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The nature of the conserved basic amino acid sequences found among 437 heparin binding proteins determined by network analysis†

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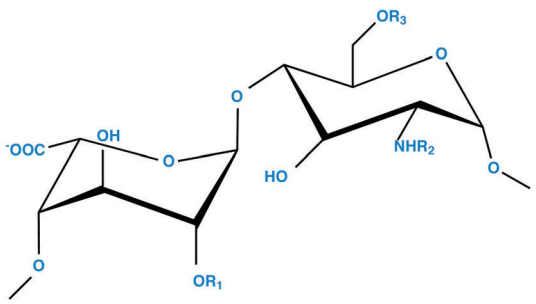
In multicellular organisms, a large number of proteins interact with the polyanionic polysaccharides heparan sulphate (HS) and heparin. These interactions are usually assumed to be dominated by charge–charge interactions between the anionic carboxylate and/or sulfate groups of the polysaccharide and cationic amino acids of the protein. A major question is whether there exist conserved amino acid sequences for HS/heparin binding among these diverse proteins. Potentially conserved HS/heparin binding sequences were sought amongst 437 HS/heparin binding proteins. Amino acid sequences were extracted and compared using a *Levenshtein* distance metric. The resultant similarity matrices were visualised as graphs, enabling extraction of strongly conserved sequences from highly variable primary sequences while excluding short, core regions. This approach did not reveal extensive, conserved HS/heparin binding sequences, rather a number of shorter, more widely spaced sequences that may work in unison to form heparin-binding sites on protein surfaces, arguing for convergent evolution. Thus, it is the three-dimensional arrangement of these conserved motifs on the protein surface, rather than the primary sequence *per se*, which are the evolutionary elements.

1. Introduction

Heparan sulfate (HS) and heparin are closely related linear polyanionic carbohydrates (Scheme 1), which are members of a class of polysaccharide known as glycosaminoglycans (GAGs).[1] Heparan sulfate is a ubiquitous element of mammalian cells and plays an important physiological role, including receptor–ligand clustering and signalling, cell-to-cell cross talk and adhesion, chemokine presentation, storage, cell adhesion and extracellular matrix (ECM) formation. Heparan sulfate is found on the surface of cells as a part of proteoglycans (HSPG), for example, syndecan and glypican,[2] as well as being an integral component of the ECM, where HS is attached to proteoglycans such as agrin and perlecan.[3] Heparan sulfate has also been found in the cell nucleus,[4,5] although the functional significance of this remains unclear.

Owing to its abundance, relatively low cost and overall structural similarity, heparin is often used as an experimental proxy for HS. Heparin is readily available as a widely used pharmaceutical anticoagulant which originates in mast cells, where the polysaccharides are stored in intracellular granules as serglycin proteoglycans. Mast cells can be stimulated to eject their granules, a process termed degranulation, through physical/chemical damage or through interaction with IgE, cytokines and others agents.



Scheme 1 General repeating disaccharide structure of HS and heparin polysaccharides; [(−4) L-IdoA α(1→4) D-GlcN α(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 residue can be replaced by its C-5 epimer, β-D-GlcA. HS has lower overall sulfation than heparin, possesses a more distinct domain structure and a higher proportion of GlcA residues.

Heparin is composed of the same disaccharide units, although in different proportions, and both HS and heparin share a common biosynthetic pathway.[6] The polysaccharides comprise alternating disaccharides of an uronic acid linked

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1→4 to α-D-glucosamine. The uronic acid can be present as β-D-glucuronic acid, or its C-5 epimer, α-L-iduronic acid, both of which can be *O*-sulfated at position 2. The glucosamine can be *O*-sulfated at positions 3 and 6, as well as *N*-acetylated, *N*-sulfated, or unsubstituted at position 2. The major repeating disaccharide unit of heparin is shown in Scheme 1. The large number of possible enzymatic modifications involved in the biosynthesis together with the non-template-driven nature of their biosynthesis, results in highly heterogeneous polysaccharides.[7,8]

The principal difference between HS and heparin resides in the organisation and content of their domain structures. The majority of the HS chain is composed of [(−4) D-GlcA α(1→4) D-GlcNAc α(1−)], disaccharide repeats containing glucuronic acid and *N*-acetyl glucosamine, exhibiting little or no sulfation. Interspersed between low sulfation domains are sequences with higher degrees of sulfation. It is in these high sulfation regions, where the majority of protein interactions are thought to occur,[9,10] that have a structure more closely resembling that of heparin. Heparin consists of around 80% of the trisulfated disaccharide, [(−4) L-IdoA2-*O*-sulfate α(1→4) D-GlcN-sulfate,6-*O*-sulfate α(1−)]. Heparin is composed of around eighty percent of this trisulfated disaccharide, making it more homogenous than HS.[7]

It is often stated that heparan sulfate and heparin interact with numerous, key proteins primarily *via* the high sulfation regions in HS/heparin. This statement is perhaps tautological, since almost all experimental investigations have involved the selection of proteins bound to HS/heparin *via* elution from a heparin column using salt that inherently selects for high charge interactions.[11] Ori *et al.*[12] compiled a list of 435, non-redundant, human HS/heparin binding proteins (HEPbps) in the HS/heparin interactome, which include members of important protein families, such as growth factors, cytokines and morphogens. Heparan sulfate is a molecule that, in some manner, choreographs signalling pathways thereby allowing information to cross the cell membrane.[11,13] Heparin binding proteins play a key role in controlling development, for example, *via* the Wnt, Hedgehog, transforming growth factor-beta and fibroblast growth factor (FGF) pathways.[14] Furthermore, HS has been implicated in diseases such as Alzheimer's,[15] cancer[16] and sexually transmitted infections.[17] Recently, Nunes *et al.*[18] performed a study to examine the role of HEPbps in pancreatic diseases, concluding that a concerted network of highly connected HEPbps was important for distinguishing between normal and diseased pancreatic tissue. Chen *et al.*[19] showed that the interaction between the cell surface HSPGs of two-breast cancer cell lines and their innate complement of HEPbps is a key component of tumourigenicity. Inhibition of the innate HEPbps of breast cancer cell lines by the addition of extraneous heparin perturbed the PI3K/Akt and Raf/MEK/ERK signalling pathways.

In evolutionary terms, HEPbps are thought to originate at the dawn of multicellular life, *via* colonies of communicating unicellular organisms. *Monosiga brevicollis* is one such organism and it is known to contain the biosynthetic machinery necessary to produce heparin/HS.[12] *M. brevicollis* also possesses receptor tyrosine kinases (RTK),[20] and the HEPbps FGF family are ligands for RTKs in metazoans. Recently, Bertrand *et al.* found orthologous genes to the FGFs in *M. Brevicollis* and proposed that FGFs and their receptors originated in a eumetazoan ancestor.[21] Finally, three GAG lyases have also been predicted in the proteome of the organism.[22] These observations indicate that what is often considered a relatively simple organism possesses the full apparatus of a HSPG-mediated cell-signalling system. Furthermore, *M. Brevicollis* possesses lyases capable of causing GAGs to be shed into the environment and is, in principle, therefore, able to interact with its neighbours *via* protein and glycan communication. Such findings support the idea that HEPbps are crucial for, and may be a defining characteristic of, multicellular animal life.

Basic amino acids in HEPbps are postulated as being key to interactions with HS/heparin. Linhardt *et al.* published a number of studies investigating the heparin binding properties of the three basic amino acids,[23–25] arginine, lysine and histidine. They concluded that the affinity between heparin and arginine is higher than that between heparin and lysine. Histidine exhibits low affinity and only at pH values at which it is protonated (below its pK_a of *ca.* 6.5). The frequency, location and structure of basic amino acids in HEPbps are consequently likely to be important determinants of their binding properties.

Heparin binding sequences (HBSs) are amino acid sequences found in HEPbps that have been shown, or are predicted to be, the domains that bind to HS/heparin. Cardin and Weintraub[26] reported two sequences, XBBBXXBX and XBBXBX in the heparin binding proteins: apo B; apo E; vitronectin; and platelet factor 4 (where B and X signify basic and hydropathic amino acids, respectively). These sequences were then used to predict HBSs in other proteins and a similar approach was used to propose the von Willebrand factor HBS – XBBBXXBBBXXBBX.[27] Subsequently, Hileman *et al.* proposed the heparin-binding consensus sequence TXXBXXTBXXXTBB (T denotes a turn), combining secondary structure information and conserved sequence information. This sequence was proposed using the crystallographic/NMR structural data for FGF-1 and -2 and transforming growth factor (TGF). A recent theory proposed by Torrents *et al.*, defines a minimal sequence, termed the "CPC clip motif" (C – cationic and P – polar residues), with this sequence working analogously to a staple; small points of contact pinning the polysaccharide to the protein.[28] Even in combination, however, these studies have only surveyed a very small fraction of HEPbps, which may be too small for global features to become apparent. By examining all HEPbp sequences, it was thought that more general, underlying similarities may emerge.

