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Structure and Function of the Glycosaminoglycan Binding Site of Chemokine Macrophage-Inflammatory Protein-1β

Witte Koopmann, Chandrika Ediriwickrema, and Michael S. Krangel

The ability of chemokines to bind to glycosaminoglycans (GAGs) on cell surfaces and in the extracellular matrix is thought to play a crucial role in chemokine function. We investigated the structural basis for chemokine binding to GAGs by using in vitro mutagenesis to identify amino acids of chemokine macrophage-inflammatory protein-1β (MIP-1β) that contribute to its interaction with the model GAG heparin. Among six basic residues that are organized into a single basic domain in the folded MIP-1β monomer, three (R18, K45, and R46) were found to contribute significantly to heparin binding. Of these, R46 was found to play a dominant role, and proved essential for the interaction of MIP-1β with both heparin and heparan sulfate in physiological salt. The results of this mutational analysis have implications for the structure of the MIP-1β-heparin complex, and a comparison of these results with those obtained by mutational analysis of the MIP-1α-heparin interaction suggests a possible structural difference between the MIP-1β-heparin and MIP-1α-heparin complexes. To determine whether GAG binding plays an important role in receptor binding and cellular activation by MIP-1β, the activities of wild-type MIP-1β and R46-substituted MIP-1β were compared in assays of T lymphocyte chemotaxis. The two proteins proved equipotent in this assay, arguing that interaction of MIP-1β with GAGs is not intrinsically required for functional interaction of MIP-1β with its receptor.


Chemokines interact with a large family of seven-transmembrane-spanning, G protein-coupled receptors on target cells (1, 3); additionally, they interact with glycosaminoglycans (GAGs) on cell surfaces, in the extracellular matrix, and in exocytic granules. Chemokines have been shown to bind to purified subfractions of heparin in vitro (4), as well as to naturally occurring GAGs such as heparan sulfate and chondroitin sulfate in the extracellular matrix in vitro (5), and on the surface of endothelial cells both in vitro (6–8) and in vivo (9). Furthermore, chemokines have been found to be stored in and released from platelet α-granules (10, 11) and T lymphocyte cytolytic granules (12) in association with GAGs.

The ability of chemokines to bind to GAGs is thought to be critical for chemokine biology. It has been proposed that the immobilization of chemokines by GAGs forms stable, solid-phase chemokine foci and gradients necessary to direct leukocyte trafficking in vivo (13, 14). Consistent with this notion, immobilized IL-8 has been shown to promote neutrophil migration both in vitro (15) and in vivo (9). Furthermore, GAG-immobilized MIP-1α and RANTES have been shown to promote leukocyte adhesion in vitro (5, 14). GAG binding may also influence chemokine structure and activity in other ways. For example, the binding of IL-8, MIP-1α, RANTES, and MCP-1 to GAGs has been shown to promote their multimerization in vitro (8). Binding of chemokines to cell surface GAGs may also serve to increase their effective local concentration, and consequently increase their binding to cell surface receptors (8). Additionally, GAG binding could potentially influence chemokine t½ in vivo. As chemokines vary in the affinity (and perhaps the specificity) with which they interact with GAGs (4), a detailed understanding of chemokine-GAG interactions may be critical to appreciate functional distinctions among chemokines that may have been judged by other criteria to be functionally redundant.

The molecular determinants of chemokine binding to GAGs have been studied in several instances. The α chemokines carry a highly basic, amphipathic carboxyl-terminal α-helix. In the cases of IL-8 and PF4, this structure has been shown to be important for binding to acidic GAGs (16, 17, 40). Additional basic residues...
within the PF4 framework have been found to interact with GAGs as well (18, 40). Among β chemokines, basic residues within the MCP-1 α-helix were found to be important for GAG binding (41). However, we (19) and others (20) previously noted that other β chemokines do not have highly basic α-helices, and that other regions of these molecules must be important determinants of GAG binding. We used in vitro mutagenesis to test the roles of individual basic amino acids of huMIP-1α, and identified three noncontiguous amino acids (R18, R46, and R48) that were each essential for heparin binding (19). A fourth residue, K45, was found to contribute as well. Similarly, a double mutation of K45 and R46 was shown to abrogate heparin binding of murine MIP-1α in another study (20). Based on a modeled MIP-1α structure, all four basic residues are predicted to lie on one face of the molecule, with the side chains of R18, R46, and R48 aligned and protruding prominently from the surface.

