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ATP-binding on Fibroblast Growth Factor 2 Partially Overlaps with the Heparin-binding Domain

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Fibroblast growth factor 2 (FGF2), an intensively studied heparin-binding cytokine, is an important modulator of cell growth and differentiation under both physiological and pathophysiological conditions. It has been shown recently that ATP binds to FGF2 and that this binding is crucial for its biological function. In this study we demonstrated that divalent cations were not necessary for binding of ATP to FGF2, but it could be demonstrated that heparin blocked the labelling of FGF2 with ATP indicating an involvement of the heparin-binding domain (aa 128–144) in ATP-binding. FGF2, bound to Heparin Sepharose, could be eluted with ATP and GTP, but not with cAMP, AMP or ADP. Successive mutation of positively charged amino acid residues located in the heparin-binding domain drastically reduced the signal intensity of [γ-^{32}P]ATP labelled FGF2 indicating that this domain is not only important for heparin binding to FGF2 but also for ATP-binding.

Key words: ATP, bFGF, complex, FGF2, heparin, heparin-binding domain.

Abbreviations: ADP, adenosine diphosphate; AMP, adenosine monophosphate; APAF-1, apoptotic protease factor 1; ATP, adenosine triphosphate; DTT, dithiotreithol; GTP, guanosine triphosphate; HSP90, heat shock protein 90; HSPG, heparin sulphate proteoglycans.

Fibroblast growth factor 2 (FGF2) affects growth, differentiation, migration and survival of various cell types (*1*). The protein has a secondary structure containing 12 anti-parallel ß-sheets, which are organized into a trigonal pyramidal structure. FGF2 contains a large number of basic residues resulting in a pI of 9.6 (*2*). Domains and sites for FGF2-heparin-binding (*3*, *4*), FGF2 dimerization (*5*, *6*), FGF2 receptor-binding (*5*, *7*, *8*) and phosphorylation of serine and threonine residues by protein kinases A and C (*9*) have been identified. The ability of FGF2 to bind ATP proved by autoradiography and mass spectrometry has been described previously (*10*, *11*).

Growth factors like FGF2 or vascular endothelial growth factor (VEGF) are referred to as heparin-binding growth factors because their biological functions are mainly maintained by heparin and heparan sulphate proteoglycans, respectively. Heparin and heparan sulphate proteoglycans are linear sulphated polysaccharides known as glycosaminoglycans (*12*). The binding of these glycosaminglycans to FGF2 induces a small but highly reproducible conformational change (*3*), and it can facilitate the self-association of FGF2 monomers into dimers and oligomers (*12–14*). Heparan sulphate proteoglycans are required for effective activation of FGF-receptors (FGFR) by their ligands; until now not only the complexes of FGF2 with FGFR1 and FGFR2 but also a ternary complex of FGF2, FGFR1 and heparin have been crystallized: heparin bridges two FGFR1 molecules to form FGFR1 dimers, which bind two FGF2 molecules (*15*). On the one hand, heparin stabilizes the binding of FGF2 to the D2-domaine of FGFR1, on the other hand, heparin forces dimerization of the receptors. A heparin-binding site on FGFR1 also has been identified providing additional evidence that a ternary complex of FGF2, FGFR1 and heparan sulphate proteoglycans exists (*16*).

Besides heparin or heparan sulphate proteoglycans, it has been proposed that binding of ATP to some growth factors is necessary for the biological activity of these growth factors (*10*). Recently, lysine 134 of the heparin-binding domain (aa 128–144) of FGF2 was identified to be essential for heparin-binding and neuroprotective activity of this growth factor (*17*). Thompson *et al*. identified the amino acid residues on the surface of the mature FGF2 protein which are important for heparin-binding (*4*). The linear sequence in FGF2 from amino acid number 128–144 accounts for 76% of the binding free energy, the residues R129 and K134 were in direct contact with sulphate anions of heparin.

In this study we examined the character of ATP-binding to FGF2 and the role of the heparin-binding domain for ATP/FGF2-complex formation.

