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New insights into the molecular interaction of the C-terminal sequence of CXCL4 with fibroblast growth factor-2

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ABSTRACT

Full-length CXCL4 chemokine and a peptide derived from its carboxyl-terminal domain exhibits significant antiangiogenic and anti-tumor activity in vivo and in vitro by interacting with fibroblast growth factor (FGF). In this study we used NMR spectroscopy to characterize at a molecular level the interactions between CXCL4 (47–70) and FGF-2 identifying the peptide residues mainly involved in the contact area with the growth factor. Altogether NMR data point to a major role of the hydrophobic contributions of the C-terminal region of CXCL4 (47–70) peptide in addition to specific contacts established by the N-terminal region through cysteine side chain. The proposed recognition mode constitutes a rationale for the observed effects of CXCL4 (47–70) on FGF-2 biological activity and lays the basis for developing novel inhibitors of angiogenesis.

Introduction

The CXCL4 chemokine (ancient terminology CXCL4) is synthesized not only in platelets or megacaryocytes but also in different other cell types including monocytes, T-cells or neutrophils [1].

Both full-length CXCL4 and a peptide derived from its carboxyl-terminal domain display a strong anti-angiogenic activity in vitro [2–5] and in vivo [6,2,5]. They suppress growth of various tumors [7,8] and metastasis [9] in vivo. This effect is related to their anti-angiogenic action and not to tumor cell proliferation [6,8,9,7]. Although CXCL4 is one of the first agents discovered to have an antiangiogenic action in ex-vivo systems [2], the specific receptor mechanisms that transduce the antiangiogenic signal of CXCL4 are still poorly understood. More recently, CXCL4 has been described to bind the chemokine receptor CXCR3-B [10]. Among of the other effects described of the molecule is notably a immunomodulatory function on T-cells [11].

We have previously shown that the CXCL4 C-terminal region 47–70, named CXCL4 (47–70), exhibiting significant antiangiogenic and anti-tumor activity in vivo and in vitro, is the minimal sequence required for angiogenesis inhibition [4,12,5]. CXCL4 (47–70) inhibits endothelial cell proliferation, migration and tube formation. Furthermore it shows in vivo activity when injected in mice xenografted with U87 glioma [7]. Shorter amino-acid sequences derived from CXCL4 (47–70) are not able to do so. CXCL4 (47–70) is also able to interact with FGF-2 and to form a 1:1 complex [4,12].

In this study, we used NMR spectroscopy to evidence direct interactions of CXCL4 (47–70) with FGF-2 and characterize the residues mainly involved in the contact area. Altogether NMR data point to a major role of the hydrophobic contributions of the C-terminal region of CXCL4 (47–70) peptide in addition to specific contacts established by the N-terminal region through cysteine side chain.

Materials and methods

Protein and peptide synthesis. Recombinant human FGF-2 was synthesized and purified as previously described [13]. CXCL4 (47–70) peptide: N_{47}GRKIC$_{52}$LDLQ$_{56}$AP$_{58}$LYKKIIKKLLES$_{70}$, the mutants C52S and Q56R, and CXCL4 (47–58) and CXCL4 (58–70) peptides were purchased from Thermo Hybaid (Ulm, Germany).

NMR samples preparation. CXCL4 (47–70), the mutants C52S and Q56R and the shorter fragments CXCL4 (47–58) and CXCL4 (58–70) were dissolved at 0.9 mM peptide concentration in 30 mM water phosphate buffer, 50 mM NaCl, pH 7 and have been analysed by ^{1}H 1D and 2D NMR techniques at different temperatures in the range 280–298 K. To all peptide solutions,

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except for C52S and CXCL4 (58–70) preparations, DTT was added at a final concentration of 4.2 mM in order to avoid the formation of dimers through the formation of disulphide bridges between the free cysteines.

