Solution Structure and Interaction with Basic and Acidic Fibroblast Growth Factor of a 3-kDa Human Platelet Factor-4 Fragment with Antiangiogenic Activity*[^S]

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Platelet factor-4 is a protein belonging to the family of ELR-negative CXC chemokines which binds to fibroblast growth factor and inhibits its mitogenic activity. Platelet factor-4 also inhibits tumor growth by mechanisms involving antiangiogenesis. Antiangiogenic activity in vitro has also been shown for the 24-residue C-terminal fragment of the protein, which decreases the affinity between basic fibroblast growth factor and its cell-surface receptor. In this study, the preferential conformation of this fragment in solution has been determined and has been found to be composed of two helical subdomains. In addition, we show that the fragment forms a specific 1:1 complex with acidic and basic fibroblast growth factors and that both subdomains are probably required for inhibition of fibroblast growth factor-driven mitogenesis. Finally, we show that the binding of the fragment alters the structure of the fibroblast growth factors, although some of such alterations do not seem related with the inhibition of mitogenic activity. Since this fragment has recently been shown to inhibit fibroblast growth factor-induced angiogenesis in vitro when injected intraperitoneally, these results are relevant for developing new antiangiogenic treatments.

Angiogenesis plays an important role during embryonic development and in many pathologies including cancer and cardiovascular and inflammatory diseases. Fibroblast growth factors (FGFs) and vascular endothelial growth factor (VEGF) family members constitute the major class of angiogenic regulators. These factors induce endothelial cell proliferation, migration, and angiogenesis in vitro and in vivo (1, 2). FGFs and VEGFs induce specific cellular responses by binding selectively to cell-surface receptors. The FGF family is presently known to include 22 closely related proteins. Acidic fibroblast growth factor (aFGF) and the subsequently discovered basic fibroblast growth factor (bFGF) are considered to be representative of the whole family (3–5). FGFs bind to two receptor classes with high and low affinity at the cell surface. The high affinity receptors (FGFR) belong to the class of the transmembrane receptor tyrosine kinases (6). FGFRs are widely distributed in all cells of mesodermal and neuroectodermal origin studied thus far, which accounts for the wide spectrum of in vitro mitogenic targets of this protein family (7, 8). The low affinity receptor of FGFs is the glycosidic moiety of the cell coat and basal membranes (9). Angiogenesis promoted by VEGF has been reported to be dependent on the endogenous expression of bFGF by endothelial cells and is therefore blocked by antibodies that neutralize to bFGF (10, 11). Consequently, there is considerable pharmacological interest in developing inhibitors with the mitogenic activity of FGFs.

Platelet factor-4 (PF4) is a CXC chemokine with high affinity for sulfated glycosaminoglycans like heparin and heparan sulfate. PF4 inhibits endothelial cell proliferation, migration, and angiogenesis in vitro and in vivo (12–14). Furthermore, the inhibitor of bFGF mitogenic activity released by activated platelets has been demonstrated to be PF4 (15). In addition, PF4 inhibits tumor growth through an angiogenesis-dependent mechanism (16–19). PF4 has been shown to bind to bFGF and to inhibit processes considered to be essential in bFGF-induced mitogenesis (bFGF binding to high affinity receptors, bFGF internalization, and activation by heparan sulfate proteoglycans (20, 21)). As the synthesis of high quality PF4 by recombinant technologies is considerably cumbersome because of its quaternary structure, and because its activation likely requires an intramonomer hydrolysis (see below), the search for small mimics of PF4 is of obvious interest.

PPF4-(47–70), PF4 fragment spanning residues 47–70 of the full-length PF4 sequence; PF4-(47–58), PF4 fragment spanning residues 47–58 of the full-length PF4 sequence; PF4-(54–70), PF4 fragment spanning residues 54–70 of the full-length PF4 sequence; RMSD, root mean square deviation; sFPF4-(47–70), scrambled form of PF4-(47–70); TOCSY, total correlation spectroscopy; TFE, trifluoroethanol; TSP, 3-trimethylsilylpropionate; VEGF, vascular endothelial growth factor; XTT, 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide; DTT, dithiothreitol; HSCQ, heteronuclear single quantum coherence.

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The monomer of PF4 consists of 70 amino acids (22). Under physiological conditions four identical polypeptide chains assemble in a defined manner, and it is presumed that the tetramer is the structure populated in vitro (23). The PF4 monomer secondary structure from the C to the N terminus consists of one α-helix (Ser7–Ala57), a three-stranded antiparallel β-sheet (Leu53–Gly48, Lys46–Pro37, Gly33–Thr25), and a strand-like element (Glu18–Asp1) that forms an antiparallel β-sheet-like structure between dimers in the tetramer (23). A PF4 form has been isolated from activated human leukocyte supernatants that exhibits a 30–50-fold greater growth inhibitory activity on endothelial cells than the commonly isolated form of the protein. This more active form is a derivative of PF4 generated by cleavage of the Thr16 and Ser17 peptide bond. The two resulting fragments remain linked by a disulfide bond (24). This unique cleavage affects the connection between the β-strand-like element and the rest of the protein, which may alter the quaternary structure of PF4 (23). Recently, it was shown by peptide sequencing that a fragment of PF4, comprising amino acids 47–70 (PF4(47–70)) decreases the affinity of bFGF for its cell-surface receptors and emulates many of the antiangiogenic properties of the full-length protein (25, 26). Inhibition of bFGF binding to its cell membrane receptor by PF4 and PF4(47–70), as well as the inhibition of bFGF mitogenic activity in the absence of heparin, has been observed in two different heparan sulfate-deficient cells (20, 26). This suggests that inhibition by PF4(47–70) of bFGF mitogenic activity in vitro is not exclusively mediated by blocking the activation of FGFs (27) by either heparin or cell-surface heparan sulfates, to which PF4(47–70) bind with high affinity (theoretical pI = 9.75 (21, 23, 26, 28, 29)), but that PF4(47–70) somehow interacts with bFGF, as in the case of PF4. Here we report the three-dimensional structure of PF4(47–70) in solution, the first biophysical characterization of the interaction of PF4(47–70) with FGFs and an evaluation of the relevance of some effects caused by this interaction for the inhibitory activity of PF4(47–70). We have recently shown that PF4(47–70) injected intraperitoneally suppresses bFGF-induced angiogenesis in vitro (26). These findings are important for developing new pharmacological approaches aimed at modulating angiogenesis.

