Comparable stabilisation, structural changes and activities can be induced in FGF by a variety of HS and non-GAG analogues: implications for sequence-activity relationships†‡

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The activities of heparan sulfate (HS) and heparin do not correlate simply with sulfation levels or sequence. The alternative hypothesis, that appropriate charge and conformational characteristics for protein binding and activity can be provided by other sequences in heparan sulfate and, possibly, also in unrelated sulfated polysaccharides, is explored. Differential scanning fluorimetry was used to measure the thermostabilisation bestowed by modified heparin polysaccharides (proxies for heparan sulfate) on fibroblast growth factor-1 (FGF-1) and fibroblast growth factor-2 (FGF-2), prototypical heparan sulfate-binding proteins, revealing varied abilities and primary sequence-activity redundancy. The effect of substitution pattern on the heparin/heparan sulfate backbone was explored using principal component analysis of ^{13}C NMR chemical shift data for homogeneously modified heparin polysaccharides revealing complex conformational effects. No simple relationship emerged between these polysaccharides, with their distinct charge distributions and geometries, and their ability to signal. Other, structurally unrelated sulfated polysaccharides were also able to support signalling. These influenced FGF stabilisation in a similar manner to the HS analogues and provided analogous cell signalling activity. For FGF-1, but not FGF-2, signaling correlated strongly with protein stabilisation and circular dichroism spectroscopy demonstrated that some non-HS polysaccharides invoked comparable secondary structural changes to those induced by heparin. Active conformations can readily be found in several heparin derivatives, as well as among non-HS polysaccharides, which comprise unrelated primary sequences, confirming the hypothesis and implying that the level of unique information contained in HS sequences may be much lower than previously thought.

Introduction

Glycosaminoglycans (GAGs) comprise diverse populations of negatively charged, linear polysaccharides, which are important components of the extracellular matrix.[1] GAGs are found on the surface of practically all mammalian cells, where they play important regulatory roles in a wide range of situations including the developing neural[2] and vascular systems,[3] stem cell differentiation[4] and development.[5] It has been postulated that GAGs, which are a late evolutionary development associated with Eumetazoa,[6] encode high information content by virtue of the huge number of combinations of different disaccharide subunits, commonly characterised in terms of sulfation pattern and also by uronic acid content,[7] as well as the observed heterogeneity of native heparan sulfate (HS). Heparan sulfate and its close structural analogue, heparin, as well as their chemically modified derivatives (Scheme 1), share an underlying repeating disaccharide unit comprising a uronic acid (either β-D-GlcA or α-L-IdoA) and α-D-glucosamine, with varying patterns of sulfation, at position-2 of the uronic acid (for iduronate denoted I-2) and/or -6 of the glucosamine (denoted A-6) units with either *N*-acetyl (*N*-Ac), *N*-sulfonamido (*N*-sulfate; NS), or free amino (NH_2) groups at A-2. Other, rarer sulfations can also occur, most notably 3-*O*-sulfation in glucosamine and 2-*O*-sulfation in GlcA residues.

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‡ Electronic supplementary information (ESI) available: Supplementary Table 1 Systematically modified heparins studied by ^{13}C NMR and analysed using principal component analysis. Supplementary Table 2 ^{13}C chemical Shift assignments (ppm) for 12 chemically modified heparins. Supplementary Table 3 Loadings derived from the principal component analysis of ^{13}C chemical shift values. Supplementary Figure 1. The principle of DSF data analysis in the presence of illustrative plant polysaccharides and visualisation of background signal of Sypro Orange. Supplementary Table 4. For each set of compounds (heparin derivatives (D1–D9) and chemically sulfated plant polysaccharides (P1–P11)), the melting temperatures of the polysaccaride-FGF complexes, recorded in triplicate, are presented. Supplementary Figure 2 The ability of heparin chemical derivatives (DX) and sulfated plant polysaccharides (PX) to support signalling through FGFR1c/FGF-1 and -2 in a BaF3 cell assay. Supplementary Table 6 Degree of sulfation of sulfated polysaccharides. Supplementary Figure 7 ^{1}H NMR spectra of the chemically sulfated polysaccharides. See DOI: 10.1039/c0ob00246a

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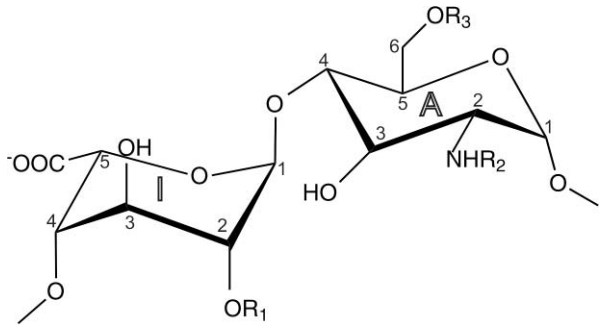

Scheme 1 The general repeating disaccharide structure of heparan sulfate and modified heparin polysaccharides; [-4) α-L-IdoA 1–4 α-D-glucosamine (1-], where R_1 = H or SO_3^-, R_2 = H/$COCH_3$ or SO_3^- and R_3 = H or SO_3^-. The α-L-IdoA can be replaced by its C-5 epimer, β-D-GlcA. For clarity, ***I*** represents iduronate and ***A*** (aminosugar) represents glucosamine. In HS, there is also a domain structure and GlcA predominates together with lower overall sulfation.