For the NMR analysis of the bound peptides, samples of 42 μM FGF-2 in the presence of 0.9 mM peptides in 30 mM deuterated buffer phosphate (95% D_2O, 5% H_2O), 4.2 mM DTT, 50 mM NaCl and pH 7.0 were prepared and analysed at 280 K.

NMR spectroscopy. All NMR spectra were recorded on a Bruker DMX 500 MHz NMR Spectrometer equipped with a triple resonance probehead, incorporating gradients in the *z*-axis. In order to assign proton resonances 2D-TOCSY, ROESY and NOESY spectra were recorded using standard sequences for each analysed peptide [14,15]. A spin lock time of 80 ms was used for TOCSY, mixing times of 150 and 250 ms were used for ROESY and NOESY spectra. Longitudinal relaxation times T1 of the peptide resonances were measured in the presence of FGF-2 protein with the standard inversion recovery method. Data points (32 K) were acquired to cover a sweep width of 10 ppm and a relaxation delay of 6.5 s was used. Data were analysed using Bruker Topspin software. T1 values were found to be rather constant along the peptide side chains in a range of 0.4–0.7 s, except for longer T1 observed for aromatic protons of Y60 showing T1 values of 1.7 and 2.0 s, respectively.

For the acquisition of STD NMR experiments a 1D pulse sequence incorporating a $T_{1\rho}$ filter to remove disturbing protein signals was used [16]. Spectra were recorded with a spectral width of 6510 Hz and 32 K data points. On-resonance irradiations were performed at different frequencies in the methyl (−236, −150 Hz) regions and off resonance irradiation was performed at −20,000 Hz, using a series of Gaussian pulses with a 1% truncation and 50 ms duration to give different total saturation times of 2, 2.5, 3, 4, 4.5 s. The selective saturation of the protein was checked by collecting a STD control spectrum in the same experimental conditions on the ligand alone which did not show any signal, thus excluding direct excitation of the peptide resonances. The duration of the $T_{1\rho}$ filter was 30 ms. STD NMR spectra were acquired with a total of 128 transients in addition to 32 scans to allow the sample to come to equilibrium. STD spectrum was obtained by subtraction, through phase cycling, of saturated fid from the reference one, obtained with off resonance irradiation.

Results and discussion

We and others have previously shown that CXCL4 and CXCL4 (47–70) are able to form complexes with FGF-2 as evidenced by biochemical and biophysical techniques [3,4,12]. In particular, gel seeving chromatography and differential centrifugation clearly evidenced molecular masses compatible with a 1:1 complex between CXCL4 (47–70) and FGF-2 [12].

In an attempt to evidence direct interactions of CXCL4 (47–70) with FGF-2 and characterize the structural basis and the nature of binding interactions of CXCL4 peptides with FGF-2, different NMR experiments were performed. Saturation Transfer Difference (STD) NMR methods [16] were applied in order to map the peptide residues making direct contacts with FGF-2. The STD spectra of CXCL4 (47–70) and its C52S and Q56R mutants in the presence of FGF-2 are shown in Fig. 1. In order to attribute the STD signals 2D experiments were recorded in the experimental conditions used for the interaction studies (water solution, pH 7). The assignment of the peptide resonances has been previously reported both in 30% TFE and in water at pH 3.4 [12] and the authors observed very few non-sequential NOEs connectivities in aqueous solution. The NMR

