Identification of an Antiangiogenic FGF2-binding Site in the N Terminus of the Soluble Pattern Recognition Receptor PTX3*

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Long-pentraxin 3 (PTX3) is a soluble pattern recognition receptor with non-redundant functions in inflammation and innate immunity. PTX3 comprises a pentraxin-like C-terminal domain involved in complement activation via C1q interaction and an N-terminal extension with unknown functions. PTX3 binds fibroblast growth factor-2 (FGF2), inhibiting its pro-angiogenic and pro-restenotic activity. Here, retroviral transduced endothelial cells (ECs) overexpressing the N-terminal fragment PTX3-(1–178) showed reduced mitogenic activity in response to FGF2. Accordingly, purified recombinant PTX3-(1–178) binds FGF2, prevents PTX3/FGF2 interaction, and inhibits FGF2 mitogenic activity in ECs. Also, the monoclonal antibody mAb-MNB4, which recognizes the PTX3-(87–99) epitope, prevents FGF2/PTX3 interaction and abolishes the FGF2 antagonist activity of PTX3. Consistently, the synthetic peptides PTX3-(82–110) and PTX3-(97–110) bind FGF2 and inhibit the interaction of FGF2 with PTX3 immobilized to a BIAcore sensor chip, FGF2-dependent EC proliferation, and angiogenesis in vivo. Thus, the data identify a FGF2-binding domain in the N-terminal extension of PTX3 spanning the PTX3-(97–110) region, pointing to a novel function for the N-terminal extension of PTX3 and underlining the complexity of the PTX3 molecule for modular humoral pattern recognition.

Pentraxins are a superfamily of proteins characterized by a pentameric structure (1). The classical short-pentraxins C-reactive protein and serum amyloid P component are acute phase proteins in man and mouse, respectively (2). Pentraxins bind various ligands and are involved in the innate resistance to microbes and scavenging of cellular debris and extracellular matrix components (1, 3).

Long-pentraxins are characterized by an unrelated N-terminal domain coupled to a pentraxin-like C-terminal domain (4). The prototypic long-pentraxin PTX3 (5, 6) is a 45-kDa glycosylated protein produced locally by mononuclear phagocytes, dendritic cells, and endothelial cells (ECs) in response to primary inflammatory signals (7). Studies in ptx3−/− mice have shown that PTX3 plays complex non-redundant functions, ranging from the assembly of a hyaluronic acid-rich extracellular matrix and female fertility to innate immunity against diverse microorganisms (8, 9). This is related, at least in part, to the capacity of PTX3 to bind the complement component C1q, the extracellular matrix protein TSG6, and selected microorganisms, activating complement activation and facilitating pathogen recognition by macrophages and dendritic cells (1, 10). Thus, PTX3 is a soluble pattern recognition receptor with unique non-redundant functions in various pathophysiological conditions (1, 10).

Fibroblast growth factor-2 (FGF2) is a potent angiogenic growth factor (11). FGF2 modulates neovascularization during wound healing, inflammation, atherosclerosis, and tumor growth (12). Several molecules sequester FGF2 in the extracellular environment and inhibit its angiogenic activity (reviewed in Ref. 12). Many of these inhibitors are produced/released locally and/or systemically, thus underlying the complex tuning of the angiogenesis process.

PTX3 binds FGF2 with high affinity and specificity. Accordingly, PTX3 inhibits FGF2-dependent EC proliferation in vitro and angiogenesis in vivo (13). Also, PTX3 inhibits FGF2-dependent smooth muscle cell activation and intimal thickening after arterial injury (14). Thus, PTX3 may contribute to the modulation of FGF2 activity in different pathological settings characterized by the co-expression of the two proteins, including inflammation, wound healing, atherosclerosis, and neoplasia (see Refs. 13 and 14 for a further discussion).

