Research

A heparin binding synthetic peptide from human HIP / RPL29 fails to specifically differentiate between anticoagulantly active and inactive species of heparin

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Abstract

Despite extensive progress in determining structures within heparin and heparan sulfate (Hp/HS) and the discovery of numerous proteinaceous binding partners for Hp/HS so far; the only detailed characterization of a specific protein-glycosaminoglycan interaction is antithrombin III (ATIII) binding to a Hp pentasaccharide containing a unique 3-O-sulfated glucosamine residue. Previously, it was reported from our laboratories that a 16 amino acid synthetic peptide derived from the C-terminus of human HIP/RPL29 (HIP peptide-1) enriched for ATIII-dependent anticoagulant activity, presumably by specifically binding the ATIII pentasaccharide. Herein, we demonstrate that HIP peptide-1 cannot enrich ATIII-dependent anticoagulant activity from a starting pool of porcine intestinal mucosa Hp through a bio-specific interaction. However, a HIP peptide-1 column can be used to enrich for anticoagulantly active Hp from a diverse pool of glycosaminoglycans known as Hp byproducts by a mechanism of nonspecific charge interactions. Thus, HIP peptide-1 cannot recognize Hp via bio-specific interactions but binds glycosaminoglycans by non-specific charge interactions.

Introduction

The serine protease inhibitor, antithrombin III (ATIII), is 1000 times more active when bound to a specific pentasaccharide sequence within the heparin / heparan sulfate (HS) chain [1]. While this sequence is found with a low frequency in HS, approximately 30% of the heparin molecules within a commercial preparation of porcine intestinal mucosa heparin (Hp), contains this pentasaccharide [2–5]. The ATIII – Hp complex inhibits the coagulation cascade by inactivating serine proteases, such as factor Xa (FXa) and thrombin. The interaction between ATIII and the Hp pentasaccharide (ATIII binding pen-

tasaccharide) is the paradigm of a bio-specific Hp-protein interaction.

Specific protein-Hp/HS interactions involving the ATIII binding pentasaccharide or related sequences have been proposed for the fibroblast growth factor receptor (FGFR) [6], and a synthetic peptide derived from the C-terminus of human heparin/heparan sulfate interacting protein / ribosomal protein L29 (HIP peptide-1) [7]. These interactions were determined partly on the basis of column chromatography experiments. Tritiated Hp with or without unlabelled Hp is applied to a column of immobilized

FGFR or HIP peptide-1 in low salt and a proportion of Hp flows through with apparently no affinity, while the remainder binds and is eluted with high salt. FGFR and HIP peptide-1 bound fractions were enriched in ATIII-dependent anti FXa activity, presumably by specifically binding to the ATIII binding pentasaccharide or a motif associated with this sequence. The proposed bio-specific affinity of FGFR for the ATIII binding pentasaccharide or related structures has recently been challenged [8]. We also report here that HIP peptide-1 does not select for anticoagulantly active molecules in Hp or heparin byproducts through bio-specific interactions.

Previous work [7] developed five lines of evidence to indicate a specific interaction between HIP peptide-1 and the ATIII binding pentasaccharide. Firstly, a large proportion of tritiated Hp flows through a HIP peptide-1 column at 0.15 M NaCl, suggesting that most of the molecules in a commercial preparation of tritiated Hp do not have a specific motif needed for binding. Secondly, Hp oligosaccharides generated by partial deaminative cleavage with nitrous acid show significant binding to HIP peptide-1 only when the length is an octasaccharide or higher which is similar to that seen for ATIII binding. Thirdly, tritiated Hp that binds with high affinity to HIP peptide-1 also binds to an ATIII column with high affinity. Fourthly, Hp separated by HIP peptide-1 chromatography is enriched in ATIII-dependent FXa inhibitory activity while low affinity species show a decrease in the same activity. Lastly, HIP peptide-1 inhibits the ability of ATIII-Hp complexes to inhibit FXa and thrombin activity, while a scrambled peptide does not. These results led us to formulate the hypothesis that HIP peptide-1 separates Hp into anticoagulantly active or inactive species by interacting with the ATIII binding pentasaccharide in a bio-specific manner.

The data presented in this paper show that fractionation of unlabelled Hp and tritiated Hp by HIP peptide-1 display dramatic qualitative and quantitative differences. Tritiated Hp that binds to HIP peptide-1 exhibits an increase in ATIII-dependent anti FXa activity over starting material while an analogous preparation of unlabelled Hp fails to do so. The differences between HIP peptide-1 fractionation of tritiated and unlabelled Hp is partly explained by differences in the charge profiles of these two pools as measured by anion exchange chromatographic analyses. The validity of using the commercially available tritiated Hp as a model for unlabelled Hp is discussed.

A second source of anticoagulantly active glycosaminoglycans (GAGs), called Hp byproducts, is used to analyze the binding specificity of HIP peptide-1. This is a byproduct from the preparation of commercial Hp and contains several GAG species, including those of the Hp/HS subclass, less sulfated than Hp [9]. Fractionation of Hp byproducts

by HIP peptide-1 chromatography yields a high affinity material that is enriched in ATIII-dependent anti FXa activity over starting material. The relative activity of the fractionated material is proportional to the salt concentration used for elution while unbound fractions are depleted in this same inhibitory activity. However, we demonstrate that this fractionation is not due to a bio-specific interaction between HIP-peptide-1 and the ATIII binding pentasaccharide but due to a non-specific, charge based mechanism resulting in the enrichment of anticoagulantly active Hp from Hp byproducts.