Heparan sulfate has become the focus of considerable interest following its implication in diverse biological and medically-related activities. These range from the largely sequence-specific interaction with antithrombin (AT) and subsequent inhibition of factor Xa, to the much less specific interaction with thrombin (factor IIa), as well as to a wide variety of other interacting proteins,[7] including fibroblast growth factors and receptors (FGF/FGFRs).[8] Early on, the broad hypothesis was that HS structure-function relationships could be interpreted in terms of primary sequence. However, it has become apparent that, of the miriad proteins with which HS and heparin interact, only a handful exhibit anything approaching structural specificity in the conventional biochemical sense. Furthermore, these structure-function conclusions have been drawn largely from experiments in which only restricted sub-sets of all possible oligosaccharide structures (*e.g.*, <0.01% hexa or ocatasaccharides of those theoretically possible) have been screened[9,10] (*e.g.* there are $>10^7$ theoretically possible sequences for hexadecasaccharides) making it difficult to reach definitive conclusions concerning sequence-activity relationships.

While the evidence shows that high levels of sequence specificity are absent, a *degree* of selectivity *is*, nevertheless, observed. Several (but certainly not all) distinct substitution patterns typically give rise to comparable activity levels. These relationships are not, however, determined by purely charge density considerations either[11] and the situation is further complicated by the fact that many activities involve interactions between HS chains and single proteins, while others require the formation of ternary complexes. The latter might be expected to involve higher levels of structural specificity. However, a clear understanding of the structure-function relationships of these molecules has, so far, proved elusive and remains the subject of much debate.

Here, we investigate an alternative hypothesis; that the structure-function relationship in HS/heparin is determined by the formation of suitable conformational and charge characteristics, that are complementary to protein binding sites, rather than by simple primary sequence *per se*. These requirements can be satisfied by multiple, but not all, distinct primary polysaccharide sequences, as long as a suitable shape and charge distribution can be generated. The conformation will ultimately derive from the primary sequence but, the complexities of modelling 3-dimensional structure and dynamics, as well as the effect of the surrounding solvent water, currently prevents its elucidation in simple terms for these charged molecules. A predicted consequence of this hypothesis is that broadly similar structural changes should be induced in proteins by the binding of mimic structures, able to support signalling, as for HS/heparin.

One approach to studying the characteristics of this system, adopted by ourselves[12–15] and others[16] has been to study a number of chemically modified polysaccharides whose homogeneous substitution patterns represent narrow regions of sequence space, regions which are, nevertheless, widely separated from each other. Simple reference to sulfation position is insufficient to delineate the connection between substitution and activity and these compounds served as probes of structure–activity relationships with particular proteins and biological systems[10] and as model compounds to assist the elucidation of the relationship between defined substitution patterns, conformation and dynamics.[13–15,17] Here, they are employed to explore the structural and activity consequences with FGF-1 and -2, then to investigate the relationship between the substitution pattern and structural effects in the polysaccharide, detected by changes in experimental ^{13}C NMR chemical shift values, which must be unravelled if the connection between substitution and activity is to be understood.

The extent to which the requirements for binding, stabilisation and activation of FGFs, are uniquely provided by the inherent characteristics of HS and its heparin analogues (which all share common underlying structural features) were then addressed by determining whether other sulfated polysaccharides of a non-GAG origin (and hence structurally distinct) could also induce comparable stabilisation and activities. If the hypothesis is correct, and many polysaccharides with diverse primary sequences can indeed support signalling, then the unique information content of HS may be much lower than the huge diversity of its primary sequence suggests. Furthermore, not only will it be possible to replicate readily the biological activities of HS but, it will be useful to know whether this is achieved by reproducing the binding and structural changes induced by HS/heparin in the relevant proteins. This could have a considerable impact on the practical use of such agents as HS mimics for pharmaceutical purposes and the understanding of selectivity.

Results and discussion

1. Interactions between modified heparin polysaccharides and FGF-1 or -2 were followed through their effects on protein thermal stabilisation and these effects were not directly related to the charge of the polysaccharide

In the interaction of GAGs with proteins, one of the plausible explanations by which GAGs enhance, or sustain particular activities, involves the stabilisation of a particular protein conformation. Most of the research relating to protein stabilisation by GAGs has been performed using heparin as a proxy for HS and it is well known that heparin can stabilize lipase,[18] or antithrombin III,[19] as well as growth factors, to sustain enzymatic function or mitogenic activity.[20]

The interactions of the modified heparin polysaccharides (D1) to (D9) (Table 1) with FGFs were followed using a recently developed thermostability assay based on differential scanning

Table 1 Compound key. **A.** The eight modified heparin derivatives and the corresponding predominant substitution patterns of the repeating disaccharide. **I** indicates iduronate, **A** glucosamine (aminosugar) while the sub- and super-scripts indicate the presence of hydroxyl (nOH) or *O*-sulfate at position-n (nS), or *N*-acetyl (NAc) or *N*-sulfate (NS). **B**. The starting materials from which each of the eleven chemically sulfated plant polysaccharides were prepared

