Identification of the Glycosaminoglycan Binding Site of the CC Chemokine, MCP-1

IMPLICATIONS FOR STRUCTURE AND FUNCTION IN VIVO

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Chemokines are small 8–10-kDa proteins that control the migration of specific leukocyte populations during inflammatory responses, hematopoiesis, and routine immune surveillance. They exert their biological effects by binding to seven transmembrane G protein-coupled receptors on leukocytes, triggering changes in the cytoskeleton and in adhesive interactions with the extracellular matrix and cell surfaces to produce locomotion (2–4). Inflammatory chemokines can also stimulate further cellular activation, resulting in destructive processes such as lysosomal enzyme release, generation of toxic products from the respiratory burst, and apoptosis (5).

Control over cell populations that are mobilized from the vasculature is determined in large part by the specific chemokines that are secreted and the corresponding receptors that are expressed on the migrating cells. To date, 45 human chemokines and 18 receptors have been discovered. They segregate into four families (CXC, CC, CX3C, and C) based on the pattern of cysteine residues in the ligands (6). They can be further classified as either inducible chemokines, which are expressed as a consequence of physiological stress and inflammation (7), or as constitutive chemokines which are the homeostatic ligands responsible for routine leukocyte trafficking and developmental processes (8, 9). In general, many chemokines bind multiple receptors and many receptors bind multiple chemokines, creating the potential for combinatorial diversity in their functions. Traditionally, the overlapping receptor-binding capacity of chemokines has been perceived as redundant (10). However, there is mounting evidence that different chemokines interacting with the same receptor may activate different sets of signaling pathways, leading to finely tuned cellular responses (11). Moreover, some chemokines act as agonists of some receptors and antagonists of others (12–15).

In addition to chemokine G protein-coupled receptor interactions, chemokines bind both to soluble glycosaminoglycans (GAGs) (16) as well as GAGs immobilized on cell surfaces and the extracellular matrix (17). It has been suggested that chemokine immobilization through the GAG interaction facilitates the formation of haptotactic chemokine gradients and enhances their concentration at the site of production, particularly in the presence of shear forces in blood vessels and draining lymph nodes. Interaction with GAGs may also provide another level of specificity and control to cell migration, beyond that defined by receptor engagement, by selective binding of certain chemokines to different types of GAGs and their diverse isoforms (18–21). Structurally, GAGs are linear polysaccharides containing repeating disaccharide sequences that vary in composition, N- and O-sulfation patterns, and linkage, thus leading to an enormous level of diversity (22, 23). The most common GAGs are heparan sulfate, heparin (a highly sulfated form of heparan sulfate), chondroitin sulfate, dermatan sulfate, keratin sulfate, and hyaluronic acid. With the exception of hyaluronic acid and heparin, GAGs are normally covalently linked to proteins as glycosaminoglycan proteoglycans (GAGPs) (16), which are then immobilized on cell surfaces in the extracellular matrix (17). In addition to the presence of GAGPs, the extracellular matrix contains many other glycosaminoglycans (GAGs) (16) as well as GAGs immobilized on cell surfaces and the extracellular matrix (17). The GAG chain length is determined in large part by the specific chemokines that are secreted and the corresponding receptors that are expressed on the migrating cells. To date, 45 human chemokines and 18 receptors have been discovered. They segregate into four families (CXC, CC, CX3C, and C) based on the pattern of cysteine residues in the ligands (6). They can be further classified as either inducible chemokines, which are expressed as a consequence of physiological stress and inflammation (7), or as constitutive chemokines which are the homeostatic ligands responsible for routine leukocyte trafficking and developmental processes (8, 9). In general, many chemokines bind multiple receptors and many receptors bind multiple chemokines, creating the potential for combinatorial diversity in their functions. Traditionally, the overlapping receptor-binding capacity of chemokines has been perceived as redundant (10). However, there is mounting evidence that different chemokines interacting with the same receptor may activate different sets of signaling pathways, leading to finely tuned cellular responses (11). Moreover, some chemokines act as agonists of some receptors and antagonists of others (12–15).

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attached to membrane bound "core" proteins, although these proteoglycans can also be processed, releasing small fragments that can have either inhibitory or activating functions (24). The presence of carboxylate and sulfate groups on the GAG chains gives rise to an overall negative charge and facilitates the presence of carboxylate and sulfate groups on the GAG chains that can have either inhibitory or activating functions (24). The proteoglycans can also be processed, releasing small fragments.

