Mutations Uncouple Human Fibroblast Growth Factor (FGF)-7 Biological Activity and Receptor Binding and Support Broad Specificity in the Secondary Receptor Binding Site of FGFs*

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The fibroblast growth factor (FGF) family plays a key role in a multitude of physiological and pathological processes. The activities of FGFs are mediated by a family of tyrosine kinase receptors, designated FGFRs. The mechanism by which FGFs induce receptor activation is controversial. Despite their structural similarity, FGFs display distinct receptor binding characteristics and cell type specificity. Previous studies with FGF-2 identified a low affinity receptor binding site that is located within a loop connecting its 9th and 10th β-strands. The corresponding residues in the other family members are highly variable, and it was proposed that the variability might confer on FGFs unique receptor binding characteristics. We studied the role of this loop in FGF-7 by both site-directed mutagenesis and loop replacement. Unlike the other members of the FGF family, FGF-7 recognizes only one FGFR isoform and is, therefore, ideal for studies of how the specificity in the FGF-FGFR interaction is conferred at the structural level. Point mutations in the loop of FGF-7 did not change receptor binding affinity but resulted in reduced mitogenic potency and reduced ability to induce receptor-mediated phosphorylation events. These results suggest that the loop of FGF-7 fulfills the role of low affinity binding site required for receptor activation. The observation that it is possible to uncouple FGF-7 receptor binding and biological activity favors a bivalent model for FGFR dimerization, and it may be clinically relevant to the design of FGF-7 antagonists. Reciprocal loop replacement between FGF-7 and FGF-2 had no effect on their known receptor binding affinities nor did it alter their known specificity in eliciting a mitogenic response. In conclusion, these results suggest that, despite the diversity in the loop structure of FGF-2 and FGF-7, the loop has a similar function in both growth factors.

The keratinocyte growth factor (FGF-7) is a member of the heparin-binding fibroblast growth factor (FGF) family with a distinctive target cell specificity (1, 2). FGF-7 is secreted by cells of mesenchymal origin and acts predominantly on cells of epithelial origin (2). FGF-7 is thought to regulate a number of biological processes in embryonal development and in adults including cell growth, differentiation, cell migration, and repair of epithelial tissues (2, 3). It is also implicated in a number of pathological conditions such as prostate and breast cancer and inflammatory bowel disease (4, 5). The involvement of FGF-7 in a variety of physiological processes and in diseases provides a strong impetus for uncovering the structural basis of its activities. Furthermore, the knowledge of the structural and functional relationship in FGF-7 is key to rational design of analogues for potential therapeutic applications.

FGFs interact predominantly with two types of receptors: a family of tyrosine kinase receptors (FGFRs) that mediate the biological activities of FGFs, and heparan sulfate proteoglycans that act as modulatory co-receptors for FGFs (6, 7). Four closely related FGFRs (FGFR1–FGFR4) have been isolated, and an additional level of complexity was created via an alternative splicing mechanism that generates FGFR isoforms with altered ligand binding properties (6). Members of the FGF family bind FGFRs with varying affinities. For example, FGF-1 binds with high affinity to the four known receptors and all the isoforms that have been studied, whereas FGF-2 binds FGFR1, FGFR2, and FGFR4 with high affinity (8–12). FGF-7 interacts only with a splice variant of FGFR2 known as the KGFR or FGFR2IIIb form, whereas FGF-2 binds this receptor with about 10–20-fold lower affinity (13, 14).

Structure-based site-directed mutagenesis in FGF-2 identified two putative receptor-binding sites as follows: a primary site that contributes most of the high affinity interaction of FGF-2 to FGFR1, and a secondary site that exhibits low receptor binding affinity but is required for receptor activation (15). The secondary site is located in a surface-exposed loop connecting the 9th and 10th β-strands of FGF-2 (16). The primary site is highly conserved among FGFs suggesting that the dominant interaction of FGFs with FGFRs is likely to be similar if not identical. On the other hand, the secondary site is highly variable among FGFs both with respect to primary sequence and length of the loop. Because FGFs interact differently with their receptors, it was suggested that the secondary binding site may confer on FGFs unique receptor binding characteristics, tissue specificity, and unique biological activities (15, 17–19). Seddon et al. (17) reported that exchanging the loop of FGF-2 with that of FGF-1 confers on FGF-2 the ability to bind the KGFR with high affinity. In view of the reported effect of mutations in the loop of FGF-2, the finding of Seddon et al. (17) implies that the binding affinity contributed by the secondary site may be weak or strong depending on the FGF type. However, this question was not studied in other members of the FGF family.