Similar to short-pentraxins, PTX3 binds C1q via the C-ter-

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2 The abbreviations used are: EC, endothelial cell; bPTX3, biotin-labeled PTX3; FCS, fetal calf serum; FGF2, fibroblast growth factor 2; MAE cell, murine aortic endothelial cell; PTX3, pentraxin 3; EGFP, enhanced green fluorescent protein; PBS, phosphate-buffered saline; mAb, monoclonal antibody.
minal pentraxin domain (15). In contrast, the short-pentraxin C-reactive protein and serum amyloid P component are inefficient FGF2 binders/antagonists despite their sequence homology with the 203-amino acid C-terminal portion of PTX3 (13). This suggests that the 178-amino acid N-terminal portion of PTX3, absent in short-pentraxins, may mediate the binding to FGF2. At present, no biological functions have been ascribed to the PTX3 N terminus. On this basis, we have investigated the ability of the PTX3 N terminus to interact with FGF2. The results identify a short FGF2-binding domain in the N-terminal extension of PTX3 spanning the PTX3-(97–110) amino acid sequence. Synthetic peptides related to this sequence bind FGF2 and inhibit its angiogenic activity in vitro and in vivo.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—Human recombinant FGF2 and PTX3 were produced and purified as described (15, 16). Synthetic human PTX3-(31–60), PTX3-(57–85), and PTX3-(107–132) peptides were provided by Primm (Milan, Italy); all the other peptides were provided by Tecnogen (Piana di Monteverna, Caserta, Italy) (high performance liquid chromatography purity ≥95%). For all peptides, the amino acid sequence is shown in Table 1; amino acid numbering starts from the methionine residue in position 1 in the PTX3 leader sequence. Rat monoclonal antibodies directed against purified human PTX3 were described previously (15, 17).

**Cell Cultures**—Fetal bovine aortic GM7373 ECs (13) were grown in Eagle’s minimal essential medium containing 10% fetal calf serum (FCS). Human embryonic kidney (EcoPack2–293) packaging cells (Clontech) were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% FCS. Balb/c murine aortic 22106 ECs (MAE cells) were obtained from R. Auerbach (University of Wisconsin, Madison, WI) and grown in Dulbecco’s modified Eagle’s medium added with 10% FCS. Human embryonic kidney (EcoPack2–293) packaging cells (Clontech) were grown in Dulbecco’s modified Eagle’s minimal essential medium containing 10% human FCS plus FGF2 (0.55 nM) in the absence or the presence of different antagonists. After 24 or 48 h, respectively, cells were trypsinized and counted in a Burker chamber.

**Expression and Purification of PTX3 Fragments**—Nterm-PTX3 and Cterm-PTX3 cDNAs were amplified from pLX-PTX3 by PCR with primers containing additional nucleotides (PTX3-N: (+) CACCGGAAACTCGGATGATTGTA; (−) TTAACCTGCGGCCGACGCCGCTCC; PTX3-C: (+) CACCCTGTAACACGCTATTTTA; (−) TTATGAAACATACTGAGCCTC). These cDNAs were cloned into the pENTR TOPO vector (pENTR Directional TOPO cloning kit; Invitrogen) and sequenced. By using the Gateway® technology (Invitrogen), Nterm-PTX3 and Cterm-PTX3 cDNAs from pENTR TOPO vector were then cloned into the pDEST17 vector, allowing the insertion of a His6 tag at the C terminus of the recombinant proteins. *Escherichia coli* BL21-AI cells (Invitrogen) were then transformed with the two recombinant plasmids and grown at 37 °C in Luria Bertani medium containing 100 μg/ml ampicillin. Recombinant protein expression was induced by overnight incubation at 30 °C in the presence of 0.2% l-arabinose. After induction, cells were resuspended in binding buffer (20 mM sodium phosphate, 0.5 mM NaCl, 10 mM imidazole, pH 7.4) and lysed by sonication. Clarified supernatants were filtered through a 0.45-μm filter and loaded onto a 3.0-ml HiTrap Immobilized Metal Affinity Column (Amersham Biosciences) with nickel for purification according to the manufacturer’s instructions. Fractions were probed for the presence of the recombinant protein by immunoblotting, and positive fractions were collected and desalted by gel filtration chromatography (Sephadex G25 column PD10; Amersham Biosciences) in PBS. Purity of recombinant proteins was higher than 90%, as assessed by SDS-PAGE followed by silver staining of the gel (see Fig. 2A, inset).