A. Heparin derivatives		B. Plant polysaccharides	
Sample	Compound	Sample	Starting material
D1	$I_{2S}\ A^{6S}{}_{NS}$ (heparin)	P1	Tylose
D2	$I_{2S}A^{6S}{}_{NAc}$	P2	Ethyl cellulose
D3	$I_{2OH}\ A^{6S}{}_{NS}$	P3	Hydroxypropylmethyl cellulose
D4	$I_{2S}\ A^{6OH}{}_{NS}$	P4	Alginic acid
D5	$I_{2OH}\ A^{6S}{}_{NAc}$	P5	Xanthan gum
D6	$I_{2S}\ A^{6OH}{}_{NAc}$	P6	Locust bean gum
D7	$I_{2OH}\ A^{6OH}{}_{NS}$	P7	Gum arabic
D8	$I_{2OH}\ A^{6OH}{}_{NAc}$	P8	Pectin
D9	$I_{2S3S}A_{6S3SNS}$	P9	i-Carrageenan
		P10	Hydroxyethyl cellulose
		P11	Glycogen type II

fluorimetry (DSF).[21] The assay exploits the ability of a hydrophobic dye, Sypro® Orange, to interact with hydrophobic core amino acid residues exposed upon denaturation, resulting in an altered emission spectrum. This provides denaturation curves from which melting temperature (Tm) values can be calculated (Supplementary Figure 1). Derivatives of heparin with distinct substitution patterns (Section 2 and Scheme 1) stabilised FGF-1 and -2 to different extents when compared to the unmodified reference compound, heparin (D1) (Fig. 1A & B).

FGF-1 and FGF-2 showed thermal stabilisation to similar extents in the presence of heparin (D1) (Supplementary Table 4). The thermal stability (*i.e.* absolute value) of FGF-2 was higher than FGF-1, consistent with FGF-2 having better defined secondary structural features than the molten globular[22] and more conformationally flexible[23] FGF-1. The extent of stabilisation for each protein was not, however, related directly to the charge density. Derivatives with similar charges had quite different effects. For example, 2-de-*O*-sulfated heparin (D3) stabilised FGF-1 and FGF-2 less than 6-de-*O*-sulfated heparin (D4), despite these two polysaccharides having the same overall charge (Fig. 1A). There was also evidence of redundancy, in that structures with distinct substitution patterns and different charges, for example, *N*-acetylated heparin (D2) and 2,6-de-*O*-sulfated heparin (D7), had similar stabilising effects on FGF-1 and FGF-2 (Fig. 1A & B).

On binding FGF-1, there was reportedly a small change in protein tertiary structure, but little change in secondary structure,[24] while the interaction between FGF-2 and heparin did alter secondary structure.[25] Nevertheless, both types of change in protein structure caused an increase in thermal stability but, this may not necessarily have resulted in the assembly of an active signalling complex. FGF-2 was, in general, more stable that FGF-1 but, interestingly, both proteins showed very similar patterns of increased stability with the chemically modified heparins, suggesting similar modes of binding.