EXPERIMENTAL PROCEDURES

Reagents—Culture plates were obtained from Costar. Bacteriological agar, yeast extract, tryptone RPMI 1640, fetal bovine serum, penicillin, streptomycin, gentamicin, and t-glutamine were from Life Technologies, Inc. Nitrocellulose filters were from Millipore; EDTA and DTT were from Sigma. Distilled water filtered through a Milli-Q water purifier fitted with an Organex column (Millipore) was used for all solutions.

Protein Preparation—The full-length bFGF (155 residues (30)) used in this study were synthesized and purified as described (31). A 139-residue form of aFGF prepared as described previously (32) was used throughout this work. Different aFGF variants were prepared using the gene coding for the 139-residue wild-type protein as described (33).

Peptide Synthesis—Fmoc (9-fluorenylmethylcarbonyl) synthesis of peptides (free C and N terminus) was carried out in a Synergy synthesizer (Applied Biosystems). Cleavage was carried out according to the manufacturer's instructions. The dried raw synthesis product was dissolved in 13 mM trifluoroacetic acid and chromatographed on a 25 cm C4 reverse-phase column (10 M sodium phosphate containing 0.3 M of NaCl, 1 mM DTT, 10 mM EDTA, was concentrated as described (36). To prepare 15N-labeled aFGF(47–70) complex, an appropriate amount of aFGF(47–70) was added to the protein just before the NMR spectra acquisition to get a 1:1 complex in a final volume 0.45 ml. Just before acquisition of NMR spectra, appropriate amounts of D2O and sodium [3-trimethylsilyl-2,2,3,3-D3]propionate (TSP) as an internal reference for 1H Hs were added. The 15Nслав-2020 values were indirectly referenced by multiplying the spectrometer frequency that corresponds to 0 ppm in the 1H spectrum, assigned to internal TSP, by 9.101329118 (37). NMR experiments were performed on a Bruker AMX-600 spectrometer. Two-dimensional homonuclear spectra were acquired in the phase-sensitive mode using the time proportional phase incrementation technique (38) with presaturation of the water signal. COSY (39) and NOESY (40) spectra were recorded using standard phase-cycling sequences. Short mixing times (150 ms) were used in the NOESY experiments in order to avoid spin–diffusion effects. TOCSY (41) spectra were acquired using the standard MLEV17 spin-lock sequence and an 80-ns mixing time. Two-dimensional heteronuclear 1H,15N HSQC (42) spectra were recorded for the 15N-labeled peptide samples and acquired by using the States-time proportional phase incrementation method (43). Water suppression was achieved either by selective presaturation or by including a WATERGATE pulse sequence (44) in the chemical pulse sequence prior to acquisition. The size of the acquisition data matrix was 2048 × 512 words in F2 and 15Nслав-2020 respectively. Prior to Fourier transformation, the two-dimensional data matrix was multiplied by a phase-shifted square-sine bell window function in both dimensions and zero-filled to 4096 × 1024 words. The phase-shift was optimized for every spectrum.

1H NMR spectra of PF4(47–70), at pH 3.4, in aqueous solution and in 15% TFE were assigned using standard two-dimensional sequential assignment methods (45, 46). The fingerprint region of PF4(47–70) was also assigned both in aqueous solution and 30% TFE at pH 6.5. The sequential NN, αN, and βN NOE connectivities used for assignment are summarized in Fig. 2. 1Hслав-2020 values (pH 3.4) are available upon request and are included as Supplemental Material.

Three-dimensional Structure Calculation—Calculations of peptide structures were carried out using the standard annealing strategy within the program DYANA 1.5 (47), from the spectra acquired at pH 3.4 because of the better signal to noise ratio obtained at acidic pH values. Distance constraints were derived from NOESY spectra acquired with 150-ms mixing time. Quantitative values of the NOE cross-peak volumes were determined by using the integration routine of the XEASY program (48). The NOE cross-peak intensities were translated into upper limit distance constraints by using the CALIBA routine (49) within the DYANA program. The CALIBA routine assumes that the NOE intensities are proportional to 1/rn, where r is the interproton distance. The exponent n was assigned a value of 6 when neither of the protons participating in a NOE belonged to a methyl group and a value of 4 if at least one methyl proton is involved. The scaling factors were chosen to reproduce the correct distance limits between pairs of protons separated by known fixed distances (H–H, H–CH3 protons, H–methylene in Val, etc.). Lower bounds between non-bonded atoms were set to the sum of van der Waals radii. In order to increase the quality of the structure, the φ angles were constrained to the range 0° to 180°, except for Gly and Asn residues.

Fluorescence Measurements—Fluorescence emission spectra were
performed in a thermostatically controlled PerkinElmer Life Sciences LS50-B spectrophotometer. Excitation and emission slit widths were 2.5 and 10 nm, respectively. Samples were excited at 295 nm. The concentration of fluorescent sample was in the linear response range of the spectrophotometer.

Urea denaturation studies were carried out as described (50). The conformational stability of the protein was estimated as $\Delta G^{\text{den}} = m[U_{13}]$, where $m$ is the average cooperativity value describing the change in $\Delta G$ with the concentration of denaturant measured for the species under study, and $[U_{13}]$ is the urea concentration at the midpoint transition. Values were estimated from the best fit values for the data to a two denaturation transition (51, 52). Use instead of the particular $m$ in each case lowers the standard error of the calculation (53).