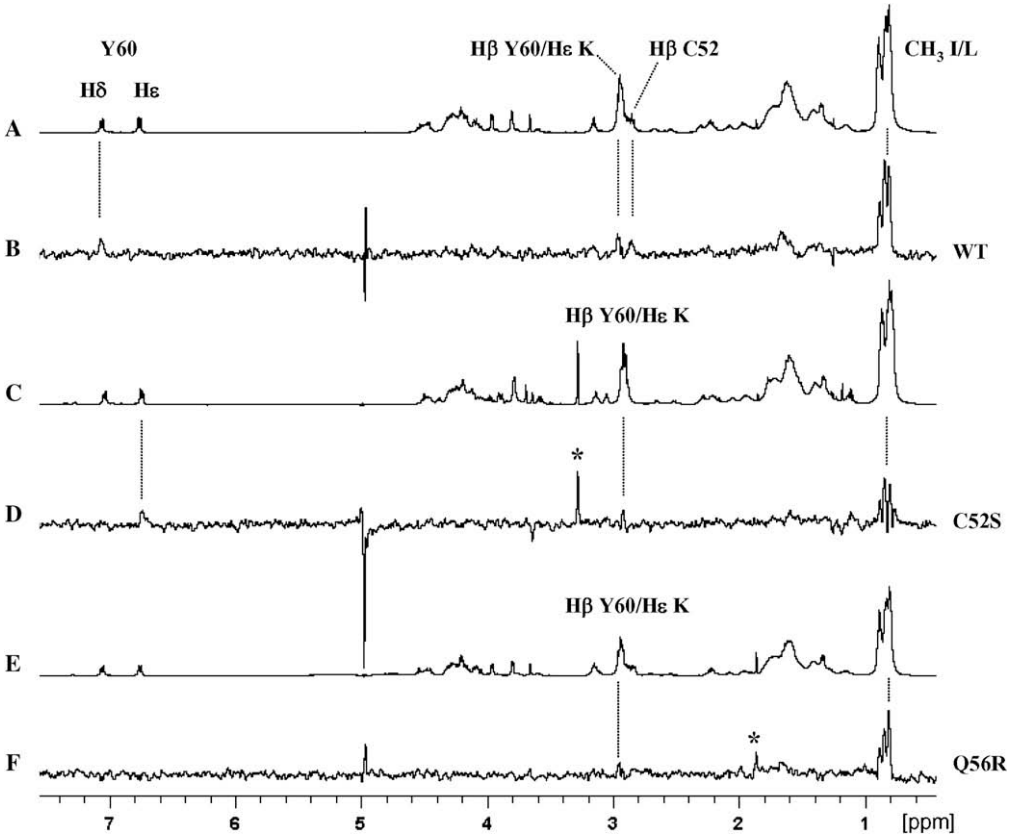

Fig. 1. STD NMR spectra of CXCL4 (47–70) (WT), C52S and Q56R mutants peptides in the presence of FGF-2. Reference ^{1}H NMR spectra of 0.9 mM CXCL4 (47–70) (WT) (A), C52S mutant (C) and Q56R mutant (E) peptides in the presence of 43 μM FGF-2 in 30 mM buffer phosphate (95% D_2O, 5% H_2O), 4.2 mM DTT, 50 mM NaCl and pH 7.0 recorded at 280 K on a 500 MHz Bruker spectrometer. ^{1}H NMR STD spectra of CXCL4 (47–70) (B), C52S mutant (D) and Q56R mutant (E) in the same experimental conditions of reference spectra (21:1 peptide: FGF-2 ratio, 4.5 s irradiation at −236 Hz). The assignment of the STD signals is reported, an asterisk marks signals due to impurities.

spectra at pH 7.0 showed an extensive spectral overlap which hampered the complete ^{1}H assignment of residues 50–51, 53 and 62–68. The analysis of the ROESY experiments showed only intra-residue and sequential cross-peaks, in line with previous results.

The STD spectrum of CXCL4 (47–70) peptide in the presence of FGF-2 indicates that both aliphatic and aromatic groups receive saturation transfer from the protein, giving rise to STD NMR signals. In particular some signals could be unambiguously attributed to Y60 aromatic protons and C52 Hβ (Fig. 1), while the main STD signals around 0.85 ppm, originating from methyl protons of leucines and/or isoleucines and the one at 2.9 ppm, relative to Y60 Hβ and/or Hε protons of lysines, could not be specifically assigned due to spectral overlap and to the presence of a high number of this kind of residues in the peptide sequence. A similar pattern of STD signals was observed in the spectra of both mutants (Fig. 1D and F) suggesting that the interaction of the three peptides with FGF-2 protein is mainly mediated by hydrophobic groups.

