Biochemical characterization of the molecular interaction between recombinant basic fibroblast growth factor and a recombinant soluble fibroblast growth factor receptor

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The extracellular domain of human fibroblast growth factor receptor (XC-FGF-R) was expressed in *Escherichia coli*. The protein was purified to homogeneity and the interaction with basic fibroblast growth factor (bFGF), its physiological ligand, was examined. Using resins on which bFGF was reversibly bound, we analysed the characteristics of the binding between XC-FGF-R and immobilized bFGF. We also investigated the stoichiometry of the binding between XC-FGF-R and recombinant human bFGF (rhbFGF) applying non-denaturing gel electrophoresis, chemical cross-linking followed by SDS/PAGE, and gel-filtration chromatography. In cross-linking

and gel-filtration chromatography experiments, a 1:1 complex between rhbFGF and XC-FGF-R was observed. The complex was separated from the non-complexed proteins using non-denaturing PAGE in the presence of 0.1 % Triton X-100. The band corresponding to the complex was recognized by specific antibodies directed against bFGF and its receptor, blotted on poly(vinylidene difluoride) membranes and submitted to sequence and amino acid analysis. The data obtained from these determinations confirmed the formation of a 1:1 complex between rhbFGF and XC-FGF-R.

INTRODUCTION

The fibroblast growth factors (FGFs) are a family of biologically and structurally related proteins that stimulate cell proliferation, cell differentiation and cell migration [1,2]. To date, seven distinct gene products of this family have been isolated including acidic FGF [3], basic FGF (bFGF) [4], the *int-2* oncogene-related protein [5], Kaposi sarcoma-related FGF [6], FGF-5 [7], FGF-6 [8] and FGF-7 [9]. Recently, the three-dimensional structure of the FGFs has been reported [10–12] but the mechanism of the interaction between FGFs and their receptors, as well as the role of polysaccharides or related molecules, is not yet clarified.

The FGF receptors (FGF-Rs) also seem to be encoded by a gene family. The receptor for FGF was originally purified and cloned from chicken [13] and it was shown to be a member of the tyrosine-kinase-receptor family [14–19]. To date, four molecular variants of FGF-R have been identified. They share a similar molecular topology with immunoglobulin-like loops in the extracellular domain, a single hydrophobic transmembrane region and a conserved cytoplasmic tyrosine kinase domain split by a short kinase insert. Interestingly other receptors of growth factors and cytokines involved in the regulation of cell growth, such as colony-stimulating factor 1 receptor [20], platelet-derived growth factor (PDGF) α receptor [21], PDGF β receptor [22], interleukin-1 receptor [23] and interleukin-6 (IL-6) receptor [24], showed that the extracellular part of the molecule was organized in immunoglobulin-like domains.

We previously expressed, purified and characterized recombinant forms of human bFGF [25–28]. Recently, we

reported the cloning [29], the expression and the characterization of the recombinant extracellular domain of an FGF-R [30].

The association between the extracellular domain of a human FGF-R (XC-FGF-R) and labelled bFGF was also investigated and a K_d value of 5–10 nM was calculated [30]. In the present work, the complex formation between recombinant human bFGF (rhbFGF) and the recombinant soluble XC-FGF-R was studied, applying different and complementary techniques. The stoichiometry and kinetics of the binding, as well as the influence of different experimental parameters, were also investigated. To reduce the possible artifactual problems due to the presence of labelled materials, the experiments were mainly performed using unlabelled molecules and avoiding the use of chemicals which could significantly modify the structure and functional features of the proteins. Knowledge of the molecular interaction between bFGF and its receptor is key to the rational design of new agonists and antagonists of angiogenesis.

MATERIALS AND METHODS

Recombinant bFGF and XC-FGF-R

A homogeneous 146-amino-acid form of rhbFGF was prepared as described previously [26]. Briefly, the cDNA encoding the 155-amino-acid form of bFGF was inserted into a plasmid used to transform an *E. coli* type-B strain. After fermentation and purification an equimolar mixture of a 154-amino-acid form and a 153-amino-acid form of rhbFGF was recovered. A homogeneous 146-amino-acid form was obtained after a controlled enzymic proteolysis of the mixture.

Abbreviations used: FGF, fibroblast growth factor; rhbFGF, recombinant human basic FGF; FGF-R, FGF receptor; XC-FGF-R, extracellular domain of human FGF receptor; DSS, disuccinimidyl suberate; BS³, bis(sulphosuccinimidyl) suberate; BSOCOES, bis[2-(succinimido-oxycarbonyloxy)-ethyl]sulphone; DTSSP, 3,3'-dithiobis(sulphosuccinimidyl) propionate; CAPS, 3-(cyclohexylamino)-1-propanesulphonic acid; PVDF, poly(vinylidene difluoride); PTH, phenylthiohydantoin; PDGF, platelet-derived growth factor; TEMED, *N,N,N',N'*-tetramethylethylenediamine; IL-2, interleukin 2; IL-6, interleukin 6; EGF, epidermal growth factor.

