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Cations Modulate Polysaccharide Structure To Determine FGF−FGFR Signaling: A Comparison of Signaling and Inhibitory Polysaccharide Interactions with FGF-1 in Solution[†]

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ABSTRACT: For heparan sulfate (HS) to bind and regulate the activity of proteins, the polysaccharide must present an appropriate sequence and adopt a suitable conformation. The conformations of heparin derivatives, as models of HS, are altered via a change in the associated cations, and this can drastically modify their FGF signaling activities. Here, we report that changing the cations associated with an *N*-acetyl-enriched heparin polysaccharide, from sodium to copper(II), converted it from supporting signaling through the fibroblast growth factor receptor (FGF-1−FGFR1c) tyrosine kinase signaling system to being inhibitory in a cell-based BaF3 assay. Nuclear magnetic resonance and synchrotron radiation circular dichroism (SRCD) spectroscopy demonstrated that the polysaccharide conformation differed in the presence of sodium or copper(II) cations. Electron paramagnetic resonance confirmed the environment of the copper(II) ion on the *N*-acetyl-enriched polysaccharide was distinct from that previously observed with intact heparin, which supported signaling. Secondary structures in solution complexes of polysaccharides with FGF-1 (which either supported signaling through FGFR1c or were inhibitory) were determined by SRCD. This allowed direct comparison of the two FGF-1−polysaccharide complexes in solution, containing identical molecular components and differing only in their cation content. Subtle structural differences were revealed, including a reduction in the level of disordered structure in the inhibitory complex.

Fibroblast growth factors (FGFs) are conserved throughout the animal kingdom. The FGF family consists of at least 22 members in mammals with manifold biological activities, which include the regulation of cell growth and differentiation, survival, migration, and homeostasis (*1*). It was discovered early in their history that FGFs have a high affinity for heparan sulfate (HS)[1], and initially, an HS analogue, heparin, was thought to stabilize the growth factor (*2*), protecting it from denaturation and proteolytic degradation. It has since been shown that HS plays an active role in FGF biology by regulating interactions with its cognate receptors (*3*, *4*).

FGFs exert their effects on cells through a family of tyrosine kinase receptors, the FGF receptors (FGFRs). These are encoded by a family of four genes named FGFR1−FGFR4. The prototypical receptor has three Ig domains (with the so-called "acid box" residing between domains D1 and D2) in the extracellular ligand binding region, a transmembrane domain, and a split tyrosine kinase domain (*5*), with the second Ig domain containing a heparin binding region (*6*). FGFR1−3 exist as multiple splice variants, the most common involving an alternate exon in the second half of the third Ig domain giving rise to the b and c isoforms. The isoforms arising from these splice variants have been shown to exhibit altered ligand binding specificity (*7*). Other splice variants eliminate the first Ig loop, although the biological consequences of this remain unknown (*8*).

Much effort attempting to understand the nature of the structure−function relationship of the polysaccharide component of the FGF−FGFR signaling system has been expended.

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[1]Abbreviations: NaHep, Na^+-heparin; CuHep, Cu^{2+}-heparin; NaNAc, Na^+-*N*-acetyl-enriched heparin; CuNAc, Cu^{2+}-*N*-acetyl-enriched heparin; FGF-1, fibroblast growth factor 1; FGFR1c, fibroblast growth factor receptor 1c; SRCD, synchrotron radiation circular dichroism; HS, heparan sulfate; GPC, gel permeation chromatography; ITC, isothermal scanning calorimetry; CD, circular dichroism; NMR, nuclear magnetic resonance; FTIR, Fourier transform infrared; GlcA, D-glucuronic acid; GlcNAc, *N*-acetyl-D-glucosamine; IdoA, L-iduronic acid; PCA, principal component analysis; GAG, glycosaminoglycan; BaF3, mouse pre-B lymphocytes; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; IL-3, interleukin-3; HPLC, high-performance liquid chromatography; RPMI, Roswell Park Memorial Institute; PBS, phosphate-buffered saline.

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Heparan sulfate is a member of the glycosaminoglycan (GAG) family of polysaccharides, which mediate a large number of biological processes, including development, embryogenesis, cell growth and division, homeostasis, and blood coagulation. Heparan sulfate is known to interact with many proteins, of which those of the fibroblast growth factor (FGF−FGFR) tyrosine kinase signaling complex are important examples. In addition to FGF and its receptor, the signaling system also requires a HS saccharide to form a signaling complex.

