Identification of Residues Important Both for Primary Receptor Binding and Specificity in Fibroblast Growth Factor-7*

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Fibroblast growth factors (FGFs) mediate a multitude of physiological and pathological processes by activating a family of tyrosine kinase receptors (FGFRs). Each FGFR binds to a unique subset of FGFs and ligand binding specificity is essential in regulating FGF activity. FGF-7 recognizes one FGFR isomorph known as the FGFR2 IIB isoform or keratinocyte growth factor receptor (KGFR), whereas FGF-2 binds well to FGFR1, FGFR2, and FGFR4 but interacts poorly with KGFR. Previously, mutations in FGF-2 identified a set of residues that are important for high affinity receptor binding, known as the primary receptor-binding site. FGF-7 contains this primary site as well as a region that restricts interaction with FGFR1. The sequences that confer on FGF-7 its specific binding to KGFR have not been identified. By utilizing domain swapping and site-directed mutagenesis we have found that the loop connecting the β4-β5 strands of FGF-7 contributes to high affinity receptor binding and is critical for KGFR recognition. Replacement of this loop with the homologous loop from FGF-2 dramatically reduced both the affinity of FGF-7 for KGFR and its biological potency but did not result in the ability to bind FGFR1. Point mutations in residues comprising this loop of FGF-7 reduced both binding affinity and biological potency. The reciprocal loop replacement mutant (FGF2-L4/7) retained FGF-2 like affinity for FGFR1 and for KGFR. Our results show that topologically similar regions in these two FGFs have different roles in regulating receptor binding specificity and suggest that specificity may require the concerted action of distinct regions of an FGF.

Fibroblast growth factors (FGFs)† constitute a family of at least 19 structurally related polypeptides that play key roles during development and morphogenesis (1), as well as several physiological and pathological situations such as wound repair, neovascularization, and tumor growth and metastasis (2, 3). The biological activities of FGFs are mediated by cell surface high affinity receptors that belong to the tyrosine kinase receptor family and cellular responses to FGFs are modulated by heparan sulfate proteoglycans that are also known as low affinity receptors for FGFs (4–7). Four high affinity receptors (FGFR1-FGFR4) that share common structural features have been identified (4, 5). The prototype FGFR is composed of an extracellular ligand-binding domain that contains three immunoglobulin-like domains (D1-D3), a transmembrane domain, and a cytoplasmic domain that bears the tyrosine kinase activity (4, 5).

Receptor binding specificity is an essential element in regulating the diverse activities of FGFs. Each of the four FGFRs binds a subset of FGFs with varying affinities, and an additional level of complexity is created via an alternative splicing mechanism that generates FGFR1-FGFR3 isoforms with altered ligand binding properties (4, 5). This phenomenon is best exemplified for the FGFR2IIB isoform, also known as the keratinocyte growth factor receptor (KGFR). This receptor is a spliced variant of FGFR2. While FGFR2 binds FGF-1 and FGF-2 with high affinity and does not interact with the keratinocyte growth factor (FGF-7 or KGF), the KGFR binds FGF-1 and FGF-7 with high affinity and FGF-2 with a 20-fold lower affinity (8, 9).

FGF-7 is unique among its family members since this growth factor has a distinctive target cell specificity. Whereas the other FGFs have broader cell type specificity, FGF-7 is secreted by cells of mesenchymal origin and acts predominantly on cells of epithelial origin (10). The unique target cell specificity of FGF-7 is dictated by virtue of the fact that unlike the other FGFs, which can interact with several FGFRs, FGF-7 can only bind to the KGFR isoform that is specifically expressed in epithelial cells (11). Because of its unique receptor binding characteristics, FGF-7 is ideal for studying how the specificity of FGF-FGFR interaction is conferred at the structural level.

