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Structure-based mutational analyses in FGF7 identify new residues involved in specific interaction with FGFR2IIIb

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Abstract Receptor binding specificity is an essential element in regulating the diverse activities of fibroblast growth factors (FGFs). FGF7 is ideal to study how this specificity is conferred at the structural level, as it interacts exclusively with one isoform of the FGF-receptor (FGFR) family, known as FGFR2IIIb. Previous mutational analysis suggested the importance of the β4/β5 loop of FGF7 in specific receptor recognition. Here a theoretical model of FGFR2IIIb/FGF7 complex showed that this loop interacts with the FGFR2IIIb unique exon. In addition, the model revealed new residues that either directly interact with the FGFR2IIIb unique exon (Asp63, Leu142) or facilitate this interaction (Arg65). Mutations in these residues reduced both receptor binding affinity and biological activity of FGF7. Altogether, these results provide the basis for understanding how receptor-binding specificity of FGF7 is conferred at the structural level.
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Key words: Fibroblast growth factor; FGF7; FGF10; Fibroblast growth factor receptor; Mutagenesis; FGF7/FGFR complex

1. Introduction

The keratinocyte growth factor (KGF or FGF7) is a unique member of the fibroblast growth factor (FGF) family. Unlike other FGFs that act on a wide spectrum of cell types, FGF7 is a specific mitogen for epithelial cells [1]. It is secreted by stromal cells and stimulates the migration, differentiation and proliferation of a large variety of epithelial cells, acting as a paracrine mediator of mesenchymal–epithelial communication [1–4]. FGF7 plays an important role in epithelial tissue repair and development and is implicated in a number of pathological conditions, including psoriasis, inflammatory bowel disease and cancer [4–7].

Two FGFs that show high homology to FGF7 have been identified: FGF10 (also known as KGF2) and FGF22 [8,9]. Like FGF7, FGF10 and FGF22 act predominantly on cells of epithelial origin. FGF22 is preferentially expressed in adult skin and is involved in hair development [9], whereas FGF10 was shown to be crucial for embryonic development as FGF10-null mice die at birth due to lack of lung and limb formation [10]. Based on their sequence homology and target cell specificity, these three FGFs are classified as the FGF7 subfamily.

The biological activities of FGFs are mediated by cell-surface high-affinity receptors (FGFRs) that belong to the tyrosine kinase receptor family. 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. Four high-affinity receptors (FGFR1–FGFR4) have been identified and alternative-splicing mechanism generates FGFR isoforms with altered ligand binding properties [11]. Unlike other FGFs that can interact with several FGFRs, FGF7 subfamily members bind only one receptor isoform, an alternatively spliced variant of FGFR2 that is specifically expressed in epithelial cells (known as KGFR or FGFR2IIIb). KGFR differs from FGFR2 in the carboxyl-terminal half of D3 that is encoded by the alternatively spliced exon IIIb. The other ligand binding domains of the receptor (D2, linker and amino-terminal half of D3) are conserved between the two receptor isoforms.

The involvement of FGF7 subfamily members in a variety of physiological processes and diseases, together with their unique specific interaction with the FGFR2IIIb, provides a strong impetus for uncovering the structural basis of their biological activities. A previous mutagenesis analysis study in which large segments were exchanged between FGF7 and FGF2 (an FGF that binds FGFR2IIIb poorly) suggested that the amino-terminal part and a region in the middle of FGF7 determine its unique receptor binding specificity [12]. Further mutagenesis analysis in FGF7 showed that residues comprising the variable β4/β5 loop are crucial for the specific recognition of FGFR2IIIb, since exchanging this loop in FGF7 with the corresponding loop of FGF2 dramatically reduced the ability of FGF7 to bind and activate the receptor. In addition, point mutations in this loop of FGF7 reduced receptor binding and ability to activate the receptor [13]. Recent resolution of the crystal structure of FGF10 bound to FGFR2IIIb showed that similar interactions occur between this growth factor and FGFR2IIIb, thus emphasizing the importance of the β4/β5 loop and the amino-terminal part of

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Abbreviations: FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; KGF, keratinocyte growth factor; KGFR, keratinocyte growth factor receptor; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

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FGF7 subfamily members for FGFR2IIIb-specific recognition [14].