A considerable amount of work has been devoted to the structural basis of FGF−FGFR interactions. Many of the data relating to the structures of putative complexes concern either FGF-1 with FGFR2c or FGF-2 with FGFR1c, for which distinct models have been proposed. Studies have revealed the formation of an asymmetric putative FGF-1−FGFR2c complex with a stoichiometry among the growth factor, receptor, and heparin-derived decasaccharides of 2:2:1 (*9*). This complex has recently been proposed to involve cooperative dimerization of FGF-1 on a single polysaccharide (heparin) chain (*10*) and oligosaccharide (*11*) during formation of the crystal complex. In contrast, a symmetrical dimer with 2:2:2 stoichiometry was proposed (*12*) between FGF-2 and FGFR1c (and the model extrapolated to include a saccharide). These models of signaling complexes derive principally from X-ray crystallographic studies and have led to the hypothesis that the specificity of this system is determined by the interaction of an FGF with its receptor, followed by the binding of an appropriate HS polysaccharide or oligosaccharide. However, this is far from established, and data acquired using other techniques, including GPC, mass spectrometry, ultracentrifugation, and ITC, provide evidence for multiple complex formation, consistent with several conflicting models (*13*).

In contrast, the formation of complexes involving FGF-1, FGFR1c, and saccharides has been the subject of less scrutiny (*14*), and no overall model or stoichiometry has, to our knowledge, been proposed. These studies did not include a heparin/HS saccharide component, rather, sucrose octasulfate, and no novel dimeric structures were observed. Recently, an NMR structure for the FGF-1−Ig2 FGFR1 complex has been reported (*15*), and this study finds two binding sites in FGF-1, coinciding with the primary (*12*, *16*) and secondary (*12*, *16*, *17*) FGF binding sites; it is proposed that this NMR structure supports the symmetric model. FGF-1 was found to bind with high affinity to FGFR1, without saccharide, but the presence of a HS analogue sugar stabilized the interaction. FGF-1 and FGF-2 undergo distinct oligomerization in the presence of GAG oligosaccharide (*18*), and the secondary structural features of both FGF-1 and FGFR1 (*14*, *19*) have been shown by a variety of techniques to consist predominantly of β-structure. FGFR1 also exhibited small amounts of α-helical structure between its Ig domains in solution NMR studies (*14*, *19*). An early report found that the addition of heparin to a solution of FGF-1 showed little evidence, by conventional CD spectroscopy, of causing substantial secondary structural change in FGF-1, although it did modify activity and confer enhanced stability (*2*).

Heparan sulfate comprises a family of linear, anionic, 1−4 linked sulfated polysaccharides, covalently attached to core proteins on the surfaces of almost all mammalian cells to form proteoglycans. Heparan sulfate also possesses a domain structure in which long stretches of GlcA-GlcNAc repeating units, termed NA domains, are interspersed with regions of higher sulfation (S domains) flanked by areas that are characterized by intermediate levels of sulfation (NS domains) and hence greater sequence diversity. Heparin, another GAG family member, is commonly used as a proxy for HS, partly because it is widely available as a pharmaceutical anticoagulant, but also because it generally exhibits activity in processes involving HS. Heparan sulfate and heparin share a common biosynthetic pathway, while heparin is generally more highly sulfated, is richer in iduronic acid, but lacks the more complex domain structure of HS. Indeed, heparin can be considered to be a highly sulfated variant of HS, resembling the NS or S domains.

There is a growing body of evidence that the biological activities of HS and heparin depend on the arrangement of, and complex interplay between, structural features (N- and O-sulfation, N-acetylation, uronic acid distribution, and the associated cation) within the polymer and that these factors determine their conformation, dynamics, interactions with proteins, and, ultimately, biological activities (*20*). The interaction between the pentasacharide sequence of heparin and antithrombin, however, remains one of the few examples in which the substitution pattern of HS/heparin governs function with a relatively high degree of sequence specificity. The structure−function relationships of most other HS−protein interactions are less well-defined. In many cases, a degree of sequence degeneracy exists, where several distinct sequences or substitution patterns exhibit similar activity, but the precise relationships among structure, conformation, and activity are yet to be fully determined. The important role of cations was recently shown, in that altering the identity of the cations (from Na to K) associated with one chemically modified heparin derivative drastically altered the outcome (cell proliferation) from nonsignaling to signaling in a cell-based assay of FGF activity, a result which was not reproduced by cations alone (*20*). Furthermore, recent reports have begun to explore the interplay between adjacent groups within HS or related molecules (*21*, *22*), often mediated by cations. An example is the interaction of heparin with Cu(II) ions, which involves a two-phase process, the first consisting of a very specific interaction with a highly sulfated disaccharide sequence [IdoA2S-GlcN(S/Ac)6S] and the second a less specific, general binding (*23*). Despite the absolute requirement for the HS polyanion to be associated with cations, this remains a relatively little studied subject, particularly in connection with protein binding and activity. The interactions between the protein components of the FGF signaling system and cations have, likewise, received relatively little attention, one exception being the interaction of FGF-1 with Cu(II) ions (*24*). Incubation of heparin and FGF-1 in the presence of Cu(II) ions resulted in the formation of dimers and higher oligomeric species, but these were unable to support signaling. It was proposed that the Cu(II) ions also altered the structure of the protein by modifying the cysteine bridges.

