

CCR2 Chemokines Bind Selectively to Acetylated Heparan Sulfate Octasaccharides*

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Chemokines participate in well documented interactions with glycosaminoglycans (GAGs). Although many chemokine amino acid residues involved in binding have been identified, much less is known about the bound regions of GAG. Heparan sulfate (HS) is the predominant cell surface GAG, and its heterogeneous nature offers proteins a variety of structural motifs with which to interact. In the present study, we describe the interactions of three CC chemokines, MCP-1/CCL2, MCP-2/CCL8, and MCP-3/CCL7, with HS-derived oligosaccharides. To this end, we generated and characterized a complex HS octasaccharide library containing 17 different octasaccharide compositions based on acetyl and sulfate group content. Electrospray ionization mass spectrometry was used to detect chemokine-HS octasaccharide complexes in the bound state, and an affinity purification protocol was used to select and identify chemokine-binding octasaccharides from the complex mixture. The results indicate that HS octasaccharide sulfation is the foremost requirement for chemokine binding. However, within octasaccharides of constant charge density, acetylation is also observed to augment binding, suggesting that there may be as yet undiscovered specificity in the chemokine-HS interaction.

Chemokines are a large class of cytokines that direct leukocyte migration during various physiological processes, including routine immune surveillance, development, and inflammation (1). These proteins exert their various functions by binding to and signaling through G-protein-coupled receptors within the leukocyte plasma membrane. Prior to signal transduction, chemokines are retained near their site of production by a separate interaction with cell surface and extracellular matrix glycosaminoglycans (GAGs).² The interaction with GAGs is thought to maintain the required chemokine concentration gradient in the face of vascular flow and draining lymph nodes (2, 3). Moreover, GAG binding was recently shown to be essen-

tial for *in vivo* chemokine activity (4). Furthermore, many chemokines are capable of forming oligomeric structures. This property also seems to be required for *in vivo* activity, and can be induced by GAG binding (4–7).

GAGs are complex, linear, anionic, polysaccharides composed of repeating disaccharide units (8, 9). GAGs are broadly classified into four families, based on sugar composition, glycosidic bond linkage, and presence of specific structural modifications. These four families are as follows: heparan sulfate (HS)/heparin, chondroitin sulfate/dermatan sulfate, keratan sulfate, and hyaluronan. Heparin and HS (along with hyaluronan) are the principle protein-binding GAGs (9). Due to differences in localization of the heparinoids (heparin and HS), HS is thought to be the more relevant protein binding GAG. Heparin is produced exclusively in mast cells, cleaved to 15-kDa fragments, and sequestered in intracellular granules. Alternatively, HS is expressed by virtually all cell types as the glycan module of HS proteoglycans (9, 10). Depending on the protein core, HS proteoglycans are either secreted or tethered to the plasma membrane, displaying their HS on the outer face (9, 11).

Both heparin and HS are synthesized as polymers of α -D-N-acetylglucosamine (α -D-GlcNAc) 1 \rightarrow 4 linked to β -D-glucuronic acid (β -D-GlcA). During synthesis, residues within the polymer can undergo several modification reactions, including N-deacetylation/N-sulfation, epimerization of D-GlcA to L-iduronic acid (L-IdoA), 2-O-sulfation of the uronic acid, and both 3- and 6-O-sulfation of the substituted glucosamine (12). With the exception of 3-O-sulfation, heparin is heavily modified by each of the previous reactions. On the other hand, HS modifications, although tissue specific, are predominantly present at low levels. Sparse modification allows HS to display greater disaccharide sequence variability than heparin, which may facilitate specific protein interactions (12, 13).

More than one hundred known heparin-binding proteins exist (11). Despite this, and due to the issue of localization, HS can be considered the major physiological interaction partner for the majority of heparin-binding proteins discovered *in vitro*. That heparin is so commonly used in biochemical assays may reflect its abundance, commercial availability, and relative simplicity when compared with HS (14). The same heterogeneity within HS that can facilitate specific protein interactions also convolutes many analyses. Thus the choice of the more tractable heparin substrate is often made, despite the coincident loss of HS sequence information. So while heparin-based studies are generally quite valuable, more detailed investigations require the utilization of HS.

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² The abbreviations used are: GAG, glycosaminoglycan; CCR2, CC chemokine receptor 2; HS, heparan sulfate; MCP-1, -2, and -3, monocyte chemoattractant proteins 1, 2, and 3; CCL2, -8, and -7, CC chemokine family ligands 2, 8, and 7; ESI, electrospray ionization; FTICR MS or FTMS, Fourier transform ion cyclotron resonance mass spectrometry; HA, hyaluronan; α -D-GlcNAc, α -D-N-acetylglucosamine; β -D-GlcA, β -D-glucuronic acid; L-IdoA, L-iduronic acid.

