Oligomeric self-association of basic fibroblast growth factor in the absence of heparin-like glycosaminoglycans

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Basic fibroblast growth factor (FGF-2) represents a class of heparin-binding growth factors that are stored in the extracellular matrix attached to heparin-like glycosaminoglycans (HLGAGs). It has been proposed that cell surface HLGAGs have a central role in the biological activity of FGF-2, presumably by inducing dimers or oligomers of FGF-2 and leading to the dimerization or oligomerization of FGF receptor and hence signal transduction. We have previously proposed that FGF-2 possesses a natural tendency to self-associate to form FGF-2 dimers and oligomers; HLGAGs would enhance FGF-2 self-association. Here, through a combination of spectroscopic, chemical cross-linking and spectrometric techniques, we provide direct evidence for the self-association of FGF-2 in the absence of HLGAGs, defying the notion that HLGAGs induce FGF-2 oligomerization. Further, the addition of HLGAGs seems to enhance significantly the FGF-2 oligomerization process without affecting the relative percentages of FGF-2 dimers, trimers or oligomers. FGF-2 self-association is consistent with FGF-2's possessing biological activity both in the presence and in the absence of HLGAGs; this leads us to propose that FGF-2 self-association enables FGF-2 to signal both in the presence and in the absence of HLGAGs.

Key words: CD spectroscopy, chemical cross-linking, MALDI MS, protein–carbohydrate interactions.

INTRODUCTION

Basic fibroblast growth factor (FGF-2) is a ubiquitously expressed member of the FGF family of growth-promoting and cell-differentiating factors [1]. FGF-2 signalling is known to be of importance in morphogenesis and neovascularization; it also has a key role in the progression of a variety of disease states [2–4].

FGF-2 signal transduction has been studied extensively [5–8], and much detail has been paid to the role that heparin-like glycosaminoglycans (HLGAGs) might have in modulating the biological activities of FGF-2 [9–12]. It has been suggested that HLGAGs participate in the formation, with FGF-2 and cell-surface FGF tyrosine kinase receptors (FGFRs), of a ternary complex that potentiates FGF-2 signalling [13,14]. In a ternary complex model for HLGAG involvement in FGF-2 signalling, a possible mechanism by which HLGAGs could mediate FGF-2 signalling is through facilitating the oligomerization of FGF. According to this mechanism, properly oriented oligomers of FGF-2 would be essential for receptor clustering, which has been shown to be essential for FGFR-mediated signalling [15,16], and concomitant signalling. However, at present, the exact role of HLGAGs in forming an active ternary complex with FGF and FGFR is unknown. Furthermore, evidence of FGF-2 signalling in the absence of HLGAGs calls into question the necessity for HLGAGs in the signalling pathway [17,18].

Because of the HLGAG-binding properties of FGF-2 and the focus on receptor clustering in FGF-2 signalling, much work has been devoted to examining the oligomerization properties of FGF-2 in the presence of HLGAGs and HLGAG analogues [19–22]. These studies have used a wide array of traditional biochemical techniques and have provided a substantial number of results suggesting that FGF-2 oligomerizes in the presence of HLGAGs. However, in a previous study of FGF-2 crystal structures, we observed that there exists a specific protein interface that allowed FGF-2 to self-associate [23]. Furthermore, we hypothesized that this interface would allow FGF-2 to self-associate in the absence of HLGAGs and that HLGAGs would serve as a platform to expedite this process.

In the present study, by using a combination of spectroscopic, chemical cross-linking and novel mass spectrometric techniques, we sought to test whether FGF-2 does possess an inherent ability to self-associate in the absence of HLGAGs. We also sought to understand the role of HLGAGs in this self-association process.

EXPERIMENTAL

Proteins and reagents

Recombinant human FGF-2 was generously provided by Scios (Sunnyvale, CA, U.S.A.). Recombinant FGF-2 with surface-exposed Cys-69 and Cys-87 mutated to serine residues (referred to hereafter as FGF-2 Cys-Ser mutant) was generously provided by Amgen (Thousand Oaks, CA, U.S.A.) [24]. The protein purity and concentration of wild-type FGF and the mutants were assessed by SDS/PAGE and ELISA; protein activity was assessed with cell proliferation assays (described below). All mutant proteins used in this study possessed activity identical with that of wild-type FGF-2. The FGF proteins were stored in 25 μl aliquots at -70 °C before use. Storage and manipulation

Abbreviations used: BS^3, bis(sulphosuccinimidyl) suberate; FGF-2, basic fibroblast growth factor; FGFR, FGF receptor; HLGAGs, heparin-like glycosaminoglycans; MALDI, matrix-assisted laser desorption ionization.