In this work, we show that altering the cation form can convert a signaling into an inhibitory saccharide and the structural effects of the two forms on the growth factor, FGF-1, are distinct. The measurement of protein secondary structure is likely to be one of the most sensitive experimental indicators of conformational change in the components of these complexes and was detected here by synchrotron radiation circular dichroism (SRCD) spectroscopy employing the purpose built CD beamline at the Daresbury Laboratory synchrotron facility (SRS, beamline 12.1), which was both highly sensitive and reproducible (*25*). The spectra were analyzed by a combination of principal component analysis (PCA), to provide an objective measure of the degree of similarity or difference between spectra, and established algorithms which were used to determine the

secondary structural characteristics of polysaccharide−cation−FGF-1 complexes (*26*, *27*). This approach provided the opportunity to detect and quantify the changes that occurred in the protein component following formation of two solution complexes consisting of FGF-1 and each cation form of the same polysaccharides, one of which [the Na form of an *N*-acetyl-enriched heparin (NaNAc)] was able to support signaling through FGFR1c and another [the corresponding Cu(II) form (CuNAc)] which was inhibitory, while both contained the same molecular constituents. Direct structural comparisons in solution could therefore be made between these components of the FGF signaling system, which led to either a signaling or an inhibitory outcome, but differed only in the identity of the associated cations. In addition, structural features of complexes of FGF-1 and other saccharides, comprising unmodified heparin in both Na and Cu(II) forms, capable of supporting signaling were examined.

EXPERIMENTAL PROCEDURES

BaF3 Proliferation Assay. BaF3 cells, which do not produce HS and which were transfected to express the three-loop FGFR1c isoform, were maintained as described previously (*28*). For the proliferation assays, 10000 cells per well were plated on a 96-well plate with culture medium (100 μL, RPMI-1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/mL penicillin-G, and 100 μg/mL streptomycin sulfate without IL-3) and were incubated with 2 ng/mL IL-3 or 1 nM FGF-1 (human, recombinant, R&D Systems) and selected modified heparin derivatives at the concentrations indicated. After 72 h at 37 °C, MTT (250 μg/mL) was added to measure the extent of cell proliferation. The cells were presented with exogenous FGF and the test polysaccharide. Cell proliferation occurs when a test compound is competent to form a functioning signaling complex with the growth factor and receptor. For inhibition assays, test polysaccharides were added in the presence of 1 μg/mL heparin. NaNAc and CuNAc were prepared from a sample of porcine intestinal heparin (Celsus), which had undergone exhaustive de-N-sulfation (*29*) and partial re-N-acetylation (55%; controlled by empirically limiting the available acetic anhydride in the re-N-acetylation step and monitoring the products by NMR) followed by exhaustive re-N-sulfation (*29*). The ^{13}C and ^{1}H NMR spectra of NaNAc and CuNAc (S1), the ^{1}H spectrum of NaNAc (S2), and the ^{1}H spectrum (HSQC) of CuHep (S3) are shown in the Supporting Information. Conversion to the Na (NaNAc) or Cu (II) (CuNAc) forms was achieved by exchange (three times) with cation exchange resin [Dowex 50W X8-400, which had been converted previously from the acid form following exhaustive washing with the relevant chloride salt (1 M)]. The M_w of the unmodified heparin was 13.8 kDa and of the N-acetylated product was 12.6 kDa, determined by gel permeation chromatography (TSK gel G2000SW$_{XL}$; 7.8 mm × 30 cm with 50 μm particle size, Supelco), eluting with water at 1 mL/min. The ion-exchanged heparin polysaccharides were desalted (twice, in HPLC grade water prior to use). Elemental analysis of NaNAc and CuNAc following exposure to RPMI medium showed the following cation content (% of total cation content): 76.2 Na, 0.9 Mg, 21.2 K, and 1.7 Ca for NaNAc and 14 Na, 20 Mg, 0.4 K, 5.1 Ca, and 60.5 Cu(II) for CuNAc. Controls for the possible activity of free Cu(II) ions have been reported previously (*20*), and the activation assay with CuNAc heparin and FGF-2 (Figure 1C) showed no signs of free Cu(II) affecting cell growth because both CuNAc and NaNAc exhibited comparable signaling activities in that assay.

Synchrotron Radiation Circular Dichroism Spectroscopy (SRCD). SRCD spectra were recorded on the purpose-built CD-12 beamline at Daresbury Laboratory, employing a quartz sample cell with a path length of 0.01 cm at 1 nm resolution between 240 and 175 nm. The CD spectra of proteins (mixed in 1:1 molar ratios) were recorded at concentrations of 26 μM in PBS, are relative to (+)-10-camphorsulfonic acid (1.0 mg/mL), and have had water and buffer background spectra subtracted. CD values are presented as molar circular dichroism (mol^{-1} cm^{-1}). There was no evidence of damage having occurred to either the polysaccharide or protein components during these experiments.

