Disulfide Bonds in Recombinant Human Platelet-Derived Growth Factor BB Dimer: Characterization of Intermolecular and Intramolecular Disulfide Linkages

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ABSTRACT: Interchain cystines of PDGF-BB dimer were characterized by Edman reaction and by SDS–PAGE analysis on the protein which was chemically cleaved at Trp-40. It was found that Cys-43 has a key role in dimer formation, asymmetrically cross-linked to a cysteine residue of another identical subunit. The remaining cystines participate in the intramolecular disulfide linkages. Pepsin digestion of PDGF-BB dimer generated several small peptides and one ubiquitous Cys-containing peptide. Sequence analyses of several Cys-containing peptides indicated the existence of three intramolecular disulfide linkages including Cys-16–Cys-60, Cys-49–Cys-97, and Cys-53–Cys-99. Two interchain disulfide bonds of Cys-43–Cys-52 between two subunits were deduced from the partial reduction and alkylation of PDGF-BB. This study provides chemically determined disulfide linkages of PDGF-BB.

Human platelet-derived growth factor (PDGF)[1] is a potent mitogen for connective tissue cells and promotes the proliferation of fibroblasts and smooth muscle cells (Robbins et al., 1983; Heldin & Westermark, 1984; Deuel et al., 1985; Ross et al., 1986). The protein has an apparent molecular mass of 30 kDa consisting of two polypeptide chains linked by disulfide bonds. There are two homologous chains termed A and B that retain 60% sequence homology (Waterfield et al., 1983; Doolittle et al., 1983; Stroobant & Waterfield, 1984; Betsholtz et al., 1986; Heldin et al., 1986; Hammacher et al., 1988; Bowen-Pope et al., 1989). These chains form active dimers represented as AA, AB, and BB. Each dimer is distributed in a variable manner depending upon tissue specificity or species difference; the AA form is detected in various types of tumor cell lines, while the AB form is predominant in human platelets, and the BB form is mainly detected in porcine platelet extracts (Stroobant & Waterfield, 1984).

Proteolytic processing at the C-terminus of gene products of the A and B chains has been reported (Waterfield et al., 1983). Both chains share a similar location of cysteine residues, resulting in eight disulfide linkages in the dimer form. It is believed that the three dimers (AA, AB, and BB) conserve similar disulfide linkages. Despite evolutionary divergence (Doolittle et al., 1983; Waterfield et al., 1983), all forms of PDGF studied to date conserve all eight homologous cysteine residues. Therefore, the disulfide bridges in the PDGF molecule have a critical role in expressing biological activity.

When PDGF dimer is treated with reducing agents, the protein loses its biological activity, suggesting that the protein conformation is disturbed by reduction of critical disulfide bond(s) (Antoniades et al., 1979; Heldin et al., 1981). The secondary structure of PDGF dimer is strongly associated with disulfide bridges. Because of the eight disulfide bonds in the dimer form and the unique amphiphilicity of the molecule, the native molecule may have unique secondary or tertiary structure. Circular dichroism spectrometry has shown that the carbon backbone chain is occupied mostly by random coil and a small percentage of α-helix (Betsholtz et al., 1986; Vogel & Hoppe, 1989). More recent Fourier-transform infrared spectroscopy studies indicate that PDGF is rich in β-structure (Prestrelski et al., 1991). On the basis of computer protein modeling, receptor binding regions or epitopes were tentatively assigned to be one of the hydrophilic regions of PDGF which is sensitive to trypsin (Vogel & Hoppe, 1989).

The determination of disulfide bonds in PDGF dimer is important for elucidating the structure–function relationships of this protein. Several attempts have been made to determine the disulfide linkages by site-directed mutagenesis of cysteine residues (Giese et al., 1987; Sauer & Donoghue, 1988). Since each single mutant of cysteines-43, -52, -53, and -99 abolished the formation of PDGF dimer, these residues are proposed to be involved in an interchain cross-linking. These cysteines were not essential for the v-*sis* gene product to act as a functional ligand for the PDGF receptor (Giese et al., 1987). Recently, disulfide linkages of PDGF-AA were proposed by Jaumann et al. (1991) using a similar approach. The proposed model contains several disulfide linkages including Cys-16–Cys-97 and Cys-49–Cys-60 for intramolecule cross-linkings, while other disulfide linkages (Cys-43–Cys-52 and probably Cys-53–Cys-99) are proposed to form the intermolecular cross-linkings. However, some of these disulfide linkages are inconsistent with experimental evidence and remain to be clarified by direct peptide analysis. We report here two dimeric cystines of PDGF-BB determined by SDS–PAGE analysis after Edman reactions. From the direct sequence analysis of the multiple digests or the chemically cleaved fragments, we have differently identified intramolecular disulfide linkages from the previous report (Jaumann et al., 1991).