The three-dimensional structures of three FGFs, FGF-1, FGF-2, and FGF-7 have been resolved (12, 13). Although there is only about 50% amino acid sequence identity between these three growth factors, their three-dimensional structures share a common structural fold, the β-trefoil scaffold, and the three structures are nearly superimposable (12, 13). However, the three FGFs display distinct receptor binding characteristics suggesting that sequence differences between FGFs regulate receptor binding specificity. Earlier mutagenesis studies by Springer et al. (14) identified two receptor-binding sites in FGF-2 that map onto diametrically opposite sides of the molecule. Based on their relative affinities for FGFR1, these sites were named high and low affinity sites. The high affinity site is comprised of a set of discontinuous residues that are clustered in a hydrophobic patch on the FGF-2 surface. These residues are highly conserved among FGFs and therefore unlikely to contribute to ligand binding specificity. The low affinity site resides within a loop that connects the ninth and tenth β-strands of FGF-2 (14). Although the loop structure and its
primary sequence vary among FGFs, β3-β10 loop exchange between FGF-2 and FGF-7 did not alter their known receptor binding specificities (15). Other investigators have proposed that a segment in the carboxyl-terminal part of FGF-7 prevents this growth factor from binding to FGFR1. Replacement of this segment with the corresponding one in FGF-1 (termed the glycine box) conferred on FGF-7 the ability to bind FGFR1 (16). However, acquisition of FGFR1 binding did not result in a concomitant loss of high affinity binding of FGF-7 to the KGFR which suggests that binding specificity is determined by more than one region of an FGF. The present work was aimed at identifying such determinants. Based on both loop exchange between FGF-2 and FGF-7 and site-directed mutagenesis in FGF-7, we found that the loop connecting the β4-β5 strands of FGF-7 participates in primary receptor binding and is critical for FGF-7/KGFR recognition. The results are discussed in light of the recently resolved crystal structure of FGF bound to FGFR (17, 18).

EXPERIMENTAL PROCEDURES

Materials—Recombinant FGF-2 and FGF-7 were produced in bacteria as described previously (15, 19, 20). NcoI (New England Biolabs) was a PerkinElmer Life Sciences product. Heparin-Sepharose CL-6B was from Amersham Pharmacia Biotech. Ni-NTA-agarose was purchased from Qiagen. Bovine serum albumin was from Roche Molecular Biochemicals. Escherichia coli BL21(DE3) cells harbor the thioredoxin (Trx) expression vector were kindly provided by S. Ishii (Tsukuba Life Science Center, The Institute of Physical and Chemical Research, Tsukuba City, Japan). The mutant FGF2-L4/7, in which the glycine box (underlined) and the sequence encoding the loop of FGF-7 (underlined) and flanking sequences from the FGF-2 gene were introduced, was used for this purpose (23).

Construction of FGF-2/FGF-7 Chimeras and Generation of Point Mutations in FGF-7—The mutant FGF-2/L4/7, in which the β4-β5 loop of FGF-2 was replaced with that of FGF-7, was created in two steps. First, two chimeric fragments were generated utilizing the polymerase chain reaction (24). Primers p1 and p2 were used to amplify the segment encoding FGF-2 residues 1–74, in which the codons encoding residues Gln65–Arg69 were replaced with the sequence encoding for the E. coli ribosome binding site (25). Then the amplified products were purified and ligated to the expression vector pKM260. For the replacement of the β3–β4 loop of FGF-7 with that of FGF-2, the FGF-7 plasmid was subjected to NcoI and SspI digestion, to remove the sequence encoding the loop. The digested plasmid was ligated to a double stranded DNA obtained by annealing two synthetic oligonucleotides, TC6CAGGAAGAGACCCACACAAAT and ATGGTGCGGCTGCGGCTTTAGCAGACATC (the sequence encoding the loop of FGF-2 is underlined). Similarly, the β4–β5 loop of FGF-7 was replaced with the corresponding loop of FGF-2 following a MscI/SnaBl digestion of the FGF-7 plasmid and utilizing the following annealed synthetic oligonucleotides: AACAGCAGAAGAGAAATTTGCGG and CCACAATTCCCTCCTCCTCTC (the sequence encoding the loop of FGF-2 is underlined). The same methodology was utilized to create the point mutants (except for the following oligonucleotide sets: T102A, GTGCAGTGGCAATGGAATTGTTG and CCACATTCCAAC-CACTCAGCAG (the sequence encoding the loop). The mutant FGF2-L4/7, in which the glycine box (underlined) and the sequence encoding the loop of FGF-7 (underlined) is complementary to the NH2-terminal part of the FGF-7 gene product, and contains artificial NcoI site 5′ to the start codon, and artificial NdeI (underlined) and SspI sites 3′ to the stop codon that contains the point mutation (underlined). A second mutated fragment, encoding for FGF-7 residues 97–194 was created using primers p3 (ATATTGGAGATACGTTGCAATGGAATTGTTG) and p4 (TTGGATCC- GTGGAAATTGTTG and CCACAAATTCCAACCTCACTGCGC) that is complementary to the COOH-terminal part of the FGF-7 gene product. Then the amplified products of the first step were annealed and amplified with primers p1 and p4. The fragment obtained was cut with NcoI and BstRI and cloned into the FGF-7 plasmid that was digested with the same enzymes. All the mutated genes were sequenced to confirm that the desired mutation had been introduced and that additional mutations had not been created during the amplification process.