The aim of this present study was to identify HBSs within all currently collated HEPbps. To do this, a sequence similarity metric paired with graph analysis[29] was employed to investigate conserved sequences within HEPbps that contain basic amino acids. The similarity between amino acid sequences was determined here using the *Levenshtein* distance (D_L).[30] *Levenshtein* distance is also called the edit distance and is defined as the minimum number of single letter elementary operations (insertions, deletions and replacements) required to convert one character string into another. This measure is used widely to compare strings of information, including in applications to protein interactions

with small ligands[31] within the field of protein interactions, which is reviewed in ref. 32. In the present work, a similarity matrix was created from the D_L's to compare the basic sequences. The similarity matrices were transformed into a graph to visualise and analyse these data and this analysis allowed strongly conserved sequences to be extracted from among the highly variable 437 HEPbps, while excluding short, core regions. It is possible that a number of these sequences work in unison to form heparin-binding domains on protein surfaces. The results are consistent with convergent evolution, in which the three-dimensional arrangement of amino acids on the protein surface is the evolutionary element, rather than the primary sequence. Furthermore, when the human proteome was searched for the sequences found in the relatively small population of verified HEPbps, it became clear that many proteins may be able to interact with heparin/HS. Indeed, this may be an innate property of extracellular proteins. This calls into question the possible control mechanism behind protein:-heparin/HS interactions; instead of considering a protein binding to a defined carbohydrate sequence, a more holistic concept should be considered.

2. Material and methods

2.1 Determination of heparin binding sequence (HBS) similarity matrices and the subsequent formation of networks

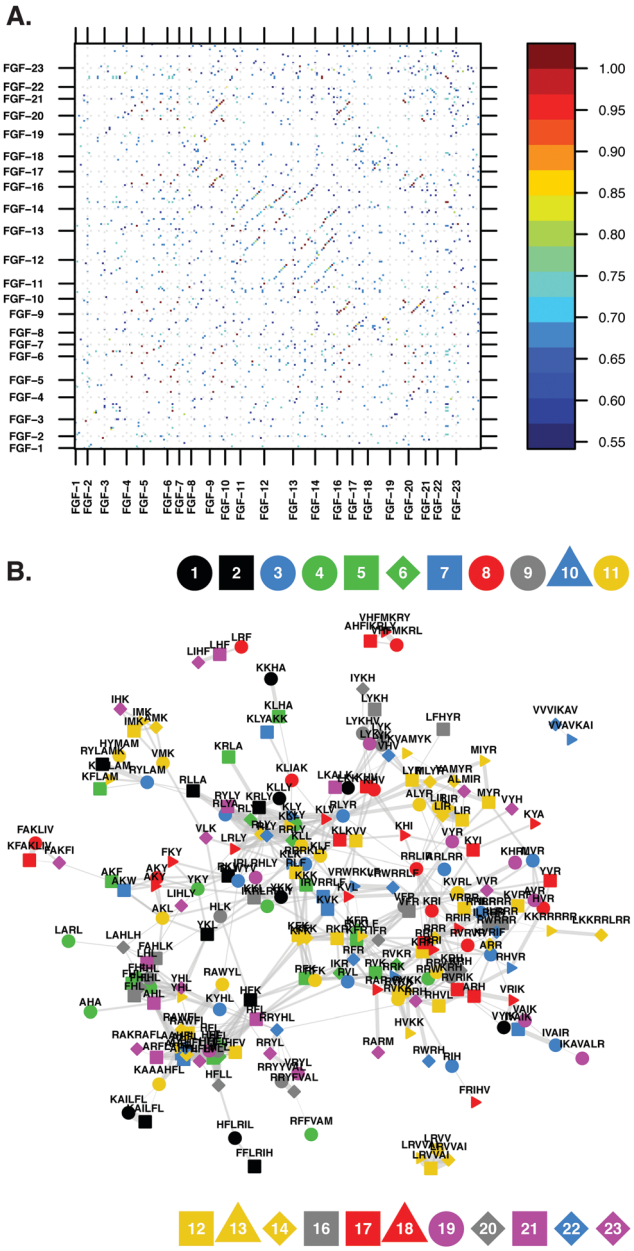
Four hundred and thirty seven HEPbp amino acid sequences were retrieved through UNIPROT.[33] The original HEPbps list[12] contained 435 proteins, from which FBS1 (Fibrosin-1, considered obsolete and removed from UNIPROT, accession number (a/no.) P62706) and IAPP (Islet Amyloid Polypeptide, a/no. P10997) were excluded. FGF11, 13, 19 and 21 (a/no. Q92914, Q92913, O95750 and Q9NSA1, respectively) were added, providing a final list containing 437 proteins.

A search was made for seven amino acid sets within the HEPbps. The sets searched for were {B,X}, {B,X,A}, {B,X,P}, {B,X,S}, {B,X,P,A}, {B,X,P,S} and {B,X,A,S}, composed of the five different types of amino acid: basic (B); hydrophobic (X); polar (P); special (S); and acidic (A) (see ESI,† Table S1 for more details). In the text, these set names are abbreviated to BX, BXA, BXP, BXS, BXPA, BXPS and BXAS. These sets are neither exclusive nor are they exhaustive. Each HBS was read serially from the N- to C-terminus to identify amino acid sequences. Sequences had a minimum length of 3 amino acids.

For the group of amino acid sequences identified from each amino acid set, a similarity matrix (Scheme 2A) was calculated using a normalised *Levenshtein* distance. The *Levenshtein* distance was defined as the minimum number of elementary character operations (insert, delete or replace a single letter) required to transform one sequence into another:

$$D_L(a,b) := \min(i(a,b) + d(a,b) + r(a,b)) \quad (1)$$

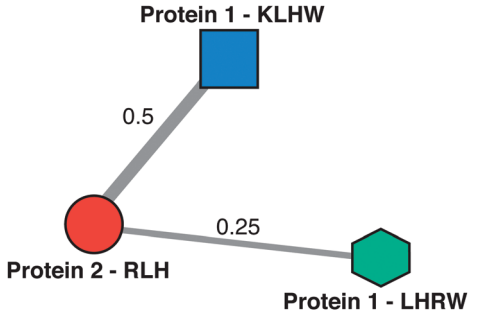
where $D_L(a,b)$ is the *Levenshtein* distance for the conversion of a to b. The terms i, d and r stand for insert, delete and replace,



Scheme 2 (A) Similarity matrix of the BX (basic and hydrophobic) amino acid-containing amino acid sequences, found in the FGF family. It is difficult to extract information from the data contained within the similarity matrix. The relationships between the proteins and basic amino acid containing sequences become clearer when the matrix is converted into a network, in the form of a graph. (B) Graph representations of the basic amino acid containing sequences found in the FGF family. The similarity cut-off (95% confidence interval of the *Levenshtein* matrix values) for the network was 0.57. The colour and shape of the vertices indicate which member of the FGF subfamily the sequence originated from, provided as a legend on the figure. The thickness of each edge is proportional to the similarity of the HBSs linked; the thicker the edge, the more similar are the vertices.

respectively. The normalised *Levenshtein* distance metric for the conversion of a into b was defined as:

$$D_Lm(a,b) := 1 - (D_L(a,b)/\max \text{ length } (a,b)) \quad (2)$$



Scheme 3 Schematic of a HEPbp HBS graph. The vertices represent a basic amino acid sequence from a heparin binding protein. The vertex contains both pieces of information. The connections between vertices is the similarity of the amino acid sequences. The width of the edges is proportional to the weight of the edge. In this analysis amino acid sequences arising from the same protein are not considered, *i.e.*, there is no edge between the vertices belonging to protein 1 in the above schematic.

where max length (a,b) is the length of the longest string, either a or b. Identical HBPs have a *Levenshtein* distance metric of 1, while entirely dissimilar sequences will have 0 *Levenshtein* distance. A 0.7 normalised *Levenshtein* distance cut-off was applied to the similarity matrix to remove dissimilar sequences.

The similarity matrices were visualised and further analysed in graph form (Schemes 2B and 3). Each node/vertex in the graph represents two properties: a sequence and the protein in which it is found. The edges of the graph are weighted by the similarity matrix (above the cut-off) or non-existent (below the cut-off). In the subsequent analysis a sequence within a protein is only compared with sequences from other proteins and not to sequences within the same protein. The graph layouts were determined using Fruchterman-Reingold and force-directed algorithms.[34]

If we consider only one component (sequence or protein) of vertex identity this reduces the number of vertices and therefore the complexity of the graphs. When there exist multiple edges between two vertices these are collapsed into one single edge with new weight defined as the sum of the weights of the original edges. A number of parameters are used to describe the resultant vertices and graphs, and are defined as:

(Vertex) degree. This is the number of edges incident at a vertex. The higher the value, then the more similar the sequence represented by the vertex is to the other sequences present in the network.

