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Investigating the mechanism of the assembly of FGF1-binding heparan sulfate motifs

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ABSTRACT

Heparan sulfate (HS) chains play crucial biological roles by binding to various signaling molecules including fibroblast growth factors (FGFs). Distinct sulfation patterns of HS chains are required for their binding to FGFs/FGF receptors (FGFRs). These sulfation patterns are putatively regulated by biosynthetic enzyme complexes, called GAGOSOMES, in the Golgi. While the structural requirements of HS–FGF interactions have been described previously, it is still unclear how the FGF-binding motif is assembled in vivo. In this study, we generated HS structures using biosynthetic enzymes in a sequential or concurrent manner to elucidate the potential mechanism by which the FGF1-binding HS motif is assembled. Our results indicate that the HS chains form ternary complexes with FGF1/FGFR when enzymes carry out modifications in a specific manner.

1. Introduction

Heparan sulfate (HS) is a linear, sulfated polysaccharide that consists of repeating units of glucosamine (GlcN) and glucuronic acid (GlcA) or iduronic acid (IdoA). As the nascent HS undergoes elongation, a series of modifications occurs on the backbone. N-acetyl glucosamine (GlcNAc) residues are N-deacetylated and N-sulfated by N-deacetylase-N-sulfotransferase (NDST), whereas GlcA residues are epimerized to IdoA by C5-epimerase. Additionally, a variety of O-sulfotransferases (OST) can add sulfate groups to the C6 (6-OST) and C3 (3-OST) carbons of GlcN residues and the C2 carbon (2-OST) of IdoA residues. It is also possible for 2-OST to add sulfate groups, albeit less preferentially, to the C2 carbon of GlcA residues [1]. To further augment this structural diversity, HS has a domain-like architecture composed of highly sulfated domains (NS domains), non-sulfated domains (NA domains), and partially sulfated domains (NA/NS domains). This immense structural complexity is believed to regulate the interactions of HS with several protein targets including growth factors and cytokines [2].

One of the most commonly studied HS–protein interactions is that of HS and FGF. The FGF family plays a major role in several fundamental biological processes including cell proliferation, cell differentiation and cell migration [3,4]. Twenty two different FGFs and four FGFR genes have been discovered in humans [4]. FGF1 (acidic FGF) and FGF2 (basic FGF) were the first FGFs isolated [5,6]. FGF1 is able to bind to all FGFRs while FGF2 can only bind to FGFR1b, 1c, 2c, 3c and 4 [7]. HS potentiates FGF signaling by acting as a co-receptor and facilitates the formation of biologically relevant HS/FGF/FGFR ternary complexes. It facilitates the dimerization of FGFRs and thereby regulates downstream signaling pathways [8–10].

There have been many studies that have investigated the structural requirements of HS–FGF interactions. It has been shown that the minimal HS sequence that can bind to FGF2 requires 2-O-sulfated IdoA and N-sulfated GlcN residues [11–13]. Highly sulfated non-reducing end HS oligosaccharides were also found to bind FGF-2 with a high affinity [14]. Similarly, short, highly sulfated, HS chains isolated from porcine liver and intestine could induce FGF-2 mediated signaling efficiently [15]. However, while the structure of the FGF binding motif has been discovered previously,

