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On the Structure of Platelet-Derived Growth Factor AA: C-Terminal Processing, Epitopes, and Characterization of Cysteine Residues†

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ABSTRACT: The complete amino acid sequence analysis of the "short" form of rPDGF-AA expressed in baby hamster kidney cells revealed the absence of posttranslationally modified amino acid. Approximately 50% of the proteins were shortened by two to three amino acid residues at the C-terminus. Trypsin treatment of BHK rPDGF-AA lead to the identification of two internal epitopes that correspond to the two previously described domains in rPDGF-BB [Vogel, S., & Hoppe, J. (1989) *Biochemistry 28*, 2961-2966]. Cysteine residues at positions 37, 46, 47, and 93, respectively, were converted by site-directed mutagenesis into serine residues, and the monomeric proteins were prepared through expression in *Escherichia coli*. None of the mutant proteins was able to dimerize, but all of them exhibited to various extents a reversible conformational change which may reflect an intermediate prefolded monomer. An intramolecular disulfide bridge between Cys-10 and Cys-91/93 was identified in these monomers. From a mixture of the mutant proteins 37 and 46, an active dimer was reconstituted, suggesting an intermolecular cysteine bridge between these two residues.

Platelet-derived growth factor (PDGF)[1] is a major mitogen in serum which promotes the proliferation of fibroblasts and smooth muscle cells in vitro (Heldin & Westermark, 1984; Deuel et al., 1985; Ross et al., 1986). The existence of two homologous PDGF chains termed A and B gives rise to the formation of three different dimeric forms: AA, AB, and BB. All these dimeric forms exist in nature with variable abundance (Waterfield et al., 1983; Doolittle et al., 1983; Betsholtz et al., 1986; Heldin et al., 1986; Hammacher et al., 1988; Bowen-Pope et al., 1989; Stroobant & Waterfield, 1984). For example, PDGF-AB is the predominant form in human platelet, whereas in porcine platelet extracts only the BB type was detected (Stroobant & Waterfield, 1984). PDGF-AA is more efficiently secreted from a variety of tumor cell lines (Heldin et al., 1986) while PDGF-BB, though expressed in significant quantities, remains membrane-associated in these cells (Robbins et al., 1985).

PDGF-AA may be synthesized in two different forms by means of a different splicing mechanism (Betsholtz et al., 1986; Hoppe et al., 1987; Rorsman et al., 1988; Bonthron et al., 1988). The two forms differ in their C-terminus (short and long form). Proteolytic processing of any of the two forms at the C-terminus like in PDGF-BB (Waterfield et al., 1983) would lead to identical termini. So far, there are no protein chemical data describing the C-terminal ending of PDGF-AA. Furthermore, the PDGF-A sequence contains a N-glycosylation consensus sequence. In light of the observation that rPDGF-AA from BHK cells (Eichner et al., 1989) exhibits some size heterogeneity, we have performed a complete protein sequence analysis showing that this heterogeneity arises from the partial cleavage of the two C-terminal residues.

The A and B forms are approximately 60% homologous, and the highest similarity is found in the center of the molecule comprising the eight cysteine residues (Betsholtz et al., 1986). One has therefore to assume a closely related tertiary structure.

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[1] Abbreviations: BHK, baby hamster kidney; SDS, sodium dodecyl sulfate.

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We have recently analyzed the epitopes in the rPDGF-BB from *Escherichia coli* by trypsin treatment (Vogel & Hoppe, 1989). This paper describes results obtained with rPDGF-AA expressed by baby hamster kidney cells (Eichner et al., 1989).

The amino acid sequence of PDGF contains eight cysteine residues that are strictly conserved among the two chains A and B. Modification or removal of any of these cysteine residues in PDGF-B abolished the formation of dimers. Analysis of mutants performed by Giese et al. (1987) and Sauer and Donoghue (1988) lead to the identification of essential and nonessential cysteine residues. It was proposed that the essential residues are involved in intramolecular disulfide bridges stabilizing a monomeric precursor form. The nonessential residues might be necessary for the formation of dimers. We describe here the effect of the modification of the four nonessential cysteine residues in PDGF-A.

Materials and Methods

Pyridylethylation and Tryptic Cleavage of the Separated rPDGF-AA Chains. One hundred micrograms of BHK rPDGF-AA was dissolved in 20 μL of 8 M urea and diluted with 20 μL of *N*-methylmorpholine hydrochloride, 0.4 M, pH 8.0. 2-Mercaptoethanol was added at a final concentration of 0.77%. After 10 min of boiling, the mixture was incubated with vinylpyridine at a final concentration of 1.1% for 90 min at room temperature. The entire sample was applied to the HPLC column. After washing out the unreacted small molecular weight products, pyridylethylated rPDGF-AA was eluted by a gradient of 14% acetonitrile in 0.1% trifluoroacetic acid to 49% acetonitrile in 0.1% trifluoroacetic acid during 90 min.

