

# Identification and Characterization of a Fibroblast Growth Factor (FGF) Binding Domain in the Cysteine-rich FGF Receptor\*

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Three distinct transmembrane glycoproteins bind fibroblast growth factor (FGF) family members. These include heparan sulfate proteoglycans, the tyrosine kinase-containing FGF receptors (FGFRs), and a cysteine-rich FGF receptor (CFR). The four FGFRs are thought to mediate FGF-signaling events but require the participation of the heparan sulfate proteoglycans to bind FGFs and transduce intracellular signals. However, a number of groups have proposed that FGF action requires events independent of FGFR activation. CFR, a high affinity FGF-binding protein, was first isolated from chicken embryos. To better understand the interactions between CFR and FGFs, we have constructed a series of CFR deletion mutants and CFR fragments. Analysis of these has identified a ~200-amino acid domain that constitutes a CFR FGF binding site. A CFR fragment of 450 residues, CFR<sub>290-740</sub>, binds FGF-2 with an affinity indistinguishable from the full-length molecule, whereas smaller fragments display greatly reduced FGF binding. Although CFR binds heparin with high affinity, an analysis of the heparin-CFR interaction failed to identify a linear sequence containing a heparin binding site. Two types of FGF binding sites were identified: an ionic strength and heparin-independent site that represents FGF binding to CFR<sub>290-740</sub> and an additional FGF binding site that is heparan sulfate-dependent and sensitive to high ionic strength. This latter site is likely to bind FGF indirectly via heparan sulfate binding to CFR. FGF-2 peptides that encompass a sequence implicated in FGF-2 binding to FGFRs also block FGF-2 binding to CFR. Our data suggest that binding of FGFs to CFR and FGFRs is mutually exclusive, since the CFR FGF binding site does not require heparan sulfate, and similar regions on FGF-2 interact with both FGFRs and CFR.

polypeptide growth factors. These include repression of skeletal muscle differentiation (1), promotion of neuronal differentiation and survival (2–4), induction of limb outgrowth (5), control of limb outgrowth and patterning (6, 7), induction of mesoderm (8), lung branching (9), control of hair follicle growth (10), inner ear development (11, 12), and induction of angiogenesis (13, 14). Three types of membrane anchored FGF-binding proteins have been identified that are likely to be involved in mediating the diverse functions of the FGF family. These include four tyrosine kinase-containing membrane glycoproteins (FGFRs) (15, 16), a large family of diverse heparan sulfate proteoglycans (17), and a cysteine-rich FGF receptor (CFR) (18). An additional secreted FGF-binding protein has also been characterized (19). Among these, the FGFRs are proposed to mediate the biological functions of the FGFs. However, these receptors require heparan sulfate proteoglycans to bind FGFs with high affinity and to transduce FGF signals (20, 21). Both binding sites for heparan sulfate present on the FGF and the FGFRs are likely to be required for FGF binding and signaling (22–27). The majority of FGF-mediated signaling events appear to require FGFRs, but additional requirements involving internalization and intracellular transport of the FGF ligand have been proposed (28–30). A role for other FGF-binding proteins in mediating FGF signaling is thus expected, since these recent reports have identified FGF receptor tyrosine kinase-independent activities of FGFs that include the stimulation of DNA synthesis (29) (29–31) and the regulation of plasminogen activator activity (32, 33). In addition, mutants of both FGF-1 and FGF-2 have revealed that the biological activities of these factors do not entirely correlate with their ability to stimulate tyrosine phosphorylation of the FGFRs (34). Thus, it is likely that additional FGF-binding proteins or receptors are involved in mediating some of the biological activities of the FGF family. A number of functions can be envisioned for these additional FGF-binding proteins. If transport of FGF into the cytoplasm is necessary for FGF function, then there are likely to be proteins involved in FGF transport and perhaps in regulation of intracellular FGF levels.

CFR was first isolated as a high affinity FGF-binding protein from chicken embryos (35). Isolation, sequencing, and characterization of the CFR cDNA revealed a protein sequence with no known homologs (18). CFR was found to bind a number of FGFs and to possess an amino-terminal signal sequence, a transmembrane domain, and a basic cytoplasmic region comprising 13 amino acids. Three other groups have since independently isolated CFR based on its identification as an FGF-binding protein (36), a medial Golgi protein (37), and a ligand for the E-selectin receptor (38). Although the function of CFR is not known, we have recently reported a role for CFR in regu-

A diverse array of functions is ascribed to the FGF<sup>1</sup> family of

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<sup>1</sup> The abbreviations used are: FGF, fibroblast growth factor; CFR, cysteine-rich FGF receptor; FGFR, FGF receptor; PAGE, polyacrylamide gel electrophoresis; RISA, radioimmunosorbent assay; wt, wild type; HA1, influenza hemagglutinin epitope; LA, CFR monoclonal an-

tibody epitope; TBST, Tris-buffered saline with Triton X-100; HBT, homogenization buffer with Triton X-100; CHO, Chinese hamster ovary.

lation of intracellular FGF trafficking (39).

To better delineate the roles of CFR in FGF action, we have constructed a series of CFR deletion mutants and CFR fragments, expressed the recombinant proteins in COS cells, and determined their FGF binding properties. In this article, we report that the binding of FGFs to CFR occurs within a 450-amino acid stretch of CFR sequence and that this binding does not require the presence of heparan sulfate. FGF-2 peptides that encompass a sequence implicated in FGF-2 binding to FGFRs also block FGF-2 binding to CFR. This result suggests that a similar region on FGF-2 may interact with both FGFRs and CFR. These data thus define an FGF binding site on CFR that is unlike the heparan sulfate-dependent FGF binding sites on the FGFRs (20, 21) and may be involved in mediating distinct CFR-dependent FGF actions (39).

#### EXPERIMENTAL PROCEDURES

**Materials**—Human recombinant FGF-2 was purified as described previously (40). Where indicated, chicken CFR was purified from chick embryos as described previously (35). Affi-Gel-activated agarose and molecular weight protein standards were purchased from Bio-Rad. Na<sup>125</sup>I and [<sup>3</sup>H]heparin were purchased from DuPont NEN. Fluoresceinated heparin was a gift from Arthur Lander (University of California, Irvine, CA) and was labeled with Na<sup>125</sup>I as described (41). The anti-hemagglutinin antigen-1 monoclonal antibody was purchased from Babco (Berkeley, CA). COS-1 cells were obtained from the American Type Culture Collection. The COS cell expression vector PHYK with an adenovirus late promoter was provided by Dr. Andrew McMahon (Harvard University). FGF-2 peptides were provided by Dr. Andrew Baird (Prizm Pharmaceuticals, La Jolla, CA). The chemiluminescence Super-signal<sup>TM</sup> CL-horseradish peroxidase substrate was purchased from Pierce.

**Affinity Purification of CFR, p70, and p45**—CFR was purified through wheat germ agglutinin-agarose and applied to and eluted from FGF-1 and FGF-2 affinity columns as described previously (35). For heparin-agarose chromatography, 300  $\mu$ l of heparin-agarose was washed with 1 ml of HBT (20 mM HEPES, pH 7.4, 0.25 M sucrose, 1 mM EDTA, 20 kallikrein aprotinin units/ml, 1  $\mu$ g/ml leupeptin, 0.05% Triton X-100) containing 2 M NaCl and then equilibrated with 5 ml of HBT containing 0.1 M NaCl. One ml of CFR, partially purified through a wheat germ agglutinin-agarose column (wheat germ agglutinin-agarose pool) was batch applied to heparin-agarose for 2 h on a rotary wheel. The resin was then packed into a column and washed with HBT containing 0.1 M NaCl and HBT containing 0.4 M NaCl prior to elution with HBT containing 0.6 M NaCl.