(Vertex) closeness. This measures the number of steps required to reach every other vertex from a given vertex. Therefore, an important vertex is close to, and can communicate rapidly with, the other vertices in a network. The closeness of a vertex is defined as the reciprocal of the sum of the distances from the vertex of interest to all of the others in the graph.

Network density. This is the measure of the total possible number of edges verses the actual number of edges in a graph. A network density of 1 equates to the number of possible edges equalling the number of actual edges. The higher the value the more similar the sequences represented by the network are to each other.

Clustering coefficient. This is the mean probability that two vertices adjacent to a vertex are themselves adjacent. This quantity is also termed transitivity and is calculated by determining the number of triangles in the entire network and dividing it by the total number of possible/theoretical triangles.

Modularity. This is a measure of the structure of a graph. It is a measure of how a network can be subdivided into modules (*i.e.*, groups, clusters or communities). A graph with high modularity has dense connections between the nodes within modules but sparse connections between nodes in different modules, *i.e.*, high intra-group degree and low inter-group degree.

Betweenness centrality. "*An important node will lie on a high proportion of paths between other nodes in the network.*" This centrality provides a measure of the control a vertex exerts over other vertices in the network.

Bonacich's centrality (also called the eigenvector centrality). "*An important node is connected to important neighbours.*" This centrality is an evolution of the degree centrality, the degree centrality awards points for connections, not all vertices are equal, however. The eigenvector centrality identifies vertices that are connected to other important vertices.

Further information regarding graphs and their analysis can be found in ref. 29.

The resultant graphs were further refined by only considering the core of the graph; the highly connected heart of the network, which was defined using the closeness centrality.

The graphs were further collapsed by translating the devolving amino acids found in the basic amino acid containing sequences into their groups: B, X, P, S or A (see ESI,† Table S1). For example, the sequence LLR is converted to XXB. The multiple parallel edges were again collapsed into one single edge with weight equal to the sum of the constituent original edges.

2.2 Computation

The analyses were performed using R 3.1.2 "Pumpkin Helmet"[35] running on a MacBook Pro (2.66 GHz Intel Core i7, 8 Gb RAM). Levenshtein distances were determined using the LevenshteinDist function from the RecordLinkage package.[36] Networks were created using the igraph package[34] and similarity matrices were plotted using the lattice package.[37] Parallel processing in R was implemented using the foreach[38] and doParallel[39] packages.

3. Results

3.1 Network description

Heparin binding protein amino acid sequences were decomposed into sequences comprising only amino acids contained within given amino acids subsets. Seven different amino acid subsets were considered, the simplest being amino acid sequences containing basic and hydropathic amino acids ({B,X} sequences). The other basic amino acid containing sequences considered were {B,X,A}, {B,X,P}, {B,X,S}, {B,X,P,A}, {B,X,P,S} and {B,X,A,S} sequences. These basic amino acid containing sequences were then compared using graphs derived from *Levenshtein* distance

metric similarity matrices. A graph is composed of edges and vertices, a vertex represents a basic amino acid sequence and the protein from which that sequence originates. Vertices are connected by edges and, if two vertices are connected by an edge, this signifies that the similarity criteria was met for those two vertices and then the weight of the edge connecting them is the similarity value. The purpose of this analysis was to identify conserved basic amino acid sequences within the HEPbps; the hypothesis being that these sequences may be characteristic for HEPbps and form the heparin binding regions of the proteins. Two parameters were used to guarantee that only conserved sequences were considered. The first was an imposed similarity cut-off, *i.e.*, two vertices were not considered to be connected if the similarity between the vertices was below the similarity cut-off. Second, the core of the graph, the highly connected heart of the network, was selected by using the closeness centrality.

3.2 Similarity cut-off

For this study the similarity cut-off for the conserved basic amino acid sequence graphs was set at 0.7. The effect of varying the similarity cut-off can be observed in ESI,† Table S2. As expected, by increasing the similarity cut-off for the networks the number of vertices, unique sequences and the number of edges decreased. This is also true for the graphs network density and the average degree of the vertices within the graphs, while the transitivity of the graphs increased with the raising of the similarity cut-off, *i.e.*, there is an increased probability that adjacent vertices of a vertex are connected.

3.3 Network core selection

Unlike the analysis of a family of highly related proteins, such as the FGF family shown earlier (Scheme 2), the networks produced from the 437 HEPbps contain many isolated vertices, which are detached from the core of the graph. These vertices belong to sequences that are not highly conserved. When community analysis, using a walktrap algorithm, was performed on these networks many communities were found, for example, the BX graph contained 566 communities, the BXP graph 1311 communities and the BXPS graph 821 communities (Table 1), with most of these communities having a low number of members and a low average degree. The walktrap algorithm used to detect communities in a network is based on a random walk; short random walks tend to stay in the same community. The number of steps used by the algorithm can be defined. In this case seven steps were used, minimising the number of communities found while maximising the modularity of the network.

To isolate the highly conserved cores of the networks, the closeness measure of vertex centrality was used. This measure finds vertices that can 'communicate' quickly with the other vertices in the graph.[29] The closeness of a vertex is defined as the reciprocal of the sum of the distance from the vertex of interest to all of the others in the graph. The closeness values for the HEPbps conserved basic amino acid sequence networks were bivariate; vertices with a higher closeness value residing in the core of the graph (ESI,† Fig. S1). After the isolated vertices were removed from the graphs, the number of communities found decreased. The majority of the communities had a large population and high average degree; for example, HEPbp BX HBS graph had 412 communities, HEPbp BXP HBS network 585 communities and HEPbp BXPS HBS graph 174 communities. Further information can be found in ESI,† Table S2 and Table 1.

3.4 Amino acid types

Historically, investigations looking for heparin-binding sequences within proteins have concentrated on amino acid types, *i.e.*, basic, hydrophobic, *etc.* For the initial survey of the graphs we adopted the same approach. The sequence that each vertex represents was converted into its amino acid type. For example, the sequence LLR was converted to XXB.

Table 1 Properties of the HBS networks constructed from 437 HEPbp

	BX	BXP	BXS	BXA	BXPS	BXPA	BXAS
	Whole network						
No. of starting sequences	10 447	15 426	15 740	13 536	14 124	14 479	16 663
No. of vertices	8987	8439	10 905	9801	3281	4724	7500
No. of unique sequences[a]	4652	6099	7281	6154	2743	3837	5798
No. of edges	121 007	39 002	82 277	81 265	4774	9771	24 852
Network density	3×10^{-3}	1.10×10^{-3}	1.38×10^{-3}	1.69×10^{-3}	8.87×10^{-4}	8.76×10^{-4}	8.84×10^{-4}
Average degree	26.93	9.24	15.09	16.58	2.91	4.14	6.63
Clustering coefficient – transitivity	0.491	0.443	0.453	0.464	0.498	0.451	0.436
No. of communities	566	1311	1288	897	821	931	1160
	Closeness selected network core						
No. of starting sequences	10 447	15 426	15 740	13 536	14 124	14 479	16 663
No. of vertices	8624	6740	9718	8875	1566	3100	5631
No. of unique sequences[a]	4348	4606	6265	5347	1315	2473	4191
No. of edges	120 742	37 800	81 409	80 598	3513	8655	23 563
Network density	3.25×10^{-3}	1.66×10^{-3}	1.72×10^{-3}	2.05×10^{-3}	2.86×10^{-3}	1.80×10^{-3}	1.49×10^{-3}
Average degree	28	11.22	16.75	18.16	4.49	5.58	8.37
Clustering coefficient – transitivity	0.491	0.443	0.452	0.464	0.483	0.446	0.435
No. of communities	412	585	780	503	174	270	363

[a] Even though a vertex is identified by the parent protein and sequence when determining the number of unique sequences, only the peptide sequences were considered.

To find important sequences, the ratio of the degree centrality, the number of vertices incident on a node – how many sequences overcome the similarity cut-off to the number of sequences, was considered (ESI,† Fig. S2). The majority of sequences within the graphs have a low degree to number ratio. This can be seen in ESI,† Fig. S2, in which the density plot of the degree to the number ratio illustrates that there are two populations. The population with the high degree to number ratio comprise shorter sequences, containing 3 or 4 amino acids. Sequences that contain special amino acids; C, G or P, contain significant sequences which are much longer, *i.e.*, in the BXPS graph vertices belonging to the sequence PSSSSPSSSPSSSSSBS have a high degree to number ratio. All of these sequences can be found in ESI,† Table S3. The total number of amino acid sequences found the various HEPbp HBS networks expressed as their amino acid type can be found in ESI,† Tables S7, S9, S11, S13, S15, S17 and S19.

