The Interaction of Heparin Tetrasaccharides with Chemokine CCL5 Is Modulated by Sulfation Pattern and pH

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Background: Chemokine-glycosaminoglycan (GAG) binding regulates leukocyte migration. Results: Heparin tetrasaccharides are examined for their ability to inhibit CCL5-CCR1 binding, and key interactions between the heparin fragments and CCL5 are identified. Conclusion: Binding modes and inhibitory capabilities depend on the extent and pattern of sulfation of the heparin fragments. Significance: Inhibition of CCL5-CCR1 binding requires heparin to interact with specific residues on the CCL5 surface.

Interactions between chemokines such as CCL5 and glycosaminoglycans (GAGs) are essential for creating haptotactic gradients to guide the migration of leukocytes into inflammatory sites, and the GAGs that interact with CCL5 with the highest affinity are heparan sulfates/heparin. The interaction between CCL5 and its receptor on monocytes, CCR1, is mediated through residues Arg-17 and -47 in CCL5, which overlap with the GAG-binding 44RKNR47 “BB” motifs. Here we report that heparin and tetrasaccharide fragments of heparin are able to inhibit CCL5-CCR1 binding, with IC50 values showing strong dependence on the pattern and extent of sulfation. Modeling of the CCL5-tetrasaccharide complexes suggested that interactions between specific sulfate and carboxylate groups of heparin and residues Arg-17 and -47 of the protein are essential for strong inhibition; tetrasaccharides lacking the specific sulfation pattern were found to preferentially bind CCL5 in positions less favorable for inhibition of the interaction with CCR1. Simulations of a 12-mer heparin fragment bound to CCL5 indicated that the oligosaccharide preferred to interact simultaneously with both 44RKNR47 motifs in the CCL5 homodimer and engaged residues Arg-47 and -17 from both chains. Direct engagement of these residues by the longer heparin oligosaccharide provides a rationalization for its effectiveness as an inhibitor of CCL5-CCR1 interaction. In this mode, histidine (His-23) may contribute to CCL5-GAG interactions when the pH drops just below neutral, as occurs during inflammation. Additionally, an examination of the contribution of pH to modulating CCL5-heparin interactions suggested a need for careful interpretation of experimental results when experiments are performed under non-physiological conditions.

Chemokines are small proteins (8–10 kDa) that guide the migration of leukocytes to the site of infection during an inflammatory immune response (1). Some chemokines are also involved in the migration of cells into tissues during tissue repair and development. Pro-inflammatory chemokines are immobilized on cell surfaces and extracellular matrices by interactions with glycosaminoglycan (GAG) chains of proteoglycans (2, 3). It is believed that this interaction with GAGs, primarily heparan sulfate (HS), allows the formation of haptotactic chemokine gradients that direct leukocytes into sites of inflammation. Recently, endogenous gradients of the chemokine CCL21 in mouse skin were visualized, and heparitinase treatment disrupted both the CCL21 gradient and dendritic cell migration (4). This and a number of other studies conducted over the last decade have provided convincing evidence that the interaction of chemokines with GAGs is a critical component of directed leukocyte migration (5). As a result, disruption of chemokine-GAG binding events have been targeted for the development of novel anti-inflammatory agents that are GAG analogs (6, 7).

Chemokines are classified according to the spacing between the first two cysteine residues in their primary sequence into four categories: C, CC, CXC, and CXC, where X indicates any other residue. These cysteine residues form characteristic intramolecular disulfide bonds that stabilize the tertiary structure, which typically consists of a disordered N terminus (N-loop), followed by a 310 helix, a three-stranded β-sheet, and a

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CCL5-heparin Interactions Depend on Sulfation Pattern and pH

helix at the C terminus (8). CCL5 (also known as RANTES, for regulated on activation normal T cell expressed and secreted) was chosen for this study because of its well characterized pro-inflammatory activity and because an interaction with GAGs has been reported to be essential for this pro-inflammatory activity (9). CCL5 binds different types of GAGs to varying degrees, but heparin and HS bind with the highest affinity (10). Upon secretion from endothelial cells and activated leukocytes, CCL5 localizes on GAGs at the site of inflammation and triggers the migration of T-cells, monocytes, basophils, eosinophils, natural killer cells, and dendritic cells (11) via engagement with one or more of its receptors, CCR1, CCR3, and CCR5, which are expressed on leukocyte cell surfaces (12).

The interaction between CCL5 and GAGs has been studied primarily using heparin as a model in vitro for the HS structures, which bind CCL5 in vivo. CCL5-heparin interactions have been probed by site-directed mutagenesis studies and shown to be mediated primarily through a highly basic RKNR motif on the surface of CCL5 located in a loop termed the “30s loop” (13) (Fig. 1). This is consistent with the fact that protein-GAG interactions generally involve interactions between the anionic sulfate or carboxylate moieties in GAGs and clusters of basic amino acids on the protein. More recent studies indicate that GAG-protein interactions are not only dependent on these linear motifs but also involve residues that can engage in hydrogen bonding and hydrophobic interactions on the three-dimensional surface of the protein (14, 15). A further complexity in characterizing GAG-CCL5 interactions is the tendency of these complexes to aggregate at physiological pH or at higher GAG concentrations (16). CCL5 naturally forms high molecular weight oligomers, in which the interaction between Glu-66 and Arg-47 of adjacent monomers plays a pivotal role (17). This role is further substantiated by the observation that the AANA motif of the second monomer in the dimer was also detected (17). This structure is shown in Fig. 1. In contrast, point mutagenesis studies, which identified the importance of the RKNR motif, were carried out under physiological conditions, at approximately neutral pH, and did not detect evidence of major contributions from outside of this motif (13). Given the highly ionic nature of heparin, it might be expected that pH could alter the preferred interaction sites between CCL5 and heparin. Accordingly, in the present work, we explored the impact of pH on the binding mode.

