THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 270, No. 37, Issue of September 15, pp. 21869–21874, 1995

Printed in U.S.A.

Glu-96 of Basic Fibroblast Growth Factor Is Essential for High Affinity Receptor Binding

IDENTIFICATION BY STRUCTURE-BASED SITE-DIRECTED MUTAGENESIS*

(Received for publication, May 25, 1995, and in revised form, July 5, 1995)

Hengyi Zhu‡, K. Ramnarayan, Jerry Anchin, Wendy Y. Miao, Arlene Sereno, Linda Millman, Jinhua Zheng, V. N. Balaji, and Manfred E. Wolff

From ImmunoPharmaceutics Inc., San Diego, California 92127

The importance of basic fibroblast growth factor (bFGF) in several pathophysiological processes has stimulated interest in the design of receptor antagonists to mitigate such effects. Of key importance in this connection is the characterization of the functional binding epitopes of the growth factor for its receptor. Based on peptide mapping and molecular dynamics calculations of the three-dimensional structure of basic fibroblast growth factor, we employed site-directed mutagenesis to investigate the effect of altering residues at positions 107, 109–114, and 96 on bFGF on receptor binding affinity. All muteins were cloned and expressed in *Escherichia coli*, purified to homogeneity employing heparin-Sepharose columns, and evaluated for receptor binding affinity. We found that replacement of residues at positions 107 and 109–114 by alanine or phenylalanine had little effect on receptor binding affinities compared with wild type bFGF, in agreement with previous evidence that bFGF residues 109–114 comprise a low affinity binding site. By contrast, substitution of Glu-96 with alanine yielded a molecule having about 0.1% of the affinity of the wild type bFGF. The affinity of the corresponding lysine and glutamine muteins was 0.3 and 10%, respectively, emphasizing the importance of a negative charge at this position. Our findings are consistent with the view that residues 106–115 on bFGF represent a low affinity binding site on bFGF. In addition, we identify Glu-96 as a crucial residue for binding to fibroblast growth factor receptor-1.

Basic fibroblast growth factor (bFGF)[1] is a potent mitogen for a wide variety of cell types of mesodermal and neuroectodermal origin (Folkman and Klagsbrun, 1987; Gospodarowicz, 1974; Gospodarowicz *et al.*, 1987; Burgess and Maciag, 1989). It has been suggested that bFGF and other members of this family may play a critical role in several pathophysiological processes, including the growth of tumors (Folkman, 1985), wound healing response (Folkman and Klagsbrun, 1987), and diabetic retinopathy (Sivalingam *et al.*, 1990). The discovery of the high affinity binding of bFGF to heparin has accelerated its purification, characterization, and cloning (Shing *et al.*, 1984; Abraham *et al.*, 1986). So far, nine members of the FGF family have been identified, including acidic FGF (aFGF; Jaye *et al.*, 1986), bFGF (Abraham *et al.*, 1986), int-2 gene product (int-2; Dickson and Peters, 1987), hst/kFGF (Delli-Bovi *et al.*, 1987; Yoshida *et al.*, 1987), FGF-5 (Zhan *et al.*, 1988), FGF-6 (Marics *et al.*, 1989), keratinocyte growth factor (KGF; Finch *et al.*, 1989), and the actin-binding protein hisactophilin (Habazettl *et al.*, 1992). DNA sequencing of members of this gene family has revealed that they possess 30–55% identity at the primary amino acid sequence level (Dionne *et al.*, 1990).

Several three-dimensional structures of acidic and basic FGFs are available. These include two acidic forms (Zhu *et al.*, 1991, 1993) and three basic forms (Zhu *et al.*, 1991; Zhang *et al.*, 1991; Eriksson *et al.*, 1991). Baird *et al.* (1988) identified two important regions on bFGF for binding to FGFR1, comprising residues 24–68 and residues 106–115. These workers showed that the sequence 24–68 has high affinity for heparin. Hence, the region 106–115 (Fig. 2) was considered the putative receptor binding site on bFGF.

Recently, the binding site for heparin/heparin sulfate on bFGF was more explicitly defined (Thompson *et al.*, 1994; Li *et al.*, 1994). Through identification of key amino acids for heparin binding on bFGF based on its three-dimensional structure and site-directed mutagenesis, these studies indicate that pure electrostatic interactions contribute only 30% of the binding free energy to the ligand receptor interaction and that hydrophobic effects, as well as other noncovalent forces such as hydrogen bonding and van der Waals packing, contribute the bulk of the free energy for this binding reaction. Heparin, although it is not absolutely required for bFGF to bind to its receptor (Shi *et al.*, 1993; Roghani *et al.*, 1994; Pantoliano *et al.*, 1994), increases the receptor affinity of bFGF (Roghani *et al.*, 1994), protects bFGF from inactivation (Gospodarowicz and Cheng, 1986) and proteolytic degradation (Saksela *et al.*, 1988; Sommer and Rifkin, 1989), and is essential for the mitogenic activity of bFGF stimulated cells (Yayon *et al.*, 1991).

In addition, Springer *et al.* (1994) identified high and low affinity receptor binding surfaces on bFGF on the basis of site-directed mutagenesis and molecular modeling. Based on their data, Tyr-103, Leu-140, and Tyr-24 on bFGF contribute significantly to the primary, higher affinity binding interaction. They found that the FGFR binding site composed of amino acids Lys-110, Tyr-111, and Trp-114 (Baird *et al.*, 1988), referred to as the putative receptor binding region, is a secondary, lower affinity.