MATERIALS AND METHODS

Expression and Purification of FGF2—Plasmid pET16b::FGF2, which directs a His-tag sequence, MetGly(His)$_{10}$, at the N-terminus, was used to overexpress FGF2 in *Escherichia coli* BL21(DE3) according

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to (*17*). In brief, for overexpression of FGF2 protein, *E. coli* BL21(DE3) harbouring pET16b::FGF2 was incubated until OD600 = 0.7. Then 1 mM IPTG was added to induce expression and incubation was continued for 4 h. Cells were collected by centrifugation (3,000*g*, 15 min, 4°C), sonicated and FGF2 was isolated from the soluble extract. This protein solution was loaded onto BioRad Econo-columns (BioRad, Hercules, CA, USA) with Ni^{2+}-NTA Agarose (Qiagen, Hilden, Germany) and eluted with buffer containing 250 mM imidazol according to the suppliers handbook. Protein containing fractions were pooled (6–7 ml) and dialysed three times against 25 mM Tris–HCl, pH 7.5. This solution was concentrated 10-fold by using Amicon Ultra centrifugal filter devices (10,000 Da molecular weight cutoff; Millipore Corporation, MA, USA). Glycerol (15%) and 0.1 mM DTT were added after ultraconcentration, and FGF2 was stored frozen in aliquots at −80°C. Site-directed mutants of FGF2 were purified accordingly.

Site-directed Mutagenesis—Site-directed mutagenesis was performed according to the protocol of the Quick Change site-directed mutagenesis kit (Stratagene, Heidelberg, Germany) using pET16b::FGF2 as template and the following primer pairs: 5′-CTGAAACGTACCGG TCAGTATGCATTGGGTTCGAAAACCGGT-3′ and 5′-GG ACCGGTTTCGAACCCAATGCATACTGACCGGTACGTT TCAG-3′ (K134A); 5′-CGTTGCTCTGAAAGCTACCGGTC AGTAC-3′ and 5′-GTACTGACCGGTAGCTTTCAGAGCA ACG-3′ (R129A in K134A-mutant); 5′-GGTACGTTGCTC TGGCAGCTACCGGTCAG-3′ and 5′-CTGACCGGTAGCT GCCAGAGCAACGTACC-3′ (K128A in R129A/K134A-double-mutant); 5′-CTGGGTTCGGCAACCGGTCCGGGT CAG-3′ and 5′-CTGACCCGGACCGGTTGCCGAACCCA G-3′ (K138A in K128A/R129A/K134A-triple-mutant); 5′-GGTCCGGGTCAGGCAGCTATCCTGTTCC-3′ and 5′-G GAACAGGATAGCTGCCTGACCCGGACC-3′ (K144A in K128A/R129A/K134A). Mutated pET16b::FGF2 was subcloned in *E. coli* BL21(DE3). Correctness of each mutation was verified by sequence analysis of the DNA fragment encompassing the mutation.

[γ-^{32}P]ATP and [α-^{32}P]ATP Labelling of FGF2—FGF2 (1.5 μg) was incubated in a volume of 15 μl containing 25 mM Tris–HCl, pH 7.5 and 6 μCi [γ-^{32}P]ATP or [α-^{32}P]ATP (GE Healthcare, Munich, Germany) (*10*). In some experiments 1 mM EDTA, 1 mM EGTA, 1 mM phenantroline, 1 μg/ml or 10 μg/ml heparin (from porcine intestinal mucosa, molecular mass ~6,000; Sigma, Taufkirchen, Germany) was added. Reactions were stopped after incubation for 15 min at 37°C by adding 5 μl sample buffer (130 mM Tris–HCl, pH 6.8, 10% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.06% bromphenol blue). Proteins were separated by 15% sodium dodecyl sulphate-polyacrylamide mini-gel electrophoresis, the gels were dried and finally, phosphorylation was detected by autoradiography or phosphoimaging using Fujifilm BAS-1800 II imager (Fuji, Japan).

2D Densitometry—Signal intensity was determined by 2D densitometry using AIDA Image Analyser software Version 3.21.001 (Raytest GmbH, Straubenhardt, Germany).