Heparan sulfate and heparin are highly heterogeneous linear polysaccharides composed of repeating 1,4-linked disaccharide units of GlcA or α-L-iduronate (IdoA) and 3-glucosamine (GlcN) (23, 24). The uronic acids may be sulfated at the 2-O-position (IdoA2S or GlcA2S), whereas the glucosamine may rarely occur as a free amine or more often as an N-sulfated (GlcNS) or an N-acetylated (GlcNAc) moiety. The GlcNS may also be O-sulfated at the 3- or 6-position, giving rise to GlcNS6S, GlcNS3S, or GlcNS3S6S. In contrast, the GlcNAc residues may be unsulfated or O-sulfated only at C6 (GlcNAc6S) (25). Heparin is produced by mast cells, and commercial heparin is obtained from tissues with abundant mast cells, such as porcine intestinal mucosa. Structurally, heparin and HS differ in sulfation patterns and the proportion of the various disaccharide units, with there being more IdoA and GlcNS in heparin as well as more overall sulfation. Common features in heparin are repeating stretches of the trisulfated disaccharide structure, IdoA2S-GlcNS6S in contrast, HS has a more organized structure with regions of low or no sulfation separating highly sulfated regions that resemble mast cell heparin. Heparan sulfate is produced by almost all cell types, and its functions in vivo are primarily to bind and present a range of different growth factors and chemokines to their cell surface receptors (14, 25).

Both CCL5 and CCR1, a major receptor on circulating monocytes, have been proposed as therapeutic targets for cancer-related inflammation (26, 27) as well as for infectious diseases (28). Residues Arg-47 and Arg-17 of CCL5 have been shown to play a crucial role in the CCL5-CCR1 binding event (29, 30), and the N terminus of CCL5 is known to be crucial for CCR1 signaling (30, 31). Although it has previously been shown that pools of heparin-derived oligosaccharides can inhibit the binding of CCL5 to its receptor, CCR1 (29), no specific sulfation pattern or motif is known to be optimal for binding to CCL5. GAG heterogeneity makes such evaluations particularly challenging and presents a role for computational methods to provide theoretical insights. A prerequisite for development of molecules that modify this interaction is the characterization of the dependence of the CCL5-CCR1 binding on the structures and sulfation properties of GAGs and GAG fragments.

Toward the goal of developing small-molecule inhibitors of chemokines, we previously determined that tetrasaccharide fragments from heparinase-digested heparin were able to inhibit both receptor binding and in vivo peritoneal recruitment in an inflammation model (17). In the present study, heparin tetrasaccharides were purified to homogeneity, assayed for their ability to inhibit CCL5-CCR1 binding in vitro, and fully structurally characterized by mass spectrometry. The interactions of these tetrasaccharides with CCL5 were then modeled and compared with those of a 12-mer model of intact heparin.

Because there are no crystal structures of CCL5 with any of the tetrasaccharides isolated in this work, computational docking was employed to generate initial structures for the three-dimensional complexes. Docking GAGs to proteins is challeng-
ing due to the internal flexibility of the GAGs, the high charge density of the GAGs, and the fact that proteins that bind GAGs often lack well defined binding pockets (32, 33). The development and testing of protocols that address the issues associated with GAG docking is an area of active research (34–38). Keeping these caveats in mind, the initial CCL5-heparin tetrasaccharide complexes were generated by docking using AutoDock Vina (39). Vina has recently gained popularity in docking carbohydrate ligands (40–42).

To aid in overcoming inaccuracies in the initial structures, as may arise from approximations associated with docking, the complexes were subjected to long (100 ns) fully solvated molecular dynamics (MD) simulations to refine the docked pose and to assess the characteristics of binding. MD simulations could also be performed under conditions that modeled the effects of low or neutral pH. Docking followed by MD simulation and subsequent binding energy evaluation is an approach that is frequently used to predict the properties of GAG-protein complexes (43–45).

The results were analyzed in terms of structural stability, preferred binding mode, and computed interaction energies. The theoretical results clearly indicated a dependence of binding site preference and interaction energy on both pH and the positions of the sulfates within the tetrasaccharides. The heparin fragments were assessed for their ability to inhibit CCL5-CCR1 binding in terms of their ability to engage certain key residues on CCL5 implicated in its interaction with the receptor. The results highlight the important contribution of computational modeling for interpreting biological data and in predicting GAG binding preferences. In addition, these studies offer a cautionary note with regard to the treatment of pH in experimental studies of GAG-protein interactions.

**Materials and Methods**

**Preparation of Heparin Oligosaccharides**

Heparin was depolymerized according to the procedure described by Chai *et al.* (46). Briefly, heparin (5 g) and albumin (4 mg) were dissolved in 50 ml of 30 mM CH₃CO₂Na containing 3 mM CaCl₂ and adjusted to pH 7 with 0.2 M NaHCO₃. Heparinase I (2 IU) or heparinase III (2 IU) (both from Grampain Enzymes, Aberdeen, UK) was added, and the mixture was incubated at 30 °C for 16 h. The mixture was boiled for 3 min, centrifuged, and then filtered (0.45 μm). Size exclusion chromatography was performed on two 90 × 2.5-cm glass columns connected in series. The first column was packed with Bio-Gel
CCL5-heparin Interactions Depend on Sulfation Pattern and pH

P6 fine, and the second column was packed with Bio-Gel P10 fine (both from Bio-Rad). The columns were eluted with 0.25 M NaCl at a flow rate of 0.5 ml/min using a Gilson HPLC (Middleton, WI), and the effluent was monitored with a refractive index detector. Data were acquired using Gilson Unipoint software. Fractions (1 ml) adjacent to the peak maxima were pooled, lyophilized, and, after reconstituting in a minimum of water, desalted on a fast desalting column (10 × 100 mm; GE Healthcare) to give pools of oligosaccharides of a uniform degree of polymerization; see our earlier publication for an example of the separation achieved (47). The desalted fragments were lyophilized, redissolved in water, and stored at −20°C. The concentration of each fragment was determined spectrophotometrically at 232 nm in 30 mM HCl using the extinction coefficient of 5500 mol⁻¹ cm⁻¹.

