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ARTICLE

^{1}H, ^{13}C and ^{15}N assignment of D2 domain of human fibroblast growth factor receptor 4

Bettina Elshorst · Krishna Saxena · Ulrich Schieborr · Harald Schwalbe

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Abstract Fibroblast growth factor receptor (FGFR) 4 has been associated with progression of melanoma, breast, head and neck and hepatocellular carcinoma and is therefore an interesting target for therapeutic intervention (Ho et al. in J Hepatol 50:118–127, 2009). The extracellular D2 domain of the FGFR4 receptor contains a heparin binding site and the main interaction site with the fibroblast growth factor. We report the sequential backbone and side chain resonance assignment of the D2 domain of human FGFR4.

Keywords NMR assignment · Fibroblast growth factor receptor 4 · D2 domain

Biological context

Fibroblast growth factor receptors are integral membrane proteins consisting of an intracellular receptor tyrosine kinase, a single transmembrane helix and an extracellular part. The extracellular part consists of three immunoglobuline-like domains (referred as D1, D2 and D3) and a short span of acidic residues called the 'acid box' placed between the D1 and the D2 domain. Alternative mRNA splicing occurs in the D3 domain of FGFR1–3 resulting in two isoforms b and c for each receptor (Ornitz et al. 1996; Mohammadi et al. 2005). Unlike FGFRs 1–3, FGFR4 is not alternatively spliced in the D3 domain (Vainikka et al. 1992). The several isoforms of the FGFR differ in the binding specificity toward the different fibroblast growth factors (FGF1–23). Binding of FGFs to their receptors is stabilised by heparin sulphate glycosaminoglycans (HSGAGs) and results in dimeric FGF/FGFR/HSGAG complexes that activate the intracellular tyrosine kinase domain by autophosphorylation. This activation triggers downstream signalling pathways such as phopholipase C-g/protein kinase C, phosphatidylinositol 3-kinase/Akt and Ras/mitogen-activated protein kinase (Schlessinger 2004).

Structural data of several extracellular domains alone (Hung et al. 2005; Kiselyov et al. 2006) and in complex with several FGF ligands are available revealing that FGF interacts with the D2, D3 and the D2–D3 linker region of the receptor (Mohammadi et al. 2005; Stauber et al. 2000; Pellegrini et al. 2000). However, no structural data of the extracellular domains of FGFR4 are available so far.

D1 functions as a competitive autoinhibitor of the FGFR-ligand interaction. This autoinhibition suggests a function of preventing spontaneous FGFR dimerisation (through a direct D2–D2 interaction) in the absence of FGF (Kiselyov et al. 2006). The acid box region within the D1–D2 linker region is engaged in electrostatic interactions with the highly basic heparin binding site of the D2 domain. Interestingly, FGFR4 has the shortest acid box of all FGFR receptors, suggesting that FGFR4 activation is only mildly autoinhibited (Olsen et al. 2004).

It was demonstrated that FGF19 is a specific ligand of the FGFR4 receptor (Xie et al. 1999). The FGF19-FGFR4 signalling is significantly increased by the interaction with co-receptor beta-klotho, a single-pass transmembrane protein highly expressed in liver and fat (Wu et al. 2007; Lin et al. 2007). Aberration of the FGF19-FGFR4 signalling might have a strong pathophysiological impact in the liver. It was demonstrated that FGFR4 regulates proliferation, anti-apoptosis and alpha-fetoprotein secretion during

B. Elshorst · K. Saxena · U. Schieborr · H. Schwalbe (✉)
Institute for Organic Chemistry and Chemical Biology, Center for Biomolecular Magnetic Resonance, Johann Wolfgang Goethe-University Frankfurt, Max-von-Laue-Str. 7, 60438 Frankfurt, Germany
e-mail: schwalbe@nmr.uni-frankfurt.de

hepatocellular carcinoma (HCC) progression in the liver. Therefore, FGFR4 is a potential target for therapeutic intervention of HCC cancer (Ho et al. 2009) and the structural investigation of the extracellular domains of FGFR4 are of great interest for drug discovery studies. The D2 domain contains both the positive charged heparin binding site and the main interaction site with FGF. Therefore, the D2 domain is the most interesting domain of the extracellular part and with a molecular weight of 13.2 kDa suitable for NMR investigations.