Along CXCL4 (47–70) sequence two regions, rich in leucines, isoleucines and lysines are present, namely 50–55, close to the interacting residue C52 and 59–68, close to the interacting residue Y60. In order to better localize the protein interaction site on PF4 sequence two shorter peptides, covering the N-terminal region, CXCL4 (47–58), and C-terminal region, CXCL4 (58–70), were synthesized and characterized by NMR. The analysis of the STD spectra of the two shorter fragments (Fig. 2) and the comparison with data obtained for the whole length CXCL4 (47–70), suggests that the C-terminal I, L, K rich region (residues 59–68) is the one mainly involved in direct FGF-2 interaction. The N-terminal fragment CXCL4 (47–58) maintains however the ability to interact with the protein through C52 and A57 side chains (Fig. 2D).

Altogether the NMR data describing CXCL4/FGF-2 interaction point to a major role of the hydrophobic contributions of the C-terminal region of CXCL4 (47–70), mediated by Y60 and leucines and/or isoleucine residues, in addition to specific contacts established by the N-terminal region through the cysteine side chain.

The observation that C52 side-chain contributes to CXCL4 (47–70) interaction with FGF-2 is in agreement with biological data on endothelial cells in which the C52S mutant does not compete for FGF-2 receptor binding or biological activity in all assays we have examined at concentrations up to 100 μM. This indicates that C52 is critical for the biological activity at least at the studied concentrations. The presence of a free cysteine seems to be important also for the biological activity of other proteins such as VEGF-C. Indeed, mutation of Cys in VEGF-C abolished its interaction with VEGFR2, only favoring binding to VEGFR3 [17].

NMR data clearly indicate the presence of direct interaction of CXCL4 (47–70) with FGF-2, however the question remains whether this interaction could represent the basis of the inhibition of FGF-2 biological activity evidenced in different assays [5]. Indeed binding studies with ^{125}I-FGF-2 in bovine capillary endothelial cells evidenced a CXCL4 (47–70) concentration dependent inhibition of FGF2-2 binding to the receptor with a maximum inhibition at 10 μM (half maximum inhibition 4 μM to low affinity binding sites and 2 μM to high affinity sites). This is in line with the inhibition of FGF-2 induced endothelial cell proliferation, migration and tube formation in vitro or ex vivo in the rat aortic ring assay. Furthermore, CXCL4 (47–70) inhibited FGF-2-induced signaling and