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The 356-amino-acid XC-FGF-R was expressed in *E. coli* and purified to homogeneity using a process described previously [30].

The protein concentration of the different samples was always referred to amino acid analysis.

Immobilization of bFGF on thiopropyl-Sepharose or heparin-Sepharose

Thiopropyl-Sepharose 6B resin (Pharmacia, Uppsala, Sweden) (1.5 ml) equilibrated with 125 mM NaCl/5 mM EDTA in 25 mM phosphate buffer, pH 7.0 (equilibration buffer), was incubated at 25 °C for 5 h with 300 µg of rhbFGF dissolved in the same buffer and then washed with 30 ml of this equilibration buffer. XC-FGF-R (200 µg) was added to FGF-thiopropyl-Sepharose, incubated overnight at 4 °C and the resin was extensively washed with the equilibration buffer. Aliquots of the resin were subsequently treated with SDS/PAGE sample buffer (with or without reducing agents) and electrophoresed on a 12.5 % polyacrylamide gel.

In other experiments, heparin-Sepharose resin (Pharmacia, Uppsala, Sweden) (1 ml) suspended in the equilibration buffer, was incubated with 200 µg of rhbFGF dissolved in the same buffer at 25 °C for 2 h. The resin was washed with 20 ml of the same buffer, incubated with 100 µg of XC-FGF-R overnight at 4 °C and washed with the equilibration buffer. In this case, aliquots of the resin were treated with reducing sample buffer before SDS/PAGE analysis.

Cross-linking

The cross-linking reactions were performed using disuccinimidyl suberate (DSS), bis(sulphosuccinimidyl) suberate (BS³), bis[2-(succinimido-oxy-carbonyloxy)ethyl]sulphone (BSOCOES), 3,3'-dithiobis(sulphosuccinimidyl) propionate (DTSSP) purchased from Pierce Chemical Co. rhbFGF and XC-FGF-R were incubated at different times, temperatures and concentrations in PBS. DSS or BSOCOES were dissolved immediately before the cross-linking reaction (100× solution in dimethyl sulphoxide) and added to a final concentration of 1 mM. In some experiments a 100 mM solution of BS³ or DTSSP in PBS was added to the incubation mixture between rhbFGF and its soluble receptor to a final concentration of 1 mM. The mixture was then kept on ice for 30 min and the reaction was stopped by the addition of an equal volume of 100 mM Tris/HCl/1 mM EDTA, pH 7.4, (DSS, BS³, DTSSP) or one-tenth of the volume of 1 M ammonium acetate (BSOCOES). The reaction products were then submitted to SDS/PAGE analyses.

SDS/PAGE

Samples were analysed by SDS/PAGE using 12.5 % polyacrylamide gels stained with Coomassie Blue R-250 or silver staining. If not specified, electrophoresis sample buffer did not contain reducing agents and the different samples were incubated at 25 °C for 15 min. In some experiments samples were boiled at 95 °C for 5 min in a buffer containing 1 % (v/v) 2-mercaptoethanol.

Immunoblotting

The proteins were subjected to SDS/PAGE, electrophoretically transferred to a nitrocellulose membrane and subjected to immunoblotting using polyclonal rabbit anti-(human FGF-R)

antibodies (provided by Dr. P. Maher, Whittier Institute, La Jolla, CA, U.S.A.) as previously described [30] or using polyclonal rabbit anti-(human bFGF) antibodies. Vectastain ABC kits (Vector Laboratories, Burlingame, CA, U.S.A.) containing biotinylated goat anti-(rabbit IgG) as secondary antibodies were used, as recommended by the manufacturers.