**Construction of CFR Mutants**—The CFR in-frame deletion mutants and fragments were made according to standard molecular cloning protocols and cloned into the COS cell expression vector PHYK. The numbers assigned to the CFR mutants are the first and last amino acids of the deleted parts ( $\Delta$ ) of the CFR reading frame or the first and last amino acids of the fragments. Deletion mutant CFR <sub>$\Delta$ 93-290</sub> was made by removing DNA sequences encoding amino acid 93–290 fragment with *MscI* digestion. The remaining DNA fragments were ligated with T4 DNA ligase. CFR <sub>$\Delta$ 219-1138</sub> was obtained by *SacI* digestion of CFR and ligation of the remaining fragments. CFR <sub>$\Delta$ 219-740</sub> and CFR <sub>$\Delta$ 740-1138</sub> were made by partial digestion of CFR cDNA with *SacI*. The constructs were obtained by purification and ligation of the respective DNA fragments. CFR <sub>$\Delta$ 496-624</sub> was constructed by *NcoI* and *BspHI* digestion to remove the DNA fragment between amino acids 496 and 624. The remaining fragments were purified and ligated to yield the deletion mutants. A CFR fragment spanning amino acids 290–740 (CFR<sub>290-740</sub>) was constructed by digesting CFR <sub>$\Delta$ 93-290</sub> with *SacI* to remove DNA sequences from amino acids 740–1138 and then ligating the remaining DNA fragments. The CFR fragment spanning amino acids 290–425 (CFR<sub>290-425</sub>) was obtained from CFR<sub>290-740</sub> by digesting it with *StuI* and *FspI*. The resulted fragments were ligated to yield CFR<sub>290-425</sub>. The CFR fragment containing residues 425–740 (CFR<sub>425-740</sub>) was made by *MscI* and *StuI* digestion of CFR<sub>290-740</sub> to remove the DNA sequence encoding amino acids 290–425. CFR<sub>290-426</sub> was made by digesting CFR<sub>290-740</sub> with *NcoI* and *EcoICRI*. The overhang from *NcoI* digestion was removed by mung bean nuclease treatment, and the fragments were ligated with T4 DNA ligase. CFR<sub>704-870</sub> was made by digesting CFR<sub>wt</sub> cDNA with *MscI* and *EcoICRI*, removing the intervening DNA, and filling in the protruding ends. Following dephosphorylation, an *FspI*-*SspI* fragment of CFR was cloned into the *MscI*-*EcoICRI* site by

blunt end ligation. All the CFR mutants and CFR fragments generated were cloned into the PHYK vector.

**Insertion of HA1 and LA Peptide Tags**—The influenza hemagglutinin epitope (HA1) tag (42) was inserted into the CFR-coding sequence as follows. Two oligonucleotides, atatgatgttctctgattatgctagctctcgcgatacgt and atccgagagcctagcacaatcaggaaacatcatatcgt, were synthesized, purified, and annealed. The annealed double-stranded DNA has compatible overhangs to *AatII*. After phosphorylation, it was inserted into the *AatII* site near the amino terminus of the CFR coding sequence. The CFR monoclonal antibody epitope (LA) was inserted into the *XhoI* site immediately in front of the coding sequence for the HA1 tag by a similar method. The LA epitope tag sequence will be described in a subsequent publication.

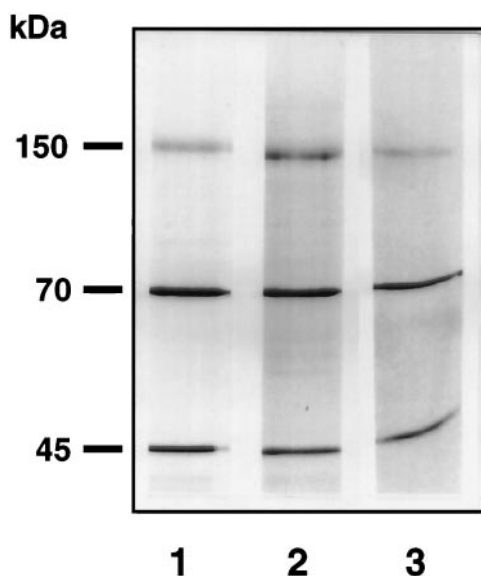
**Expression of CFR in COS Cells**—COS cells were grown and transfected as described (43). After 48 h, they were washed two times with ice-cold phosphate-buffered saline and harvested with a rubber policeman. Cells were centrifuged at 300  $\times g$  for 5 min at 4  $^{\circ}C$  and resuspended in solubilization buffer (20 mM Tris, pH 7.4, 100 mM NaCl, 4 mM EDTA, 1  $\mu$ g/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100). Membranes were solubilized by incubating samples on a rotating wheel for 30 min at 4  $^{\circ}C$ , and detergent-insoluble material was removed by centrifugation at 18,000  $\times g$  for 10 min at 4  $^{\circ}C$ . Protein concentrations were determined using the BCA assay per the manufacturer's instructions (Pierce).

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (PAGE) and Quantitation of CFR by Immunoblotting**—COS cell extracts were subjected to SDS-PAGE and electrophoretically transferred to an Immobilon membrane (Millipore, Bedford, MA). Immobilon membranes were blocked with Tris-buffered saline containing 3% nonfat dry milk and 0.05% Tween 20 (blocking buffer) for 2 h at 22  $^{\circ}C$ . The blot was incubated for 1 h with either anti-LA monoclonal antibody or anti-HA1 monoclonal antibody (12CA5) in blocking buffer. Unbound antibody was removed by washing three times for 10 min with blocking buffer. Goat anti-mouse IgG horseradish peroxidase conjugate (Promega Corp., Madison, WI) was diluted to 1:5,000 in blocking buffer and added to the blot for 1 h. The blot was subjected to three 10-min washes in blocking buffer, followed by treatment with a chemiluminescence substrate (Pierce). The membrane was immediately exposed to the chemiluminescence screen (Bio-Rad) for 10 min and quantitated by phosphorimager analysis using a Bio-Rad GS363 phosphorimager.

**Iodination of FGF-2**—Iodination of human recombinant FGF-2 was performed with chloramine T as described previously (40). Specific activities were determined by MM14 cell cycle exit assay (44) and range from 1000 to 5000 cpm/fmol.

**Radioimmunosorbent Assay (RISA) for FGF and Heparin Binding**—Anti-CFR monoclonal antibody 15E9, referred to as the anti-LA epitope tag monoclonal antibody, was purified from ascites fluid by caprylic acid precipitation (45), and 0.3  $\mu$ g/ml (100  $\mu$ l/well) was bound to the wells of a polystyrene microtiter plate in 20 mM NaHCO<sub>3</sub>, pH 9.5. Nonspecific sites were blocked with TBST (50 mM Tris, pH 7.4, with 100 mM NaCl and 0.05% Triton X-100) containing 0.5% bovine serum albumin for 2 h at 4  $^{\circ}C$ . Unbound antibody was removed by rinsing three times with TBST. COS cell extracts (30  $\mu$ g/well) were incubated in the wells for 2 h at 4  $^{\circ}C$  with equivalent amounts of CFR protein adsorbed as determined by quantitative immunoblot analysis. Nonimmunoadsorbed factors were removed by washing three times with TBST. <sup>125</sup>I-FGF-2 (200 pM), [<sup>3</sup>H]heparin (360 ng/ml or 0.02  $\mu$ Ci/well), or <sup>125</sup>I-heparin (9.5 ng/ml) was added for 2 h in the presence or absence of FGF-2 peptide competitors at 0.5 mg/ml. Unbound <sup>125</sup>I-FGF-2 or [<sup>3</sup>H]heparin was removed by washing three times with TBST. For <sup>125</sup>I-FGF-2 binding analysis, the wells were cut from the plate, and the <sup>125</sup>I cpm remaining in the wells were determined by counting in a LKB Clinigamma counter. For [<sup>3</sup>H]heparin binding analysis, 150  $\mu$ l/well Hi-Load scintillation fluid (FSA Laboratory Supplies, Loughborough, United Kingdom) was added and counted using a Wallac (Turku, Finland) Microbeta plate counter. Nonspecific binding of both assays was defined as counts per minute using mock-transfected COS cell lysates.