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of FGF-2 were performed in silicon-treated tubes. Bis(sulphosuccinimidyl) suberate (BS^3) was obtained from Pierce (Rockford, IL, U.S.A.) and stored under nitrogen in 1 mg aliquots at −70 °C before use. Monoclonal FGF-2 antibody 11.1 was kindly provided by Scios. Horseradish peroxidase-conjugated goat anti-mouse antibodies and SuperSignal ULTRA chemiluminescent horseradish peroxidase substrates were obtained from Pierce. 2-Mercaptoethanol was obtained from American Bioanalytical (Natick, MA, U.S.A.) and stored at −70 °C as 2 % (v/v) single-use aliquots. Heparin from pig intestinal mucosa was obtained in powdered form from Celsus Laboratories (Cincinnati, OH, U.S.A.). RPMI 1640 medium, calf serum and 100 × penicillin/-streptomycin/glutamine were from Sigma (St. Louis, MO, U.S.A.). For the MS experiments, sinapinic acid was purchased from Aldrich and used as received. Acetonitrile was from Jackson and Burdich.

Production of Δ28 mutant FGF-2

Some CD experiments required FGF-2 lacking the N-terminal nine-residue leader sequence and the first 19 residues (referred to hereafter as the Δ28 mutant). The Δ28 mutant was subcloned into the *Spe*I/*Nde*I sites of the multiple cloning region of a variant of Novagen pET14b plasmid (obtained from D. Ornitz) and the mutant protein was expressed in *Escherichia coli* BL21 cells. Protein expression was performed in 500 ml cultures of Luria–Bertani medium in the presence of ampicillin (100 μg/ml final concentration) at 37 °C. Isopropyl β-D-thiogalactoside (1 mM final concentration) was added when the D_{600} of the cultures had reached 1.0. After induction with isopropyl β-D-thiogalactoside for 3 h cells were harvested by centrifugation at 3500 ***g*** for 10 min at 4 °C, resuspended in a buffer containing 20 mM Tris/HCl, pH 7.9, 500 mM NaCl and 5 mM imidazole and sonicated. Crude extract was collected by centrifugation of the sonicated cell suspension at 12000 ***g*** for 20 min at 4 °C. Purification of protein was performed with a BioCAD 60 perfusion chromatography workstation (PerSeptive Biosystems, Framingham, MA, U.S.A.) with a $NiSO_4$-charged column connected in-line with a heparin-charged column. The recombinant protein was eluted at 1.4 M NaCl/10 mM Tris, desalted and concentrated in a Centricon concentrator (Amicon, Danvers, MA, U.S.A.) to a final volume of 500 μl in 10 mM sodium phosphate buffer, pH 7.0. The wild-type activity for all mutant FGFs was determined by assaying for F32 cell proliferation after 72 h of incubation with the protein in the presence of heparin [25].

MS

Sinapinic acid (approx. 10 mg/ml) in 30 % (v/v) acetonitrile was used as a matrix solution. Seeded surfaces were prepared by a modification of the method of Xiang and Beavis [26]. In brief, 1 pmol of either FGF-1 or FGF-2 was added to matrix such that the sample-to-matrix ratio was 1:10. A 1 μl sample of this was deposited on the seeded surface (stainless steel plate). After crystallization had occurred, the sample was washed with water to remove excess material, dried with a stream of nitrogen gas and placed into the mass spectrometer. Matrix-assisted laser desorption ionization (MALDI)-MS spectra were acquired in the linear mode by using a Voyager Elite reflectron time-of-flight instrument (PerSeptive Biosystems) fitted with a 337 nm wavelength nitrogen laser. Delayed extraction was used to increase resolution (25 kV, grid at 91 %, guide wire at 0.25 %, pulse delay 350 ns, low-mass gate at 2000). Mass spectra were calibrated externally with myoglobin and BSA.

CD spectroscopy

CD spectra of FGF-2 (wild-type, Cys-Ser and Δ28 mutant) were recorded on an Aviv 62DS spectropolarimeter equipped with a thermostatic temperature controller and interfaced to an IBM microcomputer. Measurements were performed in quartz cells of 0.1, 0.5, 1 and 10 mm path lengths. Spectra were recorded at 30 °C, in an average of 10 scans between 195 and 260 nm, with a 1.0 nm bandwidth, a scanning rate of 10 nm/min, a wavelength step of 0.2 nm and a time constant of 2 s. FGF-2 samples of various concentrations were prepared in 10 mM sodium phosphate buffer, pH 7.2. Cells with a shorter path length were used to record spectra of concentrated samples; cells with a longer path length were used for dilute solutions. CD band intensities are expressed as molar ellipticities, $[\theta]_M$, in degrees·cm²·dmol⁻¹.

Cross-linking reactions

It was important to ensure that the analysis of chemical covalent cross-linking would be an accurate reflection of specific FGF-2 associations and not an artifact of adventitious cross-linking or the cross-linking conditions. BS^3 was chosen for chemical cross-linking for reasons that included aqueous solubility, homobifunctional specificity for lysine residues, moderate spacer length (11.4 Å) and a variety of published uses for studying protein associations [27,28]. Control experiments were completed to ensure the specificity of the reaction. First, cross-linking reactions were completed in the presence of 2-mercaptoethanol to ensure the absence of disulphide-bonded dimers. Secondly, initial cross-linking studies were completed by using a variety of ratios of cross-linker to protein (see the Results section) to demonstrate that non-specific cross-linking was minimal and the oligomerization patterns observed were specific to FGF-2. Finally, to ensure the reproducibility of these studies, key experiments were repeated with glutaraldehyde (i.e. a different cross-linker with a different spacer length).

