

Copper and Calcium Binding Motifs in the Extracellular Domains of Fibroblast Growth Factor Receptors*

(Received for publication, November 22, 1995, and in revised form, December 22, 1995)

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High affinity fibroblast growth factor (FGF) receptors contain a cluster of acidic amino acids in their extracellular domains that is reminiscent of the calcium binding domains of some cell adhesion molecules. Based on this observation, we used a calcium blotting technique to show that FGFR-1 binds calcium and that calcium binding is not observed in a mutagenized form of the receptor that lacks the acidic box region. The acidic box also binds other divalent cations, including copper. This latter interaction appears unique since the binding of copper to FGFR-1 mediates the binding of the receptor to immobilized heparin. While this observation may help explain the angiogenic properties of copper, divalent cation binding to FGF receptors may also mediate the interaction between FGF receptors, cell adhesion molecules and other proteoglycan components of the extracellular matrix.

Basic (FGF-2)¹ and acidic (FGF-1) fibroblast growth factors are the prototypes for a family of multifunctional growth factors which have been identified in a wide variety of tissues (for reviews, see Baird and Bohlen (1990), Burgess and Maciag (1989), Wagner (1991), Fernig and Gallagher (1994)). The nine known members of this growth factor family all share some sequence homology and associate with heparan sulfate proteoglycans on the cell surface and in the extracellular matrix. FGF-1 and FGF-2 are mitogenic for a variety of different cell types, but predominantly for those of mesenchymal or neuroectodermal origin. FGFs can also modulate a number of other cellular functions such as differentiation, chemotaxis, and protease synthesis and secretion.

FGFs interact with two classes of FGF receptors; high affinity receptors which bind FGFs with picomolar affinity and are thought to mediate the cellular responses to FGF and low affinity heparan sulfate containing proteoglycans which bind FGFs with nanomolar affinity. The family of high affinity FGF receptors contains four major members (for reviews, see Givol

and Yayon (1992), Johnson and Williams (1993), Partanen *et al.* (1993), and Fernig and Gallagher (1994)), each of which exists in multiple isoforms generated by alternate splicing of their mRNAs. The four different FGF receptor genes encode proteins that are closely related and share a number of characteristic structural features which distinguish them from other tyrosine kinase receptors (Hanks *et al.*, 1988). One of these structural features is the presence of a cluster of acidic amino acids in the extracellular domain of the receptor between the first and second Ig-like loops. This acidic box is found in all FGF receptor isoforms except a variant of the keratin growth factor receptor, an isoform of FGFR-2. Nevertheless, the role of this characteristic sequence with regard to receptor function is, at present, unknown.

Initially, the acidic box was postulated to play a role in ligand binding to the receptor (Lee *et al.*, 1989). Since, FGF-2, as its original name would indicate, is quite basic, this idea made sense. Furthermore, the acidic box is either very short (Partanen *et al.*, 1991) or absent (Miki *et al.*, 1991) from forms of the FGF receptor to which FGF-2 binds poorly (Partanen *et al.*, 1991; Miki *et al.*, 1991). However, more recent studies have suggested that the ligand binding domains of the FGF receptors reside in the second and third Ig domains and do not include the acidic box (Zimmer *et al.*, 1993; Chellaiah *et al.*, 1994). In addition, in two separate studies, deletion of the acidic box from FGFR-1 had no effect on ligand binding (Byers *et al.*, 1992; Hou *et al.*, 1992).

Our approach to determining the role of the acidic box in FGF receptor function was to search for similar sequences in other proteins and to compare the role of those acidic boxes to the one in FGF receptors. Specifically, an acidic box similar to that found in the FGF receptors is found in some cell adhesion molecules such as uvomorulin (Kemler *et al.*, 1989). In this instance, the acidic box binds calcium (Ringwald *et al.*, 1987), and the calcium binding is critical to the activity of this Ca²⁺-dependent cell adhesion molecule (Ozawa *et al.*, 1990). In the experiments described below, we tested the ability of FGFR-1 to bind calcium, and, after obtaining a positive result, proceeded to explore the role of divalent cation binding in FGFR-1 function.