**Equilibrium Binding and Determination of the K<sub>D</sub> Values for <sup>125</sup>I-FGF-2 Binding**—For equilibrium binding analysis, the RISA was performed as described, except COS cell extracts containing 10  $\mu$ g of protein were used. The specifically bound <sup>125</sup>I-FGF-2 was defined as the <sup>125</sup>I-FGF-2 bound to CFR-transfected COS cell extract minus the amount bound to mock-transfected COS cell extract. The free concentration of <sup>125</sup>I-FGF-2 was determined by removing an aliquot of the incubation mixture and counting it just prior to termination of the assay. The data were plotted according to the method of Scatchard (46), and curve fitting was performed using the Ultrafit program (Biosoft, Cambridge, UK).



**FIG. 1. CFR binds to FGF-1, FGF-2, and heparin-agarose.** Partially purified CFR was applied to FGF-1-agarose (lane 1), FGF-2-agarose (lane 2), or heparin-agarose (lane 3) and eluted as described under "Experimental Procedures." Purified fractions were analyzed by SDS-PAGE. Proteins eluted from the FGF-1 and FGF-2 affinity matrices were visualized by staining with Coomassie Brilliant Blue followed by silver staining, whereas protein eluted from the heparin-agarose column was silver-stained.

**Preparation of FGF-Agarose**—FGF-agarose was prepared by coupling purified human recombinant FGF-2 to Affi-Gel 10 according to the manufacturer's instructions. Control Affi-Gel beads were made by blocking underivatized beads with ethanolamine. The beads were then equilibrated with HBT containing 0.15 M NaCl.

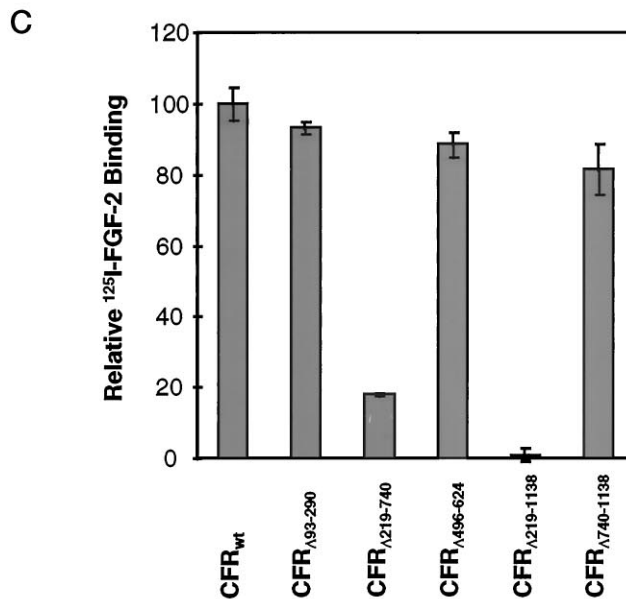
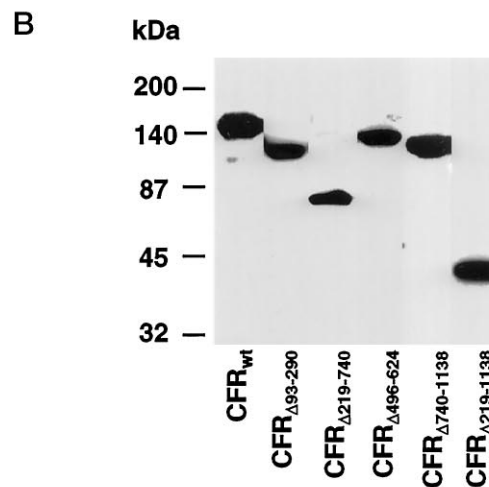
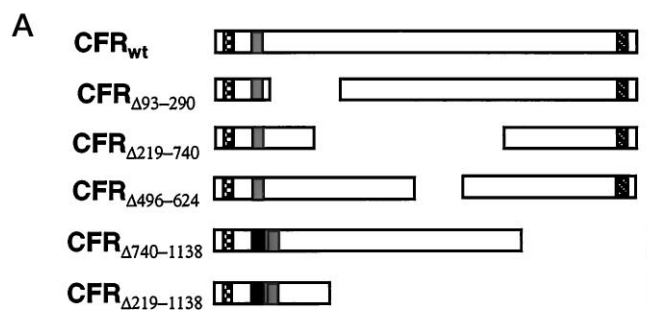
**CFR Mutant Binding to FGF-Agarose**—The FGF-agarose beads were washed twice with 2 M NaCl and then equilibrated with TBST prior to their use. COS cell lysates were diluted in 100  $\mu$ l of HBT containing 0.15 M NaCl and mixed with 20  $\mu$ l of FGF-agarose and control agarose. After incubating at room temperature for 2 h on a rotator, the beads were washed three times with TBST. 20  $\mu$ l of SDS-PAGE buffer was added to each sample, and the samples were boiled for 2 min. The supernatants were removed and subjected to SDS-PAGE. The amount of CFR present was quantified as described previously for the CFR deletion mutants by phosphorimage analysis.

**CFR Mutant Binding to Heparin-Agarose**—The heparin-agarose beads were washed twice with 2 M NaCl and equilibrated with TBST prior to use. Lysates of COS cell extracts expressing CFR mutants were diluted in 100  $\mu$ l of HBT containing 0.15 M NaCl and mixed with 20  $\mu$ l of agarose beads. After incubating at room temperature for 2 h on a rotator, the beads were washed three times with TBST. 20  $\mu$ l of SDS-PAGE buffer was added, and the samples boiled for 2 min. The samples were then separated by SDS-PAGE on 7.5% gels and analyzed as described for the binding of CFR mutants to FGF-agarose.

**RESULTS**

The CFR protein binds affinity matrices containing covalently attached FGF-1, FGF-2, or heparin, suggesting that CFR binds not only to FGFs but also to heparin (Fig. 1). Although the CFR-associated proteins of 70 and 45 kDa are adsorbed to the affinity matrices, we have previously demonstrated that CFR binds FGFs directly (35). However, these data do not determine whether FGF binding to CFR requires the presence of heparin or heparan sulfate, as is observed for the FGFRs. Therefore, we performed a detailed analysis of the CFR interactions with FGFs and heparin to further characterize the binding sites for the individual ligands.