Secondary Structural Analysis and Principal Component Analysis (PCA). Secondary structural analysis was performed using the Dichroweb website (*26*, *27*), specifically the CDSSTR algorithm, developed by Johnson (*30*) [Sreerama and Woody (*31*) package the CDSSTR algorithm with two other algorithms, SELCON and CONTIN, into a software package, CDPro; also describing the performance of the algorithms with their new reference data sets], employing reference library 4. Principal component analysis was performed on the mean-centered spectra using SPSS (SPSS UK Ltd., Woking, England), with the principal components being extracted via a correlation matrix; the varimax method was used to rotate the component axes, and components were plotted in Sigmaplot (Systat Software UK Ltd., Hounslow, U.K.). In the analysis of secondary structural changes (Table 1), differences of ≥3% were considered significant.

Nuclear Magnetic Resonance (NMR) Spectroscopy. NMR spectra were recorded as previously described (*20*). Chemical shift values were measured downfield from trimethylsilyl propionate (sodium salt) as the standard at 308 K. All proton-detected spectra were recorded on a Bruker Avance 600 instrument equipped with a cryogenic TXI 5 mm probe. Cu(II) ions (as the chloride) were added in D_2O, calculated as a percentage equivalent of the theoretical charge of heparin based on its composition (*32*, *33*). In the two-dimensional experiments, the matrix (size 2K × 320) was zero-filled to 4K × 2K by application of a squared cosine function before Fourier transformation.

Electron Paramagnetic Resonance (EPR) Spectroscopy. EPR experiments were conducted on a Bruker BioSpin EMX microspectrometer (X-band; 9 GHz) (Bruker BioSpin Ltd., Coventry, England). The spectra were recorded at room temperature (290 K) in water and are the average of 16 scans. Spectra were analyzed using Bruker WinEPR software (Bruker

Table 1: Secondary Structure Changes Observed by SRCD[a] in FGF-1 following Interaction with NaNAc and CuNAc

	H1	H2	S1	S2	T	U
FGF-1 to FGF-1 with NaNAc	0.0	4.0	−8.0	0.0	10.0	5.0
FGF-1 to FGF-1 with CuNAc	1.0	2.0	−6.0	0.0	6.0	−3.0
difference, NaNAc to CuNAc (Na to Cu)	1.0	2.0	2.0	0.0	−4.0	−8.0
FGF-1 to FGF-1 with NaHep	−1.0	5.0	−9.0	−1.0	3.0	4.0
FGF-1 to FGF-1 with CuHep	1	5.0	−13.0	−2.0	4.0	6.0
difference, NaHep to CuHep (Na to Cu)	2.0	0.0	−4.0	−1.0	1.0	2.0

[a] SRCD is highly sensitive and reproducible. Differences in secondary structural features of ≥3% were considered significant. Secondary structural changes are defined as follows: H1, regular α-helix; H2, distorted α-helix; S1, regular β-strand; S2, distorted β-strand; T, turns; U, unordered.

BioSpin Ltd.). Additional UV−vis spectra (λ = 250−800 nm) were also recorded (Molecular Devices, SPECTRA max Plus) for CuNAc and CuHep to confirm differences in coordination geometry of the Cu(II) ion, which has been reported to be in a planar tetragonal arrangement in intact heparin (*23*).

RESULTS

NaNAc and CuNAc Resulted in Distinct Signaling Outcomes through the FGF-1−FGFR1c Complex. The signaling outcome of the *N*-acetyl-enriched heparin polysaccharide in the Na (NaNAc) or Cu(II) cation form (CuNAc), through the FGF receptor tyrosine kinase signaling system, was determined with an assay employing BaF3 cells (*28*). The BaF3 cell assay consisted of mouse pre-B lymphocytes, which lacked endogenous HS and had been transfected with a chosen FGFR isoform, in this case, the three-Ig domain variant of FGFR1c. The assay involved introduction of both an exogenous test FGF and a saccharide (NaNAc, CuNAc heparin, or another derivative) into the culture medium, and if the components supported signaling, the cells multiplied and were detected by means of MTT. If, on the other hand, they were unable to support signaling, no cell proliferation was detected. In this case, a further assay in which the test saccharide was titrated in the presence of heparin, an established signaling proxy of HS (*4*), and the ability of the test saccharide to inhibit signaling thereby determined was conducted. NaNAc and CuNAc (characterized in the Supporting Information) exhibited contrasting signaling outcomes (Figure 1A). We hypothesized that this difference in activity arose from fundamental differences in the interactions between the constituents of the signaling system. In the case of NaNAc, the outcome with FGF-1 and FGFR1c was to support signaling, while in contrast, CuNAc had an inhibitory effect when competed against a known signaling polysaccharide (Figure 1B). An additional control, that of CuHep, was also included (Figure 1A). This compound, like NaHep and NaNAc, was able to support signaling. The effect of CuNAc therefore depends on the combined effect of the Cu(II) ion on the polysaccharide structure and subsequent interactions with proteins of the signaling system.