Previously, our laboratory developed a series of complementary techniques designed to screen protein-GAG interactions using a combination of electrospray ionization (ESI) Fourier transform ion cyclotron resonance mass spectrometry (FTICR MS or simply FTMS) and affinity purification with MS detection (15). These techniques rely on both the ability of ESI to transfer intact non-covalent complexes from solution to the gas phase and on the high resolving power of FTMS (16–18). The aforementioned methods were developed using a commercially available heparin octasaccharide library on the basis that octasaccharides were previously shown to be an ideal size for binding chemokine oligomers (6). In the present study we extend these and new methods of analysis toward the examination of chemokine-HS interactions.

Generation and characterization of a library of sulfate-bearing HS octasaccharides allowed us to observe the interactions of three CC chemokine receptor 2 (CCR2) ligands, MCP-1/CCL2, MCP-2/CCL8, and MCP-3/CCL7, with specific HS octasaccharides. FTICR MS was effectively used to detect the extent of sulfation and acetylation on each chemokine-bound saccharide. We observed that, although each chemokine is able to bind HS octasaccharides of moderate sulfation, they all preferentially bind to saccharides of increasing sulfation. Interestingly, among octasaccharides of constant sulfation (and charge density), each chemokine favored interactions with the more acetylated species. These observations offer new insights as to possible mechanisms of chemokine-HS binding and afford direction for future study.

EXPERIMENTAL PROCEDURES

Materials—Porcine intestinal HS was purchased from Celsus Laboratories (Cincinnati, OH), and Arixtra™ (fondaparinux sodium) was obtained from GlaxoSmithKline (Research Triangle Park, NC). Bio-Gel® P-10 gel was purchased from Bio-Rad (Hercules, CA) and prepared via the manufacturer's instructions. Heparinase III (heparitinase, EC 4.2.2.8) was a gracious gift from Prof. Ram Sasisekharan at the Massachusetts Institute of Technology. All other chemicals were purchased from Fisher Scientific. CCR2 ligands, MCP-1, MCP-2, and MCP-3, were expressed and purified as previously described (15).

HS Depolymerization—HS was prepared at a concentration of 50 mg/ml in 50 mM sodium phosphate (pH 7.5) and extensively depolymerized at 37 °C using heparinase III. Heparinase III cleaves preferentially at regions of low sulfation generating a C4–C5 double bond on the uronic acid residue of newly generated nonreducing ends (19, 20). Sulfated oligosaccharides are enriched by prolonged cleavage, which completely cleaves unsulfated regions to their disaccharide building blocks. After 20 h, the HS/heparinase III reaction was quenched by mixing 50:50 with methanol and boiling for 10 min. The product mixture was then frozen at –80 °C and freeze-dried.

HS Oligosaccharide Size-exclusion Chromatography—Depolymerized HS was redissolved in water and mixed with ammonium bicarbonate to 1 M NH_4HCO_3 final concentration. The solution was then injected onto a 170 × 1.5 cm Econo-Column® (Bio-Rad) loaded with P-10 size-exclusion gel. The chromatographic separation was performed under gravity flow conditions at room temperature in 20 mM NH_4HCO_3 . The

flow rate varied between 1 and 3 ml/h, and fractions were collected at 30-min intervals. Separation was monitored by absorbance online at 254 nm and verified offline at 232 nm in 96-well plates. All fractions were lyophilized using an Eppendorf Vacufuge™ (Eppendorf, Westbury, NY).

Preparation of HS Oligosaccharides for Analysis—Lyophilized fractions were dissolved in 100 μl of Millipore H_2O . These fractions were desalted by dialysis, at room temperature, against Millipore H_2O using a 1-kDa molecular weight cut-off Dispo-Biodialyzer™ (The Nest Group, Inc., Southborough, MA). The absorbance of each desalted fraction was recorded in 0.03 M HCl at 232 nm and converted to concentration using the extinction coefficient of $5,500 \text{ M}^{-1} \text{ cm}^{-1}$.

ESI FTICR of HS Oligosaccharides—Mass spectra of all oligosaccharides were acquired on an APEX II 7-tesla FTICR mass spectrometer (Bruker, Billerica, MA) equipped with an Apollo (Bruker) electrospray ion source using negative ion mode detection. A 60 μM solution of each desalted fraction was prepared in 50:50 H_2O :MeOH with 5 mM NH_4OH . A low concentration of NH_4OH (or NH_4OAc) is used to prevent desulfation during the ESI process (21). Samples were infused into the mass spectrometer at a flow rate of 1 $\mu\text{l}/\text{min}$ using a syringe pump (Harvard Apparatus, Holliston, MA). Nebulizing and drying gas pressures were maintained at 35 and 25 p.s.i., respectively. A capillary exit voltage of –25.8 V was used during desolvation. Approximately 128 broadband time domain transients containing 1,024,000 data points were averaged and subjected to zerofill, Gaussian multiplication, and fast Fourier transform. The parameters of the ESI source, ion optics, and ICR cell were optimized to maximize signal intensity while minimizing cation adduction and desulfation of oligosaccharides. The mass spectrum of the HS octasaccharide library was calibrated externally using the pharmaceutical GAG analog Arixtra™. All data were acquired and processed using Xmass version 6.0.0 (Bruker). Oligosaccharide composition was determined from the monoisotopic mass using HOST software (22).

