A Binding Site for Highly Sulfated Heparan Sulfate Is Identified in the N Terminus of the Circumsporozoite Protein

SIGNIFICANCE FOR MALARIAL SPOROZOITE ATTACHMENT TO HEPATOCYTES

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Circumsporozoite protein (CSP) coats the malarial sporozoite and functions to target the liver for infection, which is the first step to developing malaria. An important tissue ligand for CSP is the glycosaminoglycan heparan sulfate (HS) found on the surface of hepatocytes and in the basement membrane of the space of Disse. To better understand this efficient targeting process, we set out to identify and characterize the HS binding site(s) of CSP. We synthesized a series of peptides corresponding to five regions of Plasmodium falciparum CSP containing basic residues, a common requirement of HS binding sites, and screened them for heparin and HS binding activity. Only one of these peptides (Pf 2), which contains a motif we have named region I-plus, demonstrated both high affinity heparin/HS binding activity and the ability to block the binding of recombinant CSP to heparin-Sepharose 4B. Analysis by isothermal titration calorimetry revealed that region I-plus has a binding constant of Kd = 5.0 μM and a stoichiometry of n = 7.8 binding sites/heparin chain. Heparin binding was dependent on the amino acid sequence of region I-plus, and the binding sites on heparin/HS are contained within a decasaccharide. Furthermore, HS oligosaccharides rich in sulfate and iduronic acid content (heparin-like) are required for efficient binding. Because liver HS is exceptionally high in both these components relative to the HS of other organs, the HS structural requirements for efficient region I-plus HS binding are consistent with this peptide sequence functioning to target sporozoites to the liver for attachment to hepatocytes. Finally, the region I-plus heparin/HS binding site was also discovered for two other species that infect humans, Plasmodium malariae and Plasmodium vivax, further supporting the existence of a HS binding domain in the N-terminal portion of CSP.

Four species of the protozoan genus Plasmodium (P. falciparum, P. vivax, P. ovale, and P. malariae) cause malaria, the most devastating parasitic disease in humans. Worldwide 300–500 million people are infected yearly, leading to 1.5–2.7 million deaths caused mostly by P. falciparum (1). The complex life cycle of parasites involves several stages, which probably contributes to its ability to evade immune detection and complicates the development of vaccines. Malarial infection is initiated when as few as 10 sporozoites (2) are injected into the host blood stream by a feeding infected Anopheles mosquito, and within minutes, the sporozoites attach to and invade liver cells (3). There they divide asexually into large numbers of merozoites, which leave the liver and infect erythrocytes causing the severe symptoms of malaria. The initial rapid and specific invasion of hepatocytes is mediated by the interaction between the circumsporozoite protein (CSP), which coats the surface of the sporozoite (4), and the glycosaminoglycan (GAG), heparan sulfate (HS), found on the surface of the hepatocytes (5–7) and in the extracellular matrix in the space of Disse (8).

Numerous proteins such as growth factors, serine protease inhibitors, extracellular matrix proteins, lipoproteins, and lipolytic enzymes are also known to associate with HS as part of their normal function (9, 10), and many pathogenic organisms including Plasmodium have evolved proteins to target HS for attachment and invasion of specific host cells (11). In the last 15 years, there has been extensive characterization of HS binding sites and heparin, which is produced and stored only in mast cells, has been widely used in these studies because of its structural similarity to the more ubiquitously distributed HS. Heparin and HS are both acidic polysaccharides composed of alternating hexuronic acids and hexosamines (10, 12). Nascent chains of heparin and HS are composed of β-D-glucuronic acid (GlcA) and N-acetylgalactosamine, which are linked through a tetrasaccharide “stem” to Ser residues of a core protein. During the process of elongation, the polysaccharide chains are further modified by a series of enzymatic reactions: N-deacetylation; N-sulfation of GlcNAc (GlcNS); C-5 epimerization of GlcA to α-D-iduronic acid (IdoA) followed by sulfation generating IdoA-2-OSO₄G, GlcNS-6-OSO₄G, and trace levels of GlcNS-3-OSO₄G saccharide units. For heparin, the resulting mature chain contains >80% IdoA with >2.4 SO₄G/disaccharide. By comparison, HS is generally less extensively modified with <50% IdoA and containing a more varied sulfation pattern with regions of high sulfation associated with high IdoA content and regions of little or no sulfation associated with high GlcA content.