The minimal GAG binding motif found in huMIP-1α is conserved in other β chemokines, but may be augmented by additional basic residues. As an example, huMIP-1β contains basic residues at positions 18, 45, 46, and 48, and, in addition, at positions 19 and 22. The solved MIP-1β structure (21) indicates that the side chains of all of these residues lie in proximity on one face of the molecule, leading to the prediction that MIP-1β uses an expanded binding motif to bind more tightly to GAGs. Consistent with this idea, we have found that MIP-1β interacts more tightly with heparin than MIP-1α, as judged by the concentration of salt required to disrupt the interaction. Nevertheless, mutagenesis experiments indicate that only a subset of these residues contributes detectably to GAG binding. Interestingly, functional analysis of one MIP-1β mutant indicates that, as for MIP-1α, GAG binding is not required for functional interaction of MIP-1β with its receptor.

Materials and Methods

Molecular cloning of wild-type huMIP-1β and huMIP-1β-SEAP

huMIP-1β full-length coding region cDNA was cloned from PHA/PMA-stimulated HMC-1 human mast cells (22, 23) by RT-PCR using a primer pair derived from the 5’ and 3’ ends of the published MIP-1β sequence (24). Primers included EcoRI sites to facilitate cloning. The sequences of the primers used are: 5’-CCCGAATTCACAAATACCCGAAGCCGACAGCCAGGCGCATCCGCTCCCTCCTCTTGCGGTTTG-3’ sense and 5’-CCACGAAGCCGACAGCCAGGCGCATCCGCTCCCTCCTCTTGCGGTTTG-3’ antisense. The cDNA, which included 11 bp of 5’ untranslated sequence, was subcloned into the mammalian expression vector pCAGGS (25, 26) and sequenced in its entirety using Sequenase (United States Biochemical, Cleveland, OH). The sequence obtained was identical to the published huMIP-1β sequence Act-2 (24) with the exception of an S to G substitution at position 70. This substitution has previously been reported for another huMIP-1β isoform (27). pDREF-hyg-MIP-1β-SEAP and pDREF-hyg-MIP-1β-R46A-SEAP, which encode fusion proteins in which MIP-1β is linked via a five-amino-acid carboxyl-terminal linker to secreted alkaline phosphatase, were produced as described.6

Site-directed mutagenesis

Mutagenesis was performed by extension overlap amplification (28) using 400 ng of pCAGGS-MIP-1β as template. Note that the numbering refers to amino acid residues of the mature, secreted protein. The sequences of the mutagenic primers used are as follows: MIP-1β R18A sense, 5’-TTTACACCGCGCAAAGAGGCTCTCTCTCTTCGTGCGGTTTG-3’; MIP-1β K44A antisense, 5’-CAACACCAAGGGCGCATCCCGCTCCCTCCTCTTGCGGTTTG-3’; MIP-1β K45A antisense, 5’-CAAAAGGCGCGGCCGCTTCCGCTCCCTCCTCTTGCGGTTTG-3’.

PCR products were subcloned into pCAGGS and sequenced in their entirety using Sequenase (Amersham, Arlington Heights, IL).

Transient transfection and metabolic labeling

Transient transfection of COS-P fibroblasts with wild-type and mutant pCAGGS-MIP-1β constructs and metabolic labeling were performed exactly as described (19). Transient transfection of 293/EBNA-1 cells with pDREF-hyg-SEAP constructs was performed as previously described (29).