Analytical Ultracentrifugation—Short column (70 μl) sedimentation equilibrium experiments were performed at 283 K, either at 25,000 rpm (bFGF, aFGF, and mixtures) or 50,000 rpm (PF4 (47–70)) in a Beckman Optima XL-A analytical ultracentrifuge equipped with absorbance optics, using an An-60Ti rotor and standard (12-mm optical path) 6-channel centerpieces of Epon charcoal. Radial absorbance scans were carried out at 277–288 nm until equilibrium was reached. High speed sedimentation (50,000 rpm) was carried out afterward for base-line determination. Whole-cell apparent weight average molecular weights ($M_{\text{w,a}}$) were obtained by fitting the experimental data to the expression describing the radial concentration distribution of an ideal solute on sedimentation equilibrium using the program EQASSOC (54). Partial specific volumes of 0.73 ml/g (bFGF; 50), 0.77 ml/g (PF4-(47–70) determined from the ratio of the translational frictional coefficient ($f_T$) to the experimental time of 2 h.

Three-dimensional Structure in Solution of PF4-(47–70)—Several NMR parameters provide information on the structure of peptides in solution. The most conclusive evidence for the existence of preferred structures in peptides comes from the NOE connectivities, particularly from the non-sequential ones. Since an NOE peak between two protons is observed when they are spatially close irrespective of their sequence proximity, the presence of non-sequential NOE cross-correlations indicates significant populations of preferred conformations.

According to the $^1$H NMR spectra, the association state of PF4-(47–70) does not change when its concentration is lowered from 3.2 to 0.32 mM, where equilibrium sedimentation experiments show that PF4-(47–70) is a monomer (see below). Fig. 2A (top) shows the primary structure of PF4-(47–70) (PF4 numbering scheme) and a summary of the NOE connectivities observed for PF4-(47–70) in aqueous solution. Apart from the sequential NOE connectivities used for assignment, a number of non-sequential NOE cross-peaks, $d_{\text{N,Ni},i-2}$ and $d_{\text{N,Ni},i+3}$, were also observed. These NOEs, together with a few non-sequential $i, i + 3$, and $i, i + 4$ NOE connectivities involving side chain protons, indicate the presence of $\alpha$-helical conformations in PF4-(47–70), in agreement with the far-UV CD data. Excluding the N- and C-terminal residues, the conformational deviations of the measured chemical shifts ($\delta$-values) for the $C^\alpha$ protons from random coil values ($\Delta \delta_{\text{calcd}} = \Delta \delta_{\text{calcd}|\text{observed}} - \Delta \delta_{\text{calcd}|\text{random coil}}$ ppm) measured for PF4-(47–70) are, in general, negative and relatively large in absolute value, both at pH 3.4 and 6.5 ($\delta$ $> 0.05$ ppm; Fig. 2A, bottom). Thus, according to NOE and $\Delta \delta_{\text{calcd}}$ data, PF4-(47–70) preferably adopts $\alpha$-helical structures (64–66; Fig. 2A). According to the $\Delta \delta_{\text{calcd}}$ values of Fig. 2A, the structure of PF4-(47–70) is equivalent at pH 3.4 and pH 6.5, in agreement with the CD data.

The conformation of PF4-(47–70) was also examined in the presence of 30% TFE, where, according to CD data, the $\alpha$-helix content is close to the 100%. As expected, the number of NOEs characteristic of $\alpha$-helix, $d_{\text{N,Ni},i-2}$, $d_{\text{N,Ni},i+3}$, $d_{\text{N,Ni},i-4}$, $d_{\text{N,Ni},i+3}$, and $d_{\text{N,Ni},i+3}$, increases significantly in the presence of TFE, as do the number of NOEs involving side chain protons (Fig. 2B, top). In addition, the conformational $\Delta \delta_{\text{calcd}}$ shifts of segment 57–68 become more negative indicating that the enhancement in helix structure of PF4-(47–70) observed in the CD spectra occurs chiefly in the 57–68 segment (Fig. 2B, bottom). As in the case of PF4-(47–70) in the absence of TFE, $\Delta \delta_{\text{calcd}}$ values show that the structure of PF4-(47–70) should be essentially the same at pH 3.4 and 6.5 (Fig. 2B, bottom).

Since in aqueous solution the number of observed NOE connectivities relevant for the structure calculation is small (20 distance constraints), we first performed the calculation of the structure of PF4-(47–70) in 30% TFE. Under these conditions, the number of non-sequential NOE connectivities (141 distance constraints in total) is adequate for such structure calculations. Sequential NOE connectivities were not included in the calculations since random conformations contribute to their intensity. Fig. 3 (top) shows the stereoview of the backbone of the backbone and side chain heavy atoms of the 20 structures calculated for PF4-(47–70), and Fig. 3 (bottom left) shows a ribbon diagram of the backbone topology. Considering residues 49–68, the pairwise RMSD is 0.8 ± 0.5 Å for the backbone atoms and 1.7 ± 0.5 Å for all heavy atoms. In agreement with NOE connectivities and $\Delta \delta_{\text{calcd}}$ data of Fig. 2B, the calculated structure shows two helical regions composed of residues 50–53 and 57–67 which are linked by a short loop. The relative orientation of the two helices (Fig. 3, bottom left) is well substantiated by the unambiguous assignment of long range NOEs between protons of Asn$^{57}$ and Pro$^{68}$, Ile$^{51}$ and Pro$^{68}$, and Ile$^{51}$ and Leu$^{59}$ (Asp$^{57}$ C=H-Pro$^{58}$ C=H; Ile$^{51}$ HN-Pro$^{58}$ C=H/C=O H; Ile$^{51}$ C=H-Pro$^{58}$ C=H; Ile$^{51}$ C=H-Leu$^{59}$ HN). The 57–67 $\alpha$-helix is highly amphipathic, mostly toward the C
and 358 K (dotted line) (v/v) TFE. Protein (72/H9262 conditions. Top plot interfaced with a Nestlab RTE-110 water bath. J-720 spectropolarimeter fitted with a thermostated cell holder and HCl or NaOH. Spectra (scan speed 50 nm/min) were acquired in a Jasco the figure where pH was adjusted at the indicated values either with increases in bFGF Trp fluorescence induced by PF4-(47–70) of Trp barely increases during the first 24 h of incubation (Fig. 4 but steadily increases when incubated in the presence of PF4-(47–70). Fluorescence enhancement was prevented by DTI (200 μm; not shown) during at least the first 12 h of incubation, which clearly indicates that the fluorescence enhancement of bFGF Trp involves oxidation of Cys residues. Increases in bFGF Trp fluorescence induced by PF4-(47–70) are not prevented by strong polyamions like MIHS at relative concentrations (1:1) when it protected FGF from denaturation (not shown (50, 71)). Fluorescence enhancement seems specif-

