Identification of the Fibroblast Growth Factor (FGF)-interacting Domain in a Secreted FGF-binding Protein by Phage Display*

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Fibroblast growth factor-binding proteins (FGF-BP) are secreted carrier proteins that release fibroblast growth factors (FGFs) from the extracellular matrix storage and thus enhance FGF activity. Here we have mapped the interaction domain between human FGF-BP1 and FGF-2. For this, we generated T7 phage display libraries of N-terminally and C-terminally truncated FGF-BP1 fragments that were then panned against immobilized FGF-2. From this panning, a C-terminal fragment of FGF-BP1 (amino acids 193–234) was identified as the minimum binding domain for FGF. As a recombinant protein, this C-terminal fragment binds to FGF-2 and enhances FGF-2-induced signaling in NIH-3T3 fibroblasts and GM7373 endothelial cells, as well as mitogenesis and chemotaxis of NIH-3T3 cells. The FGF interaction domain in FGF-BP1 is distinct from the heparin-binding domain (amino acids 110–143), and homology modeling supports the notion of a distinct domain in the C terminus that is conserved across different species. This domain also contains conserved positioning of cysteine residues with the Cys-214/Cys-222 positions in the human protein predicted to participate in disulfide bridge formation. Phage display of a C214A mutation of FGF-BP1 reduced binding to FGF-2, indicating the functional significance of this disulfide bond. We concluded that the FGF interaction domain is contained in the C terminus of FGF-BP1.

Polypeptide growth factors of the fibroblast growth factor (FGF) family represent over 20 distinct proteins that are widely expressed in various tissues. FGFs regulate important developmental processes such as limb formation or mesoderm induction, induce neurite outgrowth, and stimulate the growth of new blood vessels in wound healing and tumor growth and impact on the regulation of the vascular tone [1–4]. FGF-1 (acidic FGF) and FGF-2 (basic FGF) are the best studied members of this family, FGF-1 and -2 are tightly bound to the extracellular matrix, and FGF-2 is a relatively abundant protein in numerous adult tissues, from which it can be extracted as a biologically active growth factor [5]. In support of the significance of this up-regulation of FGF-BP1, its depletion abrogates tumor growth and angiogenesis of human squamous cell carcinoma and colon cancer cells xenografted into athymic nude mice [21]. On the other hand, overexpression of FGF-BP1 in human tumor cells induces highly vascularized xenograft tumors in athymic nude mice [12]. Gene expression is up-regulated transcriptionally in cultured cells by phorbol esters as well as epidermal growth factor [13–15] during wound healing and chemical carcinogenesis of human and mouse skin [16, 17] and in kidney tissues injured by different toxic attacks [18]. Furthermore, FGF-BP1 mRNA and protein are up-regulated during early stages of colon and breast epithelial transformation [19, 20], and subsets of invasive colon, breast, and squamous cell cancers overexpress FGF-BP1 [12, 19–22]. In support of the significance of this up-regulation of FGF-BP1, its depletion abrogates tumor growth and angiogenesis of human squamous cell carcinoma and colon cancer cells xenografted into athymic nude mice [21]. On the other hand, overexpression of FGF-BP1 in human tumor cells induces highly vascularized xenograft tumors in athymic nude mice [12], and expression as a transgene in chick embryos causes vascular leakage [23]. At the protein level, FGF-BP1 protein interacts at least with FGF-1, FGF-2, and FGF-7 and enhances angiogenesis in a chicken chorioallantoic membrane assay as well as FGF signaling in fibroblasts, epithelial, and endothelial cells [18, 22, 24, 25]. These data support a significant role for FGF-BP1 in malignancies and during tissue repair.

To localize the interaction interface between FGF-2 and FGF-BP1 and gain further insight into the mechanism of action of FGF-BPs, we sought to identify a minimum interaction domain of FGF-BP1 that can retain the interaction with FGF-2 using a T7 phage display/panning system. Phage display combinatorial libraries have become invaluable tools for the identification of novel peptides or protein fragments from libraries, and the approach has been applied successfully to the selection-interacting peptide or protein ligands for antibodies [26]. FGF