The importance of the GAG interaction is supported by the fact that chemokines bind to purified glycosaminoglycans in vitro (25, 26) and to endothelial cell surface GAGs in vitro (25) and in vivo (17, 20, 27). Removal of GAGs by glycosidase treatment also causes a marked reduction in chemokine binding to receptor bearing cells (25). Recently, we showed that GAG binding was essential for the ability of MCP-1/CCL2, RANTES/CCL5, and MIP-1α/CCL4 to promote cell migration in vivo (1). This result was demonstrated with GAG-binding deficient mutants of these chemokines that had near-wild type function in chemotaxis assays in vitro; however, they could not induce cell migration in an in vivo intraperitoneal recruitment assay. In parallel, we also explored the functional relevance of chemokine oligomerization to migration in vivo, since it has been shown that although many chemokines form dimers, oligomerization is not required for receptor binding and chemotaxis in vitro (28–30). However, using monomeric variants of the same chemokines, we demonstrated that oligomerization is required for in vivo cell recruitment (1). We also showed that binding to GAGs induces oligomerization of RANTES/CCL5, suggesting that the in vivo requirements for GAG binding and oligomerization are functionally coupled.

In the present study, we explored the structural details of the interaction of MCP-1/CCL2 with the GAG heparin. By characterizing a panel of MCP-1/CCL2 mutants in several assays of heparin binding, we defined the GAG binding hotspots on MCP-1/CCL2. We also further investigated the relationship between GAG binding and oligomerization of MCP-1/CCL2 using sedimentation equilibrium and cross-linking experiments. Combining these data with previous crystallographic results supports a structural model of the MCP-1-heparin interaction. The structure suggests several ways in which GAGs could provide an additional level of fine control to chemokine function.

**EXPERIMENTAL PROCEDURES**

**Materials**—Unfractionated heparin was purchased from Sigma. Size-defined heparin oligosaccharides were from Neoparin, Inc. (San Leandro, CA). Tritiated heparin was purchased from PerkinElmer Life Sciences.

**Production of Chemokines**—MCP-1/CCL2 mutant expression constructs were made in the context of MCP-1 M64I, which will be referred to as wild type (WT). It has been demonstrated that M64I behaves identically to WT in binding and activity assays (31). The gene was constructed by standard gene synthesis techniques with optional codon usage for expression in *Escherichia coli* as previously described (28). Mutant plasmids were generated by a two-step PCR protocol or by the QuickChange method (Stratagene). After verification by sequencing, plasmids were transformed into *E. coli* TAP302 cells. Cell growth, protein expression and protein purification were carried out as previously described (28). If needed, proteins were subsequently treated with aminopeptidase (Peprotech, Rock Hill, NJ) to remove the initiating methionine and repurified by reverse-phase high pressure liquid chromatography. For all experiments except sedimentation equilibrium and cross-linking experiments, this mutant was used. Electro spray mass spectrometry was used to confirm the expected mass of the mutants.

**In Vitro Heparin Binding Assays**—Several assays were used to identify the GAG binding site on MCP-1. As an initial screen, the amount of salt required to elute WT and mutant MCP-1 from a heparin chromatography column was determined as previously described (32), with a few minor modifications. Briefly, 1 ml of 10 μM protein (in 10 mM potassium phosphate, pH 7.5) was injected onto a 5-ml heparin-Sepharose column (Bio-Rad Econopac) equilibrated in the same buffer. The column was eluted at a flow rate of 3 ml/min in 10 mM potassium phosphate, pH 7.5, using a NaCl gradient from 0 to 1 M over 30 min. The gradient was monitored using a conductivity meter, calibrated such that 0 and 1 M NaCl corresponded to 0 and 100% conductivity, respectively. This assay was performed at least three times for each mutant. To measure the contribution of various residues to the specificity of the interaction, the amount of salt required to elute mutants from a nonspecific 8-Sepha-rose column (Bio-Rad Econopac) was also determined using the same protocol and compared with the elution from the heparin-Sepharose column. The specificity index is related to ΔΔNaCl as calculated from the formula below, where the subscripts H and S refer to elution from the heparin-Sepharose or 8-Sepharose column, respectively (32),

$$\Delta \Delta [\text{NaCl}] = \Delta [\text{NaCl}]_H - \Delta [\text{NaCl}]_S$$

(Eq. 1)

where $\Delta [\text{NaCl}]_H = [\text{NaCl}]_H - [\text{NaCl}]_\text{WT}$, $[\text{NaCl}]_\text{mutant}$ and $\Delta [\text{NaCl}]_S = [\text{NaCl}]_S - [\text{NaCl}]_\text{WT}$, $[\text{NaCl}]_\text{mutant}$.

As an independent screen of the GAG binding affinity of the various mutants, a tritiated heparin binding assay was employed (1). In contrast to the Sepharose assays, which are biased toward electrostatic interactions, this assay is thought to reflect the overall binding capacity of a given protein. The assay was done by incubating increasing concentrations of chemokine with [3H]heparin (50 nCi/ml) in 100 μl of phosphate-buffered saline for 1 h at 37 °C. The solutions were transferred to 96-well plates fitted with Whatman cellulose phosphate filters and washed three times with phosphate-buffered saline under vacuum. After the addition of 50 μl of scintillant, the radioactivity was counted. Measurements were made at least in triplicate for all mutants.