3.5 Network centralities

The four centrality measures (eigenvector, degree, closeness and betweenness) give different, but related, insights into important network properties. The most informative and granular measure is the eigenvector centrality, as this identifies individual vertices that are connected to other important – highly connected – vertices. In the case of the networks being studied here, these are conserved sequences that are linked to other important conserved sequences. Table 2 contains the vertices that are in the top 1% by eigenvector centrality. These important vertices comprise a small fraction of the total number of vertices that compose the graphs; {B,X} 86 of 8987, {B,X,A} 89 of 6154, {B,X,P} 67 of 6099, {B,X,S} 97 of 7281, {B,X,P,A} 30 of 9771, {B,X,P,S} 16 of 4774 and {B,X,A,S} 56 of 5798. The sequences highlighted in the analysis of the {B,X} and {B,X,A} networks contained the amino acids L and R, with the sequence LLR (XXB) appear 33 and 22 times in the {B,X} and {B,X,A} graphs, respectively (Table 2). The important vertices found in the {B,X,P} and {B,X,P,A} networks were associated with the conserved amino acid sequence SYR (SXB), while the {B,X,S}/{B,X,P,S} networks had significant vertices containing the conserved amino acid sequence G?KG (S?BS), where ? was present as: A (X, prevalent in the {B,X,P,S} network); T, K, L, F, P (P, B, X, X, S, prevalent in the {B,X,S} network), and M (X). Finally, the sequence YCR (XSB) was important in the {B,X,S,A} network. Important sequences and the proteins that contain them as determined by the degree, closeness and betweenness centralities can be found in ESI,† Tables S4, S5 and S6, respectively.

3.6 Communities

Another means of describing a graph is by determining the number of communities/clusters that the graph contains. The number of communities found in the networks is a measure of the diversity of the sequences the graphs represent. The method used here to determine the number of communities was based on a random walk, the number of steps taken was chosen by analysing the {B,X} network and determining the modularity of the clustered networks. The number of steps that produced the lowest modularity, before the modularity of the analyses converged, was 7.

The networks formed of sequences that contain 4 different types of amino acid ({B,X,P,S}, {B,X,P,A}, and {B,X,A,S}) contain the fewest communities, BXPS, 174; BXPA, 270 and BXAS, 363. The most diverse network is formed by sequences that comprise basic, hydrophobic and special amino acids (BXS). This has 780 communities. The conserved sequences for the most significant communities can be found in Table 3. The gauge of significance used was size. The vertices that form the communities represent amino acid sequences that are very similar to each other, therefore, the greater the number of vertices that comprise a community, the more important is the conserved sequence.

The significance cut-off was the 95th percentile. The distribution of community sizes had a positively skewed distribution, the number of significant communities found for the different graphs were, {B,X}, 21 of 421; {B,X,A}, 25 of 203; {B,X,P}, 30 of 585; {B,X,S}, 39 of 280; {B,X,P,S}, 9 of 174; {B,X,P,A}, 14 of 270 and {B,X,A,S}, 18 of 363. It is interesting to note that the core of the conserved sequences from the most significant communities are relatively short, three or four amino acids long, as seen in the eigenvector analyses, corresponding to small discrete areas on a protein surface. Tables can be found in ESI† that contain the conserved sequences, amino acid entropy and amino acid frequency for the significant communities found in networks formed from the {B,X}, {B,X,A}, {B,X,P}, {B,X,S}, {B,X,P,A}, {B,X,P,S} and {B,X,A,S} amino acid sets, ESI,† Tables S8, S10, S12, S14, S16, S18 and S20, respectively.

3.7 Conserved sequences in proteins

In order to validate this approach for identifying HBSs within HEPbps, the sequences extracted for a small set of proteins were compared against their experimentally determined HBSs. Molecular schematics and tables of the predicted HBSs can be found in ESI† (Fig. S3–S5 and Tables S21–S26). This approach has previously been used to identify HS/heparin binding sequences in H5N1 haemagglutinin (influenza A virus A/Cygnus olor/Italy/742/2006).[40]

Fibroblast growth factors (FGFs) are a well-studied family of HEPbps. They are a group of 21 proteins that bind to HSPGs and FGF receptors (FGFRs) containing membrane-bound receptor tyrosine kinase. The HS binding of the family has been investigated using a mass spectroscopy "Protect and Label" strategy.[41] The approach has been used to determine the HBS for FGF-1, -2, -3, -4, -6, -7, -9, -10, -17, -18 and -20.[41–43] The principal example shown in the text is for FGF-1, colloquially termed *acidic FGF*. The network analysis method described here identifies sequences within this protein that are highly similar to sequences found in other proteins known to bind heparin/HS, see Fig. 1. The molecular representation of FGF-1 (Fig. 1), shows these conserved basic amino acid sequences creating an extended region around the protein (Fig. 1, lower network). Highlighted in this network are the amino acids (grey vertices) that are within 0.8 nm of the conserved amino acids and that arise in at least two of the seven sets: {B,X}; {B,X,A}; {B,X,P}; {B,X,S}; {B,X,P,A}; {B,X,P,S}; and {B,X,A,S}.

Table 2 Influential sequences within the HEPbp basic amino acid containing sequence networks. The table contains the significant sequences as determined by the eigenvector centrality, the number of times that particular sequence appears in the network and the proteins that contain it. The vertices were considered significant if they were in the 99th percentile

BX			BXA			BXP			BXS			BXPA			BXPS			BXAS		
Seq.	*n*	Prot.	Seq.	*n*	Prot.	Seq.	*n*	Prot.	Seq.	*n*	Prot.	Seq.	*n*	Prot.	Seq.	*n*	Prot.	Seq.	*n*	Prot.
LLR	33	5NTD	LLR	22	AACT	SYR	7	A1BG	GPKG	9	A1BG	SYR	5	FA12	GAKG	5	C1QA	YCR	4	FGF9
LLRL	5	A1BG	LRL	15	ABCBB	SYRT	1	FA12	GKG	8	A2MG	LSYR	1	FBLN7	GTKG	4	CO1A1	LYCR	3	FGF16
LMLR	3	AACT	LRLV	3	ABP1	LSYR	1	FBLN7	GLKG	7	APLP1	SSYR	1	FBN1	AGAKG	2	CO1A2			FGF20
LRL	3	ABP1	LMLR	3	APLP1	SSYR	1	FBN1	GAKG	7	APOB	ESYR	1	FBN2	KGAKG	2	CO5A1			HGF
HLLR	2	APOB	LLRL	3	APOB	KSYR	1	FBN2	GPRG	7	ATS3	SEYR	1	HGF	GKG	2	CO5A3			PLMN
ALLR	2	ATRN	LRAL	3	ATS8	SYNR	1	HGF	PGPKG	7	C1QA	SYNR	1	HMGB1	GKKG	1	CO8A			TPA
KLLR	2	CBPD	HLLR	2	BACE1	ASYR	1	IBP5	PKG	5	CAC1S	ASYR	1	ITA1	TGAKG	1	COBA1			UROK
RLLR	2	CHRD	LLDR	2	CAC1S	QSYR	1	ITA1	RGPKG	3	CCL28	QSYR	1	LAMA5	GNKG	1	COBA2			
LLRI	2	CO3	LREL	2	CBPD	SWYR	1	LAMA5	GRG	3	CO1A1	SWYR	1	LTBP1	GAKA	1	CODA1			
LALR	2	CO4A	ALLR	2	CHRD	ISYR	1	LTBP1	GPK	2	CO1A2	ISYR	1	PLMN	PGAKG	1	CO1			
LLLR	2	CO9	LDLR	2	CO3	SAYR	1	NRP1	AGPKG	2	CO3	SAYR	1	TIMP3	GLKG	1	HMGB1			
LLRH	1	COBA2	LLRE	2	CO4A			PLMN	GMKG	2	CO3A1			TPA	GAKS	1	LAMA4			
FLLR	1	COCA1	LRVL	2	CO5			TIMP3	VGPKG	2	CO5A1			TSP4	GFKG	1	MBL2			
LRLR	1	COIA1	LRLL	2	CO6A3			TPA	GPKA	2	CO5A3						PGBM			
LKLR	1	COJA1	FLLR	1	COBA2			TSP4	LPKG	1	CO6A3						Q9HCS8			
LLRK	1	COMP	LERL	1	COMT				GKKG	1	CO9A1						TSP1			
LLRV	1	COMT	LELR	1	CXCL6				KPKG	1	COBA1									
LLRF	1	CO1	LRLR	1	CYR61				PGAKG	1	COBA2									
LLRR	1	ENOA	LLRK	1	DCC				GGKG	1	COCA1									
LLRY	1	ENPP3	LRV	1	ECM2				GARG	1	CODA1									
LHLR	1	FGFP3	LKRL	1	ERBB2				GVKG	1	COEA1									
LLYR	1	FGFR4	LRFL	1	FA11				KGAKG	1	COIA1									
VLLR	1	FSTL1	LRIL	1	FBN1				GHKG	1	COJA1									
LLFR	1	HBEGF	LARL	1	FGF4				GPKGR	1	COLQ									
LLHR	1	HFE	LRLY	1	FGF18				GIKG	1	CO1									
YLLR	1	INSR	KLLR	1	FGFR4				LGPKG	1	COPA1									
		ITIH3	RLLR	1	FSTL1				MGPKG	1	CRLD2									
		LAMA1	LRLA	1	HFE				KGPKG	1	ERBB2									
		LAMA2	DLLR	1	ITIH3				GPKGL	1	FINC									
		LAMA3	LLRF	1	KALM				GPKH	1	HMGB1									
		LAMA5	LLRR	1	LAMA1				GPKC	1	IBP4									
		LGR4	LRKL	1	LAMA2				GPPKG	1	LAMA2									
		LIFR	LHLR	1	LAMA3				GFKG	1	LAMA5									
		LIPC	LRRL	1	LAMA5				VPKG	1	MBL2									
		LPHN2	FLRL	1	LGR4						MMP9									
		MET	LLYR	1	LIFR						PAIRB									
		MOT8	LALR	1	LPHN2						PCSK5									
		MRP6	LYRL	1	MET						PEBP1									
		V2	LLHR	1	MOT8						PGBM									
		NOGG	YLLR	1	MRP6						POSTN									
		PCOC2	LLRD	1	V2						S12A9									
		PCSK5			NOGG						TSP1									
		PERM			PCOC2						TSP2									
		PGBM			PCSK5						XDH									
		PGS1			PGBM															
		PLGF			PGS1															
		PRG2			PIGR															
		S12A9			PLGF															
		S22AI			PRDX4															
		SCN5A			PRELP															
		SEM5B			PRG2															
		SLIT1			PSN1															
		SLIT2			RL29															
		TEN1			S12A9															
		TE			S20A2															
		TENX			S22AI															
		THYG			SCN5A															
		TRFE			SLIT1															
		TRFL			SLIT2															
		TSP3			TE															
		TSP4			TENX															
		VGFR1			TGM2															
		WNT1			THYG															
		XDH			TRFL															
		ZPI			TSP2															
					TSP3															
					VGFR1															
					XDH															