Anion Exchange Purification of Tetrasaccharides

Anion exchange chromatography on a C₁₈ stationary phase coated with cetyltrimethylammonium ions was performed by adapting the guidelines presented by Mourier and Viskov (48). A preparative 250 × 21.2-mm, 5-µm Prep C₁₈ column (Phenomenex, Torrance, CA) was coated with cetyltrimethylammonium using 1 mM cetyltrimethylammonium bromide dissolved in 32% methanol at a flow rate of 2.5 ml/min at room temperature overnight. For analytical purposes, a 250 × 4.6-mm, 5-µm Luna C₁₈ column was prepared in a similar manner although at a lower flow rate of 1 ml/min for 4 h.

The preparative column was fitted to a preparative Gilson HPLC consisting of two model 306 pumps fitted with 25SC pump heads, a model 306 injection pump connected via a T-piece prior to the column, a model 151 UV detector fitted with 0.6-mL flow cell. Structural assignments were determined using in-house software written in MATLAB. In general, the assignment of sulfate groups and N-acetylation was determined by analysis of all possible structures that matched the sulfated tetrasaccharide composition. Details of the assignments for each of the tetrasaccharides are presented in the supplemental material.

Analytical HPLC

The C₁₈ analytical column coated with cetyltrimethylammonium ions was fitted to a Gilson high pressure liquid chromatograph comprising two model 306 pumps fitted with 10SC pump heads, a model 819 injector, a model 119 UV detector, and a model 215 liquid handler. The column was maintained at 40°C, and a flow rate of 1 ml/min was used with detection at 235 nm. Elution gradients were formed from buffer A (10 mM NaH₂PO₄, pH 7) and buffer B (10 mM NaH₂PO₄ containing 3 M NaCl, pH 7).

Mass Spectrometry Analysis

MALDI—The MALDI-TOF-MS technique used involved complexing peptides formed from arginine-glycine repeats with heparin tetrasaccharides. Detailed experimental protocols have been presented previously (47). Briefly, the basic peptide (RG)₁₉R was prepared as the trifluoroacetate salt by Auspep (Melbourne, Australia). AG-1 X2 anion exchange resin (20 mg) in the hydroxide form (Bio-Rad, Sydney, Australia) was added to an ice-cold aliquot (100 µl) of 50 µM peptide. The resulting suspension was pelleted and maintained in an ice bath. An aliquot of peptide (1 µl) was mixed with 10 mg/ml caffeic acid in 50% (v/v) acetonitrile (8 µl) and 5–100 µM sample (1 µl), and 1 µl was spotted onto a stainless steel sample plate and allowed to dry. MALDI-TOF-MS spectra were acquired in the linear mode by using a PerSeptive Biosystems (Applied Biosystems, Melbourne, Australia) Voyager reflectron time-of-flight instrument fitted with a 337-nm nitrogen laser. Delayed extraction was used to increase resolution (22 kV, grid at 93%, guide wire at 0.15%, pulse delay 150 ns, low mass gate at 2000, 50 shots averaged). Mass calibration was achieved by external calibration with the peptide calibration mixture provided by the manufacturer. The mass of the oligosaccharide was deduced by subtracting the mass of the (RG)₁₉R peptide observed for that sample.

Electron Detachment Dissociation and Collision-induced Dissociation—Mass spectrometry analyses on the tetrasaccharide fragments were performed with a 9.4T Bruker Apex Ultra Qq-FTICR instrument (Billerica, MA) fitted with an indirectly heated hollow cathode (HeatWave, Watsonville, CA) for electron generation. The sample solutions were infused at concentrations of 2–250 µM in 50:50 methanol/H₂O at a rate of 120 µl/h. Ions were generated by negative mode electrospray ionization using a metal capillary (Agilent Technologies (Santa Clara, CA), catalog no. G2427A). Precursor ions of interest were isolated in the external quadrupole and activated using both collision-induced dissociation in the collision cell and electron detachment dissociation (49) in the infinity cell. For each spectrum, 512,000 points were acquired, padded with 1 zero fill and apodized sinebell window. External calibration produced a 5 ppm mass accuracy, and using confidently assigned glycosidic bond cleavage product ions, internal calibration yielded mass accuracy higher than 1 ppm. All MS/MS products are reported using Domon and Costello (50) nomenclature. Structural assignments were determined using in-house software written in MATLAB. In general, the assignment of sulfate groups and N-acetylation was determined by analysis of all possible structures that matched the sulfated tetrasaccharide composition. Details of the assignments for each of the tetrasaccharides are presented in the supplemental material.

Equilibrium Competition Receptor Binding Assays

The assays were carried out on membranes from CHO cell transfecteds expressing CCR1, and the binding of CCL5 was
assessed using a scintillation proximity assay with $[^{125}\text{I}]$CCL5 as tracer according to the previously published method (6). Serial dilutions of the heparin tetrasaccharides, covering the range from $0.25 \times 10^{-3}$ to $1 \times 10^{-11}$ M, were prepared in binding buffer (50 mM Tris/HCl, pH 7.2, containing 1 mM CaCl$_2$, 5 mM MgCl$_2$, and 0.5% BSA). The tetrasaccharide pool and heparin (H 3400; Sigma) were also tested in the same assay. Wheat germ scintillation proximity assay beads (Amersham Biosciences) were solubilized in PBS at 50 mg/ml and diluted in binding buffer to a concentration of 10 mg/ml, and membranes of CHO cell transfectants were solubilized at 80 μg/ml in binding buffer.

Equal volumes of membrane and scintillation proximity assay bead solutions were mixed before adding them to the assay. The final membrane concentration in the assay was 20 μg/ml, and the concentration of $[^{125}\text{I}]$CCL5 was 0.05 nM. The plates (Corning, Inc.; 96-well, flat and clear bottom) were incubated at room temperature under agitation for 3.5 h. Radioactivity was counted with a β-counter for 1 min/well, and the data were analyzed using GraphPad Prism software. Data are expressed as a percentage where 100% is the value obtained for CCL5 binding in the absence of either heparin or the tetrasaccharides.