Finally, to determine apparent affinities, a solution-based isothermal fluorescence assay was carried out (33). Unfractionated porcine mucosal heparin (average molecular mass = 15 kDa) was titrated into a 1 μM solution of chemokine dissolved in phosphate-buffered saline, and the change in fluorescence emission of the single tryptophan residue (Trp28) was measured. Measurements were made on a PerkinElmer Life Sciences LS50B fluorimeter using excitation and emission wavelengths of 282 and 340 nm, respectively, and a 290-nm cut-off filter. The binding isotherms were analyzed by nonlinear regression to an equation describing a bimolecular association reaction. We describe the resulting number as an apparent affinity because of the assumptions made in the curve fitting to a bimolecular reaction, when changes in aggregation state seem to be occurring (see below).

**Peritoneal Cell Recruitment**—An in vivo peritoneal cell recruitment assay was performed as previously described (1). Briefly, 8–12-week-old female Balb/c mice (Janvier, Le Genest St. Isle, France) were injected intraperitoneally with 200 μl of sterile NaCl (0.9%, lipopolysaccharide free) or with 10 μg of chemokine diluted in the same buffer. At 18 h postinjection, mice were sacrificed, the peritoneal cavity was washed, the lavage was pooled, and the cells were counted with a hemocytometer.

**Sedimentation Equilibrium Ultracentrifugation**—Sedimentation experiments were performed on a Beckman Optima XL-A ultracentrifuge at speeds of 20,000–30,000 rpm at 25 °C. All proteins were prepared at a concentration of 6 μM (the lowest concentration that gave reasonable data) in 10 mM KPO4, 50 mM NaCl, pH 7.5, and premixed with specific molar ratios of heparin saccharide (protein/saccharide = 1:1, 2:1, 4:1, and 1:2). Size-defined heparin oligosaccharides ranging from tetrasaccharide to dodecasaccharide, as well as unfractionated heparin, were used in these experiments. Unless indicated otherwise, the MCP-1 in these experiments contained the initiating methionine. Protein concentrations were determined from the extinction coefficient (34) and molecular weight specific to each mutant. Six-channel cells were scanned every hour, at 226 nm, where none of the saccharides absorb. Samples were deemed at equilibrium when the last three scans superimposed, usually in about 10–16 h, and data were collected in five replicates with step size of 0.001 cm. Data analysis was performed using the Beckman/ Microcal Origin version 4.0 software package (Beckman Coulter, Inc., Palo Alto, CA) based on the Marquardt-Levenberg nonlinear least squares fitting algorithm. Data from each run was first fit to the ideal noninteracting single-species model,

$$A_r = A_{\text{exp}} \frac{HM/r^2 - r^2}{r^2} + E$$

(Eq. 2)

where $A_r$ represents the absorbance at radius $r$, $A_{\text{exp}}$ is the absorbance at reference radius $r_0$, $r$ is the solvent density, $\nabla$ is the partial specific volume, $r$ is the angular velocity of the rotor, M is the apparent monomer molecular weight of the solute, and $E$ is the baseline offset. Each data set was then successively fit to monomer-dimer, monomer-
tetramer, monomer-octamer, monomer-dimer-tetramer, monomer-tetramer-octamer, and dimer-tetramer-octamer self-association models, where the monomer molecular weight was fixed, and association constants for the relevant oligomeric species were allowed to vary according to the following equation:

\[
A_i = \exp(n_i \ln A_i + \ln K_{n_i} + \ln H_n M(r^2 - r_0^2)) + \exp(n_i \ln A_i + \ln K_{n_i} + \ln H_n M(r^2 - r_0^2)) + E \quad (Eq. 4)
\]

Here the stoichiometries \( n_i \) and, if relevant to the particular model, \( n_0 \), are defined by the user to describe monomer-mer, or monomer-mer-mer-mer equilibrium. \( K_{n_0} \) is the association constant for the assembly reaction of \( n_0 \) monomers; likewise, \( K_{n_2} \) is the association constant for the assembly of \( n_0 \) monomers. Time-dependent aggregation was noted if the absorbance at the midpoint of overlaid spectra decreased during the course of a run.

The monomeric mass used for the MCP-1-GAG complex was the sum of the protein monomer and the saccharide, normalized by stoichiometry and assuming 100% association. The partial specific volume of each MCP-1 mutant was determined from amino acid composition according to the method of Cohn and Edsall (35). The partial specific volume of the MCP-1-GAG complex was calculated as a mass-weighted average of the partial specific volume of MCP-1 (0.737 ml/g) and heparin (0.47 ml/g) (36), assuming 100% association. The solvent density was assumed to be 1.00 g/ml. The quality of the fit was characterized by the variance and by examination for systematic deviation of the residuals.