Non-denaturing gel electrophoresis

To analyse the formation of the rhbFGF-XC-FGF-R complex, aliquots of XC-FGF-R and rhbFGF were incubated at different times, temperatures and concentrations and treated with the sample buffer (4×) containing 0.4 % Triton X-100, 20 % (v/v) glycerol, 4 mM EDTA, and 125 mM Tris/HCl, pH 6.8. Samples were then subjected to non-denaturing electrophoresis using a resolving gel containing 10 % (w/v) acrylamide, 0.33 % bisacrylamide, 0.1 % ammonium persulphate, 0.1 % *N,N,N',N'*-tetramethylethylenediamine (TEMED), 0.1 % Triton X-100 in 375 mM Tris/HCl buffer, pH 8.8. The stacking gel contained 4.5 % (w/v) acrylamide, 0.15 % bisacrylamide, 0.07 % ammonium persulphate, 0.1 % TEMED, and 0.1 % Triton X-100 in 125 mM Tris/HCl buffer, pH 6.8. The electrode buffer contained 0.1 % Triton X-100 in 25 mM Tris/glycine buffer, pH 8.5. The gel was prerun at 4 °C for 1 h at 100 V and after sample loading electrophoresis was carried out at 4 °C for 5 h at 100 V. Gels were either stained with Coomassie Blue R-250 or electroblotted on nitrocellulose or poly(vinylidene difluoride) (PVDF) membranes.

Gel-filtration chromatography

XC-FGF-R, rhbFGF, molecular-mass markers (Pharmacia, Uppsala, Sweden) and the mixtures between rhbFGF and XC-FGF-R were subjected to size-exclusion chromatography on a Superose 6 HR 10/30 column (Pharmacia) equilibrated in 250 mM NaCl/25 mM phosphate buffer, pH 7.5. The column was eluted with the same buffer at a flow rate of 0.4 ml/min and the protein detected by recording the absorbance at 218 nm. A calibration curve was drawn on the basis of the K_{av} of the molecular-mass markers calculated according to the formula $K_{av} = (V_e - V_0)/(V_t - V_0)$ where V_e is the elution volume of the different proteins, V_0 is the void volume and V_t is the bed volume. The K_{av} of rhbFGF, XC-FGF-R and the complex formed by the two proteins was evaluated and the molecular mass estimated.

N-terminus and amino acid analysis

PVDF membranes, rinsed with methanol, were equilibrated with 10 mM 3-(cyclohexylamino)-1-propanesulphonic acid (CAPS), pH 11, in 10 % (v/v) methanol. Protein bands were transferred from gel at 25 °C by applying 150 mA for 45 min and stained with Ponceau S.

Blotted proteins were cut from the PVDF membrane and the N-terminus sequence analysis was performed on a pulsed liquid-phase sequencer model 477A (Applied Biosystems, Foster City, CA, U.S.A.) with an on-line phenylthiohydantoin (PTH) amino acid analyser.

Amino acid analysis was also conducted on PVDF membranes containing blotted protein bands. Briefly, after hydrolysis of the proteins on PVDF with 6 M HCl, the hydrolysate was derivatized with phenylisothiocyanate. All the final steps of the analysis were performed with an Applied Biosystems model 420A derivatizer equipped with an on-line model 130A h.p.l.c.

On the basis of the results of the amino acid analysis, the stoichiometry of the rhbFGF-XC-FGF-R complex was de-

terminated using a computer program written in BASIC [31] with few modifications.

RESULTS

XC-FGF-R binding to immobilized rhbFGF

We previously demonstrated [27] that all four cysteine residues of rhbFGF are present in the molecule in a completely reduced form. Of these residues only two are exposed on the surface of the protein and can be easily modified by the chemical reagents specific for the thiol groups without affecting the biological

activities. Taking into account this particular property of rhbFGF, we immobilized the molecule on thiopropyl-Sepharose, a resin with activated groups that can specifically bind the free thiols of other molecules.

On the other hand, bFGF binds non-covalently to polysaccharides and particularly to heparin with a high affinity and this peculiarity has allowed purification of the molecule with good yields and at a high degree of purity [25].

These properties have been used to selectively bind rhbFGF by disulphide bridges (thiopropyl-Sepharose) or through strong affinity binding (heparin-Sepharose) to a solid-phase system. The resins carrying rhbFGF were then used to test the ability of the purified XC-FGF-R to bind its ligand.

Aliquots of the two resins carrying the rhbFGF-XC-FGF-R complex were analysed using SDS/PAGE. XC-FGF-R was able to bind rhbFGF immobilized both on heparin-Sepharose (Figure 1, lane 3) or on thiopropyl-Sepharose (Figure 2, lanes 3 and 8). It is worth noting that when the cysteine residues of the rhbFGF were blocked through disulphide bridges to the resin, XC-FGF-R still retained the capability to bind its immobilized ligand. This observation suggests that the cysteines of rhbFGF and probably the adjacent regions of the molecule are not important for the interaction with its receptor.

On the other hand, since XC-FGF-R was able to bind heparin-Sepharose only when the resin was pre-incubated with rhbFGF, the receptor-binding site and the heparin-binding site should be located in different regions of the rhbFGF molecule.