The resolved structure also pointed to specific residues within the amino-terminal and middle part of FGF10 that are engaged in intimate interactions with FGFR2IIIb [14]. To determine whether the corresponding residues in FGF7 play a similar role in receptor recognition, we mutated these residues in FGF7, and studied the effect of these mutations on receptor binding affinity and biological activity. Altogether our results suggest that the specific recognition between FGF7 subfamily members and FGFR2IIIb is determined by homologous determinants in the ligands that interact with the isoform-specific domains in the receptor.

2. Materials and methods

2.1. Materials

$Na^{125}I$ (5000 Ci/mM) was a Du Pont-New England Nuclear product. Heparin–Sepharose CL-6B was from Pharmacia LKB Biotechnology. Ni-NTA agarose was purchased from Qiagen. Fetal calf serum (FCS) and media were purchased from Gibco-BRL. Fibronectin was from Biological Industries (Beth-Haemek, Israel). Heparin from bovine lung and other chemicals were purchased from Sigma.

2.2. Tissue culture

NIH/3T3 cells over-expressing FGFR2IIIb (NIH/KR cells) were grown in DMEM containing 10% newborn calf serum [15]. Balb/MK cells were grown in low-calcium media containing 5 ng/ml of epidermal growth factor and 10% dialyzed FCS as previously described [16]. The lymphocyte cell line BaF3 cells over-expressing FGFR2IIIb (BaF/KR) were grown in RPMI 1640 supplemented with 10% FCS and 10% IL-3 conditioned medium from WEHI-3B cells [17].

2.3. Generation of point mutations in FGF7

FGF7 mutants were generated in three steps. First, a mutated segment encoding for the FGF7 amino-terminal part was created using primer p1 (ATGGAT**CCATGG**CTTGCAATGACATGACTCCA) that is complementary to the N-terminal end of the FGF7 gene product and contains an artificial *Nco*I site (bold) 5′ to the start codon, and primers p2 (AGGAGGGGCTATAAGAGTGAGGAGACTCTTC), p3 (GGGGATATA GCAGTGAGAAGGCTCTTCTGT) or p4 (ACTTCAAAGAAGCAATTCTGGAAAACCA) that contain the appropriate point mutation (underlined: D63A, R65A and L142A, respectively). A second mutated fragment, encoding for the C-terminal part of FGF7, was created using primer p5 (TT**GGATC**CATTAAGTTATTGCCATAGGAAG) that is complementary to the C-terminal end of the FGF7 gene product and contains an artificial *Bam*HI site (bold) 3′ to the stop codon, and primers p6 (GAAGAGTCTCCTCACTCTTATAGCCCCTCCT), p7 (ACAGAAGAGCCTTCTCACTGCTATATCCCC) or p8 (TGGTTTTCCAGAATTGCTTCTTTGAAGT) that contain the appropriate point mutation (underlined: D63A, R65A, L142A, respectively). The amplified products of the first step were annealed and amplified with primers p1 and p5. The obtained mutated FGF7 gene was cut with *Nco*I and *Bam*HI and cloned into pKM260 expression vector [13,18] that was digested with the same enzymes. All 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.

2.4. Production and purification of the recombinant proteins

FGF7 and FGF7 mutants were expressed in BL21(DE3) PlysS cells [19] and purified by Ni^{2+}-nitrilotriacetic acid affinity chromatography followed by heparin–Sepharose affinity chromatography as previously described [13,18]. All mutant proteins eluted from the column at the same salt concentration as FGF7 (0.5 M NaCl). Thermal stability of the R65A mutant was determined by incubating wild-type and mutant protein at 37°C in the presence of Balb/MK cells for 16 h. The medium was then clarified by centrifugation and tested for mitogenic activity on Balb/MK cells as previously described [18].