The Same Polysaccharide (CuNAc) That Is Inhibitory with the FGF-1−FGFR1c Complex Is Capable of Signaling through the FGF-1−FGFR2c Complex. In control experiments, the BaF3 cells had an absolute requirement for FGF-1 to allow proliferation, and no polysaccharide or polysaccharide−cation combination had any effect in the absence of FGF-1. Furthermore, CuNAc (inhibitory with the FGF-1−FGFR1c complex in the presence of the signaling polysaccharide heparin) was also capable of signaling through FGFR2c and FGF-1 (Figure 1C). These results were consistent with the argument that the inhibitory activity of CuNAc with the FGF-1−FGFR1c complex was not mediated independently of the FGF receptor pathway and was specific to particular FGF−FGFR combinations.

The Inhibitory Signaling Outcome Cannot Be Reproduced by Free Copper Ions. When the polysaccharide was first exchanged exhaustively (with cation exchange resin) into the Cu(II) form, CuNAc prior to addition to the BaF3 cell medium, the outcome (inhibition) was distinct from that observed when the two components [NaNAc and free Cu(II) ions] were added to the medium separately, which led to cell death for an equivalent level of free Cu(II) ions [in both the presence and absence of the polysaccharide (results not shown)]. Furthermore, a sample of

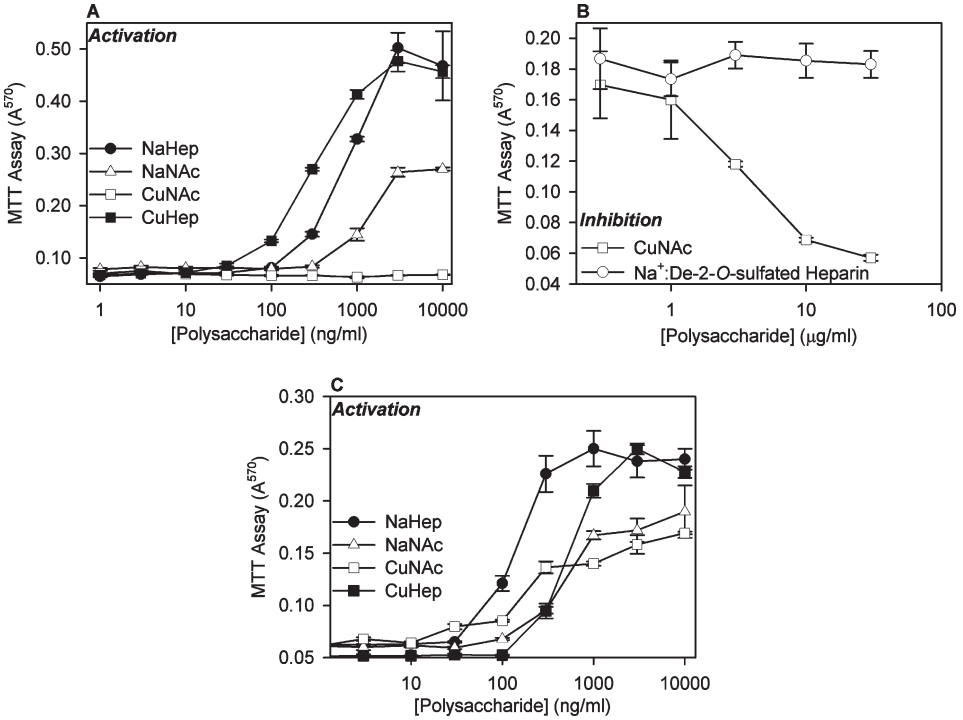

FIGURE 1: FGF-1 (1 nM) was added with increasing concentrations of heparin or polysaccharide to BaF3 cells expressing FGFR1c (A and B) or FGFR2c (C), and proliferation was assessed as described in Experimental Procedures. (A) Support of signaling through FGF-1 and FGFR1c by heparin and NaNAc and the contrasting inability of CuNAc to support it. The Cu and Na forms of intact heparin (NaHep and CuHep controls, respectively) were also both able to support signaling. (B) Inhibition by CuNAc signaling mediated by heparin (at 1 μg/mL) through FGF-1 and FGFR1c. The result of adding polysaccharide (the Na form of de-2-*O*-sulfated heparin), which is known not to inhibit NaHep-mediated FGF-1 signaling, is also shown. (C) Ability of CuNAc to support signaling of FGF-1 and receptor 2c. The effects on cell proliferation of NaHep, CuHep, and NaNAc are also shown.

