Highly Sulfated Nonreducing End-derived Heparan Sulfate Domains Bind Fibroblast Growth Factor-2 with High Affinity and Are Enriched in Biologically Active Fractions

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Human fibroblast growth factor-2 (FGF2) regulates cellular processes including proliferation, adhesion, motility, and angiogenesis. FGF2 exerts its biological function by binding and dimerizing its receptor (FGFR), which activates signal transduction cascades. Effective binding of FGF2 to its receptor requires the presence of heparan sulfate (HS), a linear polysaccharide with N-sulfated domains (NS) localized at the cell surface and extracellular matrix. HS acts as a platform facilitating the formation of a functional FGF-FGFR-HS ternary complex. Crystal structures of the signaling ternary complex revealed two conflicting architectures. In the asymmetrical model, two FGFs and two FGFRs bind a single HS chain. In contrast, the symmetrical model postulates that one FGF and one FGFR bind to the free end of the HS chain and dimerization require these ends to join, bringing the two half-complexes together. In this study, we screened a hexasaccharide HS library for compositions that are able to bind FGF2. The library was composed primarily of NS domains internal to the HS chain with minimal presence of non-reducing end (NRE) NS. The binders were categorized into low versus high affinity binders. The low affinity fraction contained primarily hexasaccharides with low degree of sulfation that were internal to the HS chains. In contrast, the high affinity bound fraction was enriched in NRE oligosaccharides that were considerably more sulfated and had the ability to promote FGFR-mediated cell proliferation. The results suggest a role of the NRE of HS in FGF2 signaling and favor the formation of the symmetrical architecture on short NS domains.

Human fibroblast growth factors (FGFs) constitute a large family comprising 22 described members with 15–85% sequence homology but versatile functions (1). Most FGFs are structurally similar at their internal core region, sharing a β sheet structure with a trigonal pyramidal architecture known as β trefoil (2, 3). However, the conformation of the loops connecting the β strands within the homologous core as well as the amino and carboxyl termini remain variable among the different FGFs (1). FGF2 (along with FGF1) is one of the most studied members of the FGF family. It is commonly referred to as basic FGF based on its isoelectric point (pI 9.6). FGF2 is biosynthesized primarily as an 18 kDa isoform; however, larger and shorter forms can arise by alternate CUG translation start site usage and proteolytic cleavage (4–6). Most of the 18 kDa form is tethered to the cell surface or the extracellular matrix (7). The higher molecular weight isoforms are targeted to the nucleus via amino-terminal extension (8). Lacking a signal sequence, FGF2 does not rely on the classic endoplasmic reticulum–Golgi pathway to reach the extracellular environment. It has been proposed that release takes place in a paracrine fashion through the damaged membranes of dying and injured cells or through non-lethal membrane disruptions (9, 10). FGF2 is involved in the development, remodeling, and pathogenesis of numerous organ systems. The pleiotropic effects of this growth factor are explained by the wide array of physiological processes it regulates including cell proliferation and differentiation, cell motility, angiogenesis, and myogenesis (11).

Extracellular FGF2 exerts its biological function through simultaneous binding to two types of receptors on the cell surface: tyrosine kinase FGF receptors (FGFRs) and heparan sulfate proteoglycan co-receptors (HSPG) (12). Four FGFRs have been identified, FGFR1–4 (13). The receptor topology contains three extracellular immunoglobulin (Ig)-like domains (D1–D3), a single transmembrane helix and a cytoplasmic domain with protein tyrosine kinase activity. Multiple splice variants of each receptor exist with alternative splicing in the extracellular domain leading to isoforms with differing specificity and affinity for FGF2 and other members of the FGF family (14). Receptor dimerization during signaling brings the cytoplasmic domains in close proximity so as to stimulate tyrosine trans autophosphorylation and initiate a downstream signaling cascade (15).