**FIGURE 2.** Anion exchange chromatography of the tetrasaccharide pool. A C$_{18}$ column coated with cetyltrimethylammonium ions was used. a, the first preparative separation at pH 3; b, preparative fractionation of the peak marked with an asterisk in a rechromatographed at pH 7. In each instance, gradients of increasing NaCl concentration (maximum 3 M NaCl) were used.
Automated Docking

Automated docking was performed using the molecular docking and virtual screening program, AutoDock Vina (39). To emulate the effects of pH on ionization states in CCL5 and the GAG fragments, the following models were generated. For low pH, the side chain in the only histidine residue (His-23) was fully protonated (net charge $= 1$), as were the carboxylates in all Glu, Asp, GlcA, and IdoA residues (net charge $= 0$). For neutral pH, His-23 was assigned zero charge (protonated only at $H$), whereas the other ionizable residues were appropriately charged.

Initial three-dimensional structures for the GAG sequences were built using the tLeap program of the AMBER 12 (51) molecular dynamics package using GLYCAM06 (52) force field, augmented for sulfate groups and unsaturated $\Delta^{4,5}$-uronate residues. Protein coordinates were obtained from the crystal structure of the human CCL5 dimer in complex with heparin-derived disaccharide (Protein Data Bank code 1U4L) (17). This model of CCL5 contains truncated N termini (3–68 variant CCL5) naturally found in serum (54). This protein model was employed for automated docking after removal of the co-crystallized ligand and all water molecules.

For docking, the entire surface of the protein was used as the search space (blind docking). This was done both to avoid biasing the subsequent analysis and because, although the $^{44}RKNR^{47}$ motif is known to modulate GAG-binding, the CCL5 crystal structure had the GAG-disaccharide placed in the groove between the CCL5 monomers (17). All glycosidic linkages and exocyclic torsion angles were allowed flexibility during docking. Docking was performed for models of CCL5 corresponding to both low and neutral pH states.

MD Simulations

Topology and coordinate files for the CCL5-heparin complexes were generated with the tLeap program, employing the Protein ff99SB (55) and GLYCAM06 (version h) (52) parameters for the protein and GAGs, respectively. The net charge on each system was neutralized with the addition of $Na^+$ or $Cl^-$ counterions, as required. The systems were solvated with TIP3P water (56) in a cubic box extending to at least 12 Å from any atom of the solute.

All MD simulations were performed with the GPU implementation of pmemd, pmemd.cuda_SPDP (57), in Amber12 (51). Energy minimization of the solvent was performed in an NVT ensemble (1000 steps of steepest descent, 24,000 steps of conjugate gradient), followed by a full system energy minimization (1000 steps of steepest descent, 24,000 steps of conjugate gradient). The systems were heated from 5 to 300 K over 60 ps in an NVT ensemble, with a weak positional restraint (10 kcal/mol-Å$^2$) on the atoms in the solute. A Berendsen type thermostat (58) with a time coupling constant of 1 ps was utilized for temperature regulation. Equilibration and production were performed at constant pressure (NPT ensemble; 1 atm), with a pressure relaxation time of 1 ps. After the heating step, the restraints were removed from the solute atoms, and the entire system was allowed to equilibrate at 300 K for 1 ns. All covalent bonds involving hydrogen atoms were constrained using the SHAKE (59) algorithm, allowing a simulation time step of 2 fs. Scaling factors for 1–4 interactions were set to the recommended values of 1.0 and 1.2 for the GAG (52) and protein (55), respectively, and a non-bonded interaction cut-off of 8.0 Å was employed. Long range electrostatics were computed with the particle mesh Ewald (PME) method. Data were collected for


![FIGURE 3. Inhibition of CCL5 binding to its receptor CCR1 by heparin tetrasaccharides. Inhibition was determined by competition equilibrium binding assays using membranes from transfected cells expressing CCR1. The six tetrasaccharides, tested to inhibit binding, are shown in comparison with the tetrasaccharide pool (DP4) and intact heparin (H-3400). The mean and S.E. (error bars) are shown for three independent replicates at each data point.](image-url)
TABLE 1

Ability of heparin tetrasaccharides to inhibit the binding of CCL5 to CCR1

For comparison, intact heparin inhibited the binding of CCL5 to CCR1 with an IC50 of 0.68 ± 0.1 μM. A three-dimensional model for a 12-mer fragment of heparin ([UA2OS-(1→4)-GlcNS6OS-α-(1→4)-IdoA2OS-α-(1→4)-GlcNS6OS] was generated as a trimer of the most highly sulfated tetrasaccharide, 2.

<table>
<thead>
<tr>
<th>ID</th>
<th>Structure</th>
<th>IC50 μM</th>
<th>Sulfation pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ΔUA2OS-(1→4)-GlcNS6OS-α-(1→4)-IdoA2OS-α-(1→4)-GlcNS</td>
<td>11.8 ± 4.8</td>
<td>3OS, 2NS</td>
</tr>
<tr>
<td>2</td>
<td>ΔUA2OS-(1→4)-GlcNS6OS-α-(1→4)-IdoA2OS-α-(1→4)-GlcNS6OS</td>
<td>25.4 ± 2.3</td>
<td>4OS, 2NS</td>
</tr>
<tr>
<td>3</td>
<td>ΔUA2OS-(1→4)-GlcNS6OS-α-(1→4)-IdoA-α-(1→4)-GlcNS6OS</td>
<td>39.1 ± 18.5</td>
<td>3OS, 2NS</td>
</tr>
<tr>
<td>4</td>
<td>ΔUA2OS-(1→4)-GlcNS6OS-α-(1→4)-IdoA-α-(1→4)-GlcNAc6OS</td>
<td>115 ± 39.2</td>
<td>3OS, 1NS</td>
</tr>
<tr>
<td>5</td>
<td>ΔUA2OS-(1→4)-GlcNS-α-(1→4)-IdoA-α-(1→4)-GlcNS6OS</td>
<td>—</td>
<td>2OS, 2NS</td>
</tr>
<tr>
<td>6</td>
<td>ΔUA2OS-(1→4)-GlcNS-α-(1→4)-IdoA-α-(1→4)-GlcNAc6OS</td>
<td>—</td>
<td>2OS, 1NS</td>
</tr>
</tbody>
</table>

a = no measurable inhibition.