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(27), anaplastic lymphoma kinase (28), FGF-1 (29), or the mapping of pathways (30). The most commonly used vectors are based on filamentous phage M13 (31). However, in M13 display, all components of the phage particle need to be exported through the bacterial inner membrane before phage assembly; thus, only protein that can be exported can be displayed on the M13 phage. Recently, phage display vectors based on lytic phage T7 have been developed (32). Since T7 phage assembly within the cytoplasm, there is no need for protein export, thus eliminating the issue of an incomplete representation of a protein library on the phage surface. In the present study, we mapped the interaction domain(s) between FGF-BP1 and FGF-2 using this T7 phage display system. We found that FGF-BP1-expressing phage bind to immobilized FGF-2 and that the FGF-BP1-expressing phage can be enriched by biopanning against immobilized FGF-2. From biopanning of phage libraries that contained different length fragments of FGF-BP1, we found that phage containing a short C-terminal fragment of FGF-BP1 bind to immobilized FGF-2. To rule out the possibility that phage portions are required for this interaction, a recombinant fusion protein containing the C-terminal fragment of FGF-BP1 and maltose-binding protein (Malt) was generated and found to bind to immobilized FGF-2 in steady-state and in kinetic studies using surface plasmon resonance. Also, FGF-2 signaling and biologic effects were enhanced. In contrast, deletion of the C-terminal domain from FGF-BP1 abolished its binding to FGF-2. Homology modeling supported these results and showed that the C-terminal residues of FGF-BP1 can form a stable domain that is sufficient for its binding to FGF.

MATERIALS AND METHODS

Cell Culture—NIH-3T3 cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% (v/v) calf serum. Transformed fetal bovine aortic endothelial GM7373 cells (33, 34) were obtained from the National Institute of General Medical Sciences, Human Genetic Mutual Cell Repository, Coriell Institute for Medical Research (Camden, NJ) and were maintained in modified improved minimal essential medium (Invitrogen) supplemented with 10% (v/v) fetal bovine serum.

Generation of FGF-BP1-expressing Phage—The human FGF-BP1 open reading frame (nucleotides 199–799) cDNA was ligated into the T7select 10-3b vector at EcoRI and HindIII sites. Phage packaging, amplification, and titration were performed according to the manufacturer’s instructions (32).

Binding of Phage to Immobilized FGF-2—Human recombinant FGF-2 (0.5 μg, Invitrogen) was diluted in 100 μl of Tris-buffered saline (TBS, 50 mM Tris-HCl, and 150 mM NaCl, pH 7.5) and incubated overnight in 96-well plates. (EIA/RIA strip plate; Corning Inc., Corning, NY) at 4 °C. Excess unbound FGF-2 was removed by washing the wells twice with TBS. Nonspecific binding was blocked by the addition of 300 μl of 5% nonfat dry milk in TBS. Specific binding was quantitated by washing the wells five times with TBS. Phage expressing FGF-BP1 or control empty phage (100 μl) were added to the wells and incubated for 1 h at room temperature. Unbound phage were removed by washing the wells five times with TBS containing 0.1% Tween 20. The bound phage were rescued by adding 100 μl of host bacteria BLT5403 (A900 at 0.6–0.8) to the wells followed by a 10-min incubation. The titer of the rescued phage was determined as described above (32). In addition, an aliquot was amplified for the next round of biopanning. For biopanning, rescued phage solution was added to 5 ml of host bacteria with 50 μg/ml penicillin and incubated overnight at 37 °C. The amplified phage was then added to a well coated with FGF-2 for the next round. The procedure was repeated several times as indicated in the respective figures. In the competition assays, different amounts of FGF-2 or EGF were preincubated with the phage for 30 min prior to addition to the wells.

Construction of Libraries Expressing Different Size Fragments of FGF-BP1—The deletion libraries were generated using “Erase-a-Base” (Promega, Madison, WI) from the 5’ or 3’ end of the FGF-BP1 open reading frame in a pSP72 vector (Promega). For the 5’ deletion library, BamHI was used to generate the 5’ overhang, which is susceptible to exonuclease III treatment, whereas KpnI generated a 3’ overhang to protect the backbone. For the 3’ deletion library, NotI and ApaI restriction sites were introduced into pSP72-FGF-BP1 with NotI close to the 3’ end of FGF-BP1. NotI was used to generate the 5’ overhang, which is susceptible to exonuclease III treatment, whereas ApaI generated a 3’ overhang to protect the backbone. Exonuclease III was added at 20 °C with an estimated erasing rate at around 90 bp/min. Enzymatic digestion of plasmids was stopped at 30-s intervals. Digested plasmids were then pooled, religated, and cloned at the EcoRI and HindIII sites into the phage 10-3b vector (see Fig. 1a). In vitro packaging generated the stock library solution, which was then used for biopanning. To generate phage expressing a C214A mutant of FGF-BP1, the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to generate a mutation of cysteine 214 (TGT) to alanine (GCT). Phage packaging, amplification, and titration were performed according to the manufacturer’s instructions.