Here, we report the sequential backbone and side chain resonance assignments of the D2 domain of the human fibroblast growth factor receptor 4, which are prerequisites for the determination of the solution structure of the FGFR4 D2 domain.

Methods and experiments

Sample preparation

The extracellular D2 domain of the human FGFR4 gene was PCR amplified and cloned into the *E. coli* vector pET21 with a C-terminal-His-tag using *Nde*I and *Xho*I restriction sites. The identity of the clones was verified by sequencing and the *E. coli* expression plasmids were transformed into BL21(DE3) Codon Plus (Stratagene) cells. Following previously published expression protocols of FGFR2 D2 (Hung et al. 2005) the D2 domain of FGFR4 was expressed in inclusion bodies, solubilised in 6 M guanidine-HCl and refolded. The purified protein sample was concentrated and exchanged into NMR buffer. Different buffer conditions were screened for the backbone assignment of FGFR4 D2. First, ^{15}N-HSQC spectra of the D2 domain monitored the pH optimum (phosphate buffer from 5.8 to 8.0). Then, numerous buffer conditions (MES, Hepes, MOPS, Bis–Tris, Acetate) in combination with different salt concentrations 50–500 mM (ammonium sulphate, sodium chloride, arginine) were tested. As a result of the buffer optimization, double labelled ^{15}N,^{13}C-NMR sample of FGFR4 D2 (0.7 mM protein in 200 mM NaCl, 25 mM bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methan (BisTris), 25 mM arginin, 0.03 % NaN_3, pH 6.0, in 90 % H_2O/10 % D_2O) was used for recording of assignment spectra. All NMR experiments were performed at 298 K on a Bruker DRX 800 MHz spectrometer with a triple-axis gradient HCN probe head and a Bruker DRX 600 MHz spectrometer equipped with a z-gradient HCN probe head.

Sequence-specific assignments of the polypeptide backbone were based on [^{1}H,^{15}N]-HSQC, HNCO, HNCA, HNCACB and CBCA(CO)NH spectra. Aliphatic side chain