2.5. Growth factor iodination, receptor binding, mitogenic assays and cell proliferation assays

Radio-iodination of FGF7 and separation from free [^{125}I]Na was performed as previously described [20]. The specific activities of radio-iodinated FGF7 was in the range of 5×10^4 cpm/ng. Cell-surface receptor binding competition assays were performed using subconfluent NIH/KR cultures in 24-well microtiter plates as described in [12,20]. DNA synthesis was measured by a [^{3}H]thymidine incorporation assay using serum-starved confluent cultures of Balb/MK cells [12,18]. For proliferation assays BaF/KR cells were seeded in 96-well microtiter plates (2×10^4 cell/well). Fresh growth factors and heparin were added every other day and viable cells were counted on day 4 or 5 after seeding.

In each experiment, each data point was performed in duplicate and each experiment was repeated at least three times. The variation between different experiments did not exceed 10%.

2.6. Molecular modeling of the FGF7:FGFR2IIIb complex

A model of the FGF7:FGFR2IIIb complex was generated by performing a superposition of all Cα coordinates from the crystal structure of free FGF7 (1QQK [21]) onto those of the bound FGF10 from the crystal structure of the FGF10:FGFR2IIIb complex (1NUN [14]) using InsightII (Accylrys). The two FGFs share an overall similar structure and superposition over all comparable Cα atoms gives an r.m.s.d. of 1.3 Å. The first 24 amino-terminal residues of mature FGF7 were not resolved in the 1QQK crystal structure and the coordinates of Asp63 are not complete, thus the side chain of this residue is not indicated in the model. It is noteworthy that an FGF7 deletion mutant lacking the 28 amino-terminal residues of the mature FGF7 polypeptide retains potent biological activity [22].

3. Results

3.1. Structure prediction of FGF7 bound to FGFR2IIIb

In order to explore the structural basis of the specific interaction between the FGF7 subfamily members and FGFR2IIIb, a theoretical structure-based model of FGF7 bound to FGFR2IIIb was generated by superimposing the coordinates of the FGF7 3D structure [21] onto the FGF10 ligand in the recently resolved structure of the FGF10:FGFR2IIIb complex [14], using the InsightII software (Accylrys). Superposition of the free FGF7 and the bound FGF10 results in an r.m.s.d. of 1.3 Å over all α carbons. Thus the two structures are generally similar, but do have some structural differences.

Similar to FGF10, FGF7 interacts with D2, linker and D3 of the receptor (see Fig. 1A). As found for other FGFs, most of the interaction with D2 is between the conserved residues Asn149, Tyr151, Leu189 and Met191 of FGF7 and the hydrophobic surface of D2 (formed by residues Lys164, Leu166, Ala168 and Phe170) and residue Arg251 in the linker domain. These observed interactions are conserved in all FGF:FGFR complexes that have been resolved [23–26], supporting the concept that these contacts provide general FGF–FGFR affinity.

On the other hand, the specificity of FGF7:FGFR2IIIb recognition is determined by interactions between variable domains in FGF7 and the C-terminal part of D3 that is unique to FGFR2IIIb. The model predicts that FGF7 interacts with three loops in D3: the βB′–βC loop, which is conserved between FGFR2 isoforms, and the βC′–βE and βF–βG loops, which are encoded by the alternatively spliced exon IIIb and are therefore specific to isoform FGFR2IIIb. As shown in Fig. 1A, the variable β4/β5 loop of FGF7 intimately interacts with the isoform-specific βC′–βE loop of D3. This finding is in agreement with previous mutagenesis analysis,