PCR Amplification of Plaques—5 μl of phage solution or a portion of top agarose of an individual plaque of interest were scraped by a pipette tip, dispersed in a tube containing 50 μl of 10 mM EDTA, pH 8.0, and heated at 65 °C for 10 min. The phage lysate served as a template in a PCR reaction with primers 5’-GGAGCTGTCGTATTTCCAGT-3’ and 5’-TTGGGGAGTTCGCGAACAT-3’ (Novagen). PCR was performed under the following conditions: 95 °C for 3 min followed by 35 cycles (95 °C for 30 s, 50 °C for 1 min, 72 °C for 1 min) and final extension at 72 °C for 6 min. 20 μl of the reaction product was analyzed on a 1% agarose gel. For sequencing, the band of interest was excised, and DNA was purified by gel extraction (Qiagen).

Generation of a Recombinant Fusion Protein Containing the C Terminus of FGF-BP1—The pMAL protein fusion and purification system (New England Biolabs) was used for production of a fusion protein with Malt released into the periplasm of bacteria, using the pMAL-p2 expression vector. The cDNA coding for the C-terminal 42 amino acids of FGF-BP1 was amplified by PCR and subcloned into the pMAL-p2 vector. Culture of transformed cells was induced with isopropyl-1-thio-β-D-galactopyranoside, leading to the expression of a 43-kDa fusion protein that reacts with anti-MBP antibodies (for example, see Fig. 3a). The respective fusion protein was purified from the bacterial periplasm by affinity chromatography on an amylose column.

Comassie Blue Staining and Western Blot Analysis—10 μl of recombinant Malt-BP1 protein were resuspended with 5X Laemmli buffer, boiled at 95 °C for 5 min, and separated by electrophoresis on a 10% gradient polyacrylamide gel. The protein was then detected by Coomassie Blue or silver staining. For immunoblotting, the fusion protein was detected with a rabbit anti-Malt antibody (New England Biolabs) that was then visualized by enhanced chemiluminescence detection using horseradish peroxidase-linked donkey anti-rabbit as the secondary antibodies (Amersham Biosciences).

Binding of Radiolabeled FGF-2 to Different Immobilized FGF-BP1 Fragments—100 ng/ml recombinant fusion protein containing the 42-amino-acid C terminus of FGF-BP1 (Malt-BP1) or of other proteins as indicated in the figures were diluted in Tris-buffered saline (TBS, 50 mM Tris-HCl and 150 mM NaCl, pH 7.5) and incubated overnight in 96-well plates (EIA/RIA strip plate; Corning Inc.) at 4 °C with constant...
rocking. Excess unbound fusion protein was removed by washing the wells twice with TBS. 125I-FGF-2 (Amersham Biosciences) was added to the wells followed by washing and measuring the bound radioactivity as described (24).

Surface Plasmon Resonance Binding Assay—Biacore 1000 (Biacore AB) and BIAeval (version 4.1, Biacore AB) were used for surface plasmon resonance measurements and kinetic analysis, respectively. Human recombinant FGF-2 in HEPES-buffered solution (0.01 M HEPES, pH 7.4, 0.15 M NaCl) was immobilized on a CM-5 sensor chip (research grade, Biacore AB) via amine coupling. An inactivated blank channel was used as a control for nonspecific binding to the sensor surface and the 42-amino-acid human N-terminal FGF-BP1 fragment fused with Malt (see above) or FBF-BP1 (R&D Systems, Minneapolis, MN) for the positive read-out. Different concentrations of either of the latter proteins in HEPES-buffered solution with 0.1% bovine serum albumin as a carrier were used in the throughput, and dissociation constants were calculated from the association rates and dissociation rates of the proteins after the wash-out. Furthermore, steady-state binding at the different concentrations was analyzed by non-linear regression analysis for a single binding isotherm as described earlier (28). Binding experiments were performed at 25.0 °C in HEPES-buffered solution.

