The Binding Surface and Affinity of Monomeric and Dimeric Chemokine Macrophage Inflammatory Protein 1β for Various Glycosaminoglycan Disaccharides

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Chemokines comprise a family of proteins that function in the immune response to recruit leukocytes to sites of infection. This recruitment is believed to be carried out by the establishment of a chemokine gradient by the binding of chemokines to sulfated polysaccharides known as glycosaminoglycans (GAGs) located on the extracellular surface of endothelial cells. In the present studies, multidimensional NMR spectroscopy was used to study the interaction of monomeric and dimeric chemokine macrophage inflammatory protein (MIP)-1β variants with a series of differentially sulfated disaccharides. The data define a GAG binding surface, including both basic and uncharged residues such as Arg18, Asn23, Val25, Thr44, Lys45, Arg46, and Ser47. Dissociation constants determined from these NMR studies consistently show for each disaccharide that dimeric wild type MIP-1β binds more tightly than monomeric MIP(9). Furthermore, analysis of the binding surface suggests that participation in the dimer of residues Met2, Gly4, and Ser1 may be responsible for this higher affinity. These studies also indicate that the specificity of MIP-1β for particular GAG disaccharides is directly related not only to the degree of disaccharide sulfation but also to the position of the sulfate moiety, with O-sulfation at position 2 of the hexuronic acid unit and position 6 of the D-glucosamine being major determinants for binding.

MIP-1β is a member of the chemotactic cytokine (chemokine) superfamily, a large group of proteins that causes chemotaxis and activation of various subpopulations of cells in the immune system (1). The activity of chemokines is believed to be mediated primarily through two types of interactions, the first of which involves chemokines binding to glycosaminoglycans (GAGs), polyanionic polysaccharides found on the endothelial surface and the extracellular matrix. This immobilization of chemokines by GAGs is likely to play a key role in the formation of chemokine gradients, which are sensed by leukocytes and trigger localization of leukocyte subpopulations to the site of infection or injury (2, 3). The other major interaction involves the tight binding of chemokines to their seven-transmembrane helix, G-protein-coupled receptors on the surface of leukocytes. This interaction causes cellular activation and mediates chemotaxis.

The chemokine superfamily has as many as 50 members (1, 4) and is divided into subfamilies on the basis of the pattern of conserved cysteine residues at their N termini. One major subfamily, the CC subfamily, has two contiguous cysteine residues and includes such members as MIP-1α, MIP-1β, RANTES, and MCP-1. Another major subfamily, the CXC subfamily, has an amino acid separating the conserved cysteines and includes such members IL-8, SDF-1, and PF-4. Two minor subfamilies also exist, the CX9C and C subfamilies, which include fractalkine and lymphotactin, respectively.

The G-protein-coupled receptor that interacts with MIP-1β is called the CC chemokine receptor 5 (CCR5), and this receptor also binds two other CC chemokines, namely MIP-1α and RANTES. CCR5 has been shown to be used by strains of HIV-1 known as the “R5 strains,” as a coreceptor, making contacts with the HIV-1 surface that allow viral entry into the cell. Consequently, it has been shown that the CCR5 ligands MIP-1β, MIP-1α, and RANTES are effective inhibitors of these strains of HIV-1 infection due to their ability to block viral access to CCR5, and in some cases, to cause the internalization and down-regulation of CCR5 upon chemokine binding (5, 6).

The interrelationship between GAG binding and receptor function in chemokines is not yet clear. We and others have shown that interaction with GAGs is not required for a chemokine to retain receptor function in vitro; chemokines retain function both on cells treated enzymatically to remove GAGs and on cells genetically modified to be unable to express surface GAGs (7–10). It has been reported, however, that the presence of GAGs on receptor-bearing cells may lead to an increased receptor sensitivity due to higher effective chemokine concentrations (7, 9). Mutagenesis to remove GAG binding residues in CCR5-binding chemokines affects receptor binding moderately.
or minimally, suggesting that the binding site for GAGs may be separate from that for the receptor (8, 11–13). This may not be the case with other chemokine receptors, because alteration of GAG binding residues in CCR1-binding chemokines significantly affects function on the CCR1 receptor (11, 12, 14). Several groups have shown that exogenous soluble GAGs compete with receptors for chemokine binding (8, 15, 16), although one group reports that a RANTES-GAG complex can bind the cognate chemokine receptor (17).

Mutagenesis and modeling studies have shown that the interaction of chemokines with GAGs is mediated by basic residues on the protein surface (8, 10–13, 15, 18–21). This is in accord with other proteoglycan-binding proteins, which use positively charged residues in ionic interactions with the polyanionic GAGs (22). Indeed, chemokines tend to bind more tightly to more highly sulfated GAGs (15). However, great variation exists within the chemokine superfamily regarding residues that are implicated in GAG binding (described in previous references and summarized in Ref. 12). CXC chemokines such as IL-8 often use basic residues located in their C-terminal α-helix (18, 23), although SDF-1 utilizes basic residues in its first β-strand (10). CC chemokines tend to use basic residues in the 40s region, although it has been reported that MCP-1 utilizes C-terminal α-helix residues (20) and mutation in MIP-1α of Arg18 as well as 40s residues affects GAG binding (14, 19). The single most important residue for interaction by MIP-1β with heparin is Arg46 (8, 13), and it has been shown that simultaneous elimination of the three positively charged residues in the 40s region of MIP-1β (K45A/R46A/K48A) completely abrogates heparin binding ability (8). Similarly, basic residues in this region have been identified as critical for GAG binding (8, 30).