![Figure 1](image)

**Fig. 1.** Molar ellipticity of PF4-(47–70) in solution at different conditions. Top plot, far-UV spectra at pH 10 (dashed line), pH 7 (solid line), and pH 3 (dotted line). Middle plot, spectra at 298 K (solid line) and 358 K (dotted line). Bottom plot, spectrum in the presence of 25% (v/v) TFE. Protein (72 μg) was in 10 mM sodium phosphate, NaCl 80 mM. Solution was buffered at 7.2 except in the experiments at the top of the figure where pH was adjusted at the indicated values either with HCl or NaOH. Spectra (scan speed 50 nm/min) were acquired in a Jasco J-720 spectropolarimeter fitted with a thermostated cell holder and interfaced with a Nestlab RTE-110 water bath. terminus, because hydrophobic (Leu, Ile, Ala, Pro, and Tyr) and charged (Lys) residues are concentrated on opposite faces (Fig. 3, bottom right). Amphipathic helices are common in protein structure and often participate in recognition processes.

A second structure computation was performed using a set of distance constraints that included those derived from the 1H NMR spectra of the protein in 30% TFE, used above, plus the nine non-sequential NOEs observed in aqueous solution that are not present when the protein is in 30% TFE. There are no significant differences between the second set of structures derived from these calculations (not shown) and those previously computed for PF4-(47–70) in 30% TFE (the RMSD between the mean structures of the two calculations are, respectively, 0.7 Å for the backbone atoms, and 1.3 Å for all heavy atoms). The populations of α-helix adopted by PF4-(47–70) in aqueous solution are 19 and 25% within the helical regions 50–53 and 57–67, respectively, and 21 and 87% in 30% TFE, as estimated from the averaged conformational δH13C13 shifts (67). These results are consistent with those estimated from far-UV CD spectroscopy (see above).

**Separate Secondary Structure of the Two Subdomains of PF4-(47–70)—** As schematized in Fig. 3, the three-dimensional structure of PF4-(47–70) consists of two helices connected by a loop constituted by residues 54–56. The C-terminal α-helix of PF4-(47–70) (residues 57–67) is also present in a full-length PF4. In PF4, this α-helix is highly solvent-exposed, whereas residues from Leu55 toward the N terminus are deeply buried in the tetramer core. To get some structural insight into the basis of PF4-(47–70) folding and inhibitory activity, we studied if the two helices could fold separately. Two partially overlapping peptides were synthesized, PF4-(54–70), corresponding to the long helix of the structure plus the N-cap (Gln56) and the residues belonging to the helix staple (Leu55 and Leu59 (68)) and PF4-(47–58) that includes the short helix (residues 50–53; Fig. 3). The far-UV CD spectrum of PF4-(54–70) indicates that it is 16% helical (not shown). The helicity increased to ~100% in 30% TFE. The good agreement between these values and those computed from the three-dimensional structure and the effect of TFE clearly demonstrate the intrinsic tendency of this region to adopt a helical fold. This does not seem to be the case with PF4-(47–58); its far-UV CD spectrum corresponds to a helix content that is essentially zero (0.8%) and increases to only 4.6% upon addition of 30% TFE. The TFE enhancement of the α-helical content of PF4-(47–58) observed in the NMR studies was also very slight (see above).

**Fluorescence of bFGF in the Presence of PF4-(47–70), PF4-(47–58), and PF4-(54–70)—** The single Trp in bFGF, as well as that in aFGF, barely fluoresces. Changes in the fluorescence of this residue closely monitor alterations in the three-dimensional structure of these proteins (69, 70) that are normally assumed to run parallel with the inactivation of bFGF. A potential interaction between PF4-(47–70) and bFGF that could cause alterations in the structure of bFGF was first studied by monitoring its fluorescence spectrum when PF4-(47–70) was incubated with bFGF at conditions where bFGF activity is inhibited. As shown in Fig. 4 (left), the fluorescence of bFGF Trp barely increases during the first 24 h of incubation (dotted line) but steadily increases when incubated in the presence of PF4-(47–70). Fluorescence enhancement was prevented byDTI (200 μm; not shown) during at least the first 12 h of incubation, which clearly indicates that the fluorescence enhancement of bFGF Trp involves oxidation of Cys residues. Increases in bFGF Trp fluorescence induced by PF4-(47–70) are not prevented by strong polyamions like MIHS at relative concentrations (1:1) when it protected FGF from denaturation (not shown (50, 71)). Fluorescence enhancement seems specif-
ically caused by PF4-(47–70) as free Cys, reduced glutathione, and scPF4-(47–70) at similar molar concentrations do not appreciably enhance the Trp fluorescence of bFGF during the first 25 h of incubation (not shown). Furthermore, incubation of bFGF with PF4-(54–70) for the same period did not cause any apparent alteration of the fluorescence (not shown), whereas peptide PF4-(47–58) produces even larger Trp fluorescence increases than that induced by PF4-(47–70) at equivalent concentrations (Fig. 4). This suggests a primary role of the free Cys residue in PF4-(47–70) in causing the alteration in the bFGF fluorescence. The Cys oxidation does not seem to require trace amounts of transition metal ions, as the experiments summarized in Fig. 4 were carried out with EDTA. Nevertheless, the presence of such ions accelerates the process, as fluorescence changes are clearly apparent at shorter times in the absence of EDTA (not shown). However, these changes can still be prevented under these conditions, for the first 12 h of incubation, by the addition of DTT to the medium (200 μM; not shown).