Heparin-Sepharose chromatography

 Supernatants containing metabolically labeled wild-type or mutant MIP-1β were mixed with supernatants containing MIP-1β-SEAP fusion protein and chromatographed on 1 ml Hi-trap heparin columns (Pharmacia Biotech, Piscataway, NJ), as described (19). The NaCl gradient used for elution was 0–800 mM; salt concentrations in individual fractions of a sample gradient were determined using a conductivity meter (WVR Model 1050). Radioactivity in each fraction was determined by liquid scintillation counting using an LKB 1290 Rackbeta scintillation counter, and by SDS-PAGE (30), followed by fluorography (31). Alkaline phosphatase activity was determined by incubation with 1 mg/ml p-nitrophenyl phosphate at 37°C and measurement of absorbance at 405 nm using an Anthos Labtech HT-2-2001 plate reader. For the experiment shown in Fig. 4, radiolabeled chemokines were first partially purified by chromatography on Hi-trap Q-Sepharose columns (Pharmacia Biotech). Peak Q-Sepharose eluate fractions were pooled and applied to 1 ml Hi-trap heparin columns in buffer containing 150 mM NaCl. Columns were washed in the same buffer, and bound chemokines were eluted with a linear 150–500 mM NaCl gradient. Flow-through and eluate fractions were pooled and analyzed by SDS-PAGE and fluorography.

Purification of wild-type and mutant MIP-1β proteins

Unlabelled wild-type and mutant MIP-1β proteins were purified from supernatants of transiently transfected COS-7 cells by sequential chromatography on Q-Sepharose and heparin-Sepharose columns. Following transfection, cells were cultured for 48 h, as described (19). The plates were then washed twice in PBS and the medium was replaced with phenol red-free DMEM (Life Technologies, Gaithersburg, MD). Cells were cultured for an additional 24 h, and the supernatants were collected. Supernatants (200 ml) were pooled and subsequently applied to 5 ml Hi-trap Q anion-exchange columns (Pharmacia Biotech) previously equilibrated in buffer B (50 mM Tris-HCl, pH 8, 5 mM EDTA) at a flow rate of 0.5 ml/min. Columns were washed with 20-column volumes of buffer B, and chemokines were eluted with a linear 0–500 mM NaCl gradient in buffer B. Peak chemokine-containing fractions were identified by SDS-PAGE and silver staining and pooled. The partially purified chemokines were diluted to 50 mM NaCl in buffer B and applied to 1 ml Hi-trap heparin columns. Columns were washed with SDS-PAGE and fluorography, pooled, and applied to 1 ml Hi-trap heparin columns in buffer containing 150 mM NaCl. Columns were washed in the same buffer, and bound chemokines were eluted with a linear 150–500 mM NaCl gradient. Flow-through and eluate fractions were pooled and analyzed by SDS-PAGE and fluorography.

Solid-phase heparan sulfate-binding assay

Wild-type and R46A mutant MIP-1β-SEAP fusion proteins were partially purified from supernatants (15 ml) of transiently transfected 293/EBNA-1 cells by chromatography over 1 ml Hi-trap Q anion-exchange columns developed with a linear 0–1 M NaCl gradient. Peak chemokine-containing eluate fractions were identified by alkaline phosphatase assay and were dialyzed against 20 mM Tris-HCl, pH 8. A solid-phase heparan sulfate-binding assay was modeled after a previously described solid-phase heparin-binding assay (33). The wells of a polystyrene 96-well plate (Corning, Acton, MA) were coated with 50 µl of 125 µg/ml bovine kidney heparin sulfate (Sigma H7640) or porcine kidney medulla heparin sulfate (34) (kind gift of Dr. Robert Linhardt, University of Iowa, Iowa City, IA, and Dr. Toshihiko Toida, Chiba University, Chiba, Japan) by incubation at 37°C for 16 h. The wells were rinsed twice with 20 mM Tris-HCl, pH 8, containing 0.05% (w/v) BSA, followed by a third rinsing with PBS. The wells were allowed to stand for 2 h at 23°C before aspiration. A total of 50 µl of 5 nM chemo kinase-SEAP fusion protein in 20 mM Tris-HCl, pH 8, and varying concentrations of NaCl were introduced into the wells for a 2-h incubation at
23°C. Wells were rinsed three times with 20 mM Tris-HCl, pH 8, containing 0.05% (w/v) BSA, and bound fusion proteins were quantified by alkaline phosphatase assay.