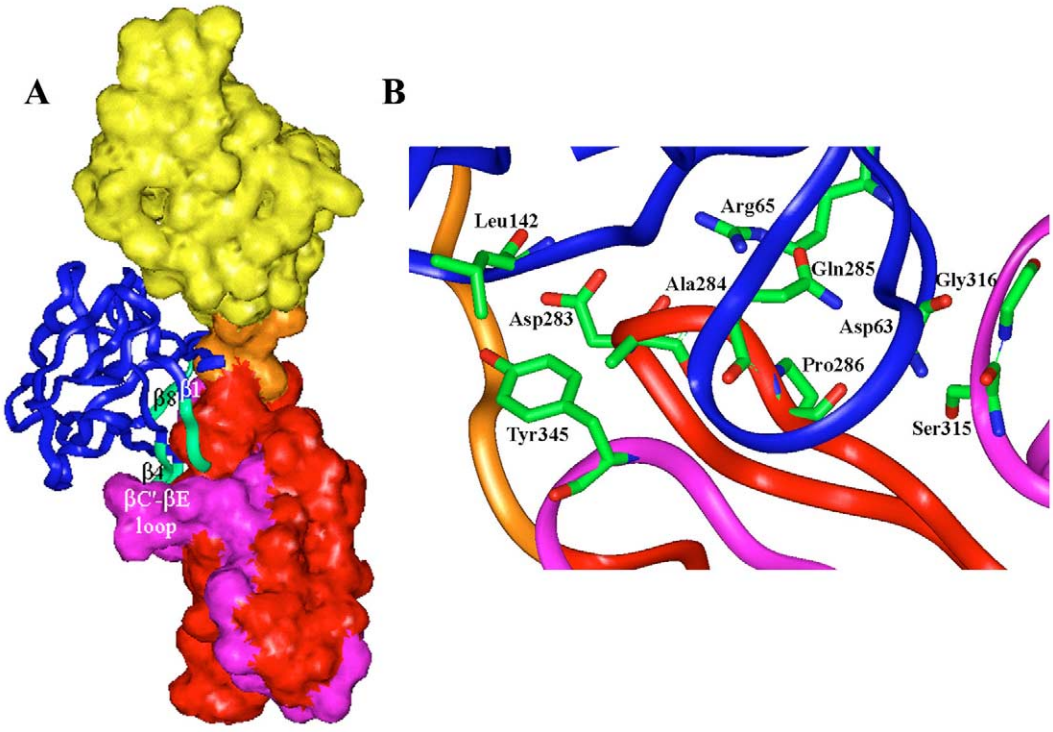

Fig. 1. A model of the FGF7:FGFR2IIIb complex was generated as described in Section 2. A: Overall structure of the FGF7:FGFR2IIIb complex. The D2, linker and D3 subdomains of the FGFR2IIIb protein are colored yellow, orange and red respectively, while FGF7 is colored blue. The alternatively spliced second half of D3 is colored purple. FGF7 regions that interact with D3 are colored green. β1, β4 and β8 of FGF7 and the βC′–βE loop of the receptor are labeled accordingly. B: Interaction domain between FGF7 and FGFR2IIIb in the vicinity of the three mutations (in CPK colored stick representation) performed in this study. Residues of FGFR2IIIb that are in putative contact with the mutated FGF7 residues are indicated as well. FGF7 and FGFR2IIIb are colored as in panel A.

which revealed that the β4/β5 loop of FGF7 is critical for the specific recognition of the FGFR2IIIb [13].

In addition, the model predicts that, as for FGF10, residues from the highly variable amino terminus and from β-strand 8 of FGF7 are involved in binding to the isoform-specific loops of D3. Thus, Asp63 from the loop preceding β1 interacts with Ser315 from the βC′–βE loop, and Leu142 from β8 interacts with Tyr345 in the βF–βG loop. In addition, the model shows that residue Arg65 from β1 contacts Gln285 in the conserved βB′–βC loop of D3. However, this residue contributes indirectly to FGF7/FGFR2IIIb-specific interaction as it is engaged in intermolecular hydrogen bonds with other residues from the amino-terminus of FGF7, allowing them to interact with the isoform-specific loops of D3. In order to verify these interactions, alanine scanning mutagenesis was performed in these key residues of FGF7 and the effect of the mutations on FGF7 biological properties was determined.

