Platelet-derived growth factor (PDGF)-C, a PDGF Family Member with a Vascular Endothelial Growth Factor-like Structure*

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Laila J. Reigstad‡, Hege M. Sande‡, Øystein Fluge‡, Ove Bruland‡, Arturo Muga†, Jan Erik Varhaug§, Aurora Martínez**, and Johan R. Lillehaug§‡‡

From the ‡Department of Molecular Biology, †Department of Pediatrics, ‡Department of Surgery, and **Department of Biochemistry and Molecular Biology, University of Bergen, Bergen 5009, Norway, and †Universidad del País Vasco, 48080 Bilbao, Spain

Platelet-derived growth factor (PDGF)-C is a novel member of the PDGF family that binds to PDGF αα and αβ receptors. The growth factor domain of PDGF-C (GFD-PDGF-C) was expressed in high yields in Escherichia coli and was purified and refolded from inclusion bodies obtaining a biologically active growth factor with dimeric structure. The GFD-PDGF-C contains 12 cysteine residues, and Ellman assay analysis indicates that it contains three intramonomeric disulfide bonds, which is in accordance with GFD-PDGF-C being a member of the cystine knot superfamily of growth factors. The recombinant GFD-PDGF-C was characterized by CD, fluorescence, NMR, and infrared spectroscopy. Together, our data indicate that GFD-PDGF-C is a highly thermostable protein that contains mostly β-sheet secondary structure and some (6%) α-helix structure. The structural model of PDGF-C, obtained by homology-based molecular modeling using the structural representatives of this family of growth factors, shows that GFD-PDGF-C has a higher structural homology to the vascular endothelial growth factor than to PDGF-B. The modeled structure can give further insights into the function and specificity of this molecule.

Platelet-derived growth factor (PDGF)† is a major mitogen and stimulant of motility of mesenchymal cells, such as fibroblasts and smooth muscle cells, but also acts on other cell types including capillary endothelial cells and neurons (reviewed in Ref. 1). PDGFS and their receptors have been extensively studied in nearly two decades, but unexpectedly, two new members of the PDGF family were recently identified by data base mining (i.e. PDGF-C (2–4) and PDGF-D (5)). The PDGFs are members of the cystine knot family of growth factors, which presently consists of at least 10 members (i.e. vascular endothelial growth factor (VEGF) (VEGF-A), VEGF-B, VEGF-C, VEGF-D, and VEGF-E from the VEGF family; PDGF-A, PDGF-B, PDGF-C, and PDGF-D from the PDGF family); and placenta growth factors PlGF-1 and PlGF-2 (reviewed in Ref. 6). Common to these factors is the cystine knot domain that contains at least eight cysteine residues perfectly conserved between the two chains (7). Two or three intramonomeric disulfide bonds exist in each monomer, and the factors dimerize in an antiparallel side-by-side mode by using two additional intramonomeric disulfide bonds (8, 9).

Tumor cells of many different human tumor types express PDGFS and PDGFR receptors (PDGFs). An autocrine stimulation of tumor cell growth may prevail (10). The VEGFs induce proliferation of endothelial cells and are involved in the development of the vascular system (angiogenesis) (11, 12) through binding to the kinase domain-containing receptor/feeder/latex kinase-1 and the Fms-like tyrosine kinase 1 receptor (13, 14).