CCR2 Ligand-HS Oligosaccharide Noncovalent Complex Assembly and Analysis—Filtration trapping assay and ESI-FTICR of non-covalent protein-oligosaccharide complexes were performed as previously described (15). Briefly, 200 μM HS octasaccharide library was incubated with 40 μM chemokine in 100 μl of 100 mM NH_4OAc . The buffer was successively exchanged with three 1-ml volumes of 200 mM NH_4OAc . A 10-kDa centrifugal molecular weight cut-off filter was employed to retain non-covalent chemokine-octasaccharide complexes while allowing free saccharides to be filtered through (Millipore). At the conclusion of filtration steps, ~100 μl of solution containing free chemokine and chemokine-octasaccharides complexes remained. This solution was infused into the ESI-FTICR mass spectrometer using positive ion detection. Acquisition parameters were the same as those previously described (15). In addition to the filtration trapping experiments, 20 μM MCP-2 was mixed with a 55 μM concentration of HS octasaccharide library in 200 mM NH_4OAc and infused directly into the ESI-FTICR mass spectrometer.

Our previous work (MS and isothermal titration calorimetry) indicates that chemokines bind oligosaccharides with low micromolar affinity (23), however millimolar binding affinities

CCR2 Ligands Interact Selectively with HS Octasaccharides

are theoretically accessible by MS-based methods (24). We also stress the requirement that non-covalent protein-ligand complexes (here chemokine-GAG) must be electrosprayed from a buffered aqueous solution. Traditional organic solvent systems employed in MS denature proteins of interest destroying their activity and binding properties. Likewise other denaturing conditions, such as low pH, have been observed to destroy chemokine-GAG binding, and the corresponding non-covalent complexes are no longer observed.

Hydrophobic Trapping/Chemokine-based Affinity Enrichment of HS Octasaccharides—Hydrophobic trapping/elution was performed as previously described with minor modifications (15). A mixture of 80 μM chemokine and 100 μM HS octasaccharide-library was prepared in 100 μl of 100 mM NH_4OAc . The solution was then loaded onto a pre-conditioned Oasis solid-phase extraction cartridge (Waters, Milford, MA). Oligosaccharides not specifically bound to protein were eluted by flushing the cartridge with three successive 1-ml volumes of 200 mM NH_4OAc . Finally the chemokine-binding HS-oligosaccharides were eluted from the cartridge-bound chemokine via a high stringency wash with 1.25 ml of 760 mM NH_4OAc and collected separately. The product solution was lyophilized, resuspended in 100 μl of water, and subject to dialysis against Millipore H_2O to remove salt impurities. After dialysis, sample volume and absorbance were recorded prior to concentrating each sample to $\sim 5 \mu\text{l}$. The samples were then diluted in 35 μl of 50:50 H_2O :MeOH with 5 mM NH_4OH and infused into the electrospray source for FTMS detection. At the time of infusion, the sample concentrations were ~ 10 – $15 \mu\text{M}$. Instrumental parameters were identical to those used for analysis of HS size-exclusion fractions.

RESULTS

Isolation of Sulfated HS Octasaccharides—HS saccharides were effectively separated by P-10 size-exclusion chromatography. The largest components included in the gel began to elute at 16 h post injection, while the disaccharides eluted last between 75 and 100 h. Within this range, three peaks contained relatively pure mixtures of hexa-, octa-, and decasaccharides (Fig. 1). Three of the fractions, 62, 63, and 64, within the DP8 peak profile, contained the same octasaccharides, in similar relative abundances, and were pooled to create a working mixture for all subsequent experiments. This mixture is referred to as the HS octasaccharide library.

ESI-FTICR Analysis of HS Octasaccharide Library—Seventeen different octasaccharide compositions were identified in the HS octasaccharide library and differed in acetyl and sulfate group content (Fig. 2 and Table 1). The library varied in sulfate content from 3 to 10 per octasaccharide, while acetyl group content ranged from 0 to 2 (Table 1). Several isomers likely exist for each composition identified, and the structure for a generic lyase-generated HS octasaccharide is illustrated in Fig. 3. Important is the fact that these compositions are indicative of successful enrichment for sulfated regions of the HS polymer, given that porcine intestinal HS is composed of $\sim 75\%$ unsulfated disaccharides (25, 26). Isolation of these regions is essential in identifying physiologically relevant ligand binding sites, as the majority of HS-binding proteins are known to bind either