Five deletion mutants were constructed that maintained the open reading frame of CFR and an intact signal peptide sequence (Fig. 2A). Detection of the deletion mutants was initially accomplished using the anti-LA monoclonal antibody. However, deletion of carboxyl-terminal regions including



**FIG. 2. Analysis of <sup>125</sup>I-FGF-2 binding to CFR and its deletion mutants in COS cells.** A, schematic diagrams of CFR deletion mutants. The CFR cDNA encodes a signal peptide (☒), a transmembrane domain (☐), and a short 13-amino acid cytoplasmic tail. The HA1 sequence (☐) was inserted following the signal peptide sequence (☒). The LA sequence (■) was inserted between the signal peptide and the HA1 sequence. B, mutants were expressed in COS cells and analyzed by SDS-PAGE and immunoblotting using the anti-LA antibody. The relative level of mutant proteins was quantitated by phosphorimage analysis. C, binding of <sup>125</sup>I-FGF-2 to CFR and its deletion mutants from COS cell extracts was performed by RISA. Nonspecific binding was defined as the amount of <sup>125</sup>I-FGF-2 bound to the mock-transfected COS cell extract. The values shown were derived by subtracting the nonspecific binding from the total binding. Bars, S.D. of triplicate determinations.

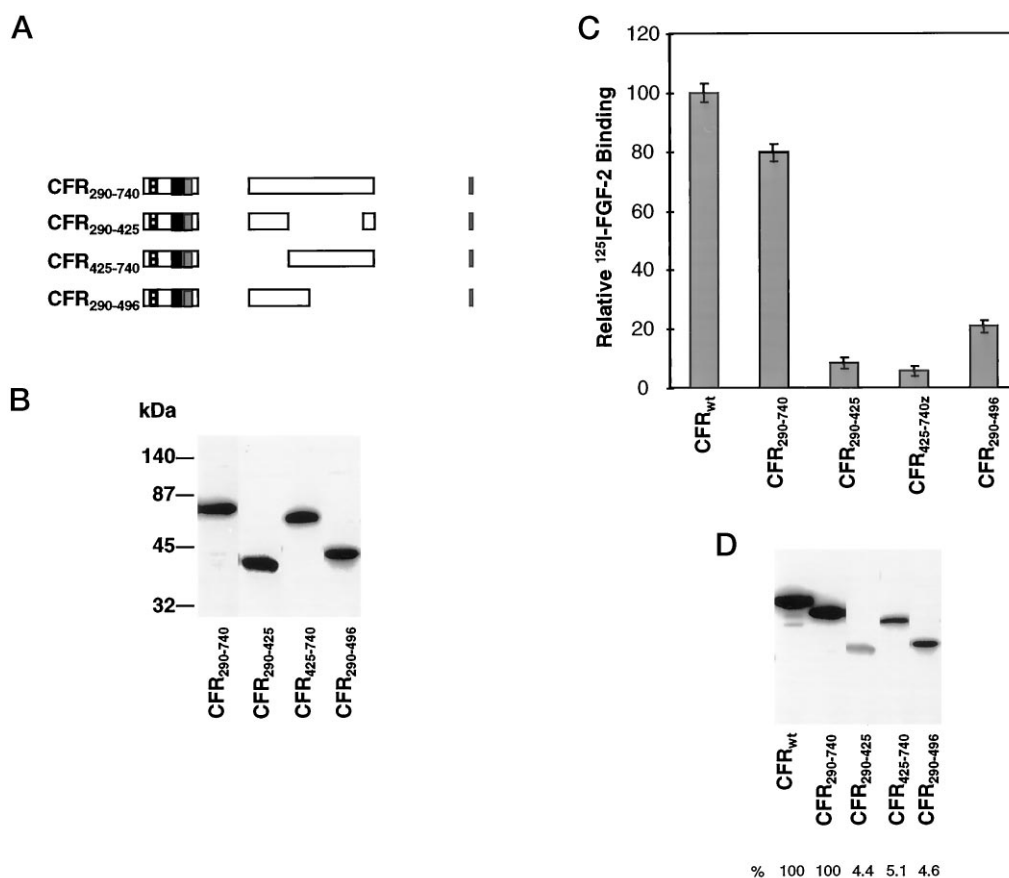


FIG. 3. Analysis of  $^{125}\text{I}$ -FGF-2 binding to CFR and CFR fragments. *A*, schematic diagrams of CFR fragments. The legend is identical to that of Fig. 2*A*. *B*, fragments were expressed in COS cells and analyzed as described for Fig. 2. *C*, binding of  $^{125}\text{I}$ -FGF-2 to CFR<sub>wt</sub> and CFR fragments expressed in COS cells was determined by RISA. Nonspecific binding was defined as the amount of  $^{125}\text{I}$ -FGF-2 bound to the mock-transfected COS cell extract. The values shown were derived by subtracting the nonspecific binding from the total binding. *D*, CFR fragments expressed in COS cells were incubated with FGF-agarose. The extracts adsorbed to FGF-agarose were washed and then boiled in SDS-PAGE sample buffer, separated by SDS-PAGE, and analyzed by immunoblotting with the anti-LA monoclonal antibody. The amount of CFR present was quantified by phosphorimage analysis.

amino acids 740–1138 or 219–1138 removed the anti-LA antibody epitope. Therefore, an HA1-tag was inserted into the mutants following the signal peptide sequence. Although the HA1 tag was satisfactory for Western analysis, it was unsuitable for analysis of  $^{125}\text{I}$ -FGF binding to CFR by RISA. Since the anti-LA antibody was originally used for RISA analysis for wild type CFR (47), we identified the 17-amino acid epitope and inserted the LA tag immediately preceding the coding sequence of the HA tag (Fig. 2*A*).

Each deletion mutant was inserted in to a COS cell expression vector and transiently transfected in to COS cells. The expression levels were determined by Western blot analysis of COS cell extracts using the anti-LA antibody (Fig. 2*B*). The Western blot analysis of CFR deletion mutants demonstrated that similar levels of protein were present, with no apparent differences in degradation of any individual mutant compared with wild type CFR (CFR<sub>wt</sub>). To normalize the levels of CFR protein present for determination of  $^{125}\text{I}$ -FGF binding, extracts were subjected to immunoblot analysis with both the anti-LA and anti-HA epitope tag monoclonal antibodies. The relative levels of CFR protein were determined by chemiluminescence quantitation of phosphorimages. The relative levels of protein detected by both antibodies were used to normalize the expression of CFR deletion mutants and CFR fragments to the level of wild type CFR expression. This procedure eliminated any potential artifacts due to differences in the expressed proteins or differences in the recognition of individual antibody epitopes.

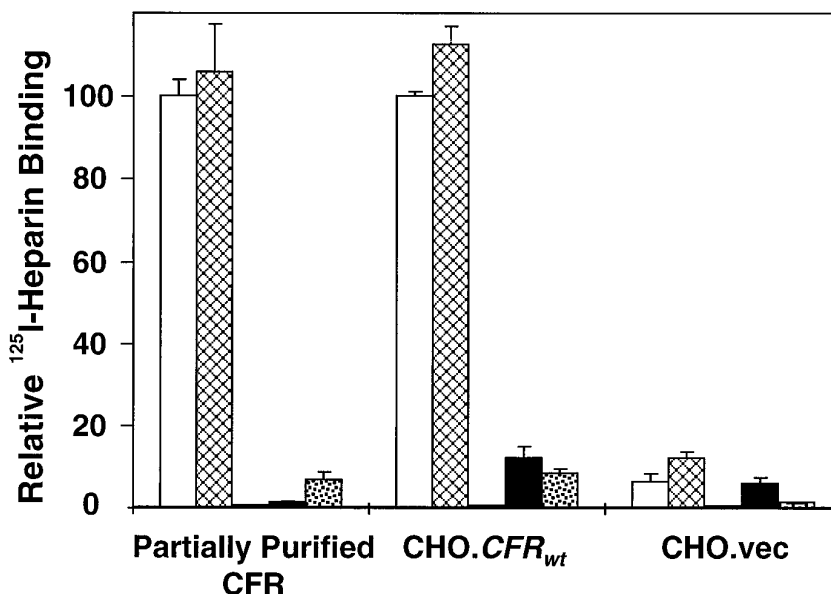
Equivalent amounts of wild type CFR and CFR deletion

mutants were analyzed for  $^{125}\text{I}$ -FGF-2 binding by RISA. Three of the deletion mutants bound  $^{125}\text{I}$ -FGF-2 similarly to wild type CFR, whereas two mutants exhibited low levels of  $^{125}\text{I}$ -FGF-2 binding (Fig. 2*C*). Typically, bound counts averaged 4000 cpm, whereas the nonspecific binding averaged 500 cpm. These data suggest that the FGF binding site on CFR lies within residues 219–496 or 624–740.