**Chemical Cross-linking of MCP-1-Saccharide Complexes**—Cross-linking between MCP-1 and heparin oligosaccharides was performed with minor modifications of the zero-length two-step procedure (37-39). The main advantage to this method is that only protein and saccharide in direct contact with each other should be cross-linked, since there is no spacer. 200 mM size-defined heparin was first activated with 6 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and 15 mM sulfosuccinimide in 10 mM MES, pH 6.0, 50 mM NaCl to convert some of the saccharide carboxyl groups into succinimide esters. The reaction was allowed to proceed at 25°C for 1 h, at which time it was quenched with 2-mercaptoethanol (20 mM final). Twenty min after quenching, 25 \( \mu \)g of MCP-1 was added in the same buffer to allow activated saccharide to react with primary amine and lysine e-amino groups of the protein, and the reaction was allowed to proceed for 2 h at 25°C (final concentration = 25 \( \mu \)g protein and 100 \( \mu \)g oligosaccharide). Laemmli buffer was then added to each sample, and samples were electrophoresed on a 12% Tris-Tricine gel. Cross-linked products were detected by silver stain.

**RESULTS**

**Identification of the GAG Binding Site on MCP-1**—Twenty-two single alanine mutants were generated to define the GAG binding site of MCP-1. To ensure that the mutations did not induce structural perturbations, only residues with solvent-accessible surface areas exceeding 30% were mutated. For all single point mutants that had reduced receptor binding affinity, we had previously recorded \( ^1H-^{15}N \) HSQC spectra to demonstrate structural integrity (28, 31). In the present study, we also recorded \( ^1H-^{15}N \) HSQC spectra of the key double mutant R18A/K19A (discussed below). For this mutant, as for the others, chemical shift changes are localized to the vicinity of the mutation. Thus, all of the mutants discussed in this paper appear structurally unperturbed (see Supplemental Material for comparison of the WT spectra and that of R18A/K19A).

The mutants were then characterized in a number of *in vitro* GAG binding assays using heparin as a representative GAG. Although heparan sulfate has been found as a cell surface proteoglycan (40), it is not the most physiologically relevant GAG with respect to surface localization. However, it is thought to be structurally and chemically similar enough to the more ubiquitous cell surface heparan sulfate to serve as a good prototype for initial investigations. In fact, almost all known GAG-binding proteins interact with the highly sulfated “S domains” of heparan sulfate, justifying its use. Furthermore, chemokine binding to extracellular matrix and other structures has been shown to correlate strongly with the avidity of binding to heparin *in vitro* (41).

We used four different assays of heparin binding to obtain the most reliable definition of the MCP-1 GAG binding epitopes. The first assay involved quantifying the concentration of NaCl required to elute chemokine from a heparin Sepharose column. The contribution of a particular residue was estimated by the decrease in [NaCl] required to disrupt heparin binding for each mutant relative to WT (\( \Delta [\text{NaCl}]_{50} \)). Whereas WT MCP-1 eluted at 546 ± 10 mM NaCl, R18A, K19A, R24A, and K49A showed decreased affinity, eluting at 387 ± 8, 448 ± 6, 414 ± 8, and 453 ± 7 mM, respectively (Fig. 1a, Table I), suggesting that these residues constitute the primary GAG binding site. No other basic residues showed a significant effect, including Lys\(^{58}\) and His\(^{66}\), residues previously reported to be critical for GAG binding (42). Other variants in the panel of mutants included a monomeric variant, P8A, and the mutant Y13A, whose ability to dimerize is greatly destabilized compared with WT MCP-1 (28). These variants exhibited a small decrease in affinity for heparin, revealing a contribution of oligomerization to GAG binding affinity.

The extended polymeric nature of GAGs, it was not surprising that no single mutation had a drastic impact on heparin binding. Thus, we generated several double and triple mutants to amplify the effects (Fig. 1a, Table I). The double mutant with the least affinity for heparin was R18A/K19A. When three of the primary GAG binding residues were mutated to make R18A/K19A/R24A, there was a further decrease in the apparent affinity. Likewise, P8A/R18A/K19A exhibited a decrease in binding relative to the double mutant, consistent with the effect of P8A alone. As expected from the elution profile of the single mutants, the double mutant K58A/H66A behaved similarly to WT. However, both double mutants R18A/K58A and R18A/H66A showed a slight decrease in the elution salt concentration relative to R18A alone, suggesting a small involvement of Lys\(^{58}\) and His\(^{66}\) in GAG binding.

To investigate the contribution of each residue to the specific interaction with heparin, binding of the mutants to a nonspecific cation exchange column (S-Sepharose) was measured for comparison with heparin Sepharose. On S-Sepharose, all mutants eluted within a narrower range of [NaCl] than from heparin-Sepharose (Table I). For most of the mutants, \( \Delta [\text{NaCl}] \) values were negative (Fig. 1b), indicating a higher affinity for the nonspecific S-Sepharose column. However, consistent with the data in Fig. 1a, the mutants R18A, K19A, R24A, and K49A had positive \( \Delta [\text{NaCl}] \) values, indicating that these residues contribute most to the specific interaction with heparin. Double and triple mutants of these residues exhibited even more positive values. The triple mutant P8A/R18A/K19A showed a significant increase in \( \Delta [\text{NaCl}] \) over the double mutant R18A/K19A, again reflecting a contribution of oligomerization to binding affinity. Single and double mutants containing Lys\(^{58}\) and/or His\(^{66}\) showed negative values. However, mutation of His\(^{66}\) in the context of R18A (R18A/H66A) resulted in a more positive \( \Delta [\text{NaCl}] \) than either mutation alone, suggesting a small contribution of H66A to the specific interaction. The charge reversal mutant R18E had a similar [NaCl]\(_{50}\) as compared with R18A/K19A but also showed slightly lower specific heparin affinity, consistent with the absence of a contribution from Lys\(^{58}\).