The physiological role of the chemokine dimer is not known, as it is possible that the chemokine dimer may be a critical mediator of GAG binding to RANTES (11, 12). A structural understanding of GAG binding by chemokines is complicated by the fact that many chemokines have been shown to form tight dimers, particularly MIP-1β, MIP-1α, and RANTES (24–27), each preferring acidic conditions and also tending to form higher order aggregates near neutral pH (26).

The physiological role of the chemokine dimer is not known, because monomeric variants of MIP-1β and other chemokines effectively bind their cognate receptor in vitro (28, 29). Because the dimer does not appear to be involved in receptor function, it is possible that the chemokine dimer may be a critical mediator of GAG binding (8, 30).

Although the structure of a chemokine in complex with a GAG has not yet been reported, the NMR spectra of PF-4, IL-8, and RANTES in the presence of saccharides have allowed some analysis of the binding sites of those proteins (12, 18, 23). In principle, NMR studies of a chemokine-GAG complex should provide a clear indication of the GAG binding site on the protein, because residues whose resonances shift in the absence of GAGs are likely involved in GAG binding in some manner. In addition, analysis of the NMR spectra of the titration of a particular GAG with a chemokine should provide the dissociation constant for the interaction. The paucity of such data for chemokines is likely related to the oligomerization that GAGs induce upon binding chemokines (30), leading to aggregates too large to be analyzed by NMR. We have found MIP-1β to be amenable to structural study with heparin disaccharides.

In the present study, NMR was used to analyze MIP-1β in the presence of eleven different disaccharides and allowed for the determination of the dissociation constant for the five found to bind the chemokine. Similarly, the GAG binding properties of a monomeric variant of MIP-1β known as MIP(9) were investigated. Combined, these studies report the binding surfaces for the monomeric and dimeric forms of the protein. Finally, using a mutant of MIP-1β that displays less of a tendency to aggregate than the wild type protein, we have obtained what we believe to be the first NMR spectra of an anti-HIV CC chemokine in the presence of a GAG near neutral pH.

**EXPERIMENTAL PROCEDURES**

**Materials**—Heparin disaccharides IV-A and II-S were obtained from Calbiochem-Novabiochem Corp. (La Jolla, CA). The following heparin disaccharides were purchased from Sigma-Aldrich Co. (St. Louis, MO): I-P, I-S, III-S, IV-S, II-H, and III-H. The heparin disaccharides II-A and III-A and the chondroitin disaccharide ADi-dis0 were purchased from V-Labs Inc. (Covington, LA). All disaccharides were used without further purification, and their structures as defined by the commercial supplier were shown in Fig. 4. Some disaccharides were synthesized, and the saccharide linkage, which will be investigated in a later study of GAG selectivity. H2O and 13C-glucose were purchased from Isotec Inc. (Miamisburg, OH), and 15NH4Cl was obtained from Martek Biosciences Corp. (Columbia, MD).

Stock solutions (10–30 mM) of disaccharides were prepared by dissolving in either H2O or D2O and kept frozen until use. The concentrations of the disaccharide stock solutions were determined based on the amount specified by the commercial suppliers. Each respective commercial source was contacted as to the magnitude of possible error in the quantity of disaccharide purchased: the error in the disaccharides obtained from Calbiochem-Novabiochem Corp. was ±1.0%; the amount purchased by Sigma-Aldrich Co. may exceed the specified quantity by a maximum of 20% (several non-binding disaccharides have the possibility of exceeding the amount specified by as much as 30%, but because these disaccharides did not bind the proteins of this study, this level of error does not pertain to our numerical results); and the amount provided by V-Labs Inc. may exceed that specified by no more than 5%.

**NMR Preparation—**The mutations of MIP-1β into the non-aggregating variant D27A/D65A/E67Q were carried out by standard methods using the gene for human MIP-1β in a variant of the Novagen pET32LIC vector as previously described (27).

**NMR Spectroscopy—**All NMR spectra were acquired at 25°C on Varian Inova 500 or 600 MHz spectrometers. Chemical shifts were referenced relative to 2,2-dimethyl-2-silapentane-5-sulfonic acid (31). Unless otherwise noted, NMR samples were prepared by dissolving lyophilized protein in a standard buffer of 20 mM sodium phosphate (pH 2.5) containing 10% H2O and 0.02% sodium azide. Data were processed using the program NmrPipe (32) and analyzed using either PIPP (33) or NMRView (34).

For disaccharide titration experiments, 1H-15N HSQC spectra were acquired at 500 MHz with 512* points in the 1H dimension and 128* points in the 15N dimension (t2), where n represents n complex points. The 1H and 15N spectral widths in these experiments were 6000.6 and 1500 Hz, respectively. These data were processed with 72*-shifted sine-bell apodization in both 1H and 15N dimensions and a final digital resolution of 3 Hz point (1) and 6 Hz point (2).

For sequential assignment of MIP-1β D27A/D65A/E67Q at pH 2.5 and 6.0, and MIP(9) at pH 2.5, CBCA(CO)NH (35) and HNCA (36) spectra were acquired. Samples of MIP-1β D27A/D65A/E67Q and MIP(9) at pH 2.5 used for assignment were 1 mM in concentration and prepared in the standard buffer. To prepare the MIP-1β D27A/D65A/E67Q and MIP(9) at pH 2.5 for use as assignment were 1 mM in concentration and were prepared in the standard buffer. To prepare the MIP-1β D27A/D65A/E67Q and MIP(9) at pH 2.5 for use as assignment were 1 mM in concentration and were prepared in the standard buffer.
samples of 0.23–0.26 mM 15N-labeled protein and 1H-15N HSQC spectra were acquired for each titration point. To ascertain binding, an initial series of spectra were acquired for each protein with every disaccharide at varying intervals between 0 and 4.0 molar equivalents of disaccharide (dilution effects were taken into account during data analysis). Titrations were continued on only those disaccharides that caused resonance shifting in the initial series of spectra until no further chemical shift changes were observed.