Results

HIP Peptide-1 chromatography of heparins
Unlabelled Hp (figure 1A) or tritiated Hp (figure 1B) was subjected to HIP peptide-1 affinity chromatography as described in the *Materials and Methods* Section. Unlabelled Hp recovered in the 0.15 M NaCl wash corresponded to 49% of the recovered material and 51% was found in the fractions eluted with 0.50 M NaCl. However, unlabelled Hp was "bleeding" from the column after extensive washing (up to 10 column volumes) with 0.15 M NaCl. Conversely, tritiated Hp eluted cleanly from the column with 68% of the recovered radioactivity in the 0.15 M NaCl wash fractions and 32% in the 0.5 M NaCl eluate. The 0.5 M NaCl eluted materials from chromatography of both Hp sources were tested in an ATIII-dependent anti FXa assay. These FXa assays (data not shown) indicated that tritiated Hp materials bound by HIP peptide-1 were enriched in anticoagulant ability over starting material while the same unlabelled Hp fraction was not. Repeated experiments where different amounts of unlabelled Hp were applied to a HIP peptide-1 column followed by analyses of bound fractions failed to show an enrichment of ATIII-dependent anti FXa ability.

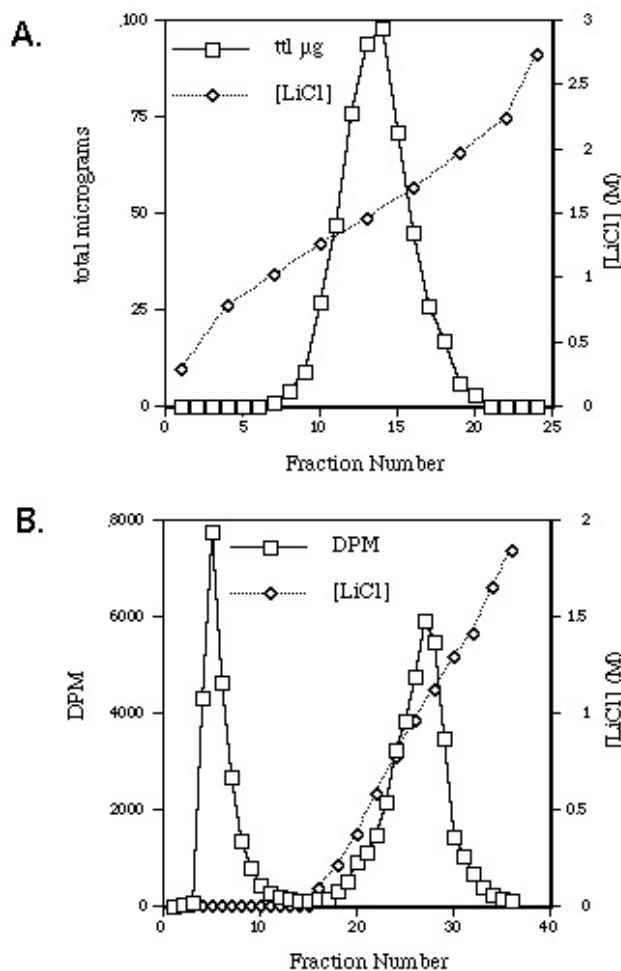### Anion exchange chromatography of unlabelled and tritiated Hp
We subsequently explored the possibility that the difference in the HIP peptide-1 binding ability of unlabelled and tritiated Hp was due to a difference in the charge density of these materials. This was tested by anion exchange chromatography of unlabelled (figure 2A) or tritiated Hp (figure 2B) with LiCl gradient elution. All of the unlabelled Hp is found to bind to an anion exchange column and elute as a single broad peak with an average (determined from three separate experiments) peak LiCl concentration of 1.5 M. The tritiated Hp preparation is found to contain a significant amount (38%) of material that does not bind to the anion exchange resin even before the start of the LiCl gradient. The bound fraction consists of 62% of the radioactivity and elutes at a peak LiCl content of 1.0 M.
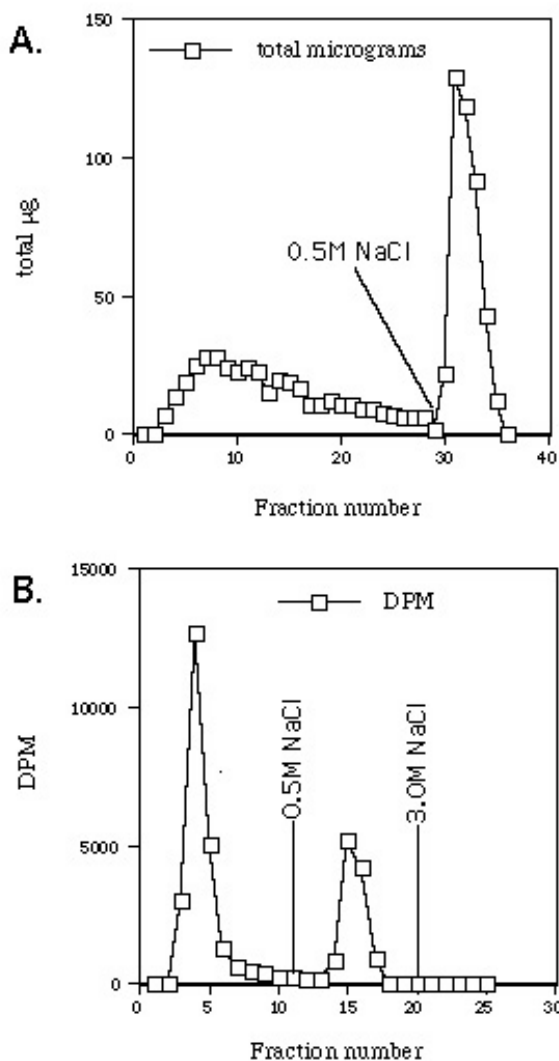
Figure 1
Fractionation of Unlabelled or Tritiated Hp by HIP Peptide-1 Affinity Chromatography. I mg of unlabelled Hp (A) or 40 ng of tritiated Hp (B) was added to a HIP peptide-1 affinity matrix, allowed to bind for 10 min and washed with 10 column volumes of PBS before elution of bound materials with high NaCl as indicated. The X-axis corresponds to fraction number, and the Y-axis corresponds to total μg (A) or DPM (B).