Efficient signaling through the high affinity receptors cannot take place unless aided by the co-receptor HSPG in the form of a ternary complex consisting of FGF, FGF2, and the heparan sulfate chains (HS). The requirement of HS for full biological activity is supported by a large body of reports (16–27). HS is a
sulfated glycosaminoglycan that constitutes an essential component of the cell surface and the extracellular matrix. It is synthesized linked to its core protein by sequential addition of a disaccharide repeat unit consisting of an N-acetylgalcosamine and D-glucuronic acid linked together through a 1–4 glycosidic linkage (10). Each disaccharide can be modified through the activity of specific Golgi enzymes by addition of sulfate groups at the N, 6-O and more rarely 3-O positions of the glucosamine and 2-O of the hexuronic acid (HexA) (28). Additionally, the \( \beta-D- \) glucuronic acid (GlcA) can be epimerized to \( \alpha-L- \) iduronic acid (IdoA). Sulfation and epimerization events tend to cluster in stretches of adjacent disaccharides units constituting the highly sulfated (NS) domains of HS. The NS domains are interrupted by unmodified N-acetylated (NA) domains and those with intermediate sulfation (NA/NS). Only a subset of the potential modification sites is modified and often the modification reactions do not proceed to completion. This, along with the lack of proofreading activity of the modifying enzymes, the presence of tissue-dependent isozymes with different specificities (29), and the presence of post-biosynthetic chain modifying enzymes such as the 6-endosulfatasases (30), leads to the high polydispersity and diverse sulfation patterns of HS. The anionic nature of HS coupled with its heterogeneity and its flexible conformation conferred by IdoA residues enables it to accommodate binding requirements for a large array of protein ligands (31). Heparin, a polysaccharide restricted to mast cells, is modified in the same way as NS domains and hence often used as an analog to HS.

Both FGF2 and FGFR sequences contain a heparin-binding domain, allowing them to interact with HS. Crystal structures of FGF2 bound to a heparin oligosaccharide show that the heparin binding domain is formed by discontinuous loops in the primary sequence that form a contiguous surface in the tertiary structure. This domain contains basic amino acid residues that interact with specific groups on the highly anionic heparinoids (32). The nature of these groups is confirmed by biochemical experiments showing that FGF2 requires the N-sulfoglucosamine and 2-O sulfate of the iduronic acid for its interaction with HS (33–38). However, there remains debate as to whether the overall sulfate density can override the specific arrangement of sulfate groups (39, 40). On the other hand, FGFR makes contact with 6-O and N-sulfate groups (41) which explains the observation that 6-O sulfate is required for mitogenicity but not for FGF2 binding (42–44).

Crystal structures have been solved for FGF1-FGFR2 and FGF2-FGFR1 complexed with fully sulfated heparin decasaccharides. Based on the crystal structures two different architectures for the ternary complex have been postulated. In the asymmetric model (Pellegrini model), two FGF1 dimerize on one heparin decasaccharide and FGFRs are bound to the FGFs and to the heparin. This gives an FGF:FGFR:heparin stoichiometry of 2:2:1 (45). Details of this model show a lack of significant interactions between the two FGF-FGFR entities of the complex. An additional striking feature is that one of the FGFRs does not show direct contact with heparin, leaving its heparin binding site unoccupied. In contrast to the asymmetric model, the crystal structure of FGF2-FGFR1 with heparin decasaccharides shows a symmetrical topology with a 2:2:2 stoichiometry (Schlessinger model). In this model, each FGF-FGFR is stabilized by contacts with one heparin decasaccharide. The two heparin molecules meet at the apex of the structure with their non-reducing ends facing each other. This brings the two half complexes together and the structure is stabilized by contacts between the two FGFRs (46). Led by the observation that only the first six sugar rings interact with proteins, the authors show that in solution, a heparin hexamer is sufficient to form the larger 2:2:2 complex. More recently, the complex formation in solution has been studied by size exclusion chromatography (SEC). The findings suggest that the 2:2:1 is favorable with oligosaccharides of 8 sugar units and larger. In contrast, the results with heparin hexamers reveal that the 1:1:1 half complex is in equilibrium with 2:2:2 full complex suggesting that this architecture is possible with high concentration oligosaccharides of this size (47, 48).