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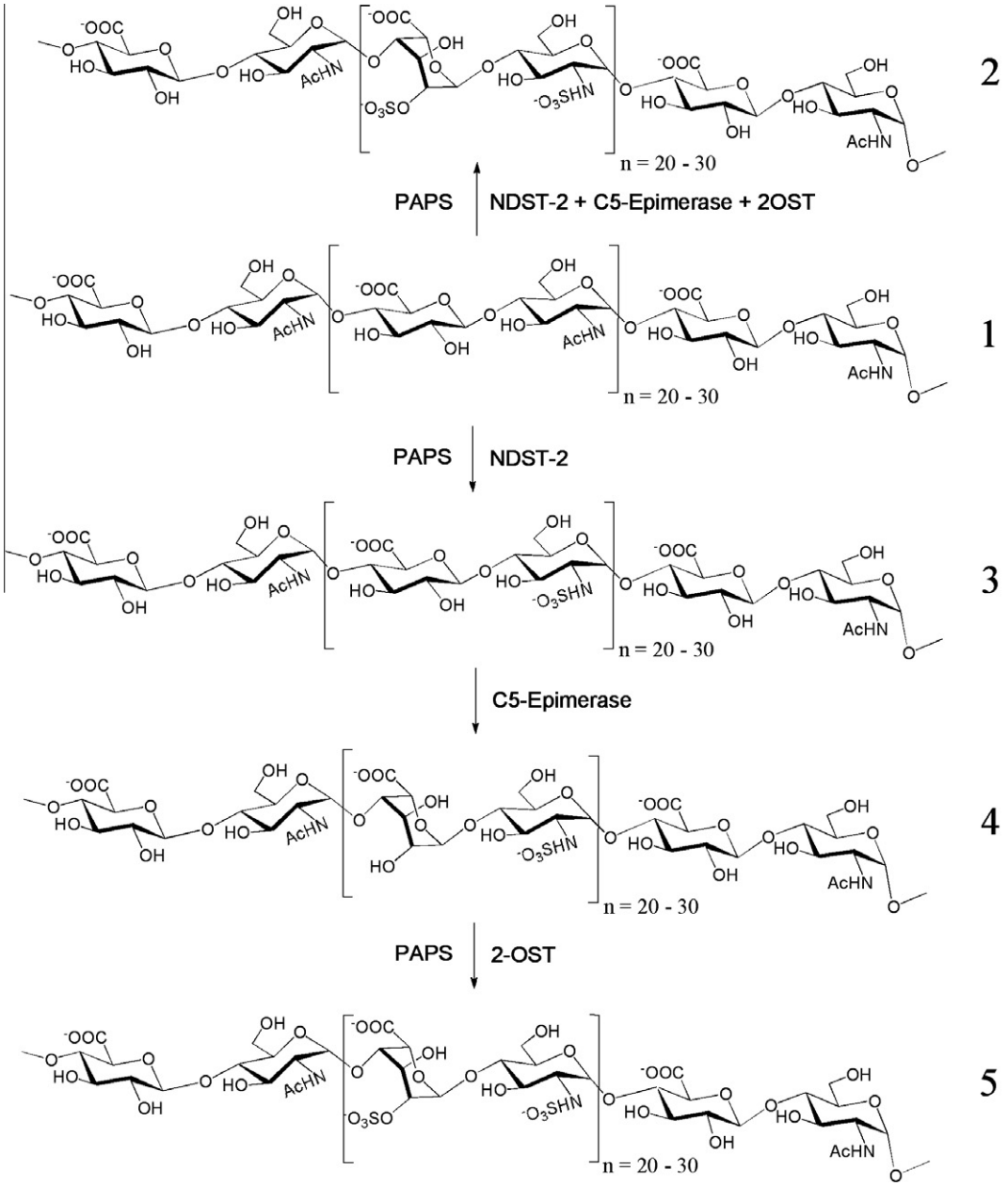
it is still unclear how such specific binding motifs arise within the intact HS chain. One proposed model points to the existence of GAGOSOMES – macromolecular enzyme complexes that reside within the Golgi where HS biosynthetic enzymes act on nascent HS chains to generate growth factor binding motifs [16,17]. However, it is unclear as to how these enzymes modify a growing HS chain. Do all the enzymes in a GAGOSOME act concurrently or sequentially on a growing HS chain to generate biologically active structures?

In this study, we aim to elucidate the natural action of HS biosynthetic enzymes by utilizing HS/FGF/FGFR interactions as a tool. To do this, we prepared N-sulfated, epimerized and 2-O-sulfated HS structures by conducting enzymatic modifications concurrently or sequentially. The resulting HS structures were examined in a gel mobility shift assay to determine their ability to form ternary complexes with FGF1 and FGFR1 or FGFR2. The results of this study outline a snapshot of the series of biosynthetic events that may take place in GAGOSOMES to generate diverse HS structures.

2. Materials and methods

2.1. Materials

Recombinant HS biosynthetic enzymes, NDST-2, C5-epimerase and 2-OST, were expressed using a baculovirus system and purified as previously described [18]. Heparitinase I, II, and III were cloned and expressed as previously described. Completely desulfated, N-sulfated heparin **6** (CDSNS) was prepared as previously described [19]. The heparosan polysaccharide **1** was prepared from *Escherichia coli* K5 strain as reported previously [20]. The DEAE-sepharose gel was purchased from Amersham Biosciences. The SAX column (250 × 4.6 mm, 5 μm particle size) was purchased from Phenomenex Inc. [^{35}S]Na_2SO_4 and Ultima-FloAP were



Scheme 1. Concurrent and sequential action of HS biosynthetic enzymes NDST, C5-epimerase and 2-OST.

purchased from Perkin Elmer Life and Analytical Sciences. [^{35}S]PAPS was prepared as reported earlier [21]. [^{32}S]PAPS was purchased from Sigma–Aldrich. HS disaccharide standards were purchased from Iduron and Sigma–Aldrich. Human FGF1, FGFR1α (IIIc) and FGFR2α (IIIc) were purchased from R&D Systems. All other reagents and solvents were obtained from Sigma–Aldrich.