Polypeptide sequences of heparin or HS binding sites are

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typically relatively short continuous sequences rich in the basic residues R and K (13), which can interact electrostatically with the anionic groups (COO⁻, OSO₃⁻) on HS. Based on the sequence alignment of several well characterized heparin/HS binding sites, three consensus sequences have been described, XBBXX, XBXXBXXB, and XBXXBXXXBXBXB (B = basic, X non-basic) (14–16), although there are many examples of heparin binding sequences for which consensus sequences have not been ascribed. It has also been proposed that the three-dimensional structure for heparin binding sites are critical with the outer basic residues tending to be spaced approximately 20 Å apart with no significant sequence similarities for the intervening residues (17).

Malarial CSPs are ~400 amino acids long organized into three domains: the N-terminal domain containing a conserved pentapeptide called region I; a highly repetitive species-specific central domain; and a C-terminal domain containing another conserved sequence called region II. This latter sequence has strong similarity to a portion of the type I cell adhesion motif called thrombospondin type I repeat (TSR), an ancient domain encoded in over 40 genes in humans (18) initially described for thrombospondin (19). It has been suggested that the region II-plus (region II plus eight downstream residues, EWSPCSCVTGNGIQQRIK) of CSP is responsible for sporozoite binding to HS on hepatocytes (20–22). However, given its extensive duplication in so many genes, the cell surface receptors for the TSR domains are probably ubiquitously distributed throughout the body, which would argue that another region in the CSP mediates the highly specific attachment of the sporozoite to hepatocytes. Also, the basic residue content and spacing for region II-plus appears atypical for a heparin/HS binding motif.

To our knowledge a systematic approach to localize the heparin/HS binding site(s) of CSP by direct heparin/HS binding assay has not been reported. In this paper we evaluate the heparin/HS binding potential of region II-plus and a series of other synthetic peptides corresponding to five different regions of *P. falciparum* CSP with a relatively high basic residue content. One high affinity heparin binding site we call region I-plus is identified in the N terminus of the CSP, which contains two copies of a heparin binding consensus sequence and the highly conserved pentapeptide, region I. The relative importance of the major side groups of heparin/HS for binding was determined. Finally, the heparin/HS binding potential of the region I-plus sequences for two other malaria-causing *Plasmodium* species, *P. malariae* and *P. vivax*, was also investigated.

**EXPERIMENTAL PROCEDURES**

**Glycosaminoglycans and Peptides—**Heparin, heparan sulfate (bovine kidney), chondroitin sulfate, dermatan sulfate, hyaluronan, and cyano- genic bromide (CNBr) were purchased from Sigma. Sepharose 4B was purchased from Amersham Biosciences. Modified heparins, low molecular weight heparins (MW 5000 and 3000), and highly sulfated HS were purchased from Seikagaku America Inc. (Ijamsville, MD) and Neoprin (San Leandro, CA). Carboxy-modified heparin was made by borohydride reduction in the presence of carbodiimide (23). Peptides were synthesized at the Advanced Protein Technology Centre, Peptide Synthesis Facility, University of Toronto (Toronto, Ontario Canada) and the Core Facility, Department of Biochemistry, Queen’s University (Kingston, Ontario, Canada). They were purified by reversed-phase high pressure liquid chromatography on an analytical C-18 column, dissolved in water (10 mg/ml), aliquoted, and stored at ~70 °C. Cysteine-containing peptides were reduced with iodoacacetamide to prevent any interdisulfide bridging and re-purified.

