Communication

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 FGFR-1 receptors may also mediate the interaction between FGF receptors, cell adhesion molecules and other proteoglycan components of the extracellular matrix.

Basic (FGF-2) 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, rinsed for 5 min with 50% ethanol, air-dried, and autoradiographed for 18–48 h. The blots were then stained with Amido Black to...
shown in Fig. 1, the extracellular domain of FGFR-1 shows bacteria to determine whether FGFR-1 could bind calcium. As combination with the recombinant extracellular domain of 45Ca2+ are not playing a role in calcium binding. However, the binding ligated) FGFR-1 (Fig. 2), indicating that the carbohydrate groups be labeled. Similar results were obtained with bacterial (unglycosylated albumin, a protein of approximately the same size, is unla-


ted. The standard assay mixture contained 60 mM KCl, 10 mM imidazole, pH 6.8, 0.2 μCi of 45Ca2+ /ml, varying concentrations (1-25 μM) of cold Ca2+, and 0.1 ng/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 °C for 10 min, the mixture was centrifuged in a Centrifree (Amicon) micropartition device for 3 min at 1000 × g. After centrifugation, aliquots of the protein solution and the ultrafiltrate were analyzed for 45Ca 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 Cu2+ on Ca2+ binding, the standard assay mixture was altered to include a fixed amount of cold Ca2+ (10 μM) and a varying concentration of Cu2+ (1-50 μM). Ca2+ 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 °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 × SDS-sample buffer. The samples were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotted with MAb6. MAb6 is a FGFR 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 FGFR receptors or their extracellular domains. We used a radioactive Ca2+ 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 45Ca2+ by this technique. Bovine serum albumin, a protein of approximately the same size, is unla-

belled. 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 45Ca2+ is competed completely by either 5 mM Ca2+ or Mn2+ (Fig. 1). Ca2+ binding to FGFR-1 is also blocked by a peptide corresponding to the acid 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 bind-

ing, 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 45Ca2+ 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 used a rapid ultrafiltration method (Fuchs, 1972) to estimate the calcium-FGFR binding constants. These studies demonstrated that both the glycosylated and nonglycosylated forms of recombinant FGFR-1 extracellular domain bind one calcium molecule with a similar affinity (Kd ~ 20 μM). 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 FGFR receptor function. Neither the addition nor the removal of Ca2+ affected either ligand binding, as determined by 125I-FGFR-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 Cu2+, rather than Ca2+, was the true divalent cation which binds to FGFRs. FGFRs 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 Cu2+. Accordingly, we first tested whether Cu2+ could block 45Ca2+ binding in the Ca2+ blotting assay. As shown in Fig. 1, Cu2+ effectively competes with 45Ca2+ in this assay, suggesting that the acidic box can bind copper as well as calcium. To explore further the interaction of Cu2+ with FGFR-1, we evaluated the effect of increasing amounts of Cu2+ on Ca2+ binding in the rapid ultrafiltration binding assay. Cu2+ efficiently reduces the binding of Ca2+ to both glycosylated and nonglycosylated FGFR-1 in a similar manner (Fig. 3), providing additional evidence for an interaction of Cu2+ 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 Cu2+. Only the wild type receptor was found to bind in a specific manner to Cu2+-charged chelating Sepharose (not shown), further supporting the idea that the acidic box region of FGFR-1 binds Cu2+.

Since the effects of copper on angiogenesis are heparin-de-
pendent, we suspected that divalent cations could play a role in modulating the interactions of FGFR 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 glyco-
ysylated receptor to heparin-Sepharose is completely eliminated if the reaction is performed in 0.5 mM NaCl (Fig. 4A), in agree-
ment 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 Ca2+ in

![FIG. 1.](http://www.jbc.org/)

![FIG. 2.](http://www.jbc.org/)
FGFR1 Binds Copper and Calcium

Fig. 3. The effect of Cu²⁺ on Ca²⁺ binding by the extracellular domain of FGFR-1 (ECD) produced in bacteria (unglycosylated) (●) or SF9 cells (glycosylated) (———). Increasing amounts of Cu²⁺ were included in the rapid ultrafiltration assay for Ca²⁺ binding along with a fixed amount (10 μM) of Ca²⁺. 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 CaCl₂ (● Ca²⁺) or 1 mM CuCl₂ (● Cu²⁺) 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 mM or 0.5 M Cu²⁺ 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, the wild type receptor, Cu²⁺ 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 FGFR receptor function. The acidic box, similar to homologous regions in other calcium-binding proteins, binds divalent cations. The binding of a specific cation, Cu²⁺, to this region modulates the interaction between the FGF receptor and heparin. It may also affect the interaction of FGFR receptors with other proteins, particularly those in the extracellular matrix.

Our results are consistent with our failure to identify a role for the FGFR 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 Cu²⁺.