The recently described PDGF-C (3, 4) is a 345-amino acid protein with a two-domain structure not previously observed in this family of growth factors. An N-terminal CUB domain extends from residue 46 to 163 (15) and a C-terminal growth factor domain (GFD) showing homology with PDGFS and VEGFs extending from residue 235 to 345. These two domains are separated by a linker region that includes a predicted protease cleavage site at the N-terminal side of the GFD. The CUB domain of PDGF-C shows highest homology to the CUB domain of neuropilins and BMP-1, which are coreceptors for certain VEGF and VEGF-B isoforms (16, 17). The CUB domain might bind to the pericellular matrix. It seems to block for receptor binding and is proteolytically cleaved off, making an active PDGF-C of the latent growth factor (3). It has been proposed that the isolated CUB domain of PDGF-C exhibits a mitogenic activity on human coronary artery smooth cells, suggesting a possible biological activity of the CUB domain in addition to its role in maintaining latency of the GFD (18). A dominant negative form of the full-length PDGF-C was recently shown to inhibit growth of cultured Ewing tumor cell (19), indicating the PDGF-C to be an important growth factor for these tumor cells. The GFD of PDGF-C (GFD-PDGF-C) shows 27–35% similarity with corresponding regions of PDGFS and VEGFs containing the conserved pattern of eight invariant cysteine residues involved in inter- and intrachain disulfide bonds similarly to the other members of the cystine knot family (3). In addition, GFD-PDGF-C has four extra cysteines (Cys52, Cys59, Cys64, and Cys107), three located between invariant cysteines 3 (Cys48) and 7 (Cys103) and one located two residues C-terminal to the eighth conserved cysteine (Cys105). The GFD-PDGF-C, but not the full-length PDGF-C protein, activates both the PDGFR α-form (3) and the PDGFR β-form of the...
The coding sequence for the growth factor domain of PDGF-C (Fig. 1A) encoding the PDGF-C C-terminal 325–345 of the full-length PDGF-C and a His<sub>6</sub> sequence added to the N-terminal was cloned into the T7 expression vector (Novagen) containing a T7 promoter, and positive clones were verified by sequencing. Large scale protein expression was performed using the BL21-Codon-Plus(DE3)-RII Escherichia coli cells (Stratagene) grown at 37 °C using the T7 19b expression vector. Expression was induced with 1 mM isopropyl-thio-β-D-galactoside at A<sub>600</sub> = 0.6 and cultured for an additional 3–6 h. Pelleted bacteria were resuspended in 10 mM Tris- HCl, pH 8.0, 1 mM EDTA, 0.1 mM NaCl (TEN buffer) (3 ml/g of bacteria cells), containing 0.8 mg of lysozyme and 4 mg of sodium deoxycholate (per g of bacteria) and French pressed (3 × 15 min, 4 °C). Inclusion bodies were collected by centrifugation (3,000 × g, 15 min, 4 °C), and the pellet was washed twice with 6 ml of TEN buffer/g of cells and centrifuged again. The content of inclusion bodies was soluble in 7 M urea, and the solubilized protein solution was almost free from proteins other than GFD-PDGF-C (Fig. 1A). The content of GFD-PDGF-C in tumor cell lines (18), we have shown elevated levels of PDGF-C mRNA in human thyroid papillary carcinoma (2). The results of our current experiments were used in a protracted expression system to express GFD-PDGF-C on a large scale in an active, soluble form. In order to obtain information on the structure and the conformational stability of this domain, we have studied the recombinant protein by CD, fluorescence, NMR and infrared spectroscopy, and molecular modeling. The biological activity of the GFD-PDGF-C was verified by assaying the stimulation of phosphorylation of p44/42 mitogen-activated protein kinase (MAPK), a kinase downstream from the PDGF- FRSs, by a cell growth assay (methyl-<sup>3</sup>H)thymidine incorporation) and by a modified in vitro “wound assay.”