Figure 1 Binding of XC-FGF-R to rhbFGF immobilized on heparin-Sepharose resin

Aliquots of heparin-Sepharose resin incubated with XC-FGF-R (lane 1) and rhbFGF (lane 2) were treated with reducing sample buffer and submitted to SDS/PAGE analysis on a 12.5% polyacrylamide gel. In lane 3, aliquots of the resin were first incubated with rhbFGF, then with XC-FGF-R and finally treated with reducing electrophoresis sample buffer before analysis. rhbFGF and XC-FGF-R standards were run in lanes 4 and 5 respectively.

Figure 2 Binding of XC-FGF-R to rhbFGF immobilized on thiopropyl-Sepharose resin

XC-FGF-R was loaded onto thiopropyl-Sepharose resin previously incubated with rhbFGF and treated with electrophoresis sample buffer, with (lane 8) or without (lane 3) 2-mercaptoethanol, before SDS/PAGE on a 12.5% polyacrylamide gel. The resin was directly incubated with XC-FGF-R and treated with non-reducing (lane 4) and reducing (lane 7) sample buffer. XC-FGF-R standards were run under non-reducing (lane 1) and reducing (lane 6) conditions. rhbFGF standards were treated with sample buffer with (lane 5) or without (lane 2) 2-mercaptoethanol before electrophoresis.

Cross-linking and SDS/PAGE analysis

rhbFGF was cross-linked with XC-FGF-R and the products of the reaction analysed on SDS/PAGE. Four different chemical cross-linkers (DSS, BS³, BSOE and DTSSP) were used and BSOE was finally selected because it gave the clearest pattern although no major difference was observed, suggesting that neither the chemical reagents nor the solvents used during the reactions influenced the complex formation (results not shown).

To investigate the stoichiometry of the binding, different receptor/ligand molar ratios were used. When 0.14 nmol of rhbFGF was incubated with 0, 0.035, 0.07, 0.14, 0.28, 0.42 and 0.56 nmol of XC-FGF-R for 2 h at 25 °C and then cross-linked with BSOE (Figure 3), the SDS/PAGE analysis showed the presence of new bands. The major band migrated with a R_f value corresponding to 87 kDa (lanes 4–7), which is similar to the sum of the apparent molecular masses of rhbFGF (23 kDa, lane 1) and XC-FGF-R (63 kDa, lane 8), indicating that one rhbFGF molecule bound one receptor molecule. Excess of XC-FGF-R did not influence the formation of the complex and the unreacted protein appeared as a 63 kDa band (lanes 5–7).

In other experiments 0.14 nmol of XC-FGF-R was incubated for 2 h at 25 °C with 0, 0.035, 0.07, 0.14, 0.28, 0.42 and 0.56 nmol of rhbFGF and then cross-linked with BSOE. Also in this case the band corresponding to the 1:1 complex was the main detected band and its formation was not influenced by the excess of rhbFGF (results not shown).

The identity of the complex was confirmed when the gels were submitted to immunoblotting and the 87 kDa band was specifically recognized by polyclonal anti-(human FGF receptor) antibodies and by polyclonal anti-(human bFGF) antibodies.

The influence of the complex concentration in solution on the stoichiometry of the binding was also electrophoretically analysed. Equimolar amounts of XC-FGF-R and rhbFGF were incubated for 2 h at 25 °C at concentrations of 6.25, 12.5, 25 and

Figure 3 SDS/PAGE analysis of the cross-linked rhbFGF/XC-FGF-R complex

rhbFGF and XC-FGF-R were incubated for 2 h at 25 °C, cross-linked with BSOCOES, submitted to SDS/PAGE analysis on a 12.5% polyacrylamide gel under non-reducing conditions and stained with Coomassie Blue R-250. rhbFGF (0.14 nmol) was incubated with 0 (lane 1), 0.035 (lane 2), 0.07 (lane 3), 0.14 (lane 4), 0.28 (lane 5), 0.42 (lane 6) and 0.56 nmol (lane 7) of XC-FGF-R. In lane 8, XC-FGF-R was incubated in the absence of rhbFGF and cross-linked as above. Positions of the molecular-mass standard proteins are reported on right. Abbreviations: C, rhbFGF-XC-FGF-R complex; R, XC-FGF-R; L, rhbFGF.