**Recombinant Circumsporozoite Protein (rCSP)—**A frozen stock of recombinant CSP was expressed in E. coli transformants expressing the *P. falciparum* CSP (27–123-NANPVDP(NANP)₃-300–411)-(H₃) was kindly provided by Dr. Ute Frevert (Department of Medical and Molecular Parasitology, New York University Medical Center). Bacteria were grown in LB broth, 0.1 mg/ml ampicillin, and 0.025 mg/ml kanamycin, and the expression of rCSP was induced with 2 mM isopropyl-ß-D-thiogalactopyranoside for 5–6 h. The rCSP was purified from a 0.1 M sodium phosphate, 6 M guanidine-HCl bacterial extract by affinity column chromatography. Purification and refolding of rCSP involved binding the bacterial extract to a 10-mL nickel-nitrilotriacetic acid-agarose column (Qiagen) and washing with 0.1 M sodium phosphate, pH 8.0, to remove the guanidine-HCl. The rCSP was then eluted from the column with 0.25 mM imidazole in sodium phosphate, and to the eluant, 0.5 M Tris-Å-glycine buffer were added and left overnight at 10 °C. Disulfide bond formation was then promoted by incubation with 0.9 mg GSSG (oxidized) for ~8 h at 10 °C. The sample was dialyzed against 20 mM Tris-HCl, 20 mM NaCl, 5% glycerol, pH 7.5 overnight at 4 °C. The dialysate was then applied to a heparin-Sepharose 4B column (5 ml) equilibrated with 20 mM Tris-HCl, 20 mM NaCl, pH 7.5. Bound rCSP was eluted with 0.5 mM NaCl buffer and dialyzed as before. Dialysate was then collected and stored at ~70 °C. The re-folded structure of rCSP was assessed by circular dichroism (CD) using a DSM 1000 CD spectrophotometer and analyzed with the CD deconvolution software CDNN, version 2.1.

**Heparin/HS Columns—**Affinity columns were generated by coupling heparin or HS to Sepharose 4B based on the method of Smith et al. (24). Sepharose 4B was washed with 36 mg/ml of water, subsequently suspended in 1 ml of water, and transferred to a beaker and placed on ice. Heparin or HS was dissolved in water (2 mg/ml) and also cooled on ice. The two solutions were mixed and the pH was adjusted to pH 10–11 with NaOH (5N). Fresh CNBr in N,N-dimethylformamide (1 mg/ml) was added dropwise to a final concentration of 31 mg/ml. The pH of the reaction mix was kept at pH 11 with the periodic addition of NaOH for 15 min and then left to stir overnight at room temperature. The gel was then washed with 20 bed volumes of water followed by 1 ml ethanamine pH 9, to block unreacted groups. The column was further washed with 10 bed volumes of (i) water, (ii) 0.1 M sodium acetate, pH 5.0, and (iii) 0.1 M NaHCO₃, pH 8.3, and then equilibrated in 20 mM Tris-HCl, 50 mM NaCl, pH 7.2. By this procedure, heparin/HS was linked through its NH₂ and –OH groups. The amount of heparin coupled to the Sepharose was 0.5–0.75 mg/ml as determined by the toluidine blue assay (24). Affi-Gel heparin was purchased from Bio-Rad.

**Affinity Chromatography on Heparin/HS-Sepharose 4B—**Peptides (30 µg–2 mg) were dissolved in 200 µl Tris-HCl, pH 7.2, and loaded onto a 2-ml heparin-Sepharose column (0.5 mg heparin/ml) connected to a high pressure liquid chromatography system (Waters) and equilibrated in the same buffer. After washing the column at 0.6 ml/min with 3 bed volumes, the column was developed with a 0–1 M NaCl linear concentration gradient (12 bed volumes) at 0.75 ml/min. The eluate was monitored continuously at 214 nm, and the absorbance was plotted against retention time (RT). Unbound peptide eluted 2.5–2.8 min after injection, and based on the RTs for the bound peptides, the NaCl concentration at which desorption took place could be calculated as follows: desorption [NaCl] / RT = (2.8–10.0 min)/32 min. Each peptide was run at least twice, and the RTs were reproducible within ± 1%.

**Binding and Competition Assays—**Binding assays were performed on a series of heparin-Sepharose columns (0.2 ml) packed in disposable 2-ml polypropylene columns (Bio-Rad) and equilibrated with 10 bed volumes of 20 mM Tris-HCl and 20 mM NaCl, pH 7.2 (elution buffer) by gravity feed. Peptides (16 nmol) were dissolved in 200 µl of equilibration buffer and applied to the columns, allowed to bind for 5 min and then washed with 7 bed volumes of the same buffer to remove unbound peptide. Bound peptide was eluted with 4 bed volumes of 20 mM Tris-HCl and 2 mM NaCl, pH 7.2, and the peptide concentration was determined by absorbance at 220 nm using a spectrophotometer. Nonspecific binding to the Sepharose 4B was <2%.