MATERIALS AND METHODS

Materials. Pepsin was purchased from Sigma Chemical Co. (Chicago, IL). TPCK-trypsin, *Staphylococcus aureus* protease V8, and endoproteinase Lys-C were from Boehringer/Mannheim (Indianapolis, IN). BNPS-skatole was obtained from Pierce Chemical Co. (Rockford, IL). All other chemicals were of highest quality.

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[1] Abbreviations: PDGF, platelet-derived growth factor; rHuPDGF-BB, recombinant human PDGF-BB; BNPS-skatole, 2-[(2-nitrophenyl)-sulfenyl]-3-methyl-3-bromoindolenine; DTT, dithiothreitol; TPCK, tosylphenylalanyl chloromethyl ketone; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; PVDF, poly(vinylidene difluoride); CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid.

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Preparation and Purification of PDGF-BB. The human recombinant PDGF-BB was prepared from *Escherichia coli* cells by a proprietary procedure to a homogeneous protein as observed by SDS–PAGE (Prestrelski et al., 1991). The specific activity of this protein was comparable to that expected for the natural product. The purified PDGF-BB dimer did not contain any free sulfhydryl residue, suggesting that all cysteine residues formed disulfide linkages.

Partial Reduction and Alkylation of PDGF. For titration experiments, the sample (20 μg) was reduced with different concentrations of DTT (0.2 and 2 mM) in 0.1 M sodium phosphate buffer, pH 7.2, for 1 h at room temperature. The total volume was adjusted to 100 μL. The sample was added to 5 μL of 4-vinylpyridine and mixed vigorously. The sample remained at room temperature overnight (12 h).

Enzyme Digestions. Pepsin digestion of native PDGF dimer was performed as follows: the protein (100 μg/100 μL) was diluted with 300 μL of 0.02 N HCl (pH 1.8), the pepsin (5 μg/5 μL) was added. The digestion was allowed to proceed 12–24 h at 37 °C without stirring, and the digested material was then subjected to reversed-phase HPLC. The digestions of native PDGF with thermolysin, trypsin, chymotrypsin, and endoproteinases Asp-N or Lys-C were performed at 37 °C for 24 h in 0.1 M Tris-HCl buffer, pH 7.3. The enzyme: substrate ratio was 1:25. A double digestion was performed as follows: the chymotryptic digestion was carried out for 24 h; then trypsin was added to the sample without any change of buffer conditions.

Chemical Cleavage of PDGF. For tryptophan cleavage, PDGF-BB dimer (100 μg/100 μL) was diluted with 100–200 μL of glacial acetic acid in an Eppendorf tube, and 1–2 mg of BNPS-skatole was added. The reaction was carried out at room temperature for 24 h in the dark. The cleavage product was initially washed 3 times with ethyl acetate (500 μL) in order to remove the excess reagents and further purified by reverse-phase HPLC using a Vydac C18 column (4.6 × 250 mm). The reduced and alkylated sample was directly treated with BNPS-skatole (1 mg) overnight at room temperature in order to cleave the Trp residue.

In situ chemical cleavage with cyanogen bromide was performed in a reaction cartridge of the ABI gas-phase sequencer. The peptide sample sequenced on a glass fiber disk precoated with Polybrene was treated with cyanogen bromide dissolved in 30 μL of 70% formic acid. The cartridge was sealed with vinyl tape, wrapped with a parafilm, and incubated at 45 °C for 1 h. The sample was unwrapped in a hood and dried under a stream of nitrogen.

Isolation of Peptide Fragments. Peptides were separated by reverse-phase HPLC. In all cases, the whole mixture was applied directly to a Vydac C18 column (218 TP54, 4.6 × 250 mm), equilibrated with 5% solvent B (0.1% TFA/90% acetonitrile). Peptides were eluted by a linear gradient from 5% solvent B to 60% solvent B over 60 min with a flow rate of 0.7 mL/min.

Other Analytical Methods. SDS–polyacrylamide gel electrophoresis for peptides or proteins were performed as described (Laemmli, 1970). Electroblotting was carried out according to the method described by Applied Biosystems, Inc. (1991). After SDS–PAGE analysis, the gel was rinsed with water and 10 mM CAPS buffer, pH 10.5. The gel was overlaid with PVDF prewashed with methanol, followed by the same buffer. Electroblotting was carried out in a cold room with 0.3 A for 2–3 h in 10 mM CAPS buffer. Staining with Coomassie Blue and destaining with 50% methanol were carried out at room temperature.

Table I: Sequence Analysis of the BNPS Fragment of Acetylated PDGF[a]

	fragment 1		fragment 2		
cycle	amino acid	pmol	amino acid	pmol	PTH-Cys (pmol)
1	Pro	141			
2	Pro	114			
3	Cys-43				
4	Val	108			
5	Glu	95			
6	Val	116			
7	Gln	85			
8	Arg	31			
9	Cys-49				
10	Ser	36			
11	Gly	57			
12	Cys-52				
13	Cys-53				
14	Asn	54			
15	Asn	65			
16b	Arg	28			
17	Asn	52	Ile	228	0
18	Val	43	Ala	188	0
19	Gln	31	Glu	121	0
20	**Cys-60**		**Cys-16**		7.4 (24%)[c]
21	Arg	29	Lys	137	3.1 (10%)
22	Pro	24	Thr	82	2
23	Thr	31	Arg	57	0

[a] Sample amount analyzed: approximately 300 pmol. [b] After 16 cycle runs of Edman degradation, the sample was in situ digested with CNBr, followed by sequence analysis. [c] A percentage of recovery was calculated on the basis of the common PTH yield from the plot curve.