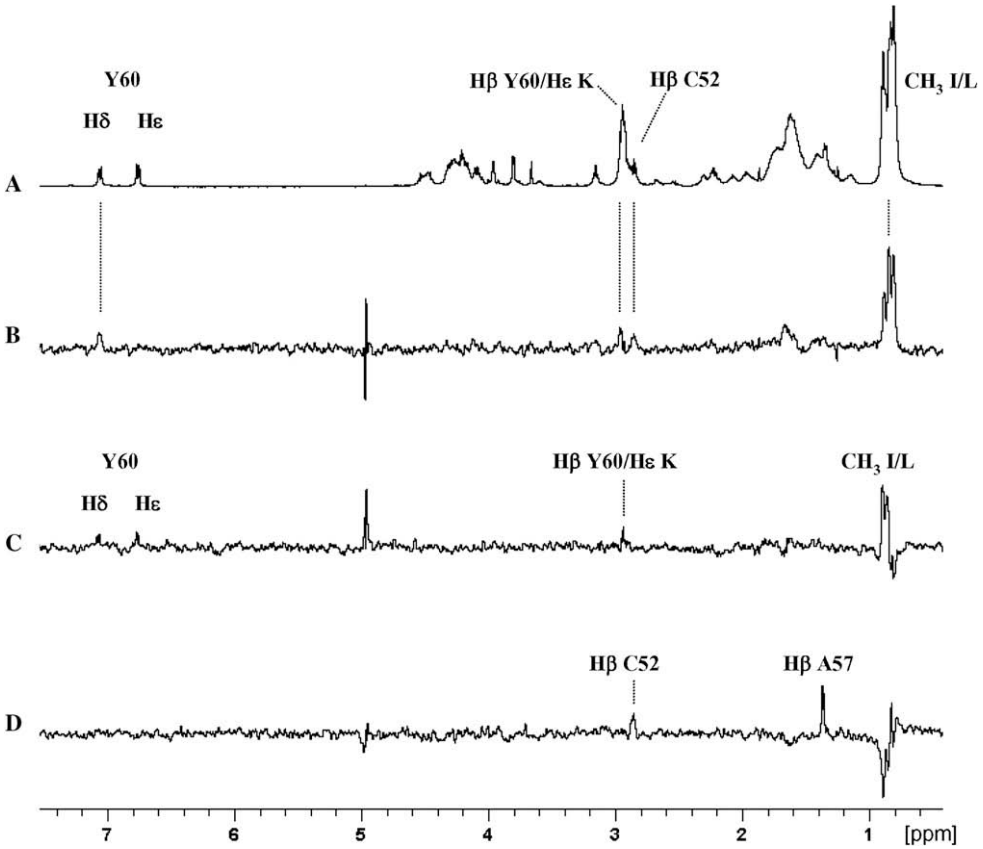

Fig. 2. STD NMR spectra of CXCL4 (47–70), CXCL4 (47–58) and CXCL4 (58–70) peptides in the presence of FGF-2. (A) Reference ^{1}H NMR spectrum of 0.9 mM CXCL4 (47–70) peptide in the presence of 43 μM FGF-2 in 30 mM buffer phosphate (95% D_2O, 5% H_2O), 4.2 mM DTT, 50 mM NaCl and pH 7.0 recorded at 280 K on a 500 MHz Bruker spectrometer. ^{1}H NMR STD spectra of CXCL4 (47–70) (B), CXCL4 (58–70) (C) and CXCL4 (47–58) (D) in the same experimental conditions of reference spectrum (21:1 peptide: FGF-2 ratio, 4.5 s irradiation at −236 Hz). The assignment of the STD signals is reported.

strongly inhibited the rate of ^{125}I-FGF-2 internalization in BCE cells in a time-dependent manner [4]. It is worth mentioning that up to now we could not evidence a direct interaction of CXCL4 (47–70) with FGF receptors (data not shown). Altogether these data reinforces the view that CXCL4 (47–70) inhibits FGF-2's biological activity by associating directly with the growth factor. However, this must be stated with caution since more recently a new variant of the chemokine receptor CXCR3, called CXCR3-B, able to interact with CXCL4 has been identified [10]. At present it is not established whether CXCL4 (47–70) is also able to interact with CXCR-3B.

NMR data indicate that CXCL4/FGF-2 interaction surface mainly involves aromatic (Y60) and hydrophobic side chains, located in the C-terminal region of CXCL4 (47–70) peptide (I63, I64, L67 and L68). Previous structural studies CXCL4 (47–70) pointed to the presence of an amphipathic helical structure in the C-terminal part of the peptide [12]. In this hypothesis the peptide side-chains identified by STD NMR studies would define a hydrophobic surface patch that could contact FGF-2 surface. The analysis of the crystal structure of FGF-2/FGFR complex [18] indicates that hydrophobic interactions dominate the interface between FGF-2 and D2 Ig-like domain of the receptor. Indeed, L165, A167, P169, and V248 of the D2 domain interact hydrophobically with the FGF-2 apolar residues Y24, F31, Y103, L140, and M142, which form a flat solvent-exposed hydrophobic surface. The mentioned FGFR residues are well conserved among the mammalian FGFRs, indicating that this hydrophobic interface represents a highly conserved interaction site for FGF family members [19]. On this basis, it seems possible to hypothesize that CXCL4 (47–70) peptide may exert its FGF-2 antagonist activity by mimicking, with Y60 and leucine and/or isoleucine residues, the hydrophobic ligand-binding region of D2, thus competing with FGFR for the binding to FGF-2.

