Insights into the role of heparan sulphate in fibroblast growth factor signalling

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Abstract
Signalling from the FGFs (fibroblast growth factors) is crucial for the correct development and homoeostasis of a wide range of cells and tissues. The FGF/FGFR (FGF receptor) signalling system forms an important paradigm for HS (heparan sulphate)–binding proteins, as both the growth factor and receptor bind to HS, and HS or heparin is an absolute requirement for full signalling. The FGF signalling system has been extremely well structurally characterized, and details of each interaction involved in forming a ternary complex of FGF–FGFR–heparin have been elucidated. Recent work has focused on a more thorough understanding of the nature of the FGF–heparin complex in particular, demonstrating that FGFs preferentially bind to similar sites on the co-receptor, and that FGF–FGFR pairs show greater specificity for heparin sulphation patterns than individual FGFs. Further work has suggested that FGF–FGFR–heparin signalling complexes contain one molecule of heparin only, and that when longer fragments of heparin are used to form FGF–FGFR–heparin complexes, multiple complexes form upon the saccharide. These observations form the basis of a model where the range of interactions that FGFs and FGFRs can form with one another and with HS may lead to the formation of complexes with more than two FGFR units. Therefore HS will be crucial to FGF signalling from the initial signalling event to the formation of large receptor clusters.

Introduction
The FGF (fibroblast growth factor) cytokine family is among the best understood of the heparin-binding growth factors [1]. The 18 signalling FGFs are involved in the development and homoeostasis of virtually every human tissue [2,3]. The cytokine signal is transmitted from the extracellular space into target cells via cell-surface protein receptors, the FGFRs (FGF receptors). The FGFRs consist of an extracellular region of three Ig-like domains (IgI, IgII and IgIII), a single transmembrane helix and an intracellular tyrosine kinase domain [3]. Following the binding of FGF to the IgII and IgIII domains [4,5], the tyrosine kinase domains of two receptors transphosphorylate one another, activating these domains to phosphorylate signalling targets [6]. The formation of an extracellular complex involving two FGFRs is therefore crucial to the signalling event (Figure 1A).

The FGFs, in addition to their protein receptor, show high affinity for the polysulphated glycosaminoglycan HS (heparan sulphate) and its analogue heparin (reviewed in [7,8]). This interaction serves to protect the FGF in the extracellular matrix, and plays a key role in its interaction with FGFRs [9]. Although the FGFs have affinity for FGFRs in the absence of heparin, heparin increases the affinity of FGF for FGFR by one order of magnitude [10,11]. The presence of HS is obligatory for a full range of signalling events associated with FGFR activation, but can be replaced by exogenously added heparin fragments, indicating the intimate involvement of HS in the activation of FGFRs by FGFs [12]. FGFRs also possess a heparin-binding activity in IgII [13], and so the signalling complex will include both FGF and FGFR interacting with HS.

Two crystallographic models have been proposed for the FGF–FGFR–heparin signalling complex (Figures 1B and 1C). Although these two structures show similar arrangements for the complex of one molecule each of FGF, FGFR and heparin, the manner in which two FGF–FGFR complexes are brought together to form the ternary complex differs drastically between the two models [16]. One model (Pellegrini model; [14]) shows the heparin as the fundamental organizing unit of the complex, dimerizing the two FGFs in the absence of any protein–protein contact (in a similar manner to a 2:1 FGF1–heparin complex observed crystallographically; [17]); the other (Schlessinger–Mohammadi model; [15]) shows additional interactions between the FGF and both members of the other FGF–FGFR complex as the main determinant of dimerization, with the heparin contributing to the interaction and providing relief from electrostatic repulsion. The latter model also suggests that two molecules of heparin are required per ternary complex. These structures have served as a tremendous stimulus to further research into the nature of the interaction of HS and heparin with the FGFs and FGFRs.

