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# Study of the interaction of the Ig2 module of the fibroblast growth factor receptor, FGFR Ig2, with the fibroblast growth factor 1, FGF1, by means of NMR spectroscopy

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**Abstract Fibroblast growth factor (FGF) receptor (FGFR) consists extracellularly of three immunoglobulin (Ig) modules (Ig1–3). Currently, there are two competing models (symmetric and asymmetric) of the FGF–FGFR–heparin complex based on crystal structures. Indirect evidence exists in support of both models. However, it is not clear which model is physiologically relevant. Our aim was to obtain direct, non-crystallographic evidence in support of them. We found by nuclear magnetic resonance that Ig2 could bind to FGF1 not only via the primary site (present in both models), but also via the secondary site (present only in the symmetric model). Thus, our data support the symmetric model.**


*Keywords:* Fibroblast growth factor; Fibroblast growth factor receptor; Interaction; Nuclear magnetic resonance; Surface plasmon resonance; Binding

## 1. Introduction

Fibroblast growth factor (FGF) receptors (FGFR) are a family of receptor protein tyrosine kinases, which regulate a multitude of cellular processes including cell proliferation, migration, differentiation and survival (for review, see [1,2]). They interact with various ligands, such as FGFs [3], heparin/heparan sulfate proteoglycans (HS) and neural cell adhesion molecules [4–6]. The FGFR-ligand interaction results in receptor dimerization and activation of the tyrosine kinase domain, which triggers the downstream cell signaling.

It was found that HS proteoglycans are required for FGF signaling [7] and the high-affinity FGF–FGFR interaction [21,22]. Based on this, several models of FGFR dimerization were proposed, which involved the FGFR immunoglobulin (Ig) modules 2 and 3. The FGFR Ig1 module (structure recently determined by Kiselyov et al. [8]) was found to have a regulatory function [9] by binding to the Ig2 module [10]. Crystallographic studies suggested two fundamentally different models of FGFR dimerization. Plotnikov et al. [11] and Schlessinger et al. [12] proposed a so-called symmetric "two-end" model. In this model, heparin interacts with both FGF and FGFR within each 1:1 FGF:FGFR complex. Heparin also interacts with FGFR of the adjoining 1:1 FGF:FGFR complex. There is also an interaction between FGFs and FGFRs of the adjoining complexes via primary (in Ig2, Ig3) and secondary (in Ig2) sites as well as a direct interaction between the Ig2 modules of the two receptors. Pellegrini et al. [13] proposed a second model, often called an asymmetric model. The main feature of the model is a heparin bridge between *trans*-oriented FGFs. In this model, each FGF binds only to one FGFR in the dimer through the primary site and there are no direct receptor-receptor contacts, and no FGF–FGFR interaction through the secondary site. Furthermore, there are absolutely no protein–protein contacts between the two FGF–FGFR complexes and the dimer is stabilized solely by heparin. It should be noted that in the symmetric model, the Ig2 module interacts with FGF through its primary and secondary sites, whereas in the asymmetric model – only through the primary site. Since the contacts identified by crystallography may be artifacts due to crystal packing, it is important to validate the presence of these contacts in solution by other methods. Indirect evidence has been obtained in favor of both models. Ibrahimi et al. [14] showed that mutations in the secondary site (present only in the symmetric model) reduced FGF10 signaling, thus supporting the symmetric model and arguing against the asymmetric model. However, mass spectroscopy and gel filtration analysis of the ternary complex indicates that both types of complexes could be formed in solution [15–17].

Here we studied the interaction between the FGFR Ig2 module and FGF in solution by nuclear magnetic resonance (NMR) with an aim to obtain a direct and non-crystallographic evidence to confirm one of the two competing models.

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*Abbreviations:* FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; HS, heparin/heparan sulfate proteoglycans; HSQC, heteronuclear single quantum correlation; Ig, immunoglobulin; NMR, nuclear magnetic resonance; NTA, nitrilotriacetic acid; PBS, phosphate buffered saline; SPR, surface plasmon resonance

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We showed by surface plasmon resonance (SPR) analysis that the Ig2 module bound to FGF with a $K_d$ value of 39 nM and demonstrated that the module bound to FGF not only via the primary but also the secondary site, which is only present in the symmetric model. Thus, our data support the symmetric model of the FGFR ternary complex.