Chemotaxis assay

The ability of wild-type and mutant MIP-1β to stimulate chemotaxis of activated human T lymphocytes was assessed using a 48-well Boyden chamber assay. Human PBMC were isolated with LSM (Organon Teknika, Durham, NC), washed four times in serum-free RPMI 1640 (Life Technologies), and resuspended in RPMI 1640 containing 10% FCS. Adherent cells were removed by two rounds of adherence to plastic tissue culture flasks for 1 h at 37°C. Nonadherent cells were cultured for 16 h at 37°C in tissue culture plates coated with a culture supernatant containing anti-CD3 Ab OKT3 (kind gift of Dr. C. Doyle, Duke University, Durham, NC). Cells were harvested, washed, and resuspended in RPMI 1640 supplemented to 20 mM HEPES-NaOH, pH 7.4, and 2% (w/v) BSA. Purified chemokines in the same medium were added in triplicate to the bottom wells of the chemotaxis chamber (NeuroProbe, Cabin John, MD) in a volume of 28 μl. The wells were overlaid with a 5 μm polyvinylpyrrolidone-free polycarbonate chemotaxis filter (NeuroProbe) that had been precoated for 45 min at 37°C with 5 μg/ml type IV collagen (Sigma, St. Louis, MO), as described (14). Cells (5 × 10^4/well) were added to the top wells and allowed to migrate for 2 h at 37°C. Filters were stained with Diff-Quik (Baxter Scientific Products, McGaw Park, IL), and cells migrated to the underside of the filter were counted in five randomly selected high power (×400) fields.

Results

huMIP-1β contains six basic residues, at positions 18, 19, 22, 45, 46, and 48. Based on the MIP-1β structure (21), all lie in proximity on one face of the molecule (Fig. 1). To determine which of the basic amino acids contributes to the ability of MIP-1β to bind to GAGs, we examined the interaction of wild-type MIP-1β and single amino acid mutants of MIP-1β with heparin-Sepharose. We expressed wild-type MIP-1β and alanine substitution mutants R18A, K19A, R22A, K45A, R46A, and K48A using a COS cell transient expression system, and harvested the supernatants of metabolically labeled cells. SDS-PAGE analysis of unfractionated COS cell supernatants revealed a 10- to 12-kDa species present in the supernatants of transfected cells that was not present in the...
supernatants of mock-transfected cells (Fig. 2A). As observed previously in similar transient expression analysis of MIP-1α (19), MIP-1β represented the most abundant radiolabeled species in these supernatants. We also expressed wild-type MIP-1β in the form of a nonradiolabeled fusion protein with SEAP by transient transfection of 293/EBNA-1 cells (29), 6 to be used as an internal control for chromatography experiments.

Supernatants of metabolically labeled and unlabeled cells were diluted to reduce the ionic strength, mixed, and applied to heparin-Sepharose columns, as described previously (19). Bound chemokines were then eluted by application of a linear salt gradient, and were identified in eluate fractions by determination of total radioactivity, by SDS-PAGE followed by fluorography, and by measurement of alkaline phosphatase activity. Co-chromatography of radiolabeled wild-type MIP-1β with unlabeled wild-type MIP-1β-SEAP fusion protein yielded peaks that essentially coeluted at 400 mM NaCl (Fig. 3). SDS-PAGE analysis across the profile confirmed that the majority of radioactivity in the peak fractions represented MIP-1β (Fig. 2B). This analysis confirmed that the SEAP moiety neither contributes to nor interferes with heparin binding by MIP-1β. By comparison with MIP-1β, MIP-1α elutes at 250 mM NaCl (19). The requirement for significantly higher salt concentrations to elute MIP-1β is consistent with a stronger interaction, perhaps mediated by additional basic residues unique to MIP-1β.

Each of the six alanine-substituted MIP-1β mutants was then co-chromatographed on heparin-Sepharose along with MIP-1β-SEAP as an internal control. Alanine substitutions in mutants K19A, R22A, and K48A had no apparent effect on binding and elution from heparin-Sepharose (Fig. 3). However, the substitutions in mutants R18A and K45A resulted in peaks of radioactivity that eluted at salt concentrations (250–300 mM) that were reduced as compared with the wild-type fusion protein. For each of the mutants, the peak radioactive fraction was confirmed to include MIP-1β as a predominant component (Fig. 2C). The profile for mutant R46A was complex in that two peaks of radioactivity were identified, one eluting at very low salt concentrations (less than 100 mM), and one eluting at a position only slightly shifted from

![Graphs showing heparin-Sepharose chromatography results](https://example.com/graph.png)
the wild-type MIP-1β fusion protein. For mutant R46A, SDS-PAGE analysis across the chromatographic profile (Fig. 2D) indicated that the first radioactive peak eluting from the column represented MIP-1β, whereas the second peak represented unrelated background proteins that were also represented, albeit at lower levels, in the wild-type elution profile (Fig. 2B). Additional experiments indicated that the relatively low ratio of MIP-1β R46A to background proteins reflected the fact that a portion of MIP-1β R46A flowed through the heparin-Sepharose column without binding (data not shown). We conclude that alanine substitutions at R18 and K45 have small but readily detectable effects on the interaction of MIP-1β with heparin, whereas an alanine substitution at position R46 has a dramatic effect on this interaction.