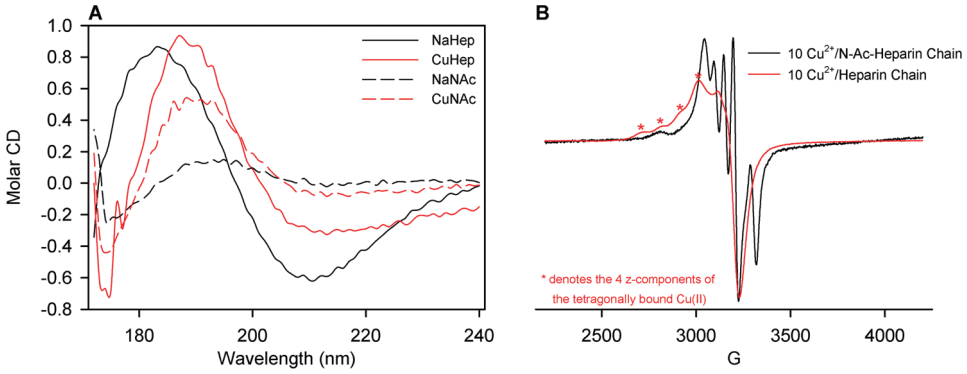

FIGURE 2: (A) SRCD spectra of NaNAc and CuNAc are distinct at equivalent molar concentrations, indicating that different chromophore environments (uronate carboxylic acids and amino sugar *N*-acetyl groups), that is, conformations, were present in each form. Also shown are the spectra of the control polysaccharides NaHep and CuHep, which also exhibited distinct conformations. (B) X-Band EPR spectra of Cu(II) ions complexed with intact heparin [in which the four *z*-components, characteristic of tetragonal Cu(II) ion coordination, were resolved (labeled with asterisks)] and *N*-acetyl-enriched heparin (CuNAc) (exhibiting a complex spectrum but lacking *z*-components). Both were recorded with 10 equiv of Cu(II) ions per polysaccharide chain, demonstrating the distinct modes of binding of the Cu(II) ion by the two polysaccharides. The interactions of intact heparin with Cu(II) ions are among the few examples of interactions between heparin derivatives and cations that are described in detail in the literature (*23*).

CuNAc introduced into the BaF3 cell medium and later recovered (following gel chromatography) retained 60% of the theoretical maximum number of Cu(II) ions (see Experimental Procedures). These experiments demonstrated that the inhibitory effects were not due to free Cu(II) ions, but these must have bound the polysaccharide before being added to the medium for the inhibitory effect on signaling to be observed.

NaNAc and CuNAc Adopt Distinct Conformations. The structural effects of Cu(II) ions binding to the *N*-acetyl-enriched polysaccharide were investigated by EPR and NMR spectroscopy, exploiting the paramagnetic nature of copper ions. Copper(II) ions possess an unpaired electron in their outer (d^9) shell. Unpaired electrons have spin quantum numbers of $\pm^1/_2$, making it possible to observe transitions excited in the microwave wavelength range using electron paramagnetic resonance (EPR) spectroscopy. In NMR spectra, positions within the structure in the proximity of the ion exhibited signal broadening, reduction in intensity, and chemical shift changes caused by altered relaxation times, dynamics, and conformations. Specific sites of interaction between the Cu(II) ion, involving the *N*-acetyl moiety, and the carboxylic acid group of the adjacent uronic acid were indicated [see the Supporting Information for CuNAc (S1)]. SRCD spectra of cation derivatives are sensitive to the conformation of the uronate residues in different cation forms of heparin derivatives (*20*), and distinct SRCD spectra, hence different conformations, were observed for NaNAc and CuNAc (Figure 2A).

The Interaction of NaNAc and CuNAc with FGF-1 Also Resulted in Distinct Secondary Structural Changes in FGF-1. The structural changes in FGF-1 observed using SRCD (with subsequent calculation of secondary structural type) following the addition of NaNAc or CuNAc (Figure 3) were subtly different for the two polysaccharides (Table 1). Other polysaccaride complexes with FGF-1, which all resulted in signaling, were included and also exhibited unique secondary structural changes in each case (Table 1). This analysis revealed secondary structural changes which were unique to the FGF-1−CuNAc complex. In particular, while levels of distorted helix (H1) and ordered strand (S1) were generally increased (H2, 2−5%) and decreased (S1, 6−12.5%), respectively, for the FGF-1−CuNAc complex, the increases in both H2 and S1 were the lowest recorded (up 2 and 6%, respectively). In all cases, little change was observed for either ordered helix (H1) or distorted

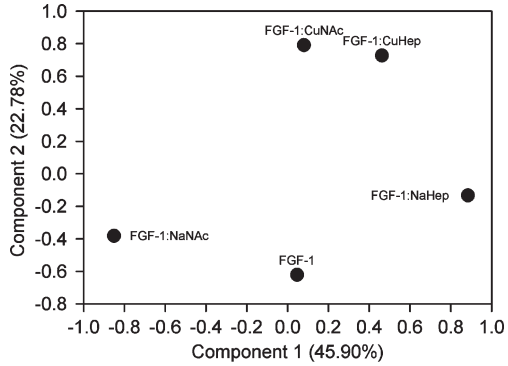

FIGURE 3: (A) PCA of the mean-centered SRCD spectra of FGF-1 and the FGF-1−NaNAc, FGF-1−CuNAc, and FGF-1−CuHep complexes highlighting their distinct protein secondary structures. The structurally distinct complexes formed between FGF-1 with NaHep and CuHep are also shown for the sake of comparison. Interestingly, all the complexes were structurally distinct, while both FGF-1−NaNAc and FGF-1−CuHep complexes were capable of signaling, indicating a level of structural degeneracy. In contrast, only the FGF-1−CuNAc complex was inhibitory. Component 2 differentiates the sodium and copper complexes; 100% of the variance is explained by four components.

strands (S2), and the proportion of turns (T) increased. The most striking difference for the FGF-1−CuNAc complex, however, was the decrease in disordered structure (3%) compared to increases (3.5−6%) for all other complexes.