Figure 4 Gel-filtration chromatography of the cross-linked rhbFGF/XC-FGF-R complex

rhbFGF and XC-FGF-R were mixed at the indicated molar ratios, incubated for 2 h at 25 °C and cross-linked with BSOCOES. The cross-linking reaction was stopped by addition of one-tenth of a volume of 1 M ammonium acetate and 10 µl of each solution were chromatographed on a Superose 6 column eluted with 250 mM NaCl in 25 mM phosphate buffer, pH 8.0, at 0.4 ml/min. Peaks were monitored for absorbance at 218 nm. The positions of the molecular-mass marker proteins (13.7 kDa, ribonuclease A; 25 kDa, chymotrypsinogen A; 43 kDa, ovalbumin; 67 kDa, BSA; 116.2 kDa, β -galactosidase) are indicated on the top.

Figure 5 Non-denaturing gel electrophoresis of rhbFGF/XC-FGF-R complex

rhbFGF and XC-FGF-R were incubated for 2 h at 25 °C, submitted to non-denaturing gel electrophoresis analysis in the presence of 0.1% Triton X-100 and stained with Coomassie Blue R-250. Samples (0.5 nmol) of rhbFGF were incubated with 0 (lane 1), 0.125 (lane 2), 0.25 (lane 3), 0.5 (lane 4), 1 (lane 5), 1.5 (lane 6) and 2 nmol (lane 7) of XC-FGF-R. In lane 8, XC-FGF-R was incubated in the absence of rhbFGF. Abbreviations: C, rhbFGF-XC-FGF-R complex; R, XC-FGF-R.

50 µM, cross-linked with BSOCOES and submitted to SDS/PAGE. Also in this case, a major 87 kDa band was observed, together with the two bands corresponding to the unreacted rhbFGF and XC-FGF-R. Only small amounts of higher-molecular-mass bands were detected, suggesting that even at high complex concentrations the 1:1 form was predominant (results not shown).

Gel-filtration chromatography

rhbFGF and XC-FGF-R were incubated at 1:0, 2:1, 1:1, 1:2, 1:4 and 0:1 molar ratio and cross-linked with BSOCOES. In Figure 4 the chromatograms of the different samples are shown. rhbFGF and XC-FGF-R appeared as 18 kDa and 61 kDa peaks respectively. At 2:1, 1:1, 1:2 and 1:4 ratios a new 77 kDa peak was observed which is in agreement with the formation of a 1:1 complex. At the same time, increasing the initial amount of XC-FGF-R in the mixture resulted in a decrease in the level of the 18 kDa peak in the chromatogram and in the appearance of a shoulder at 61 kDa, corresponding to the non-complexed XC-FGF-R excess. Also in this case no significant higher-molecular-mass peaks were observed.

Non-denaturing gel electrophoresis

Since the conditions used during SDS/PAGE dissociated the complex between ligand and receptor the association of the two molecules was studied using cross-linking experiments. However, the stabilization of the complex with chemical cross-linkers could induce structural modifications on the amino acid residues of the proteins and could interfere with the subsequent analyses.

To investigate the complex formation in solution and to separate it from non-complexed rhbFGF and XC-FGF-R, we used non-denaturing gel electrophoresis in the presence of small amounts of Triton X-100 introduced into the gel to obtain a good resolution of the bands. The use of the non-ionic detergent did not interfere with the complex formation.

When 0.5 nmol of rhbFGF was incubated with 0.125, 0.25 or 0.5 nmol of XC-FGF-R (Figure 5, lanes 2–4) only a major band (C) was detected. When the same amount of rhbFGF was incubated with 1, 1.5 or 2 nmol of XC-FGF-R (Figure 5, lanes 5–7) an additional band (R) was observed.

The same gel was subjected to immunoblotting, and anti-(FGF-R) antibodies recognized bands C and R, whereas anti-bFGF antibodies recognized only band C. These findings suggested that XC-FGF-R and rhbFGF were both present in band C and that XC-FGF-R was the only protein present in band R (results not shown).

In similar experiments, 0.5 nmol of XC-FGF-R was incubated at 25 °C for 2 h with 0, 0.125, 0.25, 0.5, 1, 1.5 and 2 nmol of rhbFGF and the samples electrophoresed under the same conditions. In this case also only C and R bands were detected (results not shown).

The rate of binding of rhbFGF to its soluble receptor was also investigated and a kinetic analysis was carried out incubating equimolar amounts of XC-FGF-R and rhbFGF at 25 °C for different times ranging from 0 to 24 h. Samples were then subjected to non-denaturing electrophoresis and the same bands and the same relative amount of each band were observed for each incubation time. This finding suggests that the binding of the two molecules is almost immediate and is stable for a long time at room temperature.