FGF and FGF–FGFR binding to heparins
Recent years have seen significant improvements in the preparation of heparin derivatives, and the sequencing of heparin fragments. These have allowed more detailed studies...
of the specificity of FGFs and FGF–FGFR complexes for sulphation patterns within heparin. Sequencing of heparin fragments binding strongly to immobilized FGFs supported the interactions observed in crystal structures, indicating that FGF1 preferentially binds to the Id02S (l-iduronic acid 2-O-sulphate)-GlcNS6S (N-sulpho-D-glucosamine 6-O-sulphate) trisulphated disaccharide [18], while FGF2 requires only an Id02S-GlcNS bisulphated disaccharide for maximal binding [19,20]. Analysis of other FGFs has suggested that FGF1 is more typical of the family, and that FGFs bind most preferentially to similar heparins to FGF1 [21]. Complementary experiments, examining the effects of selectively desulphating heparins, have suggested that the minimal sulphation requirements for binding do show some differences between the FGFs [22]. Moreover, in vivo, the FGFR plays a significant role in determining the heparins to which the FGF–FGFR complex will bind. FGF–FGFR pairs show significant differences in the heparin length and sulphation patterns required to elicit the formation of FGFR dimers, and biological activity, even between pairs with a common FGF or FGFR [23]. It therefore appears that, although there are only very moderate differences in heparin affinity between individual FGFs, the formation of complexes with FGFR has far more stringent requirements on the saccharide component of the complex: this allows for the regulation of developmental processes by alteration of cellular HS, in addition to expression changes in FGF and FGFR [24].

Biophysical analyses of FGF–FGFR–heparin interactions reveal the kinetics and stoichiometry of FGF–FGFR–heparin complex formation

The crystal structures of the FGF–FGFR–heparin ternary complex revealed the intimate involvement of heparin/HS in the formation of FGFR dimers. Several biophysical studies have used these models as bases to gain deeper insight into the role of heparin in forming interactions with FGFs and FGFRs. Surface plasmon resonance [11] determination of the rates at which complexes of FGF, FGFR and heparin form have revealed that the formation, and separation, of FGF–heparin complexes is extremely rapid in comparison with the
formation of other complexes involving these biomolecules. This suggests that, in the cellular context of large amounts of HS, the primary interaction between signalling molecules will be between the FGF and HS; that the FGF may have the opportunity to sample multiple HS sites before it interacts with FGFR; and that it is likely to bind to receptor in complex with a saccharide that is most optimal for FGF binding.

Other biophysical techniques have provided insight into the number of heparin molecules required to form a complex involving two FGFR molecules. The two crystallographic models suggest that either one or two molecules may be required to engage two FGFRs [14,15]. Analyses of complexes prepared using the methods of Pellegrini [14] and Mohammadi [4,15] (by analytical size-exclusion chromatography and analytical ultracentrifugation) suggest that species consistent with either model may be prepared from the same protein components [25]. Determination of the mass of these complexes by nanospray-MS demonstrated that complexes prepared according to either method contain one molecule of heparin (a minor product prepared according to the Schlessinger–Mohammadi method containing two molecules of heparin) [25]. It therefore appears that the stoichiometry of the FGF1–FGFR2–heparin complex is 2:2:1; that only one heparin molecule is required to engage all of the protein components in either architecture of the complex; and that there is the possibility of forming either complex in solution. The importance of HS for FGF signalling is clear: just one molecule is capable of promoting the formation of this primary signalling event.