Effect of PF4-(47–70), PF4-(47–58), and PF4-(54–70) and scPF4-(47–70) in bFGF-driven Mitogenesis—The effect of PF4-(47–70) and its two subdomains in bFGF mitogenic activity was evaluated next. As shown in Fig. 5, increasing concentrations of PF4-(47–70) reduced the mitogenic activity of bFGF to nearly undetectable levels in these assays, as described (26). Neither of the shorter peptides (PF4-(47–58) nor PF4-(54–70)) show any inhibitory activity at concentrations where PF4-(47–70) effectively inhibits bFGF (Fig. 5). PF4-(47–58) and PF4-(54–70) are also ineffective when added simultaneously at a 1:1 molar ratio (data not shown). Accordingly, neither the sequence of either short peptide nor the secondary structure of PF4-(54–70) is sufficient to evoke an appreciable inhibition of FGF-driven mitogenesis. No apparent mitogenic inhibition was observed either with scPF4-(47–70) (not shown), which clearly indicates that inhibitory activity is not due to a relatively nonspecific electrostatic binding of a redox active peptide as PF4-(47–70).

Inhibition of bFGF mitogenic activity by PF4-(47–70) does not seem to correlate exclusively with the bFGF structural

![Summary of NOE connectivities and conformational shifts of PF4-(47–70).](image)

Fig. 2. Summary of NOE connectivities and conformational shifts of PF4-(47–70). NOE connectivities of PF4-(47–70) in aqueous solution (A) and in 30% TFE (v/v) (B) are represented at the top and conformational Δδ_{Cα-H} shifts at the bottom. NMR spectra were acquired at 278 K in the first case and at 298 K in the second. Peptide solutions were at pH 3.4, unless otherwise indicated. Δδ_{Cα-H} is the difference in ppm between δ_{Cα-H (obsd.)} and δ_{Cα-H (RC)}, where δ_{Cα-H (RC)} is the δ_{Cα-H} corresponding to random coil peptides. The Δδ_{Cα-H} of Ala57, a Pro-preceding residue, was corrected for the Pro effect (−0.29 ppm) (86). The thickness of the lines reflects the intensity of the sequential NOE connectivities, i.e. weak, medium, and strong. The α-helix corresponding to the secondary structure in PF4-(47–70) appears schematized at the top of the histograms. Sequence of human PF4-(47–70) in single letter code appears at the top of the figure.
alteration reported by the enhanced Trp fluorescence. PF4-(47–58), for example, fails to induce any apparent inhibition of bFGF mitogenic activity despite being a stronger inducer of bFGF Trp fluorescence than PF4-(47–70) (Figs. 4 and 5). Furthermore, the inhibition by PF4-(47–70) of bFGF-induced mitogenesis in the presence and absence of DTT in short incubation assays (12 h, Fig. 6), where bFGF fluorescence does not appreciably change (see above), is equivalent to that in Fig. 5.

Interaction of PF4-(47–70) with aFGF—The near-absolute requirement of aFGF upon heparin for mitogenic activity and stability (50, 71, 72) makes it difficult to evaluate the effects of a PF4-(47–70) direct interaction with aFGF. Thus, we first tried to synthesize a stabilized aFGF variant that does not require heparin for mitogenesis. For bovine aFGF, it has been reported (73) that the heparin dependence can be eliminated by substituting Cys61 by Ala and His107 by Gly. Cys61 is not one of the two cysteines that are highly conserved in the FGF family. It has been suggested that the Gly for His107 substitution decreases the heparin dependence by stabilizing the three-dimensional structure of the protein because Gly fits better than His into the type I β-turn between β-strands 8 and 9 (residues 104–107 (74)). This substitution increases the stability of the protein to urea denaturation (Table I). Further substitutions at this turn to incorporate the amino acids with the highest type I β-turn propensities (74) do not improve the stability of the protein except, perhaps, in the case of Glu105 substituted by Pro in presence of the His107 to Gly mutation (aFGF3; Table I). The two most stable aFGF variants obtained

FIG. 3. Preferential three-dimensional structure of PF4-(47–70) in solution. Top, stereoview of the superposition of the best 20 structures calculated for PF4-(47–70) in 30% (v/v) TFE (black, backbone; side chains: yellow, Cys; blue, positively charged; purple, negatively charged; orange, hydrophilic; green, hydrophobic). Bottom left, ribbon representation of the topology of the backbone structure of PF4-(47–70), and right, view from the C terminus of the 57–67 α-helix best-fit backbone superposition (same color codes as above) of the 20 structures calculated for PF4-(47–70) in 30% TFE (v/v).

FIG. 4. Progressive enhancement of the Trp fluorescence of bFGF by PF4-(47–70) and PF4-(47–58). Samples were kept at 310 K in the presence of PF4-(47–70) (left) and PF4-(47–58) (right) at 18 μM. Protein (1 μM) was buffered at pH 7.0 in 90 mM NaCl, 10 mM sodium phosphate, and 5 mM EDTA. Successive scans were carried out at the beginning of the incubation (a), and after 2 (b), 4 (c), 7 (d), and 22 h (e). Dotted spectra correspond to bFGF solutions incubated for 24 h in the same conditions in the absence of PF4-(47–70) and PF4-(47–58). Dashed spectra are those of bFGF solutions after the addition of denaturing concentrations of urea (8 M).