Interestingly it has been previously reported that the CXCL4 mutant, lacking the lysine residues needed for heparin binding, retains the amphipathic alpha-helical structure, present at the carboxyl terminus in WT CXCL4 protein [8]. It is worth mentioning that this analogue lacks affinity for heparin but possesses potent angiostatic and anti-tumor activity. Our results are in agreement with these findings suggesting that the C-terminal portion of CXCL4 contains independent structural determinants for FGF-2 recognition and anti-angiogenic activity.

It is to emphasize that these data should be taken with caution with regard to full-length CXCL4. Indeed, CXCL4 (47–70) is structurally different from CXCL4 to some extent. In CXCL4 (47–70) two α-helices are present (residues 47–53 and residues 57–67) [12], while in full-length CXCL4 a single longer C-terminal α-helix is present at the level of residues 57–70. Furthermore, C52 is reduced in CXCL4 (47–70), but disulphide bonded in full-length CXCL4. However it is worth mentioning that the distal hydrophobic domain is conserved in full-length CXCL4 and thus could be involved in the interaction with FGF-2.

In conclusion, NMR results identified the amino acid residues involved in the direct interaction of CXCL4 (47–70) with FGF-2 and the proposed recognition mode constitutes a rationale for the observed effects of CXCL4 (47–70) on FGF-2 biological activity and lays the basis for the development of novel inhibitors of angiogenesis.