The physiological relevance of the two crystal models remains unclear since each sets different requirements as to where the signaling complex can assemble on the HS chain on the cell surface. The Pellegrini model suggests that on the cell surface, the signaling complex would take place on a single HS chain. However, because in this crystallographic model the two protein halves do not interact directly, the assembly of the full structure would rely on the probability that two FGF-FGFR complexes would localize at the same NS domain. In contrast, the Schlessinger model implies that 1:1 FGF-FGFR half complexes would assemble on separate HS chains. In order to minimize steric hindrance, the formation of the full signaling complex would require that the half complexes migrate to the non-reducing end (NRE) of the HS chains to promote FGFR dimerization (41).

In the present work, we use a combined SEC-hydrophobic trapping technique followed by mass spectrometric analysis to screen an HS hexasaccharide library for oligosaccharides able to bind FGF2 (49, 50). Size exclusion chromatography allowed the estimation that FGF2 binds oligosaccharides of degree of polymerization (dp) 6 and 7 in a 1:1 ratio and mass spectrometry on the intact protein-oligosaccharide complex confirmed the results. Liquid chromatography/mass spectrometry (LC/MS) was used to characterize FGF2-bound fractions that survived increasing salt concentrations. Interestingly, the high salt resistant fraction was composed primarily of highly sulfated hexa- and heptasaccharides originating from the NRE of the HS chain. The ability of this fraction to potentiate FGF2 mediated cell proliferation shed light on the involvement of the HS NRE in the formation of the ternary signaling complex in vivo and suggests that the NRE of HS might be a highly preferred site for the assembly of this complex.

**EXPERIMENTAL PROCEDURES**

*MATERIALS—Porcine intestinal mucosa HS was purchased from Celsus laboratories, Inc (Cincinnati, OH). Heparin lyase III purified from *Flavobacterium heparinum* was from IBEX (Montreal, QC). Human recombinant FGF2 was from Chiron (Emeryville, CA). Amide-80 packing material was purchased from TOSOH Bioscience LLC (Montgomeryville, PA). Dialysis cellulose acetate membranes were purchased from the Nest Group (Southborough, MA).*
Porcine Intestinal Mucosa HS Depolymerization and Size Fractionation—A quantity of 100 mg of HS was digested exhaustively with heparin lyase III in 1 ml sodium acetate buffer (pH 7.0) supplemented with calcium acetate to a concentration of 5 mM. The digestion mixture was fractionated on a preparative SEC column (170 cm × 1.5 cm; Bio-Rad) with 200 mM ammonium bicarbonate buffer flowing at 40 μl/min (51). The fraction with degree of polymerization 6 (dp6) was collected and desalted by dialysis with a 1 kDa cutoff membrane.

SEC Hydrophobic Trapping Binding Assay—FGF2 (1 nmol) was mixed with excess HS dp6 (10 nmol) and the mixture incubated for 20 min at room temperature. The reaction mixture was applied to a Superdex-peptide 3.2/30 column (GE Healthcare) coupled to a Beckman System Gold 118 solvent module (Fullerton) and the effluent monitored at 232 nm. The degassed mobile phase (100 ammonium acetate; 3% acetonitrile) was supplied at 40 μl/min. The protein fraction was collected and applied to a C18 reverse phase cartridge (MacroSpin Columns, Silica C18 Vydac, 50–450 μl, Harvard Apparatus, Holliston, MA) after column conditioning as advised by the manufacturer protocol. The hydrophobically trapped complex was washed for 3 cycles with 250 μl of different stringency ammonium acetate buffer (100 or 200 mM) and the remaining bound sugars eluted with 250 μl of 1 mM ammonium acetate.