Here we describe site-directed mutagenesis studies on the putative receptor binding region and its neighbors based on the three-dimensional structure of basic fibroblast growth factor refined by molecular dynamics. This is in contrast to the work of Thompson *et al.* (1994), Li *et al.* (1994), and Springer *et al.* (1994), which was based on the x-ray crystal structure of bFGF (Zhang *et al.*, 1991; Zhu *et al.*, 1991; Eriksson *et al.*, 1991). The

* 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.

‡ To whom correspondence should be addressed.

[1] The abbreviations used are: bFGF, basic fibroblast growth factor; aFGF, acidic fibroblast growth factor; KGF, keratinocyte growth factor; FGFR1, fibroblast growth factor receptor-1; TPA, tissue plasminogen activator; FGFR1-TPA, fibroblast growth factor receptor-1 TPA fusion protein.

crystallographically determined structure of a protein represents its static state, in which packing forces may play a major role in determining some of the conformational features. Therefore, in our work we considered it desirable to derive a solvent-immersed protein structure by computationally solvating the crystallographic bFGF structure in a bath of water and performing a molecular dynamics simulation. Structures from the dynamics trajectory were analyzed for conformational features, particularly at the putative receptor binding site. Solvent accessibility calculations were then carried out at the putative receptor binding site. The solvent accessible residues at positions 107, 109–114, and 96 were mutated to alanine or other residues. The resulting muteins were purified to near homogeneity and then compared with wild type bFGF for their receptor binding affinities to soluble FGFR1β-TPA fusion protein.

EXPERIMENTAL PROCEDURES

Materials—The human synthetic bFGF gene was purchased from R & D Systems (Minneapolis, MN). Expression vector pET11d and bacterial strain BL21(DE3) were obtained from Novagen (Madison, WI). Baculovirus transfer vector PVL1393 was obtained from Pharmingen (San Diego, CA). Magic mini-preparation kit was obtained from Promega (Madison, WI). Heparin-Sepharose was obtained from Pharmacia Biotech Inc. Heparin was purchased from Sigma. FGFR1β-TPA fusion protein was a gift of Eisai Corp. (Tsukuba-shi, Japan). ^{125}I-bFGF was obtained from DuPont NEN. Anti-bFGF monoclonal antibody was purchased from Upstate Biotechnology, Inc. Alkaline phosphatase-conjugated anti-mouse Ig antibodies were purchased from Bio-Rad. Prestained protein molecular weight standards were purchased from Life Technologies, Inc. All other chemicals were of reagent grade and were purchased from Sigma.

Molecular Dynamics Calculations—The initial model selected for the molecular dynamics simulation was the published structure of bFGF (Eriksson *et al.*, 1991) from the protein data bank (Abola *et al.*, 1987; Koetzle *et al.*, 1977) (code 3FGF). We added hydrogen atoms to these initial coordinates and minimized the system to relieve repulsive steric interactions. To evaluate solvent effects on the protein, its geometric center was determined and a spherical shell of water with a 26-Å radius was placed around it to completely immerse it in a spherical water bath. The total size of the solvated protein system was 5444 atoms. A dielectric constant of 4.0 was used in the calculations. Three stages of minimization were carried out on the system prior to the dynamics simulations. Initially, only the solvent around the protein was minimized for 500 cycles to optimize the interactions between the solvent molecules. In the second stage, 500 iterations of minimization were carried out for the total solvent/protein system to optimize the interaction between the solvent and protein. Finally, the whole system was minimized again with the SHAKE option (Ryckert *et al.*, 1977), to constrain the bonds in the system.

The molecular dynamics simulation was carried out at 300 K and 1 atm pressure. After 50 ps of equilibration, data were collected every picosecond over a period of 500 ps. Each conformer obtained at 25-ps intervals was minimized and stored for further analysis. The computations were performed using an extensively modified version (Ramnarayan *et al.*, 1990) of the AMBER program (Singh *et al.*, 1986). The time-averaged conformations resulting from the molecular dynamics calculations were analyzed to derive information for the site-directed mutagenesis studies.

Identification of Residues for Mutagenesis—Of the 124 residues in bFGF, those that are solvent-accessible were identified using solvent accessibility calculations based on the algorithm published earlier (Chothia, 1974) on the dynamic average structure. All neighbor residues within 10 Å of residues in the 106–115 loop were identified and classified into groups based on their proximity to these residues. The center of gravity of each residue was determined and used in these distance calculations. Residues within 5.0 Å were placed in one group, residues within 5.0 Å to 7.5 Å in a second group, and residues beyond 7.50 Å in a third group. Those residues around 106–115, which are either fully or partially solvent-accessible and are within 7.5 Å of any of the residues in the putative receptor binding loop (residues 106–115), were chosen for the first stage of site-directed mutagenesis.