As a control for nonspecific binding, 1H-15N HSQC spectra were acquired for WT-MIP-1 and free forms of MIP-1 (each titration, the ratio MIP-1 samples of 0.23 mM MIP-1 and MIP(9) upon titration with potassium sulfate (from a stock solution dissolved in the standard sample buffer) in the amounts of 0, 2, 4, 20, and 40 molar equivalents, respectively. The change in pH of the protein sample upon addition of disaccharides or potassium sulfate was negligible.

Backbone amide HSQC cross-peaks of the MIP-1β proteins were monitored for each titration point of every experiment with the various disaccharides. The observed chemical shift change (Δδobs) for each residue was evaluated as the weighted average of the 1H (ΔδHN) and 15N (ΔδHN) chemical shift changes as given in Equation 1 (38) as follows.

\[ Δδ_{obs} = \left(\frac{\Deltaδ_{HN}^2 + \Deltaδ_{15N}^2}{2}\right)^{1/2} \] (Eq. 1)

Because the chemical shifts observed in the presence of disaccharide are the population-weighted average of the chemical shifts of free and bound MIP-1β, Δδmax is related to the ratio of bound MIP-1β to total MIP-1β (M/B) by Equation 2.

\[ Δδ_{max} = (M/B) Δδ_{max} \] (Eq. 2)

where \( Δδ_{max} \) is the chemical shift difference between the fully bound and free forms of MIP-1β, \( M_{free} \) is the total concentration of MIP-1β, and \( M_{bound} \) is the concentration of bound MIP-1β. Assuming a 1:1 binding stoichiometry of disaccharide for MIP-1β monomer, the ratio \( M_{free}/M_{bound} \) is given by Equation 3 (39).

\[ M_{free}/M_{bound} = 0.5 \left(1 + K_{D}^{app}/D_{c}\right) \] (Eq. 3)

where \( D_{c} \) is the total concentration of disaccharide and \( K_{D}^{app} \) is the apparent dissociation constant of the MIP-1β-disaccharide complex. For each titration, the ratio \( D_{c}/M_{bound} \) and the \( Δδ_{max} \) for those residues that undergo disaccharide-induced shifts greater than the average taken over all the residues were simultaneously fit to Equations 2 and 3 using Kaleidagraph 3.5 (Synergy Software, Reading, PA) to give an apparent dissociation constant \( K_{D}^{app} \) for each residue.

To prepare samples of MIP-1β D27A/D65A/E67Q at pH 6.0 in the presence of disaccharide, lyophilized protein was dissolved in the standard sample buffer at pH 2.5, heparin disaccharide I-S was added to 1 mM, and the pH was raised to 6.0. Some precipitation occurred, but the NMR signal was observable and an 1H-15N HSQC spectrum was acquired.

RESULTS

Titration of WT-MIP-1β with Disaccharides—As WT-MIP-1β has been sequentially assigned (24), the 1H-15N HSQC NMR spectrum of the protein was used as a starting point to monitor spectral changes upon titration of the protein with ten heparin disaccharides (I-P, I-S, II-S, III-S, IV-S, II-H, III-H, II-A, III-A, and IV-A) and one chondroitin disaccharide (Di-disA-I); Fig. 4 shows the structure of these disaccharides. Fig. 1A shows the superposition of the series of spectra acquired for several points in the titration of WT-MIP-1β with heparin disaccharide I-P. Using chemical shift changes of the backbone amide 1H and 15N resonances as probes for disaccharide binding, WT-MIP-1β was found to interact with seven disaccharides: I-P, I-S, II-S, III-S, Di-disA-I, and IIIA. However, these latter two disaccharides showed so little peak movement that a \( K_{D}^{app} \) determination was impossible, and they were therefore considered to be non-binding disaccharides for the purposes of discussion. In these experiments, the resonances for all residues were monitored excluding the N-terminal Ala, six prolines not having backbone amide protons, and Leu and Val that were not observable at the protein concentrations used. The superposition of the spectra clearly indicates that a number of backbone resonances undergo considerable shifting upon addition of I-P (Fig. 1A). Most of the same residues were found to undergo shifting upon addition of I-S, II-S, III-S, Di-disA-I, and IIIA. However, these latter two disaccharides showed so little peak movement that a \( K_{D}^{app} \) determination was impossible, and they were therefore considered to be non-binding disaccharides for the purposes of discussion. In these experiments, the resonances for all residues were monitored excluding the N-terminal Ala, six prolines not having backbone amide protons, and Leu and Val that were not observable at the protein concentrations used. The superposition of the spectra clearly indicates that a number of backbone resonances undergo considerable shifting upon addition of I-P (Fig. 1A). Most of the same residues were found to undergo shifting upon addition of I-S, II-S, III-S, and Di-disA-I (data not shown). These spectra all show the clear presence of the dimer throughout the titration (27). Chemical shift changes of WT-MIP-1β for all disaccharides tested were found to fall into the fast exchange limit on the NMR time scale. Therefore, the position of each cross-peak corresponds to the population-weighted average of the bound and free chemical shifts of WT-MIP-1β. The backbone amide of Arg60 consistently displays some of the greatest peak movement during the titrations, having a \( Δδ_{max} \) of about 70 Hz upon titration with I-P, reflecting a lower limit for the exchange rate of MIP-1β with the disaccharide of \( ~450 s^{-1} (k \gg 2πDΔδ_{max}) \) (38). Because each titration shows smooth, linear peak movement (40) and because the disaccharide unit is likely to be too small to induce cooperativity between the two binding sites, each subunit of the MIP-1β dimer appears to contain an equivalent, non-cooperative GAG binding site.