Table 3 Conserved aligned sequences from the communities found in the HEPbp HBS networks. The table contains the conserved aligned sequences for the most significant communities. The measure of significance used was the size of the communities. A community was considered significant if it was in the 95th percentile

BX		BXP		BXA		BXS		BXPS		BXPA		BXAS	
Com1	----AAK--- ----XXB---	*Com1*	--RRR-- --BBB--	*Com1*	--FRY --XBX	*Com1*	--VVK--- --XXB---	*Com5*	-*G*-PGPKG--- -S-SSSBS---	*Com2*	-RDS- -BAP-	*Com1*	---GRR-- ---SBB--
Com3	---LLR---- ---XXB----	*Com3*	--FRI --XBX	*Com3*	----KKV--- ----BBX---	*Com2*	----KLL----- ----BXX-----	*Com13*	--LGR- --XSB-	*Com7*	---RS--- ---BP---	*Com2*	---KPC- ---BSS-
Com4	-----VKK -----XBB	*Com4*	---KKL--- ---BBX---	*Com7*	----LLR--- ----XXB---	*Com3*	---LR---- ---XB----	*Com16*	---GKKG ---SBBS	*Com8*	KNEE- BPAA-	*Com3*	----LLR-- ----XXB--
Com5	----RLL--- ----BXX---	*Com6*	---AKK-- ---XBB--	*Com8*	--ARR-- --XBB--	*Com4*	------KVV-- ------BXX--	*Com17*	KVL-- BXX--	*Com9*	--SLR-- --PXB--	*Com4*	----LKK- ----XBB-
Com6	----RA----- ----BX-----	*Com11*	--RAA-- --BXX--	*Com9*	---LKK--- ---XBB---	*Com13*	--KII-- --BXX--	*Com18*	---KLK- ---BXB-	*Com10*	--RVS-- --BXP--	*Com5*	--KKI-- --BBX--
Com8	-FFH-- -XXB--	*Com12*	--VLK --XXB	*Com12*	----KKK--- ----BBB---	*Com14*	-HPP-- -BSS--	*Com27*	-LLRL- -XXBX-	*Com11*	--SKK --PBB	*Com7*	----VLK- ----XXB-
Com10	--LHL-- --XBX--	*Com17*	--LVK-- --XXB--	*Com13*	---RRR--- ---BBB---	*Com15*	-PPR---- -SSB----	*Com30*	CIFK SXXB	*Com13*	--AVK- --XXB-	*Com8*	--IR--- --XB---
Com11	------K--LL-- ------*B*--XX--	*Com21*	---LR--- ---XB---	*Com16*	---RLL---- ---BXX----	*Com16*	--RVR-- --BXB--	*Com49*	-GRS- -SBP-	*Com21*	KKKS- BBBP-	*Com19*	--GGH---- --SSB----
Com13	--KKKK--- --BBBB---	*Com25*	--RLL---- --BXX----	*Com18*	----HFL- ----BXX-	*Com20*	---RRIP-- ---BBXS--	*Com60*	GRCC- SBSS-	*Com26*	SKL-- PBX--	*Com21*	------KKK-- ------BBB--
Com18	---LLK-- ---XXB--	*Com32*	--QQR- --PPB-	*Com20*	--HAA-- --BXX--	*Com21*	-CVR-- -SXB--			*Com30*	KAL-- BXX--	*Com22*	-RAA-- -BXX--
Com25	----HHL-- ----BBX--	*Com33*	-LHV-- -XBX--	*Com25*	--HLA-- --BXX--	*Com22*	--RAA--- --BXX---			*Com37*	SRR-- PBB--	*Com27*	--GGK- --SSB-
Com26	-KKR-- -BBB--	*Com34*	-QRVV-- -PBXX--	*Com29*	--EIH- --AXB-	*Com24*	--LAH- --XXB-			*Com43*	--KKL --BBX	*Com29*	--RLP-- --BXS--
Com29	--KVV-- --BXX--	*Com37*	---KKK-- ---BBB--	*Com32*	--LH--F --XB--X	*Com25*	----AAR- ----XXB-			*Com47*	---RL-- ---BX--	*Com45*	--LLH- --XXB-
Com32	---VVR--- ---XXB---	*Com39*	--KKF --BBX	*Com42*	--KEI --BAX	*Com26*	------L--KK----- ------X--BB-----			*Com49*	-NKK- -PBB-	*Com46*	--DGK- --ASB-
Com35	----LLH-- ----XXB--	*Com43*	--RSS-- --BPP--	*Com48*	-LHD-- -XBA--	*Com31*	--RRRR- --BBBB-					*Com48*	--VVR-- --XXB--
Com37	-IIR--- -XXB---	*Com46*	--KKK-- --BBB--	*Com49*	--RAA-- --BXX--	*Com32*	-CKGC -SBSS					*Com49*	---RLL-- ---BXX--
Com40	--HAA--- --BXX---	*Com48*	--ASK --XPB	*Com57*	-EER-- -AAB--	*Com34*	---RGG-- ---BSS--					*Com70*	--PRA-- --SBX--
Com42	IHH-- XBB--	*Com58*	-AAR- -XXB-	*Com58*	-FFK-- -XXB--	*Com35*	--KKKA--- --BBBX---					*Com80*	LHLL- XBXX-
Com74	--KII-- --BXX--	*Com64*	--HHL --BBX	*Com59*	--HVL-- --BXX--	*Com38*	--GHH-- --SBB--						
Com98	-HLA-- -BXX--	*Com66*	-KSQ- -BPP-	*Com64*	--RRV-- --BBX--	*Com43*	--HLG-- --BXS--						
		Com69	-KSS-- -BPP--	*Com75*	--LLH- --XXB-	*Com46*	----GPPGPKG--- ----SSSSSBS---						
		Com80	--SLH-- --PXB--	*Com83*	-FHI- -XBX-	*Com47*	--LLR-- --XXB--						
		Com83	-VVKS -XXBP	*Com101*	-IHL-- -XBX--	*Com52*	--GRC--- --SBS---						
		Com98	--KIT-- --BXP--	*Com120*	-H--VV -*B*--XX	*Com54*	--LLH--- --XXB---						
		Com99	--RNT- --BPP-	*Com326*	-AAH- -XXB-	*Com56*	--GLH- --SXB-						
		Com100	-KVT-- -BXP--			*Com60*	---RLL---- ---BXX----						
		Com113	-IYKT- -XXBP-			*Com62*	--LHL- --XBX-						
		Com120	--VRT- --XBP-			*Com63*	--CRK-- --SBB--						
		Com125	-TARK -PXBB			*Com69*	--VVR- --XXB-						
		Com161	--KVN- --BXP-			*Com76*	-----GPK---*G*---- -----SSB---S----						
						Com96	--KIG- --BXS-						
						Com97	--RHGY --BBSX						
						Com101	-IKK- -XBB-						
						Com110	-RGLPG--- -BSXSS---						