Alternatively, recombinant Nterm-PTX3 and Cterm-PTX3 fragments were purified from stable transfected Chinese hamster ovary cells. Cterm-PTX3 was obtained as previously described (15). A 570-bp fragment coding for Nterm-PTX3 was subcloned in pSG5 and transfected in Chinese hamster ovary cells. Selection was carried out with G148, and a clone secreting the predicted 17.7-kDa fragment was chosen. Culture supernatant was collected from these cells, concentrated by ultrafiltration (YM membrane, cut off 10.0 kDa; Millipore Corp., Billerica, MA) and applied on an HR 5/5 Mono Q column (Amersham Biosciences) in 10 mM Tris-HCl, pH 7.0. Nterm-PTX3 was then eluted exactly as described for the purification of intact PTX3 (15). The fraction containing Nterm-PTX3 was subjected to gel filtration on Sephacryl S-300 in PBS. Purity of recombinant proteins was assessed by SDS-PAGE followed by silver staining (not shown).

**Solid Phase Binding Assay**—FGF2 (270 nM) was immobilized onto enzyme-linked immunosorbent assay microplates as
described (13). Next, 100 μl- aliquots of PBS containing full-length PTX3, recombinant Nterm-PTX3, or Cterm-PTX3 (all at 44 nm) were incubated for 30 min at 37 °C onto the FGF2-coated wells. Then, wells were sequentially incubated for 1 h at 37 °C with a rabbit polyclonal anti-PTX3 antibody (1:2000 dilution) that recognizes both PTX3 fragments with similar efficiency in Western blot and enzyme-linked immunosorbent assay (data not shown), an anti-rabbit biotinylated antibody (1:2000), and 100 μl of streptavidin-horseradish peroxidase (1:5000, Amersham Biosciences) for 1 h at room temperature. Then, 100 μl/well of the chromogen substrate 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) were added. Absorbance values were read at 405 nm. In some experiments, 100-μl aliquots of PBS containing biotin-labeled PTX3 (bPTX3) (22 nm) were incubated for 30 min at 37 °C onto FGF2-coated wells with or without competitors. Wells were washed, and the amount of bound bPTX3 was evaluated as described (13). Alternatively, synthetic PTX3 peptides were immobilized on enzyme-linked immunosorbent assay microplate wells (200 μg/well) as described above. FGF2 (80 nm) was added, and FGF2 bound to immobilized peptides was assessed by 1 h of incubation at 37 °C with a rabbit polyclonal anti-FGF2 antibody (1:7000) followed by immunocomplex detection as described above.

PTX3 Epitope Mapping—To identify the amino acid sequence of the epitopes binding to monoclonal anti-PTX3 antibodies, 128 peptides were arrayed onto cellulose membranes by SPOT synthesis technology (19). The peptides were 13 amino acids long with a 3-amino acid frameshift. Membranes were blocked with 2% milk in Tween-TBS (MBS) for 16 h at 4 °C. After washing, the membranes were incubated for 90 min at 37 °C with monoclonal antibodies mAb MNB4 or mAb 16B5 (both at 1:1000 dilution in MBS) and then incubated for 90 min at 37 °C with rabbit alkaline phosphatase-conjugated anti-rat IgG (1:30,000; Sigma) in MBS. Color reaction was developed as described (19), and intensity of the signal was evaluated by densitometric analysis of the membrane.

BLAcore Binding Assay—A BLAcore X apparatus (BLAcore Inc., Piscataway, NJ) was used. PTX3 (2.2 μg) was allowed to react with a flow cell of a CM4 sensor chip that was previously activated with 50 μl of a mixture of 0.2 M N-ethyl-N’-(3-dimethylaminopropyl)-carbodiimide hydrochloride and 0.05 M N-hydroxysuccinimide. These experimental conditions allowed the immobilization of 5000 resonance units, corresponding to ~0.1 pmol of PTX3. Similar results were obtained for immobilization of gelatin, here used as a negative control and for blank subtraction. Increasing concentrations of FGF2 with or without synthetic PTX3 peptides were then injected in dilution buffer (PBS plus 0.005% surfactant P20, 5.0 μg/ml CaCl2 and MgCl2) over the PTX3 surface for 4 min (to allow the association of FGF2 with immobilized PTX3). The response (in resonance units) was recorded at the end of injection, and binding data were plotted with an exponential fitting program for a one-site competition model (Pristm; GraphPad Software Inc.).