To further delineate the FGF binding domain, a number of CFR fragments were constructed (Fig. 3*A*). CFR fragments were expressed in COS cells, and extracts were isolated and quantitated for CFR protein content as described for the CFR deletion mutants. Western analysis of all four CFR fragments demonstrated that the proteins were stably expressed in COS cells (Fig. 3*B*). Binding of  $^{125}\text{I}$ -FGF-2 to CFR<sub>wt</sub> and to the CFR fragments identified only one fragment (CFR<sub>290-740</sub>) that was capable of binding significant levels of  $^{125}\text{I}$ -FGF-2 (Fig. 3*C*). Although the fragments appeared stable when analyzed in COS cell extracts, more than one freeze-thaw cycle abolished  $^{125}\text{I}$ -FGF-2 binding to CFR<sub>290-740</sub>. Similar treatments did not affect binding to CFR<sub>wt</sub>. The four CFR fragments constructed were expected to narrow an FGF binding domain on CFR to approximately 200 residues. Data from the deletion analyses (see Fig. 2) suggested that the FGF binding domain was localized to a region encompassing either residues 219–496 or 740–740. However, only the CFR<sub>290-740</sub> fragment bound  $^{125}\text{I}$ -FGF-2 (Fig. 3*C*).

Several possible hypotheses could explain the lack of  $^{125}\text{I}$ -FGF-2 binding to CFR fragments smaller than CFR<sub>290-740</sub>.

FIG. 4. Heparin binding to CFR in CHO Cells. A modified RISA was used to assess the ability of CFR to specifically bind heparin. Relative heparin binding ( $\square$ ) was determined for partially purified chicken CFR and extracts prepared from CHO-K1 cells expressing CFR<sub>wt</sub> (CHO.CFR<sub>wt</sub>) and CHO-K1 cells transfected with an empty expression vector (CHO.vec). Controls include the addition of a 1000-fold excess of chondroitin sulfate ( $\circ$ ) or heparin ( $\blacksquare$ ), no  $\alpha$ -CFR monoclonal antibody ( $\blacksquare$ ), and 2 M NaCl wash ( $\square$ ).



First, the RISA requires that a monoclonal antibody interacts with and retains the CFR fragment. It is possible that smaller CFR fragments exhibit reduced FGF binding due to steric hindrance following antibody binding. Alternatively, the fragments may not possess sufficient structural stability to maintain an FGF binding site or may not be folded properly, or the binding site for FGFs may comprise non-linear amino acid sequences contained in residues 219–496 and 624–740. To distinguish between the first two possibilities, an FGF binding assay independent of the RISA was developed. COS cell extracts containing equal amounts of CFR fragments or wild type CFR were adsorbed to FGF-agarose. The amount of CFR or CFR fragment adsorbed to the affinity matrix was determined by boiling the washed beads in SDS-PAGE sample buffer, separating the fragments by SDS-PAGE, and visualizing by immunoblot analysis. The amount of CFR present was quantified as described previously for the CFR deletion mutants. These data were consistent with the RISA data in that CFR<sub>wt</sub> and the CFR<sub>290–740</sub> fragment were retained on FGF-agarose, whereas other CFR fragments were not retained (Fig. 3D). Thus, the inability of the smaller CFR fragments to bind to the affinity matrix is likely to arise from improper folding or instability of CFR fragments, resulting in proteins incapable of FGF binding.

Scatchard analysis of <sup>125</sup>I-FGF-2 equilibrium binding to CFR<sub>wt</sub> and CFR<sub>290–740</sub> revealed  $K_D$  values that were virtually identical and comparable with those previously determined by our group (35). Equilibrium binding data from three independent experiments yielded  $K_D$  values of  $1.4 \pm 0.8$  and  $1.8 \pm 0.9$  nM for CFR<sub>290–740</sub> and CFR<sub>wt</sub>, respectively. Curve fitting identified only a single class of sites when plotted according to Scatchard.<sup>2</sup> Thus, the CFR domain encompassing residues 290–740 contains a complete and intact FGF binding site. We hypothesize that CFR higher order structure is necessary for the formation of an FGF binding site, as: 1) bacterially produced CFR lacking a signal peptide and transmembrane domain does not bind FGFs; 2) denaturation of CFR by boiling abolishes FGF binding; and 3) CFR<sub>290–740</sub> possesses intact disulfide bonds, a characteristic of the wild type protein.<sup>2</sup>

Next, we examined the heparin binding to CFR to determine whether CFR bound heparin directly and to identify putative heparin binding domains. The specificity of heparin binding to the CFR complex was determined by RISA, examining <sup>125</sup>I-

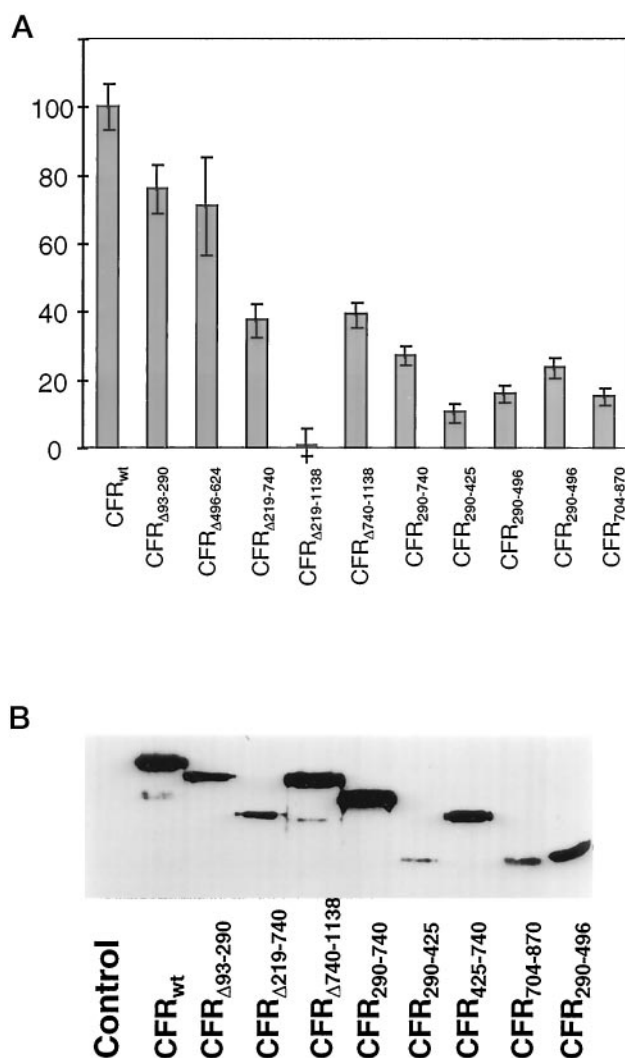
heparin binding and competition with heparin or chondroitin sulfate. CFR expressed in COS cells and partially purified CFR specifically bound heparin, since unlabeled heparin but not chondroitin sulfate competed for <sup>125</sup>I-heparin binding (Fig. 4). CFR expressed in COS cells does not contain bound CFR-associated proteins (47), suggesting a direct interaction of CFR with heparin.