Purified pyridylethylated rPDGF-AA was lyophilized and redissolved in 10 μL of 8 M urea. Ninety microliters of 100 mM Tris-HCl, pH 8.5, was added, and the solution was incubated with 5 μg of trypsin dissolved in 5 μL of 1 mM HCl overnight at 37 °C.

Trypsin Digestion. Samples (0.2–0.8 mg) of various lyophilized rPDGF-AA species were redissolved in 300 μL of 10 mM acetic acid. Three hundred micoliters of Tris-HCl (100 mM, pH 8.5) was then added. The suspension was incubated with 50 μg of trypsin dissolved in 50 μL of 1 mM HCl overnight at 37 °C. A small amount of insoluble material was removed by centrifugation.

Cleavage with 70% Formic Acid. Fifty micrograms of rPDGF-AA was dissolved in 200 μL of 70% formic acid and incubated for 72 h at 37 °C.

Isolation of Peptide Fragment. Peptides were separated by reversed-phase high-performance liquid chromatography. In all cases, the whole mixture was applied to a column (0.4 × 25 cm), Vydac 218 TP54, equilibrated in 0.1% trifluoroacetic acid. The column was washed with the same solvent until the UV absorbance at 215 nm had reached its initial value. Peptides were then eluted by a linear gradient from 0.1% aqueous trifluoroacetic acid to 35% acetonitrile in 0.1% trifluoroacetic acid during 90 min if not otherwise indicated. The flow rate was 0.7 mL/min, and fractions were collected each 1 min. The effluent was continuously monitored at 215 nm.

Analytical Methods. SDS gel electrophoresis for proteins in the molecular weight range from 10 000 to 100 000 were performed as described (Hoppe et al., 1986).

Protein sequences were determined with a gas–liquid sequenator, Model 470A (Applied Biosystems), with on-line detection using the narrow-bore column of Applied Biosystems. N-Pyridylethylated lysine-PTH eluted closely to DMPTU. It may be resolved from this peak by lowering the buffer ionic strength. Amino acid analysis was performed with a LC2000 (Biotronik). Silver nitrate staining was done according to Blum et al. (1987). Binding assays were performed in 24-well cell culture plates. Confluent-starved AKR-2B cells (400 000–500 000 cells/well) were incubated for 4 h at 4 °C with the indicated amounts of PDGF variants in MCDB 402 medium containing 1 mg/mL bovine serum albumin buffered with 20 mM HEPES at pH 7.25. Cells were then washed 5 times with 0.5 mL of phosphate-buffered saline containing 1 mg/mL bovine serum albumin. Lysis was performed with 0.5 mL of 20 mM Tris-HCl, pH 7.4, 1% Triton X-100, and 10% glycerol. An aliquot of 400 μL was used for γ-counting.

Site-directed mutagenesis, protein purification and dimerization, measurements of growth-promoting activity, and circular dichroism were performed as described (Hoppe et al., 1989, 1990).

Materials. Proteases were from Boehringer (Mannheim). Growth media were purchased from Gibco. rPDGF-AA was isolated as described from the supernatant of BHK cells and from *E. coli* (Eichner et al., 1989; Hoppe et al., 1990). All other chemicals were of highest available purity.

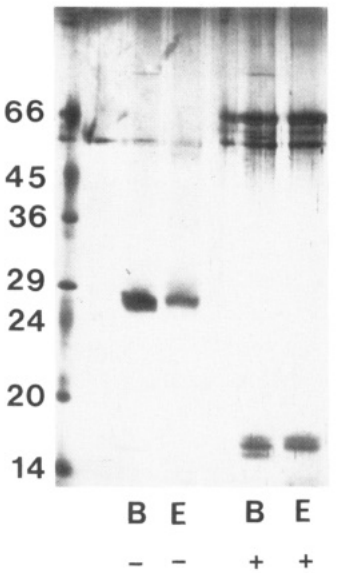

FIGURE 1: SDS gel electrophoresis of rPDGF-AA from BHK cells (0.3 μg) (B) and from *E. coli* (0.2 μg) (E) in the absence (–) or presence (+) of 2-mercaptoethanol. A 13.5% polyacrylamide gel was used. Proteins were stained with silver nitrate. The numbers indicate molecular weight standards ($\times 10^{-3}$). The stains in the high molecular weight range are due to impurities contained in 2-mercaptoethanol.

Results

Partial C-Terminal Processing in rPDGF-AA from BHK Cells. rPDGF-AA from BHK cells and from *E. coli*, which are both expressed as the "short" form, are compared by SDS gel electrophoresis (Figure 1). Evidently, in the absence of 2-mercaptoethanol, rPDGF-AA (BHK) migrates as a broader band than that from *E. coli*. After reduction by 2-mercaptoethanol, the preparation from BHK cells reveals an additional smaller band differing by ≈600 Da.