3.2. Alanine scanning mutagenesis in FGF7

The mature FGF7 polypeptide (residues 31–194) fused to

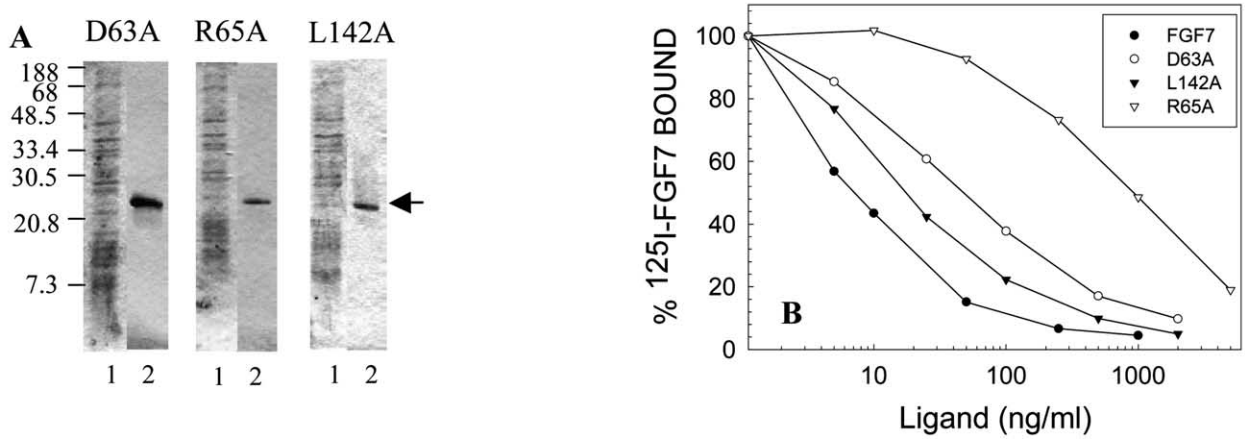

Fig. 2. Receptor binding affinity of FGF7 mutant proteins. A: 14% SDS–PAGE analysis of purification stages of FGF7 mutants. In each panel: lane 1, induced bacterial lysate; lane 2, peak fraction from heparin–Sepharose column. Molecular size markers (in kDa) are indicated on the left. The expected size of mutant FGF7 is indicated by an arrow on the right. B: Competitive binding of ^{125}I–FGF7 to NIH/KR cells was obtained by increasing concentrations of the indicated wild-type or mutant growth factors. The assay was performed as described in Section 2.