To further characterize heparin binding by MIP-1β R46A, we generated highly enriched preparations of metabolically labeled wild-type MIP-1β and MIP-1β R46A by ion-exchange chromatography on Q-Sepharose. Analysis of the Q-Sepharose eluates by SDS-PAGE revealed that radioactivity was associated almost exclusively with the MIP-1β proteins (Fig. 4A). The highly enriched preparations of MIP-1β and MIP-1β R46A were then applied to a heparin-Sepharose column in a buffer containing 150 mM NaCl, and the column was developed with a linear salt gradient. Under these conditions, a small amount of radioactivity in the wild-type preparation flowed through the column, but the vast majority bound and was eluted at 400 mM NaCl (Fig. 4B). SDS-PAGE analysis indicated that the eluate was exclusively MIP-1β, whereas the flow-through consisted primarily of higher m.w. contaminants (Fig. 4A). In contrast, MIP-1β R46A was found exclusively in the flow-through (Fig. 4, A and B). Hence, MIP-1β R46A does not stably interact with heparin when binding is assessed under conditions of physiological salt concentrations.

To ask whether the R46A mutation abrogates interaction between MIP-1β and GAGs that occur naturally on cell surfaces and in the extracellular matrix, we assessed the binding of MIP-1β and MIP-1β R46A to plastic-immobilized heparan sulfate in vitro (Fig. 5). Because sulfation levels vary among heparan sulfate chains isolated from different sources, we analyzed binding to both bovine kidney heparan sulfate, which displays a relatively low degree of sulfation, and porcine kidney medulla heparan sulfate, which displays a high degree of sulfation (34). Partially purified preparations of MIP-1β-SEAP and MIP-1β R46A-SEAP fusion proteins were incubated with the immobilized heparan sulfate preparations at several salt concentrations, and binding was assessed by alkaline phosphatase assay. In this binding assay, the signals detected in the presence of 0.75 M NaCl represent nonspecific, salt-insensitive binding. Relative to this binding, wild-type MIP-1β bound well to both bovine kidney and porcine kidney medulla heparan sulfate in subphysiological salt (30 mM), but only bound to the more highly sulfated porcine kidney medulla heparan sulfate in physiological salt. Importantly, binding of MIP-1β R46A was undetectable at both physiological and subphysiological salt concentrations, indicating that the R46A mutation abrogates the ability of MIP-1β to bind to a physiologically relevant GAG ligand.

To compare the in vitro biological activities of wild-type MIP-1β and MIP-1β R46A, we purified unlabeled MIP-1β and MIP-1β R46A from the supernatants of transiently transfected cells by ion-exchange chromatography, followed by heparin-Sepharose chromatography with loading in low salt, as described in Materials and Methods. The MIP-1β preparations obtained by this approach were highly purified (Fig. 6A). The wild-type (as well as a commercially available) MIP-1β preparation failed to induce significant increases in cytoplasmic calcium concentration in either monocytes or lymphocytes, and displayed a relatively poor chemotactic index in assays of monocyte chemotaxis (data not shown). Therefore, we assessed the ability of the wild-type and mutant MIP-1β preparations to stimulate the chemotaxis of activated human peripheral blood T lymphocytes. Both preparations displayed good chemotactic indices and yielded typical bell-shaped curves with maximal migration at about 10 ng/ml MIP-1β.
Cradle may not be appropriate to describe the huMIP-1β module III-13 with heparin (37). Hence, the notion of a cationic cradle lining the cleft were found to influence the interaction of fibronectin-

(Fig. 6B). As the activities of the two preparations were indistinguishable by this functional assay (the slight difference between the profiles at the highest concentration tested was not reproducible), we conclude that the ability of MIP-1β to bind to GAGs is unnecessary for functional interaction of MIP-1β with its receptor on T lymphocytes. We furthermore conclude that the dramatic effect of the R46A mutation on GAG binding does not result from any gross perturbation in the structure of the MIP-1β protein.