2.2. Preparation of N-sulfated, epimerized and 2-O-sulfated HS polysaccharides

All reactions were performed in a buffer consisting of 25 mM MES (pH 7.0), 0.02% Triton X-100, 2.5 mM $MgCl_2$, 2.5 mM $MnCl_2$, 1.25 mM $CaCl_2$ and 0.75 mg/ml BSA [22]. In the concurrent reaction, 20 μg of heparosan 1 was incubated with 10 μl each of NDST-2, C5-epi and 2-OST (~20 μg/ml), and with 5 μl of [^{35}S]PAPS(1×10^7CPM)/100 μg of [^{32}S]PAPS in a 200 μl reaction. The reaction was incubated for 24 h at 37 °C. The reaction was terminated by heating for 2 min at 96 °C. The samples were diluted with one volume of 0.016% Triton X-100 and loaded onto a mini DEAE-sepharose column (0.3 ml) that had been pre-equilibrated with 2 ml of wash buffer (20 mM NaOAc, 0.1 M NaCl and 0.01% Triton X-100, pH 6.0). After washing with 9 ml of wash buffer, the bound polysaccharide was eluted with 1.8 ml of elution buffer (20 mM NaOAc, 1 M NaCl, pH 6.0). The eluate was then desalted and concentrated to 100 μl final volume. In the sequential reaction, 20 μg of heparosan **1** was first incubated with NDST-2 and [^{32}S]PAPS. The sample was purified, desalted, concentrated and used as the substrate for the next reaction with C5-epimerase. Finally, the resulting product was 2-O-sulfated by 2-OST in the presence of [^{35}S]PAPS/[^{32}S]PAPS. CDSNS polysaccharide **6** was 2-O-sulfated by 2-OST in the presence of [^{35}S]PAPS or [^{32}S]PAPS and the resulting product **7** was used as a control in the gel mobility shift assay.

2.3. Disaccharide analysis of the polysaccharides

Radioactive samples were digested with heparitinase I, II, and III overnight at 37 °C and analyzed using strong anion-exchange (SAX)-HPLC coupled with an in-line radiometry/UV detector. The disaccharides were eluted with a linear gradient of 0–800 mM NaCl (pH 3.5) for 35 min and 2 M NaCl (pH 3.5) for 10 min. HS disaccharide standards were co-injected and detected at 232 nm. Non-radioactive samples were analyzed using liquid chromatography–mass spectrometry (LC–MS). Disaccharides were separated on a C18 column (0.3×250 mm, Vydac, USA) using a gradient from 0% to 100% of acetonitrile at a flow rate of 5 μl/min over 70 min. 5 mM dibutylamine was used as an ion-pairing agent. Capillary HPLC coupled to an electrospray ionization time-of-flight MS (Bruker Daltonics, USA) was used in the negative ion mode at the following conditions: cone gas 50 l/h, nozzle temperature 130 °C, drying gas (N_2) flow 450 l/h, spray tip potential 2.3 kV, and nozzle potential 35 V.