Heparin Sepharose Chromatography of FGF2—BioRad Econo-columns (BioRad, Hercules, Ca, USA) were packed with 2 ml Heparin Sepharose® 6 Fast Flow (GE Healthcare, Munich, Germany) and equilibrated with 25 mM Tris–HCl, pH 7.5. FGF2 (300 μg) was loaded onto the column and washed with 6 ml 25 mM Tris–HCl, pH 7.5 three times. FGF2 was eluted with 3 ml washing buffer containing different concentrations of nucleotides (5 or 50 mM, respectively) or washing buffer containing 150 mM NaCl. The molarity of the washing-buffer containing NaCl was calculated to give the same ionic strength as the nucleotide buffer with the highest ionic strength according to the following equation:

$$I = \frac{1}{2} * \sum (n_i)^2 * C_i$$

(with C_i, concentration; i, type of ion; n_i, charge). The eluate was concentrated using Amicon Ultra centrifugal filter devices (10,000 Da molecular weight cutoff; Millipore Corporation, MA, USA), the protein was separated by 15% SDS–PAGE and visualized by silver staining (*18*).

RESULTS

Binding of ATP to FGF2 is Independent of Divalent Cations—Previous work has shown that ATP can bind to FGF2 (*10*, *11*). Here we report that binding of ATP to FGF2 was also observed in the presence of chelators, which results in negligible concentrations of free divalent cations (Fig. 1). One millimolar EDTA, 1 mM EGTA and 1 mM phenantroline did not prevent labelling of FGF2 by [γ-^{32}P]ATP. These experiments demonstrate that ATP is associated with FGF2 in a non-metal-chelated form and that binding of divalent ions to FGF2 is not necessary for ATP/FGF2-complex formation. Consistent with these findings, addition of 0.1 mM Mg^{2+} did not increase the intensity of the labelling (data not shown). One millimolar Mg^{2+} marginally reduces the signal intensity indicating that inhibitory concentrations of divalent cations were attained (Fig. 1). These experiments point out that binding of the phosphate groups of ATP to FGF2 is based on direct coordination of positively charged amino acid residues (*e.g.* lysine, arginine) rather than on a bridging of cations between negatively charged amino acids of FGF2 and ATP.

Heparin Inhibits Binding of ATP to FGF2—The physiologically most important heparin-binding domain in the FGF2 molecule is located between amino acid residues 128–144 (*4*). Lysine 134 in this binding domain

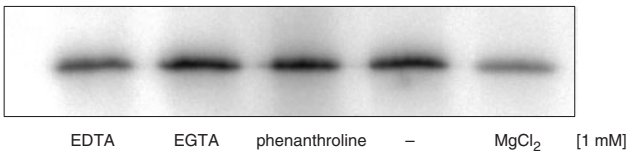

Fig. 1. **Binding of ATP to FGF2 is independent of divalent cations.** 1.5 μg of FGF2 was incubated in a volume of 15 μl containing 25 mM Tris–HCl, pH 7.5 including 1 μM of the divalent cation chelators and 6 μCi [γ-^{32}P]ATP at 37°C for 15 min. Reactions were stopped by adding 5 μl sample buffer. Proteins were separated by 15% SDS–polyacrylamide mini-gel electrophoresis, the gels were dried, and finally, ATP labelling detected by phosphoimaging.

is essential for FGF2/heparin interaction and essential for neuroprotective activity of the growth factor (*17*). Addition of heparin to the *in vitro* reaction of [γ-^{32}P]ATP or [α-^{32}P]ATP with FGF2 resulted in significant reduction of signal intensity analysed by autoradiography (Fig. 2). Heparin at a concentration of 1 μg/ml strongly reduced the formation of an ATP/FGF2-complex. This phenomenon could be observed both with [γ-^{32}P]ATP and [α-^{32}P]ATP. In contrast, mutant FGF2(K134A) did not exhibit signal reduction with 1 μg/ml heparin, indicating that the low binding affinity of heparin to this mutant, harbouring a mutation located in the heparin-binding domain (*17*), did not influence ATP association as strong as with FGF2. After addition of 10 μg/ml heparin an inhibition of the signal intensity could be observed with FGF2(K134A), too (Fig. 2).