Chicken Embryo Chorioallantoic Membrane Assay—Alginate beads (5 μl) containing vehicle or 16 pmol FGF2 with or without synthetic PTX3 peptides were prepared as described (20) and placed on top of the chicken embryo chorioallantoic membrane of fertilized White Leghorn chicken eggs at day 11 of incubation (10 eggs/experimental group). After 72 h, blood vessels converging toward the implant were counted by two observers in a double-blind fashion under a stereomicroscope (STEMI-SR, ×2/0.12; Zeiss).

RESULTS

The N-terminal Extension of PTX3 Binds FGF2—PTX3 protein is characterized by a C-terminal 203-amino acid pentraxin domain (Cterm-PTX3) and by an N-terminal 178-amino acid extension (Nterm-PTX3) (5). In the attempt to identify the antiangiogenic, FGF2-binding domain(s) of PTX3, the two PTX3 portions were assessed for their capacity to interact with FGF2.

Overexpression of full-length PTX3 results in the inhibition of FGF2-dependent proliferation in ECs due to the binding of released PTX3 to the exogenous growth factor and its sequestration in the extracellular milieu (13). On this basis, MAE cells were infected with retroviruses harboring human full-length PTX3, the PTX3 N-terminal extension Nterm-PTX3, or the PTX3 C terminus fused to the PTX3 leader sequence for secretion (sCterm-PTX3). Control cells were infected with an EGFP-harbouring retrovirus. Infected cells overexpressed and released the corresponding proteins in similar amounts (Fig. 1A) and showed a similar rate of growth under basal conditions (data not shown). However, Nterm-PTX3 overexpression caused a significant decrease in the capacity of infected cells to proliferate in response to exogenous FGF2, similar to full-length PTX3 overexpression (Fig. 1B). No inhibition was exerted by sCterm-PTX3 overexpression when compared with control EGFP-infected cells.

In a second set of experiments, conditioned media of infected MAE cells were evaluated for the capacity to affect FGF2-dependent proliferation of GM7373 ECs (Fig. 1C). As anticipated, incubation of GM7373 cells with FGF2 in the presence of the conditioned medium of Nterm-PTX3-infected or PTX3-infected MAE cells caused a significant inhibition of the mitogenic activity of the growth factor, whereas the conditioned media of sCterm-PTX3-infected and EGFP-infected MAE cells were ineffective (Fig. 1C). None of the conditioned media caused a significant inhibition of GM7373 cell proliferation triggered by 10% FCS, thus confirming the specificity of the effect (data not shown).

Accordingly, similar to full-length PTX3, purified human recombinant Nterm-PTX3 inhibits GM7373 cell proliferation triggered by FGF2, with no effect on 10% FCS-induced proliferation (Fig. 1D). No effect on FGF2 activity was exerted by the purified C-terminal PTX3 fragment.

To confirm that the FGF2 antagonist activity of Nterm-PTX3 was due to its capacity to interact directly with the growth factor, Nterm-PTX3 and Cterm-PTX3 were produced as E. coli recombinant His6-tagged proteins (Fig. 2A, inset). Full-length PTX3 and the recombinant Nterm-PTX3 fragment bind to immobilized FGF2. No interaction was observed with recombinant Cterm-PTX3 (Fig. 2A). Accordingly, a 10-fold molar excess of recombinant Nterm-PTX3 or of full-length PTX3, but not of Cterm-PTX3, prevented the binding of biotinylated PTX3 (bPTX3) to immobilized FGF2 (Fig. 2B). Taken together, these results implicate the N-terminal region of PTX3 for FGF2 interaction.
Inhibition of FGF2/PTX3 Interaction by a Monoclonal Anti-Nterm-PTX3 Antibody—The screening of a set of rat monoclonal antibodies raised against human full-length PTX3 identified antibodies mAb-MNB4 (15) and mAb-16B5 (17) that selectively bind Nterm-PTX3 and Cterm-PTX3, respectively (Fig. 3A). To map the PTX3 epitopes recognized by the two antibodies, we took advantage of the SPOT synthesis technique (19) by which 128 overlapping 13-mer peptides spanning the entire human PTX3 sequence were arrayed on a cellulose membrane. When the membrane was probed with the two monoclonal antibodies, immunocomplex detection revealed that mAb-MNB4 recognizes the epitope PTX3-(87–99) present in the N-terminal extension of PTX3, whereas mAb-16B5 recognizes the epitope PTX3-(306–312) located in the C-terminal region of PTX3 (Fig. 3B).