Protein sequences were determined with a gas-phase sequencer, Model 470A (Applied Biosystems), with on-line detection using the narrow-bore column of Applied Biosystems. PTH analysis was performed by standard procedures. In a slight modification of the elution condition, PTH-cystine eluted in front of the PTH-Tyr peak with a retention time of 14.12 min, while PTH-Tyr eluted at 14.38 min. The quantitative analysis of PTH-cystine was performed using the same calibration factor for Tyr.

Extraction of the peptide or protein from the PVDF membrane was performed by eluting with 200 μL of 70% 2-propanol/10% TFA or 100% TFA at 25 °C for 30 min. The sample was evaporated in vacuo (Savant) and subjected to SDS–PAGE as described above.

RESULTS

PTH-Cys Analysis on BNPS-Skatole Cleavage Products of Acetylated PDGF. PDGF-BB was initially acetylated to block all amino groups including the NH_2-terminal Ser, and the acetylated protein was cleaved at Trp-40 with BNPS-skatole. The sample was desalted by passing through a Vydac C18 column (data not shown) and sequenced by a gas-phase sequencer. The resulting sample gave a single sequence initiated by Pro-41. After 16 cycle runs of Edman reactions, the sample was in situ digested with CNBr on a glass fiber disk as described under Materials and Methods. The sequence analysis was continued further to cycle 23 as shown in Table I. This procedure was important to obtain the Cys residues at the same position in these fragments. When a cross-linked cystine is located at the same position as in bovine insulin, PTH-Cys is highly recovered as much as 20% of the common PTH yield. However, if the cross-linked cystine was not located at the same position in the peptides, the recovery of PTH-Cys was diminished to approximately 2–3% of the regular PTH derivatives such as Ala, Val, or Leu. In the case of PTH-Cys analysis of PDGF peptide, a significant amount of

Table II: Analysis of Lys-C- and Trypsin-Digested Peptides[a]

	peptide 1		peptide 2		peptide 3		
cycle	amino acid	pmol	amino acid	pmol	amino acid	pmol	PTH-Cys (pmol)
1	Ser	384	Thr	742	Lys	100	
2	Leu	652	Asn	429	Pro	63	
3	Gly	461	Ala	738	Ile	88	
4	Ser	244	Asn	427	Phe	59	
5	Leu	573	Phe	554	Lys	51	
6	Thr	713	Leu	1025	Lys	93	
7	Ile	514	Val	476	Ala	87	
8	Ala	453	Trp	202	Thr	158	
9	Glu	396	Pro	376	Val	168	
10	Pro	396	Pro	396	Thr	112	
11	Ala	602	Cys-43		Leu	223	3
12	Met	306	Val	282	Glu	89	4
13	Ile	357	Glu	294	Asp	52	4
14	Ala	399	Val	398	His		4
15	Glu	512	Gln	187	Leu	59	3
16	Cys-16		Arg	129	Ala	143	3
17	Lys	207	**Cys-49**		**Cys-97**		11.2 (18%)[b]
18	Thr	286	Ser	72	Lys	183	13.5 (22%)
19	Arg	100	Gly	85	Cys-99		9
20	Thr	237	Cys-52		Glu	75	8

[a] Sample amount applied to sequencer was approximately 1 nmol. [b] See footnote *c* in Table I.

PTH-Cys (24% recovery) was detected at cycle 20 which corresponds to Cys-16 and Cys-60. This demonstrates that Cys-16 and Cys-60 were cross-linked in the protein molecule.

Analysis of Endoproteinase Lys-C- and Trypsin-Digested Fragments. In order to determine other disulfide linkages, an alternative peptide fragment was generated by double digestions. PDGF was initially digested with Lys-C endoproteinase as described under Materials and Methods, followed by trypsin digestion. The resulting fragments were initiated by Ser-1, Thr-33, and Lys-81, suggesting that specific cleavages occurred at Arg-32 and Lys-80. Two fragments have cysteine residues at cycle 17, whereas an NH_2-terminal peptide contains cysteine at cycle 16. PTH-Cys analysis is shown in Table II, indicating that cycle 17 gave a significant yield of PTH-Cys, 18% PTH derivatives of the common amino acids. Since cycle 17 corresponds to Cys-49 and Cys-97, these cysteine residues were determined to be cross-linked by a disulfide bridge. Although cycle 18 showed a high recovery of PTH-Cys, it could be due to the carry-over from the previous cycle. The carry-over usually can be observed after many cycles of Edman reactions. Since a disulfide linkage including Cys-16 was already assigned by a previous study, Cys-16 can be excluded from a linkage with Cys-49 or -97. Thus, the second disulfide bond was determined to be Cys-49–Cys-97.