Figure 2
Anion Exchange Chromatography of Unlabelled or Tritiated Hp. Unlabelled Hp (A) or tritiated Hp (B) was added to 1 ml of DEAE anion exchange resin pre-equilibrated in 0.05 M Acetate pH 4.0 and allowed to bind. A LiCl gradient was directly applied to the unlabelled Hp while the tritiated Hp was washed extensively in buffer without LiCl before the start of a LiCl gradient. The X-axis corresponds to fraction number, the left Y-axis corresponds to total μg (A) or DPM (B), and the right Y-axis corresponds to LiCl concentration.
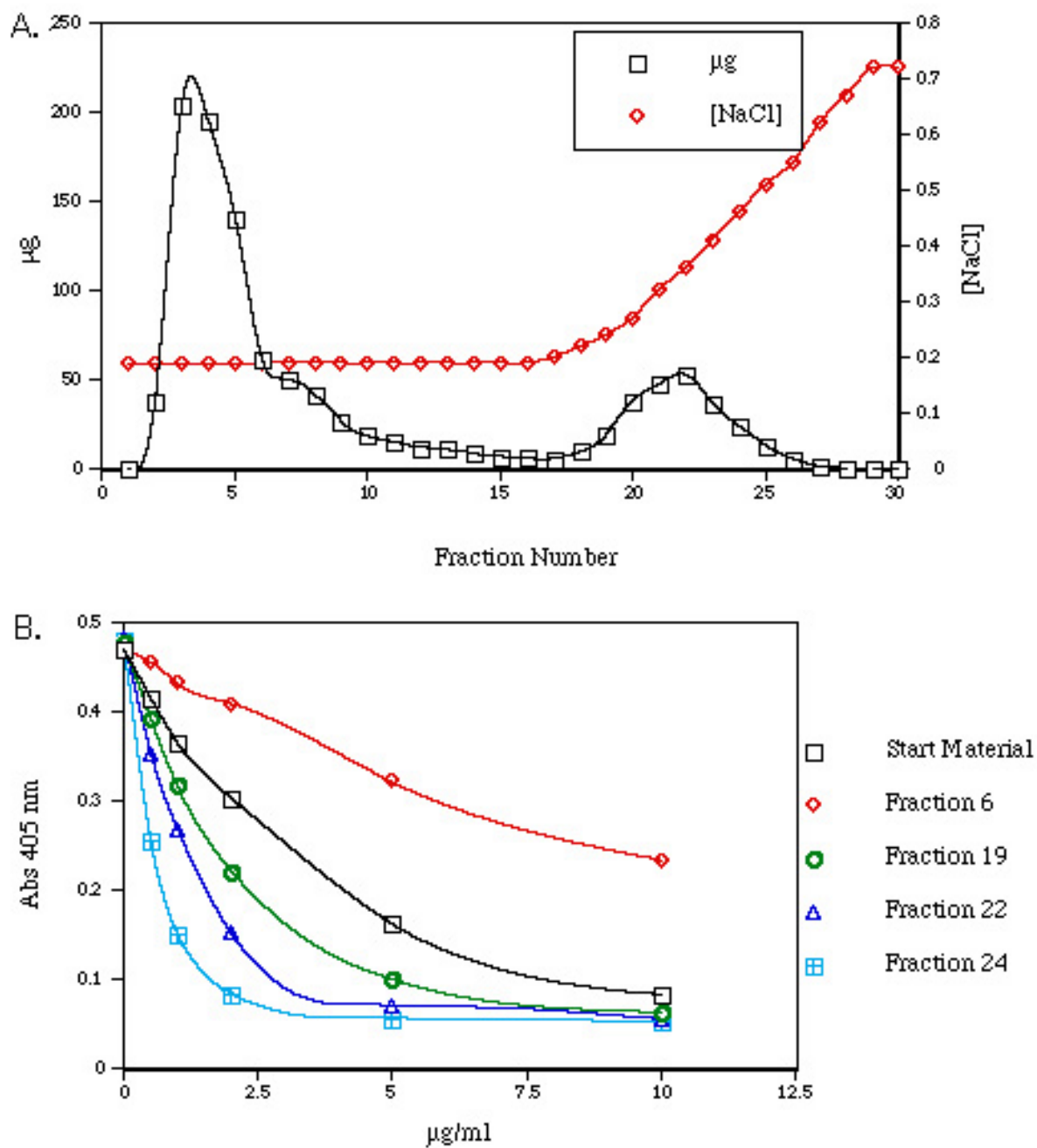
HIP Peptide-1 affinity chromatography of heparin byproducts