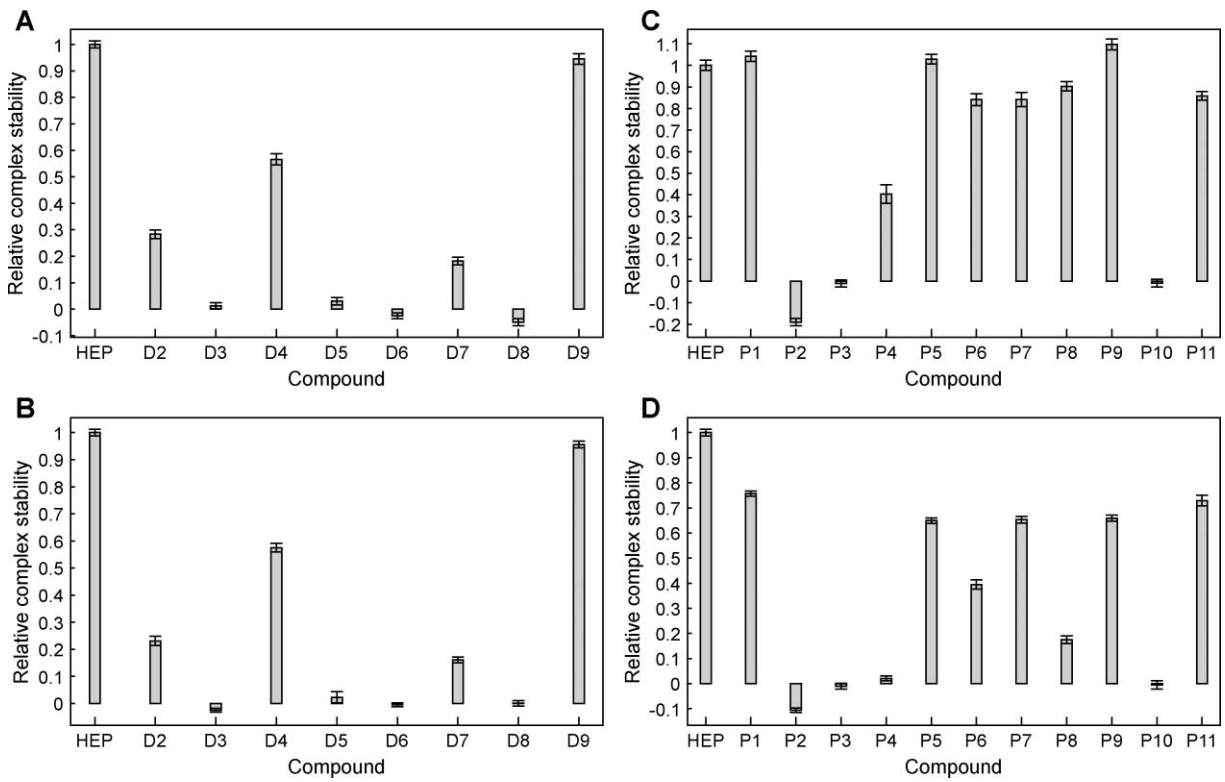

Fig. 1 Thermal stabilisation induced by modified heparins (**A** and **B**) and sulfated plant polysaccharides (**C** and **D**) on FGF-1 [Upper panels] and FGF-2 [Lower panels]. The thermal stabilities of FGF-1 and FGF-2 in the presence of different polysaccharides were determined by DSF as described in Methods. The stabilisation induced by each compound was normalised relative to those of heparin (= 1) and PBS (= 0). The stabilising effects of each polysaccharide were very similar on both FGF-1 and FGF-2 but, quite distinct in terms of the activity and the structural changes induced in the protein (Figs 3 and 4). Experiments were performed in triplicate. Results represent mean values ± SD.

2. The substitution pattern in homogeneously modified heparin derivatives influences the polysaccharide backbone at distinct positions and to different extents

The systematically modified polysaccharides, based on a heparin template and representing models of HS were first examined by ^{13}C nuclear magnetic resonance spectroscopy (NMR). Precise geometric details cannot be recovered readily from NMR data for these polysaccharides owing to the difficulty of determining coupling constants accurately and the limitations inherent in modelling heavily charged structures. However, the existence of effects and a broad indication of their extent at individual positions within the predominant repeating disaccharide units of the polysaccharide can be gained by considering the relationship between substitution (*O*-sulfation, *N*-sulfation, *N*-acetylation) and ^{13}C NMR chemical shift patterns.[26]

Statistical analysis of the ^{13}C NMR chemical shifts of a series of systematically modified heparin derivatives[14,17] employing factor analysis demonstrated that substitution with sulfate groups at I-2, A-2 or A-6, in addition to influencing chemical shift values at the positions involved directly in substitution (I-2, A-2 and A-6), also exhibited effects at distinct locations and to different extents within the repeating disaccharide unit, including the linkage positions involved in the glycosidic bonds.

A measure of the variations in ^{13}C NMR chemical shift values at the glycosidic linkages of the predominant repeating disaccharide, A-1, I-4, I-1 and A-4 (Fig. 2), were extracted from the loading values (Supplementary Table 3), obtained by factor analysis of the ^{13}C NMR chemical shift data (Supplementary Table 1 and 2). Variation at positions A-1 and I-1 was significantly dependent on the identity of the substituent at A-2 (component c2 in Fig. 2) and at I-2 (c1). Variation at A-4, on the other hand, depended on both the substituent at A-6 (c3), and at A-2, while variation at I-4 was related to which modification had occurred at I-2 (c1) and at A-2 (c2). Variation at none of the linkage positions depended heavily on the substitution condition at both I-2 and A-6 (c1 and c3), demonstrating that modifications at these positions introduced variation into the linkage positions independently of each other.

Apart from direct effects at, or near, the site of substitution, linkages flanking the glucosamine residues were influenced primarily by substitution at A-2 and A-6, but not I-2 and those flanking the iduronate residues by substitution at I-2 and A-2, but not A-6. Modification at A-6 only had a significant effect at A-4 and even this was moderate, while substitutions at either I-2 or A-2 influenced the environment at I-1. Thus, substitution at the three principle points of substitution in the repeating disaccharide had

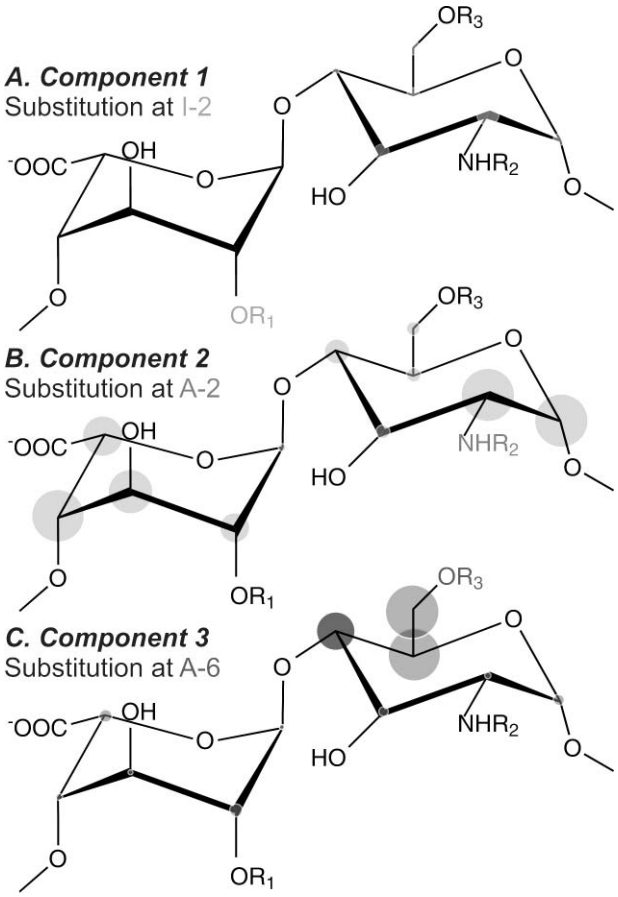

Fig. 2 A representation of the relative magnitudes (proportional to the areas of the grey circles) of the loadings associated with each of the first three principal components at each position of the disaccharide repeating unit of heparin. These were derived from component analysis of ^{13}C NMR chemical shifts for 12 modified heparin derivatives in which modifications at I-2, A-2 and A-6 and combinations there of were made.

effects on the structure which were different in extent and nature from each other.