Table 3 (*continued*)

BX	BXP	BXA	BXS	BXPS	BXPA	BXAS
			Com114 --GKK----			
			--SBB----			
			Com122 --KGP--			
			--BSS--			
			Com132 --FHL--			
			--XBX--			
			Com153 -RLA--			
			-BXX--			
			Com179 --PC-K			
			--SS-B			

The approximate length of the heparin/HS disaccharide is 0.8 nm,[7] and therefore a longer chain may lie across multiple connected vertices. These connected vertices would then form an extended heparin/HS binding domain. The 'Protect and Label' mass spectrometry performed on FGF-1 identified four heparin binding regions: KKPKLLY (amino acids (aa) 24–30); IKSTETGQYL (aa71–80); ISKKHAEKNWF (aa113–123); and VGLKKNGSCKRGPRTHYGQAILFLPL (aa124–150).[42] The analysis described above identified amino acid sequences within each of the previously identified regions in FGF-1 that interact with HS/heparin (Fig. 1).

The network analysis was also validated against the FGF-2, FGF-7, FGF-9 and FGF-18 proteins. The conserved basic amino acid sequences of these proteins are shown in Fig. 2 with the 'protect and label' mass spectroscopy hits (Table 4).[41,42] Furthermore, validation against FGF-3, -4, -6, -10, -17 and -20 are in ESI.†

The above analysis indicates that the conserved amino acid containing sequences that are found in HEPbps form a significant part of the heparin binding regions of a protein. Further illustrations of this fact include, hepatoma-derived growth factor (HDGF), lymphotactin (chemokine (C motif) ligand (XCL1)) and interleukin-10 (IL10). Solution NMR analysis of hepatoma-derived growth factor indicated that it had a primary heparin binding site and then possibly a minor binding site at the N-terminal of the protein. The primary HBS consists of K 19, 61, 72, 78 and 80, as well as R 79. The secondary site, which resides in the flexible N-terminus of the molecule is formed of R2 and R6, and K8 and K11.[44] The similarity analysis found all the members of the proposed principal binding site apart from K19. In fact, this amino acid was found by the analysis, but it only appeared once, in the BX group of amino acids. Of the minor binding site, only K11 was found to be significantly conserved, while K8 appeared once in the BXA amino acid group analysis. The network representation of HDGFs HBS highlights how the conserved basic amino acid containing sequences could come together to form the principal HBS, with the conserved sequence $_{28}ARI_{30}$ linking the primary and secondary HBSs together (ESI,† Fig. S3).

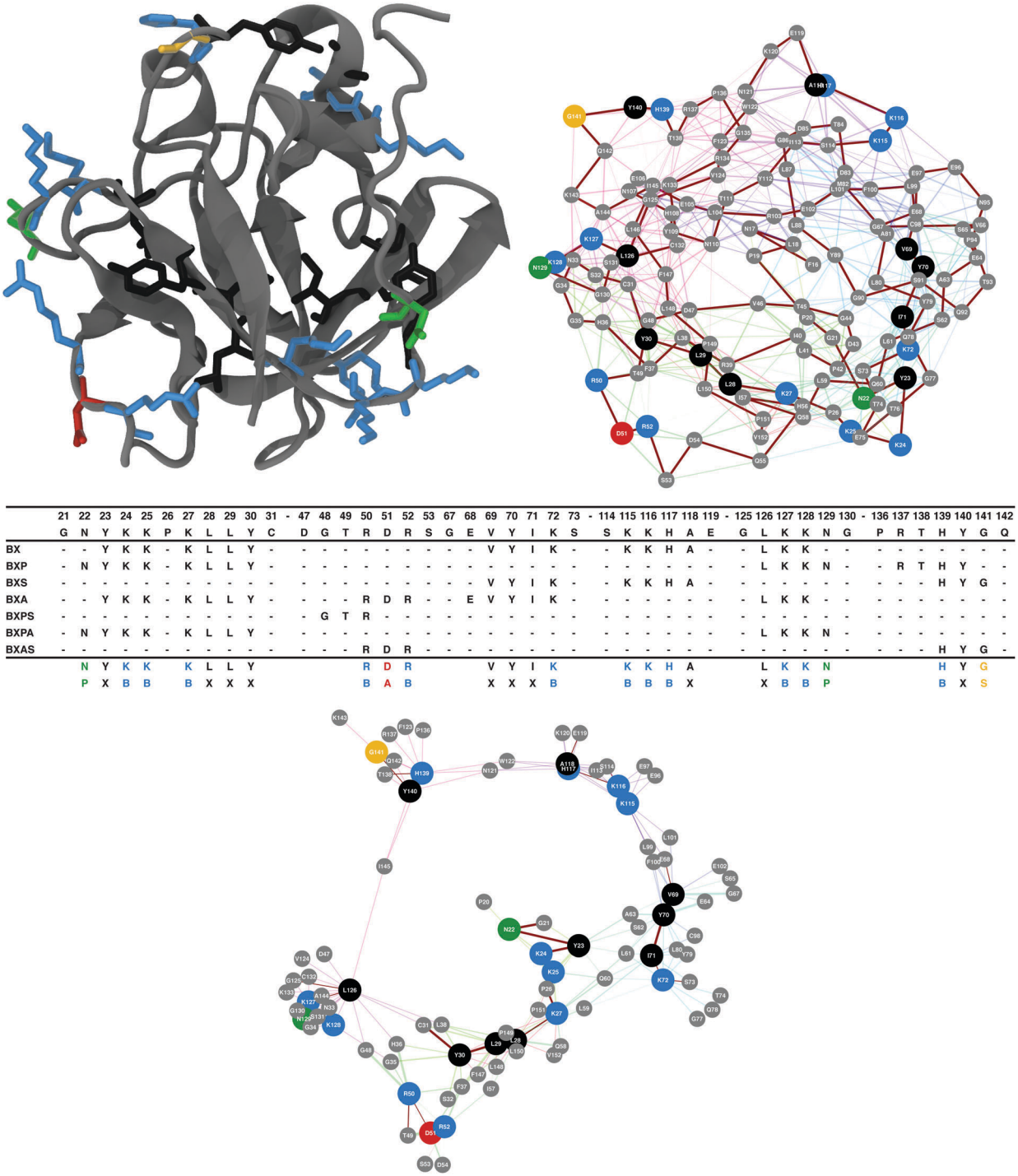
Another example is lymphotactin, a small cytokine. Petersen *et al.*[45] used backbone ^{1}H and ^{15}N chemical shift perturbations to identify the following amino acids as interacting with heparin, R39, R44, K46, K63, R64, K67, R78, R86, K87, and R91. Further use of site-directed mutagenesis identified R44 and R64 as the high affinity residues. All but three of these amino acids were identified by the similarity method employed in this manuscript, and these were K63, R64 and R70. The method was able to identify one of the high affinity binding residues and 70 percent of the total interacting residues (ESI,† Fig. S4).

The final example shown is interleukin-10 (IL-10), which is a cytokine involved in inflammation. It inhibits the production of inflammatory cytokines.[46] It has been determined by NMR that IL-10 interacts with heparin *via* a binding site that comprises resides in helix D and the adjacent DE loop.[46] The residues involved in the interaction are R120, R121, R124, R125, K135 and K137. The analyses shown in this manuscript identify all of these residues except K135. In particular, the analyses identify a domain comprising 8 basic amino acids, R42, R120, R122, R124, R125, R128, R127, H32 and H127 (ESI,† Fig. S5).

It should be noted that in the examples shown here, the FGFs, HDGF, XCL1 and IL-10, that the proteins contain conserved basic amino acid containing sequences that correspond to the experimentally determined HBSs, but there are also other conserved basic amino acid containing sequences that are found in these *bona fide* HEPbps. When heparin/HS binding studies are performed on these proteins the system may be in solution, for the case of NMR and MS studies, but this is still not the natural state of the system. Most of the proteins considered in this study are extracellular, either membrane bound or secreted in to the ECM. This environment is extremely crowded, being composed of many proteins and carbohydrates, of which proteoglycans are an important part. These additional conserved basic amino acid containing sequences found in the HEPbps may be related to the interaction of the HEPbp and its surrounds, for *e.g.*, storage of the HEPbps in the ECM or control of HEPbps diffusion through the ECM, suggesting that there are primary and secondary HBS within HEPbps. In addition to other functions such stabilising the structure of the protein. The primary sites are related to a specific biological activity, *i.e.*, the HBS related to a protein cell signal activity, while the secondary sites assist in the control and movement of the proteins though its environment. It is conceivable that a very large number of proteins interact with heparin/HS but, obviously, not all of them require heparin/HS for their biological activity.