EPR and NMR Demonstrated That Cu(II) Ions Are in a Different Environment with CuNAc or CuHep. The involvement of Cu(II) cations in converting the polysaccharide to one incapable of supporting FGF-1 signaling through FGFR1c was investigated by EPR. EPR experiments on the Cu(II) form of the polysaccharide (CuNAc) (Figure 2B) revealed that the Cu(II) environment in CuNAc was distinct from the highly sequence specific binding observed previously for intact heparin (*23*), in which the Cu(II) ion is tetragonally coordinated as shown by the four resolved *z*-components (Figure 2B). In contrast, CuNAc heparin lacks these but exhibits other complex features. Nevertheless, the Cu(II) ions clearly occupy distinct environments in the two cases. This was confirmed by UV−vis absorption spectroscopy (Supporting Information, S4), and the absorbance profiles were distinct for the CuNAc and CuHep forms.

^{1}H and ^{13}C NMR spectra (Supporting Information, S1) of CuNAc revealed distinct binding characteristics compared to the spectra of CuHep (^{1}H spectrum; see S3 of the Supporting Information and ref *23*). In CuNAc, the effects of the paramagnetic Cu(II) ions were primarily seen at the *N*-acetyl (CH_3) signals and those of the carboxylic acids (Supporting Information, S1), whereas for CuHep, a distinct pattern was observed; the ^{1}H signals arising from I-5 (adjacent to the carboxylic acid in iduronate), I-1, A-4, and A-6 were diminished during a titration of Cu(II) ions into intact heparin, indicating a distinct binding site (S3 of the Supporting Information and ref *23*).

DISCUSSION

The interaction of Cu(II) ions with *N*-acetyl-enriched heparin, CuNAc, is clearly distinct from their interaction with intact heparin (*23*) [one of the few documented cases of the detailed interactions between heparin derivatives and Cu(II) ions in the literature] in which a very structure specific interaction is evident for a low number of equivalents of Cu(II) ions per chain (< 15), followed by a second general, less specific binding. For *N*-acetyl-enriched heparin, NaNAc, the ^{13}C NMR spectrum (Supporting Information, S1) showed altered chemical shifts, most notably, for the carboxylic acid signals (176−177 ppm) and the *N*-acetyl CH_3 signals around 24 ppm. The effects of binding of Cu(II) ions on the conformations of the two polysaccharides were further demonstrated by their distinct SRCD spectra, a technique which is particularly sensitive to the conformation of the uronic acid residues (*20*). The environments of the Cu(II) ions in the two cases were shown by EPR to be different; the tetragonal arrangement of the binding site in heparin is evident from the separate *z*-components, and in *N*-acetyl-enriched heparin, the signals indicated conventional octahedral coordination.

Recent studies by X-ray crystallography of complexes of FGF-1 and FGFR1 (*14*) demonstrated the binding of FGFR1 to the FGF-1 ligand, in the absence of saccharide. Both components had high β-sheet structural content, but since this combination of FGF and receptor in the absence of a competent signaling saccharide is known to be incapable of supporting signaling, the details may be of limited relevance here. The addition of an analogue of HS, sucrose octasulfate, increased the strength of the interaction. Earlier studies employing conventional CD (*2*) showed that FGF-1 did not exhibit large conformational changes on binding heparin, although that work was conducted with relatively insensitive instrumentation. The results presented here, employing the highly sensitive SRCD technique, demonstrated modest but definite secondary structural changes, which were probably too small for conventional instrumentation of the period to measure accurately. In the same work, it was noted that FGF-1 had a higher affinity for Cu(II) ions than did FGF-2, although this was not examined in further detail.