Heparin byproducts were subjected to HIP peptide-1 affinity chromatography (figure 3A). Of the material applied, 76% was recovered in flow through fractions 1–17

and 24% bound to the HIP-peptide-1 matrix and eluted between 0.22 and 0.62 M NaCl, fractions 18–27. Fractions 6, 19, 22, and 24 were further analyzed for their ability to inhibit FXa activity via ATIII in an *in vitro* assay (figure 3B). Flow through fraction 6 exhibited a decrease in FXa inhibiting activity by 0.355X when compared to

Figure 3
Fractionation of Heparin Byproducts via HIP peptide-1 Chromatography and Determination of ATIII-dependent anti FXa Activity. (A) A subsaturating amount of Hp byproducts in PBS was applied to 3 mls of a HIP peptide-1 affinity matrix, pre-equilibrated with PBS. Unbound materials were washed from the column by 15 ml of PBS and bound materials were eluted with a 0.15 to 0.70 M NaCl gradient. 1-ml fractions were collected and the amount of material and salt content in each fraction was quantitated as in "*Materials and Methods*." The X-axis corresponds to the fraction number while the left Y-axis denotes the total amount of GAG material in each fraction. The right Y-axis gives the NaCl concentration of each fraction. (B) Fractions 6, 19, 22, and 24 were tested in an ATIII-dependent anti FXa chromogenic assay as described in "*Materials and Methods*."

starting material. All bound fractions exhibited increases in FXa inhibiting activites from 1.9X to 5.4X greater than starting material. A direct relationship between the amount of salt needed for elution from the HIP peptide-1 matrix and the fold increase in anti-FXa activity was observed.

Differential salt elution was used in HIP peptide-1 chromatography of Hp byproducts to create three pools for subsequent analyses. Initially, Hp byproducts were added to HIP peptide-1 column in 0.25 M NaCl with approximately 2% bound. The 0.25 M NaCl flow through was diluted to 0.20 M NaCl and re-applied to the HIP peptide-1 column with 9.5% of the materials binding. This bound material was named HIP 0.20 M pool. Finally, the 0.20 M NaCl flow through was diluted to 0.15 M NaCl and re-applied to the column with 14% of the materials binding (HIP 0.15 M pool) and 76% of the materials present in the final flow through (HIP flow through). As in the gradient elution of Hp byproducts, the materials bound in the presence of higher salt have an increase in FXa activity over starting material (data not shown). HIP 0.15 M pool, HIP 0.20 M pool and HIP flow through were used in subsequent analyses.

Analyses of fractionated Hp byproducts by ion exchange chromatography
Unfractionated Hp byproducts and fractions obtained by differential salt elution from a HIP peptide-1 column were applied to DEAE anion exchange columns and eluted with a gradient of LiCl as described in the *Materials and Methods* Section. No material was detected in fractions before the start of LiCl gradients. Hp byproducts displayed a very broad elution profile that is indicative of this material containing a heterogeneous population of negatively charged molecules (figure 4A). The HIP 0.20 M pool is enriched in the most negatively charged materials whereas the HIP 0.15 M pool appears to contain slightly less negatively charged materials which still elute late in the chromatography run compared to Hp byproducts starting material. Materials depleted of HIP peptide-1 binding species (HIP flow through) are completely depleted of highly negatively charged species. An ATIII affinity matrix was also used to fractionate Hp byproducts into an ATIII binding fraction with high affinity for the protease inhibitor and an ATIII non-binding fraction (see *Materials and Methods* section). These fractions were also analyzed for their negative charge properties by an ion exchange chromatography (figure 4B). ATIII binding species displayed an increased charge profile when compared to Hp byproducts. However, unlike the fraction depleted of HIP peptide-1 binding material, Hp byproducts depleted of ATIII binding sites still contain highly negatively charged species when compared to the Hp byproducts starting material. Hp byproducts depleted of ATIII binding sites were

also applied to a HIP peptide-1 column and subsequently eluted with a gradient from 0.15 to 1 M NaCl. It is noteworthy that significant material required greater than 0.25 M NaCl to be eluted (data not shown) further demonstrating that ATIII and HIP peptide-1 fractionate GAGs by different types of molecular interactions.

Capacity of a HIP peptide-1 column for binding different GAG species
The binding capacity of a HIP peptide-1 column for Hp, CS-E, DS, CS-C and bovine kidney HS (BK-HS) was analyzed. Increasing concentrations of these GAGs were incubated with a defined amount of HIP peptide-1 Sepharose and the amount of GAG bound to the gel was determined (figure 5). The curves generated show binding of Hp and CS-E to HIP peptide-1 Sepharose with saturation occurring over the same range, indicative of similar binding affinities. DS showed less binding over the same range and HS or CS-C showed no binding under the experimental conditions employed. Differences in the total amounts of materials bound were noted in parallel binding experiments with Hp as a positive control. The total amount of CS-E and DS bound at saturation were 50% and 17%, respectively, of the total amount of Hp bound at saturation. These results are consistent with the hypothesis that the HIP peptide-1 / GAG interaction depends solely on non-specific charge interactions.