To more clearly delineate the residues affected by the binding of the various disaccharides, the 1H- and 15N-weighted
average chemical shift difference between the fully bound and free forms of MIP-1β, \( \Delta \delta_{\text{max}} \), was plotted for each residue in WT-MIP-1β. Fig. 2 (A and B) gives the plots obtained for disaccharides I-P and I-S, respectively. In these plots, the average \( \Delta \delta_{\text{max}} \) taken over all monitored backbone resonances is shown as a solid line, and the average \( \Delta \delta_{\text{max}} \) plus one standard deviation is shown as a dotted line. Inspection of these plots reveals that for all binding disaccharides, cross-peaks corresponding to residues in three separate regions in the primary sequence of WT-MIP-1β undergosubstantial Significant disaccharide-induced shifting as evidenced by \( \Delta \delta_{\text{max}} \) greater than the average. The first region corresponds to the N terminus of the protein, including residues Met \(^3\), Gly \(^4\), and, for all disaccharides except III-S, Ser \(^5\) (for III-S, the shifting experienced by Ser \(^5\) was notable, but not above the average \( \Delta \delta_{\text{max}} \)). The second region having substantial chemical shift changes upon addition of disaccharide includes residues Arg \(^{19}\), Lys \(^{19}\), Arg \(^{22}\), Val \(^{25}\), Val \(^{26}\), and Tyr \(^{28}\). Because the intensity of the resonance for Leu \(^{20}\) was too weak to monitor in these experiments and residue 21 is a proline, it is unknown whether either of these residues are affected by disaccharide addition. Finally, the third region encompasses the 40s region, including residues Gln \(^{43}\), Thr \(^{44}\), Lys \(^{45}\), Arg \(^{46}\), Ser \(^{47}\), Gln \(^{49}\), Val \(^{50}\), Cys \(^{51}\), and, for disaccharides I-P and II-S, residues Phe \(^{42}\) and Lys \(^{48}\). Although each of the binding disaccharides was determined to affect almost identical residues of WT-MIP-1β indicating an overlapping binding surface, differences were noted in the magnitude of peak shifting caused by the various disaccharides with I-S > I-P > I-S having higher affinity than I-P, which in turn bound more tightly than II-S and III-S. Some precipitation occurred upon addition of I-S, which may cause error in its average \( \Delta \delta_{\text{max}} \) determination. An additional source of error in \( K_{\text{app}} \) is the concentration of disaccharide used (see “Experimental Procedures” for possible errors in amounts purchased). In the worst case, this error would cause the \( K_{\text{app}} \) to increase by 1.47-fold for disaccharides I-P and I-S. Although significant, this level of error does not affect our comparison between dimeric wild type protein and monomeric MIP(9), because the same stock solutions of disaccharide were used in these titrations.

As a control for the effect of nonspecific binding of sulfate to WT-MIP-1β, the protein was titrated with potassium sulfate to a final concentration of 10 mM. Small concentration-dependent changes in the chemical shifts of the backbone resonances of several residues were observed during the titration (data not shown). Although these resonances generally corresponded to those found to be involved in heparin binding, these shifts were significantly smaller in magnitude than those observed during the titration of WT-MIP-1β with the binding disaccharides and had not reached saturation even at 10 mM sulfate (40 molar equivalents). The small magnitude of peak shifting and the lack of saturation at 10 mM sulfate suggest that the level of nonspecific sulfate binding by wild type MIP-1β is about two orders of magnitude weaker than the binding seen for heparin disaccharides I-S and I-P.

**Titration of MIP(9) with Disaccharides**—MIP(9) is a truncated mutant of MIP-1β that begins at the ninth residue (Thr \(^9\))...
and is known to be a folded monomer in solution (27) that retains the ability to bind the CCR5 receptor (28). Using CB-CA/CO/NH and HNCA data, the assignments for the $^{1}H$-$^{15}N$ HSQC spectrum of MIP(9) were completed (data not shown). Spectral changes in the $^{1}H$-$^{15}N$ HSQC spectrum of MIP(9) were then used to monitor the titration of MIP(9) with the same eleven disaccharides used with WT-MIP-1β. 

Fig. 1B shows the superposition of the series of spectra acquired for several points in the titration of MIP(9) with heparin disaccharide I-P. Throughout the titration experiments, no indication of MIP(9) dimerization was observed as judged by the relative lack of resonance shifting for residues involved in the dimer interface of MIP-1β (27). As for WT-MIP-1β, MIP(9) was found to be in a fast exchange regime with the disaccharides (as evidenced by single resonances with chemical shifts that are the population-weighted averages of the free and bound forms of the protein) and to interact with the same set of disaccharides. A small percentage of unfolded protein is indicated by several peaks in the unstructured region of the spectrum ($^{1}H$ = 8.1–8.4 ppm). These peaks experience a decrease in the signal-to-noise ratio as the titration progresses and, for some resonances, an increase in the line width. Although this may indicate an intermediate timescale exchange process, no disaccharide-induced changes in chemical shift are seen for these peaks, so it appears unlikely that there is any specific interaction between the unstructured residues and the disaccharides. A possible explanation for the behavior of these peaks is a time-dependent aggregation or possibly a nonspecific interaction with the disaccharide, resulting in the aggregation of the unfolded MIP(9) and thereby the disappearance of the peaks.