Gene Construction and Site-directed Mutagenesis—The synthetic bFGF gene was digested with *Nco*I and *Bam*HI restriction enzymes and subcloned into pET11d vector containing T7 promoter. Site-directed mutagenesis was carried out following the Clontech method. Briefly, pET11d coding for bFGF was denatured and annealed with the *Bam*HI unique selection primer and desired mutation primers. After elongation and ligation, the mutagenic plasmid was digested with *Bam*HI for 1 h at 37 °C and transformed into BMH 71–18 mut S *Escherichia coli* strains for *in vivo* plasmid amplification. The amplified DNA was purified with a magic mini-preparation kit and digested with *Bam*HI again for the second round selection. The digested mixture was then transformed into DH5α *E. coli* strain. The desired mutation was confirmed by the dideoxy DNA sequencing method prior to expression.

Expression and Purification of Wild Type bFGF or Muteins from E. coli—Expression vectors were transformed into the BL21(DE3) *E. coli* strain. Cultures were grown to an A_{600} of 0.8 in LB medium containing 40 μg/ml ampicillin at 37 °C, and bFGF expression was induced by adding 0.4 mM isopropyl-β-D-thiogalactopyranoside to further culture at 37 °C in a thermoshaker for 4 h. The bFGF containing cell culture was then pelleted by centrifugation and lysed according to standard procedures (Seno *et al.*, 1990). The cytoplasmic fraction containing the mutein was loaded onto a heparin-Sepharose column equilibrated with buffer containing 0.6 M NaCl, 25 mM Tris, pH 7.5. After extensively washing the column with 25 mM Tris buffer, pH 7.5, containing 1 M NaCl, proteins were eluted with 25 mM Tris buffer, pH 7.5, containing 2 M NaCl. The purified protein fractions were run on 12% SDS-polyacrylamide gel electrophoresis and stained with Coomassie Blue R-250. The concentration and purity of proteins were determined with a scanning densitometer using bovine serum albumin as a standard.

Soluble Receptor Binding Assay—Soluble receptor FGFR1β-TPA fusion protein was coated on each well of a 96-well plate and incubated overnight at 4 °C. The plate was washed three times with phosphate-buffered saline, blocked with buffer containing 0.5% gelatin, 100 mM NaCl, and 25 mM Hepes, pH 7.5, and then incubated at room temperature for 1 h. For the competition assay, the soluble receptor FGFR1β-TPA-coated plate was next incubated with binding buffer containing ^{125}I-bFGF (1–20 ng/ml) and 2 μg/ml heparin in the presence of varied concentrations of unlabeled bFGF or its muteins. After incubation for 3 h at room temperature, plates were washed twice with phosphate-buffered saline, followed by phosphate-buffered saline containing 2 M NaCl, and then counted. Nonspecific binding was determined in parallel binding experiments using excess unlabeled bFGF.

RESULTS

These site mutagenesis studies were initiated to identify the critical region on bFGF for the high affinity receptor binding. Peptide mapping studies (Baird *et al.*, 1988) have shown that a peptide related to residues 106–115 of bFGF can inhibit the binding of ^{125}I-bFGF to its receptor. Subsequently, the three-dimensional structure determination of bFGF (Zhang *et al.*, 1991; Eriksson *et al.*, 1993) suggested that these residues form an antiparallel β-turn on the surface of the molecule. To identify residues on bFGF for the receptor binding, the crystal structure is first subjected to molecular dynamics treatment. The root mean square deviation of the crystal structure, and the molecular dynamics refined structure is then computed to be 2.01 Å for the α-carbon trace, 2.67 Å for the backbone atoms, and 2.98 Å for all atoms in the system, indicating that the solution structure of the protein is relatively close to the crystal structure. Since the molecular dynamics refined structure of bFGF is a more accurate presentation of the solution structure of the protein, the water-accessible surface area (in Å^2) of each residue reported here is calculated based on the solvent accessibility over the complete simulation period.

Site-directed Mutagenesis of the Putative Receptor Binding Site—Our molecular dynamics calculations indicate that residues Arg-107, Arg-109, Lys-110, Thr-112, Ser-113, and Trp-114 are solvent-accessible, Tyr-111 is partially accessible, and Tyr-106 and Ser-108 are fully buried (Table I). To evaluate whether this site is directly involved in high affinity receptor binding, we substituted each of its fully or partially solvent accessible residues with alanine or phenylalanine and determined the receptor binding affinity of the resulting mutein by radiolabeled receptor competition assay. Fig. 1 illustrates the regions in which the mutations examined in this investigation were made. Fig. 2 shows the mutated residues in the three-dimen-

TABLE I
Solvent accessibility of residues and binding of bFGF muteins to the soluble FGFR1β-TPA fusion proteins

Residues of which ASA is larger than 25 Å^2 were targeted for site-directed mutagenesis. IC_{50} value was calculated from the competitive binding of I-labeled bFGF *versus* unlabeled wild type bFGF or bFGF muteins to soluble FGFR1β-TPA fusion protein (average of two experiments). R107A indicates the Arg residue at position 107 was replaced by alanine.

Residue	ASA[a]	Mutant bFGF	IC_{50} (mutant)/IC_{50} (wild type)
Arg-107	66.0	R107A	2.8 ± 0.20
Ser-108	0.0		
Arg-109	81.1	R109A	1.45 ± 0.24
Lys-110	81.0	K110A	0.57 ± 0.12
Tyr-111	45.8	Y111F	0.86 ± 0.14
Thr-112	64.7	T112A	2.4 ± 0.56
Ser-113	64.6	S113A	2.7 ± 0.61
Trp-114	53.0	W114F	1.8 ± 0.28

[a] ASA: water-accessible surface area (in Å^2).