Discussion
Previous work [7] suggested that HIP peptide-1 could enrich for anticoagulantly active Hp on the basis of a bio-specific interaction with the ATIII binding pentasaccharide. The goal of the current study was to substantiate this hypothesis. However, an overwhelming amount of data presented here demonstrates that the underlying hypothesis is invalid. Although we can enrich for anticoagulantly active Hp by HIP peptide-1 affinity chromatography using tritiated heparin or Hp byproducts as a starting material, this enrichment is not due to bio-specific interactions. Furthermore, repeated chromatography experiments with unlabelled conventional Hp as the starting material failed to significantly enrich for ATIII binding Hp. Lastly, Zhang *et al.* [10] have recently reported that HIP peptide-1 does not protect anticoagulantly active HS from complete heparitinase digestion.

It is clear from the current data that our samples of unlabelled and tritiated Hp are not biochemically similar. A significant proportion (38%) of tritiated Hp does not bind an anion exchange column while 100% of unlabelled Hp does. We hypothesize that this sample of tritiated Hp contains 38% free label and thus has no apparent negative charge. Since the fractionation of this particular preparation of tritiated Hp over HIP peptide-1 is similar to the previously published observations, we can only
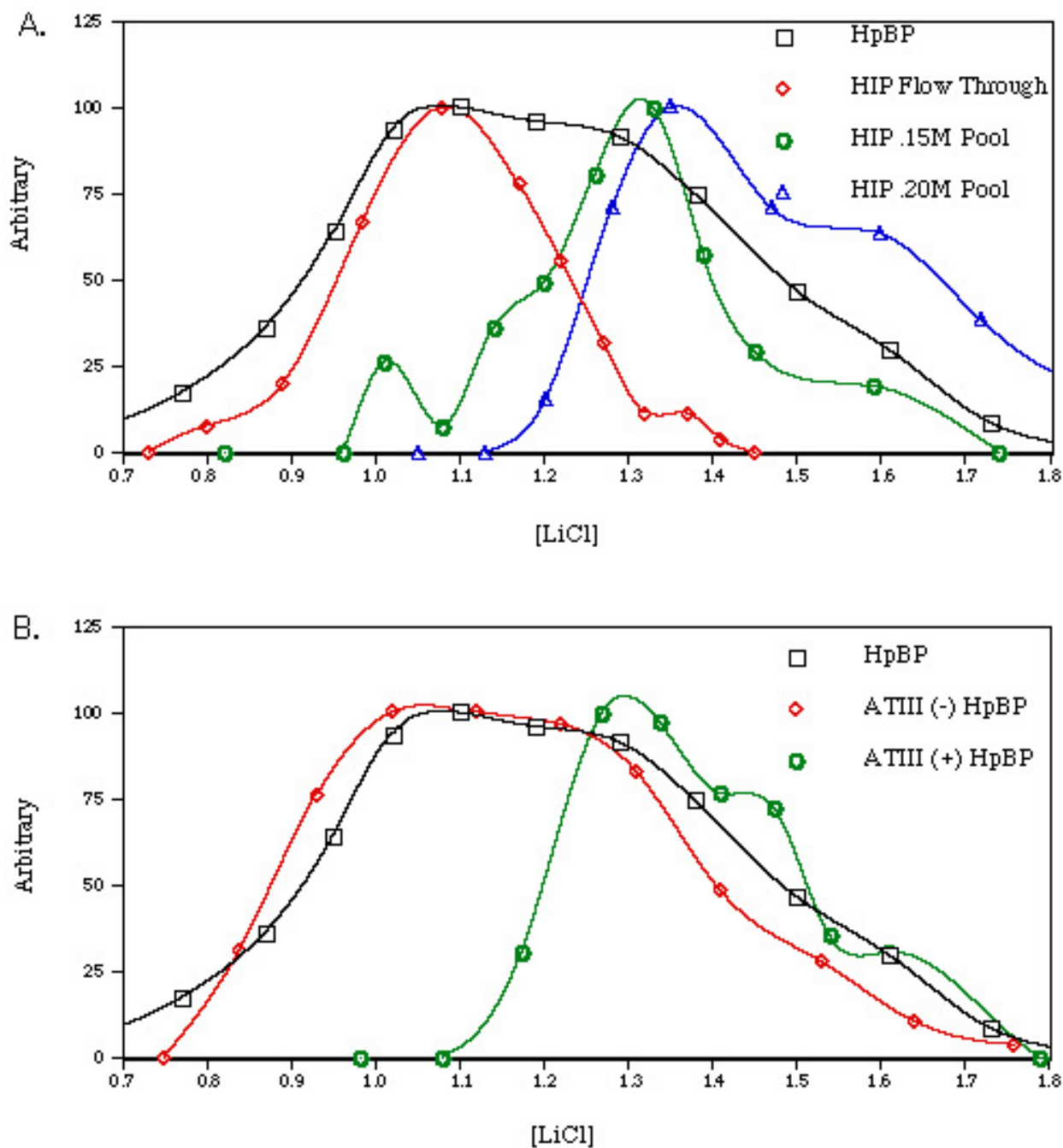
Figure 4
Elution Profiles of Hp Byproducts or Fractionated Pools from Anion Exchange Chromatography with Gradient Elution. Hp byproducts starting material (HpBP) or materials from fractionation via HIP peptide-1 (A) or ATIII (B) were applied to a 1-ml bed volume of DEAE sepharose