Since the band corresponding to the higher molecular weight comigrated with the *E. coli* rPDGF-AA, this result indicated that during maturation in the eucaryotic cell rPDGF-AA was somewhat shortened.

Sequence analysis of the entire BHK protein gave a single sequence starting with SIEE, the expected NH_2-terminus. Apparently, the heterogeneity was located at the C-terminus. Since the precise location of the assumed C-terminal processing site could not be identified from the different mobilities during SDS gel electrophoresis, we have performed a complete se-

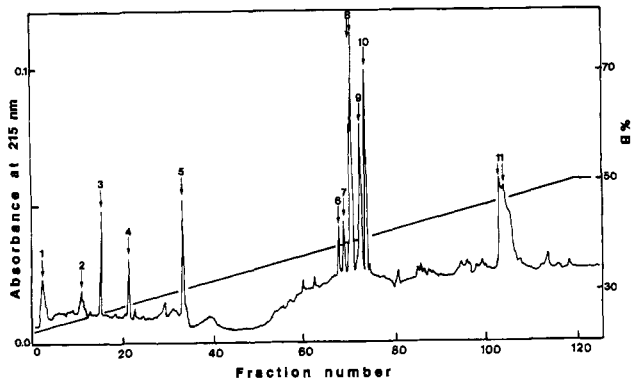

FIGURE 2: HPLC separation of peptides from rPDGF-AA after pyridylethylation and tryptic cleavage. 0.1 mg of rPDGF-AA was modified at cysteine and lysine side chains with vinylpyridine and subjected to tryptic cleavage in the presence of 0.8 M urea. Fragments were isolated by reversed-phase high-performance liquid chromatography. The amino acid sequences of all fragments were determined by gas–liquid phase sequencing. The following sequences were identified (residue number in the sequence of PDGF-A in parentheses). Buffer B = 0.1% trifluoroacetic acid in 70% acetonitrile/H_2O. (1) (59–62) VHHR; (2) (104–110) EEDTDVR; (3) (43–48) CTGCCN; (4) (49–58) TSSVKCQPSR; (5) (43–58) CTGCCNTSSVKCQPSR; (6) (1–13) SIEEAVPAVCKTR; (7) (74–84) KKPKLKEVQVR; (8) (63–73) SVKVAKVEYVR, (85–103) LEEHLECACATTSLNPDYR; (9) (85–107) LEEHLECACATTSLNPDYREEDT (D); (10) (14–21) TVIYEIPR; (11) (22–42) SQVDPTSANFLIWPPCVEVKR.

quence analysis of BHK rPDGF-AA. This analysis was also done to detect possible posttranslational modifications of amino acid residues (e.g., glycosylation, phosphorylation, acylation). This aspect might be especially relevant regarding glycosylation since the PDGF-A sequence contains a consensus sequence for N-glycosylation (Asn-48).

SH groups of cysteine residues and the ϵ-amino group of lysine side chains were modified by pyridylethylation in order to obtain an easily interpretable tryptic map after HPLC separation of the peptides (Figure 2). All major peaks were completely sequenced, and all amino acids were detected in their expected quantities, thus demonstrating that no amino acid residue was posttranslationally modified. Peak 2 contained the C-terminal peptide, indicating that the higher molecular weight form of BHK rPDGF-AA contains the entire C-terminus. The extent of removal of amino acids from the C-terminus was deduced from the sequence of peptide 9 which ended at position 107 [in some experiments, we also detected the next amino acid residue 108 (Asp)]. This species most likely represents the faster migrating band in Figure 1. Surprisingly, substantial cleavage occurred after Asn-48. Since this residue is a potential glycosylation site, we have performed a detailed analysis of this unusual tryptic attack. It was found that this cleavage only occurred when two adjacent pyridylethylated cysteine residues were present at positions 46 and 47. In summary, these results indicate that at least the major fraction of BHK rPDGF-AA is not posttranslationally modified.

Tryptic-Sensitive Sites. rPDGF-BB from *E. coli* contains two trypsin-sensitive internal domains which might be dissected without dissociation of the resistant core. To probe the structure of BHK rPDGF-AA, similar experiments were performed. Due to its lower isoelectric point, this molecule is largely insoluble at pHs required for trypsin cleavage. Though rather high ratios of trypsin to rPDGF-AA were used, we did not obtain complete digestion like with rPDGF-BB. This incomplete reaction prevented a correlation of the extent of digestion with mitogenic activities and binding affinities and likewise did not allow any conclusion regarding the connection