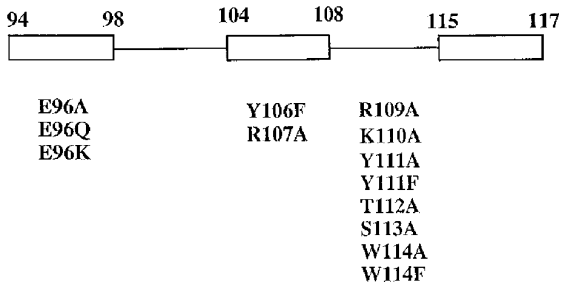

FIG. 1. **A linear representation of a partial structure of bFGF illustrating the mutations examined in this investigation.** The β strands are represented by *bars*; their starts and stops are indicated. The loops connecting the strands are represented by *straight lines*.

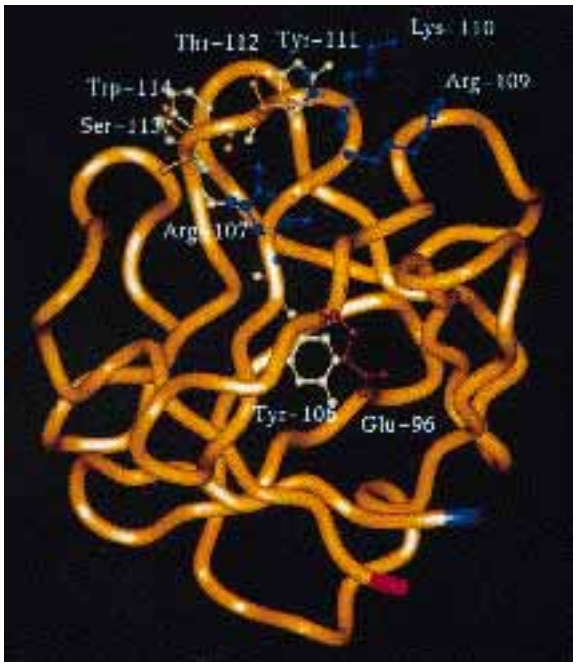

FIG. 2. **A ribbon rendering of the energy refined (see "Experimental Procedures") three-dimensional structure of basic fibroblast growth factor.** Amino acid side chains that mutated in these studies are labeled and the rest are shown in the *orange ribbon diagram* of the Cα backbone of bFGF. The N terminus of the protein is shown in *blue* and the C terminus in *red*.

sional structure of basic fibroblast growth factor. Most of the expressed recombinant proteins were partially soluble and could be purified to near homogeneity by absorption on a heparin-Sepharose column and elution with 25 mM Tris buffer containing 2 M NaCl. However, the Y111A and W114A muteins were confined to inclusion bodies, and attempts to refold the inclusion bodies were unsuccessful. By contrast, the more conservative substitution of Tyr-111 and Trp-114 with phenylalanine yielded sufficiently soluble material for purification and characterization. Our results revealed (Table I) that replacement of Arg-107, Arg-109, Lys-110, Thr-112, and Ser-113 with alanine and Tyr-111 and Trp-114 with phenylalanine gave muteins exhibiting receptor binding affinities similar to wild type bFGF. Hence, residues at positions 106–115 do not comprise a high affinity binding site.

Identification of Conserved Solvent-exposed Residues Neighboring the Residues in the 106–115 Loop—We examined the neighbors of residues (106–115) using three criteria to identify the candidates for the second round of site-directed mutagenesis. The first criterion is based on sequence homology information among the nine members of the growth factor family. Sequences from the nine members of the FGF family (Jaye *et al.*, 1986; Abraham *et al.*, 1986; Dickson and Peters, 1987; Delli-Bovi *et al.*, 1987; Yoshida *et al.*, 1987; Zhan *et al.*, 1988; Marics *et al.*, 1989; Finch *et al.*, 1989; Habazettl *et al.*, 1992) were aligned, and all fully conserved residues among all nine members of the family were identified: Leu-23, Gly-61, Val-63, Met-76, Gly-80, Leu-82, Cys-92, Phe-94, Glu-96, Tyr-103, Gly-122, Gly-127, and Phe-139. For the second criterion, all neighbors of residues 106–115 within 7.5 Å were computed. For the third criterion, solvent accessibility calculations were performed to determine which residues are buried and which are accessible to the solvent for all the identified neighbors. Only those residues that are neighbors to any of the residues (106–115), are fully conserved among the nine members of FGF family, and are solvent-exposed were selected for site-directed mutagenesis. Only Glu-96 satisfied all three criteria (Fig. 4 and Table II).

Site-directed Mutagenesis of Glu-96—Glu-96 in bFGF was replaced by alanine and purified on a heparin-Sepharose column. The interaction of E96A with the FGFR1β-TPA fusion protein was analyzed by measuring the ability of the mutant protein to compete with the binding of ^{125}I-labeled bFGF to this receptor coated on a 96-well plate. The E96A modification produced a 1600-fold decrease in IC_{50} (Table II). To further understand the nature of the interaction at this site, the polarity of this region was modified. Glu-96 was replaced by glutamine and lysine, respectively. Binding studies showed a 10-fold reduction of receptor binding properties for E96Q and almost 300-fold reduction for E96K (Table II). The effect on binding to the FGFR1β-TPA fusion protein observed for the E96A, E96Q, and E96K muteins was further confirmed in an assay employing FGFR1β expressed in insect cells (data not shown). The expression of mutein E96D failed to yield sufficient material for purification and analysis, and this analog was not pursued further.