To determine whether binding was saturable, assays were done with heparin with unmodified or modified side groups. The heparin content of the columns was determined by the toluidine blue assay (24). Competition binding assays were performed by loading peptides with increasing concentrations of soluble heparin, low molecular weight heparin, chemically modified heparins, heparan sulfate, chondroitin sulfate, dermatan sulfate, hyaluronan, or a tri-sulfated disaccharide (4-O-sulfate) for each heparin-Sepharose 4B columns (30 µg heparin/200 µl of Sepharose 4B) and incubating for 5 min. Bound peptide was assayed as previously described. All of the assays were performed at least three times, and the means ± S.D. were calculated. The concentrations for chemically modified heparins were determined by the carbazole assay (25). The molecules were for heparin and heparan sulfate modified heparin were applied to 12,000 Da with the average being 10,500 Da. For competition assays involving rCSP, the competing peptides were removed by filtration with a Microcon centrifugal filter (Mₐ = 10,000, Amicon) prior to quantitation of rCSP.
Circumsporozoite Protein Binding to Heparan Sulfate

**A**

**Heparin Binding Measured by Isothermal Titration Calorimetry (ITC)—ITC measurements were performed at 25 °C on a MicroCal™ VP-ITC. Peptides (0.1–0.2 mM in 1.4 ml of 20 mM sodium phosphate, 20 mM NaCl, pH 7.2) were titrated with 55 injections of 5 μM of heparin (0.28 mM) from a rotating syringe (430 rpm). Each injection generated a heat change, which was measured as μcal/s (thermal energy H). The area under each injection peak was determined and analyzed by an iterative nonlinear least-squares algorithm using the Origin software. The mean and range was determined.**

**B**

**RESULTS**

**A Heparin/HS Binding Site Identified in the N terminus of the CSP for P. falciparum**—Malarial parasite CSPs, including that of *P. falciparum*, are approximately 400 amino acids in length organized into three domains (Fig. 1A): the N-terminal domain, which contains a conserved pentapeptide region I; a highly repetitive central domain; and a C-terminal domain containing region II, a sequence similar to the conserved adhesive sequence, TSR. Inspection of the CSP sequence for heparin/HS binding activity are indicated (underlined and labeled Pf 1–6).  

**Peptides Pf 3, Pf 5, and Pf 4 demonstrated little or no avidity for heparin and eluted in the void fraction. Pf 1, Pf 2, and Pf 6 also bound to HS-Sepharose 4B (Fig. 2B) with Pf 2 again having the highest affinity. Because Pf 4 (region II-plus) had previously been reported by others (20–22) to bind heparin/HS, a second preparation of Pf 4 was synthesized by a different service to check for the possibility of an error in synthesis, but this second batch of Pf 4 also lacked heparin binding activity (data not shown). The lack of binding activity was not because of cross-linking of peptides by interdisulfide bonds because iodoacetamide acetylation of the cysteine residues for Pf 4 did not improve its binding activity (data not shown).**

We were interested in performing competition assays and to economize on the amount of peptide and GAG competitor, the heparin-columns were reduced in volume (2 ml) and eluted with 20 mM Tris-HCl, pH 7.2 (3 bed volumes), and then developed with a 0–1 M NaCl concentration gradient in the same buffer (12 bed volumes). Eluant was monitored continuously at 214 nm. Peptides were run individually at least twice, and composite, representative elution profiles are shown.

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We were interested in performing competition assays and to economize on the amount of peptide and GAG competitor, the heparin-columns were reduced in volume (2–0.2 ml), and the amount of immobilized heparin reduced quantitatively by serial dilution with Sepharose 4B. From preliminary tests, it became apparent that the relative binding affinities of the Pf peptides could be rapidly compared this way in more detail.