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 Ca²⁺ 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 Ca²⁺ 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 Ca²⁺, the interaction of the receptor with this divalent cation is likely to play a regulatory role in FGF receptor function. Thus, local changes in Ca²⁺ concentration could trigger a conformational change (e.g., loop formation) in the receptor. This change 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 Ca²⁺ inhibits neurite outgrowth induced by L1 and N-CAM, even though these cell adhesion molecules mediate Ca²⁺ 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 Cu²⁺ 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.

REFERENCES

Baird, A., and Bohlen, P. (1990) in Peptide Growth Factors and Their Receptors (Spong, M. B., and Roberts, A. B., eds) pp. 369–418, Springer Verlag, Berlin

Burgess, W. H., and Maciag, T. (1989) Annu. Rev. Biochem. 58, 575–606

Byers, S., Amaya, E., Munro, S., and Blaschuk, O. (1992) Dev. Biol. 152, 411–414

Chaudhuri, M. M., Moscatelli, D., and Basilico, C. (1993) J. Cell Physiol. 157, 209–216

Chellaiah, A. T., McEwen, D. G., Werner, S., Xu, J., and Ornitz, D. M. (1994) J. Biol. Chem. 269, 11620–11627

Fernig, D. G., and Gallagher, J. T. (1994) Protein Growth Factor Res. 5, 353–377

Fuchs, F. (1972) Int. J. Peptide Protein Res. 4, 147–149

Givol, D., and Yayon, A. (1992) FASEB J. 6, 3362–3369

Gullino, P. M. (1986) Anticancer Res. 6, 153–158

Hanks, S. K., Quinn, A. M., and Hunter, T. (1988) Science 241, 42–52

Hannenek, A., Maher, P. A., and Baird, A. (1995) J. Cell Biol. 128, 1221–1228

Hemseley, A., Arnheim, N., Toney, M. D., Cortopassi, G., and Galas, D. J. (1989) Nucleic Acids Res. 17, 6545–6551

Hou, J., Kan, M., Wang, F., Xu, J., Nakahara, M., McBride, G., McKeenan, K., and McKeenan, W. L. (1992) J. Biol. Chem. 267, 17804–17808

Johnson, D. E., and Williams, L. T. (1993) Adv. Cancer Res. 60, 1–41

Kan, M., Wang, F., Xu, J., Crabb, J. W., Hou, J., and McKeenan, W. L. (1993) Science 259, 1918–1921

Kenser, R., Ozawa, M., and Ringwald, M. (1989) Curr. Opin. Cell Biol. 1, 892–897

Kiefer, M. C., Baird, A., Nguyen, T., George-Nascimento, C., Mason, O. B., Boley, L. J., Valenzuela, P., and Barr, P. J. (1993) Growth Factors 5, 115–127

Kretsinger, R. H. (1980) Crit. Rev. Biochem. 8, 119–174

Lee, P. L., Johnson, D. E., Cousens, L. S., Fried, V. A., and Williams, L. T. (1989) Science 245, 57–60

Maruyama, K., Mikawa, T., and Ebashi, S. (1984) J. Biochem. (Tokyo) 95, 511–519

McAuslan, B. R., and Reilly, W. (1980) Exp. Cell Res. 130, 147–157

Miki, T., Fleming, T. P., Bartett, D. P., Rubin, J. S., Ron, D., and Aaronson, S. A. (1991) Science 251, 72–75

Ornitz, D. M., Yayon, A., Flanagan, J. G., Svahn, C. M., Lev, E., and Leder, P. (1992) Mol. Cell. Biol. 12, 240–247

Ozawa, M., Engel, J., and Kemler, R. (1990) Cell 63, 1033–1038

Partanen, J., Makela, T. P., Ervela, E., Korhonen, J., Hirvonen, H., Claesson-Welsh, L., and Alitalo, K. (1991) EMBO J. 10, 1347–1354

Partanen, J., Vainikka, S., and Alitalo, K. (1993) Philos. Trans. R. Soc. Lond. B Biol. Sci. 340, 297–303

Raju, K. S., Alessandri, G., and Gullino, P. M. (1984) Cancer Res. 44, 1579–1584

Ringwald, M., Schuh, R., Vestweber, D., Eistetter, H., Latzsch, F., Engell, J., Dutz, R., Janhig, F., Epplen, J., Mayer, S., Muller, C., and Kereir, R. (1987) EMBO J. 6, 3647–3653

Stuart, D. I., Adharya, K. R., Walker, N. P. C., Smith, S. G. Lewis, M., and Phillips, D. C. (1986) Nature 324, 84–87

Terrell, G. E., and Swain, J. W. (1991) Matrix 11, 108–114

Uno, H., Gunp, M., Dett, K., Tseng, A., and Williams, L. (1992) J. Biol. Chem. 267, 1470–1476

Wagner, J. A. (1991) Curr. Top. Microbiol. Immunol. 165, 95–118

Williams, E. J., Furness, J., Walsh, F. S., and Doherty, P. (1994) Neuron 13, 583–594

Zimmer, Y., Givol, D., and Yayon, A. (1993) J. Biol. Chem. 268, 7899–7903