Sequence Analysis of Tryptic Peptides of Acetylated PDGF. To confirm the disulfide linkage Cys-49–Cys-97, the following experiment has been done. PDGF was acetylated with acetic anhydride (10 μL) at 25 °C for 30 min in order to block NH_2-terminal Ser and Lys residues. This procedure was necessary to minimize the sequences obtained from the tryptic fragments. The digest was purified by HPLC, representing a similar profile to the native protein (data not shown). Trypsin should cleave several peptide bonds in PDGF-BB, whereas only two sequences were detected by the analysis. Table III shows the peptide sequences which were cleaved at Arg-32 and Arg-79. Although in these peptides cysteine residues were not located at the same position, a significant amount of PTH-Cys was detected in cycle 18 which corresponds to Cys-49 and Cys-97. This was probably due to the PTH carry-over from cycle 17 (Cys-49). Similarly, cycle 19 would be reflected by carry-over from cycle 18. PTH-cystine at cycle 20 was relatively low although Cys-52 and Cys-99 were present at the same position, suggesting that Cys-52 was not bonded to Cys-99. A relatively high yield of PTH-cystine obtained at cycle 21 was derived from the disulfide linkage Cys-53–Cys-99. This suggests the presence of a disulfide linkage between Cys-53 and Cys-99.

Table III: Sequence Analysis of Tryptic Fragments from Acetylated PDGF[a]

	fragment I		fragment II		
cycle	amino acid	pmol	amino acid	pmol	PTH-Cys (pmol)
1	Thr	1218	(Lys)[b]		0
2	Asn	800	(Lys)		0
3	Ala	1069	Pro	641	0
4	Asn	773	Ile	846	0
5	Phe	932	Phe	932	0
6	Leu	901	(Lys)		0
7	Val	868	(Lys)		0
8	Trp	521	Ala	709	0
9	Pro	788	Thr	572	0
10	Pro	744	Val	498	0
11	**Cys-43**		Thr	640	2
12	Val	728	Leu	495	4
13	Glu	428	Glu	428	2
14	Val	614	Asp	325	1
15	Gln	353	His	115	1
16	Arg	409	Leu	419	0
17	**Cys-49**		Ala	430	3
18	Ser	150	**Cys-97**		44 (14%)[c]
19	Gly	208	(Lys)		20 (10%)
20	**Cys-52**		**Cys-99**		19 (6%)
21	**Cys-53**		Glu	239	32 (11%)
22	Asn	136	Thr	244	20 (7%)
23	Asn	269	Val	217	10

[a] Sample amounts used: approximately 1.5 nmol. [b] Not quantitated due to acetylation of the ε-amino group of lysine. [c] See footnote *c* in Table I.

Table IV: Sequence Analysis of Trypsin-BNPS Fragments of PDGF[a]

	fragment I		fragment II		
cycle	amino acid	pmol	amino acid	pmol	PTH-Cys (pmol)
1	Pro	426	Ala	428	
2	Pro	432	Thr	311	
3	**Cys-43**		Val	318	
4	Val	908	Thr	454	
5	Glu	317	Leu	450	
6	Val	557	Glu	488	
7	Gln	325	Asp	182	
8	Arg	278	His	54	
9	**Cys-49**		Leu	363	0
10	Ser	134	Ala	433	0
11	Gly	212	**Cys-97**		14 (4%)[b]
12	**Cys-52**		Lys	276	38 (11%)
13	**Cys-53**		**Cys-99**		47 (15%)
14	Asn	143	Glu	299	38 (13%)
15	Asn	276	Thr	179	12
16	Arg	234	Val	175	

[a] Sample amounts analyzed: approximately 1 nmol. [b] See footnote *c* in Table I.

Sequence Analysis of Trypsin- and BNPS-Cleaved Fragments. Another Cys-containing peptide of PDGF was obtained from trypsin digestion, followed by BNPS-skatole cleavage. The HPLC-purified peptide was directly analyzed in a gas-phase sequencer, resulting in three major sequences. Table IV shows sequence results and PTH-cystine analysis. To simplify the data, the sequence of an NH_2-terminal fragment, S-L-G-S-L-T-I-A-E-P-A-M-I-A-E-C-K-T-R-T-E-V-F-E-I-S-R (residues 1–27), was deleted in this table. In