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§The abbreviations used are: FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; AP, alkaline phosphatase; KGFR, keratinocyte growth factor receptor; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay.
FGF-7 is ideal for studies of how the specificity of the FGF-FGFR interaction is conferred at the structural level because it recognizes only one isoform of FGFRs and because of its unique target cell specificity (2, 6). Since FGF-7 binds the KGFR with a similar affinity as FGF-1, we investigated whether the loop of FGF-7 also participates in high affinity interaction and whether it contributes to the unique receptor binding characteristics of FGF-7. To address this question we utilized two mutational approaches. First, we reciprocally replaced the loop between FGF-2 and FGF-7, and we examined how the unique receptor binding characteristics of each growth factor are segregated in the chimeras. As mentioned earlier in this section, FGF-2 binds weakly to the KGFR, and it binds strongly to all other members of the FGFR family. Second, we generated point mutations in the loop of FGF-7 and examined the effect of the mutations on receptor binding affinity and biological activity. Mutations in the loop of FGF-7 separated high affinity receptor binding from biological activity, suggesting that the loop of FGF-7 does not contribute to strong receptor binding interactions but rather functions as low affinity site required for receptor activation and subsequent signaling. Loop replacement revealed that the loops of both FGF-7 and FGF-2 have similar function.

**EXPERIMENTAL PROCEDURES**

**Materials—**Recombinant FGF-2 and FGF-7 were produced in bacteria and purified as described previously (14, 20). Na<sup>125</sup>I (500 Ci/mmol) was from NEN Life Science Products. Heparin-Sepharose CL-6B was purchased from Amersham Pharmacia Biotech. Ni<sup>2+</sup>-nitrilotriacetic acid-agarose was purchased from Qiagen. Bovine serum albumin was from Roche Molecular Biochemicals. *Escherichia coli* BL21(DE3) cells harboring the thioredoxin expression vector were kindly provided by S. Ishii (Tsukuba Life Science Center, The Institute of Physical and Chemical Research (RIKEN), Japan). Fetal and newborn calf serum and media were purchased from Biological Industries (Beth-hehemek, Israel) or from Life Technologies, Inc. Rabbit anti-FGF-2 antibodies were from R & D Systems. Fibronectin and *a*-phosphotyrosine antibodies were from Upstate Biotechnology Inc. Heparin from bovine lung, monoclonal antibodies against human secreted placental alkaline phosphatase, and all other chemicals were purchased from Sigma.

**Transformation of E. coli Cells—**The generation of E. coli cell lines expressing high levels of the KGFR gene product (designated L6/KR cells) and FGRF1 (L6/R1 cells) was described elsewhere (11). These cell lines were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. L6/KR cells were grown in low calcium in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (L6/R1 cells) was described elsewhere (11). These cell lines were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. L6/KR cells were grown in low calcium in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (L6/R1 cells) was described elsewhere (11). These cell lines were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum.

**Fibroblast Growth Factor Iodination, Receptor Binding, and Mitogenic Assays—**Growth Factor iodination, receptor binding, and mitogenic assays were performed as described previously (14, 20). Na<sup>125</sup>I (500 Ci/mmol) was from NEN Life Science Products. Heparin-Sepharose CL-6B was purchased from Amersham Pharmacia Biotech. Ni<sup>2+</sup>-nitrilotriacetic acid-agarose was purchased from Qiagen. Bovine serum albumin was from Roche Molecular Biochemicals. *Escherichia coli* BL21(DE3) cells harboring the thioredoxin expression vector were kindly provided by S. Ishii (Tsukuba Life Science Center, The Institute of Physical and Chemical Research (RIKEN), Japan). Fetal and newborn calf serum and media were purchased from Biological Industries (Beth-hehemek, Israel) or from Life Technologies, Inc. Rabbit anti-FGF-2 antibodies were from R & D Systems. Fibronectin and *a*-phosphotyrosine antibodies were from Upstate Biotechnology Inc. Heparin from bovine lung, monoclonal antibodies against human secreted placental alkaline phosphatase, and all other chemicals were purchased from Sigma.