3.8 Human proteome

The result of searching the human proteome for the conserved basic amino acid containing sequences found in the 437 HEPbp is interesting. From this analysis, two main pieces of



	21	22	23	24	25	26	27	28	29	30	31	-	47	48	49	50	51	52	53	67	68	69	70	71	72	73	-	114	115	116	117	118	119	-	125	126	127	128	129	130	-	136	137	138	139	140	141	142
	G	N	Y	K	K	P	K	L	L	Y	C		D	G	T	R	D	R	S	G	E	V	Y	I	K	S		S	K	K	H	A	E		G	L	K	K	N	G		P	R	T	H	Y	G	Q
BX	-	-	Y	K	K	-	K	L	L	Y	-		-	-	-	-	-	-	-	-	-	V	Y	I	K	-		-	K	K	H	A	-		-	L	K	K	-	-		-	-	-	-	-	-	-
BXP	-	N	Y	K	K	-	K	L	L	Y	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-		-	L	K	K	N	-		-	R	T	H	Y	-	-
BXS	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	V	Y	I	K	-		-	K	K	H	A	-		-	-	-	-	-	-		-	-	-	H	Y	G	-
BXA	-	-	Y	K	K	-	K	L	L	Y	-		-	-	-	R	D	R	-	-	E	V	Y	I	K	-		-	-	-	-	-	-		-	L	K	K	-	-		-	-	-	-	-	-	-
BXPS	-	-	-	-	-	-	-	-	-	-	-		-	G	T	R	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-		-	-	-	-	-	-		-	-	-	-	-	-	-
BXPA	-	N	Y	K	K	-	K	L	L	Y	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-		-	L	K	K	N	-		-	-	-	-	-	-	-
BXAS	-	-	-	-	-	-	-	-	-	-	-		-	-	-	R	D	R	-	-	-	-	-	-	-	-		-	-	-	-	-	-		-	-	-	-	-	-		-	-	-	H	Y	G	-
		N	Y	K	K		K	L	L	Y						R	D	R				V	Y	I	K			K	K	H	A			L	K	K	N						H	Y	G	
		P	X	B	B		B	X	X	X						B	A	B				X	X	X	B			B	B	B	X			X	B	B	P						B	X	S	

Fig. 1 Conserved basic amino acid containing sequences found in FGF-1. The table shows the sequences found for the different amino acid combinations. As a further selection criterion, an amino acid was only considered to be significant if it arose at least twice in the different amino acid groups, *e.g.*, R50 appears in the BXA, BXPS and BXAS selections. The conserved amino acids are illustrated on the molecular structure of FGF-1 (1RG8).[47] This structure was also represented as a network, the vertices of the network are the αC positions. In the bottom network, conserved basic amino acid containing residues are shown, along with any amino acid that is less than 0.8 nm away – the approximate length of a HS/heparin disaccharide. This reductionist view illustrates how the small basic amino acid containing sequences in unison can form an extended heparin-binding domain. The previously identified HBSs of FGF-1 can be found in Table 4.

information can be ascertained. The first, is the number of times a conserved basic amino acid containing sequence arose in the members of the human proteome. Unlike the earlier analysis performed, where discrete sequences were found in a set of HEPbp, this analysis searched for the sequences found in the earlier analysis in the entire human proteome. As a

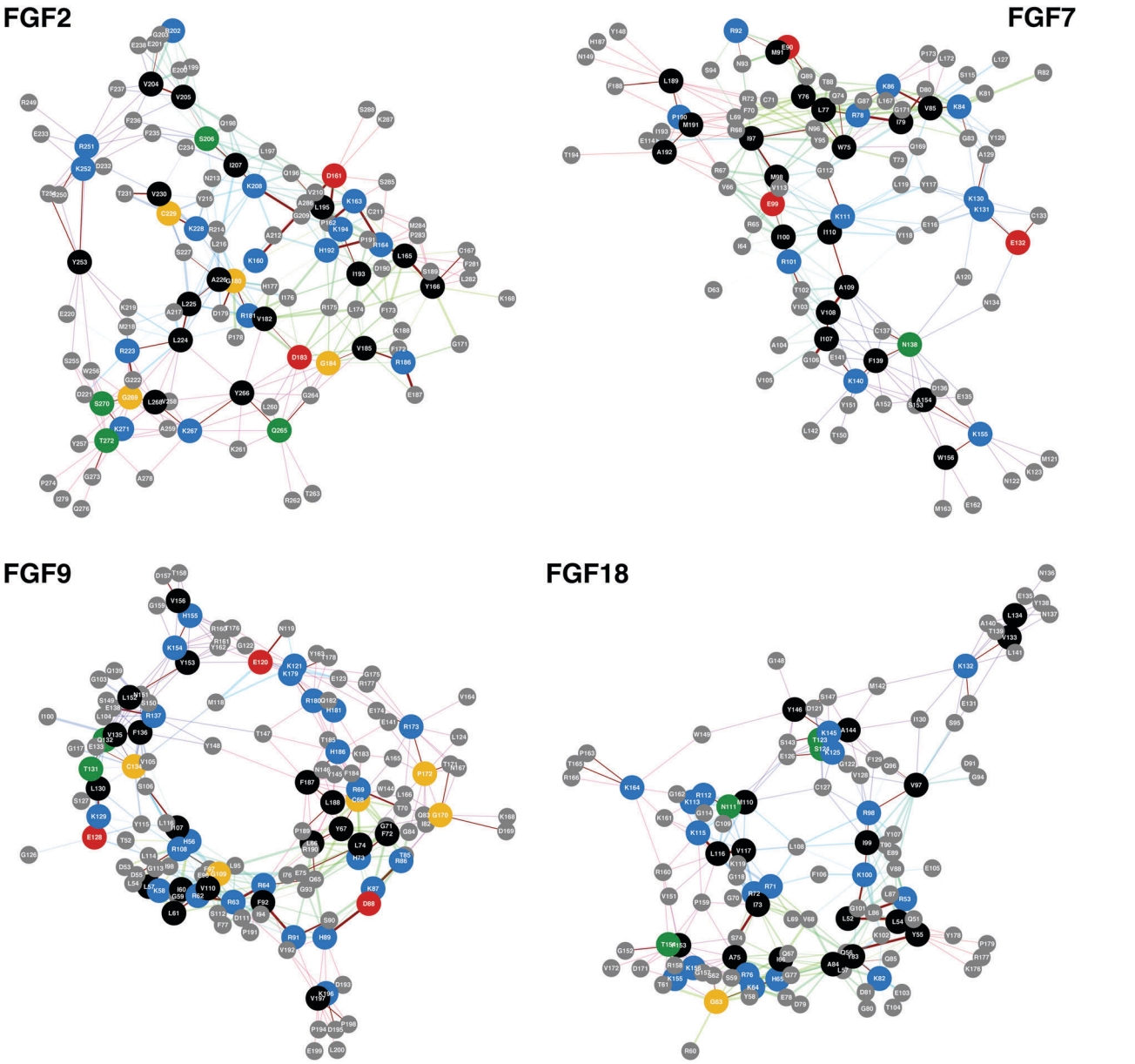


Fig. 2 Conserved basic amino acid containing sequences found in members of the FGF family. In these networks conserved basic amino acid containing residues are shown along with any amino acid that is less than 0.8 nm away, the approximate length of a HS/heparin disaccharide. The previously identified HBSs, FGF-2, -7, -9 and -18 can be found in Table 4. The PDB files used to produce the networks were, FGF-2, 1BFF;[53] FGF-7, 1QQK;[54] FGF-9, 1IHK[55] and FGF-18, 4CJM.[56]

consequence of this, overlapping sequences will be found in proteins. For example, the sequence ARLLAR could have the sequences ARL, RLL, LLAR and LAR as hits. The second piece of information is the frequency with which a specific basic amino acid containing sequence appears in the human proteome.