Heparin binding to CFR deletion mutants and CFR fragments was performed analogously to the previously described FGF binding experiments. CFR deletion mutants and fragments bind heparin but exhibit a complex pattern (Fig. 5A). At least one heparan sulfate binding site appears to be present. Binding of CFR deletion mutants and fragments to heparin-agarose also presents a complex pattern (Fig. 5B). Both assays demonstrate that alterations of CFR structure and primary sequence affect heparin binding. These data do not allow unambiguous identification of a heparin binding domain within the CFR primary amino acid sequence.

We then performed a series of experiments to determine whether the binding of CFR to FGF was dependent on heparan sulfate. CHO-K1 cells (wild type) and CHO cell mutants that do not synthesize heparan sulfate and display low levels of heparan sulfate proteoglycans (CHO677) (48) were stably transfected with CFR<sub>wt</sub> expression vectors. A CHO-K1 clone and a CHO677 clone were selected for similar levels of CFR protein expression (Fig. 6A) and subjected to <sup>125</sup>I-FGF-2 binding by RISA (Fig. 6B). The CHO677 cells exhibited 50% less <sup>125</sup>I-FGF-2 binding than the CHO-K1 cells (Fig. 6B). To confirm that the assay conditions did not alter the levels of CFR present, the cells were solubilized in SDS-PAGE buffer following the assay, and the products were analyzed by Western blotting (not shown). This confirmed that equivalent levels of CFR were present in all wells.

Heparin appears to augment FGF binding to CFR. Since a high ionic strength wash disrupts the interaction of CFR with heparin (Fig. 1), it should also abrogate heparin-dependent increases in FGF binding. Analysis of <sup>125</sup>I-FGF-2 binding by RISA in the presence or absence of heparin revealed that heparin-dependent increases in FGF binding occur (Fig. 7). Addition of heparin to extracts from CHO-K1 and CHO677 cells expressing CFR increased <sup>125</sup>I-FGF-2 binding to equivalent levels (Fig. 7). A 2 M NaCl wash following incubation with heparin and ligand reduced <sup>125</sup>I-FGF-2 binding 2–3-fold, resulting in equivalent binding to CHO-K1 and CHO677 extracts. In the absence of

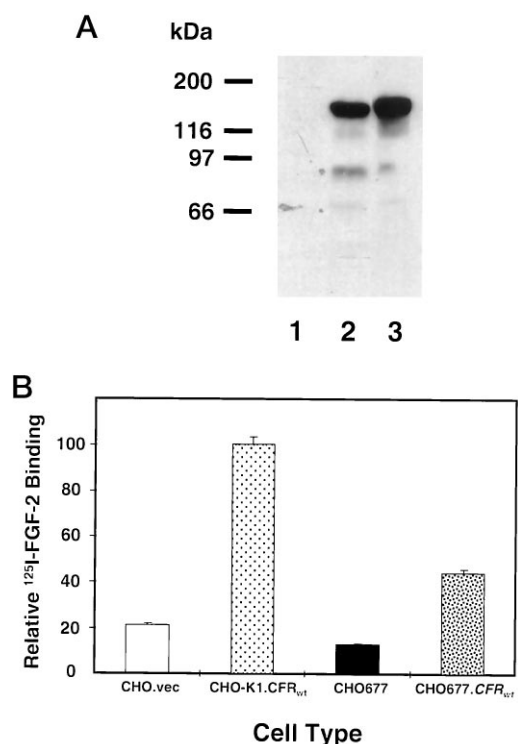
<sup>2</sup> Z. Zhou and B. B. Olwin, unpublished data.



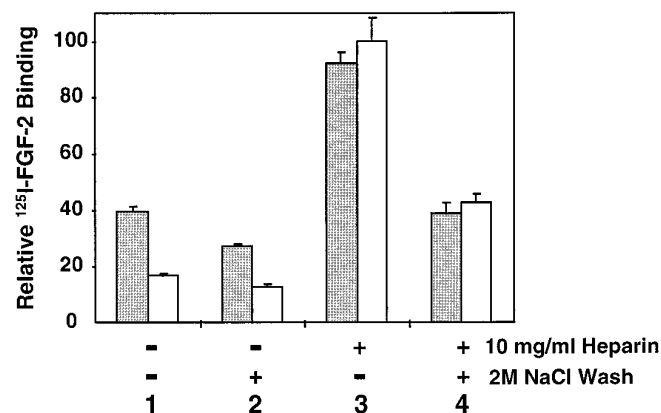
**FIG. 5. Heparin binding to CFR deletion mutants and CFR fragments.** *A*, CFR deletion mutants and CFR fragments from COS cell extracts were used for heparin binding by RISA. Equal amounts of CFR mutants and fragments were used for each assay. Nonspecific binding was defined as the amount of [<sup>3</sup>H]heparin bound to the mock-transfected COS cell extract. The values shown were derived by subtracting the nonspecific binding from the total binding. *B*, CFR fragments expressed in COS cells were incubated with heparin-agarose. The extracts adsorbed to heparin-agarose were washed and then boiled in SDS-PAGE sample buffer, separated by SDS-PAGE, and analyzed by immunoblotting with the anti-LA monoclonal antibody. The amount of CFR present was quantified by phosphorimage analysis.

heparin or heparan sulfate, we observed a single class of FGF binding sites on CFR that were not affected by high ionic strength and displayed a  $K_D$  near 1 nM. In the presence of heparin, additional FGF binding was observed that is ionic strength dependent (Fig. 7). Scatchard analysis of CFR and CFR treated with heparin followed by a high ionic strength wash revealed an increase in the number of FGF binding sites but no detectable change in affinity. Thus, it is likely that heparin binding to CFR stabilizes higher order CFR structure.

The interactions of CFR with FGFs appear to differ from those observed for the FGFR tyrosine kinases. FGF-2 peptides have been used previously to identify a critical region of FGF-2 that has been proposed to interact with the FGF receptor tyrosine kinases (49). FGF-2 peptides containing a loop sequence (residues 106–115) bind to an FGFR site that is proposed to be involved in promoting dimer formation and signaling. The peptides containing this loop sequence block FGF-2



**FIG. 6. FGF binding to CFR expressed in CHO-K1 and CHO.677 cells.** *A*, Western analysis of CFR in CHO cell extracts. Shown here are representative clones isolated following transfection with the parent vector (*lane 1*), CHO-K1 cells transfected with the CFR<sub>wt</sub> expression vector (*lane 2*), and CHO677 cells transfected with the CFR<sub>wt</sub> expression vector (*lane 3*). Equal amounts of extract protein were loaded in each lane. *B*, extracts from the indicated cell types were used to determine their relative ability to bind <sup>125</sup>I-FGF-2 in a RISA. Equivalent amounts of CFR were added from CHO-K1 and CHO677 extracts. An immunoblot analysis of the proteins remaining in the wells following the RISA confirmed that equivalent amounts of CFR were immunoadsorbed (not shown).