For the initial cross-linking studies, 20 μl of 31.6 μM FGF-2 was incubated for 15 min at room temperature in a buffer containing 50 mM sodium phosphate, 100 mM NaCl and 0.2 % (v/v) 2-mercaptoethanol, pH 7.0. A 1 μl sample of the appropriate cross-linker was added in molar ratios to protein of 10:1, 2:1, 1:1, 1:2, 1:5 and 1:10. BS^3 was diluted in 5 mM sodium citrate, pH 5.5, before addition to FGF-2. Reactions were left to continue for 30 min with BS^3 or overnight with glutaraldehyde before being quenched with the addition of 1 μl of 200 mM ethanolamine. The reaction products were subjected to SDS/PAGE [15 % (w/v) gel] without the addition of reducing agent. Proteins were then transferred to nitrocellulose at 275 mA for 45 min. Membranes were blocked with Pierce blocking buffer and incubated at room temperature overnight with monoclonal antibody 11.1. Membranes were then washed, incubated with secondary conjugate with horseradish peroxidase, developed and quantified with a Stratagene Eagle Eye II imaging system (Stratagene, La Jolla, CA, U.S.A.). Total band intensity was quantified by summing all pixels above background in identical regions of interest. All bands were then expressed as a fraction of the total intensity of the individual lane.

To quantify the ability of FGF-2 to self-associate, cross-linking studies in the absence and in the presence of heparin (1:10 ratio of heparin to protein) were performed essentially as

described above. A FGF-2 concentration range from 336 to 1.58 μM was used with a constant 2:1 ratio of BS^3 to FGF-2. Alternatively, 79 μM FGF-2 was cross-linked at various ratios of heparin to protein.

RESULTS AND DISCUSSION

MS of FGF-2

One of the hindering factors of using traditional biophysical and biochemical methods to study growth factors is that the techniques require large amounts of protein concentrated to greatly above physiological levels. Such concentrations are necessitated by the limitations of the sensitivity of these techniques. To probe the FGF-2 behaviour of physiological quantities of protein requires a technique that directly measures quantitative changes on a much more sensitive scale. MALDI time-of-flight has been used in several instances to probe protein association [29,30]. In addition, MALDI-MS complements data from other techniques, in that we can directly measure whether FGF-2 self-associates with picomoles of protein. It should be borne in mind that, after bringing the sample to dryness, the protein molecules are still highly diluted by the large excess of matrix ('matrix isolation'), allowing the material to be analysed at a concentration comparable to those found physiologically. Therefore FGF-2 was ionized in the absence of HLGAGs (Figure 1). The measured m/z for FGF-2 monomer was 17083, which was close to the theoretical m/z of 17103.

Also present in the spectrum under mild ionization conditions were higher-order oligomers, namely dimers, trimers, tetramers and pentamers (Figure 1A). Several control experiments were run to ensure that the observed oligomers for FGF-2 were not simply a result of protein aggregation. First, higher-order oligomers were seen in the mass spectrum even if FGF-2 was passed through a size-exclusion column to remove any possible aggregates before placement on the target. Expanding this point, it should be remembered that the matrix isolation of the technique will limit the possibility of non-specific aggregation. Secondly, the FGF-2 Cys-Ser mutant (i.e., the mutant unable to form disulphide-bonded aggregates) displayed the same behaviour under these conditions (results not shown). Finally, FGF-1 (run as a control), which shares a high sequence similarity, had no oligomer higher than a dimer present in the spectrum (Figure 1B). Taken together, these results point to a specific protein association between FGF-2 protomers that leads to the formation of oligomers at picomole levels of protein.

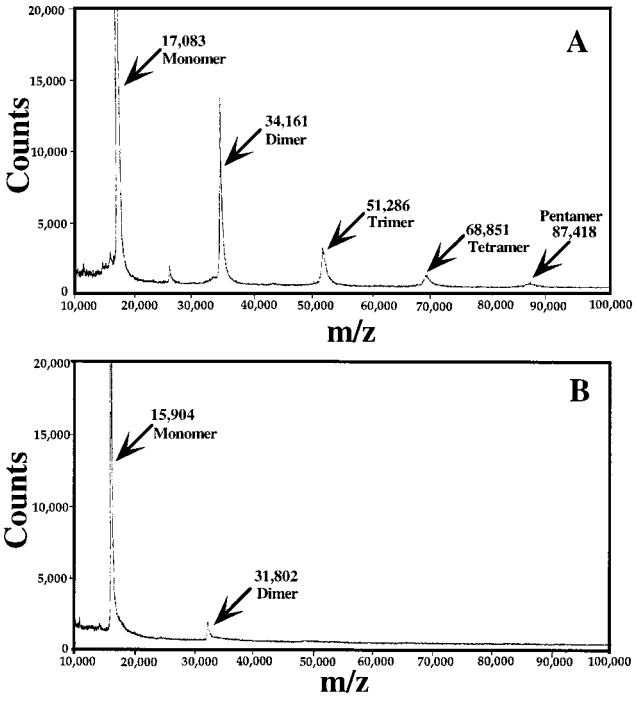

Figure 1 MS of FGF-2

(**A**) MALDI-MS spectrum of 1 pmol of FGF-2. The theoretical m/z value of $[M+H]^+$ for FGF-2 is 17102. Present in the spectrum are dimers, trimers, tetramers and pentamers. Also present is a peak at m/z 25640 corresponding to a doubly charged species for the trimer. (**B**) MALDI-MS profile of FGF-1 as a control. The theoretical m/z value of $[M+H]^+$ for FGF-1 is 15899. In contrast with FGF-2, only a weak dimer peak is seen.