Testing the binding of equimolar amounts of the Pf peptides (16 nmol) in these assays confirmed the previous result, namely that only Pf 1, Pf 2, and Pf 6 bound heparin while Pf 3, Pf 4, and Pf 5 did not (Fig. 3A). However, the amount of Pf 1 and Pf 6 binding increased in a linear fashion as the heparin concentration was increased linearly, consistent with what one would expect for low affinity or nonspecific binding. For Pf 2, binding increased in a hyperbolic manner and was saturable. Furthermore, the binding of Pf 2 to heparin appeared to involve only electrostatic interactions as NaCl washes resulted in close to 100% recovery of the bound peptide. As a control, an apoE

**Fig. 2. Screening the different P. falciparum CSP peptides for heparin/HS binding activity.** Peptides (35–45 μg) were applied to heparin-Sepharose 4B (A) or HS-Sepharose 4B column (2 ml) (B) and eluted with 20 mM Tris-HCl, pH 7.2 (3 bed volumes), and then developed with a 0–1 M NaCl concentration gradient in the same buffer (12 bed volumes). Eluant was monitored continuously at 214 nm. Peptides were run individually at least twice, and composite, representative elution profiles are shown.
peptide (residues 139–169), which contains a high affinity heparin/HS binding site (26, 27), was also tested and found to bind to heparin-Sepharose 4B in a saturable manner with an apparent affinity similar to that of Pf 2. Randomly changing the order of the amino acids in Pf 2 (random) significantly reduced the heparin binding activity, suggesting that the interaction was sequence-specific. The coupling modifications on the heparin-Sepharose (CNBr activation of /H11002OH and /H11002NH2) did not contribute or interfere with the binding because heparin Affigel columns, which use a different coupling chemistry (carbodiimide activation modifying /H11002COO/H11002groups), exhibited similar binding characteristic with these peptides (data not shown).

To confirm that Pf 2 contained the heparan/HS binding site for CSP, the ability of all of the Pf peptides to inhibit CSP binding to heparin-Sepharose was investigated. After purification from E. coli, rCSP was re-folded to generate a protein with 12.7% /H9251-helix, 37.5% antiparallel /H9252-sheet, 1.9% parallel /H9252-sheet, 22.5% /H9252-turn, and 31.7% random coil as determined by circular dichroism. The re-folded rCSP bound heparin-Sepharose saturably with high affinity (Fig. 3B). When rCSP was co-incubated with a 50 molar excess of each of the Pf peptides individually, only Pf 2 could significantly inhibit rCSP binding to heparin-Sepharose (Fig. 3B, inset). Closer examination of the Pf 2 sequence revealed that it contained a concatamer of two heparin binding consensus sequences (Fig. 3C).

**Pf 2 Binds Preferentially to Heparin over the Other Major GAGs**—With heparin immobilized on Sepharose 4B, it was possible to compare the relative binding affinities of native heparin, low molecular weight heparin (heparin 3000), HS, highly sulfated HS, chondroitin sulfate (CS), dermatan sulfate (DS), and hyaluronan by competition binding assays (Fig. 4A). Soluble heparin at a 10-molar excess over immobilized heparin was found to almost completely inhibit Pf 2 binding (95%). A similar molar excess of soluble HS over immobilized heparin showed only a 49% inhibition, whereas the same amount of highly for CSP, the ability of all of the Pf peptides to inhibit CSP binding to heparin-Sepharose was investigated. After purification from E. coli, rCSP was re-folded to generate a protein with 12.7% α-helix, 37.5% antiparallel β-sheet, 19% parallel β-sheet, 22.5% β-turn, and 31.7% random coil as determined by circular dichroism. The re-folded rCSP bound heparin-Sepharose saturably with high affinity (Fig. 3B). When rCSP was co-incubated with a 50 molar excess of each of the Pf peptides individually, only Pf 2 could significantly inhibit rCSP binding to heparin-Sepharose (Fig. 3B, inset). Closer examination of the Pf 2 sequence revealed that it contained a concatamer of two heparin binding consensus sequences (Fig. 3C).
sulfated HS inhibited binding by 70%, suggesting that highly sulfated oligosaccharide stretches on HS were important for binding. Soluble CS and DS showed approximately equal levels of inhibition (32%) well below that of heparin and HS with hyaluronan showing the least activity. Because CS and DS have a higher overall sulfate content over HS, these data indicate that Pf 2/heparin binding was not only sulfate-dependent but that full binding required the correct spacing or orientation of the sulfate groups.