Fig. 2 (C and D) shows the plots obtained of $\Delta \delta_{\text{max}}$ for the residues of MIP(9) for heparin disaccharides I-P and I-S, respectively. These plots reveal that, for the interacting disaccharides I-P, I-S, II-S, III-S, and Di-disD, residues in two distinct regions of MIP(9) experience sizeable chemical shift changes. The residues Phe$^{13}$, Tyr$^{15}$, Ala$^{17}$, Arg$^{18}$, Lys$^{19}$, and Arg$^{22}$ compose the first region of substantial shifting. Residues in and surrounding the 40s region, including Lys$^{45}$, Arg$^{46}$, Ser$^{47}$, Lys$^{48}$, Val$^{50}$, Cys$^{51}$, and in most cases Gln$^{49}$ and/or Thr$^{44}$, comprise the second region. Although many of the residues found to be involved in MIP(9) binding to disaccharides are the same as those for WT-MIP-1β, some differences were observed. The first obvious difference comes from the fact that MIP(9) lacks the first eight amino acids of WT-MIP-1β and, hence, the first region of WT-MIP-1β interaction (Met$^{4}$-Gly$^{4}$-Ser$^{5}$) can not be seen in MIP(9). The second more subtle difference involves residues surrounding Arg$^{18}$, Lys$^{19}$, and Arg$^{22}$, all of which appear to be involved in disaccharide binding for both WT-MIP-1β and MIP(9). In WT-MIP-1β, residues just C-terminal to these were seen to shift upon disaccharide addition (Val$^{25}$, Val$^{26}$, and Tyr$^{29}$), whereas in MIP(9) the residues immediately N-terminal (Phe$^{13}$, Tyr$^{15}$, and Ala$^{17}$) to this region are affected. Finally, in the 40s region Gln$^{49}$ in WT-MIP-1β was found to have one of the largest peak movements, whereas Lys$^{48}$ shifted less than or just equal to the average. In contrast, in MIP(9) Gln$^{49}$ was found to shift very little and Lys$^{48}$ was consistently the residue with the greatest chemical shift change.

The concentration-dependent disaccharide-induced chemical shift changes of individual backbone amides of MIP(9) were plotted as a function of the ratio $D_{i}/M_{i}$ for each disaccharide, and the data were fit to find $K_{D}^{pp}$ as described above. Fig. 3 (C and D) shows the fitted data for select residues in MIP(9) for the titrations with disaccharides I-P and I-S, respectively. For a given disaccharide, an average macroscopic $K_{D}^{pp}$ was calculated and is reported in Table I and Fig. 4. The $K_{D}^{pp}$ of any residue whose resonance in the HSQC experienced overlap, and residues Arg$^{22}$ and Tyr$^{62}$, which consistently exhibited an anomalously high $K_{D}^{pp}$, were not used in the calculation of the average $K_{D}^{pp}$ reported. However, these residues are included in the discussion of the binding surface (under “Discussion”). The average $K_{D}^{pp}$ varied with the disaccharide tested, with I-S and I-P binding tighter than Di-disD, which bound more strongly than II-S and III-S. MIP(9) was determined to interact less
The average macroscopic $K_{app}^{obs}$ for the MIP-1$\beta$ proteins

The average macroscopic $K_{app}^{obs}$ is determined by averaging the $K_{app}^{obs}$ found for residues whose backbone amide resonances shift greater than the average chemical shift change, as described under "Experimental Procedures" and "Results." The error shown in these values is one standard deviation of the average $K_{app}^{obs}$ calculated. These error values do not include possible error due to slightly incorrect quantities provided by the commercial suppliers (see "Experimental Procedures" and "Results").

### Table I

| Disaccharide | Average $K_{app}^{obs}$ | $\mu$ |
|--------------|-----------------------|-------|
| WT-MTP-1$\beta$ | I-P 44 ± 12 | |
|              | I-S 68 ± 15 | |
|              | II-S 307 ± 117 | |
|              | III-S 246 ± 73 | |
|              | IV-S ND | |
|              | II-H ND | |
|              | III-H ND | |
|              | II-I A ND | |
|              | III-A ND | |
|              | IV-A ND | |
|              | $\Delta\text{Di-dis}^{obs}$ 113 ± 42 | |
| MIP(9) | I-P 94 ± 23 | |
|          | I-S 129 ± 25 | |
|          | II-S 495 ± 146 | |
|          | III-S 566 ± 149 | |
|          | IV-S ND | |
|          | II-H ND | |
|          | III-H ND | |
|          | II-A ND | |
|          | III-A ND | |
|          | IV-A ND | |
|          | $\Delta\text{Di-dis}^{obs}$ 321 ± 82 | |
| MIP-1$\beta$ | I-P 51 ± 16 | |
| D27A/D65A/E67Q | I-S 140 ± 23 | |

**Notes:** ND represents those disaccharides whose average $K_{app}^{obs}$ could not be determined due to little or no interaction with the protein.

NMR Titrations of MIP-1$\beta$ with Disaccharides

The average macroscopic $K_{app}^{obs}$ was calculated for the binding of MIP-1$\beta$ D27A/D65A/E67Q with disaccharides I-P and I-S (Table I).

At pH 6.0, titrations of MIP-1$\beta$ D27A/D65A/E67Q were again conducted with I-P and I-S. As mentioned previously, at least 100 mM NaCl was required to achieve even fairly dilute NMR samples of MIP-1$\beta$ D27A/D65A/E67Q. Under these conditions, no disaccharide-induced chemical shift changes for the protein were observed upon addition of either disaccharide (data not shown), likely due to the NaCl interfering with the interaction. As an alternative approach, heparin disaccharide I-S was added directly to 0.2 mM MIP-1$\beta$ D27A/D65A/E67Q at pH 2.5 to a final concentration of 1 mM disaccharide. The pH was raised to 6.0 with no addition of NaCl, and an HSQC spectrum was acquired. Although some precipitation of the protein was noted upon raising the pH, sufficient protein remained in solution to provide an NMR signal. It is noteworthy that only 1 mM disaccharide was sufficient to keep the protein largely soluble at pH 6.0, whereas, in the absence of disaccharide, at least 100 mM NaCl was required.