When tested for the capacity to affect FGF2/PTX3 interaction, mAb-MNB4, but not mAb-16B5, prevented the capacity of bPTX3 to bind immobilized FGF2, similar to free unlabeled PTX3 (Fig. 4A). Accordingly, mAb-MNB4 abolishes the capacity of full-length PTX3 to inhibit the mitogenic activity exerted by FGF2 in GM7373 cells, whereas mAb-16B5 is ineffective (Fig. 4B). Thus, mAb-MNB4 recognizing the N-terminal PTX3-(87–99) epitope neutralizes FGF2/PTX3 interaction.

Synthetic Nterm-PTX3-related Peptides as FGF2 Antagonists—To further define the FGF2 binding region in the N-terminal extension of PTX3, we evaluated the FGF2 antagonist activity of the synthetic peptide PTX3-(82–110) that contains the PTX3-(87–99) epitope recognized by the neutralizing mAb-MNB4 (see above) together with three distinct synthetic peptides, PTX3-(31–60), PTX3-(57–85), and PTX3-(107–132), partially spanning the Nterm-PTX3 amino acid sequence (Fig. 5A and Table 1).

The four synthetic PTX3 fragments were first assessed for their
capacity to interact with FGF2. As shown in Fig. 5B, free FGF2 binds immobilized PTX3-(82–110) but not immobilized PTX3-(31–60) or PTX3-(57–85), showing only a limited interaction with immobilized PTX3-(107–132).

Next, surface plasmon resonance was exploited to assess the ability of the four peptides to affect FGF2/PTX3 interaction. FGF2 (0.8 μM) specifically binds to PTX3 immobilized to a BIAcore sensor chip but not to immobilized gelatin (Fig. 5C, upper panel). Also, injection of increasing concentrations of FGF2 (Fig. 5C, lower panel) over the PTX3 surface showed that FGF2/PTX3 interaction occurs with a kinetic dissociation constant ($k_{\text{off}}$) of $6 \times 10^{-5}$ s$^{-1}$ and a kinetic association constant ($k_{\text{on}}$) of $0.2 \times 10^3$ s$^{-1}$ M$^{-1}$, thus resulting in a $K_d$ value equal to $0.3 \times 10^{-6}$ M.

The synthetic PTX3 peptides were assessed for their capacity to sequester FGF2 in the mobile phase, thus preventing its interaction with the PTX3 surface. As shown in Fig. 5D, PTX3-(82–110) inhibits the binding of FGF2 to the PTX3 surface in a dose-dependent manner with a potency 5 times lower than that shown by free full-length PTX3 (ID$_{50}$ equal to 3.3 and 0.7 μM, respectively). No inhibition was exerted by PTX3-(31–60), PTX3-(57–85), and PTX3-(107–132) peptides (Fig. 5D and data not shown). Also, a scrambled synthetic peptide with amino acid composition equal to PTX3-(82–110) (sPTX3-(82–110), Table 1) showed a limited inhibitory effect (ID$_{50}$ equal to 3300 μM) (Fig. 5D), indicating that the primary amino acid sequence in PTX3-(82–110) is of importance for FGF2 interaction.

The capacity of PTX3-(82–110) to bind FGF2 prompted us to assess its ability to act as a FGF2 antagonist. When tested on GM7373 cells, both full-length PTX3 and PTX3-(82–110) inhibit the mitogenic activity of FGF2, whereas scrambled PTX3-(82–110), PTX3-(31–60), PTX3-(57–85), and PTX3-(107–132) peptides were ineffective (Fig. 5E).

Identification of a Minimal Linear FGF2 Binding Sequence in the N-terminal Extension of PTX3—In an attempt to identify a minimal linear FGF2 binding sequence in the PTX3-(82–110) region, three overlapping synthetic peptides,
PTX3-(82–96), PTX3-(82–101), and PTX3-(97–110) (Fig. 6A and Table 1), were evaluated for their capacity to interact with FGF2. As shown in Fig. 6B, free FGF2 binds to immobilized PTX3-(97–110), as well as to parental PTX3-(82–110) and full-length PTX3, without interacting with PTX3-(82–96) or PTX3-(82–101). Accordingly, PTX3-(97–110) binds FGF2 in the mobile phase, preventing its interaction with PTX3 immobilized to a BIAcore sensor chip (Fig. 6C). The inhibitory activity of PTX3-(97–110) was similar to that shown by the parental peptide PTX3-(82–110), whereas PTX3-(82–96) and PTX3-(82–101) were ineffective (Fig. 6C). In keeping with these observations, PTX3-(97–110), but not PTX3-(82–96) or PTX3-(82–101), inhibits the mitogenic activity exerted by FGF2 in GM7373 cells (Fig. 6D).