2.4. Gel mobility shift assay

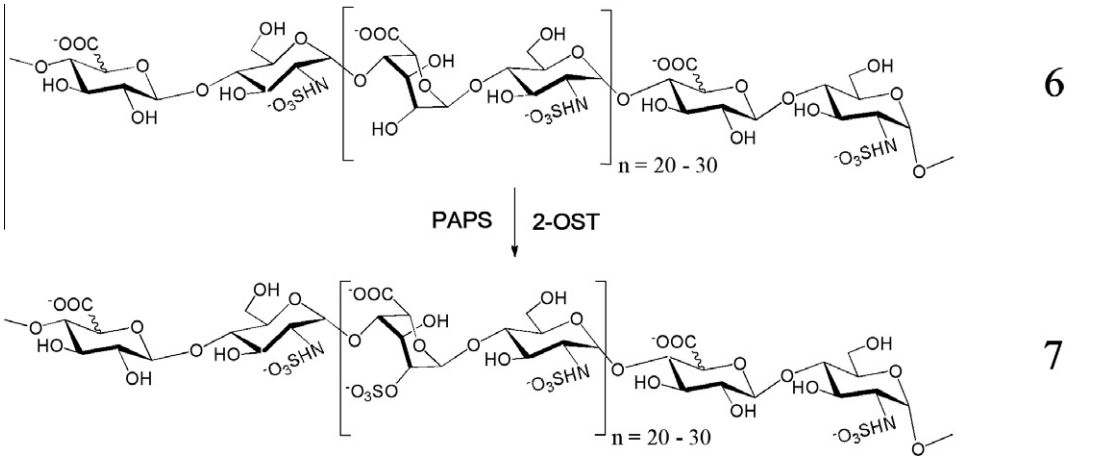
Enzymatically modified polysaccharide (1 μg), FGF (250 ng), and FGFR (500 ng) were mixed in 20 μl of binding buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 10 mM $MgCl_2$, 1.4 mM KH_2PO_4 and 12% glycerol) and incubated at 23 °C for 30 min to facilitate complex formation [22]. The entire mixture was loaded onto a native 4.5% polyacrylamide gel (20×25 cm). The gel was subjected to electrophoresis at 100 V for 6 h at 4 °C. The gel was then dried, exposed to a phosphor screen overnight and imaged by a Typhoon PhosphorImager system.

3. Results

The primary objective of the current study is to elucidate how the FGF binding motif is generated in the Golgi. In order to determine whether the different modifications present in the FGF binding motif are created by enzymatic modifications that may occur sequentially or concurrently, three different polysaccharides products were prepared in a sequential or concurrent approach as outlined in the Schemes 1 and 2 using biosynthetic enzymes:

(1) Polysaccharide **2**: Heparosan **1** was treated with NDST-2, C5-Epimerase and 2-OST all together.
(2) Polysaccharide **5**: Heparosan **1** was first treated with NDST-2, then C5-epimerase and followed finally by 2-OST.
(3) Polysaccharide **7**: Completely desulfated, N-sulfated (CDSNS) heparin **6** was treated with 2-OST in the presence of [^{35}S]PAPS to produce the polysaccharide **7** for use in the control experiment.

Polysaccharides **2**, **5** and **7** were characterized by SAX-HPLC (Fig. 1) by comparing their disaccharide compositions with the aid of co-injected disaccharide standards. While polysaccharide **2** had two radiolabeled disaccharides, ΔUA-GlcNS and ΔUA2S-GlcNS, polysaccharide **5** had only one radiolabeled ΔUA2S-GlcNS disaccharide because it was N-sulfated using non-radioactive [^{32}S]PAPS. Similarly, polysaccharide **7** only contained the radioactive ΔUA2S-GlcNS disaccharide.



Scheme 2. Enzymatic 2-O-sulfation of CDSNS-heparin polysaccharide.

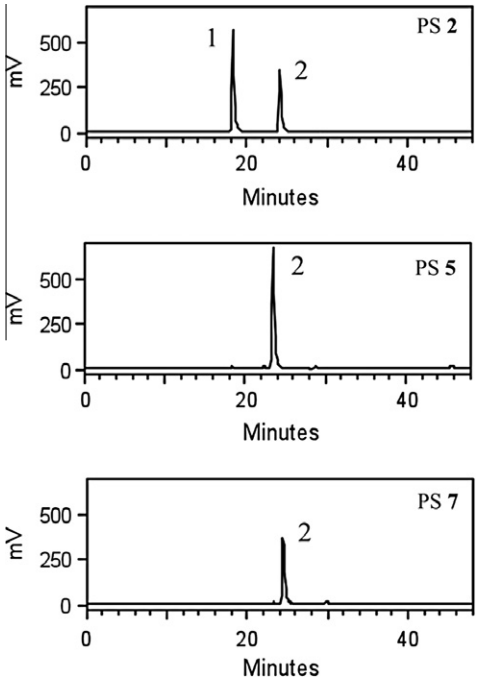


Fig. 1. Disaccharide analysis of concurrently modified polysaccharide **2**, sequentially modified polysaccharide **5** and positive control polysaccharide **7**. The disaccharide peaks were determined with the aid of co-injected disaccharide standards. (1) ΔUA-GlcNS, (2) ΔUA2S-GlcNS.