Site-directed Mutagenesis of Tyr-106—In our molecular dynamics-refined three-dimensional structure of bFGF, Tyr-106 is buried in the protein except for its phenolic hydroxyl group, which is solvent accessible. The side chain of Glu-96 forms a hydrogen bond to this phenolic hydroxyl group (Fig. 3). Significantly, Tyr-106 is highly conserved among members of the FGF family except for FGF8 (Fig. 4). To determine whether the loss of the hydrogen bond of Glu-96 contributes to the altered receptor binding of the E96A mutein, Tyr-106 was replaced with phenylalanine. We found that this single point mutein, Y106F, exhibits approximately a 5-fold loss of receptor binding activity (Table II). Thus, it seems likely that the loss of this hydrogen bond partially contributes to the reduction of receptor binding for E96A.

TABLE II
Mutations at Glu-96, Arg-97, and Tyr-106 and relative receptor binding affinities of muteins

IC_{50} values were calculated from the competitive radiolabeled receptor binding assay (average of two experiments). Tyr-106 was targeted for the mutagenesis because the phenolic hydroxyl forms a hydrogen bond to the side chain of Glu-96, which in turn contacts bulk solvent.

Residues	ASA[a]	Mutant FGF	IC_{50} (mutant)/IC_{50} (wild type)
Glu-96	59.3	E96A	1600 ± 160
		E96Q	11.4 ± 1.2
		E96K	264 ± 82
Tyr-106	0	Y106F	4.6 ± 0.38
Arg-97	96.0	R97A	2.0 ± 0.20

[a] ASA: water-accessible surface area (in Å^2).

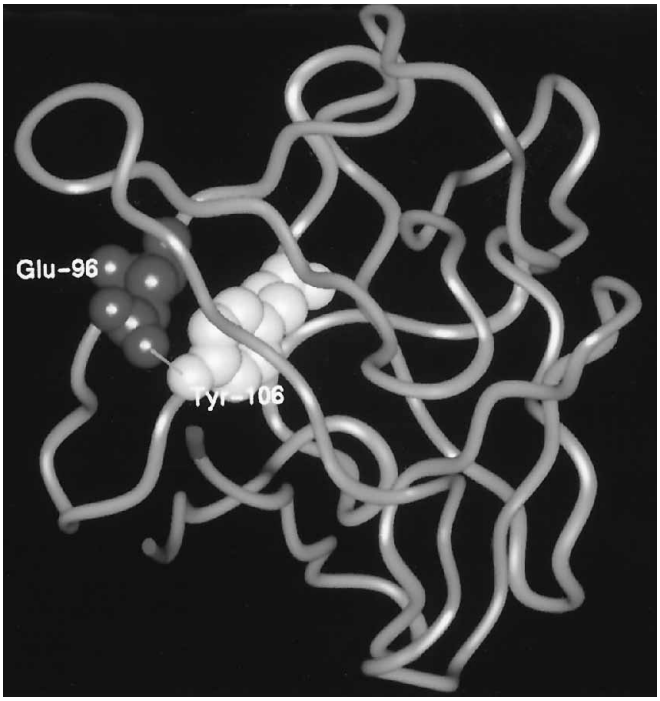

FIG. 3. **A close-up view of hydrogen bonding between the side chains of Glu-96 and Tyr-106.** The amino acid side chains for Glu-96 and Tyr-106 are displayed in *red* and *gold*, respectively, the hydrogen bond is displayed in *green*, and the remaining residues are shown in the *ribbon diagram* of the Cα backbone of bFGF.

	90						96								106
FGF1	E	E	C	L	F	L	E	R	L	E	E	N	H	Y	N	T	Y
FGF2	D	E	C	F	F	F	E	R	L	E	S	N	N	Y	N	T	Y
FGF3	A	E	C	E	F	V	E	R	I	H	E	L	G	Y	N	T	Y
FGF4	D	E	C	T	F	K	E	I	L	L	P	N	N	Y	N	A	Y
FGF5	D	D	C	K	F	R	E	R	F	Q	E	N	S	Y	N	T	Y
FGF6	E	E	C	K	F	R	E	T	L	L	P	N	N	Y	N	A	Y
FGF7	E	D	C	N	F	K	E	L	I	L	E	N	H	Y	N	T	Y
FGF8	K	D	C	V	F	T	E	I	V	L	E	N	N	Y	N	A	L
FGF9	Q	E	C	V	F	R	E	Q	F	E	E	N	W	Y	N	T	Y

FIG. 4. **Alignment of the amino acid sequences of nine members of FGF family around residue 90–106 of bFGF.** The FGF sequence was obtained from protein data bank. The alignment of the primary amino acid sequence was done using the Genalign program: aFGF, FGF1; bFGF, FGF2; KGF, FGF7. Amino acid residues for Glu-96 and Tyr-106 are shown in *boldface type*.