Phosphorylation Studies—50% confluent NIH-3T3 or GM7373 cells were serum-deprived overnight, treated with FGF-2 (Invitrogen) ± recombinant fusion protein containing the C terminus of FGF-BP1 for 5 or 10 min, respectively, and then analyzed for the induction of MAPK phosphorylation. Detection of phosphorylated ERK1/2 and actin from total cell lysates was carried out as described earlier (24), with an anti-phospho-p44/42 MAP kinase (Thr-202/Tyr-204) rabbit polyclonal antibody (Cell Signaling, Beverly, MA) and a mouse anti-actin monoclonal antibody (Chemicon International, Temecula, CA), respectively.

Proliferation Assay—Proliferation assays of NIH-3T3 cells were conducted as described earlier (24). In brief, cells were treated for 48 h with FGF-2 (1 ng/ml) (Invitrogen) ± recombinant human recombinant FGF-BP1 at 6 ng/ml (R&D Systems), and the proliferation rate was evaluated by WST-1 assay, according to the manufacturer’s instructions (Roche Applied Science).

Chemotaxis Assay—Chemotaxis was measured in a modified 96-well Boyden chamber using polycarbonate filters (8-μm pore size, Neuroprobe Inc., Gaithersburg, MD) coated with fibronectin (50 μg/ml in phosphate-buffered saline) as described in Ref. 35. Briefly, different concentrations of human recombinant FGF-2 ± recombinant FGF-BP1 (6 ng/ml) (R&D Systems) or recombinant Malt-BP1 C-terminal fragment (6 ng/ml) were added to the lower chamber, and NIH-3T3 cells (1 ×
RESULTS

FGF-BP1-expressing Phage Bind to FGF-2—Previously, we had demonstrated that FGF-1 and -2 bind to recombinant human FGF-BP1 with similar affinity and demonstrated by surface-enhanced laser desorption/ionization-mass spectrometry that recombinant human FGF-BP1 protein can specifically recognize FGF-2 even when present at low abundance in a protein mixture (24). Here we sought to identify the FGF-interacting domain of FGF-BP1, and we adapted the T7 phage display system for this since that would afford systematic mutational analysis of protein-protein interaction without having to generate and purify different recombinant protein fragments for functional analysis. First, we examined whether FGF-BP1 binds to FGF-2 in the T7 phage display system. For this purpose, we inserted the coding sequence of the FGF-interacting domain of FGF-BP1, and we adapted the T7 phage display system for this since that would afford systematic mutational analysis of the phage vector. In contrast to control phage, FGF-BP1-expressing phage, diluted 20-fold by a control phage, were recovered and purified after biopanning against immobilized FGF-2 over three rounds (not shown). Thus, we established an approach to monitor FGF interaction with FGF-BP1 by expressing the binding protein in a phage vector.

A C-terminal Fragment of FGF-BP1 Is Sufficient for FGF-2 Binding—To map the interacting domain between FGF-BP1 and FGF, we generated a library of phage that expressed different size fragments of FGF-BP1. For this, exonuclease III digestion of the FGF-BP1 cDNA contained in a plasmid vector either from the 5′ end or from the 3′ end was run for different time intervals, and the resulting fragments were pooled and then cloned into the phage vector to generate two distinct libraries. FGF-BP1 fragments resulting after different time intervals of exonuclease digestion from the 5′ end and the pooled fragments used for subcloning into the phage vector are shown as a representative example in Fig. 2, a and b. The N- and C-terminal deletion libraries were panned repeatedly against immobilized FGF-2, and the phage recovered from the panning were screened for inserts using PCR with primers flanking the inserted fragments (Fig. 1a). After panning of the C-terminal deletion library, no phage containing inserts smaller than full-length FGF-BP1 were selected, suggesting that the complete C terminus of FGF-BP1 is

10⁵/well) were added to the upper chamber. After an 18-h incubation, non-migratory cells on the upper membrane surface were mechanically removed with a cotton swab, and the cells that traversed and spread on the lower surface of the filter were fixed and stained with Diff-Quik (VWR Scientific Products, Buffalo Grove, IL). Stained migratory cells were solubilized by dissolving individual well areas in 200 μl of 0.1% citric acid/50% ethanol solution for 30 min at room temperature. Optical density of these solutions was read in a Bio-Rad Ultramark spectrophotometer at 630 nm.