Fig. 5B shows the superposition at pH 6.0 of the spectrum of MIP-1$\beta$ D27A/D65A/E67Q in the presence of I-S with a control spectrum of MIP-1$\beta$ D27A/D65A/E67Q containing 150 mM NaCl in the absence of disaccharide. This control spectrum is not ideal because it contains NaCl, which does cause peak shifts, but we were unable to obtain a control spectrum at pH 6.0 in the absence of NaCl due to extensive protein precipitation. Although small in magnitude, clear chemical shift changes are seen between the spectra at pH 6.0 in the absence and presence of heparin disaccharide. All of these shifted residues are the same as those that experienced disaccharide-induced shifts at pH 2.5. These effects would likely be more pronounced if compared with an ideal (no salt) control in the absence of heparin, because the salt required for solubility (greater than 100 mM) of the control spectrum causes some shifting of resonances in the same direction as does heparin at pH 2.5 and at pH 6 (data not shown).

**Discussion**

Disaccharide Selectivity of WT-MIP-1$\beta$ and MIP(9)—Some of the most common GAGs are heparin, heparan sulfate (related to heparin and found on the cell surface), and chondroitin sulfate. At the most basic level, GAGs are comprised of a repeating disaccharide unit of a hexuronic acid and an amino sugar. For heparin and heparan sulfate, the hexuronic acid can be either glucuronic acid or its epimer, iduronic acid, and the amino sugar is N-glucosamine. The repeating disaccharide units of GAGs have variable amounts of N- and O-sulfation and N-acetylation. Kuschert et al. (15) demonstrated that chemokines show varying degrees of selectivity for the different types of GAGs, with the highly basic RANTES demonstrating the most pronounced selectivity and the acidic MIP-1$\alpha$ the least of the chemokines tested. In addition, these studies also demonstrated that the interaction between chemokines and GAGs was dependent on GAG length and required both N- and O-sulfation for optimal binding. The same group has also demonstrated that RANTES, MIP-1$\alpha$, MCP-1, and IL-8 aggregate in the presence of heparin (30), which complicates structural analysis of chemokine-GAG interactions. However, we have...
found that MIP-1β is amenable to study by NMR with disaccharides. Our studies were limited to disaccharides available from commercial sources and so included a set of eleven such disaccharides. Fig. 4 shows the chemical structures of these sugars; unsaturation of the uronic acid unit in the disaccharides is the result of heparin lyase digestion used in the commercial production of these molecules.

Fig. 4 and Table I show the average macroscopic \( K_d^{app} \) values obtained for the binding of these disaccharides to MIP-1β and its variants. Our studies indicate that multiple sulfation of the disaccharide unit is an important factor in the binding of both dimeric WT-MIP-1β and monomeric MIP(9), because non- and mono-sulfated disaccharides were not seen to significantly bind either protein. Of the sugars tested, heparin disaccharides I-P (di-sulfated) and I-S (tri-sulfated) bound the most tightly to WT-MIP-1β with average \( K_d^{app} \) values of \(-44 \pm 12\) and \(68 \pm 15\) \(\mu\)M, respectively. The similarity of dissociation constants for I-P and I-S with WT-MIP-1β indicates that O-sulfation at the two positions that these sugars have in common (positions 2′ of the unsaturated hexuronic acid unit and 6 of the D-glucosamine unit, Fig. 4) are important in the interaction of MIP-1β with disaccharides. Because I-S shows no clear increased affinity, its additional site of N-sulfation at position 2 in the D-glucosamine unit does not appear to facilitate tighter I-S binding. Although showing weaker binding than for WT-MIP-1β, MIP(9) also bound I-P and I-S the most tightly among the disaccharides with average \( K_d^{app} \) values of \(-94 \pm 23\) and \(139 \pm 25\) \(\mu\)M, respectively. It should be noted that errors in the amount of disaccharide purchased (see “Experimental Procedures”) do not affect this conclusion or the argument regarding sulfate selectivity. For both the wild type dimer and the monomeric MIP(9), the binding of heparin disaccharides II-S and III-S was significantly weaker than for I-S or I-P. In accordance with this observation that O-sulfation at both positions 2′ and 6 is important to binding, each of these disaccharides, while being di-sulfated overall, has an N-linked sulfate at the amino moiety of position 2 of the D-glucosamine unit, with the second site of sulfation being O-linked sulfate at position 6 (II-S) or at position 2′ (III-S). The binding of these sugars also produces significantly less NMR peak movement, increasing the error in \( K_d^{app} \) measurements, leading to the larger error bars in Fig. 4 (also Table I). In support of our findings regarding the importance of O-sulfation at positions 2′ and 6, Kuschert et al. (15) found that, although both N- and O-sulfation of GAGs are required for optimal GAG-chemokine binding, enzymatic removal of O-sulfation was more deleterious to chemokine binding than the elimination of N-sulfation.

Chemokines are known to exhibit a range of selectivity for the different types of GAGs (11, 15). Because a number of chondroitin disaccharides are commercially available, we investigated the chondroitin disaccharide \(\Delta\text{Di-dis}_D\), which has a sulfation pattern identical to heparin I-P but contains a D-
galactosamine unit instead of a d-glucosamine unit. Within error, our experiments indicate that the chondroitin disaccharides ΔDis-II binds essentially as well as the heparin disaccharides I-P and I-S to WT-MIP-1β. Therefore, unlike studies using much larger heparin- and chondroitin-like GAG fragments (11, 15), where small differences in selectivity may be additive over the large number of GAG units, little sequence selectivity between heparin and chondroitin for WT-MIP-1β could be inferred using the disaccharides in our binding studies.