**FIG. 7. Effects of heparin and a 2 M NaCl wash on FGF-2 binding to CFR expressed in CHO-K1 and CHO677 cells.** Binding of <sup>125</sup>I-FGF-2 to CFR expressed in CHO-K1 (■, wild type) and CHO-677 (□, heparan sulfate-deficient) CHO cells was performed in the absence or presence of heparin. Where noted, the binding of <sup>125</sup>I-FGF-2 was followed by two 2 M NaCl washes.

binding to FGF tyrosine kinase receptors, whereas peptides lacking the sequence do not (49). A similar analysis was performed for <sup>125</sup>I-FGF-2 binding to CFR. Peptides containing the loop sequences that inhibit FGF binding to FGFRs also inhibited binding of <sup>125</sup>I-FGF-2 to CFR, whereas a FGF-2 peptide that did not contain the loop sequence did not block <sup>125</sup>I-FGF-2 binding to CFR (Fig. 8). Although our data suggest that the interactions of CFR with FGFs are different than the interac-

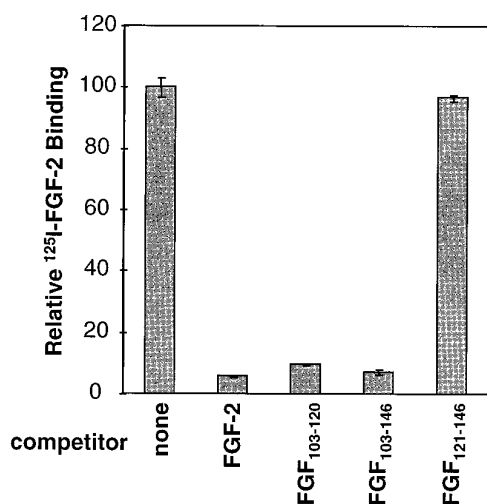


FIG. 8. **Inhibition of FGF binding to CFR by FGF-2 peptide.** Binding of <sup>125</sup>I-FGF-2 to CFR<sub>wt</sub> in the presence of FGF-2 and FGF-2 peptides were performed by RISA. Purified CFR from chicken embryos was used for this assay.

tions of FGFRs with FGFs, a similar binding site on FGF-2 may interact with both CFR and FGFRs.

#### DISCUSSION

The FGF family is known to bind to two distinct transmembrane glycoprotein complexes that include a complex of heparan sulfate proteoglycans and the tyrosine kinase-containing FGF receptors, and the CFR complex. To better understand the role(s) that CFR may play in FGF action, we constructed a series of CFR deletion mutants and CFR fragments to determine the nature of the interactions of FGFs and heparin with CFR. Analysis of deletion mutants suggested that the FGF binding domain comprises a region of ~200 amino acids from residue 290 to residue 496 and/or ~150 amino acids from residue 625 to residue 740.

CFR possesses basic stretches of amino acids that correspond well to heparin binding "consensus" sites. Consistent with this observation, we have shown that CFR is a heparin-binding protein. An analysis of heparin binding to deletion mutants and CFR fragments failed to identify a region of sequence that comprises a single binding site. However, it is clear that CFR binds heparin at physiological ionic strength, suggesting that CFR is a heparin-binding protein *in vivo*.

To better define the nature of the interactions of FGF and heparan sulfate with CFR, a series of experiments was performed to analyze the interdependence of FGF and heparin binding. Expression of CFR in a mutant CHO cell line that does not synthesize heparan sulfate provides conclusive evidence that FGF-2 does not require the carbohydrate to bind to CFR. However, addition of heparin to CFR expressed in CHO677 cells prior to incubation with FGF-2 increased FGF-2 binding by 2–3-fold. The heparin-dependent increase in FGF-2 binding is sensitive to 2 M NaCl, whereas the heparin-independent FGF binding is not sensitive to high ionic strength. Moreover, the level of FGF-2 binding in the presence of heparin can be reduced to the level of FGF binding in CHO677 cell extracts by treatment with high ionic strength. The salt treatment releases bound heparin from CFR, suggesting the presence of two types of FGF binding sites on CFR (Fig. 9). One site, identified by mutational analysis, is ionic strength-independent and resides between residues 290–740, whereas an additional site is sensitive to high ionic strength and is proposed to be bound indirectly via heparan sulfate interaction with CFR (Fig. 9). We therefore propose that these two types of FGF binding sites are

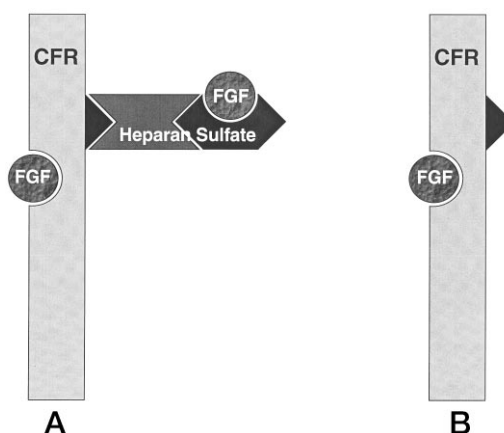


FIG. 9. **A model for FGF binding to CFR.** A model for the FGF-2 binding to CFR is presented in which two FGF binding sites are present under normal physiological conditions in the presence of heparan sulfate (a repeating carbohydrate dimer). *A*, at physiological ionic strength FGF-2 is bound directly to CFR and indirectly via heparan sulfate. *B*, at high ionic strength or in the absence of heparan sulfate, FGF-2 is bound only to the direct site on CFR. This site binds several FGF family members and is located between residues 290 and 740.

distinct as presented in our model (Fig. 9). In contrast to CFR, FGFRs are incapable of binding FGFs in the absence of heparan sulfate (20, 21, 26, 27). Heparan sulfate binding to both FGF and the FGFR appear necessary to form a functional complex (50, 51). FGFRs contain a heparan sulfate binding site that is dependent on divalent cations (27). These data demonstrate that the FGF "binding site" on FGFRs is likely to recognize a complex of FGF and heparan sulfate, whereas CFR binds FGFs independently of heparan sulfate.

To further delineate the characteristics of the FGF binding site on CFR, we examined the ability of FGF-2 peptides to block <sup>125</sup>I-FGF-2 binding to CFR. A similar study analyzing FGF-2 binding to intact cells revealed that a peptide corresponding to residues 106–115 of FGF-2 efficiently blocked FGF-2 activity, FGF-2 cross-linking, and FGF-2 binding (49). The efficacy of inhibition by the peptides on FGF-2 activity and FGF-2 cross-linking was indistinguishable from that observed for <sup>125</sup>I-FGF-2 binding to CFR. Thus, the site on FGF-2 that interacts with CFR is likely to include the site that binds to FGFRs. Thus, the FGF binding site on CFR represents the only known FGF binding site that is independent of heparan sulfate. We predict that FGF binding to CFR and to FGFRs would likely be mutually exclusive. These data are consistent with an activity of CFR we have observed in CHO cells. CFR reduces the levels of intracellular FGF-2 and FGF-1 that have been internalized from the extracellular environment (39). Import of these FGFs into the cytoplasm has been observed independently by three laboratories (28, 52–54). Two of these groups have demonstrated that import into the cytoplasm and nuclear localization of FGFs is critical for cell growth. If CFR acts to reduce the levels of intracellular FGFs, and the binding of FGFs to CFR and FGFRs is mutually exclusive as our data indicate, then CFR may modulate FGF functions that require internalized FGFs.

Analysis of CFR deletion mutants and CFR fragments for FGF binding has identified a new type of FGF binding domain that is distinct from the heparan sulfate-dependent binding site present on FGFRs. Further examination of this FGF binding site should reveal additional details of the CFR and FGF interactions. A comparison of the crystal structures for FGFR and CFR bound to FGFs will aid in the elucidation of the role(s) of CFR in FGF action.