Desulfation at position-6 of glucosamine had the smallest effect on the molecule in terms of NMR chemical shift changes and the effect was localised. In keeping with this finding, 6-de-*O*-sulfated heparin (D4) exhibited the highest stabilisation after heparin (D1) [Section 1]. These results are consistent with position-6 largely providing a location for an additional charged group, whereas desulfation at I-2 or A-2 caused additional, widespread conformational changes to the molecule (Fig. 2), which compromised their abilities to efficiently stabilise FGFs (Fig. 1A & B).

Table 2 Summary of activities of selected modified heparins with FGF-1, FGF-2 and various FGF receptors in cell signalling assays

Combination of FGF/FGF Receptor			
HS analogue	FGF-1/R1	FGF-2/R1	FGF2/R3
D1	Active[b,c]	Active[a,c]	Active[c]
D2	Weakly active[b,c]	Active[c]	Active[c]
D3	Inactive[c]	Weakly active[c]	Weakly active[c]

[a] 13. [b] 12. [c] 27.

3. The modified heparin polysaccharides exhibited varied abilities with FGF-1 or -2 to signal through FGFR and several distinct structures were able to signal through FGFR1c

In a cell based assay (a BaF3 cell line transfected with a single FGF receptor isoform) of signalling through FGFR1c, in which the ability of exogenously added saccharide and FGF to support signalling, the modified heparin polysaccharides exhibited a range of activities [Table 2]. Some heparin polysaccharides were able to support signalling through a variety of

FGF/FGFR combinations.[12,13] The abilities of intact heparin (D1), *N*-acetylated heparin (D2) and ido-2-de-*O*-sulfated heparin (D3) are illustrative and are summarised in Table 2. (D1) is active in all 3 combinations of FGF and receptor, while (D2) and (D3), despite having the same overall charge density, exhibit distinct activities. For example, with FGF-1/FGFR1, these are active and inactive respectively¶. It is known that (D2) and (D3) differ conformationally from heparin, (D1), and from each other. For example, NOE experiments[12] showed that conformation around the A-1 · · · I-4 glycosidic linkage changed considerably; the relative distances between A-1 hydrogen atoms and those at I-3 and I-4 changing, together with the disappearance in (D2) of a hydrogen bond between the *N*-sulfate and hydroxyl at I-3 present in (D1).[15] On the other hand, the iduronate residue in (D3) has a distinct conformation from both (D1) and (D2)[17] and altered geometry around the glycosidic linkages, $O_g \cdots$ I-4 and I-1 $\cdots O_g$.[14]

Polysaccharides had broadly similar abilities to stabilise FGF-1 and -2 (Fig. 1A & B) although the outcomes in terms of signalling were distinct, suggesting that with FGF-1, stabilisation is sufficient, while for FGF-2, additional interactions involving both FGF receptor and polysaccharide are involved.

Signalling activity is clearly not related simply to charge density, or the presence of particular sulfate groups but, to more subtle conformation and charge characteristics. Heparin derived polysaccharides with different sequences and clearly distinct conformations can exhibit similar activities, supporting the notion that a level of sequence-activity redundancy exists in heparin derivatives and by implication, also in HS. Whatever the requirement of a particular protein is for charge distribution and conformation, it can clearly be satisfied by a number of distinct structures. This led us to investigate the possibility that other non-GAG sulfated polysaccharides may also possess the requisite characteristics and to investigate their effects on FGF stabilisation and conformation.

4. Interactions between FGF-1 and chemically sulfated plant polysaccharides, with primary sequences distinct from GAGs, were analogous to those with heparin derivatives; stabilisation of FGF-1 correlating strongly with the ability to signal through FGFR1c

To investigate whether the structural requirements for FGF binding, stabilisation and activation could only be satisfied by HS or GAG analogues, or whether these features could be mimicked by other sulfated polysaccharides with distinct primary structures (*i.e.* constituent monosaccharide linkages and geometries), a series of plant polysaccharides with primary sequences distinct from GAGs (Table 1) were prepared. The ability of these to stabilise both FGF-1 and -2 (Fig. 1C & D) and their capacity to support signalling through FGFR1c were then examined (Supplementary Figure 2).

For FGF-1, several but not all, sulfated plant polysaccharides were able to stabilise the protein and this ability was highly correlated with the capacity to support signalling through FGF-1 and FGFR1c (Fig. 3A). In contrast, all the sulfated plant polysaccharides were able to support signalling through FGF-2, but did not all stabilise it (Fig. 3B). This demonstrated that the structural requirements of FGF-1 and FGF-2 were not restricted solely to heparin/HS or analogous GAGs but, could also be satisfied by diverse sulfated sequences with different underlying primary sequences. In addition, their associated conformational, or charge properties and an ability to stabilise FGF-1 were requirements for signalling. For FGF-2, the structural requirements for signalling were more relaxed than for FGF-1 (for the compounds prepared here); all of the compounds prepared showed some ability to support signalling (Fig. 3), but not to induce stabilisation (Fig. 1D), consistent with signalling through FGF-2 being dependent on mechanisms other than stabilisation of the protein structure.