Computer-assisted Homology Modeling—A Profile Library Search (FUGUE search; v2.s.07 (36)) against Homologues Structure Alignment Data base (HOMSTRAD) (37) identified the NMR structure of porcine PEC-60 (PDB entry 1pce) as a suitable template to model a three-dimensional structure of the C-terminal 42 residues of human FGF-BP1. PEC-60 is a small disulfide-rich protein belongs to a serine proteinase inhibitors of the Kazal family (38). Using the alignment from FUGUE, the model three-dimensional structure of the FGF-BP1 fragment was calculated using SWISS-MODEL (39) and refined using Insight II (version 2000.1, Accelrys).

Statistics—Prism 4 for Mac (GraphPad Inc. San Diego) was used for analysis of variance of studies with multiple treatments and repeats. Non-linear regression analysis was applied in the calculation of the apparent $K_d$ from the steady-state binding data (Fig. 4b) using an equation describing a single site binding isotherm ($B = B_{max}/(L + K_d)$) as described previously (28) with a correction for free $L$. 

\[ B = \frac{B_{max}}{L + K_d} \]
required for FGF-2 binding and recovery of panned phage (Fig. 2c). In contrast, from panning of the N-terminal deletion library, four distinct phage groups containing PCR fragments of ~600, 550, 350, and 250 bp were recovered and purified further by repeated rounds of panning (Fig. 2d, arrowheads). Selection of individual plaques, amplification, and sequencing of the respective inserts showed that the smallest insert (clone 17; Fig. 2e) contained only the C-terminal 42 amino acids of FGF-BP1 (see Fig. 7a). Binding to FGF-2 by individual phage revealed that the C-terminal 42-amino-acid fragment that is contained in phage clone 17 are sufficient for binding to the immobilized FGF-2 bait (Fig. 2f). It is intriguing that this short fragment presented as a fusion protein with the phage gene 10 appears to bind better to FGF-2 than the full-length FGF-BP1 (clone 17) (Fig. 2f). The deletion mutant protein was produced and purified, and sequencing of the respective inserts showed that the smallest insert (clone 17; Fig. 2f) contained only the C-terminal 42 amino acids of FGF-BP1 as a positive control. As a negative control, the Malt fusion partner at background levels and cannot be competed by cold FGF-2 (Fig. 3a). The immobilized fusion protein specifically bound to radiolabeled FGF-2 in a dose-dependent manner (not shown). Also, binding of FGF-2 radioligand to immobilized recombinant full-length FGF-BP1 (24) was competed by the addition of the Malt-BP1 C-terminal protein (Fig. 3b), indicating overlapping binding sites.

The Malt fusion protein contains a Factor X cleavage site that allows for a release of the C-terminal FGF-BP1 fragment (Fig. 3c). After purification of the Factor X-digested protein by fast protein liquid chromatography, the immobilized C-terminal FGF-BP1 still binds FGF-2, whereas the Malt component does not show binding above control (Fig. 3d). To detect other potential FGF-binding domains in FGF-BP1, we generated a deletion mutant that lacks the C-terminal domain ("BP1 del C-term"). The deletion mutant protein was produced and purified, and we found that FGF-2 radioligand binding to this deletion mutant is only at background levels and cannot be competed by cold FGF-2 (Fig. 3d, BP1 del C-term).

Binding of the C-terminal FGF-BP1 Fragment to FGF-2 Using Surface Plasmon Resonance—To test the direct interaction between FGF-2 and the FGF-BP1 protein, biosensor analyses (Fig. 4) were carried out with the C-terminal 42-residue fragment of FGF-BP1 and full-length FGF-BP1 as a positive control. As a negative control, the Malt fusion partner of the C-terminal fragment was analyzed with the same procedure and showed no binding to FGF-2 (not shown). $K_d$ values of 53 ± 5 and 82 ± 4 nM were calculated from the binding kinetics of the C-terminal fragment to FGF-2 in two different experiments, and these values were indistinguishable from those obtained for the full-length FGF-BP1. Fig. 4a shows a representative set of association and dissociation curves of the C-terminal fragment at 1 and 10 μM with immobilized FGF-2 as the bait. Furthermore, steady-state binding data from a representative experiment are shown in Fig. 4b. From these analyses, we conclude that the 42-amino-acid C-terminal portion of FGF-BP1 contains the domain that defines the binding affinity for FGF-2.