FIG. 5. Inhibition of bFGF-driven mitogenesis by PF4-(47–70) and its two subdomains. BaF3/FR1c-11 cells in the presence of increasing concentrations of PF4-(47–70) (○), PF4-(47–58) (●), or PF4-(54–70) (■) were challenged, except in ●, by a mitogen concentration (60 ng/ml) that elicits a cell proliferation 85% of the maximum obtained in bFGF concentration-proliferation dependence assays (two consecutive duplications of the population after 48 h). The experiments were repeated four times with similar results.
The mitogenic activities of the remaining aFGF forms reported here are more dependent on heparin than aFGF7 (Fig. 7). Similar effects were observed when the experiments were carried out in the presence (10 μg/ml) of the heparin-functional analog MIHS whose chemical structure is well defined and whose binding site on the aFGF surface has been mapped by 1H NMR spectroscopy (50, 62) (half-inhibition at 5 μg/ml in the presence of MIHS versus 7 μg/ml in its absence). As in the case of bFGF, scPF4-(47–70) does not appreciably inhibit mitogenesis induced by aFGF7 under the conditions of the assays carried out with PF4-(47–70) (not shown).

by substitutions in the loop between β-strands 8 and 9 (aFGF3 and aFGF4) do indeed show a higher mitogenic activity in the absence of heparin than the wild-type protein (Fig. 7).

The substitution of Ser for Cys has been also reported to be responsible for most of the differences in three-dimensional geometry between Cys and Ser (the increased half-life previously reported for these mutations was most likely due to the elimination of sulphydryl chemistry (76)). Human aFGF has a non-conserved Cys, Cys131. This residue is approximately at the center of β-strand 11, which links with β-strand 10 to form a β-antiparallel hairpin (50). Alignment of the very similar human and bovine aFGF sequences (11 substitutions (77)) shows that in the bovine sequence Ser substitutes for Cys in the β10-β11 hairpin, and Arg and Leu substitute for Ser130 and Arg133, respectively. As in the case of bFGF, scPF4-(47–70) and aFGF5-(47–70) do not appreciably inhibit mitogenesis induced by aFGF7 under the conditions of the assays carried out with PF4-(47–70) (not shown).
The effect of PF4-(47–70) on the fluorescence of aFGF7 was subsequently tested. aFGF7 does not show any apparent Trp fluorescence in native form, as in the case of bFGF and wild-type aFGF (Fig. 4 (69)). However, in contrast to the case of bFGF reported above, incubation of aFGF7 for 24 h at 310 K with PF4-(47–70) does not elicit any appreciable Trp fluorescence increase, either in the presence or absence of DTT plus EDTA (data not shown).

**Formation of a PF4-(47–70):FGF Complex**—We first studied whether incubation of bFGF in the presence of PF4-(47–70) induces the formation of some sort of bFGF-PF4-(47–70) complex. As a first step, molecular sieving performed in gels equilibrated with buffers containing 50 μM PF4-(47–70), a concentration that fully inhibits bFGF mitogenic activity (Fig. 5), resulted in bFGF eluting earlier than when PF4-(47–70) is absent (Fig. 9). Differences in the elution time correspond to an apparent $M_r$, difference of +4200, a value that may reflect a bFGF-PF4-(47–70) complex. As a first step, molecular sieving performed in gels equilibrated with buffers containing 50 μM PF4-(47–70), a concentration that fully inhibits bFGF mitogenic activity (Fig. 5), resulted in bFGF eluting earlier than when PF4-(47–70) is absent (Fig. 9). Differences in the elution time correspond to an apparent $M_r$, difference of +4200, a value that may reflect a bFGF-PF4-(47–70) complex.

To understand the structural basis of the alteration in migration rates observed in molecular sieving chromatography, a series of analytical ultracentrifugation studies were carried out. We first determined the equilibrium sedimentation profiles of the solutions of PF4-(47–70), bFGF, and a mixture of both at inhibitory concentrations of PF4-(47–70) (40 μM; molar excess of PF4-(47–70) over bFGF of 4:1). The profiles for the first two correspond to sedimenting particles with respective $M_r$ values of $M_w$ of $M_w$ of $M_w$ and $M_w$ and $M_w$. These values are quite close to the molecular weights calculated on the basis of their amino acid composition (2785 and 17,200). In the case of the two compound mixture, the sedimenting profile was that of a particle with a $M_w$ of $M_w$, a value corresponds to a 1:1 complex of PF4-(47–70) and bFGF (Fig. 10A). The free bFGF is undetectable in the ultracentrifuged mixture of the growth factor and PF4-(47–70), indicating that the dissociation constant of the complex lies in the submicromolar range, in agreement with mitogenesis data (see above). No appreciable association with bFGF was detected when PF4-(47–70) was substituted by scPF4-(47–70) (not shown). Omission of DTT from the bFGF and PF4-(47–70) mixture results in the precipitation of both from the solution. This is not the case when the compounds are ultracentrifuged separately, where equivalent results were obtained with and without DTT (not shown).

**Retention Time (min)**

![Graph showing differential absorbance](image)

**Fig. 8. Effect of heparin and PF4-(47–70) on BaF3/FR1c-11 cell proliferation induced by aFGF7. Left plot, proliferation of BaF3/FR1c-11 cells induced by aFGF7 in the presence (○) and the absence (■) of heparin (50 μg/ml). Right plot, effect of PF4-(47–70) (▲) on the proliferation of BaF3/FR1c-11 cells induced by 60 ng/ml of aFGF7 in the absence of heparin.▼, effect of PF4-(47–70) on quiescent cells in the absence of heparin. Heparin was used at the concentrations where its maximum activating effect is observed.**