Although sulfation appears to influence the binding of disaccharides to dimeric WT-MIP-1β and monomeric MIP(9) in similar manners, the main difference in affinity that our studies reveal between these proteins is a clear trend of WT-MIP-1β binding more tightly than MIP(9). Although there is some overlap in the error range of the two proteins in Fig. 4 and Table I, this is eliminated if only the NMR resonances with the largest magnitude of disaccharide-induced chemical shift changes (and therefore the least amount of error) are used in the calculations.

The GAG Binding Surface of the Wild Type MIP-1β Dimer—The GAG binding surface on WT-MIP-1β was characterized by 1H-15N HSQC NMR spectroscopy using 15N-labeled protein and a total of eleven different heparin and chondroitin disaccharides. The changes in these spectra upon disaccharide titration could result from the direct interaction of the amide group with the GAG, the participation of the side chain of an amino acid (resulting in a shift of its backbone amide resonance), or be due to a general shifting of the structure in that region of the protein. Spheres in Fig. 6A represent the backbone amide nitrogen atoms of all the residues in the dimer of WT-MIP-1β that are demonstrated by the present titration studies to have above-average disaccharide-induced resonance movement. As is evident from Fig. 6A, the distal surfaces of the protein are involved in GAG binding, whereas the intervening regions of the dimer, including much of the dimer interface, and the C-terminal α-helices are unaffected. Fig. 6B shows a single monomeric subunit of the wild type protein with the same residues highlighted. The binding surface contains the basic residues Arg18, Lys45, Arg46, and Lys48 that surround a cleft that includes residues Val15, Val26, Tyr28, Gln43, Thr44, Ser47, Gln49, Val50, and Cys51. In addition, although not included in Fig. 6A or 6B, the side chain of Asn23 (found directly in the binding cleft) is likely to be part of the binding surface, because its side-chain amide resonances were significantly shifted in the presence of disaccharide.

Basic residues at positions corresponding to Arg18, Lys45, Arg46, and Lys48 in MIP-1β are conserved in both MIP-1α and RANTES, the latter three forming a BBXB motif (B representing basic residues), which has been shown to be a common heparin binding motif for a number of proteins (22). For MIP-1β, the involvement of Arg18, Lys45, and Arg46 in heparin binding is consistent with previous studies (8, 13) that demonstrated that mutagenesis of any of these residues in MIP-1β can diminish heparin binding ability, although each mutation alone does not have a large effect. Studies have reported similar results for MIP-1α (14, 19), whereas only the conserved residues in the 40s region (44, 45, 47) are important for RANTES (11, 12). Additionally, the backbone amide resonances of Lys45, not conserved in either RANTES or MIP-1α, and Arg46, not conserved in MIP-1α, were found to undergo considerable disaccharide-induced chemical shift changes in the present studies, but mutation of both of these residues in MIP-1β (13) and modification of the residue corresponding to Arg46 in RANTES (11) appeared to show no effect on heparin binding.

To understand the involvement of Met3, Gly4, and Ser5, the binding of GAGs to MIP-1β must be approached from the perspective of the MIP-1β dimer. Fig. 6B depicts the residues in the monomeric unit of WT-MIP-1β that are affected by disaccharide binding, showing that residues 3 through 5 are located on the opposite end of the subunit from the GAG binding site. This suggests the cluster Met3-Gly4-Ser5 is not involved with GAG binding to its own monomer. On the other hand, in the context of a dimer these residues are located such that they are in close proximity to the conserved basic residues on the other monomer unit as shown in blue in Fig. 6C, and it appears these residues form part of the GAG binding cleft.
affected by GAGs (Fig. 2, A and B). Interestingly, these residues (shown in green in Fig. 6D) are at the back of the GAG binding site (using approximately the same orientation shown in Fig. 6C), on the same side of the site as the dimer residues Met3, Gly4, and Ser5, which form the back of the pocket in the wild type protein.

Since MIP(9) is a monomer, it lacks the full binding pocket provided by the dimer. This not only potentially provides a rationale for the trend toward lower GAG affinity for the monomer as compared with the dimer, but it also appears that residues Phe13, Tyr15, and Ala17 may be recruited in MIP(9) to help form the pocket or are structurally affected due to the elimination of the dimer residues. Furthermore, often the single largest peak movement in monomeric MIP(9) is displayed by residue Lys48, which contrasts with the wild type dimer where this residue is only perturbed somewhat by GAG binding. The location of Lys48 in the wild type dimer is within 5 Å of the dimer residues 3–5, so in the MIP(9) monomer the lack of these residues may cause an increased role for Lys48 in binding GAGs.

MIP(9) is a truncation mutant and does not contain residues 1–8, but if our structural interpretation is correct the lower binding affinity and difference in titration spectra found for MIP(9) are expected to be observable in other MIP-1β monomeric variants that do contain residues 3–5. The N-terminal residues of full-length monomers should not be effective at binding GAGs, because, unlike in the dimer, their distance from the GAG binding site precludes participation in GAG binding. Preliminary titration studies have been conducted on a functional full-length monomeric variant, MIP-1β P8A (28), with heparin disaccharide I-P. These titration studies of MIP-1β P8A with I-P indicate that nearly identical residues exhibit disaccharide-induced chemical shift perturbations as were found for MIP(9), including residues 13, 15, 17, 18, 19, 45–48, and 62 among others.2 The relative overall magnitudes of the perturbations were consistent with those of monomeric MIP(9) rather than that for the wild type dimer. In addition, the affinity of P8A for the I-P disaccharide is even weaker than that for monomeric MIP(9).2 Due to the lack of a dimer in this variant, there is considerable N-terminal heterogeneity in P8A, but the major peaks for residues Met3, Gly4, and Ser5 (which are involved in GAG binding in the dimer) do not shift in the P8A monomer upon GAG titration, consistent with our structural model that the chemokine dimer itself causes the involvement of the residues. However, minor peaks in P8A that have chemical shifts consistent with residues 3 through 5 do shift about an average amount upon titration with I-P (Δδmax ranging from 0.03 to 0.06). The magnitudes of these perturbations are less than seen with the wild type protein, where the resonance for Gly4 (Δδmax = 0.11) consistently displays some of the greatest disaccharide-induced shifting observed for the protein. Work continues on this and other monomeric variants (data not shown).