**EXPERIMENTAL PROCEDURES**

**Cloning, Expression, and Purification of the Growth Factor Domain of PDGF-C**—The coding sequence for the growth factor domain of PDGF-C (Fig. 1A) encoding the PDGF-C C-terminal 325–345 of the full-length PDGF-C and a His<sub>6</sub> sequence added to the N-terminal was cloned into the T7 expression vector (Novagen) containing a T7 promoter, and positive clones were verified by sequencing. Large scale protein expression was performed using the BL21-Codon-Plus(DE3)-RII Escherichia coli cells (Stratagene) grown at 37 °C using the T7 19b expression vector. Expression was induced with 1 mM isopropyl-thio-β-D-galactoside at A<sub>600</sub> = 0.6 and cultured for an additional 3–6 h. Pelleted bacteria were resuspended in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.1 mM NaCl (TEN buffer) (3 ml/g of bacteria cells), containing 0.8 mg of lysozyme and 4 mg of sodium deoxycholate (per g of bacteria) and French pressed (3 × 1000 g, 15 min, 4 °C) and the pellet was washed twice with 6 ml of TEN buffer/g of cells and centrifuged again. The inclusion bodies were then dissolved in 7 M urea in either TEN buffer or in 25 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.15 mM NaCl, pH 4.0, as indicated, and incubated at 50 °C for 40 min. Unsolubilized material was discarded by centrifugation, and the solution was adjusted to either 1 mM DTT or 10 mM β-mercaptoethanol, as indicated. The protein was refolded by dialysis and stepwise removal of urea followed by an overnight dialysis step in the absence of urea. Aggregated protein was removed by centrifugation (17,000 × g, 20 min at 4 °C). When indicated, the renatured protein sample was dialyzed against and stored on 2.5 mM KCl, 0.1 mM NaCl, pH 4.3. Solubilization and purification of the protein was analyzed by SDS-PAGE run at constant voltage of 200 V for 35 min in 14.5% (w/v). 4.3. Solubilization and purification of the protein was analyzed by SDS-PAGE run at constant voltage of 200 V for 35 min in 14.5% (w/v).

**Circular Dichroism**—CD measurements were performed on a Jasco J-810 spectropolarimeter equipped with a Jasco 423S Feltier element for temperature control. Protein samples (25 μM) were prepared in different buffers as indicated. The data were analyzed using the Standard Analysis program provided with the instrument. The amount of secondary structure elements was estimated by the CDNN program that is a part of the CDNN analysis program provided with the instrument. Circular dichroism was performed at 222 nm of the reference. The excitation cell holder and 1-cm path length quartz cells. The excitation and emission slits were 3 and 5, respectively. All spectra were corrected for the solvent absorbance emission. The samples were prepared on 2.5 mM KAc, 0.1 mM KCl, pH 4.3.

**Infrared Spectroscopy**—Spectra were recorded in a Nicolet Magna II 550 spectrometer equipped with a MCT detector, using a demountable liquid cell (Harrick Scientific, Ossining, NY) with calcium fluoride windows and 50-μm spacers. Samples (4 mg/ml) were lyophilized in 2.5 mM KAc, 0.1 mM KCl, pH 4.3, and prior to IR measurements they were resuspended in deuterium oxide and centrifuged to remove any insoluble material (20 min at 15,000 × g). Deconvolution of the original amide I band has been described previously (26). Thermal analysis was performed in the rapid scan mode within the temperature interval 20–80 °C, using a scan rate of 1 °C/min and recording one spectrum every 2 °C. A tungsten-iodine lamp was focused onto the window, and the cell was placed into a thermostatted cell mount.

**Nuclear Magnetic Resonance**—Lyophilized samples of GFD-PDGF-C for 1H NMR experiments were dissolved in 25 mM NaH<sub>2</sub>PO<sub>4</sub> pH 4.0, containing 50 mM NaCl, 10% D<sub>2</sub>O (1.2 mM protein; 0.5 ml). NMR spectra were also taken of protein samples containing 0.2–0.4 mM dimer at pH 7.5. The pH of the samples was checked with a glass microelectrode and was not corrected for isotope effects. NMR experiments were acquired on a Bruker AMX-600 pulse spectrometer operating at 600.13 MHz for the proton at a probe temperature of 298 K. One-dimensional 1H NMR spectra were run with water suppression by Pulse Sculpting (27) and “W5” pulse train (28). Following parameters were set: 2-μs delay time, 150-μs “W5” pulse train parameter, 713.908 Hz spectral width. 32 scans were acquired. Data were processed using XWIN-NMR (Bruker) on an SGI work station.

**Molecular Modeling**—Structural models for GFD-PDGF-C were prepared by sequence alignment modeling using the Homology module of InsightII 2000 (Accelrys).