**Fig. 9. Representative gel-sieving chromatograms of bFGF in the presence of PF4-(47–70) and in its absence.** The column (Superdex-75 PC 3.2/30; Amersham Pharmacia Biotech) was equilibrated with either (dotted plot) 150 mM NaCl and 10 mM sodium phosphate (pH 7.2) or (solid plot) the same buffer containing 50 μM PF4-(47–70). Injection volume was 20 μl. Flow rate was 50 μl/min, and temperature was 298 K. Time origin was the beginning of the injection of 0.6–2 nmol of bFGF. The injection sample also contained 50 μM PF4-(47–70) in the case of the solid plot. Arrows indicate the position of the size standards as follows: 67 kDa, albumin; 43 kDa, ovalbumin; 25 kDa, chymotrypsinogen; 13.7 kDa, ribonuclease A (Amersham Pharmacia Biotech Low Molecular Weight Gel Filtration Calibration Kit). Chromatography was carried out in a Smart System equipped with 50-ml pumps and a μPeak Monitor (Amersham Pharmacia Biotech).
the global hydrodynamic behavior of the complex slightly deviates from that expected for rigid spherical proteins \((f/f_0 = 1 \text{ (80)})\), such as free bFGF (81). The departure from the spherical behavior could result from increased hydration or expansion (80). Hydration alone could only account for this deviation if it reaches abnormally high values (4 g of water/g of protein). Assuming moderate hydration, the results indicate that the protein becomes less spherical upon PF4-(47-70) binding. Differences in \(f/f_0\) ratios between bFGF and the PF4-(47-70)-bFGF complex sedimenting solutions could account for the value between 1 and 2 estimated for the stoichiometry of the PF4-(47-70)-bFGF complex from the molecular sieving chromatography. In the case of mixtures of bFGF and scPF4-(47-70), an \(s_{20,w}\) value of 2.04 S was obtained, a value equivalent to that of samples containing exclusively bFGF.

The ultracentrifugation experiments carried out with bFGF were repeated with aFGF7. In this case, no precipitation of the protein in the presence of PF4-(47-70) was observed between 200 and 310 K when both DTT and EDTA were omitted from the solution. Sedimentation profiles at equilibrium of aFGF7 in the presence and the absence of PF4-(47-70) (molar ratio of PF4-(47-70) to aFGF7 of 4:1; 310 K; no DTT; no EDTA) correspond to sedimenting particles with \(M_w\) of \(\sim 18,500\) and \(\sim 15,700\), respectively (Fig. 10B). These values are quite close to the \(M_w\) calculated on the basis of amino acid composition for a complex with a 1:1 stoichiometry of aFGF7 and PF4-(47-70) (18,534) and aFGF7 (15,750), respectively. Equivalent results were also obtained between 70 and 500 mM NaCl concentrations. Addition of MIHS, at a molar ratio where it protects FGF activity (62), did not substantially alter the sedimentation profile of the aFGF7/PF4-(47-70) mixture (Fig. 10B). Ultracentrifugation experiments did not reveal any appreciable interaction between PF4-(47-70) and MIHS under these conditions (not shown).

Sedimentation velocity experiments carried out with aFGF7 in the presence and absence of PF4-(47-70) also yielded equivalent results to those obtained with bFGF. Those profiles present single sedimentation boundaries in each case that, according to the equilibrium sedimentation experiments, correspond to the sedimentation of aFGF7 and the complex PF4-(47-70)-aFGF7, respectively (data not shown). Values of \(s_{20,w}\) computed from those profiles (2.08 ± 0.05 S for aFGF7 and 1.95 ± 0.04 S for the PF4-(47-70)-aFGF7, respectively) show that the formation of the complex is accompanied by a slight alteration in the overall shape of the sedimenting particle in the order of that for bFGF. An equivalent \(s_{20,w}\) value (1.94 ± 0.04 S) was obtained at one-third the concentration at the same aFGF7:ligand ratio.

As in the case of bFGF, the sedimentation velocity results obtained with aFGF7 also indicate an alteration in the molecular sphericity of the sedimenting particle upon PF4-(47-70) binding. A partial assignment of \(^1^H\)-\(^1^N\) HSQC spectra of \(^1^N\)-labeled aFGF7 free and bound to PF4-(47-70) shows significant differences in the location of cross-peaks corresponding to backbone HN resonances: (free versus bound, \(^1^N\)/\(^1^H\) \(\delta\) values in ppm) Gly\(^33\): 105.67/8.43 versus 106.37/8.43; His\(^55\): 113.80/6.34 versus 113.65/6.33; His\(^65\): 117.50/9.78 versus 117.10/9.80; Gln\(^57\): 120.05/7.13 versus 120.22/7.2; Lys\(^117\): 120.55/8.76 versus 120.21/8.75. These differences suggest an alteration of the aFGF7 backbone upon PF4-(47-70) binding. Some of these changes may correlate with the inhibition of mitogenic activity by PF4-(47-70), as they correspond to backbone groups at the aFGF face recognized by the cell-surface receptors (Gly\(^33\), His\(^55\), and Gln\(^57\) (62, 82)).
50% to reach the maximum helical content (Ref. 83 and references therein). In the case of PF4-(47–70), the helix content reaches 100% in 25% TFE. In addition, the far-UV CD data indicate that a high helix content (46%) is maintained over a wide range of conditions in the absence of TFE. Deviations of the measured chemical shifts (δ-values) for the C¹H protons from random-coil values (ΔδC¹H) provide additional information on the presence and nature of the preferred conformation adopted. These deviations are negative in helices and positive in extended or β-sheet conformations (64–66). These values show that the helical propensity of PF4-(47–70) extends throughout its sequence even in the absence of TFE. ¹H NMR spectroscopy data are in agreement with the far-UV CD spectra of PF4-(47–70).