MIP-1β Binding to Heparin Disaccharide I-S at pH 6.0—Although several chemokines are naturally monomeric (39, 43), many chemokines form dimers even at low concentrations (28, 29). Several CC chemokines, including MIP-1α, MIP-1β, and RANTES, self-associate further to form high molecular weight aggregates in a pH-dependent manner (25, 26), which precludes study of their GAG binding properties at physiological pH by NMR methods. Due to the tendency of WT-MIP-1β and MIP(9) to self-associate, the disaccharide titration studies of these MIP-1β proteins were conducted at pH 2.5, where WT-MIP-1β has been shown to be a stable dimer (24) and MIP(9) a monomer (27). The question is therefore raised whether the

[2 Melissa A. McCormack, Craig K. Cassidy, and Patricia J. LiWang, unpublished.]

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**Fig. 6.** The disaccharide binding site of MIP-1β. Magenta spheres in A and B represent the backbone amide nitrogen atoms of residues of WT-MIP-1β shown by NMR to be affected by disaccharide binding on the dimeric and monomeric units, respectively. The structure used for WT-MIP-1β was determined by NMR at pH 2.5 (24). C, a close up of the proposed binding surface of WT-MIP-1β. Magenta spheres symbolize the backbone amide nitrogen atoms of residues originating from the monomeric unit shown, and those in blue correspond to residues Met3, Gly4, and Ser5 of the other monomer in the dimer. D, the NMR structure determined for the monomeric mutant MIP-1β F13A (42) onto which the GAG binding surface for the monomeric MIP(9) is mapped in magenta spheres. Residues truncated in MIP(9) are shown in light blue at the N terminus. Shown as green spheres are the backbone amide nitrogen atoms of residues 13, 15, and 17, which are affected by disaccharide binding to monomeric MIP(9) but are not affected in dimeric WT-MIP-1β.
GAG binding surface on MIP-1β determined at pH 2.5 accurately portrays the surface under physiological conditions. To answer this question, a mutant of MIP-1β was sought that would not form high molecular weight aggregates at physiological pH. Neutralization of charged residues on the surfaces of MIP-1γ, MIP-1α, and RANTES has been reported to decrease their tendency for self-association at physiological pH (8, 26).

For example, we have shown that the triple mutant MIP-1β K45A/R46A/K48A does not exhibit pH-dependent aggregation at pH 7.0 (8), but this mutant could not be used in the present studies because these mutations adversely affect heparin binding. Studies conducted by Czaplewski et al. (26) demonstrated that a single mutation of either of the acidic residues at positions 26 or 66 in MIP-1α and their counter residues in MIP-1β (Asp27 or Glu67) and RANTES (Glu26 or Glu66) significantly disaggregated the chemokines (26). In our hands, significant salt concentrations were still required to obtain sample concentrations above 0.1 mM for any single point mutant (data not shown). To obtain a MIP-1β mutant that was soluble to NMR-amenable concentrations at pH 6.0, we constructed the mutant MIP-1β D27A/D65A/E67Q.

Fig. 5B shows the spectrum of MIP-1β D27A/D65A/E67Q in the presence of heparin disaccharide IS at pH 6.0, with no salt present except 20 mM sodium phosphate, 0.02% sodium azide. As stated previously, we are unable to obtain a pH 6.0 control spectrum of MIP-1β D27A/D65A/E67Q in the absence of NaCl, so Fig. 5B shows the superposition of the spectrum of the MIP-1β D27A/D65A/E67Q disaccharide IS complex at pH 6.0 with the spectrum of MIP-1β D27A/D65A/E67Q at the same pH in the presence of 150 mM NaCl. This superposition clearly shows chemical shift changes for resonances of the same residues that were seen to shift upon addition of disaccharide to both this mutant and to WT-MIP-1β at pH 2.5. Therefore, the surface determined at pH 2.5 is consistent with the available spectrum at pH 6.0, indicating that studies performed at pH 2.5 are a valid assessment of the surface under physiological conditions.

Biological Significance of Disaccharide Binding Results—The GAG binding surface of MIP-1β forms a clear cleft (Fig. 6C) that involves some of the residues (such as Arg18 and Arg46) that also participate in receptor binding (44). The overlapping nature of the receptor binding surface and the GAG binding groove is consistent with results that GAGs can compete with the receptor for chemokine binding (8, 11, 16). Mutations in the groove is consistent with results that GAGs can compete with the spectrum of MIP-1β B at pH 6.0, with no salt concentrations were still required to obtain sample concentrations above 0.1 mM for any single point mutant (data not shown). To obtain a MIP-1β mutant that was soluble to NMR-amenable concentrations at pH 6.0, we constructed the mutant MIP-1β D27A/D65A/E67Q.

We have used NMR to determine the affinity of wild type MIP-1β and a monomeric variant MIP(9) for several GAG disaccharides. This work has delineated a GAG binding cleft in MIP-1β and shown the importance of GAG-sulfation at the 2′ and 6′ positions of the hexuronic acid and the d-glucosamine units, respectively. In addition, a possible role for the dimer in forming part of the GAG binding site in the chemokine is revealed.

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