The influence of temperature, pH and ionic strength on complex formation was examined with the same electrophoretic method. After incubation of the same molar amount of the receptor and the ligand for a period of 2 h, the complex was equally stable at temperatures ranging from 4 °C to 25 °C. On the contrary, most of the complex was lost at temperatures higher than, or equal to, 37 °C, possibly due to precipitation before electrophoresis (results not shown). Further, when XC-FGF-R and rhbFGF were incubated at pH values lower than 5.0, no band corresponding to the complex was observed and the two molecules were present totally in their non-complexed form. On the contrary, from pH 6.0 to pH 9.0, the complex was stable (results not shown). At an ionic strength corresponding to 2 M NaCl in 25 mM phosphate buffer at pH 7.5, the complex formation was completely inhibited, from 2 M to 1.5 M NaCl it was gradually reduced, and from 1.5 M to 0 the complex was shown to be stable (results not shown).

Sequence and amino acid analysis of the complex

The XC-FGF-R–rhbFGF complex (band C) was separated from the non-complexed proteins (band R) using non-denaturing gel electrophoresis and electroblotted on PVDF membranes. Thereafter, the band corresponding to the complex was cut out and subjected to N-terminal-sequence analysis. As expected from previous immunoblotting results, double sequences were obtained from the blotted band C, indicating that the new band was indeed the rhbFGF–XC-FGF-R complex. On the basis of the ratios of the sequence yields of leucine released from the third and the fourth Edman cycle, belonging to rhbFGF and XC-FGF-R respectively, a 1:1 stoichiometry can be assigned to the complex.

The amino acid analysis of the blotted band C confirmed the presence of the two molecules in a 1:1 ratio. A computer program based on the least-squares method [31] was applied to the experimental values of 14 amino acids compared with the predicted composition. Also in this case the calculated rhbFGF/XC-FGF-R molar ratio was 0.92, supporting the hypothesis of an equimolar complex.

Specificity of the binding between rhbFGF and XC-FGF-R

Non-denaturing gel electrophoresis was also used to investigate the specificity of the binding between XC-FGF-R and rhbFGF.

A cytokine (IL-6), a protein with a basic isoelectric point similar to rhbFGF (prourokinase) and a growth factor [epidermal growth factor (EGF)] were incubated for 2 h at 25 °C in PBS with an equal molar amount of XC-FGF-R and subjected to non-denaturing gel electrophoresis. In all the experiments, XC-FGF-R did not form any detectable complex with the proteins examined since the soluble receptor band was always present with the same intensity and no band corresponding to hypothetical complexes appeared.

DISCUSSION

In the study reported here, we have analysed the FGF-binding properties of the recombinant extracellular domain of an FGF receptor expressed in *E. coli*.

Similar studies with soluble fragments of other cytokine, growth factor and hormone receptors were recently presented. In particular, the ligand–receptor interaction was analysed in experiments *in vitro* in the case of interleukin 2 (IL-2) [32,33], human growth hormone [34], interferon γ [35], tumour necrosis factor [36,37], EGF [38,39] and PDGF [40]. It has been proposed that ligand binding induces conformational changes, with dimerization or oligomerization of the receptors that undergo rapid activation of tyrosine kinase activity and cause phosphorylation of specific substrates in the intracellular compartments [41]. However, in the case of EGF, it was shown that activation of tyrosine kinase did not require receptor oligomerization [42]. A 1:1 receptor–ligand stoichiometry was also reported for IL-2 [32,33]. Recently, it was proposed that the activation of the intracellular tyrosine kinase domain of the FGF-Rs occurred in response to dimerization induced by ligand binding [43,44].

An analysis of the receptor–ligand interaction in cell culture models is often complicated by the presence of different molecular species which can interfere with the mechanisms of binding.

In the present work, we investigated the molecular basis of the interaction between FGF and its receptor using a simplified cell-free model in solution. Based on cross-linking techniques followed by SDS/PAGE analysis or gel-filtration chromatography we isolated the complex formed between XC-FGF-R and rhbFGF and we showed that the two proteins were present in the complex in their monomeric form. Non-denaturing gel electrophoresis coupled to sequence and amino acid analysis of the blotted complex confirmed a 1:1 ratio. Using this technique we excluded denaturing or reducing agents from the analysis of the complex and we were able to separate the complex from the two non-complexed proteins without using cross-linking reagents. The composition of the complex obtained in such a way was then examined, reducing possible artifacts due to chemical modifications or radiolabelled materials. Since one rhbFGF molecule appeared to bind one XC-FGF-R molecule, we can speculate that only one receptor-binding site should be present on the ligand. The same assumption should be deduced for the FGF-binding site of the soluble receptor. Crystallographic studies of the complex will further elucidate this point.