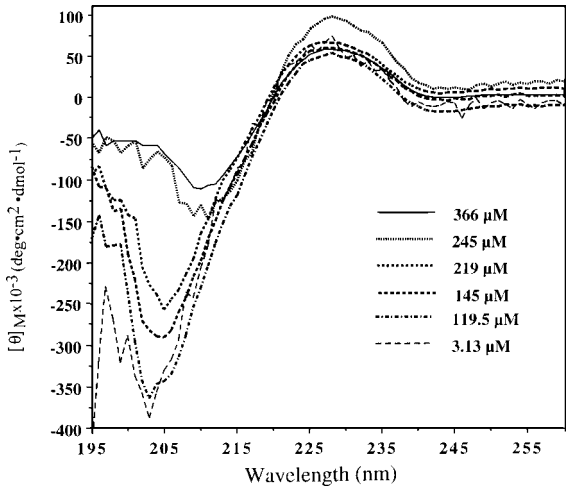

Figure 2 CD spectra of FGF-2 as a function of concentration

The spectra are characterized by a positive band at approx. 227–230 μM that does not change as a function of FGF-2 concentration, whereas there is a significant shift in both the intensity and the wavelength of the negative band.

CD spectroscopy of FGF-2

Although MALDI-MS provided a direct measure of protein associations, the technique is limited in its ability to observe concentration-dependent phenomena over a wide concentration range. CD spectroscopy is commonly used as a first technique to observe protein secondary structure. However, the molar ellipticity and the position of CD bands can be exploited to follow concentration-dependent association of peptides and proteins [31–33]. The typical CD spectrum of FGF-2 exhibits a weak positive band at 227–230 nm and an intense negative band at 202 nm [21]. CD spectra of FGF-2 at concentrations from 366 to 3 μM are shown in Figure 2. Both the bands in the CD spectra at concentrations below 120 μM were consistent with those reported previously [24]. Neither the λ_{max} nor the molar ellipticity of the weak positive band was concentration-dependent. However, the position (wavelength) as well as the intensity of the negative band changed significantly as a function of FGF-2 concentration (Figure 2). A plot of the molar ellipticity (or the λ_{min} wavelength) of the negative band as a function of FGF-2 concentration is shown in Figure 3. Below 119 μM, the negative band was static; neither the λ_{min} nor the molar ellipticity changed

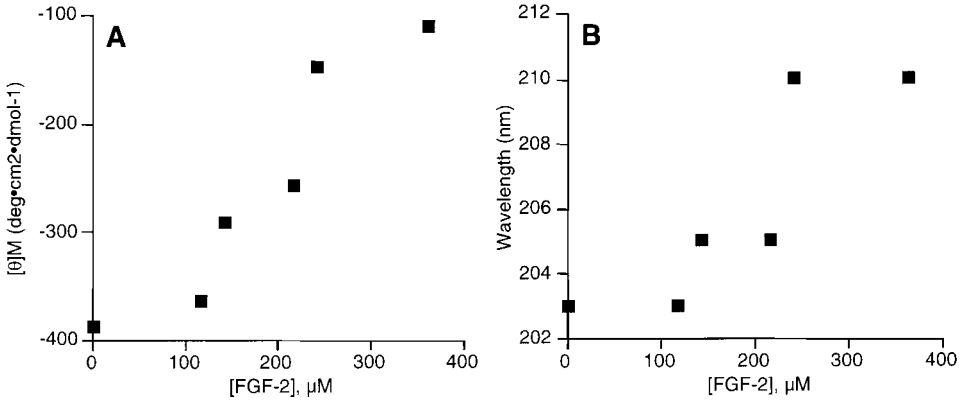

Figure 3 Changes in the CD spectra of the negative band

(**A**) Variation in molar ellipticity with FGF-2 concentration. The molar ellipticity at the negative band in Figure 2 is plotted as a function of the FGF-2 concentration. The large observed shift in molar ellipticity is indicative of changes in the self-association state of FGF-2. (**B**) Variation in the wavelength of the negative band with FGF-2 concentration. Shifts occur at the same concentrations as seen in Figure 2(A) and are indicative of changes in protein ordering or self-association.

($[\theta]_M = 380000$ degrees·cm^2·dmol^{-1}). However, above 119 μM the negative band shifted towards a longer wavelength (λ_{min} shifted to 205 nm) and the molar ellipticity of this band decreased to approx. 280000 degrees·cm^2·dmol^{-1}. A further increase in the FGF-2 concentration shifted the negative CD band to a longer wavelength (λ_{min} further shifted to 210 nm) with a significant decrease in the molar ellipticity. The simultaneous transitions in the position as well as the intensity of the negative band indicate a concentration-dependent change in the protein environment.