Production and Purification of the Recombinant Proteins, Analysis of Secondary Structures, and Thermal Stability—All the FGFs used in this study were expressed as His-tagged products in E. coli as previously reported (15). FGF-7 and all the FGF-7 mutants were expressed in BL21(DE3) plys S cells (25). FGF-2 and the FGF2-L4/7 mutant were co-expressed with E. coli thioredoxin (Trx) in BL21(DE3) cells (15, 26). This enzyme increases the solubility of eukaryotic proteins in E. coli, thus increasing the protein yield. Purification of the recombinant proteins was carried out by Ni2+-nitrilotriacetic acid affinity chromatography followed by heparin-Sepharose affinity chromatography as described previously (15). All mutant proteins eluted from the column at the same salt concentration as the parental molecules (0.5 M NaCl for the FGF-7 mutants, 1.5 M NaCl for the FGF-2 mutants).

Circular dichroism (CD) spectra was employed for analyzing differences in secondary structure and in thermal stability of mutant proteins that displayed lower receptor binding affinity or biological potency compared with wild type proteins (27) (see Tables I and II). Thermal stability was also determined by incubating wild type and the mutant proteins at 37 °C for 16 h and testing the effect of incubation on receptor binding affinities compared with fresh proteins as described previously (15).

Growth Factor Iodination, Receptor Binding, Mitogenic Assays, and Protein Tyrosine Phosphorylation—Radiiodination of FGF-2 and FGF-7 and separation of the radiolabeled growth factors from free Na125I was performed as described previously (21). The specific activities of radiiodinated growth factors were in the range of 1–2 x 105 cpm/ng. Cell surface receptor binding competition assays were performed using subconfluent cultures in 24-well microtiter plates as described (19, 21).

In vitro kinase assays were performed using 96-well enzyme-linked immunosorbent assay plates coated with a soluble extracellular domain of the mouse KGFR fused to secreted placentan alkaline phosphatase (KR/AP) essentially as described (15, 28).

DNA synthesis was measured by an [3H]thymidine incorporation assay using serum-starved confluent cultures of NIH/3T3 or Balb/Mk cells and protein tyrosine phosphorylation experiments were performed utilizing serum starved subconfluent cultures of NIH/3T3 (15, 19).
FGF-2 (amino acids Gln 65-Ala-Glu-Glu-Arg69) resulted in a dramatic reduction in the binding affinity to KGFR. Half-maximal competition for $^{125}$I-FGF-7 binding was observed at about 20 and 2500 ng/ml FGF-7 and the FGF7-L4/2 mutant, respectively (Fig. 2A). Similar results were obtained using a cell-free binding assay with a soluble extracellular domain of KGFR (data not shown). The reduction in receptor binding affinity of the mutant was not due to an apparent change in the protein secondary structure as the CD spectra of FGF-7 and the mutant protein were identical and, in addition, the mutant displayed wild-type affinity for heparin (Table I, and data not shown).

We next examined the mitogenic potency and ability to induce tyrosine phosphorylation of cellular substrates by the FGF7-L4/2 mutant. As shown in Fig. 2C, the reduction in receptor binding affinity was accompanied with a significant reduction in mitogenic potency (ED$_{50}$ of 800 and 1 ng/ml for mutant and FGF-7, respectively). In addition, the maximal mitogenic response that was induced by the mutant was about 2-fold lower than that induced by FGF-7. In agreement with these results, the mutant did not induce tyrosine phosphorylation of cellular substrates such as MAP kinase or FRS2 (29, 31) at a concentration (100 ng/ml) where it failed to stimulate a mitogenic response (Fig. 2D).

Interestingly, the replacement of the loop did not confer on FGF-7 the ability to bind or activate FGFR1. The mutant failed to compete with FGF-2 for binding to the L6/R1 cells (Fig. 2B) and was not mitogenic to NIH/3T3 even at concentrations as high as 1 µg/ml (data not shown).