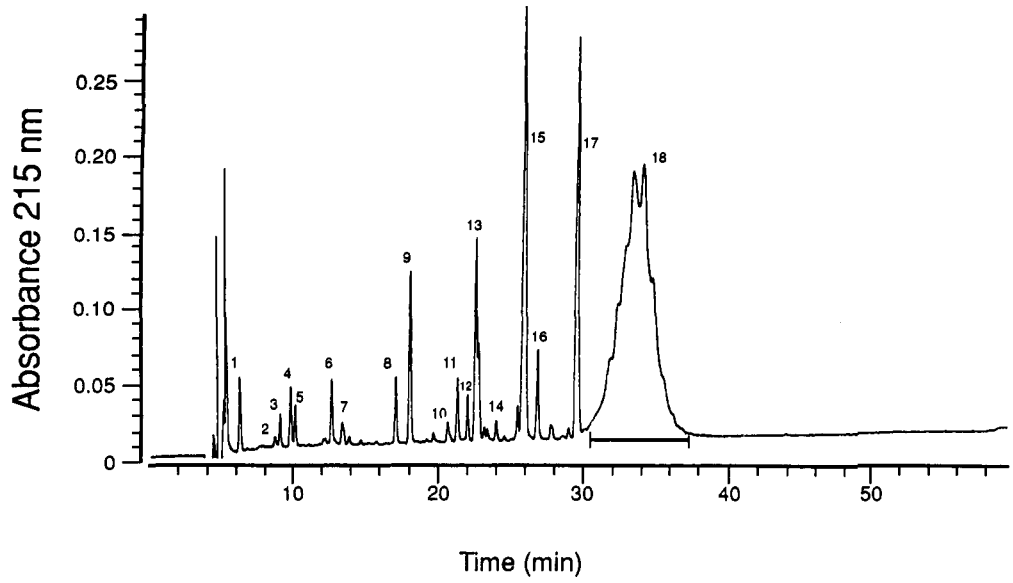

FIGURE 1: HPLC peptide map of the peptic digest of intact PDGF. The native sample (100 μg) was digested with pepsin (5 μg) in 0.02 N HCl, pH 1.8, for 24 h at 37 °C. The broad peak which eluted at 30–37 min was collected and analyzed for determination of disulfide linkages.

this analysis, PTH-cystine was significantly detected in cycles 12–14, corresponding to Cys-52, Cys-53, Cys-97, and Cys-99. The results suggest that two disulfide linkages including Cys-52–Cys-97 or Cys-53–Cys-99 are possible according to the PTH recovery, whereas considering the previous results and the relative yield of PTH-Cys in this analysis, Cys-53–Cys-99 linkage is more likely than the other. A relatively high yield of PTH-Cys at cycle 11 might be due to the disulfide linkage Cys-49–Cys-97 assigned previously.

Sequence Analysis of the Peptic Fragment. Intact PDGF-BB (100 μg) was digested with pepsin in order to isolate different Cys-containing peptides. The typical HPLC map is shown in Figure 1, exhibiting several short peptides and one ubiquitous core peptide. Sequence analyses of all peptide peaks suggested that most of the short peptides were derived from the surface of the protein due to vigorous proteolysis, whereas the broad peak that appeared at 35 min contained three or four Cys-containing peptides. This core material could not be further purified because of association through the multiple disulfide linkages. A direct sequence analysis of the core material (peak 18) is shown in Table V, revealing that the sample contained basically three peptides; peptides 1, 2, and 3; peptide 4 contained one additional Leu residue at the NH_2-terminus to peptide 3 due to the partial proteolytic cleavage. Although the apparent PTH recovery from peptide 3 was as low as 50% of the other peptides, the total recovery of PTH from peptides 3 and 4 was equivalent to those for either peptide 1 or peptide 2. From a significant recovery of PTH-Cys in cycle 12 (13.2 pmol, corresponding to 2% recovery of regular PTH), one would expect that the Cys-49 likely links one of the cysteines-16, -43, -97, or -99. Together with our previous assignments and site-directed mutagenesis results (Sauer & Donoghue, 1988), the disulfide linkage Cys-49–Cys-97 is most likely.

Determination of a Dimeric Cysteine Residue. PDGF-B contains only one tryptophan residue per subunit, i.e., Trp-40. The tryptophan cleavage product of PDGF must still be associated by internal or external disulfide linkages. The chemical cleavage yields useful information on the state of Cys-43 which was proposed to be a dimeric cysteine residue. The Trp cleavage product of PDGF-BB dimer was obtained by treatment with BNPS-skatole in 70% acetic acid medium. The product was finally purified by HPLC using a Vydac C18 column (Figure 2). The broad peak eluting at 37–48 min was combined and evaporated for the following experiments. SDS–PAGE analysis of the products is shown in Figure 3A, indicating that the BNPS-treated protein migrated