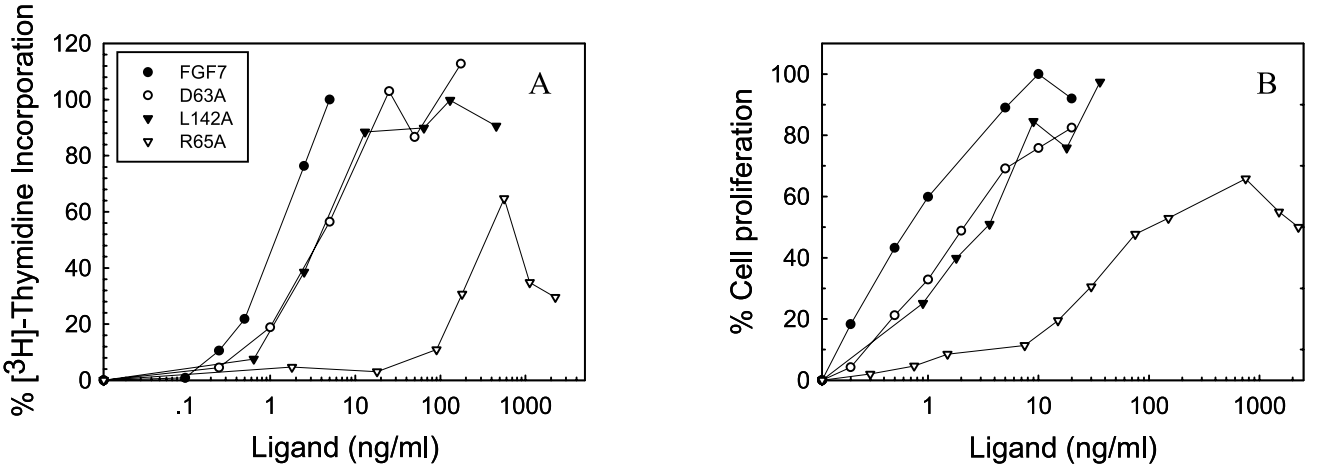

Fig. 3. The biological properties of FGF7 mutants. A: Serum-starved Balb/MK cells were stimulated with increasing concentrations of FGF7 and FGF7 mutant proteins. 16 h later, [^{3}H]thymidine was added for 6 h and incorporation was determined as described previously [12,20]. Maximal fold increase was 25 for FGF7. B: BaF/KR cells were washed with RPMI 1640 medium and seeded at a density of 2×10^4 cells/well in 96-well microtiter plates in growth medium lacking IL-3. The cells were grown in the presence of indicated concentrations of ligands and 2.5 μg/ml heparin. The number of viable cells in the presence of FGF7 was 227 000 cells/well (about 11-fold stimulation).

an amino-terminus hexa-histidine (His_6) tag was employed for the mutagenesis analysis. As previously reported, this tag does not change the biological properties of FGF7 [18]. The mutant proteins were expressed in BL21(DE3) plysS cells as described in Section 2. The cells were lysed and mutant proteins were purified from the soluble fraction by two consecutive purification steps: Ni^{2+}-nitrilotriacetic acid affinity chromatography followed by heparin–Sepharose affinity chromatography. The mutant proteins eluted from the heparin–Sepharose column at the same salt concentration as parental FGF7 (0.5 M NaCl). The purified mutant proteins were homogeneous as evaluated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis (see Fig. 2A).

The effect of point mutations in FGF7 on FGFR2IIIb binding affinity was examined by binding competition assays in NIH/3T3 cells over-expressing the receptor [15]. As shown in Fig. 2B, substitution of Asp63 or Leu142 with alanine resulted in six- and three-fold reduction, respectively, in receptor binding affinity, whereas mutating Arg65 to alanine severely compromised the ability to bind FGFR2IIIb. Half-maximal competition of ^{125}I–FGF7 binding occurred at 1000 ng/ml of the R65A mutant compared with 7 ng/ml of native FGF7.

Next, we examined whether the mutations affected the ability to activate FGFR2IIIb, by testing the ability of mutant proteins to stimulate DNA synthesis in Balb/MK cells that naturally express FGFR2IIIb, and to induce proliferation of BaF3 cells that were engineered to over-express the receptor (BaF/KR). As shown in Fig. 3, the biological activity of the mutant proteins in both cell types correlated with their binding affinity characteristics. Mutants D63A and L142A exhibited three- to five-fold reduction in their ability to induce a mitogenic response in Balb/MK cells (Fig. 3A) and proliferation in BaF/KR cells (Fig. 3B) compared to native FGF7. The mitogenic activity of mutant R65A in Balb/MK cells was 200-fold lower compared to native FGF7 (EC_{50} of 1 ng/ml and 200 ng/ml, respectively). Moreover, the R65A mutant achieved only 60% of the maximal mitogenic response produced by native FGF7. This mutant exhibited a similar dramatically reduced proliferative potency in BaF/KR cells. The correlation between reduction in receptor binding affinity and biological activity of the three mutants indicates that the mutations had no effect on their thermal stability. Nevertheless, the thermal stability of mutant R65A was confirmed as described in Section 2. The thermal stability assay, the location of the three mutated residues on the surface of FGF7 [21] and the fact that the three mutant proteins retained wild-type affinity for heparin, strongly suggest that these mutations do not affect the global structure of the proteins.