On the basis of these considerations, a mechanism in which the cellular receptor in its monomeric form interacts with an FGF monomer can be hypothesized. Alternatively, an oligomerization of FGF could occur *in vivo* after interaction of the growth factor with metal ions [27,45], polysaccharides [44] or related molecules [46], and an FGF oligomer could induce a dimerization of the cellular receptor that could be a crucial point for signal transduction.

Another interesting remark comes from the study of the binding of XC-FGF-R to immobilized rhbFGF. In fact, rhbFGF

bound to heparin-Sepharose resin is able to bind the soluble receptor, suggesting that the heparin-binding site and the receptor-binding site are located on different regions of the FGF molecule. On the other hand, binding of rhbFGF to thiopropyl-Sepharose through disulphide bridges between thiol groups of cysteine residues of the protein and activated thiol groups of the resin, did not significantly interfere with the binding of rhbFGF to the soluble receptor. This finding supports the hypothesis that the thiol groups of the cysteine residues in rhbFGF are not involved in the recognition mechanisms of the receptor [27].

In conclusion, in our studies *in vitro* we investigated the main characteristics of the complex between a soluble FGF-R and rhbFGF. The influence of some parameters (time, temperature, pH, ionic strength and concentration) was also examined.

These results may be of interest for a more accurate structural analysis of the receptor-ligand interaction and can be useful for studies of the mechanism of action *in vivo* of FGFs and their receptors.