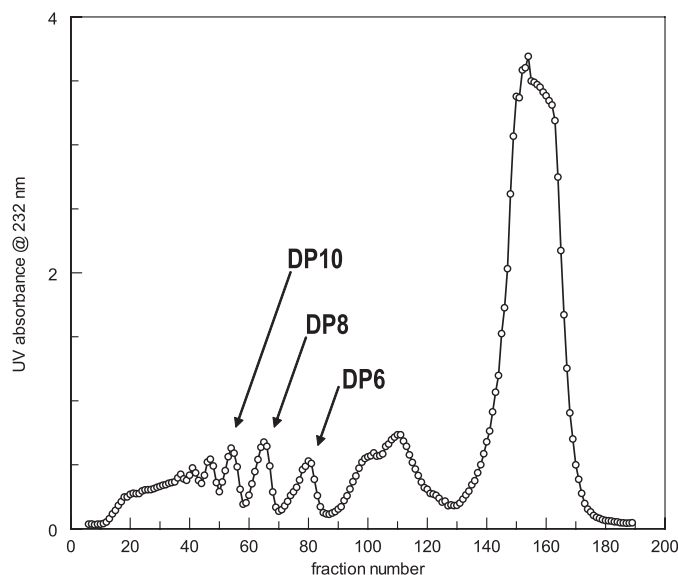


FIGURE 1. Separation of heparinase III-generated HS oligosaccharides. The chromatogram was obtained offline in 96-well UV-transparent plates following P10 size-exclusion chromatography of cleavage products. Peaks corresponding to deca-, octa-, and hexasaccharides are denoted.

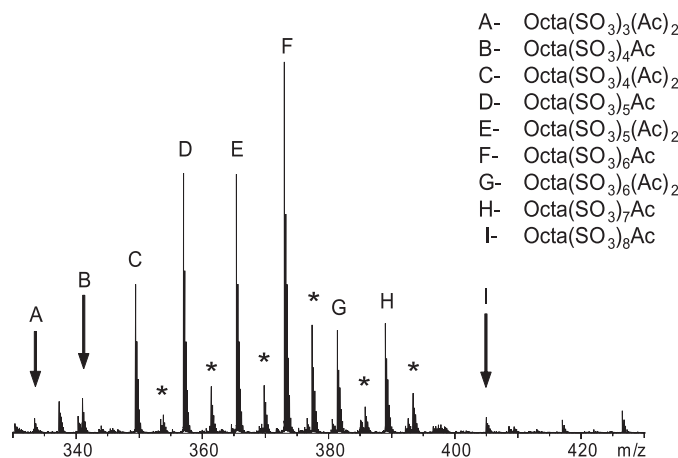


FIGURE 2. Negative mode ESI-FTICR mass spectrum of a mixture of fractions 62, 63, and 64 (HS octasaccharide library). The most abundant octasaccharides (5^- charge state) are denoted. Asterisks denote Na^+ adducts. Lower intensity ions requiring magnification are not labeled but are listed in Table 1.

the sulfate-rich S-domains or their flanking mixed sequences (11, 27, 28).

Chemokine-HS Library Filtration Trapping Assay—The chemokines, MCP-1, MCP-2, and MCP-3, were individually incubated in buffered solutions containing the HS octasaccharide library. Each resulting mixture was placed in the sample compartment of a 10-kDa molecular weight cut-off centrifugal filter, and the buffer was exchanged three times over ~ 1.5 h. During this time, non-bound octasaccharides were washed through the filter and discarded. Unfortunately, MCP-2 consistently precipitated from solution during this procedure. However, both MCP-1 and MCP-3 remained in solution for MS analysis. The final solutions ($\sim 100 \mu\text{l}$) contained both free chemokine and non-covalent complexes between each chemokine and members of the HS octasaccharide library as determined by ESI-FTICR MS.

TABLE 1

Saccharide compositions identified in the HS octasaccharide library

Saccharide	$m/z_{\text{observed}}^{-5}$ charge state	$M_{\text{theoretical}}$	M_{Observed}	Δppm
Octa(SO ₃) ₃ (Ac) ₂	333.45214	1672.29516	1672.29709	1.15
Octa(SO ₃) ₄ Ac	341.04135	1710.24142	1710.24314	1.01
Octa(SO ₃) ₅	348.63054	1748.18768	1748.18908	0.80
Octa(SO ₃) ₄ (Ac) ₂	349.44361	1752.25198	1752.25445	1.41
Octa(SO ₃) ₅ Ac	357.03279	1790.19824	1790.20035	1.18
Octa(SO ₃) ₆	364.62215	1828.1445	1828.14712	1.43
Octa(SO ₃) ₅ (Ac) ₂	365.43492	1832.2088	1832.21099	1.20
Octa(SO ₃) ₆ Ac	373.02412	1870.15506	1870.15699	1.03
Octa(SO ₃) ₇	380.61355	1908.10132	1908.10414	1.48
Octa(SO ₃) ₆ (Ac) ₂	381.42645	1912.16562	1912.16865	1.58
Octa(SO ₃) ₇ Ac	389.01574	1950.11188	1950.11509	1.65
Octa(SO ₃) ₈	396.60502	1988.05814	1988.06151	1.70
Octa(SO ₃) ₇ (Ac) ₂	397.41800	1992.12244	1992.12637	1.97
Octa(SO ₃) ₈ Ac	405.00712	2030.0687	2030.07199	1.62
Octa(SO ₃) ₉	412.59628	2068.01496	2068.01776	1.35
Octa(SO ₃) ₉ Ac	420.99848	2110.02552	2110.02877	1.54
Octa(SO ₃) ₁₀	428.58797	2147.97178	2147.97625	2.08