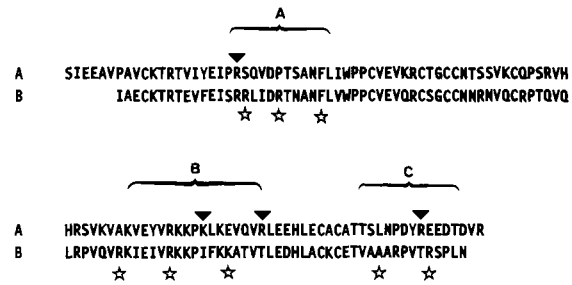

FIGURE 3: Comparison of tryptic-sensitive sites in rPDGF-AA from BHK cells and rPDGF-BB from *E. coli*. Arrows, trypsin cleavage sites in rPDGF-AA; stars, trypsin-sensitive sites in rPDGF-BB (Vogel & Hoppe, 1989). A, B, and C indicate trypsin-sensitive domains.

of the cysteine residues. As shown in Figure 2, trypsin cleaved at all arginine residues in monomeric pyridylethylated rPDGF-A. To further demonstrate that trypsin cleavage is depending on the conformation, we have performed the same analysis with S-sulfonated monomeric rPDGF-A from *E. coli* (data not shown). As expected, trypsin cleaved at all basic residues including lysine, and the digestion was complete.

BHK rPDGF-AA was treated with trypsin, excised peptides were separated by reversed-phase chromatography, and different fractions were analyzed by SDS gel electrophoresis. A trypsin-resistant core with a molecular weight reduced by about 2–3K was isolated and subjected to sequence analysis in order to determine the sites and the extent of digestion. Typically, four sequences were detected at different ratios showing that the trypsin cleavage was not complete. For quantification, the amounts of suitable PTH-amino acids were determined. For comparison, the NH_2-terminal sequence was set to 100%: (1) SIEEAVPVC... (100%); (2) LEEHLECACA... (33%); (3) LKEVQVRLEE... (40%); (4) SQVDPTSANF... (47%). To substantiate these findings, the core protein was dissociated by 2-mercaptoethanol, and peptides were pyridylethylated. A further aim of these studies was to analyze other cleavage sites which might escape detection by sequencing the entire core protein, e.g., cleavage in the cluster of basic amino acid residues and at the C-terminus. Typically, peptides in the molecular weight range of 2–8K were recovered. Interestingly enough, one peptide with the sequence LEEHLECACATTSLNPDYR was isolated ending at position 102 (Arg). As our results (see above) suggest that the processed C-terminus ends at position 107 or 108 in the intact BHK rPDGF-AA, trypsin had apparently also cleaved (at least partially) at position 102. Thus, like in rPDGF-BB, the C-terminal part of rPDGF-AA is accessible to proteases.

A comparison of the tryptic-sensitive domains in rPDGF-AA and rPDGF-BB is given in Figure 3. Remarkably, the three sensitive domains were very similar. In one case, trypsin cleaved at identical positions; in the other cases, juxtaposed basic residues were attacked.

Cleavage at Asp-25–Pro-26. We have recently proposed that the NH_2-terminal segment including Cys-10 is bridged to the central cysteine-rich segment. This suggestion was based on the observation that despite the complete dissection of domain A in rPDGF-BB the NH_2-terminal segment remained attached to the core protein. Since a complete digestion in rPDGF-AA could not be achieved, no conclusions can be drawn from these experiments.

It has been reported by Heldin and co-workers that by mild acid treatment PDGF-AA is cleaved, most likely at the acid-labile Asp-25–Pro-26 bond (Östmann et al., 1988). On the basis of the shift in electrophoretic mobility of the acid-treated unreduced PDGF-AA, they proposed a symmetrical

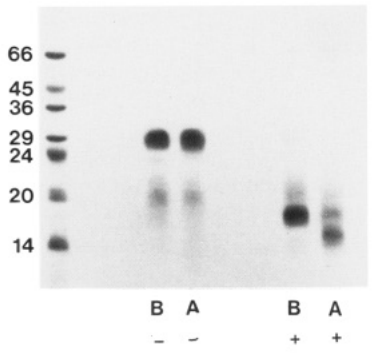

FIGURE 4: Cleavage by mild acid treatment of BHK rPDGF-AA. BHK rPDGF-AA was treated with 70% formic acid for 72 h at 37 °C. An aliquot was dried and analyzed by SDS gel electrophoresis. (B) untreated rPDGF-AA; (A) acid-treated rPDGF-AA; (±) ±2-mercaptoethanol.

arrangement of the disulfide bridges; i.e., Cys-10 forms a bridge with Cys-10 (Östman et al., 1988). Figure 4 shows the effect of mild acid treatment on BHK rPDGF-AA. In the presence of 2-mercaptoethanol, a new band with a reduced molecular weight appeared. From the intensity, it was estimated that cleavage occurred to about 60–70%. In a symmetrical arrangement, this should have led to the liberation of a peptide of $M_r \approx 5000$ under nonreducing conditions. Apparently, such a peptide is not detected during SDS gel electrophoresis. We have used different gel systems (Schägger & Jagow, 1987) and different staining procedures but never detected such a peptide. As peptides might escape detection by SDS gel electrophoresis, we have used HPLC to demonstrate that no fragment was released by mild acid treatment (data not shown). To prove that cleavage has occurred at Asp-25, the HPLC-purified acid-treated rPDGF-AA was sequenced. As expected, an additional sequence was detected starting with PTSA.... This sequence appeared with about 70% of the intensity of that of the NH_2-terminus, thus further establishing that mild acid treatment opens the peptide chain at Asp-25–Pro-26 without liberating a fragment.