**Construction of FGF-2/FGF-7 Chimeras and Generation of Point Mutations—**Thioredoxin expression vector were kindly provided by S. Ishii (Tsukuba Life Science Center, The Institute of Physical and Chemical Research (RIKEN), Japan). Fetal and newborn calf serum and media were purchased from Biological Industries (Beth-hehemek, Israel) or from Life Technologies, Inc. Rabbit anti-FGF-2 antibodies were from R & D Systems. Fibronectin and *a*-phosphotyrosine antibodies were from Upstate Biotechnology Inc. Heparin from bovine lung, monoclonal antibodies against human secreted placental alkaline phosphatase, and all other chemicals were purchased from Sigma.

**RESULTS**

**Production and Purification of the Mutant Proteins—**The mutant proteins were produced and purified as described above. The FGF-2 mutant was expressed in BL21(DE3) cells (23). The FGF-7 mutants, with the exception of the FGF-7 mutant in which the loop was replaced with the analogous loop of FGF-2 (designated FGF7/II), were expressed in BL21(DE3) pLys S cells (23). The FGF7/II mutant unlike all the other mutants was accumulated in inclusion bodies. To obtain this mutant protein in a soluble form, we co-expressed it with *E. coli* thioredoxin. This enzyme increases the solubility of eukaryotic proteins in *E. coli* (24).

**Construction of FGF-2/FGF-7 Chimeras and Generation of Point Mutations in FGF-2 and FGF-7—**The polymerase chain reaction technique was utilized to generate the mutant proteins (25). Primers p1 (GCCATGCAACAAGAAATAACACACCTGTTGTTGTGTGCTTTAATCAAAAGGGG) contains the nucleotide sequence corresponding to the loop of FGF-2 (underlined) and flanking sequence from the FGF-7 gene containing a unique NsiI site (bold letters). Primer p2 (TCTGATCCATTAAAGTGATTGCATCAGGAGG) is complementary to the carboxyterminal 3′ end of the FGF-7 gene. Primer p3 (GCCATGCAACAAGAAATAACACACCTGTTGTTGTGTGCTTTAATCAAAAGGGG) contains the nucleotide sequence corresponding to the loop of FGF-2 (underlined) and flanking sequence from the FGF-7 gene containing a unique NsiI site (bold letters). To generate the point mutations in the loop of FGF-7, amplification was carried out using primer p2 in combination with each of the following primers: K155E, CATATGCACTGCTGAATGGCACACAAC; W156A, CATATGCACTGCTGAATGGCACACAAC; N159A, CATATGCACTGCTGAATGGCACACAAC; AT; N139, CATATGCACTGCTGAATGGCACACAAC; GGGGAAAATTG; E162A, CATATGCACTGCTGAATGGCACACAAC; GGGGAAAATTG; E162A, CATATGCACTGCTGAATGGCACACAAC; GGGGAAAATTG; E162A, CATATGCACTGCTGAATGGCACACAAC; GGGGAAAATTG. The amplified fragments were each cloned into the A/T cloning vector (26), and then plasmids containing the desired mutation were digested with NsiI/BamH1 for FGF-7 and AgeI/BamH1 for FGF-2 to liberate the mutated fragments. Each fragment was purified and ligated to the remaining coding region of the corresponding parental molecule in the pKM260 expression vector. The mutated genes were sequenced to confirm that the desired mutation was obtained and that additional mutations were not created during the amplification process.

**Production and Purification of Soluble Human KGFR (KGFR/AP)—**A cDNA fragment encoding the entire extracellular domain of the mouse KGFR (13) was cloned into the A/T cloning vector to produce an in-frame fusion of KGFR with secreted placental alkaline phosphatase (AP) (27). This plasmid was co-transfected with the selectable Neo<sup>R</sup> marker into NIH/3T3 cells. Conditioned medium from G418-resistant colonies was screened for AP activity (27). The clone that produced the highest level of AP activity was expanded and used for the collection of conditioned medium.