EXPERIMENTAL PROCEDURES

Reagents—Recombinant FGFR-1 expressed in Sf9 cells was obtained from P. Barr (Kiefer *et al.*, 1991). For expression of the extracellular domain of FGFR-1 in bacteria, the extracellular domain was cloned, using PCR, into the pMal vector (New England Biolabs) and expressed as a fusion protein with maltose-binding protein. The fusion protein was purified on amylose resin, cleaved overnight with factor IX, and then collected as the unretained fraction following a second passage over amylose resin. The acidic box, including the sequence from nucleotides 300–325, was deleted by PCR mutagenesis (Hemsley *et al.*, 1989), and the deletion was confirmed by dideoxy DNA sequencing. The sense primer for this mutagenesis was TCC-TCT-TCA-GAG-GAG-AAA-GAA, and the antisense primer was CGA-GGA-GGG-GAG-AGC-ATC. The mutagenized receptor was expressed and purified as described above.

Ca²⁺ Blotting—⁴⁵Ca²⁺ blotting was carried out essentially as described (Maruyama *et al.*, 1984). Briefly, 5–20 µg of protein were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose in the absence of SDS, and then rinsed in 60 mM KCl, 10 mM imidazole-HCl, pH 6.8, with several changes over 60 min. The transfer was incubated with 2 µCi of ⁴⁵Ca²⁺/ml for 40 min at 22 °C in the same buffer, rinsed for 5 min with 50% ethanol, air-dried, and autoradiographed for 18–48 h. The blots were then stained with Amido Black to

* This work was supported by National Institutes of Health Grant DK 18811. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: FGF, fibroblast growth factor; PCR, polymerase chain reaction; PBS, phosphate-buffered saline.

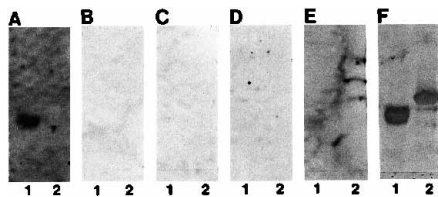


FIG. 1. $^{45}\text{Ca}^{2+}$ blotting of the extracellular domain of FGFR-1 produced in Sf9 cells (lane 1) and bovine serum albumin (lane 2), alone (A) or in the presence of 5 mM CaCl_2 (B), 5 mM MnCl_2 (C), 100 $\mu\text{g/ml}$ acidic box peptide (D), or 5 mM CuCl_2 (E). F shows the Amido Black staining of one of the panels. Similar results were obtained in three independent experiments of identical design.

assess the amounts of protein loaded in each lane.

Ca^{2+} Binding—To estimate Ca^{2+} binding, a rapid ultrafiltration method for the determination of calcium-protein binding constants was used (Fuchs, 1972). The standard assay mixture contained 60 mM KCl, 10 mM imidazole, pH 6.8, 0.2 μCi of $^{45}\text{Ca}^{2+}/\text{ml}$, varying concentrations (1–25 μM) of cold Ca^{2+} , and 0.1 mg/ml recombinant extracellular domain of FGFR-1 produced in either bacteria or Sf9 cells in a total volume of 0.5 ml. After incubation at 22 $^{\circ}\text{C}$ for 10 min, the mixture was centrifuged in a Centrifree (Amicon) micropartition device for 3 min at $1000 \times g$. After centrifugation, aliquots of the protein solution and the ultrafiltrate were analyzed for ^{45}Ca by liquid scintillation counting, and the amount of calcium bound to the extracellular domain of FGFR-1 was calculated as described (Fuchs, 1972). To assess the effect of Cu^{2+} on Ca^{2+} binding, the standard assay mixture was altered to include a fixed amount of cold Ca^{2+} (10 μM) and a varying concentration of Cu^{2+} (1–50 μM). Ca^{2+} binding was then assayed as described above.

Heparin-Sepharose Binding—For the heparin-Sepharose experiments, 10 μg of recombinant protein was combined with 25 μl of heparin-Sepharose (1:1) in a total volume of 100 μl . The mixture was rotated for 1 h at 22 $^{\circ}\text{C}$, washed twice with 0.1% Triton X-100 in 50 mM NaCl, 10 mM HEPES, pH 7.4, once with PBS, and eluted with $2.5 \times$ SDS-sample buffer. The samples were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotted with McAb6. McAb6 is a FGF receptor monoclonal antibody which was prepared using the extracellular domain of FGFR-1 expressed in Sf9 cells as an antigen.