Since neither treatment with proteases nor chemical cleavage lead to the isolation of cysteine-containing dipeptides, we developed an alternative strategy to identify disulfide bridges. By site-directed mutagenesis, the following four cysteine residues were converted into serine residues, Cys-37, -46, -47, and -93, and mutant proteins were expressed in *E. coli*. These four residues were previously characterized as nonessential. Though unable to dimerize, these mutant proteins still exhibited a significant transforming activity (Giese et al., 1987; Sauer & Donoghue, 1988).

The effect of treatment of the four S-sulfonated monomeric mutant proteins with the dimerization buffer is shown in Figure 5. None of the mutant proteins alone formed dimers (Figure 5A). However, mutants 37 and 46 were almost quantitatively converted to a more compact component indicated by their higher mobility during SDS gel electrophoresis. This mobility change is readily reversed by reduction with 2-mercaptoethanol, indicating that disulfide bonds have been formed. The mobility of mutant protein 47 is almost not affected, whereas about 30–50% of mutant protein 93 is converted into the compact structure.

If mixtures of the mutant proteins were incubated with the dimerization buffer (Figure 5B), only mutant proteins 37 and 46 dimerized. Further experiments performed in the presence of monomeric S-sulfonated PDGF-B revealed that only mutant proteins 37 or 46 formed heterodimers of type AB.

These three mutant dimers were purified to homogeneity by ion-exchange chromatography and further characterized.

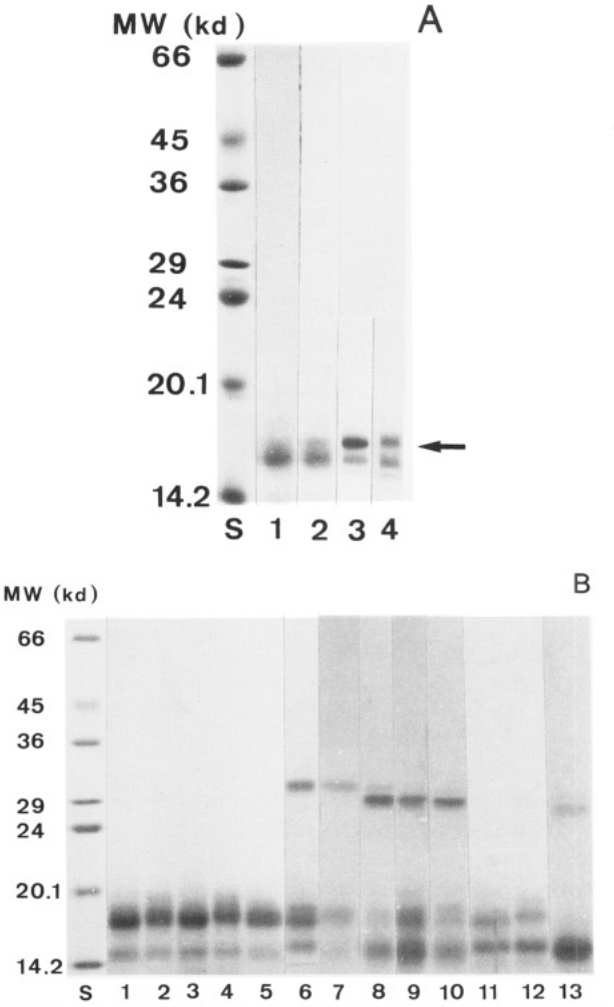

FIGURE 5: Effect of the mutation of individual cysteine residues on the formation of dimeric PDGF. Purified S-sulfonated monomeric mutant proteins were subjected to the dimerization reaction. The reaction was performed with 5 mM reduced glutathione and 0.5 mM oxidized glutathione in 50 mM Tris-HCl/3 M urea, pH 7.5. Aliquots of the reaction mixture were analyzed by SDS gel electrophoresis. (A) Individual mutant proteins. (1) mutant 37; (2) mutant 46; (3) mutant 47; (4) mutant 93; (S) molecular weight standards ($\times 10^{-3}$). The arrow indicates the position of the untreated S-sulfonated mutant proteins. The lower molecular weight species is indicative for a more compact structure. (B) Mixtures of PDGF variants. The following mixtures of mutant proteins were analyzed: (1) 37/47; (2) 37/93; (3) 46/47; (4) 46/93; (5) 47/93; (6) 37/46; (7) unmodified PDGF-A; (8) PDGF-A/PDGF-B; (9) 37/PDGF-B; (10) 46/PDGF-B; (11) 47/PDGF-B; (12) 93/PDGF-B; (13) PDGF B.