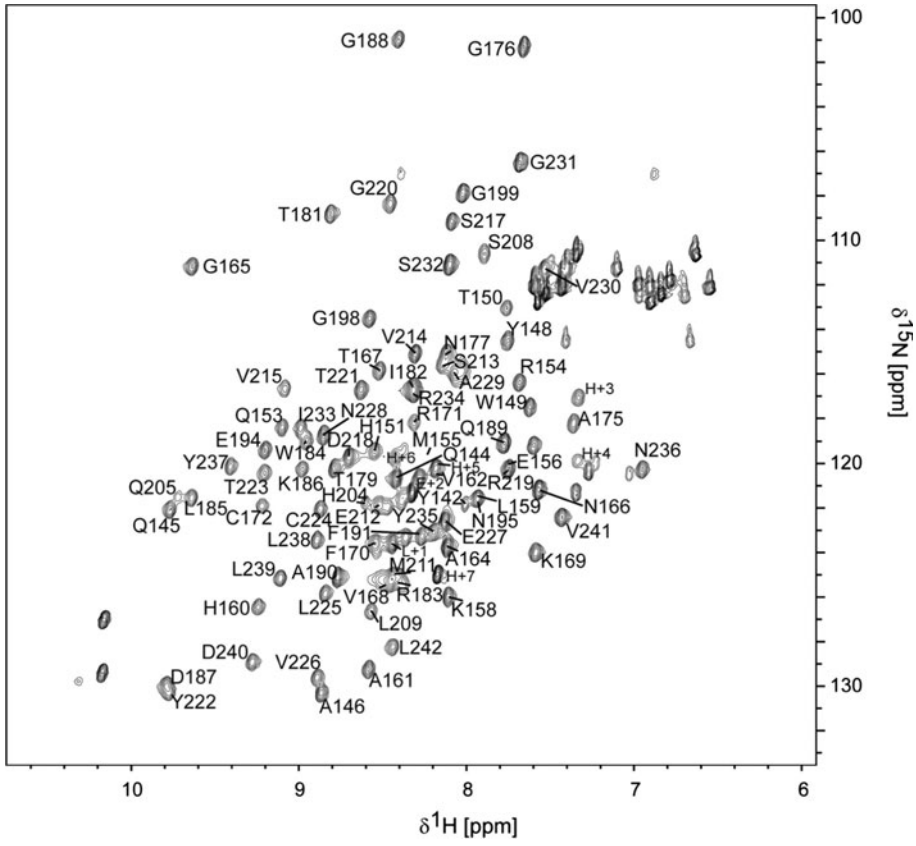

Fig. 1 [^{1}H,^{15}N]-HSQC spectrum of FGFR4 D2 with annotations of the assigned residues. Annotations with "+" correspond to residues in the His-tag region. 95 % of all resolved peaks were assigned. Based on the sequence of FGFR4 D2 84 % of the backbone amide resonances were assigned. The NMR sample contained 0.7 mM ^{15}N,^{13}C-FGFR4 D2, 25 mM BisTris, pH 6.0, 200 mM NaCl, 25 mM arginin, 0.03 % NaN_3, in 90 % H_2O/10 % D_2O

resonances were assigned from the combined information content of the CC(CO)NH, H(CC)(CO)NH, HBHA (CO)NH, HNHA and ^{15}N-edited NOESY-HSQC spectrum.

The protein sample was freeze dried and transferred into 100 % D_2O buffer. A set of [^{1}H,^{15}N]-HSQC spectra was recorded after different time periods after dissolving the protein in D_2O and residual amide proton resonances of residues which are involved in hydrogen bonds determined. Further NMR experiments for side chain assignment ([^{1}H,^{13}C] HMQC, CCH-TOCSY, HCCH-TOCSY) and for NOE assignments were recorded (^{13}C-edited NOESY-HMQC). For the assignment of aromatic side chains, 2D-TOCSY and 2D-NOESY spectra were recorded. For all NMR experiments standard BRUKER pulse programs were used.

All NMR data were processed using XWIN-NMR (BRUKER) software including linear prediction and apodization by shifted sine-bell functions. For the analysis of the spectra, the software CARA (Keller 2004) was used.

Assignment and data deposition

The [^{1}H,^{15}N]-HSQC spectrum of ^{15}N,^{13}C-labelled FGFR4 D2 (Asn138 to Leu242) domain is shown in Fig. 1. The spectrum is well-dispersed and contains 93 cross peaks. The analyzed construct consists of 114 amino acids with 8 prolines. An assignment of the FGFR4 D2 resonances could be achieved to the following extend: 76.2 % N, H_N 82.4 %, C_α 90.5 %, H_α 88.5 %, C_β 87.6 %, H_β 66.3 %, C_γ 61.1 %, H_γ 32.8 %.

The backbone resonances of Asn138 to His140, Lys157, Ala174, His192, Gly193, Arg196, Ile197, Ile200 to Arg203, His206, Trp207, and Val210 could not be assigned. Most of these not assigned residues are part of the region His192 to Val210, which consist of two short helical loops flanking the β-sheet 7 in the homolog FGFR2 D2 structure. In the same region, two residues could not be assigned in the NMR structure determination of FGFR2 D2 (Hung et al. 2005). NMR relaxation measurements published for the homologous FGFR2 D2 domain (Hung et al. 2005) indicate that the helical loop after the β-sheet 7 is either very flexible or is conformationally heterogeneous. Analogously, it can be assumed that a conformational flexibility in the intermediate NMR time regime leads to signal broadening of FGFR4 D2 amide resonances beyond detection. Variation of buffer conditions did not lead to an increase of amide signals.

Secondary structure elements of FGFR4 D2 are predicted on the basis of the chemical shift values of the assignments with the program TALOS (Cornilescu et al.