RESULTS

If the acidic box region of FGFR-1 binds calcium, then it should be possible to detect calcium binding to either intact FGF receptors or their extracellular domains. We used a radioactive Ca^{2+} blotting technique (Maruyama *et al.*, 1984), in combination with the recombinant extracellular domain of FGFR-1 expressed in either insect cells (Kiefer *et al.*, 1991) or bacteria to determine whether FGFR-1 could bind calcium. As shown in Fig. 1, the extracellular domain of FGFR-1 shows strong labeling with $^{45}\text{Ca}^{2+}$ by this technique. Bovine serum albumin, a protein of approximately the same size, is unlabeled. Similar results were obtained with bacterial (unglycosylated) FGFR-1 (Fig. 2), indicating that the carbohydrate groups are not playing a role in calcium binding. However, the binding of $^{45}\text{Ca}^{2+}$ is competed completely by either 5 mM Ca^{2+} or Mn^{2+} (Fig. 1). Ca^{2+} binding to FGFR-1 is also blocked by a peptide corresponding to the acidic box region (FGFR-1 125–133) (Fig. 1). These data indicate that FGFR-1 can bind calcium, and perhaps other divalent cations, and that this binding may be mediated by the acidic box.

To explore further the role of the acidic box in calcium binding, this region was deleted from FGFR-1 using PCR-based mutagenesis. The mutant was expressed in bacteria and purified, and the calcium binding activity of the mutant protein was compared with that of the wild type protein. As shown in Fig. 2, when equal amounts of the wild type and mutant proteins are subjected to $^{45}\text{Ca}^{2+}$ blotting, only the wild type protein is labeled. These data provide strong support for the hypothesis that the acidic box mediates divalent cation binding by FGFR-1.

To characterize further the calcium binding of FGFR-1, we

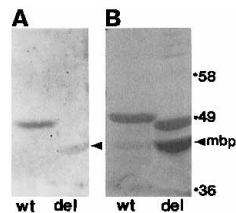


FIG. 2. $^{45}\text{Ca}^{2+}$ blotting of the extracellular domain of FGFR-1 produced in bacteria. Wild type (wt) receptor and the acidic box deletion mutant (del) were examined for Ca^{2+} binding (A), and then the same blot was stained with Amido Black to confirm equal protein loading in both lanes (B). The lower band indicated with an arrowhead in B is maltose-binding protein, which proved difficult to separate completely from the mutant receptor. Similar results were obtained in three independent experiments of identical design. Molecular weights ($\times 10^{-3}$) are indicated at right.

used a rapid ultrafiltration method (Fuchs, 1972) to estimate the calcium-FGFR-1 binding constants. These studies demonstrated that both the glycosylated and unglycosylated forms of recombinant FGFR-1 extracellular domain bind one calcium molecule with a similar affinity ($K_d \sim 20 \mu\text{M}$). In contrast, when the mutant receptor was tested in the same assay, no specific binding of calcium was detected.

Several experiments were carried out to determine a role for calcium binding in FGF receptor function. Neither the addition nor the removal of Ca^{2+} affected either ligand binding, as determined by ^{125}I -FGF-2 binding to whole cells, or signal transduction, as monitored by changes in protein tyrosine phosphorylation (data not shown). Because of the role copper plays in regulating angiogenesis (for review, see Gullino (1986)), we tested the possibility that Cu^{2+} , rather than Ca^{2+} , was the true divalent cation which binds to FGFRs. FGFs have been clearly implicated (for reviews, see Baird and Bohlen (1990), Burgess and Maciag (1989), Wagner (1991), and Fernig and Gallagher (1994)) in the control of angiogenesis, further supporting the notion that the physiological divalent cation affecting FGFR-1 could be Cu^{2+} . Accordingly, we first tested whether Cu^{2+} could block $^{45}\text{Ca}^{2+}$ binding in the Ca^{2+} blotting assay. As shown in Fig. 1, Cu^{2+} effectively competes with $^{45}\text{Ca}^{2+}$ in this assay, suggesting that the acidic box can bind copper as well as calcium. To explore further the interaction of Cu^{2+} with FGFR-1, we evaluated the effect of increasing amounts of Cu^{2+} on Ca^{2+} binding in the rapid ultrafiltration binding assay. Cu^{2+} efficiently reduces the binding of Ca^{2+} to both glycosylated and nonglycosylated FGFR-1 in a similar manner (Fig. 3), providing additional evidence for an interaction of Cu^{2+} with the acidic box region of FGFR-1. In yet other experiments, we tested the ability of the wild type and mutant receptors to bind specifically to chelating Sepharose charged with Cu^{2+} . Only the wild type receptor was found to bind in a specific manner to Cu^{2+} -charged chelating Sepharose (not shown), further supporting the idea that the acidic box region of FGFR-1 binds Cu^{2+} .