100 ns for each CCL5-heparin tetrasaccharide (1–6) system at neutral and low pH conditions. For CCL5-heparin dodecasaccharide (7), the simulations were performed at slightly acidic (protonated histidine residues) and neutral pH conditions, and data were collected for 200 ns each.

Postprocessing of the MD simulations was performed using the ptraj (60) module of Amber, and graphical representations of the results were generated with the R package (61) and VMD (62).

Binding Energy Calculations

Energetic postprocessing of the trajectories to obtain binding free energies and per-residue contribution of binding free energies was done with the single-trajectory MM-GBSA method (63, 64) in a continuum solvent model using the MMPBSA.py script (65) from the AMBER 12 package. All water molecules were removed from the complexes, and solvation energies were approximated through the modified generalized Born model (igb = 2) (66, 67). A dielectric constant of 4 was used to model the interior of the solute, because a higher internal dielectric constant for proteins with highly charged binding interfaces has been shown to be appropriate for accurate free energy calculations (68).

For each of the CCL5-heparin tetrasaccharide simulations, energetic convergence was estimated by extracting 100 frames from each of the 40 equal parts (2.5 ns each) of the trajectory and performing a binding free energy calculation. This, along with the root mean square deviation of the tetrasaccharides through the course of the simulations, was used as an indicator of convergence. Consequently, for each of the CCL5-heparin tetrasaccharide simulations, the first 40 ns from the production run were discarded to allow the heparin tetrasaccharide to form stable interactions with the protein. From the last 60 ns of the production run, 6000 frames were extracted and used for more accurate binding energy and per-residue decomposition calculations using MM-GBSA (igb = 2) with an internal solute dielectric of 4. For the CCL5-heparin dodecasaccharide complexes, first 50 ns of the 200-ns production run were discarded, and 10,000 frames were employed for the final calculation of binding free energies and per-residue decomposition. To obtain interaction energies for CCL5-disaccharide complex (Protein Data Bank code 1U4L), CCL5 dimer bound to the disaccharide in the groove mode as well as CCL5 dimer bound to the disaccharide through the 44RKNR47 motif from the crystal unit cell were subjected to a short implicit solvent minimization, followed by single frame MM-GBSA energy calculation, with settings similar to those described above.

Contact Area Calculation

Contact areas between the heparin disaccharide and CCL5 dimer in the groove-binding mode as well as between the disaccharide and the lobes of the opposing CCL5 dimer were calculated using NACCESS (69) using appropriate radii for sugar atoms.

Electrostatic Potential Calculation

To calculate the electrostatic potential on the surface of CCL5 at neutral and low pH, the input files containing the appropriate atomic charge and radii were prepared using the PD2PQR server (70, 71), which uses PROPKA (72–75) for assigning the protonation states of the residues. The electro-
CCL5-heparin Interactions Depend on Sulfation Pattern and pH

The heparinase-digested heparin fragments were separated into pools of oligosaccharides, each with a uniform degree of polymerization, and the tetrasaccharide pool was subjected to anion exchange chromatography to purify individual structures. An example of the separation profile achieved for the tetrasaccharide pool at pH 3 is presented in Fig. 2a. Additional separation at pH 7 was employed for further resolution (Fig. 2b).

The tetrasaccharides resolved from the pool were tested for their ability to inhibit the binding of CCL5 to its receptor CCR1, expressed on membranes prepared from CHO cells transfected with CCR1 (Fig. 3). Some tetrasaccharides had markedly better inhibitory activity than the tetrasaccharide pool (DP4) from which they were isolated, although none matched the activity of intact heparin (Fig. 3). Calculation of IC50 values from the inhibition data revealed that three tetrasaccharides (1, 2, and 3) were significantly better at inhibiting the binding of CCR1 than the tetrasaccharide pool (DP4 IC50 = 183 ± 18 µM) (Table 1). A fourth tetrasaccharide (4) was a relatively weak inhibitor, and the remaining two failed to achieve 50% inhibition at the concentration ranges tested (Fig. 3 and Table 1).

MALDI-TOF-MS analyses of the tetrasaccharides tested in the biological assays indicated that the sulfate content varied among the fragments (Fig. 4), with inhibitory activity requiring at least four sulfate groups (Fig. 4). However, the data also indicated that the sulfation pattern affected activity and that solely...
the presence of four sulfate groups (as in 5) was not sufficient to achieve inhibition.

Accurate structural elucidation of highly sulfated GAGs by MS is challenging due to the labile nature of the sulfate half-ester bonds, because they can readily be cleaved during ionization and ion activation stages. The decomposition of sulfate groups is more pronounced in heparin oligomers due to the high sulfate density. Such molecules require careful control of ion activation to retain the sulfate modifications while providing the necessary fragmentation of glycosidic bonds and pyranose rings. We have shown that, with the aid of Na\(^+\)/H\(^+\) exchange coupled with collision-induced dissociation (77), it is possible to obtain detailed structural information on even highly complex heparin oligomers like arixtra (78). Electron detachment dissociation has also been shown to produce highly informative tandem mass spectra with minimal sulfate loss. Here, assignments of the sulfation positions in the tetrasaccharides (Table 1) were obtained using collision-induced dissociation and electron detachment dissociation MS (for details, see the supplementary data). Cross-ring cleavages and glycosidic bond products were used to assign sites of sulfation.