**Biological Activity**—The biological activity of GFD-PDGF-C was assayed both by a cell growth assay (methyl-<sup>3</sup>H)thymidine incorporation) and by mediation of phosphorylation of p44/42 MAPK downstream of the PDGF receptors, as well as by an in vitro “wound assay.” In the cell growth assay of methyl-<sup>3</sup>Hthymidine (Amersham Biosciences) incorporation, confluent cultures of mouse 3T3 fibroblasts were seeded in serum-free DMEM-CM-1 medium (Clonetics, Kibbutz, Israel) for 24 h upon stimulation with equal amounts of either the recombinant GFD-PDGF-C or PDGF-AA (Sigma). Triplicate wells were used for each agent tested and also for the wells not stimulated with any agents. methyl-<sup>3</sup>HThymidine (1 μCi/ml) and cold thymidine (1 μM final concentration) were added to each well 8, 10, and 24 h before termination of each experiment. At the end of the labeling period, DNA was collected by trichloroacetic acid precipitation (29), and the radioactivity was determined using a scintillation counter. The biological activity of the recombinant PDGF-C was then tested for mediation of phosphorylation of p44/42, MAPK. Serum-starved mouse 3T3 fibroblasts were stimulated with the recombinant refolded GFD-PDGF-C and with PDGF-AA and epidermal growth factor (both from Sigma) in comparable amounts. Stimulation was carried out for 15–60 min. Total cell protein was then isolated, separated on a 12.5% SDS-PAGE, and immunoblotted. The effect of stimulation was verified by assaying induction of MAPK phosphorylation using anti-phospho-p44/42 MAPK monoclonal antibody (New England Biolabs, Inc., Beverly, MA) and anti-Erk1 polyclonal antibody (Santa Cruz Biotechnology). The anti-Erk1 polyclonal antibody was used as a control for equal amounts of protein loaded in each well. In the in vitro wound assay (30), cell monolayers were wounded by scratching a line with a plastic scriber, and after washing with phosphate-buffered saline, the cells were incubated in serum-free RPMI 1640 medium with 0.1% bovine serum albumin and with or without refolded GFD-PDGF-C. The cells were fixed and stained with Giemsa stain.

**RESULTS**

**Expression, Purification, and Renaturation**—Several small scale experiments were performed in order to achieve an optimum expression level and to obtain maximal GFD-PDGF-C solubility. In the absence of denaturant, there was no soluble recombinant GFD-PDGF-C in the supernatant of French press-treated bacterial suspensions (cleared cell lysate) (Fig. 1A). Approximately 60–70% of the recombinant protein present in inclusion bodies was soluble in 7 M urea, and the solubilized protein solution was almost free from proteins other than GFD-PDGF-C (Fig. 1A). For large scale purification, two equally effective protocols were used to generate refolded GFD-
PDGF-C from solubilized inclusion bodies. First, the recombinant His_{10}-tagged GFD-PDGF-C was purified and refolded under denaturing conditions by affinity chromatography on nickel-nitrilotriacetic acid silica resin (Qiagen). The solubilized protein in TEN buffer, containing 7 M urea and 10 mM β-mercaptoethanol, was bound to the nickel-nitrilotriacetic acid column at pH 8.0, and renaturation by dialysis to remove urea. Shown is the renatured, refolded GFD-PDGF-C after centrifugation (13,000 × g, 20 min): supernatant (lane 1) and pellet (insoluble fraction) (lane 2). C, effect of reducing conditions on the oligomeric structure of GFD-PDGF-C. Cleared supernatant of the cell lysate was treated under reducing (10 mM β-mercaptoethanol) (lane 1) and nonreducing conditions (no β-mercaptoethanol in the sample buffer) (lane 2). Unsoluble fraction (pellet) was treated under reducing (10 mM β-mercaptoethanol) (lane 2) and nonreducing conditions (no β-mercaptoethanol in the sample buffer) (lane 4).