Circular dichroism spectra of rPDGF-AA and protein 37/46 were identical (Figure 6), and those of 37B or 46B resembled closely that of rPDGF-AB. All the spectra are characterized by a strong negative ellipticity at 200 nm. It should be mentioned that these spectra are markedly different from those of monomeric PDGF (data not shown, Hoppe et al., 1989, 1990). In Figure 7, the biological properties of the mutant dimers are summarized. In all instances, the mutant proteins were active though in some cases 5–10-fold higher concentrations were needed.

As shown in Figure 5, treatment with the dimerization buffer induced a conformational change in the mutant monomeric protein. To investigate whether such prefolded monomers may reflect true precursor molecules, we combined the prefolded mutant proteins 37 and 46 and followed the time course of association (Figure 8). Indeed, the rate of forming dimers was about 20 times faster in comparison with the control experiment (dimerization of S-sulfonated monomers).

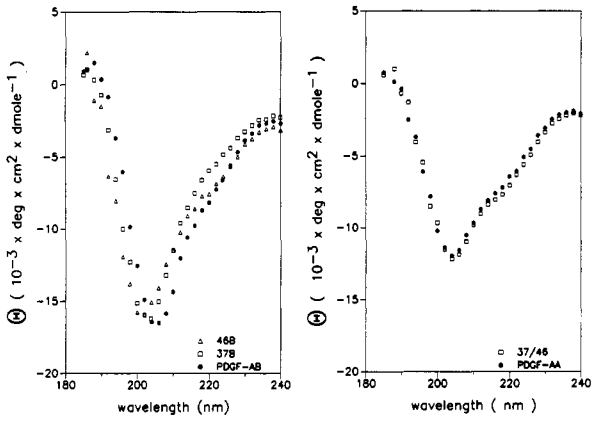

FIGURE 6: Circular dichroism measurement of PDGF variants from *E. coli*. PDGF variants (0.1 mg/mL) were dissolved at pH 6, and circular dichroism spectra were recorded in the range from 190 to 240 nm with a JASCO 600 CD spectrometer. Protein content was determined by amino acid analysis.

Similar observations were made regarding the association of the prefolded monomers 37 or 46 with PDGF-B (data not shown). As a first approach, we suggest from these experiments that the "prefolded" monomers obtained may be considered as precursors containing properly arranged intramolecular disulfide bonds.

These precursor proteins (37 or 46) were treated with trypsin, and peptide fragments were separated by HPLC. In both proteins, an additional peak appeared compared to the pattern of the S-sulfonated monomers (data not shown). Both amino acid analysis and amino acid sequence analysis showed the presence of two peptides, one from residue 1 to 11 and the other from residue 85 to 103, in this new peak. The first peptide contains the amino terminus and cysteine residue 10, the second one contains the carboxy terminus with cysteine residues 91 and 93. Sequence analysis of the dipeptide did not identify to which of the two C-terminal cysteine residues the amino-terminal residue 10 was bound. It should be noticed that the recovery of the dipeptide was rather low. The reason for this low recovery might be an instability during purification and analysis. Likewise, we detected only insignificant amounts of another dipeptide that contains cysteine residues 43 and 54 (data not shown). [This connection was proposed by Giese et al. (1987) and Sauer and Donoghue (1988).]

Discussion

There are now three species of PDGF (AA, AB, and BB) (Waterfield et al., 1983; Doolittle et al., 1983; Betsholtz et al., 1986; Heldin et al., 1988; Hammacher et al., 1988; Bowen-Pope et al., 1989) without considering possible isoforms of A, interacting with at least two types of receptors (Heldin et al., 1988; Hart et al., 1988; Nistér et al., 1988; Matsui et al., 1989; Claesson-Welsh et al., 1989a,b; Gronwald et al., 1989; Seifert et al., 1989). The reason for such a complexity is unclear, but there is increasing evidence that the two types of receptors transmit different signals into the cell. So far, most biological effects can be attributed to the B receptor, e.g., IP_3 release, Ca^{2+} mobilization, contraction, membrane ruffling, and chemotaxis (Hart et al., 1988; Nistér et al., 1988; Block et al., 1989). The A receptor might activate different phospholipases leading to the generation of diacylglycerol without the liberation of IP_3 (Sachinidis et al., 1990). The availability of recombinant PDGF expressed in various organisms will certainly lead to a better understanding of the signal transduction processes induced by the various isoforms.