Liquid Chromatography/Mass Spectrometry—HS oligosaccharides were separated by amide hydrophilic interaction chromatography (HILIC) with on-line mass spectrometric detection. The column used was packed in house with 3 μm bead Amide-80 packing material (TOSOH Biosciences, Montgomeryville, PA) into a 150 μm × 15 cm capillary. The oligosaccharides were trapped on a 250 μm × 5 cm column for 3 min at 5 μl/min flow rate of 30% A. The trapping event allowed direct injection of the bound fractions highly concentrated in salt without prior desalting. The oligosaccharides were eluted from the column with a gradient starting at 30% and ending at 65% solvent A over a period of 15 min at a flow of 1 μl/min delivered by a Waters NanoAQuity (Milford, MA). Solvent A consists of 50 mM ammonium formate, pH 4.4. Solvent B is 95% acetonitrile and 5% A.

Mass spectrometric analysis was accomplished using a Thermo-Fisher Scientific (West Palm Beach, FL) LTQ Orbitrap mass spectrometer operating in the negative high resolution mode. The instrument ionization source was interfaced to an automated Triversa Nanomate robot (Advion Biosystems, Inc., Ithaca, NY) operating with a spray voltage of −1.4 kV. The instrument was tuned using Arixtra (GlaxoSmithKline, Ithaca, NY) operating with a spray voltage of 1.4 kV and nitrogen back pressure 1.8 PSI. The MS data files were calibrated offline and mass accuracy was better than 5 ppm.

RESULTS
Size Exclusion Chromatography of the FGF2-dp6 Complex—To generate a size-defined library of degree of polymerization 6 (dp6), porcine intestinal mucosa HS was digested with heparin lyase III (HL III). HL III was chosen because of its specificity for digesting NA and NA/NS domains, leaving the highly sulfated NS domains that are important in protein binding intact (53). The digestion mixture was separated using SEC and the hexasaccharide fraction collected and desalted. Bacterial heparin lyases digest HS by cleaving the glucosamine (GlcN)-GlcA bond at the reducing end of the GlcN residue. The enzymes cut the linkage by an eliminative mechanism, leaving a C4-C5 unsaturation on the GlcA of the cleaved bond. The features of this endolytic mechanism allow the determination of the position of an oligosaccharide in the context of the original HS chain. Unsaturated hexasaccharides are ones that have been generated by a cleavage of the lyase and hence are internal to the HS chain. In contrast, saturated hexasaccharides occur due to a single cleavage of the HS and therefore reside at the NRE of the chain. Saturated heptasaccharides (dp7) can also originate from the NRE of chains terminating with a GlcN residue. The HS chain may contain multiple NS domains internal to the chain.

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but one NRE. Only a fraction of the HS chains in a mixture terminate with a NRE NS domain of dp6/7. Hence, the hexasaccharide library contains abundant unsaturated internal NS domains and very low abundance NRE saturated dp6. Additionally, due to the low resolution of the SEC chromatography, low levels NRE dp7 are also expected to occur in the library.

To estimate the binding ratio of FGF to hexasaccharides, FGF2 was incubated with the purified HS dp6 library, and the mixture fractionated using an analytical SEC column (Fig. 1). Ammonium acetate was chosen as the mobile phase because of its volatility and hence compatibility with subsequent mass spectrometric analysis. The absorbance of the eluant was monitored at 232 nm. This wavelength was used to monitor lyase generated oligosaccharides as their C4-C5/HexA resonance absorb at this value. However, proteins are also known to absorb strongly at this wavelength, which enables their simultaneous detection. The column used had an optimal separation for peptide and proteins ranging from 3 to 70 kDa. The FGF2 used in the experiments was a short isoform of 16.4 kDa, hence the free and complexed proteins fell within the optimal SEC separation range. The column was calibrated with low molecular weight standards, and a calibration curve generated to estimate the molecular weight of the complex forming in solution (data not shown). Fig. 1 shows the SEC chromatogram of the free protein compared with the protein and dp6 reaction mixture. Whereas the free FGF2 eluted around the same time as the ribonuclease A standard (13.7 kDa), the FGF2-dp6 complex eluted between ribonuclease A and the carbonic anhydrase standard (29 kDa). Oligosaccharides eluted at an earlier time than expected due to their highly sulfated nature and hence large hydrodynamic volume. By extrapolation from the calibration curve, the molecular weight of the free FGF was estimated to be 14.0 kDa while that of the FGF2-dp6 complex 18.3 kDa. This suggested that the complex formed with an FGF2:dp6 stoichiometry of 1:1.