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References

[1] L. Lasagni, R. Grepin, B. Mazzinghi, E. Lazzeri, C. Meini, C. Sagrinati, F. Liotta, F. Frosali, E. Ronconi, N. Alain-Courtois, L. Ballerini, G.S. Netti, E. Maggi, F. Annunziato, M. Serio, S. Romagnani, A. Bikfalvi, P. Romagnani, PF-4/CXCL4 and CXCL4L1 exhibit distinct subcellular localization and a differentially regulated mechanism of secretion, Blood 109 (2007) 4127–4134.
[2] T.E. Maione, G.S. Gray, J. Petro, A.J. Hunt, A.L. Donner, S.I. Bauer, H.F. Carson, R.J. Sharpe, Inhibition of angiogenesis by recombinant human platelet factor-4 and related peptides, Science 247 (1990) 77–79.
[3] C. Perollet, Z.C. Han, C. Savona, J.P. Caen, A. Bikfalvi, Platelet factor 4 modulates fibroblast growth factor 2 (FGF-2) activity and inhibits FGF-2 dimerization, Blood 91 (1998) 3289–3299.
[4] V. Jouan, X. Canron, M. Alemany, J.P. Caen, G. Quentin, J. Plouet, A. Bikfalvi, Inhibition of in vitro angiogenesis by platelet factor-4-derived peptides and mechanism of action, Blood 94 (1999) 984–993.
[5] M. Hagedorn, L. Zilberberg, R.M. Lozano, P. Cuevas, X. Canron, M. Redondo-Horcajo, G. Gimenez-Gallego, A. Bikfalvi, A short peptide domain of platelet factor 4 blocks angiogenic key events induced by FGF-2, FASEB J. 15 (2001) 550–552.
[6] R.J. Sharpe, H.R. Byers, C.F. Scott, S.I. Bauer, T.E. Maione, Growth inhibition of murine melanoma and human colon carcinoma by recombinant human platelet factor 4, J. Natl. Cancer Inst. 82 (1990) 848–853.
[7] T. Tanaka, Y. Manome, P. Wen, D.W. Kufe, H.A. Fine, Viral vector-mediated transduction of a modified platelet factor 4 cDNA inhibits angiogenesis and tumor growth, Nat. Med. 3 (1997) 437–442.
[8] T.E. Maione, G.S. Gray, A.J. Hunt, R.J. Sharpe, Inhibition of tumor growth in mice by an analogue of platelet factor 4 that lacks affinity for heparin and retains potent angiostatic activity, Cancer Res. 51 (1991) 2077–2083.
[9] D.L. Kolber, T.L. Knisely, T.E. Maione, Inhibition of development of murine melanoma lung metastases by systemic administration of recombinant platelet factor 4, J. Natl. Cancer Inst. 87 (1995) 304–309.
[10] L. Lasagni, M. Francalanci, F. Annunziato, E. Lazzeri, S. Giannini, L. Cosmi, C. Sagrinati, B. Mazzinghi, C. Orlando, E. Maggi, F. Marra, S. Romagnani, M. Serio, P. Romagnani, An alternatively spliced variant of CXCR3 mediates the inhibition of endothelial cell growth induced by IP-10, Mig, and I-TAC, and acts as functional receptor for platelet factor 4, J. Exp. Med. 197 (2003) 1537–1549.
[11] P. Romagnani, L. Maggi, B. Mazzinghi, L. Cosmi, L. Lasagni, F. Liotta, E. Lazzeri, R. Angeli, M. Rotondi, L. Fili, P. Parronchi, M. Serio, E. Maggi, S. Romagnani, F. Annunziato, CXCR3-mediated opposite effects of CXCL10 and CXCL4 on TH1 or TH2 cytokine production, J. Allergy Clin. Immunol. 116 (2005) 1372–1379.
[12] R.M. Lozano, M. Redondo-Horcajo, M.A. Jimenez, L. Zilberberg, P. Cuevas, A. Bikfalvi, M. Rico, G. Gimenez-Gallego, Solution structure and interaction with basic and acidic fibroblast growth factor of a 3-kDa human platelet factor-4 fragment with antiangiogenic activity, J. Biol. Chem. 276 (2001) 35723–35734.
[13] L. Van den Berghe, I. Mortier, C. Zanibellato, F. Amalric, H. Prats, B. Bugler, FGF-2 dimerization involvement in growth factor mediated cell proliferation but not cell differentiation, Biochem. Biophys. Res. Commun. 252 (1998) 420–427.
[14] D.G. Bax, MLEV-17-based two-dimensional homonuclear magnetization transfer spectroscopy, J. Magn. Reson. 65 (1985) 355–360.
[15] T.L. Hwang, A.J. Shaka, Multiple-pulse mixing sequences that selectively enhance chemical exchange or cross-relaxation peaks in high-resolution NMR spectra, J. Magn. Reson. 135 (1998) 280–287.
[16] M. Mayer, B. Meyer, Group epitope mapping by saturation transfer difference NMR to identify segments of a ligand in direct contact with a protein receptor, J. Am. Chem. Soc. 123 (2001) 6108–6117.
[17] V. Joukov, V. Kumar, T. Sorsa, E. Arighi, H. Weich, O. Saksela, K. Alitalo, A recombinant mutant vascular endothelial growth factor-C that has lost vascular endothelial growth factor receptor-2 binding, activation, and vascular permeability activities, J. Biol. Chem. 273 (1998) 6599–6602.
[18] M. Mohammadi, S.K. Olsen, O.A. Ibrahimi, Structural basis for fibroblast growth factor receptor activation, Cytokine Growth Factor Rev. 16 (2005) 107–137.
[19] A.N. Plotnikov, S.R. Hubbard, J. Schlessinger, M. Mohammadi, Crystal structures of two FGF-FGFR complexes reveal the determinants of ligand-receptor specificity, Cell 101 (2000) 413–424.