**Biological Activity**—The biological activity of refolded GFD-PDGF-C was determined by assaying GFD-PDGF-C-mediated phosphorylation of p44/42 MAPK, a kinase downstream from the PDGF receptors. Serum-starved 3T3 cells were treated with recombinant renatured GFD-PDGF-C or with commercially available PDGF-AA and epidermal growth factor, and a strong increase in p44/42 phosphorylation was obtained with each of the three factors (Fig. 2), thus identifying a biological signal transduction activity of the expressed and renatured GFD-PDGF-C preparation. Also, when GFD-PDGF-C activity was assayed by a modified in vitro “wound assay” (30), a well defined migratory activity into the cell-free space was observed. Similarly, a significant increase in [methyl-^3H]thymidine incorporation (5–8-fold over untreated controls, compared with 7–10-fold for PDGF-AA added at equal amounts) into DNA was observed in 3T3 cells stimulated with the recombinant protein (data not shown), indicating cell proliferation, increased motility, and DNA synthesis and providing evidence of biological active GFD-PDGF-C.

**Circular Dichroism**—The purified and urea-solubilized GFD-PDGF-C shows a far UV CD spectrum with a minimum at around 215 nm, and after renaturation of the protein by elimination of the urea, the spectrum shows an even broader minimum with slightly decreased ellipticity (Fig. 3A). Similar spectra are obtained in the pH range 4.0–7.5. As estimated by the CDNN neural network procedure (25), this spectrum corresponds to a content of secondary structure of −6 ± 1% α-helix, 52 ± 4% β-sheet, 17 ± 1% β-turn, and 25 ± 2% random coil. The near-UV CD spectrum of the renatured protein shows a broad positive transition centered at about 260 nm in Fig. 3B, which most probably corresponds to disulfide bonds in a strained conformation, i.e. with a CβSS torsion angle deviating significantly from 90° (32). The positive signal around 260 nm diminishes upon incubation with 5 mM DTT at 25 °C and almost disappeared when the GFD-PDGF-C was reduced with 5 mM DTT for 5 min at 95 °C (Fig. 3B). Upon heating the protein at 95 °C in the absence of DTT, the strained orientation of the S–S bonds is preserved in the protein, indicating a high conformational stability. The high stability of GFD-PDGF-C is also manifested by the elevated midpoint melting temperature (>100 °C) of the renatured protein in the pH range 4.0–7.5 (Fig. 3C). Indeed, both the far-UV and the near-UV spectra of the heat-treated (95 °C) protein at nonreducing conditions were similar to the spectra of the renatured protein at 25 °C (Fig. 3, A and B). The presence of urea in the protein samples decreases its thermal stability, and, in fact, the renaturation of the pro...
tein achieved by the removal of urea parallels the increase in thermal stability of the protein (Fig. 3C).

Fluorescence Emission Spectroscopy—GFD-PDGFC contains one Trp residue at position 39, which is conserved in PDGF-B (Trp40). The tryptophan fluorescence emission spectrum of the recombinant renatured GFD-PDGFC (excitation at 295 nm) shows a maximum at 342 nm (Fig. 4A) and a quantum yield of 0.09, indicative of a solvent-exposed residue. Fluorescence studies also showed that this residue is solvent-exposed in PDGF-B (emission maximum at 347 nm) and that denaturation of the protein results in a large red shift (the emission maximum increases to 356 nm) (33).

**NMR Studies of GFD-PDGFC**—In an attempt to obtain structural information on the GFD-PDGFC in solution, preliminary NMR experiments were performed. At concentrations of about 1 mM GFD-PDGFC, pH 3.0–4.0, and temperatures between 25–50 °C, the line widths of the NMR resonances were too broad, indicating some aggregation of the protein at these conditions. A significant improvement of the spectral quality was obtained when the pH of the sample was changed to pH 7.5 by dialysis, lyophilization, and resuspension in 50 mM NaCl, 25 mM NaH2PO4, 100% D2O, pH 7.5, and the protein concentration was adjusted to 0.2–0.5 mM (Fig. 4B). Two-dimensional TOCSY and NOESY spectra could be recorded at this slightly alkaline pH (7.5), but they were not of enough quality to attempt a complete assignment of the signals. Future NMR investigations of the three-dimensional structure of GFD-PDGFC will require isotopic labeling of the protein. Nevertheless, some structural information can be inferred from the NMR spectrum, since most of the signals appearing between 6.0 and 5.0 ppm probably correspond to low field shifted Hα protons, indicating a positive differential shift (i.e. δ
Structural Characterization of Recombinant PDGF-C