in .05 M acetate, pH 4.0. Columns were washed with 10 column volumes of acetate buffer before application of LiCl gradients. All material bound to DEAE column and 100% eluted with LiCl. One-ml fractions were collected and analyzed for GAG and LiCl content as in "*Materials and Methods*." Arbitrary values of 100 were set for the elution peaks for each of the column runs with every other point multiplied by the same factor. The resulting plots are of LiCl concentration (X-axis) versus arbitrary amount of GAG (Y-axis).
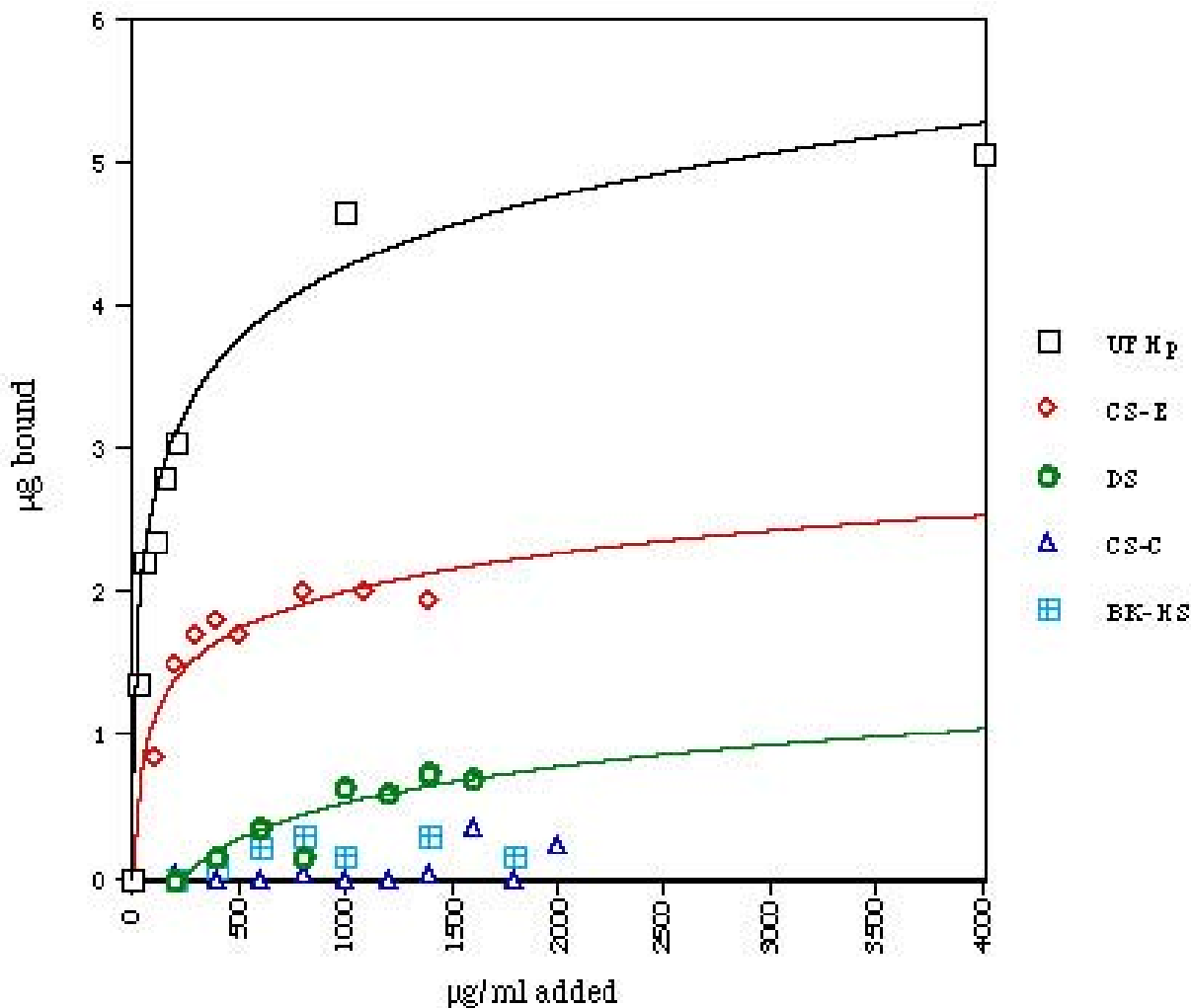
Figure 5
Binding Curves of GAGs to HIP Peptide-1. Dilutions of Hp, CS-E, DS, CS-C or Bovine kidney-HS (BK-HS) were added in 400 μl PBS to 100 μl of a 1:1 slurry of HIP peptide-1 sepharose : PBS. After a 10-minute incubation, the tubes were washed with three serial dilutions of 500 μl PBS. Bound materials were eluted with 2 M NaCl and quantified as in "*Materials and Methods*." Values shown are the total amounts bound (Y-axis) versus the concentration of the material added (X-axis). Hp, CS-E, and DS were fitted to a logarithmic curve with r squared values of 0.81 or greater.

conclude that the initial preparation also contained significant amounts of "free label". The presence of "free label" would explain the repeated enrichment of ATIII-dependent anti FXa ability seen in tritiated Hp. Rather than enriching for the ATIII binding pentasaccharide by HIP peptide-1 chromatography of tritiated Hp, this chroma-

tography enriches for Hp, leaving behind "free label". This would result in an apparent enrichment of activity per DPM in bound tritiated Hp materials and a depletion of activity per DPM in unbound tritiated Hp materials.