At high protein concentrations, it is important to eliminate the plausibility of covalent oligomer formation due to disulphide bond formation between the surface-exposed cysteine residues of native FGF-2. The disulphide bond is expected to exhibit a CD band due to n–σ* transition, normally observed in the region between 240 and 300 nm [34]. For FGF-2, the CD signal in this region was constant throughout the concentration range used in this study, suggesting that there was no disulphide bond formation. In addition, the changes in the position and intensity of the negative CD were readily reversible (on dilution of FGF-2), indicating no covalent bond formation. Taken together, these results indicate that the observed changes in the CD spectra of FGF-2 do not involve the formation of disulphide bonds or FGF-2 denaturation.

To demonstrate further that disulphide-bonded FGF-2 molecules are not responsible for the observed trends in the CD profile, the CD spectra were recorded as a function of concentration for the FGF-2 Cys-Ser mutant, which does not contain any solvent-accessible cysteine residues. The FGF-2 Cys-Ser mutant exhibited a concentration-dependent CD transition similar to that of wild-type FGF-2 (results not shown). This confirms that the observed CD changes are not due to disulphide formation.

It is known from X-ray structures and NMR data [22,23,35] that the 19 N-terminal amino acids of the FGF-2 molecule are highly disordered. To explore whether changes in the conformation of this region contribute to the observed effects in the CD spectra, an FGF-2 mutant lacking the N-terminal region (Δ28 mutant) was used. The spectra of the Δ28 mutant exhibited identical shifts to the wild-type spectra (results not shown), establishing that the N-terminal disordered region is not responsible for the changes in the CD spectra.

It should be noted that the molar ellipticity of a CD band is expected to be independent of concentration as long as the molecular environment is the same. Therefore the changes in the position and molar ellipticity of the negative CD band suggest a concentration-dependent reversible change in the protein environment. From the reversible nature of the transitions and the similarity of the CD spectra of the wild-type, Cys-Ser mutant and Δ28 mutant FGF-2 proteins, we conclude that the reversible changes in the CD spectrum do not arise from protein aggregation, from disulphide bond formation between FGF-2 molecules or from the N-terminal disordered region. The most likely explanation for the trends in the CD spectrum of FGF-2 is a concentration-dependent protein association of FGF-2. Because HLGAGs and their fragments display very active CD spectra, it was not possible to explore the role of HLGAGs in the self-association with this technique. Therefore chemical cross-linking was employed.

Chemical cross-linking of FGF-2

To investigate further the attributes of the concentration-dependent protein association or FGF-2 self-association and the role of HLGAGs in this process, we used chemical cross-linking methods. Chemical cross-linking with glutaraldehyde and BS^3 was chosen for the specificity of the reaction as well as for their well-precedented use for studying specific protein associations [36–38] (see also the Experimental section). In this study it was of importance to ensure that all FGF-2 cross-linking was an accurate reflection of specific protein self-associations and was not due to artifacts or adventitious cross-linking. In addition, it was essential to ensure that the results obtained from this study were generally applicable (i.e. were not condition-specific). Therefore the extent of oligomerization of FGF-2 over a range of ratios of FGF-2 to cross-linker was measured (Figure 4B). At a limiting cross-linker concentration (high ratio of protein to cross-linker), the fraction of FGF-2 dimers (oligomers) reached a plateau, indicating specificity of the cross-linking reaction. However, in the presence of excess cross-linker, a significant amount of non-specific cross-linking reaction seemed to occur, increasing the overall fraction of oligomers (Figure 4B). Because