4. Discussion

Receptor binding specificity is essential for regulating the multitude of biological activities of FGFs. Among FGFs, FGF7 exhibits the highest degree of receptor binding specificity and is therefore ideal to study how specificity is conferred at the structural level. In the present study, we took advantage of the recently resolved FGF10:FGFR2IIIb complex in order to identify new residues in FGF7 that contribute to its unique interaction with FGFR2IIIb, and to corroborate the results from previous mutagenesis studies.

We found that mutations in Asp63 from the loop preceding β1 and Leu142 from β8 of FGF7 reduce FGFR2IIIb binding and activation by about an order of magnitude, whereas mutation in Arg65 from β1 has a more profound effect, reducing these activities of FGF7 by two orders of magnitude. The theoretical model of the FGF7:FGFR2IIIb complex suggests that these residues interact with the D3 domain of the receptor. It is noteworthy that Asp63 and Arg65 are located in a region that was previously shown by domain swapping to be involved in FGFR2IIIb recognition [12]. The importance of Asp63 in conferring on FGF7 subfamily members with their specific recognition of FGFR2IIIb is further underlined by the fact that this residue is uniquely conserved in the FGF7 subfamily and mutating the corresponding residue in FGF10 (Asp76) was found to similarly reduce the mitogenic activity of FGF10 in Balb/MK cells [14]. Interestingly, Leu142 is unique to FGF7 as in other FGFs there is an arginine residue

in this position. In spite of this difference, mutating this arginine in FGF10 (Arg155) reduced the mitogenic activity of FGF10 in Balb/MK cells to an extent similar to that observed by mutating Leu142 in FGF7. The crystal structure of the FGF10:FGFR2IIIb complex suggests that Arg155 of FGF10 forms hydrophobic contacts with the βF–βG loop [14]. This observation, together with the present findings, suggests that hydrophobic contacts are important in the interaction of this residue with FGFR2IIIb in the FGF7 subfamily.

The dramatic effect of the R65A mutation on FGFR2IIIb binding affinity and biological activity indicates that this residue plays a key role in receptor recognition. The crystal structure of FGF7 and our model suggest that the striking effect of the R65A mutation may result from the fact that, in addition to interacting with D3, Arg65 is capable of forming intramolecular hydrogen bonds that may be required for maintaining the local conformation of the amino-terminus of FGF7, allowing residues from this domain (including Asp63) to interact with the receptor. Moreover, the position of residue Arg65 in close proximity to the β4/β5 loop suggests that it may be responsible for the correct positioning of the β4/β5 loop in relation to D3 of the receptor. Therefore, mutating this residue may have affected interactions of other residues of FGF7 with the receptor.

In addition to the above-described interactions, the theoretical model of FGF7:FGFR2IIIb is in agreement with previous mutagenesis data, and together they unveil the structural basis of FGF7 receptor binding specificity. Thus, domain swapping and site-directed mutagenesis have identified the important involvement of the β4/β5 loop of FGF7 in FGFR2IIIb recognition. The model predicts that this loop interacts with the isoform-specific βC′–βE loop of D3. The homologous domain of FGF10 was shown to similarly interact with this isoform-specific loop of the receptor in the FGF10:FGFR2IIIb complex [14], emphasizing the vital role of the β4/β5 loop in the specific interaction between FGF7 subfamily members and FGFR2IIIb.

In conclusion, we have identified new domains in FGF7 that are important for the specific recognition of FGFR2IIIb. The present results complement previous mutational analyses and together these findings reveal the structural basis for one of the most specific ligand–receptor interactions in the FGF family, the interaction of FGF7 with FGFR2IIIb. In addition, the fact that homologous regions in FGF7 and FGF10 are engaged in similar specific contacts with the receptor suggests that all the members of the FGF7 subfamily utilize the same domains to bind FGFR2IIIb.

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