Competition of ATP and Heparin at the FGF2 Binding Site—The absence of classic ATP-binding sites and clear evidence for ATP-binding to FGF2 (*10*, *11*, *17*) indicates an unusual ATP-binding domain of FGF2. The dominant heparin-binding domain (aa 128–144) of FGF2 consists of positively charged amino acids (K128, R129, K134, K138 and K144), which possibly could bind to one or more phosphate groups of nucleotides (*4*, *19*). Therefore, we hypothesized that this binding domain is not only able to bind heparin but is also able to bind to or, at least, is involved in the binding of ATP or other nucleotides. In a chromatographic approach we analysed the elution pattern of FGF2 from Heparin Sepharose with different nucleotides at different concentrations. In a first approach, 300 μg FGF2 was loaded onto Heparin Sepharose and was retained via its heparin-binding domain. Buffer containing 150 mM NaCl did not elute FGF2 from the column (Fig. 3A), whereas FGF2 could be eluted with 50 mM ATP (pH 7.5). The ionic strength of the NaCl-buffer (150 mM NaCl) was identical to the ionic strength of the nucleotide buffer (50 mM ATP), so that ionic interactions, leading to unspecific elution of the protein, can be excluded. In a second approach, FGF2 was eluted successively from Heparin Sepharose by different nucleotides (50 mM, respectively). Figure 3B shows that the growth factor can be eluted from Heparin Sepharose solely with nucleoside triphosphates (Fig. 3A: ATP; Fig. 3B: GTP). It was not possible to elute FGF2 with cAMP, AMP or ADP, respectively. In addition, incubation with 6 μCi [γ-^{32}P]ATP and 1 mM non-radioactive ATP inhibited radiolabelling of the growth factor after gel electrophoresis and phosphoimaging (data not shown) indicating that radioactive ATP is displaced by untagged ATP.

Mutagenesis of the Heparin-binding Domain of FGF2 Reduces ATP-binding—To investigate the importance of positively charged amino acids in the heparin-binding domain of FGF2 (aa 128–144) for ATP-labelling, we performed site directed mutagenesis and successively replaced all lysine and arginine residues with alanine. The single point mutation K134A in FGF2 resulted in a marginal decrease of [γ-^{32}P]ATP-labelling, whereas the double mutation of FGF2(K134A/R129A) resulted in 50% reduction of the signal strength (Fig. 4). Residues R129 and K134 were in direct contact with sulphate anions of heparin (*19*), therefore it might be possible, that these residues were also able to interact with the negatively charged phosphate groups of ATP. A drastic reduction of the autoradiographic signal intensity could be observed with the triple-mutant FGF2(K128A/R129A/K134A). The signal intensity was reduced to ~24% compared

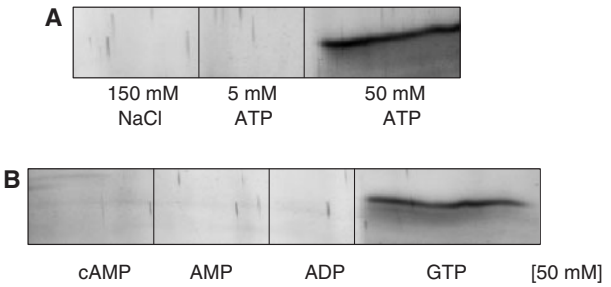

Fig. 3. **Heparin Sepharose chromatography of FGF2.** Columns were packed with 2 ml heparin Sepharose 6 Fast Flow and equilibrated with 25 mM Tris–HCl, pH 7.5. Protein solution (300 μg FGF2) was loaded onto the column and washed with 6 ml 25 mM Tris–HCl, pH 7.5 three times. FGF2 was eluted with 3 ml washing buffer containing 5 or 50 mM ATP (A) or 50 mM cAMP, AMP, ADP and GTP (B), respectively, or washing buffer containing 150 mM NaCl. The eluate was concentrated using Amicon Ultra centrifugal filter devices and the protein was separated by 15% SDS–polyacrylamid mini-gel electrophoresis and visualized by silver staining.