Investigating the Importance of the $-\text{COO}^-$ and $-\text{OSO}_3^-$ Groups on Heparin for Binding—We investigated the structural aspects of heparin that might be critical for binding using chemically modified heparins in which Glc/IdoA-COO$^-$ groups were reduced and GlcNSO$_3^-$, IdoA-2-OSO$_3^-$, GlcN-6-OSO$_3^-$ sulfates were chemically removed (Fig. 4B). Complete desulfation of heparin rendered it a poor competitor indicating that full binding activity was dependent on sulfation at one or more positions on the heparin chain. Furthermore, the spacing of the $-\text{OSO}_3^-$ was probably important for Pf 2/heparin binding because neither a tri-sulfated disaccharide (hexuronic acid (2-OSO$_3^-$)-GlcNSO$_3$ (6-OSO$_3^-$)) derived from heparin nor taurine (NH$_3$-C$_2$H$_4$-OSO$_3$G) (data not shown) at approximately equimolar SO$_3^-$ content to that estimated for the amount of heparin used could compete for binding. The importance of
COO<sup>-</sup>, NSO<sub>3</sub>, 6-OSO<sub>3</sub>, and 2-OSO<sup>-</sup> groups for binding were also assessed individually. A lack of 2-OSO<sub>3</sub> appeared to have the least effect on inhibition (68%), whereas the other three modifications, NSO<sub>3</sub>, 6-OSO<sub>3</sub>, and COO<sup>-</sup> had similar effects on inhibition (56–61%).

Heparin Binding Sites Are Also Found in Other CSPs—The conservation of the CSP heparin/HS binding site (PF 2) in other species of malaria, which infect humans, was also examined. CSP protein sequences for *P. falciparum*, *P. vivax*, *P. malariae*, and *Plasmodium simiovale* were aligned using the ClustalW program (Fig. 5A). The latter species infects monkeys but is believed to be similar to *P. ovale* (28), which infects human and for which the CSP sequence is unknown. The overall sequence identity was low (<10%) with significant similarity localizing only to region I and II. However, sequences rich in basic residues were also found adjacent to region I for the other CSPs. Peptides corresponding to this region on *P. vivax* (Pv 2, residues 71–95) and *P. malariae* (Pm 2, residues 83–102) were synthesized, and their heparin binding activities were evaluated (Fig. 5). Both peptides bound heparin with apparent affinities similar to that observed for a apoE peptide containing a high affinity heparin binding site (residues 139–169) (26, 27). The region I-plus peptide was also the only peptide tested that contained a concatamer of two consensus sequences for heparin binding sites.

**DISCUSSION**

There is good evidence that sporozoite attachment and invasion of hepatocytes requires the malarial CSP and specifically its region II-plus domain (7, 20). It is also clear that at least one of the cell surface ligands for CSP is HS (6, 29, 30), and there have been a number of studies suggesting that region II-plus (region II plus eight downstream residues) serves as the actual HS binding site (21, 22, 31). However, in these studies, the HS binding activity of region II-plus was determined indirectly in cell culture after HS-lyase digestion by showing diminished binding in Chinese hamster ovary cells deficient in proteoglycan synthesis and by heparin and HS prevention of proteolytic digestion of CSP. To get more detailed sequence and binding kinetics information regarding the HS binding site(s), we set out to investigate the heparin binding activity of region II-plus and a series of peptides corresponding to different regions of CSP containing basic residues, a known requirement of heparin binding sites (9, 10, 13).

**Fig. 6.** The CSPs for *P. malariae* and *P. vivax* also contain heparin/HS binding sites. A, heparin-Sepharose 4B elution profiles for CSP peptides, residues 71–96 for *P. vivax* (Pv 2), residues 83–102 for *P. malariae* (Pm 2), and residues 82–100 (Pf 2) for *P. falciparum* were run as in Fig. 2. B, peptide binding to serial dilutions of heparin-Sepharose 4B were run as in Fig. 3B. Similar to that observed for a apoE peptide containing a high affinity heparin binding site (residues 139–169) (26, 27), the region I-plus peptide was also the only peptide tested that could inhibit rCSP binding to heparin-Sepharose, further confirming its identity as a HS binding site. Closer examination of the region I-plus sequence (residues 82–100) revealed that it contained a concatamer of two consensus sequences for heparin binding. The lack of heparin binding activity observed with region II-plus (Pf 4), although surprising, was not unexpected given its low basic residue content. Also, region II-plus contains two of four Cys residues found in CSP, which would normally form disulfide bonds stabilizing its final native structure. Hence, the conformation of region II-plus domain in its reduced form would probably have a very different structure from its native disulfide-bonded version as part of the full-length CSP. Interestingly, replacement of three of the four Cys residues with Ala residues did not affect binding to heparin columns but did abrogate binding to cells (32). Other recent reports provide good evidence in support of our data that region I-plus is the HS binding site on CSP. Rathore et al. (33) have demonstrated that deletion of region II sequences did not prevent the HS-dependent binding of CSP to hepatocytes. Ying et al. (31) found that a synthetic peptide corresponding to region I could inhibit the binding of recombinant CSP to HepG2 cells almost as
effectively as region II-plus. In addition, they found that heparin and HS could prevent the cleavage of recombinant CSP at region I by the endoproteinase Arg-C.