**Growth Factor Iodination, Receptor Binding, and Mitogenic Assays—**Radioiodination of FGF-2 and FGF-7 and separation of the radiolabeled growth factors from free Na<sup>125</sup>I was performed as described previously (11). The specific activities of radioiodinated growth factors was in the range of 1–2 × 10<sup>6</sup> cpm/μg. For the binding competition experiments, NIH/3T3 cells in 24 well microtiter plates were plated 3 times with ice-cold Dulbecco's phosphate-buffered saline, and then tracer levels of radiolabeled ligand (1–2 ng/well) were added in HEPES binding buffer in the presence of increasing concentrations of unlabeled competitor (0–1 μg/ml) (28). Incubation was performed for 2 h on ice, and analysis of specifically bound growth factor was done as described previously (29).

**Solid phase binding assays were performed using a soluble extracellular domain of the mouse KGFR fused to secreted placental alkaline phosphatase (KGFR/AP) essentially as described (30). Briefly, 96-well ELISA plates were first coated with monoclonal antibody directed against alkaline phosphatase. Then, conditioned medium from NIH/3T3 cells expressing KGFR/AP (0.15 AP optical density units/min) was added to the antibody-coated wells to immobilize the receptor. Binding was performed with 1–2 ng/well 125I-labeled FGF-7 in the presence of increasing concentrations of competitors for 2 h at room temperature in 100 μl of binding buffer. Free 125I-labeled FGF-7 was removed by extensive washing, and estimation of bound FGF-7 was done following solubilization with 0.2 M NaOH. All the experiments were done in duplicate and were repeated at least three times. Nonspecific binding was less than 10% of the total binding. DNA synthesis was measured by [3H]thymidine incorporation assay using serum-starved confluent cultures of NIH/3T3 or Balb/MK cells as described previously (14, 31).
saline and solubilized in cold HEPES buffer containing 1% Triton X-100 and protease and phosphatase inhibitors (12). The lysate was cleared by centrifugation, and the clarified lysate (150 μg) was diluted in sample buffer and resolved by 7% SDS-PAGE. Proteins were then transferred to Immobilon-P membrane and immunoblotted with monoclonal anti-phosphotyrosine antibody as described previously (12).

RESULTS

Structure Prediction of FGF-7—In order to obtain a more detailed insight into the three-dimensional structure of FGF-7, a model was built based on the experimentally determined structures of FGF-1 and, in particular, FGF-2 (16) (see Fig. 1). The 29 amino-terminal residues of FGF-2 were not resolved in the crystal structure. Therefore we did not include residues 2–32 of FGF-2 in our structure model (amino acids are numbered from the FGF-7 initiation codon; residues 1–31 encompass the signal peptide). It is noteworthy that a FGF-7 deletion mutant lacking the 28 amino-terminal residues (residues 32–58 of FGF-7 in our structure model (amino acids are numbered from the FGF-7 initiation codon; residues 1–31 encompass the signal peptide). It is noteworthy that a FGF-7 deletion mutant lacking the 28 amino-terminal residues (residues 32–58 of FGF-7) retains potent biological activity (20). The model was generated as described in the legend to Fig. 1. Based on this model we predicted that residues 154–163 (AKWTHINGGEM) form the loop connecting the 9th and 10th β-strands of FGF-7. Superimposition of the loop region of FGF-7 and FGF-2 is shown in Fig. 1, A and B. The amino-terminal part of the loop is structurally similar in both growth factors (residues 154–157 and 118–121 in FGF-7 and FGF-2, respectively). Then the loops diverge from each other and re-}

**FIG. 1.** Predicted three-dimensional structure of FGF-7. A, proposed FGF-7 secondary structure based on homology with FGF-2 (16). B, the three-dimensional structure of FGF-2 and the FGF-7 model were superimposed, and the loop connecting their 9th and 10th β-strands is shown. C, a close up of the loop region. Indicated residues are the ones mutated in this study. Yellow, FGF-2; gray, FGF-7. The model was initially generated using the protein modeling package BRAGI (43) by the following steps: (a) exchange of modified amino acids based on the principle of maximal steric retainment of side chain; (b) replacement of the loop connecting the 9th and 10th β-strands in FGF-2 (Arg118- Trp123) by the corresponding region in FGF-7 (44, 45); (c) energy minimization with restraints on the core Cα atoms; (d) limited protein dynamics with restraints (using AMBER 4.1 (46)).