In this work, highly reproducible SRCD spectra [recorded on the sensitive CD (SRS beamline 12) at Daresbury Laboratory] were analyzed via an extensive protein CD reference library containing spectra from more than 70 structurally diverse soluble proteins (*26*, *27*, *30*, *31*). This library contains a broad distribution of all the major secondary structural types. The improved sensitivity of SRCD for the estimation of secondary structure over conventional CD instruments results from the lower wavelengths accessed by the SRCD instrument and the very high, but stable, photon flux of the synchrotron source. The practical lower-wavelength limit with quartz cell windows is 168 nm; however, the effective cutoff also depends on the buffer (PBS), and this restricts the effective limit to 173 nm. At these lower wavelengths, β-sheet-rich and unstructured sequences, in particular, are more clearly differentiated because their characteristic spectra diverge (*34*). The analysis of spectra through their principal components allowed several complex spectra to be compared simultaneously and their degree of similarity to be determined objectively. Both polysaccharides (NaNAc and CuNAc) were found to influence the conformation of FGF-1, causing increases in the levels of distorted α-helix (4% for NaNAc vs 2% for CuNAc), and the main difference between them was that the signaling form (NaNAc) affected the proportion of turns (10% for NaNAc vs 6% for CuNAc) and unordered structure more than the inhibitory from (CuNAc) (5% for NaNAc vs −3% for CuNAc). Interestingly, the secondary structural properties of the signaling FGF-1−CuHep complex are more similar to those of the FGF-1−CuNAc complex, which was inhibitory, than they are to those of NaNAc, which also supported signaling, indicating that a level of degeneracy exists in terms of the structural consequences of interactions between FGF-1 and saccharides which are capable of supporting signaling. It was interesting that the FGF-1−CuNAc complex exhibited distinct structural changes from FGF-1 compared to the other complexes (which all supported signaling). In particular, the FGF-1−CuNAc complex uniquely contained a lower proportion of unordered structure, raising the intriguing possibility that inhibitory FGF-1−polysaccharide complexes may contain less disordered secondary structure.

The fact that CuNAc was also capable of supporting signaling through a different receptor (FGFR2c) with FGF-1 excludes the possibility that the Cu(II) ions damage FGF-1 in some way, for example, by modifying disulfide bridges, thereby preventing successful signaling. Furthermore, control experiments, in which FGF-1 was excluded, failed to result in cell proliferation in the presence of any combination of polysaccharide and cation. These findings argue against the possibility that the inhibition observed with CuNAc was due to interference with another signaling mechanism, or with events downstream of the formation of an inhibitory complex. The possibility of detecting phosphorylation of the receptor complex or other subsequent events [e.g., MAPK, Crk, or PLC-γ (*35*, *36*)] would seem to offer little assistance in resolving this because the absence of such events neither confirms nor refutes the formation of an inhibitory complex which may not, in any case, be subject to phosphorylation. Furthermore, phosphorylation could occur with a complex that is deficient in some other way and that prevents it performing a proper signaling function.

The FGF signaling pathway, HS, and Cu(II) ions have all been associated with angiogenesis (*37*). Free Cu(II) ions are very toxic in living systems, and mammals possess Cu(II) binding proteins such as metallothionein to regulate them. However, it has been noted that the serum level of Cu(II) ions, which normally varies between 10 and 25 μM, becomes elevated considerably during tumorigenesis and pregnancy (*38*). Moreover, HS rich in Ido2S-GlcNAc, which is also prevalent in the NAc-rich heparin used in these studies, has been reported in endothelial basal lamina (*39*). The interactions among Cu(II) ions, heparin derivatives (acting here as proxies for HS), and the FGF signaling system, which result in inhibition, are of both academic and practical interest, although whether polysaccharides with the structures studied here and in association with high levels of either Na or Cu(II) ions exist in nature is unknown.

The findings reported here also suggest that an additional level of control may, at least in principle, exist in this signaling system, one that exploits the influence of cations and is brought to bear via the polysaccharide. It should be pointed out that delineating the binding preferences for various cations by these polysaccharides is not simple because in most cases their location cannot be detected directly but must be inferred, usually from NMR spectroscopic data. Recent experiments, however, have demonstrated that Cu(II) ions exhibit marked selectivity for a particular sequence in intact heparin (*21*, *23*).

The challenge of resolving the issue of stoichiometry in these complexes remains, and the problem of detecting and quantifying particular complexes in a mixed population will need to be addressed (*40*). The deconvolution of structural changes in the two protein components of the complex, as well as the removal of the interference of the polysaccharide during spectral observations, is the subject of ongoing work (*22*). It should be noted that this analysis cannot provide information concerning the distribution or positions of structural changes in three dimensions or of the overall stoichiometry. The importance of the order of interactions between the components also warrants closer scrutiny.

Here, we have shown in principle that putative signaling and inhibitory complexes comprising identical molecular constituents, but varying in the identity of their associated cations, can be formed. This and other recent reports, in which Cu(II) complexes of hyaluronic acid, a related GAG, have been shown to influence endothelial cell behavior (*41*) and stimulate tissue vascularisation (*42*), further highlight the potential of specific inhibitors of particular growth factor−receptor combinations for experimental, biotechnological, or medical purposes.

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SUPPORTING INFORMATION AVAILABLE

^{13}C (NaNAc and CuNAc) and ^{1}H (NaNAc and CuHep) NMR spectra and UV−vis spectra of CuHep and CuNAc. This material is available free of charge via the Internet at http://pubs.acs.org.

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