We considered it important to investigate some basic structural features of BHK rPDGF-AA, since alteration, e.g., glycosylation or processing, might be relevant regarding the

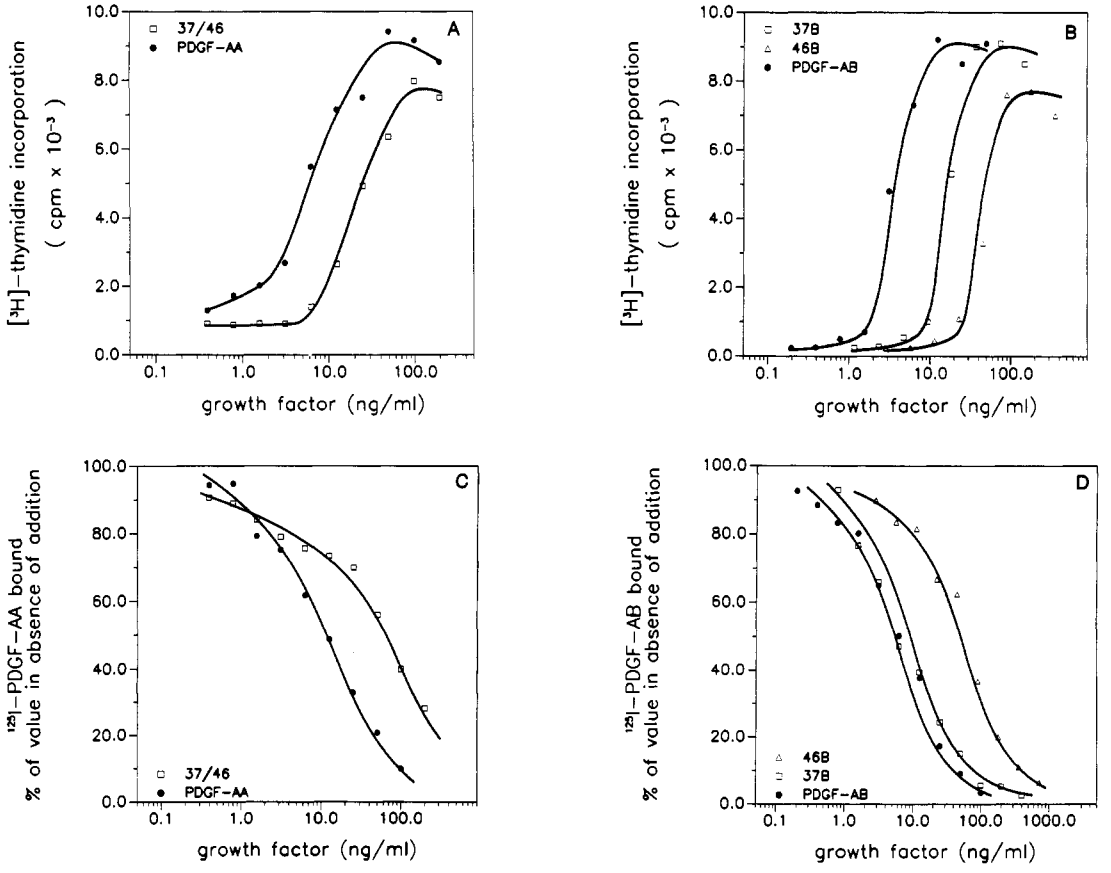

FIGURE 7: Biological properties of various PDGF dimers. (A, B) Growth-promoting activity; (C, D) competition against the respective nonmodified radioiodinated dimer (5 ng/mL).

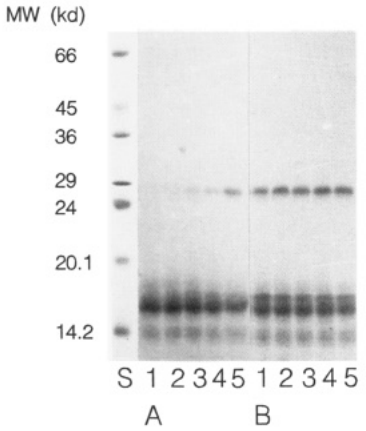

FIGURE 8: Kinetics of the formation of dimers between mutants 37 and 46. (A) Both S-sulfonated monomers were mixed. (B) S-Sulfonated monomers were separately treated with the dimerization buffer for 16 h and were then mixed at stoichiometric ratios. After (1) 10 min, (2) 30 min, (3) 1 h, (4) 2.5 h, and (5) 4 h, aliquots of reactions were analyzed by SDS gel electrophoresis.

biological activity of this PDGF form. There have been some reports claiming glycosylation of PDGF isolated from human platelets (Czyrski et al., 1984; Deuel et al., 1981). Biosynthetic studies with PDGF-B have shown a glycosylation of the preform that is split off during maturation (Robbins et al., 1985). In contrast to PDGF-B, the sequence of the mature PDGF-A contains a N-glycosylation consensus sequence. In view of the fact that PDGF-AA can now be also produced from *E. coli* and yeast, it was important to know whether a mammalian host would use this site. Our data show that this site was neither glycosylated nor were other posttranslational modifications detectable.