Table V: Sequence Analysis of the Peptic Core from PDGF[a]

	peptide 1		peptide 2		peptide 3		
cycle	amino acid	pmol	amino acid	pmol	amino acid	pmol	PTH-Cys (pmol)
1	Ile	5095	Leu	6496	Glu	1361	0
2	Ala	3562	Val	3214	Asp	677	0
3	Glu	2023	Trp		His	250	0
4	**Cys-16**		Pro	2141	Leu	784	0
5	Lys	1158	Pro	2111	Ala	933	0
6	Thr	1101	**Cys-43**		**Cys-97**		2.3
7	Arg	630	Val	2008	Lys	704	4.1
8	Thr	1035	Glu	1477	**Cys-99**		3.2
9	Glu	1000	Val	1842	Glu	457	2.4
10	Val	1296	Gln	1254	Thr	497	3.4
11	Phe	432	Arg	840	Val	946	5.3
12	Glu	540	**Cys-49**		Ala	506	13.2*
13			Ser	385			8.0
14			Gly	655			2.5

[a] A duplicate run has been performed using a gas-phase sequencer, ABI Model 470A. PTH-cystine was detected at 14.12 min, in front of PTH-Tyr (14.38 min). In our analysis, PTH-Ser at cycle 13 also gave a trace of the same product, resulting in a slightly higher value. The asterisk shows a significant increase of PTH-cystine.

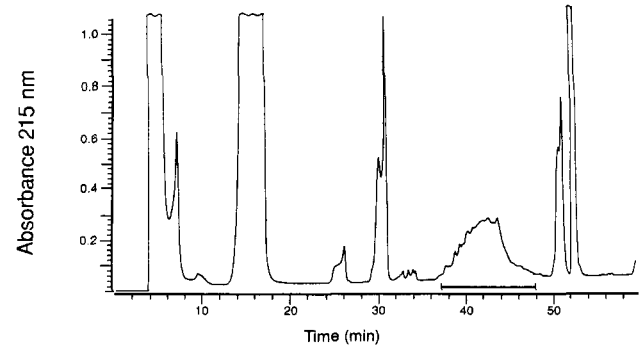

FIGURE 2: Purification of BNPS-skatole-cleaved PDGF. The reaction mixture was directly subjected to reversed-phase HPLC using a Vydac C18 column (4.6 × 250 mm). The cleavage product was eluted as described under Materials and Methods.

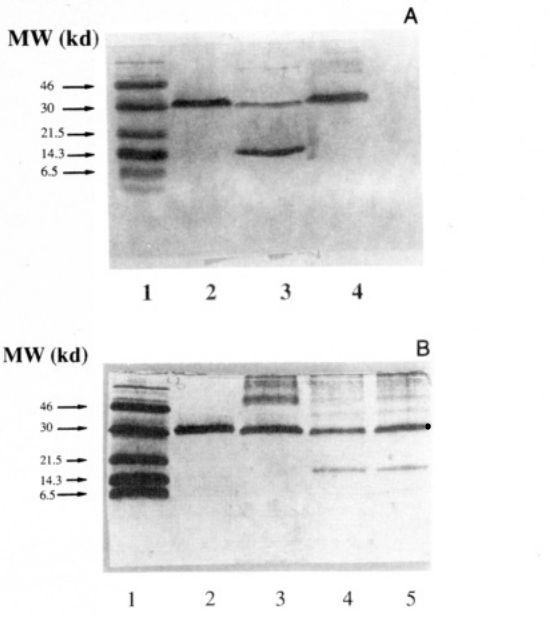

FIGURE 3: SDS–PAGE of BNPS-PDGF before or after Edman degradation. (A) Before Edman degradation: lane 1, standard markers; lane 2, intact PDGF-BB dimer; lane 3, reduced PDGF; lane 4, BNPS-treated PDGF. (B) After Edman degradation: lane 1, standard markers; lane 2, native PDGF; lane 3, BNPS-PDGF after 2 cycles of Edman reaction; lane 4, BNPS-PDGF after 4 cycles of Edman reaction (70% 2-propanol/10% TFA extraction); lane 5, BNPS-PDGF after 4 cycles of Edman reaction (100% TFA extraction).

at an identical position to that of the native protein. BNPS-skatole did not disturb any of the dimeric disulfide linkages, so the products might preserve the native disulfide structure.

Sequence analysis of the BNPS-treated PDGF showed two NH_2-terminal sequences: one is the NH_2-terminal sequence initiated by Ser-1 and the other beginning from Pro-41. The results indicate that specific cleavage of Trp-40 has occurred without any disturbance of the disulfide bonds. The yield of the cleavage was usually 70–80%, and the remaining portion still contained the uncleaved protein. Since the cleaved and uncleaved proteins show similar molecular masses (30 kDa), these fragments could not be separated by either reverse-phase HPLC or gel filtration. To determine whether Cys-43 is involved in dimerization or not, the BNPS product was directly sequenced by the gas-phase sequencer without Polybrene. After two or four cycles of Edman reactions, the samples were extracted from the PVDF membrane with 70% 2-propanol/10% TFA and 100% TFA solution. Both samples were analyzed by SDS–PAGE under nonreducing conditions. Figure 3B shows the results demonstrating that the BNPS products after two cycles of Edman reactions still exhibited a dimer form of 30-kDa molecular mass, while the sample after four cycles of Edman reactions revealed the monomer form in addition to the dimer form which might indicate the uncleaved protein. The sample after 10 cycles of Edman reactions also showed the same result as the second sample (lane 4 in Figure 3B) (data not shown). These SDS–PAGE results suggested that Cys-43 was mainly involved in dimerization of PDGF-BB. However, there are two possibilities in forming a dimer due to the two types of disulfide linkages such as Cys-43–Cys-43 or Cys-43–Cys-?. The sequence results of the BNPS fragments suggest that Cys-43 asymmetrically links to another cysteine residue from a different subunit since only a trace amount of PTH-Cys was detected in cycle 3. If Cys-43 intermolecularly links with Cys-43 of another subunit, the recovery of PTH-Cys in cycle 3 should be as high as 20%.