The structures of VEGF (VEGF-A) (Protein Data Bank accession code 1VPF) (38) and PDGF-B (Protein Data Bank accession code 1PDG) (39) were used as templates to construct a structural model of GFD-PDGF-C. In the alignment of these three proteins, the sequence identity was 26% for PDGF-B and 28% for VEGF, whereas the sequence similarity was 40 and 51%, respectively (Fig. 6). The model of GFD-PDGF-C obtained using the Homology module of InsightII 2000 (MSI) was similar to that obtained by the Swiss-Pdb Viewer in conjunction with SWISS-MODEL (40). Both methods rendered three-dimensional models of acceptable quality, as evaluated by PROCHECK (41) and Whatatch (42).

Fourier Transform Infrared Spectroscopy—The original and the deconvolved spectra of GFD-PDGF-C are shown in Fig. 5A. The conformationally sensitive amide I band, 1700–1600 cm\(^{-1}\), displays an intense band component at 1625 cm\(^{-1}\) coming from ionized acidic side chains and residual acetate buffer, that hampers the estimation of the relative intensity of the amide I band components. Nevertheless, the spectrum clearly indicates that the protein folds into a \(\beta\)-structure. There is no spectral evidence for the existence of \(\alpha\)-helical conformational structures, although it cannot be ruled out that a weak helical signal (1653–1643 cm\(^{-1}\)) may hide below the intense \(\beta\)-component. Interestingly, the intensity of the residual amide II band at around 1545 cm\(^{-1}\) is negligible, indicating that the NH groups of the protein readily exchange with the solvent and therefore that the protein core is highly accessible. This characteristic, together with the width of the amide I band, argues against the presence of protein aggregates. If this were the case, the amide I band would be narrower, as expected from immobilized protein segments forming part of the aggregates, and these segments would, at least partially, be shielded from the solvent. The temperature dependence of the amide I band absorption maximum supports this interpretation (Fig. 5B). Intermolecular aggregates usually give rise to a component at around 1620 cm\(^{-1}\) that slightly shifts downwards with increasing temperatures (36, 37). The observed temperature-induced upward displacement of the absorption maximum of the amide I band (from 1625 to 1633 cm\(^{-1}\)) reinforces the interpretation that GFD-PDGF-C folds into a \(\beta\)-structure and does not aggregate under these experimental conditions.

Structure Prediction of GFD-PDGF-C by Molecular Modeling—The structures of VEGF (VEGF-A) (Protein Data Bank accession code 1VZV) (43) as template (16% sequence identity with GFD-PDGF-C) did not have any influence on the final predicted structure. The model of GFD-PDGF-C showed three intramonomeric cystine bonds (Fig. 7A), and, as seen by the overall structural alignments of GFD-PDGF-C with both VEGF and PDGF-B (Fig. 7, B and C), the similarity between VEGF and GFD-PDGF-C is higher than between GFD-PDGF-C and PDGF-B.

Based on the dimeric structure of both VEGF-AA (38) and PDGF-BB (39), a structural model of the dimeric form of GFD-PDGF-C was also constructed (Fig. 8). The two cysteine residues Cys\(^{32}\) and Cys\(^{54}\) form the interdisulfide bonds between the monomers, giving a dimer of the GFD-PDGF-C. The \(\alpha\)-helix of one monomer stretches over the other monomer, contributing to the stability of the dimer. The areas most probably involved in the binding to the Fms-like tyrosine kinase 1 receptor, based on the structure of VEGF-AA complexed with domain 2 of the receptor (44), are indicated in Fig. 8. The location of the solvent-exposed Trp\(^{25}\), responsible for the intrinsic fluorescence of GFD-PDGF-C (Fig. 4A), is also shown in Fig. 8.