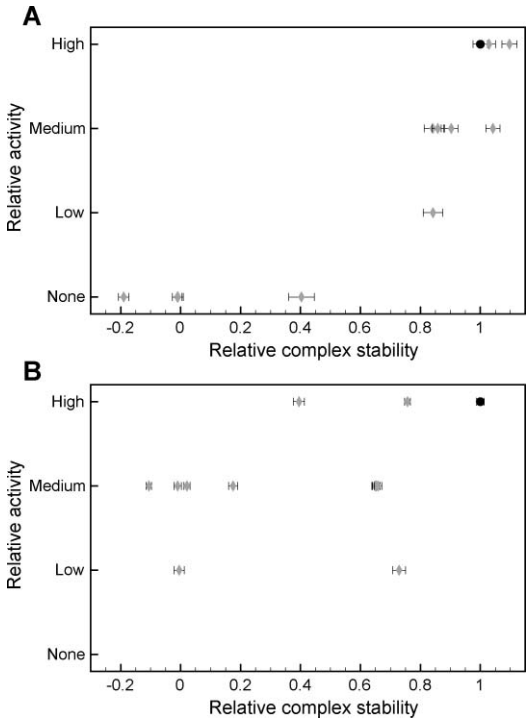

Fig. 3 Correlation of stability and activity bestowed by sulfated plant polysaccharides on FGF-1 (**A**) and FGF-2 (**B**), where the strongest activity (stabilisation defined as 1) is bestowed by heparin (D1) (labeled as a black circle). Activity refers to the ability to support cell proliferation in BaF3 cells transfected with FGFR1c in the presence of exogenous FGF-1 and the test polysaccharide. (Supplementary Figure 2) Compound identities for FGF-1 grouped in terms of activity: none; P2, P4, P10. Low; P7. Medium; P1, P6, P8, P11. Strong; P5, P9. For FGF-2: Low: P10, P11. Medium: P2, P4, P5, P7, P8, P9. Strong: P1, P6.

5. Comparable secondary structural changes were induced in FGFs, particularly FGF-2, by structurally diverse sulfated polysaccharides

No simple correlation was evident between signalling and changes in CD spectral features upon polysaccharide binding to FGF-1, (Fig. 4A), while complete correlation was observed between the ability of a polysaccharide to stabilise and its ability to signal. This suggests that complex formation, comprising FGF-1:FGFR1c:polysaccharide, required the structural stabilisation of FGF-1, (implying a strong interaction between FGF-1 and polysaccharides) for signalling to occur. Stabilisation of FGF-1

¶ In this paper, all derivatives were initially in the sodium salt form; altering the associated cations can also influence both conformation and activity.[13–15,17,27]

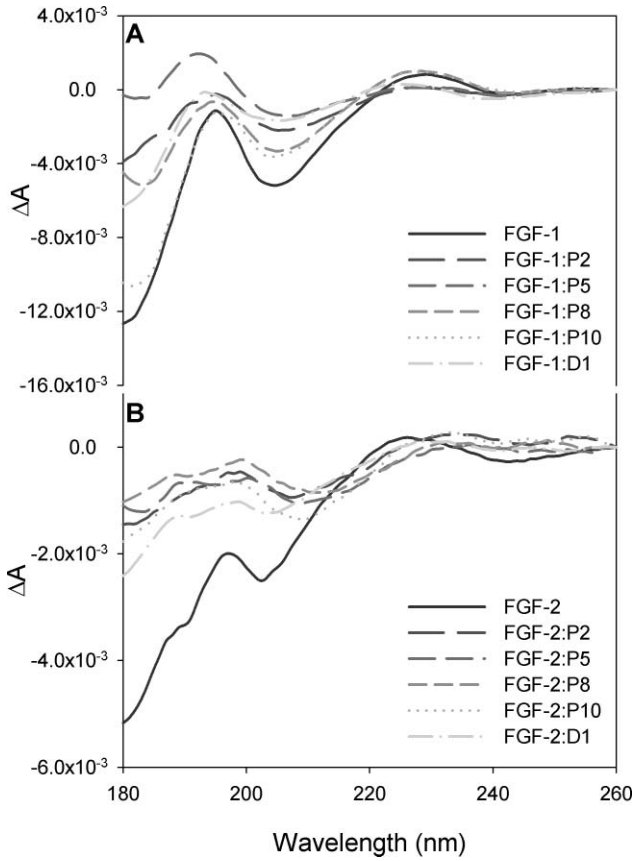

Fig. 4 SRCD spectra (180–260 nm) of FGF-1 (**A**) (recorded at 0.9 mg ml^{-1} in phosphate buffer) and FGF-2 (**B**) (recorded at 0.5 mg ml^{-1} in phosphate buffer) with a selection of sulfated plant polysaccharides (at 1/5th molar equivalence of the polysaccharide with respect to the protein). All spectra were normalised at 260 nm and smoothed using the Savitzky–Golay method, 12 point window, using a second order polynomial.

correlated strongly with activity above a threshold level (Fig. 3A) but, it does not induce identical secondary structural changes in FGF-1 (Fig. 4A) for (P5) and (P8), for instance, which are both active. In contrast, FGF-2 exhibited more consistent secondary structural changes upon interacting with the sulfated plant polysaccharides (Fig. 4B). Seemingly, FGF-2 did not require thermal stabilisation to signal but, relatively consistent secondary structure does result from the binding of sulfated polysaccharides, irrespective of the level of signalling (Fig. 4B). The controlling mechanism is obviously related to the binding of this complex to the third component of the signalling complex, FGFR, rather than either the ability to bind and stabilise FGF-2, or to form a particular secondary structure.