Table VI: Sequence Analysis of Pyridinylethylated PDGF[a]

	sequence I		sequence II		PTH-PECys (pmol)[b]	
cycle	amino acid	pmol	amino acid	pmol	A	B
1	Ser	49	Pro	182	0	0
2	Leu	213	Pro	180	0	0
3	Gly	172	**Cys-43**		26 (15%)	20 (40%)
4	Ser	102	Val	159	0	3
5	Leu	173	Glu	136	0	0
6	Thr	111	Val	127	0	0
7	Ile	165	Gln	118	0	0
8	Ala	171	Arg	116	0	0
9	Glu	148	**Cys-49**		0	0
10	Pro	128	Ser	46	0	0
11	Ala	135	Gly	61	0	0
12	Met	97	**Cys-52**		13 (13%)	12 (40%)
13	Ile	110	**Cys-53**		5	4
14	Ala	104	Asn	43	0	0
15	Glu	73	Asn	69	0	0
16	**Cys-16**		Arg	20	0	0
17	Lys	30	Asn	57	0	0
18	Thr	35	Val	48	0	0
19	Arg	30	Gln	41	0	0
20	Thr	32	**Cys-60**		0	0

[a] Sample amounts applied to sequencer: approximately 10 μg. [b] PTH-PECys was calculated using a calibration factor for PTH-Pro. Columns A and B show reducing conditions using 0.2 and 2 mM DTT, respectively.

Table VII: Summary of Possible Disulfide Linkages in PDGF-BB

	Cys-16	Cys-43	Cys-49	Cys-52	Cys-53	Cys-60	Cys-97	Cys-99
Cys-16	X							
Cys-43	–[a]	X						
Cys-49	–	–	X					
Cys-52	–	yes	–	X				
Cys-53	–	–	–	no[b]	X			
Cys-60	yes	–	–	–	–	X		
Cys-97	no	no	yes	–	–	–	X	
Cys-99	–	–	–	no	yes	–	no[b]	X

[a] Not analyzed. [b] A linkage between these cysteines can be excluded due to steric hindrance.

We found that bovine insulin possessing cross-linked Cys-7 gave 20–30% recovery of PTH-Cys, compared with the regular PTH derivatives (Haniu et al., 1992).

Partial Reduction and Alkylation of PDGF. To obtain information regarding the nature of disulfide bonds in PDGF, several sets of partial reduction of PDGF were performed with different concentrations of DTT under native conditions. Partial reduction of PDGF was initially analyzed by SDS–PAGE, revealing that PDGF-BB dimer could be readily reduced with 0.2 mM DTT and turned to monomeric form (data not shown). It suggests that initially reduced disulfide linkages are involved in dimer formation. After reduction, the PDGF sample was alkylated with 4-vinylpyridine and treated with BNPS-skatole in order to examine the reduced cysteine residues. Table VI shows the sequence results, including analysis of PTH-(pyridinylethyl)cysteine. Edman cycles 3 and 12 corresponding to Cys-43 and Cys-52 gave a significant amount of PTH-(pyridinylethyl)cysteine, whereas other cysteine residues did not show. The ratio of the relative percentage yield of these modified residues at Cys-43 and -52 is approximately 1:1. Reduction with 2 mM DTT supported this result. From these results, it is concluded that Cys-43 and Cys-52 participate in dimer formation. As mentioned earlier, a dimeric Cys-43 should not be linked to Cys-43 of another subunit, but links only Cys-52 of another subunit.