In contrast with PTX3-(97–110), the shorter overlapping peptides PTX3-(97–104) and PTX3-(100–110), spanning the 97–110 amino acid sequence, did not prevent the binding of free FGF2 to PTX3 immobilized to a BIAcore sensor chip and were unable to inhibit GM7373 cell proliferation triggered by FGF2 (data not shown). Thus, PTX3-(97–110) represents a minimal linear FGF2 binding amino acid sequence in the N-terminal extension of PTX3.

Synthetic Nterm-PTX3-related Peptides Inhibit the Angiogenic Activity of FGF2—The capacity of Nterm-PTX3-related peptides to affect FGF2-induced neovascularization in vivo was assessed in the chicken embryo chorioallantoic membrane assay (Fig. 7). Alginate beads adsorbed with FGF2 (16 pmol/embryo) exert a potent angiogenic response when compared with beads adsorbed with vehicle (macroscopic vessels converging toward the implant being equal to 44 ± 7 and 11 ± 5 vessels/embryo, respectively). In keeping with the in vitro observations, the FGF2-dependent angiogenic response was significantly reduced (28 ± 5 vessels/embryo, p < 0.05 by analysis of variance) by the addition of 3.0 nmol PTX3-(82–110) peptide in the FGF2 implants (Fig. 7). Accordingly, 80 nmol PTX3-(97–110) caused a 50% inhibition in the angiogenic response triggered by FGF2; no effect was exerted by PTX3-(82–96) (data not shown).

DISCUSSION

Pentraxins are evolutionary conserved soluble pattern recognition receptors (1). The pentraxin superfamily can be divided into two structural subfamilies: the classic short-pentraxins,
which include C-reactive protein and serum amyloid P component, and the novel long-pentraxins, their prototypic member being represented by PTX3 (1, 4).

PTX3 comprises a pentraxin-like C-terminal 203-amino acid domain and an N-terminal 178-amino acid extension with no significant homology with any other protein. The two domains are encoded by distinct exons in the ptx3 gene (5). Previous observations had shown that PTX3 binds FGF2 without showing any significant interaction with other growth factors, cytokines, and chemokines, including other members of the FGF family (for further discussion see Ref. 13). PTX3 inhibits the angiogenic activity exerted by FGF2 on ECs (13) and suppresses FGF2-dependent smooth muscle cell activation (14), thus representing an endogenous FGF2 inhibitor and a candidate for the design of novel FGF2 antagonists.

Here we have shown that FGF2 interaction is mediated by the N-terminal extension on PTX3. Also, neutralizing monoclonal antibodies and synthetic PTX3-related peptides identify the amino acid linear sequence PTX3-(97–110) as responsible for this interaction. These conclusions are based on the following experimental evidence: (i) the short-pentraxins C-reactive protein and serum amyloid P component are inefficient FGF2 binders (13) despite their sequence homology with the PTX3 C terminus (4); (ii) retroviral transduction of the Nterm-PTX3 fragment, but not of sCterm-PTX3, inhibits the mitogenic activity of FGF2 in ECs; (iii) recombinant Nterm-PTX3, but not Cterm-PTX3, binds to immobilized FGF2, prevents PTX3/FGF2 interaction, and inhibits FGF2 activity in ECs; (iv) the monoclonal antibody mAb-MNB4, mapping the linear epitope PTX3-(87–99), prevents FGF2/PTX3 interaction and abolishes the FGF2 antagonist activity of PTX3 in ECs; (v) synthetic peptides PTX3-(82–110) and PTX3-(97–110), but not other peptides based on different regions of the PTX3 N terminus, prevent FGF2/PTX3 interaction by binding FGF2, thus inhibiting FGF2-dependent EC proliferation in vitro and angiogenesis in vivo.