**Binding of multiple FGF–FGFR units to a single saccharide**

The importance of HS as an organizing unit in this complex is highlighted by the confirmation that a single saccharide is sufficient for the formation of two types of 2:2 FGF–FGFR ternary complexes. The role of heparin in these complexes raises the question of how many FGFs might bind to a single HS chain in vivo. In particular, crystallographic models for FGF dimerization have shown FGFs dimerized by their binding to a single heparin saccharide, with no protein–protein contact [14,17]. In the context of the cellular environment, where HS is abundant and there are likely to be a good number of potential binding sites for FGFs (especially at low cytokine doses), it is not clear why such a complex would form: what would drive the FGFs to localize on opposite sides on a single HS stretch, such that the FGFRs will be juxtaposed in a manner sufficient to induce the activation of their tyrosine kinase domains? Assessment of the effect of adding excesses of heparin samples to preparations of the Pellegrini complex has shown that, in the presence of a 5-fold excess of heparin, the complex is still stable [26]. Similarly, FGF1 predominantly forms 2:1 FGF1–heparin complexes even in the presence of over 10-fold excess of heparin [27]. There is little sign of the FGFs, or FGF–FGFR pairs separating on to separate saccharides, as would be likely in the absence of some driving force for the co-localization of the two FGFs at the same point on the saccharide. These observations have suggested that the binding of a second FGF1 molecule to a saccharide of sufficient length is more favourable than the binding of this FGF1 to free heparin: and that the binding of the one FGF1 monomer to heparin may pay the entropic price for the loss of flexibility of the saccharide, allowing the more favourable binding of the second molecule [26]. It remains to be seen whether this tendency is shared by all FGFs, but this suggests an effective route to the nucleation of ternary complexes upon HS.

The HS chain differs considerably from heparin fragments: it is far longer (100–300 saccharides), has variable sulphation, with multiple sulphate-rich S-domains [7,8], and so may have rather different FGF-binding properties from short heparin fragments. As a step towards a treatment of these longer polysaccharides, the effects of longer fragments of heparin on the formation of FGF1–FGFR2–heparin complexes were examined [26]. Fragments as short as 16 saccharides permitted the formation of a discrete species larger than the 2:2:1 FGF1–FGFR2–heparin complex; and the proportion of chains capable of forming this complex rises with increasing heparin length. Further analysis of this larger species revealed that this is a 4:4:1 FGF1–FGFR2–heparin complex, and suggests that two copies of the minimal complexes observed crystallographically are forming on a single heparin saccharide. Furthermore, it appeared from MS analysis of this complex that the most highly sulphated chains were the most competent to form these larger complexes (as might be expected for FGF1). These observations suggest that multiple FGF complexes might form on one HS saccharide in the cell, and that these might be one driving force towards the initiation of aggregates of many FGFRs co-localized on the cell surface to generate the signalling plaques that have been observed with other tyrosine kinase receptors (e.g. [28]).

**Perspective**

The role of HS in the initial complex that activates FGFRs in vivo remains a matter of considerable interest and debate. This initial complex represents an excellent target for the inhibition of the action of a single pathologically acting FGF, and a regulatory role for HS in development is becoming increasingly apparent. Since the elucidation of two crystallographic models for the ternary complex, the role of HS in the physiological complex has become better appreciated. It has become apparent that many of the FGFs bind most favourably to similar patterns of modifications on the heparin backbone, and that the FGF–FGFR combination has considerably more specificity for heparin sequence than either component alone. The search for the most favourable site appears to be driven by the capability of the FGF to rapidly bind to (and dissociate from) heparin, allowing this molecule to sample a wide selection of the available HS locations on the cell. The role of heparin as an organizer of the FGF–FGFR complex is demonstrated by the capacity of one molecule of heparin, not only to form 2:2 FGF1–FGFR2 complexes in either the Pellegrini or Schlessinger–Mohammadi architectures, but also to associate two FGF1
molecules in the absence of protein–protein interactions, and
even to form higher order complexes if the saccharide has
sufficient length and sulphation.

The progress towards a thorough understanding of the
FGF–FGFR–HS complex identifies areas that are still poorly
explained. Although it has become clear that the FGF–FGFR
combination is likely to be the determinant of HS affinity,
there is a need for more structures of FGF–heparin, and FGF–
FGFR–heparin complexes, to allow the molecular basis for
the different affinities to be determined. Finally, a change
in the focus of research from heparin fragments to HS chains
will open the understanding of interactions that may be
masked by the short length and high homogeneity of heparin
fragments. These routes will provide progress towards the
full range of FGF–FGFR–HS interactions that contribute
to the control of FGF-related signalling throughout human
development and adulthood.

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