Mass Spectrometry (MS) of the FGF2-dp6 Complex.—To confirm the stoichiometry of the complex that forms in solution and identify the nature of the hexasaccharides bound, the SEC purified FGF2-dp6 complex was collected and analyzed by direct infusion using a high mass accuracy, high resolution mass spectrometer. Mass spectrometric analysis showed that the bacterially expressed protein was a mixture of at least four different isoforms that differed from each other by one or a few amino acids (Fig. 2A). It was unclear whether these isoforms were generated at the synthesis stage, post-synthetically or during purification. We refer to the most abundant 16.4 kDa isoform as isoform A. The mass spectrum of the SEC collected FGF2-dp6 complex showed a complex pattern due to the number of HS compositions that bind FGF2, the initial presence of multiple protein isoforms and the presence of cation adducts. Fig. 2B shows the most abundant charge state of the protein distribution (+8) in the 2100–2285 Da interval with assignments for unambiguous m/z values. For every FGF2-dp6 complex, the protein component is referred to by the corresponding isoform letter and the saccharide component by a numerical code. In this code the numbers correspond to the groups in the following code: [ΔHexA,HexA,GlcN,SO3,Acetate]. The most abundant binding compositions were the singly acetylated hexasaccharides with 4 and 5 sulfate groups ([1,2,3,4,1] and [1,2,3,5,1]). Because of the higher abundance of A compared with the other isoforms, other binding compositions were detectable in complex with A such as [1,2,3,6,1]. Surprisingly, NRE dp7, minor oligosaccharides in the library, were detected in complex with A as shown by the saturated compositions [0,3,4,6,1] and [0,3,4,7,1]. All these oligosaccharides (dp6 and
dp7) bound FGF2 at a 1:1 ratio, consistent with the previous SEC molecular weight estimations.

The unexpected presence of NRE compositions among the binding oligosaccharides suggested that these low abundance oligosaccharides competed effectively with the high abundance internal NS domains for binding to FGF2 and thus that their affinity is high. To evaluate the affinity of these oligosaccharides, we performed a similar experiment where SEC was performed with a higher concentration of salt (200 mM ammonium acetate) in the mobile phase. Analysis of the 200 mM resistant FGF2-dp6 complex fraction by MS showed that at these SEC conditions, the 1:1 complexes formed by FGF2 and the NRE compositions \([0,3,4,6,1], [0,3,4,7,1], \) and \([0,3,4,8,1]\) were enriched over the rest of the complexes formed by other oligosaccharides (Fig. 2C). The lower signal-to-noise ratio is a consequence of the high salt concentration during direct infusion. In summary, MS analysis of intact protein FGF2-dp6 complexes showed that under low stringency SEC conditions, internal NS domains were the most abundant binders of FGF2 (Fig. 2B) whereas at higher stringency conditions NRE NS domains were enriched (Fig. 2C). Additionally, FGF2 formed a 1:1 FGF2-oligosaccharide complex with HS dp6 and dp7.