Due to the rather high similarity of the A and B chains, all isoforms should adopt the same type of tertiary structure. We have recently shown by circular dichroism spectroscopy that indeed all forms including PDGF isolated from platelet contain the same proportions of secondary structures (Vogel & Hoppe, 1989; Hoppe et al., 1990). Here we have shown that some basic structural features are conserved, i.e., that the NH_2-terminal segment is bridged to the trypsin-resistant core and similar epitopes are present. By using various peptides from PDGF-B, it was shown that domain B might interact with the receptor (Vogel & Hoppe, 1989). On the other hand, recent data showed that domain A is responsible for the transforming and secretory properties of the PDGF isoforms (Larochelle et al., 1990). Both domains might be closely together in the dimer, forming a discontinuous epitope that is recognized by an inhibitory monoclonal antibody (Larochelle et al., 1989). If the sequences of the two respective domains in PDGF-A and -B are compared, the high content of conserved basic residues is evident, but the overall homology is less than 50%, significantly lower than the homology of the entire polypeptide chain. One could imagine that basic residues contribute to the recognition by all receptors and that specificity is brought about by the nonhomologous amino acids in those regions.

The role of cysteine residues in PDGF-B has been analyzed by using an eucaryotic expression system exploiting the ability of PDGF-B (sis) to transform fibroblasts. Modification of any of the eight cysteine residues abolished the formation of dimers, but only four residues were essential for the transformation activity. It was suggested that these residues (Cys-10, -43, -54, and -91) are involved in essential intramolecular disulfide bonds (Giese et al., 1987; Sauer & Donoghue, 1988). The other cysteine residues, especially those at position 37 or 46, respectively, might be mutated without significant loss in

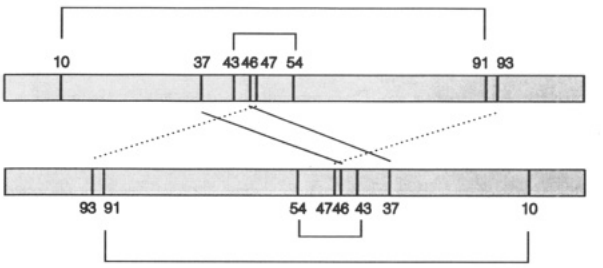

FIGURE 9: Model for the connection of disulfide bridges in PDGF-AA. Bridges 10–91 and 37–46 were analyzed in this paper. The disulfide bond 43–54 was suggested by Giese et al. (1987) and Sauer and Donoghue (1988).

transforming potency. Unfortunately, the applied system did not allow the protein chemical analysis of a mutant protein and the simultaneous expression of two mutant proteins within one cell. We took advantage of the synthesis of monomeric PDGF-A in *E. coli* which makes detailed protein chemical studies feasible. As expected, none of the mutant proteins alone was able to form dimers, but from a mixture of the two mutant proteins 37 and 46, an active dimer was reconstituted. If one assumes an intermolecular disulfide bond between Cys-37 and -46 in the respective dimer, the modification of any of the two residues would abolish the formation of two disulfide bonds. In the dimer 37/46, only one bond would be missing; the other still might be formed. We therefore conclude that residues 37 and 46 are involved in pairwise intermolecular disulfide bonds.

The analysis of "prefolded" monomers lead to the identification of an intramolecular bond between Cys-10 and Cys-91 or -93. Considering the results of Giese et al. (1987) and Sauer and Donoghue (1988), this bridge should be between Cys-10 and -91. The other intramolecular bridge containing the remaining essential cysteine residues must then extend from residue 43 to residue 54. The connections between the remaining nonessential residues Cys-47 and -93 remains less clear. Since we have not detected dipeptides containing these residues during the analysis of prefolded monomers, we suggest that these residue might be involved in intermolecular bonds.

Figure 9 represents a model for the connection between the 16 cysteine residues in a PDGF-AA dimer. This model explains the failure of tryptic digestion or formic acid cleavage to generate cysteine-containing peptides since cleavage between residues 10 and 37 or 54 and 91 as it occurs with trypsin or formic acid will not dissect the molecule. The detailed knowledge of the disulfide connections will significantly reduce the number of plausible structures (Vogel & Hoppe, 1989; Robson et al., 1985). The present results together with molecular modeling techniques might be considered as a first step toward the analysis of the three-dimensional structure of PDGF.

Registry No. Blood platelet derived growth factor AA (baby hamster kidney A chain reduced), 121273-01-6; blood platelet derived growth factor AA (baby hamster kidney), 132017-22-2.

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