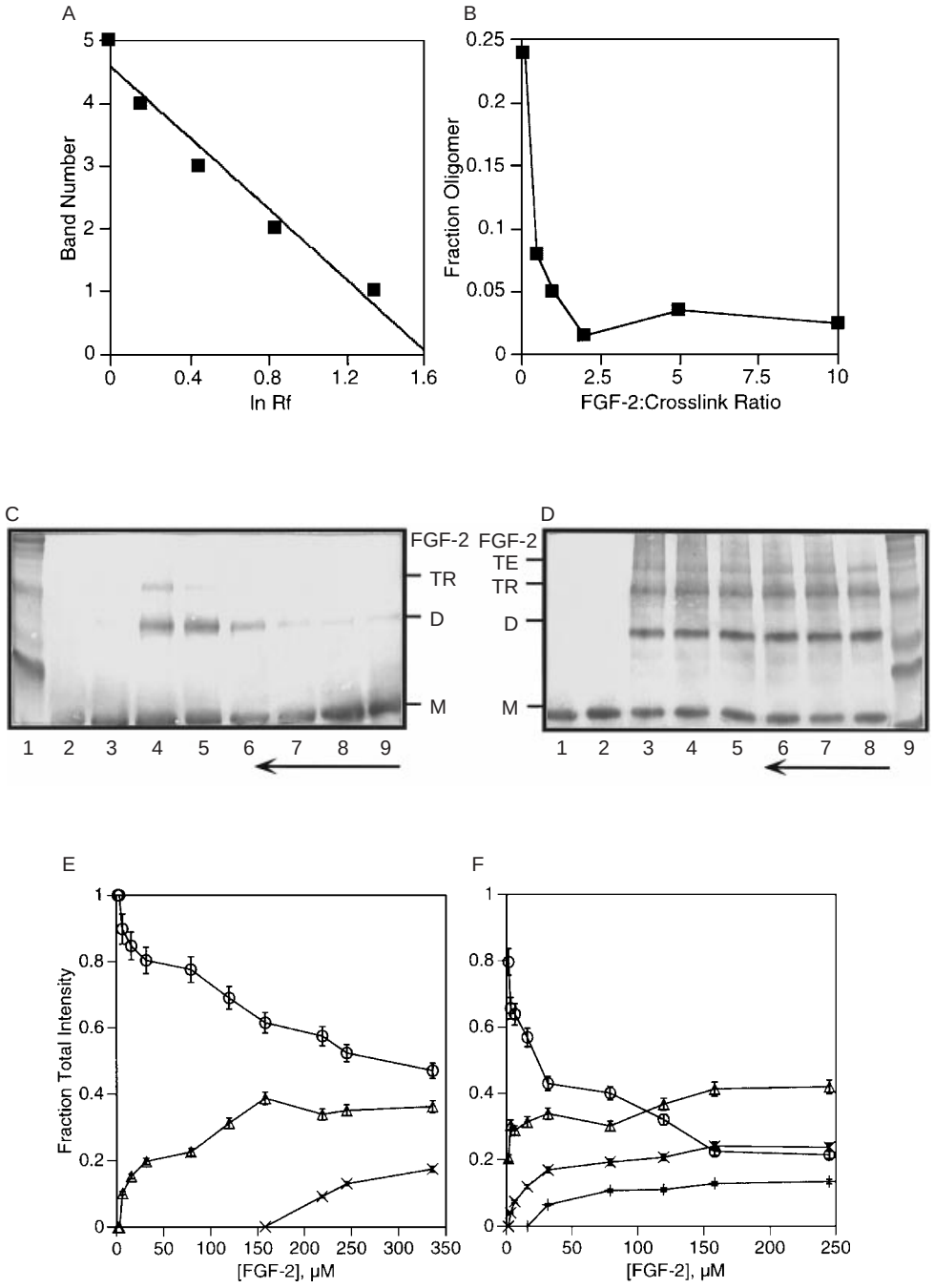

Figure 4 Chemical

(**A**) R_F plot of FGF-2 oligomers. A representative gel of FGF-2 cross-linking in the presence of heparin was used to plot the logR_F of individual bands against the band number. The linearity of the plot confirms that the bands are sequential oligomers of FGF-2. (**B**) Effect of BS^3 concentration on FGF-2 oligomerization. The flat line observed at all except the lowest ratio of FGF-2 to BS^3 is indicative of specific cross-linking reactions. (**C**) Representative blot of FGF-2 cross-linking in the absence of heparin. A range of FGF-2 concentrations was cross-linked in the absence of heparin. Each lane had 523 ng of total protein loaded. Lane 1, molecular mass standards (111, 77.0, 48.2, 33.8, 28.6 and 20.5 kDa); lanes 2 and 3, 336 μM FGF-2 with and without 0.2% (v/v) 2-mercaptoethanol respectively; lanes 4–9, respectively 336, 245, 219, 158, 145 and 119.5 μM FGF-2 cross-linked with a 2:1 ratio of BS^3 to FGF. A transfer artifact is observed in the monomer bands in lanes 6 and 7. Note that dimers are present at all cross-linked concentrations, and trimers are visible at the two highest concentrations. M, D and TR refer to the positions of the monomer, dimer and trimer bands; the arrow indicates increasing concentrations. (**D**) Representative blot of cross-linking of a range of FGF-2 concentrations in the presence of a 1:10 molar ratio of heparin to FGF-2. Each lane had 523 ng of total protein loaded. Lanes 1 and 2, 336 μM FGF-2 with and without 0.2% (v/v) 2-mercaptoethanol respectively; lanes 3–8, respectively 336, 245, 219, 158, 145 and 119.5 μM FGF-2 cross-linked with a 2:1 ratio of BS^3 to FGF; lane 9, molecular mass standards (111, 77.0, 48.2, 33.8, 28.6 and 20.5 kDa). Note that tetramers are clearly visible at all concentrations and that higher-order oligomers taper off at the lower concentrations. M, D, TR and TE refer to the positions of the monomer, dimer, trimer and tetramer bands; the arrow indicates increasing concentrations. (**E**) BS^3 cross-linking of FGF-2 in the absence of heparin. This is a representative graph from three independent cross-linking experiments. The normalized intensity of the different oligomer and monomer bands [monomers (○), dimers (△) and trimers (×)] of FGF-2 in the Western analysis is shown as a function of FGF-2 concentration. Note that at low FGF-2 concentrations there was 100% monomer and that the amount of monomer decreased with increasing FGF concentration. (**F**) BS^3 cross-linking of FGF-2 in the presence of heparin. This is a representative graph from three independent cross-linking experiments. The pattern of oligomerization is shown in the presence of heparin at a constant molar ratio of 1:10 (heparin to protein). Here, dimers were present even at the lowest protein concentration; tetramers (+) were present above 15.8 μM FGF-2. Note that the relative abundances of the dimers and trimers on saturation did not differ significantly from the plots without heparin.