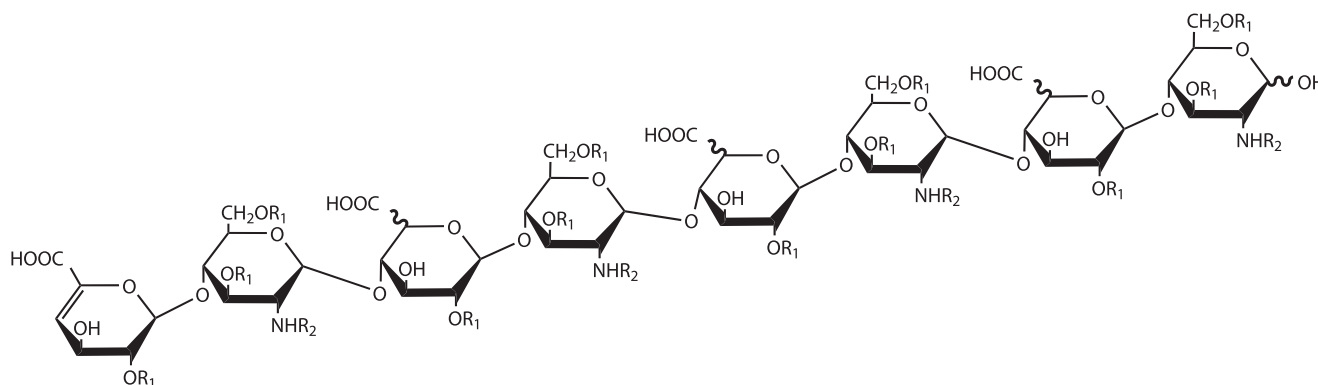
FIGURE 3. Generic structure for a lyase-generated HS octasaccharide. $R_1 = \text{H}$ or SO_3H ; $R_2 = \text{Ac}$, SO_3H , or H .

TABLE 2

Summary of chemokine-HS octasaccharide non-covalent complexes observed in the filtration trapping assay (MCP-1 and -3) and by direct infusion (MCP-2)

Chemokine mass (monomer)	Oligomerization state		Composition of bound HS-octasaccharides	
	Alone	HS-bound	Sulfate content	Acetyl group content
<i>Da</i>				
MCP-1/CCL2 (8662.5)	Monomer/dimer equilibrium	Dimer	5 6 7	1 and 2 1 and 2 1 and 2
MCP-2/CCL8 ^a (8909.7)	Monomer/dimer equilibrium	Dimer	6 7	1 1
MCP-3/CCL7 (8951.6)	Monomer	Monomer	6 7 8 9	1 and 2 1 and 2 1 0

^a The concentrations employed to observe non-covalent complexes between MCP-2 and the HS-octasaccharides by direct infusion were significantly lower than those used in the filtration trapping assay for MCP-1 and -3, and presentation of these results together is not meant for direct comparison.

MCP-3 is a monomer in solution and bound octasaccharides as a monomer. MCP-1, normally in monomer/dimer equilibrium in solution, bound HS octasaccharides only as a dimer (Table 2), consistent with previous binding studies using heparin (15). Both chemokines, MCP-1 and MCP-3, bound to HS octasaccharides containing moderate to relatively high sulfation. MCP-3 was observed in complex with octasaccharides of six compositions; (*i.e.* 6–9 sulfates and 0–2 acetyl groups, Fig. 4, Table 2). MCP-1 was also observed in complex with octasaccharides of six compositions. The sulfate content for MCP-1-bound saccharides observed by filtration assay ranged from 5 to 7, and acetyl groups from 1 to 2 (Table 2). In both cases, the most intense ions corresponding to non-covalent assemblies could be observed alone as well as complexed with a Na⁺ ion.

Although MCP-2 precipitated under the conditions employed in the filtration trapping experiment, this chemokine could be mixed with the HS octasaccharide library, albeit at lower concentrations, and directly infused into the electrospray source for MS detection. In this instance, MCP-2 was observed as a dimer bound to octasaccharides with 1 acetyl group and both 6 and 7 sulfate groups (Table 2). It is worth noting that these saccharides were also present in the most abundant complexes with MCP-1 and MCP-3 after filtration trapping.