## 2. Materials and methods

### 2.1. Production and purification of recombinant proteins

The Ig2 module of mouse FGFR1 consists of a His-tag, AGHHHHHH, and amino acids 140–251 (Swissprot P16092). The Ig2 module was produced as previously described [5]. In brief, the protein was expressed in KM71 strain of yeast *Pichia pastoris* (Invitrogen). For the $^{15}$N-labeled protein $^{15}$N-labeled ammonium sulfate was used as a nitrogen source. After expression, the supernatant (which contains protein) was loaded on an affinity chromatography column ($Ni^{2+}$-nitrilotriacetic acid (NTA) resin, Qiagen, Holland) and washed overnight with 800 ml. of the 20% glycerin and 1 M NaCl in phosphate buffered saline (PBS) (pH 7.4). Afterwards the protein was eluted from the column by 0.25 M imidazole in PBS (pH 7.4). The protein was deglycosylated by Endo HF enzyme (New England Biolabs, USA) for 4–6 h at room temperature and purified by size-exclusion chromatography in PBS on Superdex 75 HiLoad 16/60 column (GE Healthcare, USA). The protein purification was performed using AktaFPLC instrument (GE Healthcare, USA).

### 2.2. Surface plasmon resonance (SPR) analysis

Binding analysis was performed using a BIAcore 2000 instrument (GE Healthcare, USA). Experiments were performed at 25 °C with PBS (pH 7.40) used as a running buffer and a flow-rate of 20 μl/min. The Ig module 2 of FGFR1 was immobilized on the sensor chip CM5 using an amine coupling kit (GE Healthcare, USA) in three steps: activation, protein immobilization and blocking. 20 μl of the activation solution were used for the chip (CM5) activation. Then, 12 μl of 20 μg/ml protein in 10 mM sodium phosphate buffer (pH 6.0) were used for immobilization and the chip was blocked by 35 μl of blocking solution (during immobilization 5 μl/min flow-rate was applied). For analysis, FGF1 was injected simultaneously into a flow-cell with the immobilized FGFR Ig2 module and into a control flow-cell (activated and blocked in the same way as the one used for immobilization) with no protein immobilized. The unspecific binding was subtracted from the sensorgram, and the resulting curve was used for analysis.

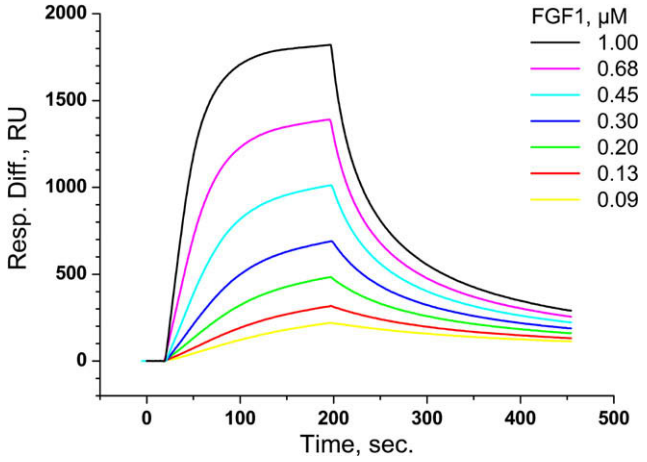


Fig. 1. SPR analysis of the binding between the FGFR Ig2 module and FGF1. Binding of soluble FGF1 at the indicated concentrations to the immobilized Ig2 module of FGFR1 is shown. Measurement of the FGF1 binding at all of the indicated concentrations was performed nine times.

### 2.3. NMR measurements

The following samples were used for recording of NMR spectra: $^{15}$N-labeled 0.1 mM Ig2 module of FGFR1 with or without addition of 0.05, 0.10 or 0.50 mM FGF1. PBS (pH 7.40) was used as a buffer. The $^{15}$N-heteronuclear single quantum correlation (HSQC) spectra were recorded using the standard set-up provided by ProteinPack. The spectra were processed by NMRPipe [18] and analyzed by Pronto3D [19]. The NMR experiments were performed using Varian Unity Inova 750 and 800 MHz spectrometers. All spectra were recorded at 25 °C.

## 3. Results and discussion

In order to study interaction between the Ig2 module of mouse FGFR1 and FGF1, the two proteins were produced in a yeast expression system of *P. pastoris* and in *Escherichia coli*, respectively. The proteins were found to be correctly folded as judged by one-dimensional NMR analysis.

### 3.1. SPR analysis of the interaction between FGF1 and Ig module 2 of FGFR1

To test whether the recombinantly expressed Ig2 module of FGFR was capable of binding to FGF1, SPR analysis was used. As appears from Fig. 1, FGF1 was capable of binding to Ig2 module with a dissociation constant ($K_d$) of 39 ± 8 nM, which is in agreement with the 65 nM $K_d$ value for the interaction between the Ig2 module of human FGFR and FGF1 (determined by isothermal titration calorimetry) [20], thus giving us evidence that both recombinant proteins are functionally active. When FGF1 was immobilized, soluble Ig2 module bound with a $K_d$ value 38 ± 33 nM (data not shown).