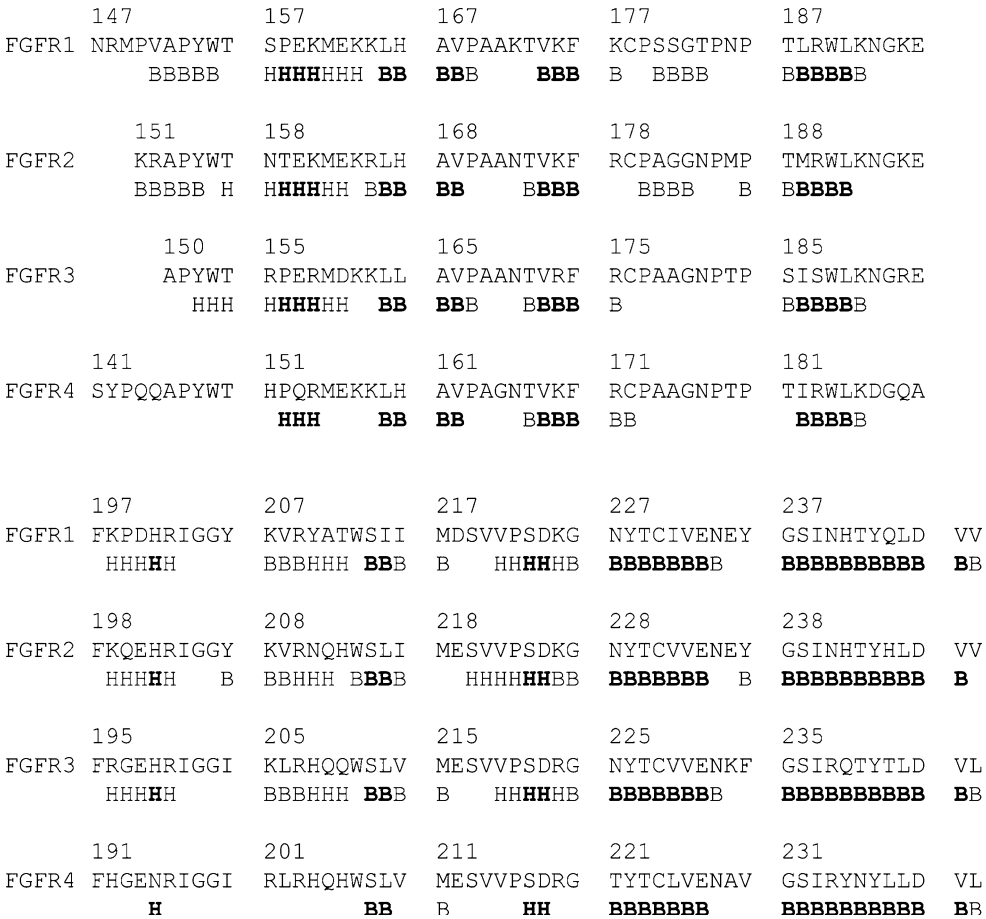

Fig. 2 Amino acid sequence of the FGFR4 D2 construct and the analogue primary sequences in FGFR1, FGFR2 and FGFR3. Secondary structure elements found in structures of FGFR1–3 [pdb codes 1EVT (Plotnikov et al. 2000), 3DAR (Brown et al., unpublished) and 3GRW (Qing et al. 2009)] are marked as "*H*" for helical and "*B*" for beta sheet. TALOS predictions of the secondary structure based on HN, N, Hα, Cα, Cβ and C' chemical shifts (Cornilescu et al. 1999) is indicated for FGFR4. Secondary structures that are found in all receptors 1–4, are symbolized in *bold letters*. Not all expected secondary structure elements were found, but the predicted secondary structure is in agreement with the related structures over the entire sequence

1999). A detailed comparison of the predicted secondary structure elements of FGFR4 D2 with conformations of the related receptors FGFR1 D2, FGFR2 D2 and FGFR3 D2 is given in Fig. 2. Not all of the expected secondary structure elements could be predicted. Nevertheless, all the found predictions are in agreement with the structures of the related proteins. The chemical shift values for ^{1}H, ^{13}C and ^{15}N resonances of D2 domain of FGFR4 receptor have been deposited at the BioMagResBank (http://www.bmrb.wisc.edu) under accession number 17620. According to the investigated FGFR4 D2 construct, the numbering of amino acids in the deposited file starts with Ser5, which corresponds to Ser141 in the numbering of the complete human receptor FGFR4.

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