The effect of the reciprocal loop replacement on the biological properties of FGF-2 is shown in Fig. 3. The mutant protein (designated FGF2-L4/7) retained wild type receptor binding affinity for both FGFR1 and KGFR (panels A and B). In fibroblasts the mitogenic activity of this mutant was identical to that of FGF-2 (Fig. 3C). By contrast, in keratinocytes the mutant induced a 2–3-fold higher level of DNA synthesis than FGF-2 (Fig. 3D). Thus, loop exchange affected KGFR-mediated responses, but had no effect on binding affinity or ability to activate FGFR1.

Site-directed Mutagenesis in the β4-β5 Loop of FGF-7—Of the five residues comprising the β4-β5 loop of FGF-7 (Gln$^{65}$-Ala-Glu-Glu-Arg$^{69}$), three are potentially charged, one is polar and one is hydrophobic. This is quite different than the situation in the corresponding loop of FGF-7 where four of the five residues are hydrophobic (Arg$^{101}$-Thr-Val-Ala-Val$^{105}$). The dramatic reduction in receptor binding affinity of the FGF7-L4/2 mutant may have resulted from the change in the residues comprising the loop or from a strong charge conflict imposed by the predominantly charged residues of FGF-2. To examine these two possibilities, we substituted four of the five residues in the loop of FGF-7 with the corresponding residues of FGF-2 (Thr$^{102}$ to Ala, Val$^{103}$ to Glu, Ala$^{104}$ to Glu, and Val$^{105}$ to Arg). In addition, we substituted Arg$^{101}$, Val$^{103}$, and Val$^{105}$ to alanine. The mutations did not significantly affect secondary structure or thermal stability of the mutant proteins (see Tables I and II).
Fig. 2. The biological properties of the FGF7-L4/2 mutant. Panels A and B, 125I-FGF-7 was bound to L6/KR cells (panel A) and 125I-FGF-3 was bound to L6/R1 cells (panel B). Binding was competed by increasing concentrations of the indicated wild type or mutant growth factors. The assay was performed as described under “Experimental Procedures.” Panel C, the mitogenic activity of the FGF7-L4/2 mutant was determined in Balb/MK cells as described in the legend to Fig. 1. Maximal counts/min were 58,000 for FGF-7. Panel D, serum-starved subconfluent cultures of NIH/KR cells were stimulated for 10 min with serum-free medium alone (Lane 1) or the same medium containing 100 ng/ml FGF7-L4/2 (Lane 2) or 10 ng/ml FGF-7 (Lane 3). Equal amounts (150 µg) of clarified cell lysate were separated on 7% SDS-polyacrylamide gel electrophoresis, and immunoblotted with anti-phosphotyrosine antibody, as described previously (29). The position of molecular weight markers is indicated on the left and that of FRS2 (p90) and MAPK (p42) on the right.

| Protein       | Two-dimensional analysis from 185 to 250 nm |
|---------------|--------------------------------------------|
|               | α-Helix | β-Sheet | β-Turn | Random coil |
| FGF-7         | 1       | 62      | 28     | 9           |
| FGF7-L4/2     | 3       | 59      | 32     | 7           |
| R101A         | 3       | 62      | 27     | 8           |
| T102A         | 0       | 57      | 34     | 9           |
| V103A         | 1       | 61      | 28     | 9           |
| V105R         | 0       | 66      | 31     | 3           |
| V105A         | 2       | 64      | 28     | 7           |

The CD spectra were analyzed using the CONTIN program to estimate the proteins secondary structures from circular dichroism (41).

DISCUSSION

FGFs share a high degree of amino acid sequence similarity and the structures of three FGFs, resolved so far, are nearly superimposable (12, 13). Despite this structural similarity, FGFs display distinct receptor binding characteristics. In the present study we utilized domain swapping and site-directed mutagenesis to identify domains and residues that participate in determining the receptor binding specificity of FGF-2 and FGF-7. FGF-7 interacts only with the KGFR whereas FGF-2 binds with high affinity to FGFR1, FGFR2, and FGFR4 and with lower affinity to KGFR (21, 22, 32).