Hydrophobic Trapping and Elution of Chemokine-bound Saccharides—Octasaccharides of various compositions were retained in this assay based on their affinity for each chemokine under investigation. By comparing the spectrum of the library alone (acquired at 15 μM) to each spectrum acquired after che-

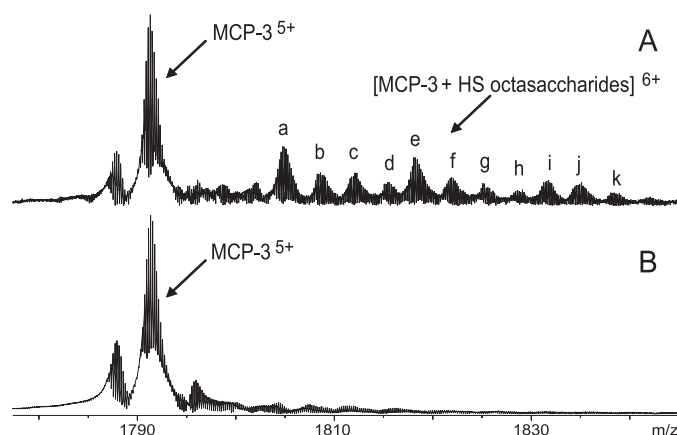


FIGURE 4. ESI-FTICR mass spectrum of the chemokine MCP-3 with and without HS octasaccharides; sample workup provided in methods. A, mass spectrum displaying non-covalent complexes formed between the chemokine MCP-3 and several members of the HS octasaccharide library. Non-covalent complexes are denoted by lowercase letters; as follows: a, MCP-3 + Octa(SO₃)₆Ac; b, MCP-3 + Octa(SO₃)₆Ac + Na⁺; c, MCP-3 + Octa(SO₃)₆(Ac)₂; d, MCP-3 + Octa(SO₃)₆(Ac)₂ + Na⁺; e, MCP-3 + Octa(SO₃)₇Ac; f, MCP-3 + Octa(SO₃)₇Ac + Na⁺; g, MCP-3 + Octa(SO₃)₇(Ac)₂; h, MCP-3 + Octa(SO₃)₇(Ac)₂ + Na⁺; i, MCP-3 + Octa(SO₃)₈Ac; j, MCP-3 + Octa(SO₃)₈Ac + Na⁺; and k, MCP-3 + Octa(SO₃)₉. B, mass spectrum of the chemokine MCP-3 alone, prepared and run under the same conditions as A.

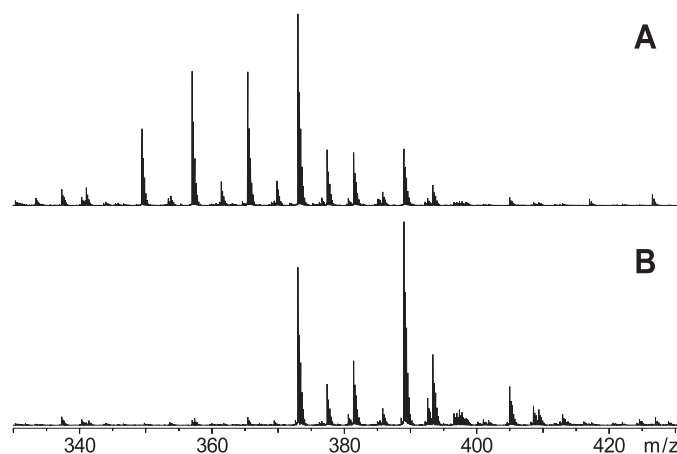


FIGURE 5. Negative mode ESI-FTICR mass spectra of native and enriched octasaccharides. A, mass spectrum of the HS octasaccharide library acquired at 15 μm. B, mass spectrum of HS octasaccharides after hydrophobic trapping based chemokine (MCP-3) affinity enrichment. Assignments provided in Table 1.

chemokine-based affinity enrichment, a clear shift to octasaccharides of higher mass was observed (Fig. 5). The HS octasaccharides are present at different concentrations in the library due to factors, including differential occurrence of sequences in intact HS as well as the preference of the heparinase III cleavage reaction. The oligosaccharide mixtures obtained from hydrophobic trapping assay are also relatively complex. To comparatively evaluate these two mixtures, the mass spectral peak intensities from the hydrophobic trapping experiments (*i.e.* post enrichment) were compared with the same components present in the HS octasaccharide library (prior to enrichment). The quotient, $\text{Intensity}_{\text{peak A-HS-library}} / \text{Intensity}_{\text{peak A-hydrophobic trapping}}$, is defined as -fold intensity change. Plotting -fold intensity change *versus* octasaccharide mass clearly shows that selective enrichment correlates with increasing mass (Fig. 6).