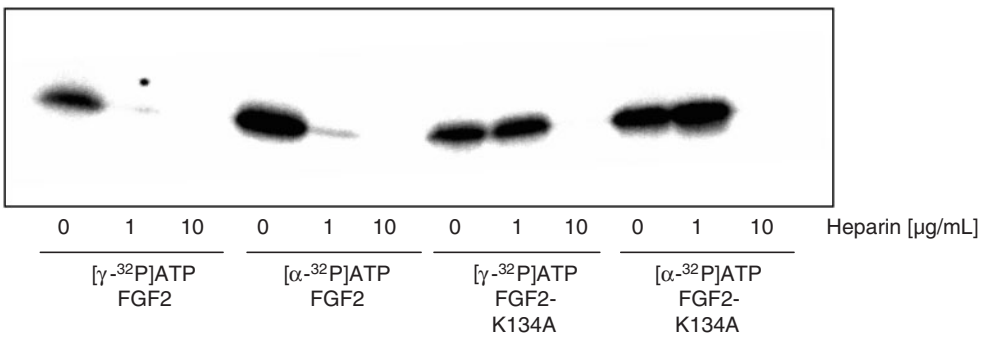

Fig. 2. **Effect of heparin on [α-^{32}P]ATP-labelling and [γ-^{32}P]ATP-labelling of FGF2 and FGF2(K134A).** FGF2 and FGF2(K134A) (1.5 μg, respectively) were incubated in 25 mM Tris–HCl (pH 7.5) with 6 μCi [α-^{32}P]ATP or [γ-^{32}P]ATP including heparin at indicated concentrations at 37°C for 15 min. Proteins were separated by 15% SDS–polyacrylamide mini-gel electrophoresis, the gels were dried, and finally, phosphorylation was detected by phosphoimaging.

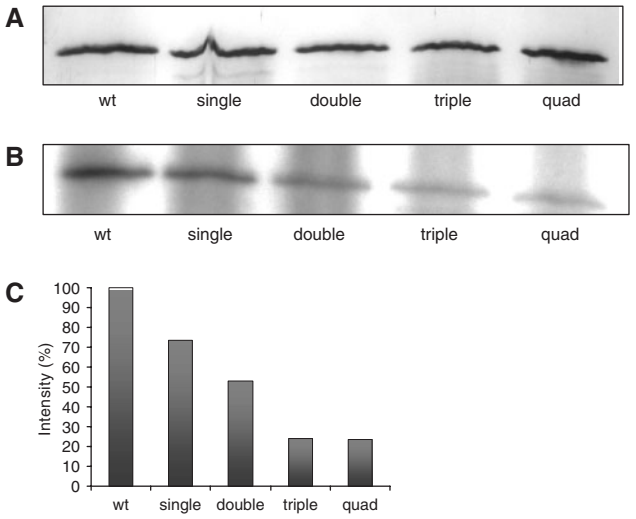

Fig. 4. **Intensity of autoradiographic signals of different FGF2-variants with mutations in the heparin-binding domain of FGF2 labelled with [γ-^{32}P]ATP.** (A) Silver staining of FGF2 and FGF2-variants (1.5 μg, respectively). (B) [γ-^{32}P]ATP-labelling of FGF2 and FGF2-variants (1.5 μg, respectively) carried out for 15 min as described in MATERIALS AND METHODS section. (C) Intensity of the autoradiographic signals was determined by AIDA Image Analyser 2D Densitometry software (Raytest GmbH, Straubenhardt, Germany). The signal intensity of wt-FGF2 was set to 100%. Wt, FGF2; single, FGF2(K134A); double, FGF2(R129A/K134A); triple, FGF2(K128A/R129A/K134A); quad, FGF2(K128A/R129A/K134A/K138A).

with FGF2, indicating that these three amino acid residues were not only important for heparin-binding (*4*, *17*) but also for formation of the FGF2/ATP-complex. These results are in good accordance with the results of the Heparin Sepharose elution experiments (previous section). The quad-mutants FGF2(K128A/R129A/K134A/K138A) (Fig. 4) and FGF2(K128A/R129A/K134A/K144A; data not shown) did not show an advanced signal reduction compared to the triple-mutant, indicating that lysine 138 and lysine 144 are assumed to be only marginally involved in ATP-binding to FGF2.