Discussion

huMIP-1β includes six basic residues that are found in two segments of the linear amino acid sequence (R18, K19, R22 = segment 1; K45, R46, K48 = segment 2), and are organized into a single basic domain in the folded MIP-1β monomer. Segment 2 conforms precisely to the BBXB pattern of basic residues implicated as a heparin-binding motif in other studies (35), whereas segment 1 displays a related BBXXB pattern. Interestingly, the basic domain in the folded MIP-1β structure takes a form that appears related to the cationic cradle implicated in the binding of lactoferrin (36) and fibronectin (37) to heparin. This structure consists of six basic residues arranged with three on each side of a central cleft. For MIP-1β, residues 18 and 19 appear to line one side of a cleft, and residues 48, 46, 45, and 22 the other side. This organization suggests that all six residues could play significant roles in mediating interactions between MIP-1β and heparin. Our data indicate otherwise: R46 is apparently critical for the stable interaction of MIP-1β with heparin, R18 and K45 play secondary roles, and K19, R22, and K48 have roles that, if significant, are not detectable in our assays. In contrast, mutations in all six residues lining the cleft were found to influence the interaction of fibronectin module III-13 with heparin (37). Hence, the notion of a cationic cradle may not be appropriate to describe the huMIP-1β heparin binding site.

HuMIP-1α, which elutes from heparin-Sepharose at lower salt concentrations than huMIP-1β, carries basic amino acids at positions 18, 45, 46, and 48, but lacks the basic amino acids at positions 19 and 22 that are contained within huMIP-1β. If huMIP-1β residues K19 and R22 were responsible for the substantially different salt elution properties of huMIP-1β and huMIP-1α, we should have detected an effect of mutations at these positions in our assays. Furthermore, our data present an apparent paradox in that the more avid interaction of MIP-1β with heparin seems to be mediated by fewer basic residues (three rather than four). Recent experiments by Hoogewerf et al. (8) may suggest an explanation. These investigators found that several chemokines bound to immobilized heparin as tetramers, whereas MIP-1α bound as a dimer. If MIP-1β (which was not examined in that study) were to bind as a tetramer, binding of the MIP-1β tetramer to GAGs would have contributions from 12 basic residues (3 per monomer), whereas the MIP-1α dimer would have contributions from 8 basic residues (4 per monomer), providing an adequate explanation for the distinct heparin-binding properties of the two molecules.

Why might MIP-1β residues R18, K45, and R46 be selectively involved in the interaction with heparin? Residues R18 and R46 are notable in that their side chains are aligned with each other and protrude directly out from the surface of the MIP-1β monomer in a fashion that can easily be envisioned to interact with heparin (Fig. 1). Although the side chain of K45 is not aligned similarly, R18, K45, and R46 do form a relatively focused basic patch on the surface of the molecule. Interestingly, in a MIP-1β dimer, the basic patches of the two monomers face away from each other on opposite ends of the molecule. If MIP-1β binds to heparin as a multimer (either a dimer or tetramer), the positioning of the basic patches would suggest that the heparin chain wraps around the MIP-1β multimer.

Of the remaining basic residues in MIP-1β, R22 is probably not involved in heparin binding because it extends away from the basic patch formed by R18, R48, and K45. A clear explanation for the apparent absence of involvement by K19 is less certain. On the other hand, the absence of a significant role for K48 can probably be best understood as a function of its orientation in the MIP-1β dimer (21). Whereas R18, K45, and R46 create a pair of basic patches on opposite ends of the dimer, the K48 residues of each monomer face toward each other on the concave surface of the dimer. As such, this surface of the dimer most likely plays no role in heparin binding. Given this conclusion, it is striking that residue R48 plays an important role in heparin binding by the highly homologous MIP-1α (19). This might be consistent with distinct quaternary structures for the MIP-1β-heparin and MIP-1α-heparin complexes.