The C Terminus of FGF-BP1 Positively Modulates FGF-2 Signaling—We next sought to determine whether the C-terminal domain is sufficient to enhance FGF-2-induced effects. We had found previously that full-length recombinant FGF-BP1 enhances FGF-2 signaling in NIH-3T3 fibroblasts (24) and GM7373 endothelial cells (41). We thus generated a dose response of FGF-2 in these cells using MAP staining and Western blotting with an anti-Malt antibody showed that the purity of the preparation was >80% (Fig. 3a). The immobilized fusion protein specifically bound to radiolabeled FGF-2 in a dose-dependent manner (not shown). Also, binding of FGF-2 radioligand to immobilized recombinant full-length FGF-BP1 (24) was competed by the addition of the Malt-BP1 C-terminal protein (Fig. 3b), indicating overlapping binding sites.
FIGURE 7. Domains and structure of FGF-BP1. a, alignment of known FGF-BP1 proteins from human (GenBank™ accession number NP_005121), mouse (NP_032035), rat (NP_072125), bovine (NP_776762) and chicken (XP_420773). Identical (*) and conserved (: or /) amino acids are indicated. The heparin-binding domain (underlined), as reported in Ref. 42, and the FGF-binding domain (red) are highlighted.

b and c, predicted structure of the C-terminal 42 residues of FGF-BP1 at two viewing angles rotated by 90° (MOLMOL, version...
kinase phosphorylation as a read-out (24). MAP kinase phosphorylation induced by FGF-2 was significantly enhanced by the addition of the FGF-BP1 C-terminal protein (Fig. 5). This effect is seen using a phospho-specific MAPK antibody (Fig. 5, a and c) and after blotting for anti-phospho-tyrosine (pY) and quantitation of the phospho-MAPK band (Fig. 5b). The FGF-2-enhancing effect of the C-terminal fragment of FGF-BP1 is most pronounced at low concentrations of FGF-2, as also observed by us previously for the full-length protein (24). Finally, a significant increase of FGF-2-mediated NIH-3T3 fibroblast proliferation (Fig. 6a) and migration (Fig. 6b) was induced by the C-terminal FGF-BP1 fragment, at levels identical to those obtained with the full-length FGF-BP1 protein. From these data, we propose that the FGF-binding domain in FGF-BP1 carries all of the properties necessary to modulate the signaling and biological efficacy of FGF-2 ascribed to the full-length protein.

The C Terminus of FGF-BP1 Represents a Distinct Domain in Computer-assisted Homology Modeling—A BLAST search within the known three-dimensional structures deposited in the PDB did not find any molecules with significant amino acid sequence identity with the FGF-BP1 fragment. The lack of known distinct domains in FGF-BP protein is also reflected in the assignment of the whole protein as a single conserved domain, pfam06473.1, in the NCBI conserved domain data base. Since the data presented here show a distinct FGF interaction domain in the FGF-BP protein, we used a structure homology recognition program based on environment-specific substitution tables, FUGUE (36), to find a structural alignment that would take this beyond the simple amino acid sequence identity. From this, we identified the NMR structure of porcine PEC-60 (PDB entry 1pce) as a suitable template to model a three-dimensional structure of the C-terminal 42 residues of human FGF-BP1. PEC-60 is a small, disulfide-rich protein that belongs to a serine proteinase inhibitors of the Kazal family (38) and was identified in our homology modeling with more than 95% confidence. The resulting model structure of the FGF-BP1 fragment is shown in Fig. 7, b and c. PEC-60 has three disulfide bridges connecting its 6 cysteine residues 28–60, 11–42, and 20–39. Among them, 3 cysteines align well with the modeled FGF-BP1 structure, and the disulfide bridge 11–42 in PEC-60 is seen as a disulfide bridge Cys-197/Cys-234 in the FGF-BP1 C terminus (Fig. 7, b and c, red), with the other disulfide bridge attributed to Cys-214/Cys-222 (Fig. 7, b and c, yellow). The disulfide bridge formations in the human FGF-BP1 C-terminal fragment match with the pattern shown earlier by protein analysis of bovine FGF-BP1 (40).