### 3.2. NMR analysis of the interaction between FGF1 and Ig2 module of FGFR1

Resonance assignment of the mouse Ig2 module of FGFR has previously been described [10]. It should be noted that the linker region connecting the C and D β-strands of the Ig2 module of human FGFR is very flexible in solution and adopts different conformations in the solution [20] and crystal [11–13] structures of FGFR1. In the mouse Ig2 module of FGFR1, resonances corresponding to this linker region (residues 200–213) are completely missing, which could be caused by either an intermediate exchange between the alternate linker conformations, enhanced flexibility compared to the human module, or maybe due to both of these effects.

The interaction between the Ig2 module and FGF1 was studied by NMR spectroscopy, which allows identification of residues in the vicinity of the binding sites. The $^{15}$N-HSQC spectrum of a $^{15}$N-labeled protein records the one bond coupling of the H–N bond, and it can be used as a useful tool for monitoring site specific perturbations. The chemical shift changes of the signals provide a method for identification of the amino acid residues whose NMR signals are perturbed by the binding of another molecule. $^{15}$N-HSQC spectra of 0.1 mM Ig2 module were recorded in the presence of 0, 0.05, 0.1 or 0.5 mM FGF1. Addition of FGF1 led to either line broadening, chemical shift changes or disappearance of the NMR signals for certain residues (see Fig. 2). The recorded changes of chemical shifts after addition of 0.1 and 0.5 mM FGF1 are shown in Fig. 3. As the highest change of the chemical shift was 0.23 ppm, the residues with the signals disappearing are indicated in Fig. 3 by a 0.3 ppm change of the chemical

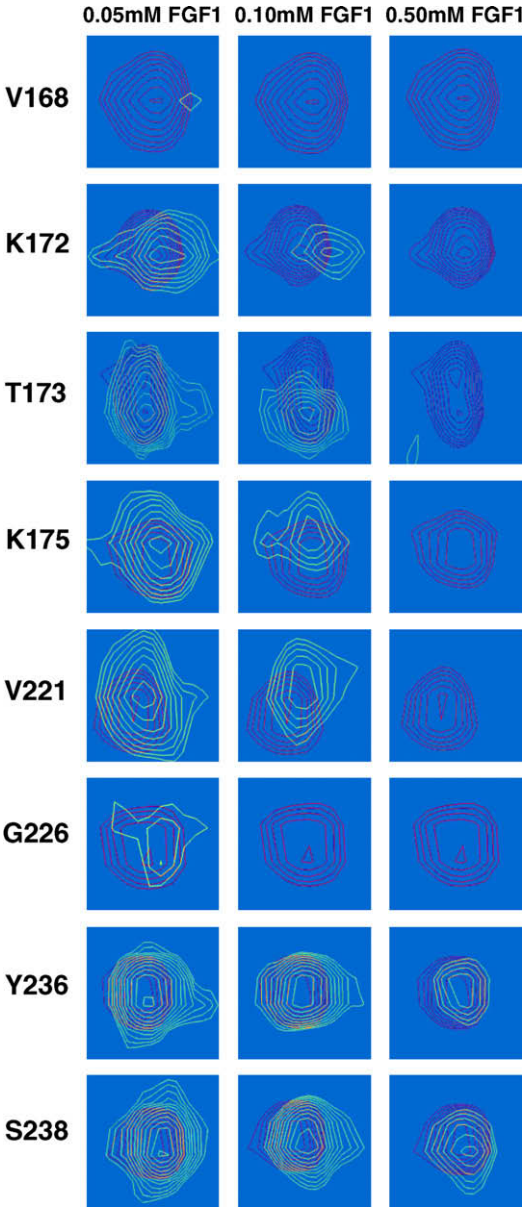


Fig. 2. Effect of FGF1 on NMR signals of the FGFR Ig2 module. $^{15}$N-HSQC spectra of the representative residues of 0.1 mM $^{15}$N-labeled FGFR Ig2 module in the absence (in red) or presence (in yellow) of FGF1 at the indicated concentrations are shown. The spectra in the presence of FGF1 are shown as overlapping the reference spectrum (in the absence of FGF1).