Since the effects of copper on angiogenesis are heparin-dependent, we suspected that divalent cations could play a role in modulating the interactions of FGF receptors with components of the extracellular matrix. In order to test this idea, we examined FGFR-1 binding to heparin-Sepharose in the absence or presence of divalent cations. As shown in Fig. 4, only low levels of either glycosylated or nonglycosylated FGFR-1 bind to heparin-Sepharose when evaluated in PBS. The binding of glycosylated receptor to heparin-Sepharose is completely eliminated if the reaction is performed in 0.5 M NaCl (Fig. 4A), in agreement with previous results (Kan *et al.*, 1993), whereas the binding of nonglycosylated receptor to heparin-Sepharose is not affected by salt (Fig. 4B). The presence of 1 or 5 mM Ca^{2+} in

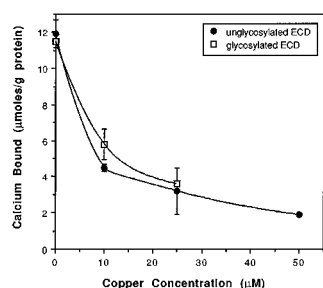


FIG. 3. The effect of Cu^{2+} on Ca^{2+} binding by the extracellular domain of FGFR-1 (ECD) produced in bacteria (unglycosylated) (●—●) or Sf9 cells (glycosylated) (□—□). Increasing amounts of Cu^{2+} were included in the rapid ultrafiltration assay for Ca^{2+} binding along with a fixed amount ($10 \mu\text{M}$) of Ca^{2+} . Each point was tested in duplicate. The results shown are from a single experiment. Similar results were obtained in three independent experiments of identical design.

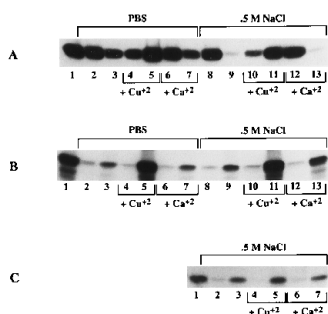


FIG. 4. Heparin-Sepharose binding of the wild type (A and B) extracellular domain of FGFR-1 produced in Sf9 cells (A) or bacteria (B) and the acidic box deletion mutant produced in bacteria (C). Heparin-Sepharose binding was carried out in PBS or 0.5 M NaCl in the absence of divalent cations (lanes 2, 3, 8, and 9) or in the presence of 1 mM CaCl_2 ($+\text{Ca}^{2+}$) or 1 mM CuCl_2 ($+\text{Cu}^{2+}$) as described under "Materials and Methods." In all cases, lane 1 is the receptor prior to the addition of heparin-Sepharose; lanes 2, 4, 6, 8, 10, and 12 show the receptor remaining in an aliquot (25%) of the supernatant following precipitation with heparin-Sepharose, and lanes 3, 5, 7, 9, 11, and 13 show the total amount of receptor that binds to heparin-Sepharose. Similar results were obtained in five independent experiments of identical design.

the binding buffer has no effect on the interaction of either glycosylated or nonglycosylated receptor with heparin-Sepharose. In contrast, the addition of 1 or 5 mM Cu^{2+} to the binding buffer significantly increases the level of FGFR-1 binding to heparin-Sepharose in both PBS and 0.5 M NaCl (Fig. 4). The mutant receptor which lacks the acidic box shows a slightly increased level of basal heparin binding in the absence of divalent cations (Fig. 4C). However, unlike the wild type receptor, Cu^{2+} has little or no effect on the interaction of the mutant receptor with heparin-Sepharose (Fig. 4C). These observations provide additional evidence that the acidic box binds copper and suggest that this interaction plays an important role in mediating the binding of the receptor to extracellular matrix.

DISCUSSION

The results presented here provide the first indication of a specific role for the acidic box in FGF receptor function. The acidic box, similar to homologous regions in other calcium-binding proteins, binds divalent cations. The binding of a specific cation, Cu^{2+} , to this region modulates the interaction between the FGF receptor and heparin. It may also affect the interaction of FGF receptors with other proteins, particularly those in the extracellular matrix.