When the starting material used for fractionation on a HIP peptide-1 column is Hp byproducts, a charge dependent increase in ATIII-dependent anti FXa activity for bound materials is seen. Hp byproducts are a very heterogeneous mixture of GAGs. Due to the nature of the Hp/HS biosynthetic pathways, the ATIII binding pentasaccharide is formed preferentially in highly negatively charged regions [1]. Our hypothesis is that HIP peptide-1 chromatography does not enrich for anticoagulant activity from Hp byproducts on the basis of a bio-specific interaction with the ATIII binding pentasaccharide but rather due to non-specific charge interactions. This hypothesis was substantiated by comparing the charge characteristics of Hp byproducts depleted of HIP peptide-1 or ATIII binding activity. Also, the binding ability of HIP peptide-1 for Hp byproducts depleted of ATIII binding activity was determined. Firstly, elution profiles from anion exchange chromatography show that ATIII binding species have an increased charge profile when compared to starting material, but the ATIII-depleted materials are only slightly shifted to a less negatively charged profile compared to starting material. In contrast, HIP peptide-1 depleted Hp byproducts are essentially depleted of its most negatively charged species. This shows that highly negatively charged species within Hp byproducts are associated with, but not sufficient for ATIII interaction while HIP peptide-1 has a simple charge requirement. Secondly, HIP peptide-1 binds some ATIII depleted Hp byproducts indicating that the ATIII binding pentasaccharide is not essential for HIP peptide-1 binding. The inherently high negative charge of ATIII binding species [11,12] and the charge heterogeneity of Hp byproducts makes the HIP peptide-1 separation of Hp byproducts into pools with low and high anticoagulant activity possible. In contrast, the relatively homogeneously charged Hp preparations tested are refractory to a similar separation.

Binding studies suggest that HIP peptide-1 has a selectivity for GAGs of the Hp/HS subclass [13]. This is demonstrated by the inability of CS-A, DS, CS-C, KS, and HA to act as effective competitors for HIP peptide-1 binding to tritiated Hp. Additionally, HIP peptide-1 is found to bind subsets of cell surface JAR cell HS and DS, suggesting differentiation within a class of GAG. Binding potential is increased in pools of JAR cell HS or DS that have longer length and higher sulfation content. Likewise, Hp byproducts bound in the presence of 0.20 M have increased size (D.E.H. and M.H. unpublished observations) and charge when compared to Hp byproducts bound in 0.15 M NaCl. The new interpretation of this data is that HIP peptide-1 binds GAGs via a threshold charge and not due to inherent bio-specificity. This is further supported by the determined hierarchy of binding potential; Hp > CS-E > DS > CS-C = BK-HS, which mimics the charge density order of the GAGs; suggesting that the sulfation content is the most important factor in the interaction with HIP peptide-1 and not the subclass of GAG.

Theoretically, it should be possible to create linear peptides that can specifically bind to 'sequences' within the linear GAG chain. Our current knowledge on Hp/HS binding motifs has come from an examination of Hp/HS binding proteins that identified the XBBXBX and XBBBXXBX motifs where X is an uncharged or hydrophobic amino acid and B is a basic amino acid [14]. It is important to note that HIP peptide-1 (CRPKAKAKAKAKDQTK) does not exactly correspond to either of these motifs yet binds Hp/HS. However, studies have shown that concatamers of peptides that conform to these consensus motifs have binding affinity proportional to the number of subunits [15] and can reverse the antithrombotic activity of Hp *in vivo* [16]. A natural example of a concatamer of Hp/HS binding motifs is human HIP/RPL29. This protein is highly basic with 29.5% of Lys/Arg content distributed evenly throughout the protein. Studies with deletion mutants of human HIP/RPL29 show that Hp/HS binding ability increases with the length of deletion mutant, irrespective of domain [17]. Concatamers of Hp/HS binding sequences may be a common mechanism of protein/GAG interactions. Future work will be aimed at identifying novel proteins and peptide sequences that specifically interact with ATIII binding Hp/HS.

Materials and Methods
Materials
Porcine intestinal mucosa heparin (product number H3393), bovine kidney heparan sulfate, dermatan sulfate, and chondroitin sulfate C, were purchased from Sigma. Chondroitin Sulfate E was purchased from CalBiochem. Tritiated heparin (0.44 mCi/mg) was purchased from NEN life science products. DEAE resin was purchased from Pharmacia. Heparin byproducts were obtained from Scientific Protein Laboratories (division of Viobin corporation) Waunakee, WI. Human blood plasma was obtained from the Houston Blood Center (Houston, TX).

HIP peptide-1 affinity chromatography
HIP peptide-1 affinity resin was prepared as in Liu et al., [7]. One mg Hp or 40 ng tritiated Hp was applied to HIP peptide-1 affinity resin in 0.15 M NaCl, phosphate buffer, allowed to bind for 10 min and washed with 10 column volumes of 0.15 M NaCl phosphate buffer. Then 0.5 M NaCl was used to elute any bound Hp or tritiated Hp. A final elution with 3 M NaCl was employed for tritiated Hp. Hp byproducts were applied to 3 mls of a HIP peptide-1 affinity matrix in PBS, washed with 15 mls PBS, and eluted w/ a 0.15 M to 0.70 M NaCl gradient. Material for the bulk separation of Hp byproducts were initially added in 0.25 M NaCl, under subsaturating conditions, washed with ten column volumes of 0.25 M NaCl and finally

eluted with 1 M NaCl. Subsequent experiments for the bulk separation of Hp byproducts took the flow through from the previous salt separation and diluted them to 0.2 M, and then 0.15 M for the serial separation of materials with decreasing stringency. Five different column runs were done at each step to create HIP 0.20 M, HIP 0.15 M and HIP flow through pools. One ml fractions were collected and salt concentrations were determined against a standard curve using a conductivity meter.