With the goal of determining how the sulfation pattern of these tetrasaccharides affects their inhibitory activity, three-dimensional structures of the heparin fragments bound to CCL5 were generated using a combination of molecular docking and molecular dynamics simulation. The docking employed crystallographic data for CCL5, obtained at low pH, in complex with a sulfated disaccharide (Protein Data Bank code 1U4L). Because the inhibition experiments were carried out at pH 7.2, the effect of pH on the predicted modes of interaction was also examined. In addition to the six tetrasaccharides, a dodecasaccharide with the repeating trisulfated disaccharide pattern of the CCL5 monomers was modeled to assess the binding of longer heparin fragments.

**Molecular Docking**—Automated docking of 1–6 was performed using AutoDock Vina using the entire surface of the protein as the search space. Docking consistently placed all tetrasaccharides in the groove between the CCL5 monomers (Fig. 5), in agreement with the placement of the disaccharide fragment in the crystal structure. Changing the protonation states of the ionizable side chains to that expected at pH 3.5 did not change the general distributions of the tetrasaccharide poses (Fig. 5).

The ranking of the theoretical binding energies from docking was not consistent with the IC\(_{50}\) data, although the predicted affinities were significantly enhanced at low pH (Table 2), as might be expected on the basis of enhanced electrostatic interactions between the anionic ligand and the protein surface (Fig. 6). The predicted binding energies varied by less than 1 kcal/mol for each of 1–6, whereas the IC\(_{50}\) data indicated that tetrasaccharides with the same overall degree of sulfation inhibited the CCL5-CCR1 interaction at neutral pH with varying efficacy (Table 1). Nor was there a pronounced enhancement of the binding energy for the more highly sulfated ligand 2. In the groove-based ligand alignment produced by docking, the tetrasaccharides do not interact directly with the known 44RKNR47 heparin-binding motif (Table 3) or with residue Arg-17, reported to be important for the binding of CCL5 and CCR1 (30).

Because these docking experiments were unable to identify the biologically known binding motif or discriminate between ligands, a representative complex for each CCL5-tetrasaccharide interaction was selected and subjected to MD simulation to more accurately model the effects of molecular dynamics, solvation, and pH.

**MD Simulation**—Within the first 30 ns of simulations performed at neutral pH, tetrasaccharides 1–5 diffused out of the groove (Fig. 7) and created new interactions, including with Arg-17 and the 44RKNR47 motifs (Fig. 8). Non-inhibiting tetrasaccharide 6, which has the lowest degree of sulfation, remained bound in the groove throughout the 100-ns MD sim-

### TABLE 3

| Distances between any sulfate group in 1-6 and the RKNR motif as a function of pH |
|---|---|---|---|---|---|
| 1 | 2 | 3 | 4 | 5 | 6 |
| **Neutral pH** | | | | | |
| 1–6 | 20.4 (12.4, 32.4) | 17.1 (9.9, 24.6) | 17.0 (5.5, 25.0) | 18.8 (10.9, 26.2) | 17.5 (10.7, 26.2) | 18.1 (10.6, 26.2) |
| **Low pH** | | | | | |
| 1–6 | 16.3 (5.7, 27.6) | 21.8 (12.2, 32.5) | 19.1 (5.2, 26.8) | 17.3 (5.8, 24.5) | 17.3 (8.4, 26.1) | 17.8 (8.3, 26.1) |

**FIGURE 7.** Root mean square deviation of 1–6 with respect to their starting position over the course of the MD simulations (100 ns) at low (red) and neutral (blue) pH. All tetrasaccharides except for 6 move out of the initial docked site and make interactions with residues outside of the groove of the CCL5 dimer.
**CCL5-heparin Interactions Depend on Sulfation Pattern and pH**

**Summary of the per-residue theoretical interaction energies (kcal/mol) (MM-GBSA) for 1-6 at neutral pH for residues in the 44RKNR47 motif and other key residues (those that contribute more than 1 kcal/mol to the interaction energy)**

Most significant energetic contributions to binding are shown in bold/italics.

<table>
<thead>
<tr>
<th>GAG</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>kcal/mol</td>
<td>kcal/mol</td>
<td>kcal/mol</td>
<td>kcal/mol</td>
<td>kcal/mol</td>
<td>kcal/mol</td>
</tr>
<tr>
<td>Neutral pH</td>
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<td>4OS, 2NS</td>
<td>3OS, 2NS</td>
<td>3OS, 1NS</td>
<td>2OS, 2NS</td>
<td>2OS, 1NS</td>
</tr>
<tr>
<td>44RKNR47 A</td>
<td>−1.3</td>
<td>−1.3</td>
<td>−8.6</td>
<td>−2.8</td>
<td>−13.3</td>
<td>−1.7</td>
</tr>
<tr>
<td>44RKNR47 B</td>
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<td>−14.1</td>
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<td>0.0</td>
<td>−1.2</td>
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<td>−1.0</td>
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<td>Arg-17 B</td>
<td>−8.6</td>
<td>−8.0</td>
<td>−0.6</td>
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<td>0.0</td>
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<tr>
<td>Total MM-GBSA</td>
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<td>−60.1</td>
<td>−52.9</td>
<td>−47.8</td>
<td>−36.4</td>
<td>−61.9</td>
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</tbody>
</table>

* Total from both monomers, conformational entropic effects not included.

In contrast, for the simulations corresponding to low pH, all of the heparin tetrasaccharides remained bound in the groove between the CCL5 monomers in the dimer, in similar positions as obtained from docking (Fig. 8).

The origin of the differences in the behavior of the complexes at neutral and low pH conditions can be understood in terms of the difference in the distribution of positive potential on the surface of the protein (Fig. 6). At neutral pH, the 44RKNR47 motifs have a higher positive charge density as compared with other regions of CCL5, and consequently the negatively charged heparin fragments are attracted to these regions. The binding of 6 to the groove between the two RANTES monomers at neutral pH may be explained by its low degree of sulfation and, consequently, weaker attraction to the 44RKNR47 motifs. Subsequent analyses were performed only on the complexes simulated at neutral pH unless noted otherwise.