The Biological Properties of the FGF-2 and FGF-7 Loop Replacement Mutants—The 155 amino acid form of FGF-2 and the mature FGF-7 polypeptide (residues 31–194) were employed in the present study. The amino acid composition of the loop replacement mutants is as follows: FGF-21–117, FGF-7154–163, FGF-2124–155 (FGF2/L7); FGF-731–153, FGF-2118–123, FGF-7164–194 (FGF7/L2). The generation, expression, and purification of the mutant proteins is described under “Experimental Procedures”. Both mutants retained wild type affinity for heparin (data not shown). Binding competition experiments and [3H]thymidine incorporation assay were utilized to assess the receptor binding properties and mitogenic potencies of the mutants.

The binding experiments were performed in L6E9 cells that individually express FGFR1 and the KGFR and display a similar number of receptor binding sites (11, 12). As shown in Fig. 2, replacement of the loop of FGF-2 with that of FGF-7 slightly enhanced the binding affinity of the mutant to both FGFR1 and the KGFR as compared with the parental FGF-2 molecule (Fig. 2, A and C). The reciprocal mutation slightly reduced binding affinity of the mutant (FGF-7/L2) to the KGFR, but the mutant did not acquire the ability to bind FGFR1 (Fig. 2, B and C). Taken together, these results indicate that exchanging the loop between FGF-2 and FGF-7 did not have a significant effect on their receptor binding properties.

The mitogenic capacity of the mutants was assessed in NIH/3T3 fibroblasts and in the keratinocyte cell line Balb/MK. FGF-2 is highly mitogenic to NIH/3T3 cells and displays a significantly lower activity in Balb/MK keratinocytes, whereas FGF-7 is highly mitogenic to keratinocytes but cannot stimulate a mitogenic response in NIH/3T3 cells (14, 32). In agreement with the results of the binding assays, the FGF2/L7 chimera retained the target cell specificity of FGF-2 in eliciting a mitogenic response. Furthermore, the extent of the mitogenic activity was indistinguishable from that of FGF-2. Thus, both proteins induced an efficient and identical level of DNA synthesis in NIH/3T3 cells (half-maximal response was observed at a concentration of about 2 ng/ml, Fig. 3A) and were similarly
As expected from its receptor binding properties, the reciprocal mutant FGF7/L2 induced a mitogenic response in Balb/MK but not in NIH/3T3 cells (Fig. 3, A and C). However, despite its FGF-7-like affinity for the KGFR, the mitogenic activity of the mutant was significantly lower compared with that of FGF-7. Half-maximal response was obtained at 70 and 1 ng/ml FGF7/L2 and FGF-7, respectively (Fig. 3C). Nevertheless, at higher concentrations, the mutant induced a maximal level of DNA synthesis comparable with that of FGF-7.

**Fig. 2.** Receptor binding properties of the loop replacement mutants, FGF2/L7 and FGF7/L2. Binding was carried out in L6E9 cells expressing the KGFR (L6/KR) or FGFR1 (L6/R1). 125I-FGF-7 was bound to L6/KR cells, (A and B), and 125I-FGF-2 was bound to L6/R1 cells (C). Binding was competed by increasing concentrations of the above indicated parental and mutant growth factors. The assay was performed as described under “Experimental Procedures.” These results are representative of at least three different experiments.

**Fig. 4.** Stimulation of tyrosine phosphorylation in intact NIH/KR cells by FGF-7 and the FGF7/L2 chimera. Serum-starved subconfluent cultures of NIH/KR cells were incubated for 10 min with and without the indicated concentrations of FGF-7 and the FGF7/L2 chimera. Lysates were prepared and cleared by centrifugation. Equal amounts (150 μg) of clarified lysate were separated on 7% SDS-PAGE and immunoblotted with anti-phosphotyrosine antibody, as described previously (12). The position of molecular weight markers is indicated on the left.