DISCUSSION

Several attempts have been made to define residues that participate in the FGF-FGFR interactions. An early peptide mapping approach by Baird *et al.* (1988) suggested that residues 106–115 are involved in receptor binding. Later, studies of the three-dimensional structure of bFGF revealed that part of this putative receptor binding peptide forms a loop consisting of residues 109–114 on the surface of the molecule (Eriksson *et al.*, 1993). In addition, Seno *et al.* (1990) studied the action of truncated N- and C-terminal forms of bFGF on mitogenesis and heparin binding. Their results indicate that an essential part of bFGF for receptor binding is present within the sequence Asp-41 to Ser-100.

Recently, Pantoliano *et al.* (1994) described multivalent ligand-receptor binding interactions in the fibroblast growth factor system based on isothermal titration calorimetry and molecular modeling. Their results indicate that the ^{110}KYTSW^{114} loop is a low affinity binding site required for receptor dimerization *in vitro* and mitogenic signal transduction *in vivo*. In a subsequent publication, Springer *et al.* (1994) defined the primary high affinity FGFR binding site as comprising the four hydrophobic amino acids (Tyr-24, Tyr-103, Leu-140, and Met-142) and the two polar residues (Arg-44 and Asn-101).

In our work, we employed site-directed mutagenesis based on the dynamic structure of bFGF to determine which region is responsible for high affinity binding to FGFR1β. Substitution of residues at positions 107 and 109–114 by alanine or phenylalanine gave muteins with similar receptor binding affinities compared with wild type bFGF. To extend these studies, we identified the neighboring side chain Glu-96, which is solvent accessible and conserved among the nine member of FGF family (Fig. 4 and Table II). Upon replacement of Glu-96 with alanine, a 1000-fold decrease in receptor binding was observed. This result suggests that the conserved glutamic acid residue in other members of FGF family may also play an important role in high affinity receptor binding. To address the question of whether the drastic loss of receptor binding affinity of E96A mutein is due to a global conformational change, we considered the following evidence. First, like wild type bFGF, E96A binds tightly to heparin-Sepharose, which is the hallmark of specific interaction between bFGF and heparin (Thompson *et al.*, 1994). Second, the E96A mutein can be precipitated with specific monoclonal antibodies that recognize only the native form of bFGF (data not shown). Finally, molecular dynamics studies revealed no global change for the E96A mutein (data not shown).

To elucidate the nature of the interaction between Glu-96 and FGFR1β, the anionic Glu-96 was changed to neutral glutamine. The resulting mutein showed a 10-fold reduction in receptor binding, suggesting that electrostatic interaction is involved in the receptor binding. Furthermore, substitution of Glu-96 with a cationic residue, lysine, gave more than a 300-fold reduction of receptor binding, providing further evidence that electrostatic interaction in this region is an important component of receptor binding.

To investigate whether the internal hydrogen bond between Glu-96 and the phenolic hydroxyl in Tyr-106 seen in our energy-refined structure of bFGF (Fig. 3) is important for the high affinity receptor binding of bFGF, we replaced Tyr-106 with phenylalanine. The resulting mutein, Y106F, had an almost 5-fold reduction of receptor binding affinity, suggesting that an internal hydrogen bond contributes to the receptor binding affinity.

Two likely mechanisms by which the loss of this hydrogen bond could negatively affect receptor binding were evaluated. Although destabilization of the protein structure of a bFGF mutein which lacks a buried hydrogen bond between Glu-96 and Tyr-106 might be expected, the behavior of the correspond-

ing Y106F mutein, in which this hydrogen bond is also absent, does not support this possibility. Y106F mutein is a stable molecule that can be overexpressed at 37 °C and like wild type bFGF, binds tightly to a heparin-Sepharose column at 22 °C. Moreover, Y106F has only a 5-fold reduction in receptor binding, indicating that the dramatic loss of receptor binding activity of E96A is due principally to the loss of the carboxylate group of Glu-96, rather than to the loss of the buried hydrogen bond.

It is more probable that the Y106F mutein possesses a higher entropic state in comparison with wild type bFGF, owing to the gain in the freedom of the rotation of the carboxylate group of Glu-96 in the absence of hydrogen bond formation with the phenolic hydroxyl group of Tyr-106. As has been pointed out (Somers *et al.*, 1994; Kossiakoff *et al.*, 1994), the entropic states of the unbound partners are of importance in other hormone-receptor protein-protein interactions. In the present instance, by contrast to the Y106F mutein, the lower entropic state associated with restricted rotation due to hydrogen bonding is already present in unbound wild type bFGF, and a correspondingly smaller entropy loss, with consequent greater affinity, is therefore resulting from receptor binding.

Recently, Ornitz *et al.* (1995) described the crystal structure studies of the complex between bFGF and a synthetic trisaccharide. On the basis of their results, these workers suggested that a pair of trisaccharide binding sites derived from basic amino acid residues on bFGF (sites 2 and 2′) may be involved in the oligomerization of bFGF. In consideration of the experimentally determined crystal structure of the complex, site 2 is formed by Glu-96, Arg-97, and Arg-60, whereas site 2′ comprises Arg-72, Lys-77, Arg-81, and Lys-86. According to these authors, site 2′ may have a higher affinity for the trisaccharide than site 2, owing to shorter hydrogen bond lengths in site 2′ relative to site 2. Since this crystal structure differs considerably from the arrangement postulated by Pantoliano *et al.* (1994) for the bioactive complex between heparin and bFGF, it is pertinent to inquire which of the two our present results support.