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## REFERENCES

- Linkhart, T. A., Clegg, C. H., and Hauschka, S. D. (1981) *Dev. Biol.* **86**, 19–30
- Togari, A., Baker, D., Dickens, G., and Guroff, G. (1983) *Biochem. Biophys. Res. Commun.* **114**, 1189–1193
- Walicke, P., Cowan, W. M., Ueno, N., Baird, A., and Guillemin, R. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 3012–3016
- Morrison, R. S., Sharma, A., DeVellis, J., and Bradshaw, R. A. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 7537–7541
- Cohn, M. J., Izipisua-Belmonte, J. C., Abud, H., Heath, J. K., and Tickle, C. (1995) *Cell* **80**, 739–746
- Niswander, L., Tickle, C., Vogel, A., Booth, I., and Martin, G. R. (1993) *Cell* **75**, 579–587
- Fallon, J. F., López, A., Ros, M. A., Savage, M. P., Olwin, B. B., and Simandl, B. K. (1994) *Science* **264**, 104–107
- Slack, J. M., Darlington, B. G., Heath, J. K., and Godsave, S. F. (1987) *Nature* **326**, 197–200
- Peters, K., Werner, S., Liao, X., Wert, S., Whitsett, J., and Williams, L. (1994) *EMBO J.* **13**, 3296–3301
- Hebert, J. M., Rosenquist, T., Gotz, J., and Martin, G. R. (1994) *Cell* **78**, 1017–1025
- Represa, J., Leon, Y., Miner, C., and Giraldez, F. (1991) *Nature* **353**, 561–563
- Mansour, S. L., Goddard, J. M., and Capecchi, M. R. (1993) *Development (Camb.)* **117**, 13–28
- Wilkie, A. O., Morriss-Kay, G. M., Jones, E. Y., and Heath, J. K. (1995) *Curr. Biol.* **5**, 500–507
- Klagsbrun, M., and D'Amore, P. A. (1991) *Annu. Rev. Physiol.* **53**, 217–239
- Johnson, D. E., and Williams, L. T. (1993) *Adv. Cancer Res.* **60**, 1–41
- Mason, I. J. (1994) *Cell* **78**, 547–552
- Kjellen, L., and Lindahl, U. (1991) *Annu. Rev. Biochem.* **60**, 443–475
- Burrus, L. W., Zuber, M. E., Lueddecke, B. A., and Olwin, B. B. (1992) *Mol. Cell. Biol.* **12**, 5600–5609
- Wu, D. Q., Kan, M. K., Sato, G. H., Okamoto, T., and Sato, J. D. (1991) *J. Biol. Chem.* **266**, 16778–16785
- Yayon, A., Klagsbrun, M., Esko, J. D., Leder, P., and Ornitz, D. M. (1991) *Cell* **64**, 841–848
- Rapraeger, A. C., Krufka, A., and Olwin, B. B. (1991) *Science* **252**, 1705–1708
- Guimond, S., Maccarana, M., Olwin, B. B., Lindahl, U., and Rapraeger, A. C. (1993) *J. Biol. Chem.* **268**, 23906–23914
- Maccarana, M., Casu, B., and Lindahl, U. (1993) *J. Biol. Chem.* **268**, 23898–23905
- Turnbull, J. E., Fernig, D. G., Ke, Y., Wilkinson, M. C., and Gallagher, J. T. (1992) *J. Biol. Chem.* **267**, 10337–10341
- Walker, A., Turnbull, J. E., and Gallagher, J. T. (1994) *J. Biol. Chem.* **269**, 931–935
- Kan, M., Wang, F., Xu, J., Crabb, J. W., Hou, J., and McKeehan, W. L. (1993) *Science* **259**, 1918–1921
- Patstone, G., and Maher, P. (1996) *J. Biol. Chem.* **271**, 3343–3346
- Bouche, G., Gas, N., Prats, H., Baldin, V., Tauber, J. P., Teissie, J., and Amalric, F. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 6770–6774
- Imamura, T., Engleka, K., Zhan, X., Tokita, Y., Forough, R., Roeder, D., Jackson, A., Maier, J. A. M., Hla, T., and Maciag, T. (1990) *Science* **249**, 1567–1570
- Wiedlocha, A., Falnes, P. Ø., Madhus, I. H., Sandvig, K., and Olsnes, S. (1994) *Cell* **76**, 1039–1051
- Lin, Y. Z., Yao, S. Y., Veach, R. A., Torgerson, T. R., and Hawiger, J. (1995) *J. Biol. Chem.* **270**, 14255–14258
- Isacchi, A., Statuto, M., Chiesa, R., Bergonzoni, L., Rusnati, M., Sarmientos, P., Ragnotti, G., and Presta, M. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 2628–2632
- Rusnati, M., Dell'Eza, P., Urbinati, C., Tanghetti, E., Massardi, M., Nagamine, Y., Monti, E., and Presta, M. (1996) *Mol. Biol. Cell* **7**, 369–381
- Burgess, W. H., Shaheen, A. M., Ravera, M., Jaye, M., Donohue, P. J., and Winkles, J. A. (1990) *J. Cell Biol.* **111**, 2129–2138
- Burrus, L. W., and Olwin, B. B. (1989) *J. Biol. Chem.* **264**, 18647–18653
- Soulet, L., Chevet, E., Lemaitre, G., Blanquaert, F., Meddahi, A., and Barritault, D. (1994) *Mol. Reprod. Dev.* **39**, 49–55
- Gonatas, J. O., Mourelatos, Z., Stieber, A., Lane, W. S., Brosius, J., and Gonatas, N. K. (1995) *J. Cell Sci.* **108**, 457–467
- Stegmaler, M., Levinovitz, A., Isenmann, S., Borges, E., Lenter, M., Kocher, H. P., Kleuser, B., and Vestweber, D. (1995) *Nature* **373**, 615–620
- Zuber, M. E., Zhou, Z., Dodge, N., and Olwin, B. B. (1996) *J. Cell. Physiol.* in press
- Rapraeger, A. C., Guimond, S., Krufka, A., and Olwin, B. B. (1994) *Methods Enzymol.* **245**, 219–240
- Lee, M. K., and Lander, A. D. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 2768–2772
- Wilson, I. A., Niman, H. L., Houghten, R. A., Cherenon, A. R., Connolly, M. L., and Lerner, R. A. (1984) *Cell* **37**, 767–778
- Munro, S., and Pelham, H. R. (1987) *Cell* **48**, 899–907
- Olwin, B. B., and Hauschka, S. D. (1986) *Biochemistry* **25**, 3487–3492
- Steinbuch, M., and Audran, R. (1969) *Arch. Biochem. Biophys.* **134**, 279–284
- Scatchard, G. (1949) *Ann. N. Y. Acad. Sci.* **51**, 660–672
- Deleted in proof
- Esko, J. D. (1991) *Curr. Opin. Cell. Biol.* **3**, 805–816
- Baird, A., Schubert, D., Ling, N., and Guillemin, R. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 2324–2328
- Pantoliano, M. W., Horlick, R. A., Springer, B. A., Van Dyk, D. E., Tobery, T., Wetmore, D. R., Lear, J. D., Nahapetian, A. T., Bradley, J. D., and Sisk, W. P. (1994) *Biochemistry* **33**, 10229–10248
- Rapraeger, A. C. (1995) *Chem. Biol.* **2**, 645–649
- Baldin, V., Roman, A. M., Bosc, B. I., Amalric, F., and Bouche, G. (1990) *EMBO J.* **9**, 1511–1517
- Cao, Y., and Petterson, R. F. (1993) *Growth Factors* **8**, 277–290
- Imamura, T., Oka, S., Tanahashi, T., and Okita, Y. (1994) *Exp. Cell Res.* **215**, 363–372