Characterization of Bound Hexasaccharides by LC/MS—Intact complex analysis by direct infusion MS is a useful approach when studying protein-oligosaccharide stoichiometry and identifying binding compositions in simple protein-oligosaccharide mixtures. In this particular case, the presence of multiple protein isoforms, multiple binding structures and multiple adducts yields very complex spectra where only few \(m/z\) values can be unambiguously assigned to a structure. Furthermore, the SEC buffer salt present in the infused samples decreases the detection sensitivity in the high resolution mode and many complexes might not survive the different steps of mass spectrometric analysis, which leaves many structures undetected (54). In addition, quantitative analysis is not amenable to direct infusion due to signal irreproducibility. For these reasons, another approach was adopted to analyze the compositions of the binding HS oligosaccharides. In this approach we used an SEC-hydrophobic trapping protocol whereby the FGF2-dp6 complex fraction was purified using SEC and subsequently trapped using a C-18 cartridge that binds the protein component of the complex through hydrophobic interactions. The C-18 bound complex was washed repeatedly and the bound oligosaccharides liberated from the complex using high salt elution (1M) while the protein remained bound to the hydrophobic resin (49, 55). The composition of the eluted oligosaccharides was analyzed by LC/MS. This methodology offered the advantage of removing the complex protein mixture and yielding enhanced sensitivity for oligosaccharide detection and quantification (56).

The distribution of compositions in the dp6 library is shown in Fig. 3. The library consisted of unsaturated singly acetylated...
compositions where the content of sulfate ranged from 2–6 with the [1,2,3,3,1] and [1,2,3,4,1] compositions accounting for 68% of the total. A second, lower abundance series of compositions with unacetylated structures holding 3–7 sulfates was detected. The NRE oligosaccharides consisting of saturated dp6 and dp7 accounted collectively for less than 1% of total abundance.

After the library was incubated with FGF2 and subjected to the binding protocol including a 100 mM salt wash step, the wash-resistant oligosaccharide fraction was eluted and characterized. The compositional distribution differed markedly from that of the library. It was characterized by the disappearance of the low sulfation content compositions such as [1,2,3,2,1] and [1,2,3,3,1], paralleled with an enrichment of those with high sulfation content such as [1,2,3,5,1] and [1,2,3,6,1] (Fig. 3, A and B). Furthermore, the NRE oligosaccharides that fell under the limit of detection in the library were present in the 100 mM resistant-bound fraction and were represented by [0,3,3,6,1], [0,3,3,7,1], and [0,3,4,6,1]. Additional binding compositions were identified as a result of the high sensitivity of the LC/MS technique and these include [1,2,3,5,0], [1,2,3,6,0], [0,3,3,6,1], and [0,3,3,7,1].

To confirm the results of Fig. 2C where NRE oligosaccharides were enriched at 200 mM salt stringency SEC separation, the SEC hydrophobic trapping protocol was repeated with a 200 mM salt wash and the resistant oligosaccharides were analyzed by LC/MS. The drastic difference in the compositional profile of the 200 mM resistant bound fraction compared with the library is shown in Fig. 4. As the saturated oligosaccharides originating from the NRE of the HS chain accounted for less than 1% in the library, they were strongly enriched in the bound fraction constituting 73% of the total binders. The compositions identified in the intact complex ([0,3,4,6,1], [0,3,4,7,1], and [0,3,4,8,1] in Fig. 2C) ranked among the most abundant binders identified by LC/MS. Additional potent NRE binders were identified using LC/MS such as [0,3,3,8,0] and [0,3,3,9,0]. The most highly sulfated internal NS domains could also bind FGF2 with high affinity; however, they showed a lower level of enrichment compared with NRE NS domains.

In conclusion, the LC/MS data showed that although internal NS domains within the HS chain were able to bind FGF2 at 100 mM stringency, only those with the highest sulfation content, present in the lowest abundances in the library, were able to survive at 200 mM salt concentration. The NRE dp6 and dp7 that represented a minimal fraction of the library (less than 1%) were present at a much higher relative abundance in the bound fractions suggesting their ability to compete with internal NS domains for binding which in turn is suggestive of their high