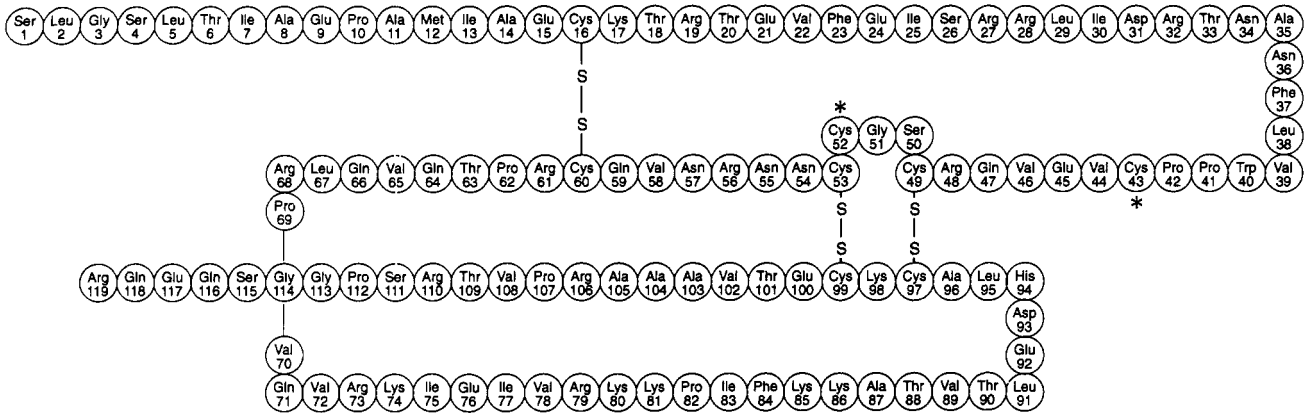

FIGURE 4: Disulfide structure of PDGF-BB. Two disulfide bridges, Cys-43–Cys-52, form an asymmetrical dimer. Intramolecular disulfide bonds Cys-16–Cys-60 and Cys-49–Cys-97 were newly reported in this paper. Highly charged regions (residues 15–32 and 73–94) are significantly solvent-exposed, suggesting an interaction with receptor or antibody.

DISCUSSION

PDGF has important biological activity and is structurally complicated. There are three active forms due to the different combinations created by disulfide linkages of the two subunits. Because of its unique function in wound-healing and proliferation of muscle cell, an elucidation of the relationship between structure and function of PDGF is important. Although the primary structure of PDGF has been determined by cDNA or protein sequence analysis (Waterfield et al., 1983; Johnsson et al., 1984; Betsholtz et al., 1986; Jaumann et al., 1991), secondary and tertiary structures including S–S linkages have not been fully determined.

Although there are some difficulties in detection of the PTH derivative of the cystine residue, we showed the existence of several disulfide bonds in the PDGF-BB molecule by means of direct sequence analysis (Table VII). Our proposed model (Figure 4) shows two identical dimeric cystines consisting of Cys-43 and Cys-52 in PDGF dimer. SDS–PAGE analyses demonstrated that dimer formation relied upon Cys-43 since the monomer form was effectively generated after removal of this residue by Edman reaction. Therefore, since a linkage between Cys-53 and Cys-99 was identified as intramolecular cross-linking by our study, it is unlikely that these cysteines participate in dimer formation as reported for PDGF-AA (Jaumann et al., 1991). A mutation of Cys-99 may cause abolishment of a dimer form as well as Cys-53 due to steric hindrance or some conformational instability. It is known that mutants of cysteine residues (Cys to Ser) significantly change the charge properties (unpublished data),[2] suggesting that site-directed mutagenesis of some critical residues may cause a conformational change of the protein.

An essential disulfide linkage between Cys-16 and Cys-97 or Cys-60 was previously uncertain (Vogel & Hoppe, 1989). Since a site-directed mutagenesis study suggested that Cys-16, -49, -60, and -97 are essential for transforming activity, two disulfide linkages can be expected to be formed between these cysteine residues. A recent model indicated the disulfide linkage between Cys-16 and Cys-97 in PDGF-AA (Jauman et al., 1991), whereas our results demonstrated that functional disulfide linkages were Cys-16–Cys-60 and Cys-49-Cys-97 in PDGF-BB. The model proposed by Jaumann et al. (1991) is not clear whether two or four dimeric S–S linkages between two monomers exist.

[2] R. Rush and M. F. Rohde, unpublished results.

Our assignment is consistent with the results of Giese et al. (1987) with regard to one dimeric linkage of Cys-43–Cys-52 as proposed by Sauer and Donoghue (1988). However, a linkage of Cys-49–Cys-60 proposed previously (Jaumann et al., 1991) was not identified by this study. Our results suggest that Cys-49 is linked to one out of Cys-16, -97, or -99, probably Cys-97, but unlikely to be Cys-60.

Chemical determination of disulfide linkages of the PDGF dimer was difficult because of the resistance to proteolytic digestion. If a heterogeneity of recombinant PDGF dimer is created due to alternate disulfide linkages during expression (Canova-Davis et al., 1991; Violand et al., 1991), the elucidation of the three-dimensional structure of rHuPDGF may be difficult. From a site-directed mutagenesis study, there is still unclear evidence including two forms of monomer or dimer created by single and double mutants (Giese et al., 1987; Sauer & Donoghue, 1988). Also, two different electrophoretic forms of the monomers were noted: Mf (monomer, fast) and Ms (monomer, slow). These forms can be explained as the same protein existing in different conformations, whereas another possibility may exist that the PDGF molecule may be refolded by different disulfide linkages.

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