The caveat to the Sepharose assays is that they are biased toward detecting residues involved in electrostatic interactions, which may not be the sole contributor to GAG binding. Therefore, we also carried out a tritiated heparin binding assay. In Fig. 2, representative binding curves for WT and select mutants are illustrated. The single point mutants R18A and K19A showed reduced affinity, whereas the double mutant R18A/K19A was severely impaired in heparin binding (Fig. 2a).
As expected from the Sepharose assay, K49A and R24A also showed reduced binding capacity, as did the monomeric mutant P8A (Fig. 2b). Additionally, we discovered that if the initiating methionine was not removed, the apparent affinity for heparin was increased. For example, H66A had reduced heparin binding capacity compared with WT, but (Met+)H66A has WT affinity (Fig. 2c). Similarly, K69A showed a similar binding capacity as WT, whereas the capacity of (Met+)K69A...
exceeded that of WT (not shown). These results were not detected in the Sepharose chromatographic assays due to their insensitivity to interactions other than electrostatics. The major discrepancy between the Sepharose and the tritiated heparin binding assays is that K58A, H66A, and K58A/H66A did not show a reduction in heparin binding compared with WT in the Sepharose assay, whereas they did in the tritiated heparin assay (Fig. 2), as previously reported (42).

Using an isothermal fluorescence titration (IFT) assay, we were able to obtain apparent dissociation constants (Kd values) for the interaction of the mutants with unfractionated heparin (Fig. 2c, Table I). The Kd for WT MCP-1 was 1.55 μM, in good agreement with a previous report (42). The data also reconfirm that Arg18, Lys19, Arg24, and Lys49 are all critically involved in the interaction to an extent that accurate Kd values were not measurable (Kd values >10 μM) for these single point mutants. P8A again showed a reduced affinity relative to WT, whereas the addition of the N-terminal Met increased the affinity. Mutation of some of the other basic residues resulted in WT or slightly greater affinity, which was somewhat surprising but nevertheless discounts the mutated residues as GAG-binding epitopes. The major discrepancy again involved mutants containing K58A and H66A. In contrast to the tritiated heparin assay, the fluorescence assay indicates that K58A behaves like WT, and H66A has a slightly higher affinity than WT, more in line with results from the Sepharose assays.

As a final physiological test, we examined several of the mutants in an in vivo intraperitoneal recruitment assay (1). Consistent with the small effects observed for single point mutants in the in vitro binding assays, these mutants showed virtually no effect on recruitment in vivo compared with wild type (Fig. 3). The exception to this was P8A, which was unable to recruit, as previously demonstrated (1), although P8A recruits as well as WT in vitro (28). The double mutant, R18A/K19A, also showed little capacity to induce migration, despite the fact that it also recruits nearly as well as WT in vitro (1). Interestingly, K58A/H66A showed a greatly diminished ability to induce cellular recruitment, confirming a contribution of Lys58 and His66 to GAG binding.

Integrating all of the data, it is clear that Arg18, Lys19, Arg24, and Lys49 constitute the primary GAG binding residues. The Sepharose and IFT experiments suggest that Lys58 and His66 contribute only a small amount to the interaction. However, the tritiated heparin assay and the in vivo data clearly demonstrate that these residues are involved, although perhaps to a lesser extent.

From a technical perspective, the results also suggest that performing a single assay to define GAG binding epitopes may yield data that underestimates or overestimates the contribution of specific residues, depending on the assay. However, we do not presently have a complete explanation for the discrepancies between the assays.

Sedimentation Equilibrium Suggests That a Heparin Octasaccharide Induces Tetramer Formation of MCP-1—To fur-
ther explore the relationship between GAG binding and oligomerization, we performed sedimentation equilibrium experiments on MCP-1 and several mutants in the presence of size-defined saccharides. MCP-1 alone (6 μM) fits well to a monomer-dimer equilibrium as previously described (Fig. 4a).

However, upon the addition of a heparin octasaccharide, oligomerization was significantly enhanced (Fig. 4b). An octasaccharide was the largest molecular weight heparin that remained entirely in solution in the presence of WT MCP-1 under the solution conditions used, and in the presence of unfractionated heparin, the complex precipitated almost immediately. When fit to a single species, the molecular weight determined from the sedimentation curve using either a 2:1 or 1:1 molar ratio of 6 μM MCP-1 to octasaccharide was ~91.5 and 30.1 kDa, respectively, approaching a tetrameric species (expected tetramer molecular mass, 44.8 and 40.0 kDa). However, the residuals were poor, indicating that the model is an inaccurate description of the macromolecular species present (Fig. 4g).