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REFERENCES

- Thomas, K. (1987) FASEB J. **1**, 434–440
- Gospodarowicz, D., Ferrara, N., Schweigerer, L. and Neufeld, G. (1987) Endocr. Rev. **8**, 95–114
- Jaye, M., Howk, R., Burgess, W., Ricca, G. A., Chui, I.-M., Ravera, M. W., O'Brien, S. J., Modi, W. S., Maciag, T. and Drohan, W. N. (1986) Science **233**, 541–545
- Abraham, J., Mergia, A., Whang, J., Tumolo, A., Friedman, J., Hjerrild, K., Gospodarowicz, D. and Fiddes, J. (1986) Science **233**, 545–548
- Jakobovits, A., Shackelford, G. M., Varmus, H. E. and Martin, G. R. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 7806–7810
- Delli Bovi, P. D., Curatola, A. M., Kern, F., Greco, A., Ittmann, M. and Basilico, C. (1987) Cell **50**, 729–737
- Zhan, X., Bates, B., Hu, X. and Goldfarb, M. (1988) Mol. Cell. Biol. **8**, 3487–3495
- Marics, J., Adelaide, J., Raybaud, F., Mattei, M. G., Coulier, F., Panche, J., de Lapeyriere, O. and Birnbaum, D. (1988) Oncogene **4**, 335–340
- Finch, P. W., Rubin, J. S., Miki, T., Ron, D. and Aaronson, S. A. (1989) Science **245**, 752–755
- Zhu, X., Komiya, A., Chirino, S., Faham, G., Fox, M., Arakawa, T., Hsu, B. and Rees, D. (1991) Science **251**, 90–93
- Eriksson, E., Cousens, L., Weaver, L. and Matthews, B. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 3441–3445
- Zhang, J., Cousens, L., Barr, P. and Sprang, S. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 3446–3450
- Lee, P. L., Johnson, D. E., Cousens, L. S., Fried, V. A. and Williams, L. T. (1989) Science **245**, 57–60
- Pasquale, E. B. and Singer, S. J. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 5449–5453
- Safran, A., Aaron, A., Orr-Urtreger, A., Neufeld, G., Lonai, P., Givol, D. and Yarden, Y. (1990) Oncogene **5**, 635–643
- Johnson, D. E., Lee, P. L., Lu, J. and Williams, L. T. (1990) Mol. Cell. Biol. **10**, 4728–4736
- Reid, H. H., Wilks, A. F. and Bernard, O. (1989) Proc. Natl. Acad. Sci. U.S.A. **87**, 1596–1600
- Ruta, M., Howk, R., Ricca, G., Drohan, W., Zabelshansky, M., Laureys, G., Barton, D. E., Francke, U., Schlessinger, J. and Givol, D. (1988) Oncogene **3**, 9–15
- Mansukhani, A., Moscatelli, D., Talarico, D., Levytska, V. and Basilico, C. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 4378–4382
- Coussens, L., VanBeveren, C., Smith, D., Chen, E., Mitchell, R. L., Isacke, C., Verma, I. M. and Ullrich, A. (1986) Nature (London) **320**, 277–280
- Matsui, T., Heidaran, M., Miki, T., Popescu, N., LaRochelle, W., Kraus, M., Pierce, J. and Aaronson, S. (1989) Science **243**, 800–803
- Yarden, Y., Escobedo, J. A., Kuang, W. J., Yang-Feng, T. L., Daniel, T. O., Tremble, P. M., Chen, E. Y., Ando, M. E., Harkins, R. N., Francke, U., Fried, V. A., Ullrich, A. and Williams, L. T. (1986) Nature (London) **323**, 226–232
- Sims, J. E., March, C. J., Cosman, D., Widmer, M. B., Robson-MacDonald, H., MacMahan, C. J., Grubin, C. E., Wignall, J. M., Jackson, J. L., Call, S. M. et al. (1988) Science **241**, 585–589
- Yamasaki, K., Taga, T., Hirata, Y., Yawata, H., Kawanishi, Y., Seed, B., Taniguchi, T., Hirano, T. and Kishimoto, T. (1988) Science **241**, 825–828
- Isacchi, A., Caccia, P., Cauet, G., Bertolero, F., Bergonzoni, L., Roletto, F., Ziliotto, R., Garofano, L., Jacob, C., Sarmientos, P. et al. (1990) Chimicaoggi **6**, 72–74
- Betbeder, D., Caccia, P., Nitti, G., Bertolero, F., Sarmientos, P., Paul, F., Monsan, P., Cauet, G. and Mazué, G. (1991) J. Biotechnol. **21**, 83–92
- Caccia, P., Nitti, G., Cletini, O., Pucci, P., Ruoppolo, M., Bertolero, F., Valsasina, B., Roletto, F., Cristiani, C., Cauet, G. et al. (1992) Eur. J. Biochem. **204**, 649–655
- Isacchi, A., Statuto, M., Chiesa, R., Bergonzoni, L., Rusnati, M., Sarmientos, P., Ragnotti, G. and Presta, M. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 2628–2632
- Isacchi, A., Bergonzoni, L. and Sarmientos, P. (1990) Nucleic Acids Res. **18**, 1906
- Bergonzoni, L., Caccia, P., Cletini, O., Sarmientos, P. and Isacchi, A. (1992) Eur. J. Biochem. **210**, 823–829
- Antoni, G. and Presentini, R. (1989) Anal. Biochem. **179**, 158–161
- Wang, F. and Pan, Y. (1991) Anal. Biochem. **198**, 285–291
- Lambert, G., Stura, E. and Wilson, I. (1989) J. Biol. Chem. **264**, 12730–12736
- Cunningham, B., Ultsch, M., Devos, A., Mulkerrin, M., Clauser, K. and Wells, J. (1991) Science **254**, 821–825
- Fountoulakis, M., Juranville, J., Maris, A., Ozmen, L. and Garotta, G. (1990) J. Biol. Chem. **265**, 19758–19767
- Pennica, D., Kohr, W., Fendly, B., Shire, S., Raab, H., Borchardt, P., Lewis, M. and Goeddel, D. (1992) Biochemistry **31**, 1134–1141
- Loetscher, H., Gentz, R., Zulauf, M., Lustig, A., Tabuchi, H., Schlaeger, E., Brockhaus, M., Gallati, H., Manneberg, M. and Lesslauer, W. (1991) J. Biol. Chem. **266**, 18324–18329
- Hurwitz, D., Emanuel, S., Nathan, M., Sarver, N., Ullrich, A., Felder, S., Lax, I. and Schlessinger, J. (1991) J. Biol. Chem. **266**, 22035–22043
- Lax, I., Mitra, A., Ravera, C., Hurwitz, D., Rubinstein, M., Ullrich, A., Stroud, R. and Schlessinger, J. (1991) J. Biol. Chem. **266**, 13828–13833
- Heldin, C., Ernlund, A., Rorsman, C. and Roennstrand, L. (1989) J. Biol. Chem. **264**, 8905–8912
- Yarden, Y. and Ullrich, A. (1988) Annu. Rev. Biochem. **57**, 443–478
- Koland, J. and Cerione, R. (1988) J. Biol. Chem. **263**, 2230–2237
- Ueno, I., Gunn, M., Dell, K., Tseng, A. and Williams, L. (1992) J. Biol. Chem. **267**, 1470–1476
- Ornitz, D., Yayon, A., Flanagan, J., Svahn, C., Levi, E. and Leder, P. (1992) Mol. Cell. Biol. **12**, 240–247
- Engel, D. and Maciag, T. (1992) J. Biol. Chem. **267**, 11307–11315
- Middaugh, R., Mach, H., Burke, C., Volkin, D., Dabora, J., Tsai, P., Bruner, M., Ryan, J. and Marfa, K. (1992) Biochemistry **31**, 9016–9024