the fraction of oligomers began to increase near a ratio of FGF-2 to BS^3 of 1:2.5, all cross-linking experiments reported here were performed at a ratio of 1:2. To ensure the specificity and reproducibility of the reactions reported here, selected experiments were completed with glutaraldehyde. These reactions gave results identical to those from BS^3 cross-linking (results not shown). Glutaraldehyde was chosen to confirm cross-linking specificity because it is also a lysine-specific cross-linker but the length of its cross-link is about one-quarter that of BS^3.

All cross-linking reactions were performed under reducing conditions in the presence of 2-mercaptoethanol to ensure that there was no non-specific disulphide aggregation of FGF-2. Figure 4(D) (lanes 1 and 2) show the results for 245 μM FGF-2 with (lane 1) and without (lane 2) 2-mercaptoethanol. The control lanes were incubated under the same conditions as the experimental lanes but were treated with citrate buffer (BS^3 buffer) without cross-linker. As a further demonstration of the lack of disulphide-bonded FGF-2 oligomers, cross-linking studies were performed with FGF-2 Cys-Ser mutant and yielded patterns identical (results not shown) to those for wild-type FGF-2.

Concentration-dependent self-association of FGF-2

A wide range of FGF-2 concentrations (keeping a ratio of FGF-2 to BS^3 of 1:2), in the presence or absence of heparin, was chosen for the cross-linking studies. Representative blots of cross-linking experiments are shown in Figures 4(C) and 4(D). The presence of sequential oligomers was confirmed by plotting $\log R_F$ against band number (Figure 4A). The fractions of FGF-2 monomer and oligomers formed during cross-linking, with and without heparin, across FGF-2 concentrations are shown in Figures 4(E) and 4(F) respectively.

In the absence of heparin, FGF-2 consistently self-associates to form oligomers in a concentration-dependent fashion. The fraction of dimers increases with increasing FGF-2 concentration, with a corresponding decrease in the amount of monomer. At approx. 150 μM FGF-2, the fraction of dimers saturates at 40 % of the total intensity. Further increases in the FGF-2 concentration result in the appearance and concentration-dependent increase in FGF-2 trimers. Tetramers or higher-order oligomers of FGF-2 are not observed at this concentration range in the absence of heparin. The fraction of monomers decreases (with a concomitant increase in the oligomer population) and levels off at approx. 50 % of the total protein. These results indicate that FGF-2 does in fact self-associate, even in the absence of heparin. Furthermore, as discussed below, the data from Figure 4(E) can be used to determine some preliminary thermodynamic parameters for the association of FGF-2 in the absence of heparin.

It has previously been observed that in the presence of heparin, FGF-2 can be cross-linked into dimers and oligomers [20,25,39]. On the basis of the results presented above, we investigated the effect of heparin on FGF's ability to self-associate with the use of the same cross-linking methodology. At a fixed ratio of FGF-2 to heparin, the amount of oligomers formed saturated on increasing the FGF-2 concentration. A comparison of Figure 4(F) (FGF association in the presence of heparin) with Figure 4(E) (FGF association in the absence of heparin) indicates that heparin had a marked effect on the oligomeric self-association of FGF-2. As shown in Figure 4(F), the monomer population decreased twice as steeply with increasing FGF-2 concentration, compared with monomer in Figure 4(E). Furthermore, dimers are observed in Figure 4(F), even at the lowest FGF-2 concentration studied. The amount of dimer, shown in Figure 4(F), increased sharply and saturated at the same level as in Figure 4(E). Although the amount of dimer represented approx. 40 % of the total protein in both Figures 4(E) and 4(F), the dimer saturated at a much lower FGF-2 concentration (5 μM) in the presence of heparin. Trimers were observed at 3.13 μM; tetramers, which were not observed even at 336 μM in the absence of heparin, were observed at 15.8 μM FGF-2. Trimer and tetramer fractions also increased with increasing FGF-2 concentration and saturated as observed for the dimer. At saturation, the trimer represented approx. 20 % of the total, in both the presence and the absence of heparin. The effect of heparin therefore seems to shift the oligomerization process towards lower concentrations of FGF-2 without affecting the maximum fraction of dimers or trimers seen. Furthermore, the pattern and trends of oligomerization of FGF-2 Cys-Ser mutant are the same when compared with the wild-type FGF-2 (results not shown), establishing that the bands observed were due to specific, non-covalent FGF-2 self-interactions.