¹H NMR spectroscopy allowed computation of the relatively well defined α-helical three-dimensional structures of PF4-(47–70) in 30% TFE schematized in Fig. 3. Subsequently, the computation was repeated using a set of distance constraints that included those derived from the ¹H NMR spectra of the polypeptide in 30% TFE plus the non-sequential NOEs observed only in aqueous solution. As there are no significant differences between this new set of structures and those previously computed for PF4-(47–70) in 30% TFE, it seems reasonable to conclude that the family of folds adopted by this polypeptide in aqueous solution should not differ substantially from those adopted in 30% TFE. A direct calculation of the structure in aqueous solution was not attempted because the number of observed NOE connectivities relevant for structure calculations was small in aqueous solution. It should be pointed out that NOEs cannot be interpreted in terms of a unique structure in the case of short polypeptides in solution, due to the conformational averaging (Ref. 84 and references therein). However, computation of a limited number of structures compatible with NOE constraints provides a way to visualize the conformational properties of the family of folds preferably adopted by that peptide in solution. The determination of the three-dimensional structure at high resolution of the PF4-(47–70)-FGF complex will likely reveal if the conformation preferentially adopted by PF4-(47–70) in solution still persists when it is bound to FGF. The structural characterization of the complex should also help us to ascertain the relevance of the family of conformations preferentially adopted by PF4-(47–70) in solution for its inhibitory activity.

The experiments reported here show that PF4-(47–70) binds FGF, as was suggested by previous cell biology studies (20, 25, 26). The sedimentation results show that a consistent change in overall molecular shape occurs upon formation of the FGF-PF4-(47–70) complex. This change could be due just to the binding of PF4-(47–70) or, in addition, to an alteration of the folded conformation of FGFs upon formation of the complex. Modification of the environment of atoms deeply buried into the core of the FGF three-dimensional structure, according to the ¹H,¹⁵N HSQC data, suggests that the binding of PF4-(47–70) really alters the folded conformation of FGF. Furthermore, as some of the differences in δ-values of free αFGF and αFGF bound to PF4-(47–70) correspond to residues that are at opposite ends of the core of the molecule, the folding alteration seems to affect the whole protein. Inhibition of FGF-driven mitogenesis by PF4-(47–70) does not appear to be a direct consequence of growth factor precipitation, since this is a singular feature of bFGF that can be prevented by DTT without impairing the inhibitory activity of PF4-(47–70). For the same reasons, structural changes inherent to the fluorescence enhancement of bFGF Trp do not reflect PF4-(47–70) inhibition of mitogenic activity. It cannot be ruled out that PF4-(47–70)-induced precipitation could be an additional factor accounting

**DISCUSSION**

Experiments reported previously showed that PF4 binds to bFGF and inhibits processes considered to be essential in bFGF-induced mitogenesis (20, 21). They suggested as well that a direct interaction of PF4-(47–70) with bFGF could be also responsible of the inhibition of this last protein mitogenic activity. Here we show that PF4-(47–70), a peptide with a strong helical propensity, complexes with FGFs, inducing changes in its structure that may account for the reported decrease in affinity between bFGF and its cell-surface receptors in the presence of PF4-(47–70) (25, 28). We also show that PF4-(47–70) also induces structural changes, specific to certain forms of FGF, that do not seem related with the inhibition of its mitogenic activity.

Peptides with pronounced helical propensity, especially amphipathic, usually require a TFE concentration between 30 and
for the inhibition of heparin-induced bFGF dimerization, bFGF internalization, and bFGF-driven mitogenesis in some in vivo and in vitro experiments (Fig. 5) (25, 26).

A fluorescence enhancement of bFGF Trp occurs when the growth factor is incubated at the conditions of pH, temperature, and PF4-(47–70) concentrations used in the mitogenic assays. The single Cys of PF4-(47–70) seems quite specifically involved in this fluorescence increase, which probably involves the formation of disulfide bonds. The single Trp in bFGF is highly conserved in the FGF family including aFGF. In contrast to what is commonly assumed, the data reported here show that there are conformation(s) of mitogenically active bFGF that are highly fluorescent. This case resembles others described for aFGF; several forms of aFGF having both high mitogenic activity and high Trp fluorescence have been described (69). Actually, the fluorescence maximum of Trp of urea-denatured bFGF is red-shifted relative to the mitogenically active protein (Fig. 4), suggesting that these two fluorescent bFGFs are different structural species. Equivalent differences have been also reported for aFGF (69).

Potential interferences of polyanions, substances that are very common at the cell surface, in the interaction of PF4-(47–70) with FGFs were investigated using aFGF and MIHS as a model, except in the case of the Trp fluorescence enhancement, which seems a peculiarity of bFGF. MIHS is a heparin functional analog in aFGF-driven mitogenesis with a well defined chemical structure, and consequently, it is a better choice for biophysical studies (especially for ultracentrifugation) than heparin whose commercial preparations are rather heterogeneous. The three-dimensional structure of the aFGF-MIHS complex has been solved by NMR (50, 62, 85). According to the experiments reported here, polyanions do not seem to interfere significantly in the interaction between PF4-(47–70) and FGF at the structural and functional levels.

The structural data reported here can be used to propose a minimal structural model for the antiangiogenic activity of PF4. Comparison of the reported three-dimensional structure of human PF4 (23) with the PF4-(47–70) solution structure determined here shows that the C-terminal alpha-helix of PF4 (Ala57–Ser70) (23), which is highly exposed to the solvent, is conserved in PF4-(47–70). Residues preceding to Ala57 in PF4 form a loop and a beta-strand that, starting at Leu55, are buried into the tetramer core (23). Thus, on the basis of the PF4-(47–70) data reported here, the alpha-helix between Ala57 and Ser70 is the structural motif of PF4 responsible for its antiangiogenic activity. Nevertheless it is possible that the reported three-dimensional structure of PF4 (23) may not correspond to the actual antiangiogenic form of the protein, since Gupta et al. (24) isolated a considerably more antiangiogenic form of PF4. According to their report (24) PF4 may require the hydrolysis of the peptide bond between Thr16 and Ser17 for its antiangiogenic activity. An advance of such studies is of interest. An advance of such studies is of interest.

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