GAG binding activity of CSP is specific for heparin and highly sulfated HS species (34). Binding assays using isothermal titration calorimetry have demonstrated that heparin had the highest affinity for CSPs ($K_d = 0.16$ μM and a stoichiometry of $n = 3.75$/heparin chain) over HS, CS, and DS. They also showed that heparin and porcine liver HS, when compared with porcine intestinal mucosa HS, chondroitin sulfate E, DS, and chondroitin sulfate A, were the only two GAGs that could effectively block CSP binding to HepG2 cells in culture. For region I-plus, we observed a major class of binding site with $K_d = 5.0$ μM and a stoichiometry of $n = 7.8$/heparin chain. Despite its lower affinity, the region I-plus peptide was still able to competitively inhibit binding between rCSP and heparin. The lower binding affinity of the region I-plus over that of the full-length CSP may be attributed to a partial loss of binding site structure. In addition, CSP has been reported to oligomerize, resulting in an improved affinity (21), a phenomenon unlikely to happen with a short peptide. Multivalency resulting from protein aggregation has been observed to enhance the affinities of other protein-heparin/HS (35).

We found that heparin required both COO$^-$/ and OSO$_3^-$ side groups for full binding activity with region I-plus. This is supported by an earlier study in which soluble heparin required both these side groups for efficient inhibition of recombinant CSP binding to HepG2 cells (29). Furthermore, the spacing of charged groups on HS was important for binding to region I-plus because CS and DS were poor competitors even though they both have higher sulfate contents than HS. Tri-sulfated disaccharide and taurine did not compete for binding, also arguing that the oligosaccharide binding to region I-plus requires COO$^-$ and OSO$_3^-$ side groups at the appropriate spacing. Because low molecular weight heparin ($M_r = 3000$) was able to compete for region-I-plus/heparin binding, it appears that the minimum oligosaccharide required for binding is probably a decasaccharide or less. This again is in agreement with the observation that heparin decasaccharide but not tetrasaccharides could effectively inhibit recombinant CSP binding to HepG2 cells (34). The minimal binding sequence for fibroblast growth factor-2 and antithrombin is a pentasaccharide (10).
For fibroblast growth factor-2, there is a requirement of GlcNSO$_3$ residues and at least one IdoA-2-OSO$_3$ residue within this pentasaccharide.

Given that HS is found on all of the cell surfaces and in the extracellular matrix, liver HS must have unique characteristics for it to act as the principle target molecule for CSP. HS on hepatocytes is reported to have the highest degree of sulfation, averaging 1.34 SO$_3$/disaccharide compared with HS from other cell types such as endothelium (0.57 SO$_3$/disaccharide) (36). Also, the distal 60% of the HS chains in liver are highly sulfated at $\sim$2 SO$_3$/disaccharide, which approaches that seen for heparin (2.4 SO$_3$/disaccharide). In addition, the highest concentration of highly sulfated HS is probably found in the space of Disse where extracellular matrix HSPG is primarily secreted by Stellate cells. These cells reportedly synthesize eight times 35SO$_4$ per HS chain (37). In addition to high levels of sulfation, N-SO$_3$ heparin (2.4 SO$_3$/disaccharide) corresponded to this region for both Pm and Pv, suggesting that an identity, domains rich in basic residues were located immediately upstream of region I for all four species. Synthetic peptides corresponding to this region for both Pm and Pv demonstrated saturable high affinity binding to heparin, suggesting that region I-plus was functionally conserved. Finally, the importance of region I-plus for liver attachment may be exemplified by receptor-associated protein, a specific blocker of lipoprotein (Protein Discovery Facility, Queen’s University) for the CD analysis, and Libby Haur and Ruth Tan for their able technical assistance.

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