We have shown that modification at A-6 had little effect on the chemical shifts of the other positions (Fig. 2) within the molecule and, consequently, relatively little influence on geometry, suggesting that sulfation at A6 provided the ability to bind FGFR,[9] by supplying an additional, suitably disposed, charge. The sulfated plant polysaccharides that fail to signal may not provide the correct charged scaffold preventing signalling complexes comprising FGF-2, the polysaccharide and FGFR1c to form.

Conclusion

The observation that, for FGF-1, there was no correlation between substitution pattern, or conformation in heparin derivatives for effective stabilisation and signalling, demonstrated that a degree of latitude exists in the structural requirements of binding and activation. A number of sulfated plant polysaccharides with distinct underlying structures and substitution patterns were also able to bind and stabilise FGF-1, and did so in a similar manner to the heparin derivatives. Stabilisation correlated very closely with the ability to support signalling through FGFR1c. This situation contrasted sharply with that for FGF-2, in which all of the plant polysaccharides, but not all heparin derivatives, supported signalling and was independent of their ability to stabilise the protein. There was remarkable similarity between the stabilising influence of individual polysaccharides on FGF-1 and -2 (Fig. 1), while the resulting activities were distinct and only the activities of FGF-1, but not FGF-2, correlated closely with stabilisation. This is consistent with the formation of a signalling complex involving FGF-1 that relies primarily on protein stabilisation, while for FGF-2, additional interactions with the receptor are required.[9] These findings show that the mechanisms of signalling through FGF-1 and -2 are distinct and also that the requirements for binding and activating these FGF/FGFR combinations are rather loose, in terms of conformational and charge properties, being considerably more relaxed for FGF-2 than FGF-1. However, the results obtained with the heparin derivatives (D1) to (D9) illustrate that stabilisation and signalling are not simply related to charge density. Clearly, the structurally diverse range of sulfated plant polysaccharides are able to fulfil the role normally played by HS/heparin in the cell signalling system with FGF-1 and FGFR1c. The extent of stabilisation induced in FGF-1 by the sulfated plant polysaccharides correlated strongly with their ability to signal through FGFR1c. Furthermore, the primary sequence, backbone structures and linkage geometries of these polysaccharides were quite distinct from those in HS/heparin and from each other, indicating that many potential geometric and charge distribution arrangements can satisfy binding (in the case of FGF-2) or, binding and stabilisation (in the FGF-1 case) to allow signalling. Active conformations can readily be found among both heparin derivatives and sulfated plant polysaccharides comprising many, unrelated primary sequences, confirming the hypothesis that appropriate charge and conformational characteristics for protein binding and activity can be provided by other sequences in HS and also by unrelated polysaccharides. This calls into question the level of unique information that HS sequences contain and backs-up earlier work in which structural redundancy in HS analogues was predicted and modeled.[28]

FGF-1 is known to have a disordered structure, often referred to as "molten globular" comprising ~55% β-strands and ~20% turns, ~10% α-helix and ~15% un-ordered stretches,[29] and undergoes a small change in tertiary structure [24] when binding sulfated polysaccharides. The structural requirements of the associated anionic saccharide for signalling through FGF-1 or FGF-2 with FGFR1c, which were examined here, clearly showed considerable latitude. An important consequence is that not only is it possible to employ non-GAG analogues of HS with FGFs, which has been demonstrated amply by previous work[30] but, that these structurally diverse polysaccharides function in essentially the same way as

heparin derivatives. In the case of FGF-1/FGFR1c, they appear to work predominantly through FGF-1 stabilisation. Recently, it has also been shown that the activities of FGF-1, in which different degrees of thermal stability and heparin binding had been introduced through mutations, correlated with their thermal stability.[31] Stabilisation of FGF-1 has also been observed with non-GAG polyanions previously and related to charge.[32] The results demonstrated that the effects, while lacking exquisite primary sequence specificity, are not simply related to charge density; rather, they suggest a redundant system with relaxed structural requirements. The authors note that non-specific interactions have been proposed previously as influencing polysaccharide protein interactions for heparin and anti-thrombin.[33]

These findings confirm that non-GAG analogues of HS can be used as competitors in a wide range of situations in which FGF signalling (and potentially other activities) is involved (examples include cell proliferation, angiogenesis, cell differentiation and growth), having similar structural effects on proteins as HS/heparin, and offers the possibility of targeted activities for selected HS-related activities, with favourable off-target activities. The mechanisms of FGF signalling are controversial and complex,[34] but the findings presented here argue in favour of a considerable degree of structural tolerance (in terms of the polysaccharide involved) in the formation of FGF signalling complexes and show that this tolerance can be exploited.