FIGURE 3. LC/MS analysis of low stringency wash resistant-bound fraction. A, collected FGF-dp6 complex eluting from the SEC column was trapped onto a C-18 column, washed with 100 mM ammonium acetate and eluted by high salt. The bound oligosaccharides were analyzed for their sulfate and acetate content and quantified HILIC LC/MS. The upper panel shows the LC/MS spectrum of the HS library while the lower one shows that of the 100 mM wash resistant-bound fraction. In the bound fraction, low sulfation NS domains such as [1,2,3,2,1] and [1,2,3,3,1] disappear in favor of the higher sulfation NS domains. NRE originating NS domains such as [0,3,3,6,1] are also enriched to 10% of total binders. B, quantification of the abundances of the compositions shown in A.
affinity to FGF2. Consistent with this conclusion, these oligosaccharides were the most abundant compositions able to resist a high stringency salt wash.

Biological Activity of the Bound Fractions—The binding experiments undertaken in this study involved only two components of the biologically active ternary complex, namely FGF2 and HS. To examine whether the bound fraction had the ability to form an active ternary complex with both FGF2 and FGFR, we evaluated the ability of these samples to support FGF2 mediated cell proliferation using BaF-32 cells (19). BaF-32 lymphoblastoid cells are deficient for HS synthesis and have been engineered to express FGFR1c. BaF-32 cell proliferation is dependent on interleukin-3, but this requirement can be substituted with FGF2 and an exogenous supply of heparinoids that have the ability to support the formation of active FGF2-FGFR1 complexes. The specific activity/pmol of the library was calculated based on a cell growth dose response curve. The unfractionated dp6 library showed low activity, detectable only at very high dosage (supplemental Fig. S1). In comparison with the unfractionated library, the specific activity of the 100 mM resistant bound fraction was 2.8-fold higher (Fig. 5). The 200 mM resistant bound fraction, composed predominantly of NRE hexa- and heptasaccharides, was 7-fold more potent in inducing cell proliferation compared with the unfractionated library. Consistent with literature reports with oligosaccharides of this size, the activities of the bound fractions failed to reach the levels of activation of the full-length HS controls (57). The results showed a positive correlation between the relative amount of NRE NS domains present in the tested fraction and the ability to form biologically active ternary complexes and induce cell proliferation.
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DISCUSSION

In this study, two complementary strategies were used to screen for FGF2 binders in a library of HS hexasaccharides. The library was generated using HL III cleavage to preserve the NS domains that are the biologically relevant entities as they constitute the main protein binders in the HS chain (58). In the first strategy, FGF2 was incubated with the library and the FGF-dp6 complex separated from non binders by SEC. Using mass spectrometry, the intact complexes were studied for their binding components and stoichiometry. The results showed that FGF2 binds hexasaccharides and heptasaccharides in 1:1 ratio (Figs. 1 and 2). The stoichiometry was consistent with in solution complex formation of FGF1 and FGF2 with heparin hexasaccharides as reported previously (48, 59). At low salt stringency SEC chromatography, the most abundant complexes formed contain internal NS domains whereas at high stringency the NRE NS domains are dominant (Fig. 2, B and C). The SEC-hydrophobic trapping technique was used as a second strategy to corroborate the previous findings (Figs. 3 and 4). To our knowledge, complex formation of FGF2 with dp7 has not been studied due to the low abundance of this oligosaccharide species and hence inability to be purified. An odd number NS domain generated by lyase cleavage originates from the NRE of an HS chain terminating with a GlcN residue. One possibility is that some HS chains terminate with a GlcN residue at the chain elongation step of the biosynthetic pathway. Alternatively, the chain could be trimmed post-synthetically by heparanase, an endo-glucuronidase that exposes the glucosamine residue at the newly formed NRE of the chain (60, 61).