When we allowed for self-association, MCP-1-octasaccharide fit best to a monomer-tetramer-oligomer equilibrium model (Fig. 4, b and g), and (Met-)MCP-1-octasaccharide fit best to a monomer-dimer-tetramer equilibrium model (not shown). In both cases, the data also fit reasonably well to a monomer-tetramer equilibrium model (Fig. 4g), suggesting that the tetramer is present in considerable quantity (there is little monomer under these conditions).

The addition of saccharides smaller than an octasaccharide showed a decreasing ability to induce tetramers of MCP-1. WT MCP-1-hexasaccharide (1:1) fit best to a monomer-dimer-tetramer equilibrium, but with less tetramer content. MCP-1-tetrasaccharide (1:1:1) fit best to a single species with a molecular mass of 17.9 kDa, assuming no bound saccharide (expected dimer molecular mass, 17.6 kDa); if we assume that the saccharide binds, the fit is worse (observed mass, 16.0 kDa; expected dimer mass, 20.0 kDa). For experiments with MCP-1 in the presence of decasaccharide and dodecasaccharide, time-dependent aggregation occurred, the extent of which correlated with chain length. Still, sufficient amounts of complex remained in solution in both cases to permit curve fitting to different association models. Both fit best to a monomer-tetramer-oligomer, whereas models with a dimer component (e.g. monomer-dimer; monomer-dimer-tetramer) fit poorly. Together, these data suggest that MCP-1 oligomerizes into higher order assemblies on longer GAG chains, with the tetramer as a discrete fundamental substructure.

The mutant P8A is monomeric in solution, even above millimolar concentrations (Fig. 4c). In contrast to WT MCP-1, it remained predominantly monomeric upon the addition of equimolar octasaccharide (Fig. 4d). In the presence of the longer decasaccharide, P8A fits best to a single species with molecular mass of 13.4 kDa (expected mass assuming 100% binding is 11.8 kDa), indicating that it remains primarily monomeric as well.

The double mutant R18A/K19A was dimeric in the absence of heparin, as has been observed for WT MCP-1. Upon the addition of stoichiometric amounts of octasaccharide, R18A/K19A remained largely unaffected (Fig. 4, e and f), consistent with in vitro assays that all demonstrate its impaired heparin binding ability. By contrast, the K58A/H66A mutant behaved far more like WT, showing evidence of oligomerization in the presence of equimolar octasaccharide (data not shown). The measured mass was 26.7 kDa if forced into a single species, somewhat smaller than the molecular weight determined from fitting WT to a single species (although the fit had poor residuals). The best fit was to a monomer-dimer-tetramer equilibrium. These data confirm that although Lys58 and His66 play a role in the GAG interaction, they contribute less than Arg18 and Lys19.

Cross-linking Experiments with WT MCP-1 and Oligosaccharides Support Tetramer Formation—To confirm observations from the sedimentation equilibrium experiments, we captured MCP-1-GAG complexes using a zero-length two-step cross-linking assay specifically designed for protein-GAG interactions (37–39). Fig. 5 shows the products of cross-linking reactions between WT (Met+)MCP-1 and heparin oligosaccharides of different lengths ranging from tetramers (dp4) to dodecamers (dp12) (where dp represents degree of polymerization). In agreement with sedimentation equilibrium observations, we observe a discrete species corresponding to the MCP-1 tetramer in the presence of dp8, dp10, and dp12 (lanes 6–8) heparin oligosaccharides, but not in the presence of dp4 or dp6 (lanes 4 and 5) heparins. This result confirms that the octamer is the minimum saccharide length required to promote substantial tetramer formation under our experimental conditions. We also observed the formation of a small amount of discrete octamers in the presence of octasaccharide and longer saccharides, also consistent with sedimentation data. Last, the cross-linking reaction of MCP-1 in the presence of unfractionated heparin (molecular mass, ~15 kDa; average length, ~dp50) produces a smear of large molecular weight complexes with a very intense band at the top of the gel, most likely consisting of clusters of tetramers and octamers on the GAG chain like “beads on a string.”

DISCUSSION

In this study, we identified residues that contribute to the interaction of MCP-1 with glycosaminoglycans by employing several in vitro heparin binding assays. In order of importance, the key residues are Arg18 > Arg24 > Lys19 > Lys49. The role of Lys and Arg residues is consistent with contacts between protein and heparin observed in the crystal structures of complexes and the Arg-Lys-rich sequences of peptides selected from random libraries with heparin or heparan sulfate (22). Whereas the trinitiated heparin binding assay and the in vitro recruitment assay identified Lys58 and His66 as contributors to GAG binding, the sedimentation, IFT, and Sepharose data suggest that they contribute less to the interaction than the other residues.