Previous studies suggested that a region in FGF-7 comprised of residues 91–110 may be important for receptor recognition (19). This region spans the carboxyl-terminal half of the loop connecting the β3 and β4 strands, the β4 strand, and the β4-β5 loop. In the present study it was shown that exchanging the β4-β5 loop of FGF-7 with the corresponding loop from FGF-2 dramatically reduced both the affinity of FGF-7 to its receptor and its biological potency. In contrast, the exchange of the β3-β4 loop of FGF-7 with the homologous loop from FGF-2 was without effect. Neither of the loop exchange mutants of FGF-7 acquired the ability to bind FGFR1. Single amino acid substitutions in residues comprising the β4-β5 loop in FGF-7 lowered receptor binding affinity and mitogenic activity. Together, these findings establish the importance of the β4-β5 loop of FGF-7 for its high affinity receptor binding and specific recognition of KGFR. The finding that reciprocal loop replacement did not confer on FGF-7 the ability to bind FGFR1 is expected in view of the reported observation of Luo et al. (16). These

The affinity of the mutants for KGFR was examined in both cell-free binding competition experiments which utilize a soluble form of the extracellular domain of KGFR fused to a secreted human placental alkaline phosphatase (designated KR/ AP) and in cells expressing KGFR. Similar results were obtained in both assays. The mitogenic activity of the mutants was examined in Balb/MK cells. The results of both assay types are shown in Table II. With the exception of mutant A104E, that retained wild type receptor binding affinity and mitogenic potency, all the other mutations affected both properties. Substitution of Arg101 or Thr102 with alanine reduced receptor binding affinity and mitogenic potency to a similar extent (about a 5-fold reduction), whereas mutations in the valine position are important. This may explain why substitution with arginine had a less deleterious effect than the V103A mutant. Substitution of Arg101 or Thr102 with alanine reduced receptor binding affinity and mitogenic potency. Interestingly, the effect of substituting Val103 with the corresponding residue of FGF-2 was more pronounced: this mutant displayed about a 2.5-fold reduction in binding affinity and 22-fold compared with a 6-fold reduction in mitogenic potency. These results suggest that four of the five residues in the β4-β5 loop of FGF-7 contribute to receptor recognition and that the presence of valine residues may be essential for FGF-7/KGFR recognition. The findings that substitution with another hydrophobic residue affected receptor binding and biological potency of the valine 103 and valine 105 mutants suggest that the side chain length and position are important. This may explain why substitution with arginine had a less deleterious effect than the V103A mutant.

| Protein       | Reduction in binding affinity | Reduction in mitogenic potency |
|---------------|-------------------------------|-------------------------------|
| FGFR1         | 35%                           | 50%                           |
| FGFR2         | 40%                           | 60%                           |
| FGFR3         | 50%                           | 75%                           |

The position of molecular weight markers is indicated on the left and that of FRS2 (p90) and MAPK (p42) on the right.
FIG. 3. The biological properties of the FGF2-L4/7 mutant. Panels A and B, binding competition assays were performed on L6/R1 cells (panel A) or L6/KR cells (panel B). Panels C and D, the mitogenic activity of the indicated ligands was determined in NIH/3T3 cells (panel C) and Balb/MK cells (panel D), as described in the legend to Fig. 1.

| Table II | Receptor binding and mitogenic activity for FGF-7 mutants |
|----------|----------------------------------------------------------|
| FGF-7 mutant | KGFR binding<sup>a</sup> (IC<sub>50</sub>mutant/IC<sub>50</sub>FGF-7) | Mitogenic activity<sup>b</sup> (ED<sub>50</sub>mutant/ED<sub>50</sub>FGF-7) | Thermal transition point<sup>c</sup> °C |
| R101A | 5 ± 0.53 | 5 ± 1.4 | 54 |
| T102A | 4 ± 0.42 | 4.5 ± 0.78 | 53 |
| V105E | 2.5 ± 0.16 | 17 ± 0.1 | 54 |
| V105A | 2 ± 0.28 | 6 ± 0.74 | ND<sup>d</sup> |
| A104E | 0.95 ± 0.07 | 0.9 ± 0.09 | 52 |
| V105R | 2 ± 0.54 | 5.5 ± 0.80 | 58 |
| V105A | 2 ± 0.87 | 22 ± 3.93 | 54 |

<sup>a</sup> IC<sub>50</sub> value was calculated from the competitive binding of <sup>125</sup>I-labeled FGF-7 versus unlabeled wild type FGF-7 or FGF-7 mutants to soluble KR/AP fusion protein (an average of at least three experiments for each mutant). The IC<sub>50</sub> value for FGF-7 determined from a large number of independent experiments was 15 ng/ml.

<sup>b</sup> ED<sub>50</sub> values were calculated from at least three separate mitogenic assays on Balb/MK cells, as described in the legends to Fig. 1. ED<sub>50</sub> value for FGF-7 was 1.2 ng/ml.

<sup>c</sup> Apparent T<sub>m</sub>. The midpoint temperature in the thermal transition curve obtained by following changes in CD signal at 205 nm. T<sub>m</sub> for FGF-7 was 58 °C.