Sequential and concurrent modifications were carried out in the presence of [^{32}S]PAPS so that we could utilize LC-MS analysis to estimate the non-sulfated disaccharide content (Fig. 2). MS data suggested that the amount of ΔUA2S-GlcNS disaccharide was significantly higher in both the sequentially modified polysaccharide **5** and the positive control polysaccharide **7** in comparison to the concurrently modified polysaccharide **2.**

Once the polysaccharides were characterized, they were tested in a gel mobility shift assay to determine whether they could form the ternary complex with FGF1 and FGFR1 or FGFR2 (Fig. 3). A shift in the mobility of the radiolabeled-polysaccharide indicates the

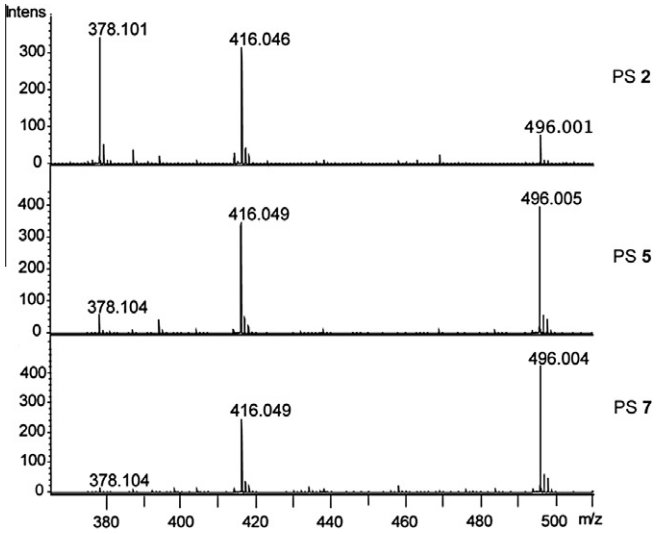


Fig. 2. MS spectra of disaccharides from concurrently modified polysaccharide **2**, sequentially modified polysaccharide **5** and positive control polysaccharide **7**. The following disaccharides were detected: ΔUA-GlcNAc (*m*/*z* 378.1), ΔUA-GlcNS (*m*/*z* 416.0) and ΔUA2S-GlcNS (*m*/*z* 496.0).

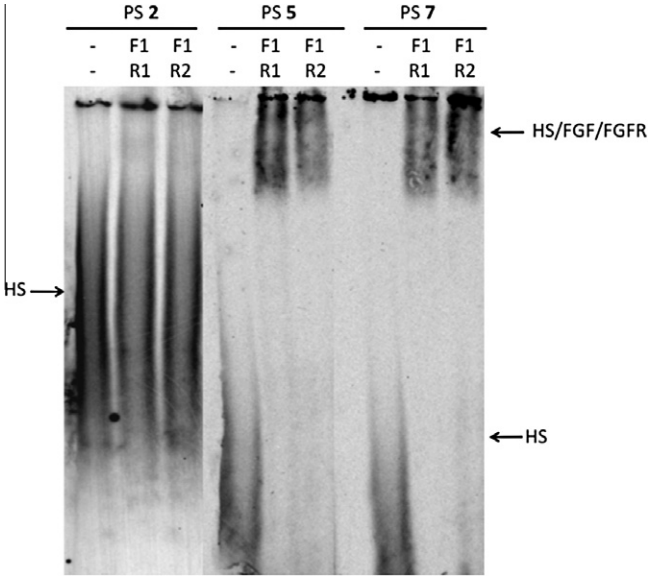


Fig. 3. Gel mobility shift assay to test the formation of the HS/FGF/FGFR ternary complex. Polysaccharide **2**, **5** and **7** were used in combinations with FGF1 (F1) and FGFR1 (R1) or FGF1 and FGFR2 (R2). A shift in the mobility of radio-labeled polysaccharides indicates ternary complex formation. Only polysaccharides **5** and **7** could form the ternary complex with FGF1/FGFR1 and FGF1/FGFR2.

formation of the ternary complex. Based on the data shown in Fig. 3, only polysaccharides **5** and **7** could form the ternary complex significantly with FGF1/FGFR1 and FGF1/FGFR2.