**Fig. 3.** Mitogenic activity of the FGF2/L7 and FGF7/L2 chimeras. Serum-starved NIH/3T3 cells (A) and Balb/MK cells (B and C) were stimulated with increasing concentrations of the above indicated parental and mutant proteins. 16 h later, 3H-thymidine was added for 6 h, and incorporation was determined as described previously (14, 31). Maximal counts/min were 116,000 for FGF-2 (A) and 57,000 or 49,500 for FGF-7 (B and C, respectively). These results are representative of at least three different experiments.

Less mitogenic in Balb/MK cells compared with FGF-7 (Fig. 3B).

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**Induction of Tyrosine Phosphorylation by the FGF7/L2 Mutant**—Because receptor activation is a prerequisite for subsequent signaling events, it was possible that the reduced mitogenic potency of mutant FGF7/L2 is due to inefficient activation of the KGFR. To examine this possibility, we compared the ability of the mutant protein and FGF-7 to induce tyrosine phosphorylation of cellular substrates. Cells expressing the KGFR were incubated with FGF-7 or the FGF7/L2 chimera. Cell lysates were then prepared and analyzed by Western blotting for tyrosine-phosphorylated proteins. Both FGF7/L2 and FGF-7 similarly enhanced tyrosine phosphorylation of several substrates including p150, p115, p90, and p42 (Fig. 4) that were previously identified as phospholipase Cγ, KGFR, SNT, and mitogen-activated protein kinase, respectively (12, 33, 34). Thus, FGF7/L2 efficiently activated the KGFR even at concentrations where it could barely stimulate a mitogenic response.

The observations that the FGF7/L2 retained wild type affinity for receptor and heparin, and was as active as FGF-7 in inducing tyrosine phosphorylation, suggest that it is properly folded, and it is capable of activating the KGFR as efficiently as the wild type protein. Because the mitogenic assay requires long term incubation, a possible explanation for the lower mitogenic potency of FGF7/L2 could be susceptibility to proteolytic enzymes released by the target cells or thermolability that is manifested only during prolonged incubation at 37 °C. Both possibilities were examined. Apparently, the parental and mutant proteins were resistant to proteolytic degradation when incubated on target cells in serum-free conditions (Fig. 5A). However, when both the wild type and the mutant protein were incubated at 37 °C for 16 h and then tested for the ability to bind the KGFR, only FGF7/L2, but not FGF-7, lost significant biological activity (see Fig. 5B). Heparin, which stabilizes FGFs (7), did not protect the mutant from loss of biological activity.2

These observations suggest that the FGF7/L2 mutant is thermolabile. The fact that fresh mutant protein was mitogenic and

2 D. Ron, unpublished results.
could stimulate a maximal response comparable with that observed with FGF-7 (see Fig. 3) suggests that the loss of its activity upon incubation at 37 °C may be gradual. It is noteworthy that a mutant of FGF-7 in which three residues (Thr157–Asn159) from the loop were deleted retained wild type affinity for receptor and heparin but was thermolabile. This could stimulate a maximal response comparable with that observed with FGF-7 (see Fig. 3) suggests that the loss of its activity upon incubation at 37 °C may be gradual. It is noteworthy that a mutant of FGF-7 in which three residues (Thr157–Asn159) from the loop were deleted retained wild type affinity for receptor and heparin but was thermolabile.2 Because the loop replacement has also reduced the overall length of the loop, these observations could imply that the length of the loop in FGF-7 may be critical for stability.