PTX3 binds the complement component C1q via its C-terminal domain (15). Accordingly, C1q does not compete for FGF2 binding to PTX3,3 thus confirming that distinct domains are involved in the interaction with the two ligands. PTX3 also binds apoptotic cells, extracellular matrix protein(s), and selected microorganisms, but their binding domain(s) on the

![FIGURE 6. PTX3-(97–110) peptide as a FGF2 antagonist. A, schematic representation of PTX3-(82–110)-spanning peptides. B, wells coated with the indicated PTX3 peptides (200 μg/well) were incubated with FGF2 (80 nM), and the amount of bound FGF2 was evaluated. Data, expressed as percentage of the amount of bound FGF2 to PTX3-coated wells, are the mean ± S.D. of three independent experiments in triplicate. C, FGF2 (0.8 μM) was injected over a PTX3-coated BIAcore sensor chip in the presence of increasing concentrations of PTX3-(82–110) ( ), PTX3-(97–101) ( ), PTX3-(82–101) ( ), or PTX3-(82–96) ( ). The response was recorded and binding data were plotted as described for Fig. 5D. R² was 0.91 and 0.94 for the FGF2 antagonists PTX3-(82–110) and PTX3-(97–110), respectively. D, GM7373 cells were incubated with FGF2 (0.55 nM) in the absence or in the presence of the indicated PTX3 peptides (all at 66 μM). Data, expressed as percentage of the proliferation observed in GM7373 cells incubated with FGF2 only, are the mean ± S.D. of three independent experiments in triplicate.](#)
PTX3 molecule are still uncharacterized (reviewed in Ref. 1). The possibility that PTX3 fragments with restricted binding specificity and different impact on innate immunity may be generated in vivo will require further experimentation.

Modeling of the human PTX3 N terminus is limited by the fact that no structural data are available for the N-terminal portion of long-pentraxins. We performed consensus secondary structure prediction of this region on the PredictProtein server (www.cubic.bioc.columbia.edu/predictprotein) using different algorithms. They predict four α-helix regions in the PTX3 N terminus spanning amino acid residues 55–75 (α1), 78–97 (α2), 109–135 (α3), and 144–170 (α4). Moreover α2, α3, and α4 have the propensity to be in a coiled-coil structure (as predicted by Coils2; www.ch.embnet.org/software/COILS_form.html). This structure comprises two or more α-helices that wind around each other to form “supercoils.” The putative minimal linear FGF2 binding region 97–110 is predicted in a loop region that comprises the end of the α2-helix (Glu102), a β-turn on residues Ala104-Pro105-Gly106-Ala107, and the first two residues of the α3-helix (Ala109-Glu110). Interestingly the same β-turn is predicted for PTX3 protein of different species (data not shown). It seems possible to hypothesize that this exposed region may interact with the FGF2 molecule. The other members of the long-pentraxin subfamily (including apedin, neuronal pentraxins NP1 and NP2, and neuronal pentraxin receptor NPR (4)) show a low level of amino acid sequence similarity in their N-terminal domain (~10% for PTX3 versus NP1). Accordingly, both genetic and biological features of the different long-pentraxins point to the hypothesis that they exert distinct, unique biological functions (4). It is therefore not surprising that the FGF2 binding region PTX3-(97–110), as well as the broader PTX3-(82–110) domain, does not show any significant homology with the corresponding regions in the other members of the long-pentraxin subfamily, thus suggesting that the FGF2 binding capacity is a unique function of PTX3. Further experiments are required to address this hypothesis.

The role exerted by FGF2 in various pathophysiological conditions is not limited to its angiogenic activity. Indeed, FGF2 stimulates the migration and proliferation of fibroblasts during wound healing and of smooth muscle cells during atherosclerosis (21, 22) and restenosis (23). Also, it favors neuronal cell survival and glia cell proliferation in the injured central nervous system (24). In all these conditions, the concomitant production of PTX3 (25, 26) may modulate the activity exerted by FGF2. Accordingly, PTX3 inhibits FGF2-dependent smooth muscle cell activation in vitro and intimal thickening after arterial injury in vivo (14).

In conclusion, we have demonstrated for the first time that PTX3 N terminus is involved in FGF2 interaction. PTX3 is a multifunctional soluble pattern recognition receptor at the crossroads between innate immunity, inflammation, matrix deposition, and female fertility. The identification of the functional motifs involved in ligand interaction will help to elucidate the mechanism(s) of action and complex functions of PTX3.

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