We have selected for high affinity binders by using a salt gradient. Sulfation density was positively correlated with affinity and biological activity. Both bound fractions show higher levels of activation than the initial library but the 200 mM resistant-bound fraction is 2.5-fold more potent than the 100 mM resistant-bound fraction (Fig. 5). This is significant since 100 mM resistant fraction contains the same highly sulfated binders enriched in the 200 mM fraction in addition to lower affinity and lower sulfation internal NS domains. The lower activity of the 100 mM resistant fraction can be explained by the fact that the lower affinity and sulfation internal NS domains present in high abundance, are capable of binding FGF2 but do not bind its receptor to form functional ternary complexes. When these domains: [1,2,3,4,1], [1,2,3,5,1], [1,2,3,6,1], and [1,2,3,5,0]- are washed away by the 200 mM salt stringency they are less likely to out-compete the low abundance but high affinity NS domains and inhibit their proliferative potential.

The high affinity bound fraction is enriched in NRE constituents (75%) but also highly sulfated internal NS domains ([1,2,3,6,0], [1,2,3,7,0], [1,2,3,8,0], [1,2,3,5,1], and [1,2,3,6,1]). This is significant in light of the in vivo implication of the Schlessinger model of the ternary complex with a 2:2:2 FGF2:FGFR1:HS stoichiometry. Within the constraints of the linear HS chains, such architecture cannot be possible unless occurring at the NRE of two HS chains. Although the symmetrical crystal structure was generated with decasaccharides, the authors show that a hexasaccharide is the smallest fragment capable of forming the ternary structure (41, 46). Many subsequent studies using a range of different length oligosaccharides corroborated this model (59, 62). To date, the literature shows a paucity of information concerning the NRE domains due to their rarity within HS oligosaccharide libraries. Hence, it is not possible to purify NRE domains in sufficient quantities for biological assays (63, 64). In addition, NRE domains lack the unsaturated uronic acid chromophore, and thus it is necessary to chemically modify them to facilitate optical detection during purification. Preserving the native structure of oligosaccharides is of importance as their biological activity might be compromised by the chemical introduction of a bulky label.

The dramatic enrichment of NRE derived oligosaccharides that bind FGF in a 1:1 ratio in the high activity fraction in this work is of great interest because it complements observations that implicated NRE oligosaccharides in the formation of the 2:2:2 complex at the end of the HS chain. The sulfate distribution seen at the NRE is noteworthy. In fact, the dp6 library compositional distribution in Fig. 3 shows that the average sulfation per disaccharide is 1.3 for the internal NS domains. For NRE NS domains this value is 2.2 approaching the average levels of sulfation of heparin estimated at 2.7 sulfate groups per disaccharide unit (65). In concordance with our findings, recent studies highlighted the highly sulfated nature of the HS NRE that seems to be a conserved feature of HS chains among different organs (63, 64). Interestingly, one of the most abundant NRE hexasaccharide contains 9 sulfate groups, a density that is not observed in internal oligosaccharide domains of the HS chain. It is known that fully sulfated heparin dp6 are the highest inducers of mitogenesis among hexasaccharides; however, the in vivo relevance of such sulfation levels were of debate as heparin is not the physiologically relevant protein modulator of FGF (59). Our results show that such high sulfation can occur at the ends of HS chains.

The contribution of the internal NS domains to the activity of the 200 mM resistant bound fraction cannot be ruled out unless the bound fraction is totally devoid of unsaturated NS domains. In this case, total depletion of internal NS domains would require a salt stringency that breaks the ionic interactions of compositions holding up to eight sulfate groups. Due to the overlap of sulfate distribution between the internal and NRE oligosaccharides, such conditions will also wash-away NRE structures of the same sulfate density that are major constituents of the 200 mM resistant bound fraction. In the event that internal oligosaccharides contribute to the biological activity of the 200 mM bound fraction, their in vivo significance in the context of sustaining the dimerization of FGF and its receptor remains unclear, given that they bind FGF with a 1:1 ratio.

Until more sensitive purification techniques and biological assays are available to allow purification and testing the activity of the individual NRE oligosaccharides, the present results suggest the involvement of the NRE in FGF signaling. The highly sulfated nature of NRE domains and enrichment in highly active fractions strengthens the hypothesis implicating small NS domains residing on the free ends of HS chains and binding with 1:1 stoichiometry in FGF signaling (59, 62).

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