Site-directed Mutagenesis in the Loop of FGF-7—The above described results indicated that the loop of FGF-7 can functionally substitute for the corresponding loop of FGF-2. These results suggested that, similar to the situation described for FGF-2, the loop of FGF-7 also functions as a low affinity receptor binding site required for biological activity but not for high receptor binding affinity (15). To examine this possibility, we generated point mutations in the loop of FGF-7 and tested their effect on receptor binding and biological activity. Residues Trp156, His158, Asn159, and Glu162 were each replaced with alanine, and residue Lys155 was replaced with glutamic acid. All the mutants retained FGF-7 affinity for heparin and were eluted from a heparin-Sepharose column at the same salt concentration as FGF-7 (0.4–0.5 M NaCl, data not shown). The affinity of the mutants for the KGFR was examined in a cell-free binding assay utilizing a soluble form of the extracellular domain of the KGFR fused to a secreted human placental alkaline phosphatase (designated KR/AP). The fusion protein was adsorbed to ELISA dishes coated with an anti-alkaline phosphatase antibody and was used in a quantitative binding assay. Thus, radiiodinated FGF-7 was bound to the immobilized soluble extracellular domain of the KGFR in the presence of increasing concentrations of each mutant protein. All the FGF-7 mutants displayed wild type-like affinity for the KGFR (Fig. 6A). Similar results were obtained in binding experiments utilizing cells that express the KGFR (data not shown). By contrast, three out of the five mutants had lower mitogenic potency as compared with FGF-7 (Fig. 6B). The mitogenic activity of the K155E and H158A mutants was reduced by 3- and 2-fold, respectively, and that of the W156A mutant was more than 70-fold lower. Each of the three mutants with reduced mitogenic potency was thermally stable, as their affinity for receptor binding and biological activity remained unaffected following 16 h incubation at 37 °C. Moreover, none was susceptible to proteolytic degradation when incubated on target cells in serum-free conditions (data not shown). In addition, no difference in the temperature dependence of intrinsic Trp fluorescence (35) of four out of the five mutants (K155E, H158A, N159A, and E162A) and FGF-7 was found (data not shown).

Since the mutation at Trp156 has the strongest impact on mitogenic activity, we tested whether the lower mitogenic potency of the mutant protein correlates with reduced ability to
assay was performed as described in the legend to Fig. 3. B, response curve of Balb/MK cells to FGF-7 and the W156A mutant. The A phorylation by the FGF-7-W156A mutant.

...on the legend to Fig. 4. The position of molecular weight markers is indicated of tyrosyl-phosphorylated proteins were performed as described in the...trations of FGF-7 or the W156A mutant. The incubation and detection...cells were incubated in the absence or presence of the indicated concen...changed the loop between FGF-2 and FGF-7, and we tested...mitogenic response. It was recently (15, 16). The corresponding region in the other family members segregated in the mutants. The residues comprising the loop in...has mostly tryptophan (similar to FGF-2) or methionine (similar to FGF-7). Therefore, whereas there is variability in both length and sequence of the loop, there exists either identity or similarity in residues that, in FGF-2 and FGF-7, contribute the most for biological activity. Based on this observation we propose that the loop functions as a secondary receptor-binding site throughout the FGF family. The findings that the loop of FGF-7 can functionally substitute for the loop of FGF-2 in...stimulate tyrosine phosphorylation of cellular substrates. As shown in Fig. 7, FGF-7 stimulated a significant level of phosphorylation already at a concentration of 10 ng/ml (Fig. 7B). This concentration is about 2-fold higher than that required for maximal stimulation of a mitogenic response (Fig. 7A). The W156A mutant induced a barely detectable level of phosphorylation at a concentration (15 ng/ml) where its mitogenic activity was weak (compare Fig. 7, A and B). Stimulation of phosphorylation by the mutant was observed at concentrations that induce half-maximal and maximal mitogenic response (90 and 300 ng/ml, respectively). However, even at these high concentrations, the extent of phosphorylation was lower as compared with FGF-7.

FIG. 7. Stimulation of mitogenic response and tyrosine phosphorylation by the FGF-7-W156A mutant. A, mitogenic dose-response curve of Balb/MK cells to FGF-7 and the W156A mutant. The assay was performed as described in the legend to Fig. 3. B, NIH/KR cells were incubated in the absence or presence of the indicated concentrations of FGF-7 or the W156A mutant. The incubation and detection...Figure 1, B and C). H158 in FGF-7 is located in the loop region that differs in structure between the two growth factors, whereas Trp123 in FGF-2 is superimposed on Met163 in FGF-7 and both terminate the loop. Met163 of FGF-7 may be important for maintaining the conformation of FGF-7 as its substitution with alanine affected the folding of the protein (18). Thus, the secondary binding site of FGF-2 and FGF-7 overlap but is not identical. However, the results of the loop exchange suggest that FGFRs can accommodate the differences.