Calculation of K_a values for self-associated FGF-2 oligomers

Association constants were calculated for FGF-2 self-association in the presence and the absence of heparin, assuming an isodesmic model in which the K_a of monomer to monomer (dimer) is assumed to be the same as the K_a of monomer to dimer (trimer), and so on. We fit the values of the cross-linking data to the equation:

$$C_0 = C_1/(1-K_aC_1)^2$$

where C_0 is the total FGF-2 concentration and C_1 is the concentration of the monomer. Plotting the data, we arrived at K_a values of 2.0 ± 0.3 and $10.6 \pm 1.3\ mM^{-1}$ for FGF-2 in the absence and the presence of heparin respectively. It should be noted that these values are approximately three orders of magnitude lower than the reported values of K_a for FGF-2 to heparin [40].

Varying the ratio of heparin to FGF-2 to investigate excluded-volume effects on FGF-2 oligomerization

To examine the possibility that the increase in oligomerization in the presence of heparin was due to excluded-volume effects, we varied the molar ratio of heparin to FGF-2 between 1:100 and 10:1 (Figure 5). An FGF-2 concentration of 79 μM was chosen because there was a pronounced difference in the fraction of FGF-2 oligomers seen with and without heparin. Figure 5 shows a very specific effect and not a continual increase as one would expect from an excluded-volume effect. The fraction of oligomer peaked with the addition of heparin at 1:10, then decreased sharply to zero. This shows that the molar ratio of heparin to FGF-2 directly influences the degree of FGF-2 oligomerization. This shape of curve is intuitively obvious because only at a ratio at which heparin is completely saturated with FGF-2 molecules would one expect optimal oligomerization.

Furthermore, as a control, dermatan sulphate was added at various concentrations to 79 μM FGF-2. Unlike heparin, dermatan sulphate had no bell-shaped effect on FGF-2 oligomerization (results not shown). In addition, as expected for excluded-volume effects, at very high concentrations of dermatan sulphate there were very small amounts of non-specific cross-linking of FGF-2.

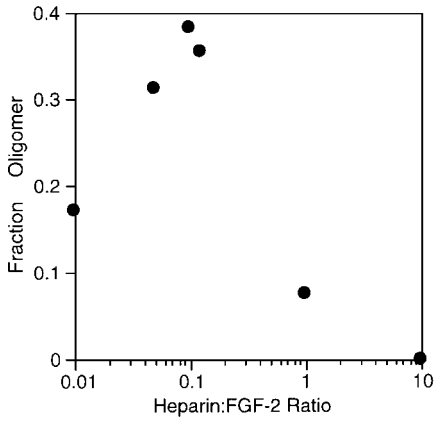

Figure 5 Variation of the molar ratio of heparin to protein

This 'bell-shaped' graph shows the relative abundance of oligomers of 79 μM FGF-2 at different ratios of heparin to protein. From the level of dimer in the absence of heparin at a ratio of 1:100, the amount rose swiftly to approx. 3-fold as much oligomer as background at a ratio of 1:10. When the molar ratio was increased further the level decreased steeply to zero.

FGF-2 self-association and biological significance

The absolute requirement of HLGAGs for FGF-2 biological activity has been the subject of debate. We and others have observed that FGF-2 is able to bind to FGFR and induce signalling even in the absence of HLGAGs, albeit at a much higher FGF-2 concentration and at a much lower signalling level *in vitro* [17,18]. Support for the importance of HLGAGs in FGFR signalling during embryonic development has recently been obtained in *Drosophila* (X. Lin, personal communication). An analysis of mutant embryos in which HLGAG biosynthesis is blocked revealed that the FGFR-dependent migration of mesodermal and tracheal cells fails to occur properly. In addition, FGFR-dependent downstream events were not detected. However, ectopic overexpression of an FGF ligand in these mutants led to the partial recovery of FGFR activity. In an oligomerization-driven model, this last finding is consistent with excess FGF's bypassing the requirement of HLGAGs to activate FGFR, supporting a hypothesis that FGFs are able to undergo self-association in the absence of HLGAGs, whereas HLGAGs considerably stabilize such association.

FGF-2 self-association is consistent with FGF-2's possessing biological activity both in the presence and in the absence of HLGAGs. Therefore the results from the present study and our model of 'side-by-side' FGF-2 self-association, taken together, lead us to propose that FGF-2 self-association enables FGF-2 to signal both in the presence and in the absence of HLGAGs. In such a model, the sites for FGF self-association, HLGAG binding and receptor binding are all separated on the surface of FGF, such that long-chain HLGAGs can act as a platform for oligomerization of FGF, leading to further stability of the FGF oligomers and consequently to FGFR oligomerization.

Further work will include the experimental determination of residues involved in the FGF-2 self-association interface and the expansion of similar biochemical studies to other FGF family members. To this end, preliminary one-dimensional NMR experiments have yielded data consistent with concentration-dependent FGF-2 self-association, namely global line-broadening coupled with increased line-broadening and shifts of specific resonances (results not shown). Although preliminary studies have shown marked differences between the modes of oligomerization between FGF-1 and FGF-2 [41], it remains to be seen what this means biologically, and how it affects the rest of the FGF family members.

In summary, the present study shows that FGF-2 has a natural tendency to self-associate specifically and that HLGAG is not essential for this self-association but serves to potentiate FGF's ability to self-associate.

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