Fig. 6, b and c, shows the GAG binding residues mapped onto the surface of the MCP-1 monomer in comparison with the residues previously identified as being important for receptor binding and signaling (31, 43). There is significant overlap
between the residues that comprise the receptor binding and the GAG binding site. Fortunately, single mutations of Arg18 and Lys19 to alanine have only a small effect (<5-fold) on receptor binding and activity (31). Thus, it was possible to specifically design a double mutant, R18A/K19A, with greatly impaired GAG binding, while preserving its receptor binding ability (IC50 = 1.4 nM compared with 0.08 nM for WT). With this mutant, we previously showed that although the protein retained robust activity in vitro, it was unable to stimulate cell migration in vivo, confirming the essential role of MCP-1-GAG interactions (1).

Using the monomeric mutant of MCP-1, P8A, we previously...
showed that in addition to GAG binding, chemokine oligomerization is required for in vivo function (1). PSA is as active as WT in binding and chemotaxis assays in vitro, but it does not recruit in vivo. The question is why. Intuitively, a higher affinity interaction should be achievable by oligomerization either through an avidity effect, with more than one GAG binding domain contributing to the interaction with GAG polymers, or by the formation of oligomerization-induced GAG binding motifs. Indeed, PSA binds less well to heparin than WT; however, the reduction in affinity is only a factor of five. Along this line, reduction does not provide a strong explanation for the inability of the mutant to recruit cells in vivo. This point is particularly borne out by the fact that the single mutations of Arg18, Lys19, Arg24, and Lys49 all show more pronounced effects on affinity for heparin than WT, but that a tetrameric variant of RANTES/CCL5 was unable to cause cell migration in vivo but that a tetrameric mutant elicited wild-type chemotaxis in vivo (1). The presence of both dimerization motifs within a CC chemokine calls into question the prevailing notion that CC chemokines only oligomerize via CC-like dimer interfaces and CXC chemokines only oligomerize via CXC-like dimer interfaces. A third, unique, interface is generated by the confluence of all four subunits (Fig. 7, a and d).

Interestingly, if we highlight the GAG binding hotspots onto the surface of the tetramer, they map out a large positively charged surface that encircles the tetramer (Fig. 7, d–f). This pattern is only formed by a tetrameric assembly and provides a compelling recognition surface for a linear sulfated biopolymer like heparin (Fig. 7g). The key GAG binding residues (Arg18, Lys19, Arg24, Lys49) lie along the interface defined by the four subunits, whereas Lys60 and His66 are in the C-terminal helices that comprise part of the CXC interface. Thus, it appears that GAG binding stabilizes the interaction between subunit interfaces that are not normally associated in solution. The highly charged and complementary nature of heparin with the GAG binding site on MCP-1 suggests a large component of electrostatic stabilization. We can also rationalize the finding that retention of the N-terminal Met increases the apparent affinity for heparin. In the context of the tetramer, the Met is in close proximity to the GAG binding surface (Fig. 7g) and could have direct, favorable interactions with heparin. This is consistent with the crystal structure of RANTES/CCL5 in complex with disaccharides, which shows an interaction between the protein N terminus and the saccharide. Alternatively, it may stabilize the tetramer, which would indirectly increase the affinity for heparin by reducing the entropic cost of GAG-induced tetramer assembly.

We used an octasaccharide to capture the fundamental substructure of the MCP-1-GAG interaction by sedimentation, because longer GAGs cause precipitation. Additionally, because of the small size of the octasaccharide, we could discount the possibility that the tetramer is actually two pairs of dimers bound to the GAG (note the comparison of the length of the dodecasaccharide to the GAG binding site in Fig. 7g). With stoichiometries of 1:1 and 2:1 MCP-1/octasaccharide, tetramer formation was observed, whereas at 4:1 the amount of tetramer decreased; thus, we believe the stoichiometry of the complex is 2:1 MCP-1/octasaccharide. This result can be rationalized in view of the structure of the tetramer and GAG binding epitopes, where an octasaccharide would be sufficient to span the major site (Arg18, Lys19, Arg24, and Lys49), one bound to each of the interfaces formed by the four subunits. It is also consistent with the fact that shorter GAGs are incapable of inducing tetramer formation. However, longer GAGs on the order of 20 show higher affinity interactions for other chemokines like PF4/CXCL4 and interleukin-8/CXCL8. Thus, in principle, a single long GAG chain could wrap around the surface of the tetramer, as has been suggested for PF4/CXCL4 (47).

This structure is consistent with several proposed functional
roles of GAG binding and oligomerization. Fig. 7, g–i, shows a comparison of the GAG and receptor-binding surface in the context of the tetramer. As mentioned above, residues that contribute most to GAG binding also contribute to receptor binding. Thus, it seems unlikely that monomeric MCP-1 could bind to GAGs and its receptor concurrently. However, oligomerization could provide a mechanism for simultaneous interaction by binding of GAGs to some of the subunits of the tetramer and receptor binding to other subunits. In this “hand-off” model, binding of the receptor would then require disassembly of the chemokine oligomer to allow the receptor to fully interact with the entire ligand binding site, since key CCR2 binding residues are buried in the oligomerized state (see below). Alternatively, oligomerized chemokines on GAGs may simply serve as cell surface or soluble reservoirs of ligand (Fig. 8). Whether surface-bound or soluble, the tetramer may facilitate accumulation on GAGs at higher density than either monomers or dimers, which in turn could lead to quantitative differences in receptor engagement and signaling output.