**Binding Free Energy (MM-GBSA) Analysis**—The tetrasaccharide interaction energies ranged from −71.0 to −36.4 kcal/mol. These values are considerably higher than would be expected experimentally, due in part to the omission of conformational entropic effects in the computational analysis (79). Theoretical entropic contributions were not computed because they were unlikely to have converged over the time scale of the simulations (68). However, because of the similarity of ligand sizes and compositions, the relative trends in MM-GBSA values are expected to be less sensitive to entropic differences. Per-residue binding energy decomposition identified the protein residues that contributed most significantly to binding of the tetrasaccharides and revealed differences in the binding modes of each tetrasaccharide (Table 4 and Fig. 9).

As expected from the structural data, the 44RKNR47 motifs contributed most strongly to ligand binding. The tetrasaccharides generally interacted preferentially with only one of the motifs in the dimer, with the exceptions of 3, which appeared to alternate in its interactions between the two domains, and 6, which remained bound in the interdomain groove. In addition to the interactions with the 44RKNR47 motifs, the tetrasaccharides frequently formed additional interactions with residues from the N terminus of the opposite domain (see boldface values in Table 4).
Although contacts with the N termini made only a small contribution to the binding of 2, 3, and 5, they played a more significant role for 1, 4, and 6. Because 6 remained bound in the groove region, the predominant contribution to its binding came from the N termini.

Residues Arg-47 and -17 of CCL5 are known to play a crucial role in the CCL5-CCR1 binding event (29, 30), whereas the N terminus of CCL5 is crucial for CCR1 signaling (30, 31). Based on the per-residue MM-GBSA data, each ligand interacts with these residues to varying degrees. Specifically, Arg-47 contributed more than 5 kcal/mol to the binding of tetrasaccharides 1, 2, 3, and 4 (see boldface values in Table 4), whereas Arg-17 contributed significantly to binding of the top two inhibitors, 1 and 2.

In terms of the overall interaction energies, the binding of 1–5 could be ranked in the same order as their inhibitory capabilities (Table 1), with 1 being both the tightest binder and most potent inhibitor. However, interaction energy alone was not sufficient to define a strong inhibitor. This is illustrated by the behavior of 6, which was predicted to bind tightly to CCL5, but because it remained in the interdomain groove and did not interact very strongly with either Arg-47 or -17, it could not inhibit CCR1 binding. The binding modes for each tetrasaccharide are presented in Fig. 10 and indicate that different sulfation patterns lead to variations in the preferred CCL5 contact regions, even for tetrasaccharides that contain the same number of sulfate groups.

Broadly, the binding affinity was predicted to increase with increasing number of sulfate groups, with the contribution of specific sulfate groups in defining the interaction being subtle. Specifically, the energetic contributions of individual sulfate groups ranged from −0.8 to −5.5 kcal/mol and frequently

FIGURE 9. Direct interactions between the sulfate and carboxylate groups of 1–6 and key residues from CCL5 at neutral pH. Interactions present for more than 20% of the simulation time, using a 3.5 Å distance cut-off between interacting atoms, during the last 60 ns of each simulation, are presented. Contributions to the interaction energy from the sulfate groups on each residue as well as the total per-monosaccharide contribution are indicated.
accounted for approximately half of the total energetic contribution of the monosaccharide. Carboxylate groups generally contributed only weakly to binding, relative to the sulfate groups. Frequently, the O-sulfate group in the terminal uronic acid as well as the carboxylate group of the iduronic acid formed hydrogen bonds with the Arg-47 residues; however, there was no clearly discernible correlation between specific hydrogen bonds and affinity.

Docking and MD Simulation of a Model Heparin Dodecasaccharide—To examine how a longer heparin fragment would interact with CCL5 and inhibit CCL5-CCR1 interaction, a dodecasaccharide consisting of a repeating sulfation pattern similar to 2 was generated, docked to CCL5, and subjected to MD simulation. This sulfation pattern was chosen because it is the most common structure in heparin. At neutral pH, as in the case of the smaller heparin fragments, docking placed the dodecamer in the groove of the CCL5 dimer. As seen with the tetrasaccharides, during the MD simulation, the dodecamer drifted out of the groove to form new interactions with the RKNR47 motifs. However, the larger heparin fragment interacted with both RKNR47 motifs simultaneously, spanning the 40s loops. It remained in this orientation for the remainder (~150 ns) of the simulation (Fig. 11). Our findings for the binding mode of a longer heparin sequence match the predictions of Vivès et al. (80) for the binding of heparin oligosaccharide (17 monomers) to CCL5.

Per-residue binding energy decomposition identified that residues Arg-47 and -17 make significant contributions to the binding of the dodecasaccharide to CCL5 (Table 5). Direct engagement of these residues by a longer heparin oligosaccharide demonstrates its potential efficacy as an inhibitor of CCL5-CCR1 interactions.

Several GAG binding proteins that contain histidine residues close to the GAG binding sites have previously demonstrated a heightened GAG binding response under slightly acidic conditions (81–84). Both CCR1 and CCL5 are up-regulated in many renal inflammatory conditions (85), and blocking CCR1 activity has been shown to reduce inflammation. CCL5 contains one histidine residue in each domain, in the 310-helix that lies close...
The likelihood that a basic charge at position 23 enhances GAG interaction, when the pH is likely to drop slightly below neutral.

were charged. These findings suggest a potential role for His-23 as a contributor to CCL5-GAG interactions at sites of inflammation, when the pH is likely to drop slightly below neutral. The likelihood that a basic charge at position 23 enhances GAG binding has similarly been indicated by a H23K mutant, which was found to bind GAGs with higher affinity than wild-type CCL5.