Amino acid sequence alignment of the 19 members of the FGF family indicates that an aromatic residue (tyrosine, tryptophan, or histidine) is always present at the position that corresponds to Trp156 in FGF-7. At the end of the loop, FGFs have mostly tryptophan (similar to FGF-2) or methionine (similar to FGF-7). Therefore, whereas there is variability in both length and sequence of the loop, there exists either identity or similarity in residues that, in FGF-2 and FGF-7, contribute the most for biological activity. Based on this observation we propose that the loop functions as a secondary receptor-binding site throughout the FGF family. The findings that the loop of FGF-7 can functionally substitute for the loop of FGF-2 in...DISCUSSION

FGFs are known to interact differently with their signaling receptors (6). FGF-7 is an unusual FGF member in that it recognizes only one FGFR isoform and acts predominantly on cells of epithelial origin (2). Studies in FGF-2 identified a secondary receptor binding site that is located in the crystal structure within a loop connecting its 9th and 10th β-strands (15, 16). The corresponding region in the other family members is highly variable, and it is, therefore, thought to contribute to the specificity of ligand-receptor interaction. It was recently proposed that this region predominantly confers receptor binding specificity to FGF-7 (18). In the present study, we exchanged the loop between FGF-2 and FGF-7, and we tested how their unique receptor binding characteristics and known cell type specificities in eliciting a mitogenic response were segregated in the mutants. The residues comprising the loop in FGF-7 were defined from a computerized model of its 3-dimen...Figure 1, B and C). H158 in FGF-7 is located in the loop region that differs in structure between the two growth factors, whereas Trp123 in FGF-2 is superimposed on Met163 in FGF-7 and both terminate the loop. Met163 of FGF-7 may be important for maintaining the conformation of FGF-7 as its substitution with alanine affected the folding of the protein (18). Thus, the secondary binding site of FGF-2 and FGF-7 overlap but is not identical. However, the results of the loop exchange suggest that FGFRs can accommodate the differences.

Amino acid sequence alignment of the 19 members of the FGF family indicates that an aromatic residue (tyrosine, tryptophan, or histidine) is always present at the position that corresponds to Trp156 in FGF-7. At the end of the loop, FGFs have mostly tryptophan (similar to FGF-2) or methionine (similar to FGF-7). Therefore, whereas there is variability in both length and sequence of the loop, there exists either identity or similarity in residues that, in FGF-2 and FGF-7, contribute the most for biological activity. Based on this observation we propose that the loop functions as a secondary receptor-binding site throughout the FGF family. The findings that the loop of FGF-7 can functionally substitute for the loop of FGF-2 in...DISCUSSION

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receptor activation suggest that the putative secondary binding site displays broad specificity for FGFRs.

Our results also have implications for the mechanism of ligand-induced FGFR dimerization. Earlier studies with FGF-2 suggested that one ligand molecule binds to two molecules of FGFR1 in a mechanism similar to that described for growth hormone (15, 36, 37). According to this mechanism FGF is a bivalent ligand containing two asymmetric sites for receptor binding, a primary and a secondary site. Binding of one receptor molecule to the primary site on FGF leads to the recruitment of a second receptor molecule through binding to the secondary site. In this model heparin stabilizes ligand receptor complexes. Others suggested that a heparin-linked FGF dimer facilitates receptor dimerization (stoichiometry of 2:2FGF:2:FGF). According to this mechanism heparin is absolutely required for receptor dimerization (stoichiometry of 1FGF-7:2KGFR (40). Ligand bivalence and broad specificity may lead to signal diversification because it enables a given FGF to induce receptor heterodimerization through the putative secondary site. This may be possible because many cell types express several distinct FGFRs (10, 11, 41, 42).

In summary, the present study suggests that the loop connecting the 9th and 10th β-strands of FGF's functions as a low affinity/broad specificity determinant that is required for the activation of FGF receptors. The ability to separate heparin and primary receptor binding from mitogenic activity in FGF-7 favors a bivalence model for receptor dimerization and suggests that it will be feasible to generate growth factor antagonists by mutational approaches.

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Mutations Uncouple Human Fibroblast Growth Factor (FGF)-7 Biological Activity and Receptor Binding and Support Broad Specificity in the Secondary Receptor Binding Site of FGFs

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