**DISCUSSION**

Our main aim was to study the structure of the growth factor domain of the novel PDGF-C protein. This protein domain has low solubility in bacterial expression systems, most likely due to its 12 cysteine residues. Therefore, we first established methods for large scale (milligram amounts) expression, purification, and renaturation of the recombinant GFD-PDGF-C protein. The system of choice was expression in E. coli using the pET 19b vector with a His tag placed in the N terminus. Although almost no soluble protein was obtained in the cell lysates, it was possible to renature and purify the protein in one step on a nickel-nitrilotriacetic acid column and in a stepwise dialysis procedure. Both [methyl-\(^3\)H]thymidine incorporation and mediation of phosphorylation of MAPK downstream of the PDGF receptors and the in vitro “wound assay” showed biological activity of the recombinant GFD-PDGF-C protein, indicating that it was refolded correctly into its native structure. To our knowledge, to date no structural characterization has been performed on GFD-PDGF-C, possibly because in previous studies only microgram amounts have been purified (3, 4).

Li et al. (3) grouped PDGF-C within the PDGF family, since it bound to the PDGF \(\alpha\)-receptor after proteolytic cleavage and separation from the N-terminal CUB domain. Later it was shown that GFD-PDGF-C binds to the PDGF \(\alpha\beta\)-receptor as well (4, 45) but not to the VEGF receptors. Nevertheless, recent studies suggest that PDGF-C can also possess VEGF-like activity (18), and, in fact, the CUB domain of this factor shows highest homology to the CUB domain of neuropilins and BMP-1, which are coreceptors for certain VEGF isoforms (16, 17). Moreover, the existence of an as yet unknown receptor specific for PDGF-C cannot be ruled out.

Based on our CD experiments, the secondary structure of GFD-PDGF-C includes about 52% \(\beta\)-sheet structure. This large
Cys18 yellow sulfur is colored in content. This high content of characteristic of members of the cystine knot family (43). The both PDGF-B (39) and VEGF (44) (Fig. 7) and seems to be residues are indicated with two dots or one dot, depending on the degree of similarity. The first conserved cystine bond (1) corresponds to Cys18–Cys26, the second (2) corresponds to Cys91–Cys100, and the third corresponds to Cys56–Cys100, whereas the intermonomeric cystine bonds (I) correspond to Cys62–Cys84.

**Fig. 6.** Sequence alignment of GFD-PDGF-C with PDGF-B and VEGF (VEGF-A). The signs above and the histogram below the alignment indicate the degree of similarity between the sequences. The stars (and highest bars) indicate identity, whereas homologue properties of the residues are indicated with two dots or one dot, depending on the degree of similarity. The first conserved cystine bond (1) corresponds to Cys18–Cys26, the second (2) corresponds to Cys91–Cys100, and the third corresponds to Cys56–Cys100, whereas the intermonomeric cystine bonds (I) correspond to Cys62–Cys84.

**Fig. 7.** Model of the three-dimensional structure of GFD-PDGF-C based on sequence alignment with PDGF-B and VEGF. 
A, α-helices are shown in red, and β-sheets are shown in turquoise. Cysteine residues are numbered and shown in a stick representation, sulfur is colored in yellow, and nitrogen and oxygen are colored in blue and red, respectively. The first intramonomeric cystine bond is Cys18–Cys26, the second is Cys91–Cys100, and the third is Cys56–Cys100. The two intermonomeric cysteines making the dimer are Cys62 and Cys105. B, overall backbone structural alignment of GFD-PDGF-C (turquoise) with PDGF-B (violet); root mean square deviation = 4.42 Å for backbone atoms. C, overall backbone structural alignment of GFD-PDGF-C (turquoise) with VEGF (orange); root mean square deviation = 0.23 Å for backbone atoms.