Experimental part

Protein and polysaccharide preparation

Recombinant FGF-1 (Uniprot Accession: P05230; residues: 16-155) and FGF-2 (Uniprot Accession: P09038-2; residues: 1-155) were expressed in C41 *E.coli* cells using a pET-14b system (Novagen, Merck Chemical Ltd, Nottingham, UK) and FGF-2 was purified as described previously.[35] The same procedure was applied for the purification of FGF-1. The same heparin stock (Celsus Lab, Cincinnati, OH, USA; batch number) was used in the assay and for the production of modified derivatives.

Differential scanning fluorimetry

The experiment was performed using 7500 Fast Real Time PCR System (software version 1.4.0) (Applied Biosystems, Foster City, CA, USA). The samples were subjected to the heating cycle as described previously.[21] Briefly, to the Fast Optical 96 Well Reaction Plate (4 °C) were aliquoted in the following order: Dulbecco's Phosphate-buffered Saline without $CaCl_2/MgCl_2$ (Gibco-Europe, Paisley, UK), the polysaccharide and protein stock solutions (10 × in HPLC-grade water and 50 mM Tris pH 7.5 2M NaCl, respectively) and a freshly prepared 100 × water based solution of Sypro® Orange 5000 × (Invitrogen, Paisley, UK). The final volume of the reaction mix was 10 µL per well, where the polysaccharide, protein and 100 × Sypro Orange constituted 10 vol.% each. Final concentration of the protein was 10 µM. For chemically modified heparin derivatives and plant polysaccharides assays, the effective concentration of heparin was chosen (10 µM corresponding to 175 µg mL^{-1}), and all other compounds were used at the same w/v ratio (175 µg mL^{-1}). After sealing, the plate was gently vortexed and directly analysed in a real time PCR instrument. The heating cycle comprised of 120 s pre-warming step at 31 °C and subsequent gradient between 32 and 81 °C in 99 steps of 0.5 °C every 20 s. Data were collected using the calibration setting for TAMRA™ dye detection (λ_{ex} 560 nm; λ_{em} 582 nm).

DSF data analysis

The DSF data were analysed using Plot v. 0.997 software for Mac OS X (plot.micw.eu) by application of an exponential correlation function approximation of the first derivative for each melting curve. For each variant three distinct melting curves were analysed. The maxima of three distinct derivatives were used to calculate the mean Tm (melting temperature) and standard deviation of each variant. Subsequently, the data were normalised to compare any stabilisation effect of assayed heparin derivatives with heparin. To perform normalisation (Tnorm), each compound was characterised by the difference between the value of Tm for the protein in PBS alone and Tm in the presence of the compound according to the formula (1),

$$\text{Tnorm} = \text{Tm(x)} - \text{Tm(PBS)} \quad (1)$$

where Tm(x) is the mean Tm of the protein with the assayed compound and Tm(PBS) is the mean Tm of the protein in PBS alone. Next, the comparison of stabilisation potency *versus* polysaccharide was obtained from the formula (2),

$$\text{Relative complex stability} = \text{Tm(x)} - \text{Tm(PBS)}/\text{Tm(hep)} - \text{Tm(PBS)} \quad (2)$$

where Tm(x) is the mean Tm of the protein on addition of the assayed compound, Tm(PBS) is the mean Tm of protein in PBS alone and Tm(hep) is the mean Tm of the protein in the presence of heparin. The relative stabilising effect of protein in PBS was set as 0, while the relative stabilising effect of heparin was set as 1.

CD spectroscopy

CD spectra were recorded at Beamline 23, Diamond synchrotron, using quartz cuvette (0.2 mm, QS cuvette, Hellma). Polysaccharides were added to 1/5th molar equivalence, maintaining the original protein concentration, and background signals from the buffer and air were subtracted. The spectra were collected with the units mdeg, and converted to ΔA by dividing by 3290.

Analysis of ^{13}C NMR data

^{13}C NMR chemical shift values for a library of chemically modified heparin polysaccharides were recorded as described previously[15] (Supplementary Table 1 and 2) and were analysed by factor analysis, with factors being extracted through principal components. The loadings were derived from the analyses reporting the effective change in chemical shift when a modification was made at specific positions in the molecules; the modifications causing the change were identified by the component regression scores.

Chemical sulfation of plant polysaccharides

The 8 chemically modified heparin derivatives were prepared and characterised essentially as described.[15] *O*-Sulfation of plant polysaccharides was achieved employing methyl sulfonate and chlorosulfonic acid essentially as described.[36] The products were

dialysed extensively (7 kDa cut-off membrane) against distilled water and characterised by ^{1}H NMR [Supplementary Fig. 3].

BaF cell assay

BaF3 cells, which do not produce their own heparan sulfate, were transfected with a single FGFR isoform, here FGFR1c. Their proliferation was measured in the presence of an exogenous test FGF (either FGF-1 or FGF-2) and sulfated polysaccharide.[37] Briefly, 10 000 cells/well were plated onto a 96 well plate with 100 µl of culture medium [RPMI-1640 with 10% foetal calf serum, 100 U mL^{-1} penicillin-G, 2 mM L-glutamine and 100 µg mL^{-1} streptomycin sulfate without IL-3] and then incubated with 2 ng mL^{-1} IL-3, 1 nM test FGF (–1 or –2) and the test polysaccharide at a range of concentrations. MTT was added after 72 h incubation at 37 °C and cell proliferation determined by reading the absorbance at 570 nm and correcting for background.

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