<sup>d</sup> ND, not determined.

authors showed that a stretch of amino acid residues within the carboxy-terminal part of FGF-7 restricts interaction with FGFR1. Therefore, exchanging its β4-β5 loop with that of FGF-2 would not be expected to alter affinity for FGFR1 because the inhibitory sequence remains.

The crystal structures of FGF-2 bound to FGFR1 and FGF-1 bound to FGFR2 were recently resolved and both support a role for the β4-β5 loop in receptor binding and specificity (17, 18). These structures suggest that the β4-β5 loop in each ligand make contacts with the carboxy-terminal half of Ig-III domain (D3) in each receptor. This region in FGFRs is clearly important for ligand binding and shows specificity toward the different FGF members (9, 33–36).

Unlike the significant effect of loop replacement on the efficiency of recognition between FGF-7 and KGFR, the reciprocal replacement (the β4-β5 loop of FGF-7 in FGF-2) had no effect on the binding of FGF-2 to FGFR1 or its biological potency in cells expressing this receptor. In addition, this exchange did not increase the binding affinity of FGF-2 for KGFR but resulted in a higher maximal level of mitogenic activity in keratinocytes compared with FGF-2. These results are in agreement with previous data obtained from a chimera containing residues 1–54 of FGF-2 and residues 91–194 of FGF-7, which contains the β4-β5 loop (19). Mutations that increase biological potency without effect on receptor binding affinity were reported for EGF and IL-1 (37, 38). These studies revealed that biological potency is determined not only by receptor binding affinity but also by post-receptor binding events including receptor internalization, degradation, or recycling. It remains to be determined whether the increased biological potency of the FGF-2 containing the FGF-7 loop is due to similar reasons.

Examination of the contacts in the crystal structure between the β4-β5 loop of FGF-2 and the D3 domain of FGFR1 may help explain why loop exchange in FGF-2 did not alter the affinity for FGFR1. In the crystal structure, many of the contacts are between backbone atoms and many of the side chains are exposed to the solvent (17). In addition, the region of the receptor that contacts the loop is quite hydrophobic. Theoretical modeling studies, in which the FGF-2 loop replacement mutant was examined within the context of the crystal structure, showed that the side chains in the FGF-7 loop would jut out into the solvent and not interfere with binding contacts (data not shown). The hydrophobic nature of the contact region in the receptor could also stabilize its interaction with the loop of FGF-7, which also contains predominantly hydrophobic residues.

While the β4-β5 loop of FGF-7 can fully substitute for that of FGF-2 for interaction with FGFR1, the reciprocal loop replacement dramatically reduced the affinity of FGF-7 for KGFR. In fact, the affinity of the FGF-7 with the FGF-2 loop for KGFR was significantly lower compared with that of native FGF-2. This suggests that the nature of FGF-7 loop/KGFR interaction...
is quite different than that of FGF-2/FGFR1 or FGF2/KGFR. From our mutational analysis, it seems that both charge and length of side chain are critical for loop interaction with KGFR.

In analogy with the situation reported for FGF-7 (16), FGF-2 may contain inhibitory sequences that do not allow an enhancement of affinity for KGFR following loop exchange. Another possibility is that the β4-β5 loop of FGF-2 does not normally interact with KGFR and this can explain why loop exchange in FGF-2 did not enhance affinity for KGFR. Interestingly, point mutations in the β4-β5 loop of FGF-7 (T102A and V103E) that reduced its binding to KGFR are naturally present in the same position in the β4-β5 loop of FGF-1. Since FGF-1 binds KGFR quite efficiently, we assume that the presence of alanine and glutamic acid in FGF-1 does not interfere with receptor binding because the loop of FGF-1 also does not interact with KGFR. Probably, other regions in FGF-1 interact with KGFR to compensate for this lack of loop binding. The effect of point mutations in KGFR on ligand binding also supports the idea that the loop interaction may exist in other FGF-FGFR complexes. After this manuscript was submitted, the crystal structure of FGF1/FGFR2 and FGF-2/FGFR1 (17, 18). The diversity in amino acid composition of the β4-β5 loop throughout the FGF family suggests that a lack of loop/receptor interaction may exist in other FGF-FGFR complexes. After this manuscript was submitted, the crystal structure of FGF1/FGFR1 was published and it was shown that in this complex the β4-β5 loop of FGF-1 does not engage in binding to FGFR1 (39).

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