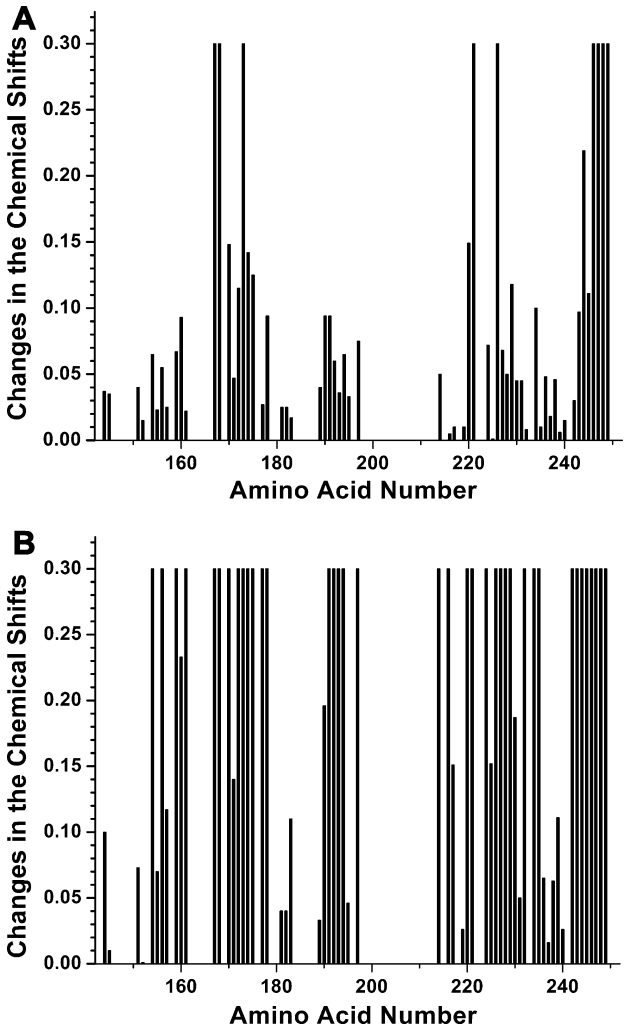


Fig. 3. Identification of the Ig2 module's residues involved in binding to FGF1. Changes in chemical shifts of 0.1 mM $^{15}$N-labeled Ig2 module after addition of 0.10 (A) and 0.5 (B) mM unlabeled FGF1. The change of the chemical shift was calculated using the following expression: ((5*ΔH)^2 + (ΔN)^2)^0.5, where ΔH is the change of the $^{1}$H chemical shift and ΔN is the change of the $^{15}$N chemical shift. Bars in diagrams representing chemical shifts 0.3 ppm does not represent the actual chemical shifts, but correspond to amino acids with disappeared signals (strongest effect).

shift. As can be seen from Fig. 3B, addition of 0.5 mM FGF1 led to disappearance of most the signals, which indicates that the exchange between the bound and free form of Ig2 module is intermediate on the NMR time scale. The residues with the changes of the chemical shifts of greater than 0.1 ppm or completely disappeared signals after addition of 0.1 mM FGF1 (Fig. 3A) were considered to be significantly perturbed. Mapping of the significantly perturbed residues is shown in Fig. 4, and as can be seen from the figure, the perturbed residues ($A^{167}$, $V^{168}$, $A^{170}$, $A^{171}$, $K^{172}$, $T^{173}$, $V^{174}$, $K^{175}$, $F^{176}$, $V^{220}$, $V^{221}$, $G^{226}$, $T^{229}$, $Q^{244}$, $L^{245}$, $D^{246}$, $V^{247}$, $V^{248}$, $E^{249}$) are located in two clusters on the opposite sides of the module, thus supporting the notion that the Ig2 module has two binding sites for FGF. According to the crystal structure of the FGFR-FGF dimer [11,12], the primary binding site of the Ig2 module consists of residues $L^{165}$, $A^{167}$, $P^{169}$ and $V^{248}$, and as can be seen from Fig. 4A, these residues are located approximately in the middle of the cluster of residues perturbed by the FGF1 binding. The secondary site in the crystal structure of the Ig2 module consists of residues $P^{199}$, $D^{200}$, $I^{203}$, $G^{204}$, $G^{205}$, $S^{219}$ and $V^{221}$ [11,12]. Unfortunately, residues $P^{199}$, $D^{200}$, $I^{203}$, $G^{204}$ and $G^{205}$ are located in the flexible part of the module (C–D linker) whose NMR signals are absent in the spectrum of the mouse Ig2 module (see above). However, as can be seen from Fig. 4B, the second cluster of the perturbed residues is immediately adjacent to the residues of the secondary site and partly overlaps it (see Fig. 4B), which indirectly provides evidence that these residues are involved in binding to FGF1 in solution. It should be noted that it is not possible to completely exclude a possibility that binding to one of the