The median values for the number of different basic amino acid containing sequence types found per protein in the human proteome were: 42 BX, 61 BXA, 69 BXP, 64 BXS, 67 BXPA, 32 BXPS and 70 BXAS. With the 99th percentile values being: 213 BX, 333 BXA, 398 BXP, 316 BXS, 311 BXPA, 156 BXPS and 347 BXAS. The unique list, a combination of all proteins with more than or equal to the 99th percentile for the different amino acid combinations, contained 329 proteins, which can be found in ESI,† Table S27. Of these 329 proteins, 17 are found in the 437 HEPbps that were originally analysed, they are: APOB, apolipoprotein B-100; ATS9, a disintegrin and metalloproteinase with thrombospondin motifs 9; CAC1S, voltage-dependent L-type calcium channel subunit alpha-1S; CO6A3, collagen alpha-3 (VI) chain; COCA1, collagen alpha-1 (XII) chain; FBN1, fibrillin-1; LAMA1, laminin subunit alpha-1; LAMA2, laminin subunit alpha-2; LAMA3, laminin subunit alpha-3; LAMA5, laminin subunit alpha-3; NAV2, neuron navigator 2; PGBM, basement membrane-specific heparan sulfate proteoglycan core protein; STAB2, stabilin-2; TEN1, teneurin-1; TENX, tenascin-X; THYG,

Table 4 Heparin binding regions of the FGF family previously identified by the '*protect and label*' mass spectrometry method.[41] Amino acids that are highlighted in bold were found in the similarity analysis, appearing at least twice in the different amino acid groups

	Amino acid sequence	Start aa	End aa	Ref.
FGF-1	**KK**P**K**LLY	24	30	Xu *et al.* protect and label ms[42]
	I**K**STETGQYL	71	80	
	IS**KKH**AEKNWF	113	123	
	VGL**KK**NGSCKRGPRT**H**YGQAILFLPL	124	150	
FGF-2	**K**DP**KR**LYCKNGGFF	160	173	Ori *et al.* protect and label ms[41]
	LAMKEDG**R**LL	216	225	
	VALKRTGQY	258	266	
	KLGS**K**TGPGQKAIL	267	280	
FGF-7	YL**R**IDKRG**KVK**GTQEM**K**NNY	76	95	Xu *et al.* protect and label ms[42]
	LAMNKEGKLY	119	128	
	ASA**K**WTHNGGEMF	152	164	
	VALNQKGIPVRGKKTKKEQKTA**H**F	165	188	
FGF-9	**H**LEIFPNGTIQGT**RK**DHS**R**F	73	92	Xu *et al.* protect and label ms[42]
	KHVDTGRRY	154	175	
	VALNKDGTP**R**EGTRT**KRH**QKF	164	184	
	T**H**FLPRPVDPD**K**VPELY	185	201	
FGF-18	RIHVENQT**RAR**DDVSRKQL	34	52	Xu *et al.* protect and label ms[42]
	G**RR**ISARGEDGD**K**Y	70	83	
	GSQV**RIK**GKETEFYL	94	108	
	CMN**RKGK**LVGKPDGTS**K**ECVF	109	129	
	T**KK**GRPRKGP**K**TRENQQDVHFM	154	175	
	MKRYPKGQPELQ**K**PF	175	189	

thyroglobulin and VWF, von Willebrand factor. Many of these are integral components of the extracellular matrix. For example, STAB2 is a large transmembrane receptor that acts as a scavenger for heparin and other GAGs, which may assist in maintaining tissue integrity by supporting extracellular matrix turnover. If the 437 *bona fide* HEPbp are considered as a whole, they have a higher median number of basic amino acid containing sequences than the whole human proteome, apart from sequences comprised of BXPA amino acids and the 99th percentiles values are all higher: median – 45 BX, 66 BXA, 75 BXP, 74 BXS, 58 BXPA, 34 BXPS and 77 BXAS and 99th percentile – 240 BX, 352 BXA, 429 BXP, 445 BXS, 327 BXPA, 191 BXPS and 462 BXAS. If one makes the selection criteria a little milder, the 95th percentile, then that pushes the number of proteins up to 1518, which is approaching ~14% of the human proteome. This suggests that many proteins found in humans possibly interact with HS/heparin. This is not an absolute measure of heparin binding, as the analysis finds overlapping sequences. It does though provide a measure of the propensity of a protein to interact with heparin/HS.

These data support the conjecture that if many proteins can bind to these polyanions, then the mechanism of control may not lie at the level of the protein, but in the sequences found in the polysaccharide chains. This would go some way to explaining why so much energy has been committed to produce the many HS/heparin biosynthetic enzymes (4 enzymes for chain initiation, 2 enzymes for chain extension and 16 enzymes for chain modification – a total of 22 enzymes for a single polysaccharide chain).

The significant conserved basic amino acid containing sequences all appear more than 2000 times (99th percentile, 2857 BX, 3271 BXP, 2826 BXS, 3083 BXA, 3816 BXPA, 3818 BXPS and 3361 BXAS) in the human proteome. The median value was considerably lower than that (median – 42 BX, 69 BXP, 64 BXS, 61 BXA, 67 BXPA, 32 BXPS and 70 BXAS). All the significant sequences were tri-peptides. The sequences on the whole contained either arginine or lysine, with only two histidine-containing sequences found in the significant populations, HLL and LLH. The unique list, a combination of all sequences with more than or equal to the 99th percentile for the different amino acid combinations, contained 98 sequences, as follows; ARR, KLA, LAK, LLR, LKL, KAA, RLA, KLK, RRA, ARL, KKL, RAA, RVL, KRK, LRR, ALK, LRA, RAL, RKL, KVL, ALR, LRK, LKA, RLL, LKK, KAL, KLL, RLR, AAR, RKK, ARA, VLK, LLK, KKK, RRL, LRL, LAR, LKR, KRL, VLR, HLL, LRV, RRR, EKK, EER, ELK, KEE, KLE, KEL, EKE, LEK, EEK, ELR, LKE, ERL, REE, LER, LRE, EKL, RLE, KEK, LKD, REL, RSS, LQR, LRS, SSR, RLS, LSR, SRL, KSL, SLR, LKS, RSL, LQK, LSK, SLK, SRS, RLG, GRR, LRG, GLR, GRG, RLP, RLK, PRP, LRP, RGL, PRL, LPR, GRL, PPR, KLR, GKL, LVK, LLH, LGK and LGR.

4. Conclusions

These analyses indicate that basic regions, and therefore heparin binding sites within HEPbps, are highly variable, containing only small conserved motifs at the heart of the HBS. It is likely that many of these small basic sequences work in unison *via* multiple heparin binding sites on a protein surface. This implies that there is agility and leeway in the composition of the complementary protein binding surface, comparable to the latitude observed in binding sequences of HS. These data preclude the notion of there

being a single, universal HBS in the family of HEPbps, since many amino acid sequence combinations are able to fulfil the same role.

Considering basic amino acid sequences found within HEPbps is a first step to understanding the biochemistry of these interactions. There are other facets to the interaction between heparin/HS and their binding proteins that are likely to have influence, including post-translational modifications, GAG heterogeneity, cationic association and the possibility that, in some cases, HEPbps may be active independent of the presence of heparin/HS.

Differing post-translation modification has been shown to regulate the interaction between the protein and carbohydrate. For example, glycosylation of the protein ligand in FGFR-1 alters the affinity of the interaction.[48] Not all GAGs are equivalent however. Both heparin and HS are heterogeneous polysaccharides with their disaccharide sequences dependent on the organ from which they originate.[49] As polyanionic polysaccharides, both heparin and HS are associated with different cations that modify their conformation.[50] For example, it has been shown that a biologically inactive carbohydrate is activated by the addition of the appropriate cation.[51] In some cases (*e.g.*, FGF-1 and -2) heparin/HS dependant signalling pathways have been stimulated by non-GAG materials, including sulfated plant polysaccharides. For these FGFs, such proxy-GAG carbohydrates only need to either thermally stabilise or induce the correct conformational change in the HEPbp for signalling to be maintained.[52]

It is difficult to rationalise an explicit control mechanism for systems regulated by protein and HS/heparin interactions. The innate elasticity of the HBSs within HEPbps, coupled to the heterogeneity found in heparin and HS precludes this. Instead of focusing on the interaction between a single protein and HS/heparin to understand biological processes, these analyses may indicate that a holistic view, taken over all the molecular interactions may be more appropriate. Specifically, they indicate that HEPbps interact with HS/heparin in a multitude of ways, and in complex networks, which enables them both to perform many tasks and for these capabilities to be both interdependent in complex ways but, also backed-up by robust systems. The network analyses above utilise a multi-dimensional technique to interrogate this multi-faceted interactome.

These multi-dimensional network analyses of HEPbp sequences have identified HBSs on a family-wide scale. They have indicated that HBSs may be composed of multiple, small, independent basic amino acid stretches that work in unison to form the HBS regions. A single universal HBS is therefore unlikely; rather many arrangements of amino acids may fulfil the same task. These observations lead to two logical inferences: that HEPbps possess an agility in their heparin/HS interactions; and that there may be a higher degree of convergent evolution in HBPs than previously thought. These analyses provide both an insight and springboard into the HEPbp, heparin and HS interactomes, as well as a validated technique for investigating protein sequences at a phenotypic level.

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