We note first that the structures of the bFGF-oligosaccharide complexes obtained experimentally by Ornitz *et al.* (1995) may not reflect the structure of the putative bioactive complex. Moreover, we conclude for several reasons that the drastic decrease we observe in the binding of the E96A mutein to FGFR1 cannot be attributed to the impaired heparin binding, as would be required by the experimental structure of Ornitz *et al.* (1995). First, computer modeling[2] indicates that substitution of Glu-96 with lysine does not appear to interfere with the hydrogen bond formation between the conformationally mobile side chain of Lys and a negatively charged trisaccharide or heparin moiety. This is at variance with the nearly 300-fold reduction we found for the receptor binding of the E96K mutein compared with wild type bFGF (Table II), which cannot therefore readily be explained on the basis of the impaired heparin binding.

A similar situation exists in the case of Arg-97, which forms a hydrogen bond with the trisaccharide in the Ornitz structure. On the one hand, we found that replacement of Arg-97 with alanine, which is incapable of forming such a hydrogen bond, gives the mutein R97A, which binds tightly to the heparin-Sepharose and requires 25 mM Tris buffer containing 2 M NaCl for elution. On the other hand the mutein R97A has nearly undiminished affinity for the receptor (Table II). These data argue against the possibility that the Ornitz structure is representative of the bioactive complex.

[2] K. Ramnarayan, H. Y. Zhu, and M. E. Wolff, unpublished results.

Last, FGF receptor dimerization is thought to proceed by a similar molecular mechanism for both bFGF and aFGF. Alignment of amino acid sequences in aFGF and bFGF reveals that the residues comprising the heparin binding site 2′ on bFGF correspond to Gln-63, Asp-68, Leu-72, and Gln-77 on aFGF, none of which are either basic or conserved residues (Eriksson *et al.*, 1993). This argues against the possibility that these particular residues on aFGF interact with trisaccharide or heparin and likewise makes it difficult to reconcile the Ornitz model with the induction of FGF receptor dimerization by aFGF.

The site-directed mutagenesis studies in this paper are consistent with the current view that region 106–115 is not the high affinity receptor binding site (Springer *et al.*, 1994). In addition, we have identified Glu-96 as a newly discovered crucial residue for binding to FGFR1β by a electrostatic interaction that is not predicted by the previous model (Pantoliano *et al.*, 1995). If we include Glu-96 as a extended high affinity site, the surface area becomes 520 Å^2. Since Glu-96 is situated approximately between high and low affinity sites, this may indicate that other residues between Glu-96 and the high affinity site are involved in the high affinity binding. Currently, we are examining residues in the neighbors of Glu-96.

Our results presented here do not support the possibility that the crystal complex between bFGF and trisaccharides examined by Ornitz is representative of a bioactive complex. These studies will aid in the understanding of the molecular interactions between ligand and receptor that is critical for the structure-based design of small molecule antagonists.

Acknowledgments—We thank Dr. Wallace L. McKeehan for the scDNA of FGFR1β. We are indebted to Drs. Edward T. Maggio, Shiniji Yoshitake, Isao Saito, Takanori Kawamura, Takatoshi Kawai, Seymour Mong, Ming Fai Chan, and V. Y. Yalamoori for helpful discussions.