Our results are consistent with our failure to identify a role for the FGF receptor acidic box in either ligand binding or signal transduction. Since receptor activation and consequent

signal transduction is thought to require receptor dimerization (Ueno *et al.*, 1992), it is unlikely that the acidic box plays a role in receptor-receptor interactions. Instead, there is now considerable evidence for an interaction between FGF receptors, cell adhesion molecules (Williams *et al.*, 1994), and the extracellular matrix (Hanneken *et al.*, 1995). Although some matrix binding is mediated by heparan sulfate proteoglycans, there is also evidence for heparin-independent interactions with extracellular matrix (Hanneken *et al.*, 1995). All of these interactions could be modulated by divalent cations, particularly Cu^{2+} .

Although our data suggest a role for copper in FGF receptor-matrix interactions, they do not preclude the possibility that calcium could also mediate a subset of receptor interactions, distinct from those that are copper-dependent. Thus, interactions with heparin could involve copper whereas interactions with other extracellular matrix components might require Ca^{2+} or even another, as yet unidentified, divalent cation. Further studies will be needed to sort out the physiological relevance of these different interactions.

Two types of calcium binding sites in proteins are known. The better known calcium binding site is the EF hand (Kretsinger, 1980), whereas the sequence in the FGF receptors resembles the calcium binding sequence found in α -lactalbumin (Stuart *et al.*, 1986). In both cases, these sites form loops which coordinate around the divalent cation. Thus, it is likely that the acidic box also forms a loop in the presence of Ca^{2+} or other divalent cations. Such a loop could stabilize the receptor in a conformation conducive to interaction with extracellular matrix components or other proteins. Given the calculated affinity of the acidic box for Ca^{2+} , the interaction of the receptor with this divalent cation is likely to play a regulatory role in FGF receptor function. Thus, local changes in Ca^{2+} concentration could trigger a conformational change (e.g. loop formation) in the receptor. This change then could be detected by other proteins at, or near, the cell surface. Divalent cations could also directly mediate protein-protein interactions. These potential functions of calcium binding domains are consistent with the proposed structural role for the acidic box (Chaudhuri *et al.*, 1993).

There is some recent evidence demonstrating that cell adhesion molecules such as N-cadherin, N-CAM, and L1 can interact with FGF receptors and, in so doing, activate the FGF signaling pathway (Williams *et al.*, 1994) that leads to neurite outgrowth. It is interesting to note that an antibody to the acidic box region was shown to block FGF receptor activation by these cell adhesion molecules. Furthermore, a reduction in extracellular Ca^{2+} inhibits neurite outgrowth induced by L1 and N-CAM, even though these cell adhesion molecules mediate Ca^{2+} independent adhesion. Thus, it is possible that these interactions of cell adhesion molecules with the FGF receptor are regulated by divalent cation binding to the acidic box. Since several domains of the FGF receptor are implicated in the interaction between FGF receptors and cell adhesion molecules, divalent cation binding to the acidic box may simply stabilize a tertiary conformation that is conducive to the interaction between FGF receptors and cell adhesion molecules.

The data presented here on the interaction of FGFR-1 with divalent cations may help to resolve a controversy in the literature regarding the ability of FGFR-1 to interact directly with heparin (Kiefer *et al.*, 1991; Ornitz *et al.*, 1992; Kan *et al.*, 1993; Fernig and Gallagher, 1994). Thus, these differences in experimental results could be due to the absence or presence of low levels of Cu^{2+} in the preparations of recombinant receptor used in the various studies.

Finally, the results presented above may serve to consolidate

a large body of work that has described the angiogenic activities of copper. A number of studies (for review see Gullino (1986)) have shown how copper can promote neovascularization and how heparin can acquire angiogenic properties when bound to copper. Although the mechanism whereby this divalent cation and glycosaminoglycans induce vascular growth remains unclear (McAuslan and Reilly, 1980; Raju *et al.*, 1984; Terrell and Swain, 1991), perhaps it is through their synergistic ability to interact with FGF receptors. If so, changes in the ionic milieu may play a critical role in regulating the cellular response to FGF, equally important as that of matrix.

Taken together, all of these results suggest that the acidic box region of FGF receptors plays an important role in their interaction with cell adhesion molecules, extracellular matrix, and heparin.

Acknowledgments—We thank Dr. D. Engler for technical suggestions and Drs. D. Schubert and A. Baird for critical reading of the manuscript.

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