It has long been argued that chemokine binding to GAGs may be important for stabilization of chemokines in the form of solid-phase gradients for presentation to passing leukocytes (13, 14). However, recent experiments have suggested that there is even greater and more fundamental involvement of GAGs in chemokine function. In support of this notion, enzymatic removal of cell surface GAGs results in a significant reduction in the apparent affinity of RANTES for its cell surface receptor (8). Heparan sulfate synergizes with RANTES to inhibit HIV-1 replication in monocytes (12). Furthermore, removal of cell surface GAGs has been shown to block the inhibitory effect of RANTES on HIV-1 replication in PM1 T cells (38) and to block intracellular Ca2+ signaling in PBL (39). GAG-induced RANTES oligomerization and a GAG-induced increase in local RANTES concentration could be critical to potentiate the interaction of RANTES with its specific receptor (8), or GAGs could represent a fundamental component of the receptor-ligand complex. In contrast, although an IL-8 mutant that binds poorly to GAGs displays reduced activity in a neutrophil chemotaxis assay that is thought to depend on both receptor binding and solid-phase immobilization, the same mutant displays normal activity in a neutrophil elastase release assay that is thought to depend on receptor binding only (9). Further, heparin binding mutants of MCP-1 stimulated normal Ca2+ signaling and chemotaxis

FIGURE 6. Chemotactic activity of wild-type MIP-1β and MIP-1β R46A. A, Purified wild-type MIP-1β and MIP-1β R46A were prepared as described in Materials and Methods and analyzed by 15% SDS-PAGE, followed by silver staining. B, Chemotaxis of activated human T lymphocytes was assessed using a Boyden chamber. The number of cells migrated was presented for one of two comparable experiments. The shaded bar represents background migration with no added chemokine.
of THP-1 cells (41). Different chemokines may therefore be differentially dependent on GAG binding for efficient interaction with their receptors.

We recently showed that a singly substituted MIP-1α mutant that fails to interact with heparin displays wild-type binding to CCR1 and wild-type Ca2+ signaling (19). Although a two-amino-acid heparin-binding mutant of MIP-1α generated by Graham et al. failed to bind to and signal through CCR1 (20), it seems likely that in this case the mutation coincidentally disrupted either an adjacent or distal receptor binding site. Importantly, both studies found wild-type MIP-1α to bind normally to CCR1 on GAG-deficient CHO cells. Thus, GAG binding is not critical for the functional interaction of MIP-1α with its receptor.

The results presented in this work indicate that the same is true for MIP-1β. Wild-type MIP-1β and MIP-1β R46A were indistinguishable in assays of T cell chemotaxis. From this we infer that the two proteins interact indistinguishably with one or more cell surface receptors on activated T cells, and that these interactions do not depend on cell surface GAGs. Interestingly, Oravecz et al. found that heparitinase digestion of PM1 T cells resulted in a dramatic inhibition of the antiviral effect of MIP-1β, a result that can be interpreted to indicate that cell surface GAGs facilitate the interaction of MIP-1β with its receptor (38). Assuming that the T cell chemotactic and antiviral effects of MIP-1β are both mediated by CCR5, the mechanistic implications of the present study and that of Oravecz et al. (38) are clearly distinct. It remains possible that heparitinase treatment of cells modulates the antiviral activity of MIP-1β by a mechanism that is more complex than that originally envisioned.

We note that our results do not necessarily imply that receptor binding and activation by MIP-1β occur independently of GAGs in all instances. These parameters may vary according to both the biological assay and the receptor involved. In addition, because we found that MIP-1β interacts preferentially with highly sulfated GAGs, any dependence of GAGs for binding or activation may vary according to tissue and/or cell type.

In conclusion, the work presented in this study dissects the heparin binding site of huMIP-1β, and provides evidence that the cationic cradle motif previously proposed to mediate the interaction of GAGs with fibronectin and lactoferrin does not accurately describe the GAG binding site of MIP-1β. In addition, the description of a non-GAG-binding mutant MIP1β that stimulates chemotaxis in vitro extends our earlier findings with MIP-1α, in that the capacity to bind to GAGs and to bind to and stimulate chemokine receptors can be functionally uncoupled for both molecules. GAG binding may nevertheless be critical for activities of these chemokines that depend specifically on their immobilization. Experiments designed to address the role(s) of GAG binding to chemokine biology in vivo are in progress.

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