**Examination of the CCL5-Disaccharide Complex**—Given our data on the contribution of pH to the binding mode of heparin tetrasaccharides, we re-examined the crystal structure of the CCL5-disaccharide complex as presented by Shaw et al. (17). Interestingly, in the CCL5-heparin disaccharide complex obtained at low pH (17), packing of the protein dimers into the crystallographic unit cell enables the disaccharide to interact simultaneously with the groove region in one dimer and with the lobes containing the 44RKNR47 motif in another (Fig. 12). It is unclear whether in solution the heparin fragment would prefer to interact with the groove region or with the RKNR motif. The contact area between the disaccharide and the two CCL5 dimers shows that the disaccharide makes a larger contact (222 Å²) with the protein in the groove-binding mode than with the lobes of the opposing dimer (177 Å²). In addition, MM-GBSA binding energy calculations indicate a preference for the groove interaction mode (ΔΔG = −3.4 kcal/mol). The packing of the protein-disaccharide complexes in the unit cell makes it difficult to say whether the low pH employed in the crystallization protocol is responsible for the placement of the ligand in the groove. However, in the context of our MD simulations of the CCL5-tetrasaccharide complex, a pH effect is one possible explanation.

**Conclusions**—This study integrated multiple experimental and computational methods to elucidate details of the interactions between CCL5 and heparin fragments with varying sulfation pattern and lengths. The modeling data suggested that the positions where heparin fragments bind CCL5 are influenced by the pattern of sulfation as well as the degree of sulfation. The effects of changes in pH on the interaction of heparin tetrasaccharides with CCL5 were monitored by ligand docking followed by MD simulation. Although AutoDock Vina has been reported to perform reasonably well in carbohydrate docking (40–42), in the case of the highly charged GAG-protein complexes studied here, it was unable to differentiate between changes in the surface charges of CCL5 at the two pH conditions for placement of the GAGs. It is worth noting that the scoring function in AutoDock Vina does not consider partial charges and instead utilizes an internal method for detecting the potential contributions from hydrogen bonds (39). It is unclear to what extent that approach is able to treat the highly

### TABLE 5

<table>
<thead>
<tr>
<th></th>
<th>Neutral pH</th>
<th>Slightly acidic pH</th>
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<tbody>
<tr>
<td>44RKNR47 A</td>
<td>−27.9 kcal/mol</td>
<td>−27.8 kcal/mol</td>
</tr>
<tr>
<td>44RKNR47 B</td>
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<td>−40.6 kcal/mol</td>
</tr>
<tr>
<td>N terminus A</td>
<td>−12.5 kcal/mol</td>
<td>−14.8 kcal/mol</td>
</tr>
<tr>
<td>N terminus B</td>
<td>−2.9 kcal/mol</td>
<td>−5.8 kcal/mol</td>
</tr>
<tr>
<td>Arg-47 A</td>
<td>−8.3 kcal/mol</td>
<td>−14.0 kcal/mol</td>
</tr>
<tr>
<td>Arg-47 B</td>
<td>−14.0 kcal/mol</td>
<td>−16.1 kcal/mol</td>
</tr>
<tr>
<td>Arg-17 A</td>
<td>−1.9 kcal/mol</td>
<td>−1.9 kcal/mol</td>
</tr>
<tr>
<td>Arg-17 B</td>
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<td>−12.4 kcal/mol</td>
</tr>
<tr>
<td>His-23 A</td>
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</tr>
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</tr>
<tr>
<td>Chain A</td>
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</tr>
<tr>
<td>Chain B</td>
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<td>−73.4 kcal/mol</td>
</tr>
<tr>
<td>Total MM-GBSA*</td>
<td>−135.2 kcal/mol</td>
<td>−182.2 kcal/mol</td>
</tr>
</tbody>
</table>

*Total from both monomers, conformational entropic effects not included.

FIGURE 11. Stable pose for dodecamer-CCL5 complex during the MD simulations at neutral (blue) and slightly acidic (red) pH. Residue Arg-17 (cyan) and Arg-47 (green) are shown in a CPK model.
CCL5-heparin Interactions Depend on Sulfation Pattern and pH

ionic interactions associated with glycosaminoglycans. In addition, the predicted poses from docking were not in agreement with biological expectations, in contrast to the data from MD simulations, where, during the simulations, the change in ionization states markedly altered the positions where most, but not all, of the heparin fragments were found to bind CCL5.

Changes in the surface charge distribution of CCL5, as would result from changes in pH, alter the preferred GAG-binding sites. At low pH, the GAG tetramers preferred to interact within the groove between the CCL5 dimer, whereas at neutral pH, most tetrasaccharides preferentially interact with residues outside the groove. Theoretical interaction energy analysis was able to identify the key residues on CCL5 and the key GAG epitopes that are important for binding. These data indicate that variations in the sulfation patterns in GAG fragments that share the same overall level of sulfation affect the nature of their interactions with CCL5, leading to variations in affinity and in the site(s) on the protein most favored for binding. This was also shown to be the case for two tetramers having the same overall sulfation but a different pattern of sulfation. This suggests that the GAG-CCL5 binding interface differs according to the fine structure of the GAG fragment. The computed interaction energies allowed the tetrasaccharides that demonstrate the best experimental IC₅₀ value for inhibition of CCL5-CCR1 interaction through engagement of residues Arg-17 and -47.

Collectively, the data presented here prompt re-evaluation of the current view that proteins can bind several GAG structures of similar overall charge density (91). To date, this discussion has focused on the affinity of the GAG-protein interaction with little or no attention being given to the possibility that different GAG structures of similar overall charge density may bind to different amino acids within the binding face and thereby exhibit different biological activities. In the case of CCL5, this is particularly important because it provides a mechanism for potentially fine tuning the activity of a chemokine that interacts with several receptors and has thus been described as redundant. The extracellular HS proteoglycan content is known to be modulated under inflammatory conditions (e.g. chronic exposure to an allergen increases airway HS proteoglycan levels) (53), and although HS structural modifications during inflammation have not yet been defined, they are likely to occur through the activities of the 6-O-endosulfatas. Hence, CCL5 could be activated, inhibited, or directed to bind a receptor that is not influenced by GAG binding, depending on the pH and the structure and quantity of HS in that tissue locality. This has significant implications for the development of GAG-related therapeutics.

References

CCL5-heparin Interactions Depend on Sulfation Pattern and pH