4. Discussion

The FGF family members play a major role in various biological processes including organogenesis, wound healing, and nervous system development and function [4]. Disrupted FGF signaling is also present in a variety of human pathologies including Crouzon's syndrome, Pfeiffer's syndrome, and Apert's syndrome [3]. It is well known that heparan sulfate acts as a co-receptor for FGF/FGFR mediated cell signaling [8–10]. Various studies have reported that both specific sulfation patterns and the extent of sulfation of HS are key parameters that determine the formation of the HS/FGF/FGFR ternary complex [11–13,15]. However, it is still unclear how the FGF binding motif on HS is assembled in the Golgi. Therefore, this work aims to elucidate whether the FGF binding motif is assembled by enzyme actions that occur sequentially or concurrently.

In this investigation, three different enzymatically synthesized polysaccharides were utilized for binding studies with FGF1 and FGFRs. The obtained data confirmed that NDST-2, C5-epimerase and 2-OST can act on heparosan concurrently. When acting concurrently, these enzymes did not generate a significant amount of the disulfated ΔUA2S-GlcNS disaccharide. However, when the enzymes were added sequentially, this disaccharide was abundant in the modified product.

After the structural characterization of the synthesized products, a gel mobility shift assay was performed with FGF1 and FGFR1 or FGFR2 to determine which polysaccharides could form the ternary complex. Surprisingly, only polysaccharides **5** and **7** could form the ternary complex with FGF1/FGFR1 and FGF1/FGFR2. While it is possible that polysaccharide **2** may form a few weak complexes that are intangible in this gel mobility shift assay, it is evident that polysaccharide **5** has significantly higher binding affinity compared to polysaccharide **2** resulting in tangible complexes under the electrophoretic conditions. Differential binding ability of these polysaccharides with FGF/FGFR may perhaps be

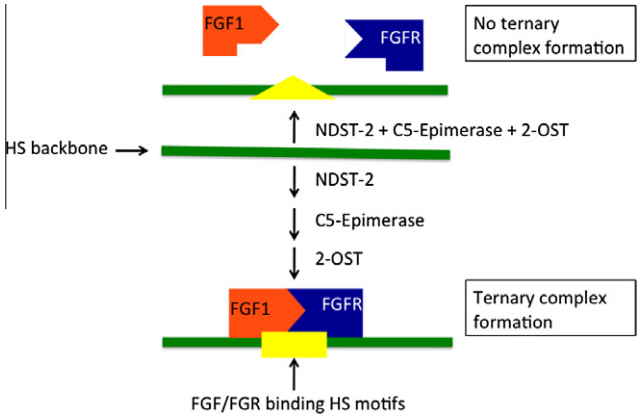


Fig. 4. Plausible schematic model of the assembly of FGF/FGFR binding HS motifs. HS chains can form ternary complexes with FGF and FGFR only when they are modified by HS biosynthetic enzymes in a sequential manner.

quantitatively deduced through sophisticated biophysical methods such as surface plasmon resonance. Interestingly, an earlier study has shown that more potent ATIII-binding HS anticoagulant structure is generated when HS biosynthetic enzymes act concurrently [18]. The current study points to a fundamental difference in the sulfation patterns produced by HS biosynthetic enzymes when they act sequentially or concurrently on the polysaccharide backbone. Natural heparan sulfate has a domain-like organization whereby some segments of the chain are highly sulfated (NS domains), some segments have little to no sulfation (NA domains), and some segments are partially sulfated (NA/NS domains). By sequentially modifying heparosan, it is likely that the resulting polysaccharides **5** and **7** have an extended sulfation pattern that mimics natural HS whereas polysaccharide **2** has a more random sulfation pattern that is not present in the natural FGF1-binding HS domain. Furthermore, polysaccharide **2** was found to migrate slower than polysaccharide **5** during gel electrophoresis, indicating that the overall sulfation density of the polysaccharide **2** is much less than that of the polysaccharides **5** and **7**.

Based on the results from this study, we can predict that the production of the FGF1/FGFR binding motif proceeds in a sequential manner in the Golgi. As the nascent HS chain passes through GAGOSOMES, it is modified in a specific order by HS biosynthetic enzymes (Fig. 4). However, the factors that modulate this orderly action remain unknown. A number of factors can affect the order of modification including: the specific location of these enzymes, the limited concentration of PAPS or the effect of sulfation patterns on the enzymatic action of other sulfotransferases. Future studies will further probe the biosynthesis of the FGF/FGFR binding motif in HS by using fluorescence assisted colocalization experiments to track nascent HS chains as they are modified by GAGOSOMES.

Acknowledgements

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