DISCUSSION

Besides two large groups of ATP-binding proteins, the kinases and the ATPases with more or less defined ATP-binding domains (Walker A, Kinase 1 or Kinase 2), some other ATP-binding proteins are described in the literature, *e.g*. hormones, metallothionein, heat shock proteins, chaperons and actins (*20–24*). Growth factors have been identified as ATP-binding proteins quite recently (*10*, *11*). Nerve growth factor (NGF), brain-derived growth factor (BDNF) and FGF2 are associated with ATP and are probably biologically active only if ATP is bound.

In vivo, ATP competes with heparan sulphate proteoglycans (HSPGs) for binding to FGF2 in the extracellular matrix, where the concentration of HSPGs is high. However, at least after tissue injury or after anoxic depolarization in the course of cerebral ischemia high ATP release and a high local ATP-concentration could be observed (*25*, *26*).

In contrast to the situation for most kinases, divalent cations are not necessary for ATP-binding to FGF2 (Fig. 1): the binding of ATP to FGF2 is independent from the availability of cations like magnesium- or manganese-ions, which has been demonstrated by the addition of different chelators. Therefore, it is more likely that ATP is bound to FGF2 via direct interaction of positively charged amino acids of FGF2 with negatively charged phosphate groups of ATP rather than interaction of negatively charged amino acids with phosphate groups of ATP via cations. However, unconsidered in this context are interactions of FGF2 with ribose or adenine-units of ATP, which might additionally participate in binding (*27*).

First hints that the heparin-binding domain of FGF2 [aa 128–144, (*4*)] is involved in ATP-binding have been derived by experiments in which heparin at concentrations of 1 μg/ml abolishes the labelling of FGF2 with [γ-^{32}P]ATP as shown here. FGF2(K134A), which does not bind heparin (*17*), is labelled with ATP not only at heparin-concentrations of 0 μg/ml but also at heparin-concentrations of 1 μg/ml. This indicates that heparin can abolish the labelling of FGF2 by ATP presumably by blocking the binding domain for ATP of FGF2 or by dimerization of two FGF2 molecules. Furthermore, labelling of FGF2 with [α-32P]ATP as well as [γ-^{32}P]ATP demonstrated binding of ATP to FGF2 and give no hints for hydrolysis of ATP after binding as described for other ATP-binding proteins like HSP90 (*28*) or APAF-1 (*29*). Addition of 1 mM non-radioactive ATP to FGF2 incubated with [γ-^{32}P]ATP eliminates radiolabeling of the growth factor (data not shown) demonstrating reversibility of complex formation. The ATP/FGF2 complex remains stable upon SDS–PAGE analysis as long as the temperature is lower than 95°C.

The competition of ATP and heparin for the heparin-binding domain of FGF2 has also been shown by different elution experiments of FGF2 from Heparin Sepharose. Heparin Sepharose is widely used in chromatography to purify heparin-binding proteins. To elute such proteins from Heparin Sepharose colums, high concentrations of NaCl (*e.g*. 2 M) are used to generate high ionic strength leading to separation of the proteins from the matrix. In this study we used concentrations of ATP up to 50 mM to elute FGF2 from Heparin Sepharose colums. Interestingly, a solution of NaCl (150 mM) with the same ionic strength as a 50 mM ATP-solution, did not lead to elution of the protein from the column (Fig. 3A). These points out that heparin and FGF2 compete for the same binding domain or at least for parts of it. FGF2 also can be eluted from Heparin Sepharose with GTP (Fig. 3B), but not with ADP or AMP. The existence of three phosphate residues seems to be important for binding of nucleotides to FGF2. Two phosphate residues (ADP) or one residue (AMP) are not sufficient for elution of FGF2 from Heparin Sepharose.

Mutants of FGF2 with positively charged amino acids of the heparin-binding domain exchanged against uncharged residues were analysed for their [γ-^{32}P]ATP-binding behaviour. Double- and triple-mutants of important residues (R128, K129, K134) of the heparin-binding domain of FGF2 revealed a significantly reduced signal intensity that indicates a reduced ATP-binding ability

and thus a similarity of the mechanisms by which FGF2 binds to ATP or heparin, where positively charged amino acids play an important role and pointing out a presumed 'dual use'-domain for heparin- and ATP-binding to FGF2.

This study presents data that ATP and heparin compete for a more or less common binding site on the FGF2 molecule. In addition, the results shed some light on the role of heparin and the 'new player' ATP in FGF2 activity.

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