REFERENCES

Abola, E. E., Bernstein, F. C., Bryant, S. H., Koetzle, T. F., and Weng, J. (1987) in *Crystallographic Databases-Information Content, Software Systems, Scientific Applications* (Allen, F. H., Bergerhoff, G., and Sievers, R., eds) pp. 107–132, Data Commission of the International Union of Crystallography, Cambridge, United Kingdom
Abraham, J. A., Whang, J. L., Tumolo, A., Mergia, A., Friedman, J., Gospodarowicz, D., and Fiddes, J. C. (1986) *EMBO J.* **5,** 2523–2528
Ago, H., Kitagawa, Y., Fujishima, A., Matsuura, Y., and Katsube, Y. (1991) *J. Biochem.* (*Tokyo*) **110,** 360–363
Baird, A., Schubert, D., Ling, N., and Guillemin, R. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 2324–2328
Burgess, W. H., and Maciag, T. (1989) *Annu. Rev. Biochem.* **58,** 575–606
Chothia, C. (1974) *Nature* **248,** 338–339
Delli-Bovi, P., Curatola, A. M., Kern, F. G., Greco, A., Ittmann, M., and Basilico, C. (1987) *Cell* **50,** 729–737
Dickson, C., and Peters, G. (1987) *Nature* **326,** 833
Dionne, C. A., Crumley, G., Bellot, F., Kaplow, J. M., Searfoss, G., Ruta, M., Burgess, W. H., Jaye, M., and Schlessinger, J. (1990) *EMBO J.* **9,** 2685–2692
Eriksson, A. E., Cousens, L. S., Weaver, L. H., and Matthews, B. W. (1991) *Proc. Natl Acad. Sci. U. S. A.* **88,** 3441–3445
Eriksson, A. E., Cousens, L. S., and Matthews, B. (1993) *Protein Sci.* **2,** 1274–1284
Finch, P. W., Rubin, J. S., Miki, T., Ron, D., and Aaronson, S. A. (1989) *Science* **245,** 752–755
Folkman, J. (1985) *Adv. Cancer Res.* **43,** 175–203
Folkman, J., and Klagsbrun, M. (1987) *Science* **235,** 442–447
Gospodarowicz, D. (1974) *Nature* **249,** 123–127
Gospodarowicz, D., and Cheng, J. (1986) *J. Cell. Physiol.* **128,** 475–484
Gospodarowicz, D., Ferrara, N., Schweigerer, L., and Neufeld, G. (1987) *Endocrinology* **46,** 187–204
Graves, B. J., Hatada, M. H., Hendrickson, W. A., Miller, J. K., Madison, V. S., and Satow, Y. (1990) *Biochemistry* **29,** 2679–2684
Habazettl, J., Gondol, D., Wiltscheck, R., Otlewski, J., Schleicher, M., and Holak, T. A. (1992) *Nature* **359,** 855–858
Jaye, M., Howk, R., Burgess, W., Ricca, G. A., Chiu, I.-M., Ravera, N. W., O'Brien, S. J., Modi, W. S., Maciag, T., and Drohan, W. N. (1986) *Science* **233,** 541–544
Koetzle, T. F., Williams, G. J. B., Meyer, E. F., Brice, M. D., Rodgers, J. R., Kennard, O., and Shimanouchi, Tasumi, M. (1977) *J. Mol. Biol.* **112,** 535–542
Kossiakoff, A. A., Somers, W., Ultsch, M., Andow, K., Muller, Y. A., and DeVos, A. M. (1994) *Protein Sci.* **3,** 1697–1705
Li, L., Safran, M., Aviezer, D., Böhlen, P., Seddon, A. P., and Yayon, A. (1994) *Biochemistry* **33,** 10999–11007
Marics, I., Adelaide, J., Raybaud, F., Mattei, M. G., Coulier, F., Planche, J., de Lapeyriere, O., and Birnbaum, D. (1989) *Oncogene* **4,** 335–340
Ornitz, D. M., Herr, A. B., Nilsson, M., Westman, J., Svahn, C., and Waksman, G. (1995) *Science* **268,** 432–436

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
Ramnarayan, K., Rao, B. G., and Singh, U. C. (1990) *J. Chem. Phys.* **92,** 7057–7067
Roghani, M., Mansukhani, A., Dell'Era, P., Bellosta, P., Basilico, C., Rafkin, D. B., and Moscatelli, D. (1994) *J. Biol. Chem.* **269,** 3976–3984
Ryckert, J. P., Ciccotti, G., and Berendsen, H. J. C. (1977) *J. Comput. Phys.* **23,** 327–334
Saksela, O., Moscatelli, D., Sommer, A., and Rifkin, D. B. (1988) *J. Cell Biol.* **107,** 743–751
Seno, M., Sasada, R., Kurokawa, T., and Igarashi, K. (1990) *Eur. J. Biochem.* **188,** 239–245
Shi, E., Kan, M., Xu, J., Wang, F., Hou, J., and McKeehan, W. L. (1993) *Mol. Cell. Biol.* **13,** 3907–3918
Shing, Y., Folkman, J., Sullivan, R., Butterfield, C., Murray, J., and Klagsbrun, M. (1984) *Science* **223,** 1296–1299
Singh, U. C., Weiner, P. K., Caldwell, J. W., and Kollman, P. A. (1986) *AMBER 3.0*, University of California, San Francisco
Sivalingam, A., Kenney, J., Brown, G. C., Benson, W. E., and Donoso, L. (1990) *Arch. Ophthalmol.* **108,** 869–872
Somers, W., Ultsch, M., DeVos, A. M., and Kossiakoff, A. A. (1994) *Nature* **372,** 478–481
Sommer, A., and Rifkin, D. B. (1989) *J. Cell. Physiol.* **138,** 215–220
Spivak-Kroizman, T., Lemmon, M. A., Dikic, I., Ladbury, J. E., Pinchasi, D., Huang, J., Jaye, M., Crumley, G., Schlessinger, J., and Lax, I. (1994) *Cell* **79,** 1015–1024
Springer, B. A., Pantoliano, M. W., Barberal, F. A., Gunyuzlu, P. L., Thompson, L. D., Herblin, W. F., Rosenfeld, S. A., and Book, G. W. (1994) *J. Biol. Chem.* **269,** 26879–26884
Thompson, L. D., Pantoliano, M. W., and Springer, B. A. (1994) *Biochemistry* **33,** 3831–3840
Yayon, A., Klagsbrun, M., Esko, J. D., Leder, P., and Ornitz, D. M. (1991) *Cell* **64,** 841–848
Yoshida, T., Miygawa, K., Odagiri, H., Sakamoto, H., Little, P. F. R., Terada, M., and Sugimura, T. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 7305–7309
Zhan, X., Bates, B., Hu, X., and Goldfarb, M. (1988) *Mol. Cell. Biol.* **8,** 3487–3495
Zhang, J., Cousens, L. S., Barr, P. J., and Sprang, S. R. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 3446–3450
Zhu, X., Komiya, H., Chirino, A., Faham, S., Fox, G. M., Arakawa, T., Hsu, B. T., and Rees, D. C. (1991) *Science* **251,** 90–93
Zhu, X., Hsu, B. T., and Rees, D. C. (1993) *Structure* **1,** 27–34