As stated, the number of *N*-acetyl groups observed (42.01 Da each) ranged from 0 to 2, contributing between 0 and 84.02 Da

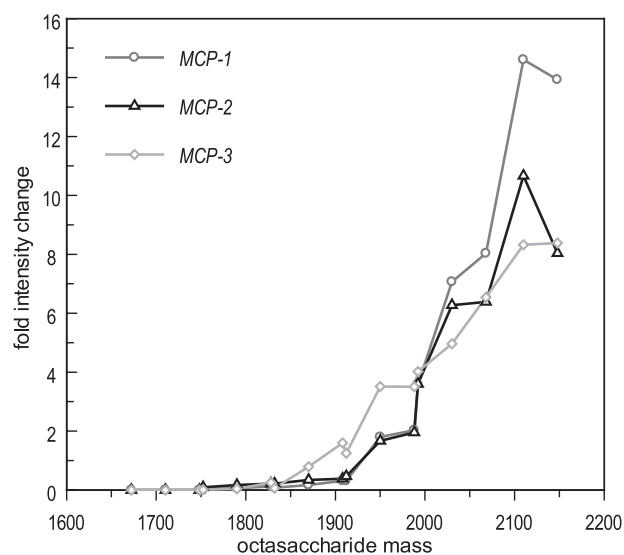


FIGURE 6. Binding preference correlates with octasaccharide mass. Absolute intensity from each peak in the hydrophobic trapping/affinity enrichment mass spectra was divided by the intensity of the corresponding peak in the HS library to generate -fold intensity change. This value is then plotted *versus* the octasaccharide mass. Chemokines used for enrichment are indicated. Peak intensities include Na⁺ or H₂O adducts, if present.

to the mass of any given octasaccharide. The number of sulfate modifications (79.97 Da each) varied from 3 to 10, contributing between 239.91 and 799.7 Da to octasaccharide mass. Thus increasing octasaccharide mass is primarily due to sulfation and selective octasaccharide enrichment correlates with that sulfation. However, detailed analysis reveals that, among octasaccharides of equal charge density (*i.e.* sulfation), acetylation is observed to augment binding. In conjunction with more highly sulfated species, octasaccharides with 7 sulfate groups were detected after affinity enrichment for all three chemokines. Within these saccharides, three variations in the *N*-acetyl group content were present, Octa(SO₃)₇, Octa(SO₃)₇Ac, and Octa(SO₃)₇(Ac)₂. Despite identical charge density, each chemokine bound these saccharides differentially, with the more highly acetylated octasaccharides experiencing the greatest enrichment (Fig. 7). This relationship was also observed for octasaccharides with 5, 6, 8, and 9 sulfate groups and lends credence toward the concept that forces beyond electrostatics are involved in chemokine-GAG interactions.

DISCUSSION

The work presented herein illustrates interactions of three CCR2 chemokines, MCP-1/CCL2, MCP-2/CCL8, and MCP-3/CCL7, with HS, a physiological ligand. The majority of studies addressing protein-HS interactions are undertaken using heparin or heparin-derived oligosaccharide substrates. Reasons for the use of heparin may include its abundance, commercial availability, and relative simplicity when compared with HS (14). Although studies of this nature may be informative, and useful for method development, the fact remains that the majority of heparin-binding proteins may never come in contact with heparin *in vivo* (11). Heparin is known to possess less structural and sequence diversity than HS (13) and, likewise, is devoid of the domain architecture observed in HS (14, 28). Thus, HS presents greater possibilities for specific binding

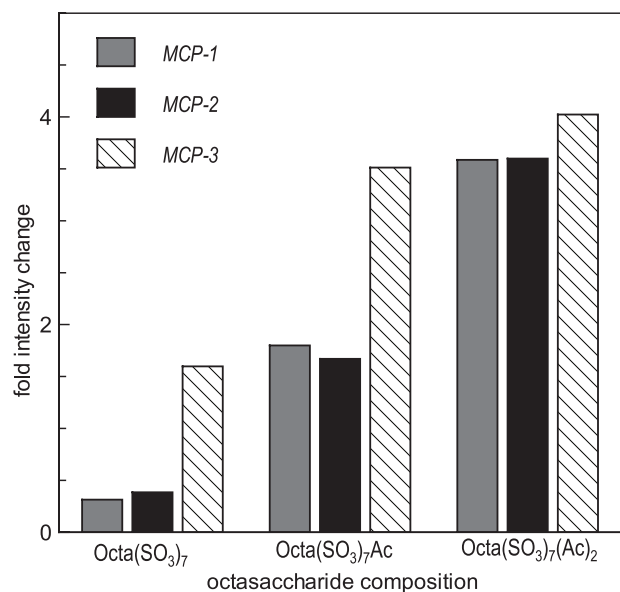


FIGURE 7. Effect of acetyl groups on enrichment of moderately sulfated octasaccharides. The -fold intensity change after affinity enrichment is plotted for octasaccharides with 7 sulfate groups and 0, 1, or 2 acetyl groups. Chemokines used for enrichment are indicated.

interactions with a multitude of proteins and provides experimental results that are more readily extrapolated to a physiological context.

In this study, we have identified the detailed compositions of chemokine-bound HS octasaccharides and obtained information on their sulfate and acetyl group content. GAG-binding proteins generally bind to a relatively small stretch of the intact polymer (12), yet many studies involving chemokine-GAG interactions have focused on the use of bulk GAG polymers or nondescript oligosaccharide substrates (30–32). The results presented herein provide a more detailed description of chemokine binding as it may occur with intact HS.