The percentage of β-sheet is also corroborated by Fourier transform infrared spectroscopy and by NMR studies in which signals corresponding to low field shifted Ha protons show a positive differential shift characteristic of proteins with high β-sheet content. This high content of β-sheet structure is also found in both PDGF-B (39) and VEGF (44) (Fig. 7) and seems to be characteristic of members of the cystine knot family (43). The β-sheet structure and the cystine knot confer a high conformational stability to the proteins. Thus, as seen with GFD-PDGF-C, the midpoint melting temperature was found to be >95 °C, and the thermal treated protein (at 95 °C for 1 h) still retains a high degree of secondary structure, even when the disulfide bridges of the cystine knot motive have been cleaved by reduction. This high stability could be suggestive of extensive aggregated β-structure, but this does not seem to be the case for the recombinant refolded GFD-PDGF-C, which shows a dimeric structure both by size exclusion chromatography and by SDS-PAGE in the absence of reducing agent. Moreover, its dispersed 1H NMR spectrum, its intrinsic fluorescence spectrum, resultant from a solvent-exposed Trp39, and its Fourier transform infrared spectroscopic properties are also typical of a folded nondenatured and nonaggregated protein. The results from all of these spectroscopic techniques support the homology-base modeled structure of GFD-PDGF-C (Figs. 7 and 8). The modeled structure contains 50% of β-sheet secondary structure, and about 6% of α-helical secondary structure located at a short helix at the N-terminal of the protein. The presence of this α-helical content is also indicated by the CD measurements. Whereas PDGF-B is devoid of α-helical structure (Fig. 7B) (39), VEGF contains about 12% helix structure distributed in two α-helices per monomer (Fig. 7C) (44). The good structural alignment between the modeled structure of GFD-PDGF-C and VEGF (the root mean square deviation for the backbone atoms between the modeled structure of GFD-PDGF-C is 0.23 and 4.42 Å when compared with the structure of VEGF and PDGF-B, respectively (Fig. 7)) also provides evidence for a large structural closeness between GFD-PDGF-C and VEGF. Moreover, as seen in the sequence alignment (Fig. 6), the four proline residues in GFD-PDGF-C are conserved in VEGF (only two with PDGF), and their position is coincident in the three-dimensional structures. Prolines are important in defining the overall conformation of the proteins and are usually found in
the flanking segments of protein-protein interaction sites (46). Although the structures of the PDGFR-α and PDGFR-β receptors are not known, it is evident that the receptors of the PDGF family may all use domain 2 for factor binding in a way analogous to the structure of VEGF8–109 in complex with domain 2 of Fms-like tyrosine kinase 1 receptor (47, 48). According to this complex structure, the putative receptor-interacting regions in the modeled dimeric structure of GFD-PDG-C (shown in Fig. 8) are flanked by the conserved proline residues. As seen in this structure, a region including Trp39, Leu79, and Arg120 might be an important binding site for the PDGF-α- or β-receptor. Amino acid sequence alignments (see, for instance, Fig. 1 in Ref. 3) show that PDGF-A and PDGF-B contain a similar sequence. Trp60, Val78, and Arg79, that is not found in the members of the VEGF family. With these structure considerations in mind, it is possible that the PDGF-like specificity of PDGF-C is associated with the residues in regions supposed to interact with the receptor, despite the similarity in overall structure to the growth factor domain to VEGF.

It is also interesting to note that an insertion of three amino acid residues (Asn51,Cys52–Ala53, numbering for the GFD) is specific for PDGF-C but is not present in the other members of the PDGVEGF family. The insertion occurs close to the loop-2 region that is thought to be involved in receptor binding. Moreover, three out of the four extra nonconserved cysteine residues in the GFD-PDG-C sequence (i.e. Cys60, Cys64, and Cys107) are positioned forming a cluster close to the cystine knot, indicating their putative involvement in further binding with coreceptors, with or without intramolecular disulfide bonding. Only mutagenesis and binding experiments may elucidate the receptor specificity of PDGF, and our modeled structure of the growth factor domain may contribute to provide the frame for a rational choice of the residues to be mutated.

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Laila J. Reigstad, Hege M. Sande, Øystein Fluge, Ove Bruland, Arturo Muga, Jan Erik Varhaug, Aurora Martínez and Johan R. Lillehaug

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