane B) or pSVBSer58 (lane BSer58) as well as medium from mock-transfected cells (lane -) were immunoprecipitated with an antiserum against PDGF-BB and then analyzed by SDS-gel electrophoresis. The relative amounts of expressed PDGF-Bstop and PDGF-BstopSer58 were determined by a Phosphorimager and found to be 3.5:1. B, the conditioned media from the experiment described for A were tested at different dilutions for the ability to compete with 125I-PDGF-BB for binding to the β-receptor. Analyses of conditioned media from cells transfected with PDGF-Bstop (○) and PDGF-BstopSer58 (■) and of medium from mock-transfected cells (◼) are shown. The curve for PDGF-BstopSer58 is adjusted to reflect the relative amount of protein expressed, determined as described for A. A standard curve with known amounts of PDGF-BB is indicated (□).

FIG. 3. Analysis of PDGF-BstopSer58. A, conditioned media from [35S]cysteine-labeled COS cells transfected with pSVBstop (lane B) or pSVBSer58 (lane BSer58) as well as medium from mock-transfected cells (lane -) were immunoprecipitated with an antiserum against PDGF-BB and then analyzed by SDS-gel electrophoresis. The relative amounts of expressed PDGF-Bstop and PDGF-BstopSer58 were determined by a Phosphorimager and found to be 3.5:1. B, the conditioned media from the experiment described for A were tested at different dilutions for the ability to compete with 125I-PDGF-BB for binding to the β-receptor. Analyses of conditioned media from cells transfected with PDGF-Bstop (○) and PDGF-BstopSer58 (■) and of medium from mock-transfected cells (◼) are shown. The curve for PDGF-BstopSer58 is adjusted to reflect the relative amount of protein expressed, determined as described for A. A standard curve with known amounts of PDGF-BB is indicated (□).

FIG. 4. Schematic illustration of interchain and intrachain disulfide bonds in PDGF-BB. The cysteine residues (C) are numbered from the NH2 terminus.

4). We showed previously that a mutant in which the 2nd and 4th cysteine residues were mutated to serine residues, thus creating a PDGF molecule without interchain disulfide bonds, retained 20–30% of the β-receptor binding activity (12). In contrast, when the 1st and 6th or the 3rd and 7th cysteine residues were mutated, no product that was recognized by antibodies made against PDGF-BB was synthesized. This indicates that these two disulfide bonds are necessary for the correct folding or stability of PDGF-BB. These results are consistent with previous analysis of the transforming activity of PDGF B-chain mutants in which individual cysteine residues were mutated; the 2nd, 4th, 5th, and 8th cysteine residues were dispensable for transforming activity, whereas the transforming activity was lost when the 1st, 3rd, 6th, or 7th cysteine residue was mutated (19, 20).

Taken together, the results from this and previous studies suggest the possibility that PDGF activity can be obtained in a molecule containing only 4 cysteine residues, forming disulfide bonds between the 1st and 6th and the 3rd and 7th cysteine residues. Although procedures have been described for the in vitro folding and dimerization of PDGF produced in bacteria (21), a PDGF form with only 4 cysteine residues should be easier to recover with high yield in a properly folded form. Since recombinant PDGF, obtained from mammalian cell expression systems, has been shown to accelerate the healing of skin ulcers (22), PDGF might be clinically useful; thus, more efficient expression systems for recombinant PDGF are highly warranted.

PDGF contains many cysteine residues; moreover, 5 of them are concentrated over an 18-amino acid region that is very resistant to proteolysis. Therefore, the conventional technique for the localization of disulfide bonds, i.e. the iso-
lation of disulfide-bonded fragments after proteolytic cleavage of the molecule, proved not to be successful.\(^2\) As an alternative, we introduced methionine residues in the PDGF molecule to make it susceptible to CNBr cleavage at specific sites. SDS-gel electrophoresis analyses under nonreducing conditions of \(^{35}\)S-cysteine-labeled CNBr-cleaved material made it possible to deduce the localization of the disulfide bonds in PDGF. Since the migration distance in SDS-gel electrophoresis of small peptides does not always correlate with their size, particularly under nonreducing conditions, our analysis aimed only at determining whether the CNBr-cleaved molecule was held together in one disulfide-bonded complex or not. The method outlined in this study should be generally applicable to the assignment of disulfide bonds in other molecules.

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