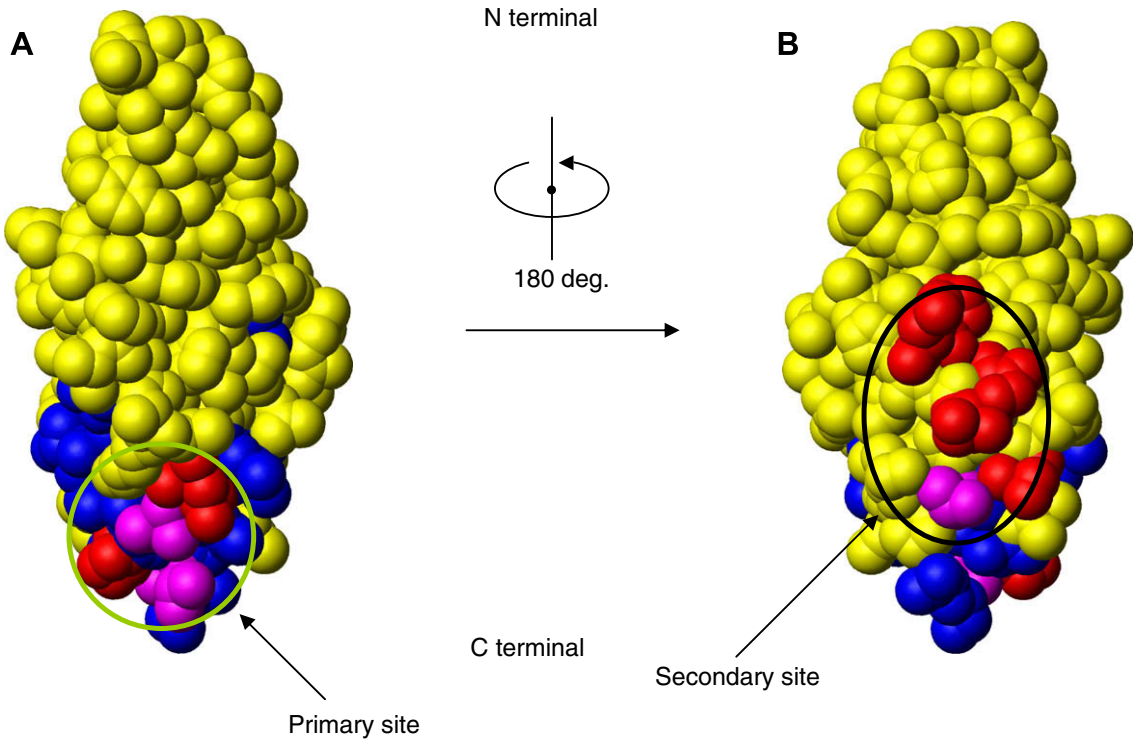


Fig. 4. Mapping of the residues of the FGFR Ig2 module perturbed by binding to FGF1 onto the module's structure. Blue and magenta – residues of the Ig2 module perturbed by the FGF1 binding. Magenta – perturbed residues which are also involved in binding to FGF1 as seen in the crystal of the ternary FGF–FGFR–heparin complex [11–12]. Red – non-perturbed residues which are involved in binding to FGF1 as seen in the crystal.

clusters observed in this study leads to a rearrangement of the protein backbone that results in perturbation of the residues from the other cluster. If we assume that this is the case, then binding of FGF1 to just one cluster of the Ig2 module is expected to affect substantially the backbone conformation for most of the module's residues, because addition of 0.5 mM FGF to the module results in disappearance of most of the module's NMR signals. However, the crystal structures of the Ig2 module from the ternary FGF–FGFR–heparin complexes are very similar to those from the FGFR not bound to FGF [11,12], which makes this assumption (that the second cluster appears due to coincidental rearrangement of the module's backbone upon binding to the first) unlikely.

Thus, we have by NMR obtained direct non-crystallographic evidence that the Ig2 module of FGFR1 has two distinct binding sites for FGF1. One of these sites overlaps with the primary site of the Ig2 module for FGF, and the other one partly overlaps with the secondary site, which supports the symmetric model of the ternary FGFR-FGF-heparin complex. Our results are consistent with those of Ibrahimi et al. [14] who showed that mutations in the secondary site (present only in the symmetric model) reduced FGF10 signaling and those of Canales et al. [23] demonstrating that heparin analogues not capable of inducing FGF1 dimerization (as required by the asymmetric model) can substitute natural heparins in FGF1 mitogenesis assays.

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