GAG quantitation

A Blyscan (Biocolor, Ltd.; Belfast, Ireland) assay was used for the quantitation of GAGs in experiments using unlabelled GAGs. The assay is based on the specific binding of the cationic dye; 1,9-dimethyl methylene blue to sulphated GAGs [18]. A standard curve of the relevant material was made during each quantitation that had a correlation coefficient of 0.96 or greater. Unknown solutions were diluted to <0.5 M NaCl where applicable and quantitation performed in at least duplicate. Quantitation of tritiated Hp was performed by liquid scintillation counting.

Anion exchange chromatography for the determination of GAG charge

A 1 ml DEAE column was pre-equilibrated in 0.05 M Acetate pH 4.0 and GAGs applied [19]. Columns were washed in acetate buffer for 10 column volumes before the start of LiCl gradients ranging from 0 to 2 M LiCl. In some experiments where it was previously determined that 100% of the GAGs bound to the column, washing steps were omitted and LiCl gradients started at fraction one. One-ml fractions were collected and quantified in the Blyscan assay against a standard curve or radioactivity counted by liquid scintillation counting. A standard curve for LiCl was also made and read on a conductivity meter enabling the conversion of experimental conductivity readings to LiCl concentration. Data from these experiments were analyzed by assigning the maximum peak of elution an arbitrary value of 100. All other values were multiplied by the same factor and elution profiles from LiCl concentration (X-axis) versus arbitrary (Y-axis) were made. A line running through the transformed elution profiles 50 arbitrary value was made and the two intersection points of LiCl concentration were noted. These two intersection values were averaged to determine the LiCl concentration for elution peaks. The LiCl concentration for elution peak in experiments where multiple elution profiles were made, was an average of the individual averages.

FXa activity of GAG fractions

Clinical kits (Sigma, St. Louis, MO) were used in the determination of ATIII – dependent Hp activity in FXa inhibition as described in the corresponding instructions except that Hp / Hp byproducts materials were added in place of plasma. Briefly, this kit uses ATIII, FXa, and a chromogenic substrate for FXa to measure Hp concentrations in blood. This assay has been utilized to measure the relative activity of fractionated Hp byproducts to accelerate the inactivation of FXa by ATIII-Hp complexes as measured by comparisons in ATIII-GAG – dependent inhibition of substrate cleavage measured at 405 nm. Glycosaminoglycan containing solutions were diluted to 0.15 M NaCl before use in the assay. Enrichment or depletion of FXa activity was determined by identifying the concentration of Hp byproducts at which FXa activity was inhibited $1/2$ maximally ($1/2$ max_{inh}). The $1/2$ max_{inh} concentration of the starting material was divided by the $1/2$ max_{inh} concentration of the sample to determine fold increase in FXa activity over starting material.

Antithrombin III affinity chromatography

Antithrombin III was purified from human plasma as described in Wickerhauser and Williams [20]. The purified ATIII was then linked to CNBr activated Sepharose in the presence of excess N-acetylated Hp as in Höök *et al.*, [2]. Hp byproducts were applied to the ATIII column in PBS, washed with 10 column volumes and bound material eluted in phosphate buffer containing 3 M NaCl. Flow through materials was subjected to ATIII chromatography 5 times. After the third time, no bound material was detected. Thus, th5 times ATIII flow through is completely depleted of ATIII binding sites. This was confirmed by a lack of activity in the FXa assay (data not shown).

GAG binding assays

Multiple tubes containing 100 μl of a 1:1 suspension of HIP peptide-1 Sepharose in PBS were assembled. Four hundred μl of UF Hp (Sigma), bovine kidney heparan sulfate (Sigma), chondroitin sulfate-C (Sigma), dermatan sulfate (Sigma), or chondroitin sulfate-E (CalBiochem), was added in a range of concentrations from 50 μg/ml to 1500 μg/ml. The mixtures were incubated at room temperature for thirty minutes after an initial vortexing. The tubes were then centrifuged at 16,000 × G for 5 min and the liquid aspirated, leaving resin in the tube. Then, 500 μl of PBS was added to the resin bed, vortexed, centrifuged, and liquid aspirated. This cycle was done three times to ensure a 1:1000 final dilution of initially added GAGs. After a final aspiration, 50 μl of 2 M NaCl in phosphate buffer was added, releasing any bound material into the liquid phase. Aliquots of this material were quantified by the Blyscan assay and μg/ml GAG added (X-axis) versus total μg GAG bound (Y-axis) plots were made.

Abbreviations

heparin / heparin from porcine intestinal mucosa – Hp; factor Xa – FXa; antithrombin III – ATIII; heparan sulfate – HS; chondroitin sulfate – CS, dermatan sulfate – DS, heparin/heparan sulfate interacting protein / ribosomal

protein L29 – HIP/RPL29; heparin/heparan sulfate interacting protein / ribosomal protein L29 peptide-1 – HIP peptide-1; glycosaminoglycan – GAG; phosphate buffered saline – PBS

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