GAG binding and oligomerization may also be involved in modulating the activity of chemokines. As shown in Fig. 7, h and i, the N termini and Tyr13, the key signaling residues in MCP-1, are much less exposed in the context of the tetramer compared with the monomer (Fig. 6b); thus, GAG binding and tetramer formation could have an inhibitory effect on receptor binding and transendothelial migration as observed in vitro (18, 48). Sequestration of the N terminus could also have a protective effect by inhibiting proteolytic processing of the N terminus (49), an important mechanism for regulating chemokine function and receptor specificity. Finally, oligomerized chemokines could, in principle, transmit different signals than monomeric chemokines to receptor-bearing leukocytes (50, 51), or via cell surface GAGs on the endothelium (52). In this regard, RANTES/CCL5 has been shown to cause cellular activation in a G protein-coupled receptor-independent fashion, by binding to the GAG chains of CD44 (52). Several of these functional roles are illustrated in Fig. 8, all or some of which may dynamically regulate chemokine function depending on the local microenvironment.

Comparison of the GAG binding site of MCP-1 and other chemokines reveals a great deal of diversity in the range of their binding affinities, ability to discriminate between different GAGs, and spatial distribution of GAG binding epitopes (32, 41, 53–55). In contrast to MCP-1, the GAG binding site of interleukin-8/CXCL8 is localized primarily to the C-terminal helix (residues Arg65, Lys64, Lys67, and Arg68 and Lys20 in the N-loop) (32). Considering the length dependence of GAG binding affinity and the distribution of the GAG epitopes in the context of the dimer, a horseshoe-like binding surface for GAGs was proposed (32) and subsequently refined by docking stud-
Fig. 7. Structural views of the MCP-1/CCL2 tetramer (Protein Data Bank number 1dol) generated with Pymol (46), with each subunit color-coded red, green, yellow, or cyan and functional epitopes highlighted as described. a, ribbon diagram showing the unique tetramer interface in front where all four subunits associate. b, same as a but rotated 90° to show CXC-like dimer subunits in front and back. c, same as a but rotated 90° to show CC dimer subunits in front and back. d, surface topology model of the MCP-1/CCL2 tetramer in the same orientation as a, e. Same as d but rotated 90° to show CXC-like dimer subunits in front and back. f, same as d but rotated 90 degrees to show CC-like dimer subunits in front and back. In d-f, the GAG binding epitopes are highlighted in blue. g, surface topology model in the same orientation as d but with the GAG binding epitopes highlighted in blue and Met⁰ highlighted in marine. To the right is the NMR structure of a fully sulfated dodecasaccharide (Protein Data Bank number 1hpn) to show the size comparison relative to the GAG binding surface and the suitability of the surface for binding linear sulfated GAGs. Sulfate groups are highlighted in yellow. h, i, the receptor binding epitopes are highlighted for comparison with the GAG binding epitopes. h, same orientation as d and g but with the receptor (CCR2) binding epitopes highlighted. The color coding is the same as in Fig. 6b. The N-terminal signaling residues are much more sequestered in the context of the tetramer than in the monomer. i, same orientation as in c and f so that the CC dimer subunits are in front and back. Additional receptor binding epitopes are observed on the CC subunit interfaces, whereas, as seen in f, there are no GAG binding epitopes on this face. Again, the N-terminal signal residues are more protected, and Tyr¹³, also required for chemotaxis, is largely buried.
solution of the crystal structure revealed an MCP-1/PF4-like tetramer (57). Additionally, two other crystal forms revealed a novel tetrameric assembly containing a 12-stranded $\beta$-sheet. Like MCP-1/CCL2, the tetramers may be inducible in solution by GAG binding, and the different types of tetramers may have different GAG specificities. These emerging structural and thermodynamic details of chemokine-GAG binding support the hypothesis that the interaction may provide an additional layer of specificity to the localization of chemokines and regulation of their function.

In summary, there is accumulating evidence that interactions with GAGs of the extracellular matrix and cell surfaces may have a profound effect on cellular responses to chemokines. Given the postulated specificity of chemokine-GAG interactions, the cell type-specific expression of different GAGs, and their dynamic modification during inflammation, disrupting these interactions may prove a valuable approach for the treatment of a wide variety of diseases. Indeed, certain pathogens like Yersinia and Staphylococcus recruit heparyn and other sulfated polysaccharides as broad spectrum binding sites that block immunologically important proteins, including chemokines (58).

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Glycosaminoglycan Binding Site of MCP-1/CCL2