Reasons for generation of a HS octasaccharide library based on extensive depolymerization of intact HS using heparinase III (heparitinase, EC 4.2.2.8) are multifold. Heparinase III catalyzes the formation of a double bond at each newly generated non-reducing terminus during cleavage (20). Double bond formation facilitates appraisal of the cleavage reaction, quantification, and monitoring of product chromatography without further derivatization. Heparinase III also preferentially cleaves saccharides at regions of low sulfation (19), leaving the sulfated regions involved in protein interactions largely intact. Finally cleavage with heparinase III serves to remove an otherwise high concentration of interfering non-sulfated HS oligosaccharides, by sequentially cleaving them to disaccharides. The reaction products can then be effectively separated by size-exclusion chromatography to generate size-defined HS oligosaccharide libraries.

Although 17 different octasaccharide compositions were identified in our HS octasaccharide library, each composition likely represents an isomeric population with varying functional group distribution between individual saccharides (Fig. 3). Nevertheless, the exact mass measurements made possible by ESI-FTICR and have facilitated characterization of these HS octasaccharides based on sulfate and acetyl content, which

might otherwise have been impossible using low resolution mass spectrometry or other techniques. Within our library, a diverse representation of sulfated and acetylated octasaccharides is present. This diversity allows us to catalog the differences in chemokine binding based on these HS structural elements.

The chemokines, MCP-1, MCP-2, and MCP-3, were observed in complex with multiple HS octasaccharide substrates. It is evident that each of these chemokines is capable of binding modestly sulfated saccharides. To our knowledge, this is the first example of these chemokines binding to such substrates, as previous studies were performed with highly sulfated heparin oligosaccharides or nondescript full-length GAGs (6, 15, 30). In accord with our previous study, each of the chemokines took on specific oligomeric states in these interactions with GAG (Fig. 3 and Table 2) (15). Oddly, MCP-2 precipitated at the higher concentrations employed in the filtration assay. Nevertheless, the HS-binding characteristics of this protein could be observed at lower concentrations. MCP-2 shares 80% homology and 62% identity with MCP-1, including several key GAG-binding residues Arg-18, Lys-19, Arg-24, and Lys-49 (6, 29), and given that they both form dimers in complex with GAG, it is not surprising that each displays a similar overall HS-binding profile.

A cursory view of the data presented in Fig. 6, and previous studies, might lead to the presumption that nonspecific electrostatic interactions drive chemokine HS interactions. However, the sensitivity of our assays and the innate selectivity of the CCR2 chemokines allowed us to demonstrate that, although sulfation may be the principal requirement, acetylation augments chemokine binding. That chemokines might interact specifically with GAG structural elements is not a new concept (1, 30), but to our knowledge this is the first time *N*-acetylation has been linked to CCR2 chemokine-GAG binding. Defining the mechanism by which *N*-acetylation facilitates this interaction deserves further investigation.

Within an HS octasaccharide there are 16 positions (12 hydroxyl groups and 4 amine groups) that may be covalently modified by sulfation. Each of the 4 amine groups can either remain *N*-acetylated, as they are during chain synthesis, or can be *N*-deacetylated/*N*-sulfated (rare free amines also exist). In an octasaccharide with a specified number of randomly distributed sulfate groups, the presence an *N*-acetyl group increases the likelihood of that *O*-sulfation is present. For instance, when one acetyl group is present, the sulfate groups may be distributed among the remaining 3 *N*-positions and all 12 potential sites of *O*-sulfation. Thus the probability of finding *O*-sulfation increases slightly when compared with an octasaccharide bearing no acetylation (12/15 versus 12/16).

For the CCR2 chemokines, both octasaccharide sulfation and acetylation correlate with binding. Because each *N*-acetyl group present increases the probability that the sulfates are on the *O*-positions, this may indicate that *O*-sulfation is more relevant than *N*-sulfation in CCR2 ligand binding. If so, this conclusion would be in agreement with the results obtained using chemically modified full-length heparin as a substrate (30). However, the intriguing possibility that a favorable interaction occurs between a chemokine and one or more *N*-acetyl group(s) of the saccharide cannot be ruled out. Whether acety-

lation plays an active or a passive role in chemokine binding, the observation that it affects binding would have been occluded by the use of heparin substrates, in which acetylation is scarce.

We have shown that, when given a choice of oligosaccharide substrates, three CC chemokines (MCP-1, MCP-2, and MCP-3) preferentially bind octasaccharides of increasing sulfation. Although highly sulfated sequences of HS seem to be preferred substrates, we have also shown that these chemokines bind acetylated species with some specificity. These data raise the issue of the importance of acetylation to chemokine binding. Although sulfation may be considered the foremost requirement, Fig. 7 provides unambiguous evidence that the HS acetyl groups are also somehow involved in binding/selection. Whether distinct HS modification patterns are involved, remains to be determined. It should be noted that the preference of these chemokines for highly sulfated saccharides could mask their ability to bind to saccharides of lesser charge bearing only a set of specifically positioned sulfate and acetyl groups. Such minimally modified saccharides would likely be of therapeutic interest, and future studies will be aimed at detecting whether they exist.

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