The Role of Cysteine Residues in the FGF-binding Domain of FGF-BP1—There are 4 cysteine residues within the C-terminal FGF-binding domain that are conserved in vertebrate FGF-BP1s (Fig. 7a), and they have been shown to form defined disulfide bonds in bovine FGF-BP1 (Fig. 7e) (40). Two of these 4 C-terminal cysteines (positions Cys-214 and Cys-222 in human FGF-BP1) are also conserved in the recently discovered human FGF-BP2, whereas the equivalent of human Cys-234 is missing in the FGF-BP2 family. From this, we reasoned that mutation of Cys-214 or of Cys-222 to alanine could reveal to what extent this conserved disulfide bridge is crucial for the FGF interaction. We generated phage expressing C214A mutant FGF-BP1 and found that its binding to immobilized FGF-2 was reduced by about 50% relative to wild-type FGF-BP1 (Fig. 7d). This supports a significant contribution of this disulfide bridge to the proper folding of FGF-BP1 and interaction with FGF.

DISCUSSION

FGF-BP1 is a secreted protein that can mobilize FGFs from the extracellular matrix and deliver them to the cell surface receptors to exert their biological function. FGF-BP1 is highly regulated during malignant progression and tissue injury and appears to enhance FGF signaling during tissue repair, angiogenesis, and tumor growth. In our present study, we mapped the FGF interaction domain of FGF-BP1 to the C-terminal 42 amino acids using a T7 phage display system. Several independent approaches used here supported the hypothesis that this fragment contains the complete FGF interaction domain of FGF-BP1. (i) The fragment competes for FGF-2 binding to the full-length FGF-BP1 (Fig. 3b), and (ii) a mutant FGF-BP1 protein lacking this C-terminal fragment is unable to bind FGF-2. (iii) Furthermore, panning of the phage library containing C-terminally deleted FGF-BP1 fragments did not result in the recovery of any FGF-binding phage (Fig. 2c), suggesting that an intact C-terminal portion is crucial for binding to FGF. The distinct FGF interaction domain identified here and the heparin-binding domain described by others (42) are the most conserved portions of the FGF-BP1 proteins from different species (Fig. 7a), suggesting a significant role for both regions. The fact that the C-terminal fragment of FGF-BP1 on its own was able to enhance FGF-2-mediated biochemical and biological cellular events suggested that the interaction with heparan sulfate or with the FGF-receptor at least in a short term in vitro experiment in cultured cells is not critical for the function of FGF-BP1. There may be a distinct role for a third conserved region in this protein encompassing amino acids 57–90 in human FGF-BP1, although this may be part of the heparin interaction domain (42). The role of the N-terminal portion of FGF-BP1 is a matter of conjecture. This portion could be involved in protein storage, trafficking, or stability, and FGF-BP1 half-life may well be regulated by a domain contained in the N-terminal portion of the mature protein.

Our computer modeling showed that the C terminus of FGF-BP1 and the PEC-60 protein share some structurally common characteristics and are both secreted proteins (43). Interestingly, despite structural similarity to pancreatic secretory trypsin inhibitor, PEC-60 has no trypsin activity and its biological function is largely unknown, although some reports suggest involvement in dopamine and insulin regulation (43). A structural alignment of the FGF-BP1 C-terminal fragment with pancreatic secretory trypsin inhibitor (Kazal type) in complex with trypsinogen (PDB entry 1TGS) (44) suggests that the large protruding loop in the FGF-BP1 fragment (Fig. 7c, to the left) may be involved in the FGF-BP1 and FGF-2 interaction.

Since the C-terminal FGF-BP1 fragment can enhance signaling and the biological activity of FGF, the structural information generated here could be valuable for facilitating the design and development of small molecules that mimic this activity of FGF-BP1, e.g. for the use in the treatment of spinal cord injury (45, 46), wound healing, or angina pectoris (47). Conversely, an inhibitor designed to block the interaction of FGF-BP1 with FGF could be useful in the treatment of cancers that overexpress FGF-BP1. Indeed, in recent studies, we identified monoclonal antibodies to FGF-BP1 that inhibit its effect on FGF-2 (48). Finally, this study demonstrated that the use of phage display to obtain information on protein-protein interaction of extracellular proteins could be a significant first step not only in delineating an interaction...
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domain but also in developing drugs based on the structural information obtained.

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