Chemokines bind to receptors of the seven-transmembrane type on target cells and also bind to glycosaminoglycans (GAGs), including heparin. In this study, we have sought to identify structural motifs mediating binding of the β-chemokine macrophage inflammatory protein-1α (MIP-1α) to GAGs. Alignment of β-chemokine amino acid sequences revealed the presence of several highly conserved basic amino acids, and molecular modeling predicted that the side chains of three of the basic amino acids fold closely together in MIP-1α. Site-directed mutagenesis was used to change the conserved basic residues in MIP-1α to alanines, and both wild-type and mutant proteins were produced in a transient COS cell expression system. Wild-type MIP-1α bound to heparin-Sepharose, while three of the mutants, R18A, R46A, and R48A, failed to bind. Mutant K45A eluted from heparin-Sepharose at lower NaCl concentrations than wild type, while the binding of K61A, with a mutation in the C-terminal α-helix, was indistinguishable from that of the wild-type protein. To determine whether GAG-binding capacity is required for receptor binding and cell activation, we performed competition radioligand binding and calcium mobilization experiments using one of the non-heparin-binding mutants, R46A. R46A bound as efficiently as wild-type MIP-1α to CCR1 and was equally active in eliciting increases in intracellular free calcium concentrations. Our data define a GAG binding site in MIP-1α consisting of three noncontiguous basic amino acids and show that the capacity to bind to GAGs is not a prerequisite for receptor binding or signaling in vitro.

The initiation of a focal inflammatory response requires a tightly regulated sequence of events. Circulating leukocytes must attach to endothelium, become arrested and activated, and undergo extravasation to reach inflammatory foci. The molecular processes underlying these events are beginning to be understood (for reviews, see Refs. 1 and 2). A cascade of events, including selectin-mediated rolling attachment of leukocytes to endothelium, integrin-mediated arrest of leukocytes, and the provision of directional cues for migration by chemottractants all are crucial for the development of an inflammatory response. The chemokines are a family of small chemottractant cytokines implicated in the attraction and activation of a variety of leukocytes (3–6). They may be broadly divided into two main classes. α-Chemokines, also known as CXC chemokines due to the spacing of two conserved cysteine residues by a single amino acid, are mainly active on neutrophils. The β- or CC-chemokines, in which the first two conserved cysteines are adjacent, are mainly active on monocytes and lymphocytes.

It is clear from both in vivo and in vitro studies (3–6) that chemokines provide directional cues for the migration of leukocytes to inflammatory sites. What is not clear, however, is the mechanism by which the chemokine concentration gradient required for chemotaxis arises. A soluble chemokine gradient would not be expected to be stable, particularly under conditions of blood flow in the circulation. It has been suggested, rather, that an immobilized, substrate-bound gradient of chemokines is responsible for the chemotaxis of leukocytes (7, 8). For example, the α-chemokine IL-8,1 in the solid phase, has been demonstrated to induce migration of neutrophils in vitro (9). Furthermore, it is known that chemokines bind to glycosaminoglycans (GAGs) (10); binding of chemokines to GAGs, either at the surface of endothelial cells or in the extracellular matrix, might thus serve to establish an immobilized chemokine gradient and “present” the molecules to leukocytes in vivo. In support of this idea, the β-chemokine MIP-1β, immobilized by attachment to solid phase GAG, is capable of stimulating leukocyte adhesion (11). Similarly, Gilat et al. (12) have shown that MIP-1β and RANTES bind to an ex vivo extracellular matrix preparation in a heparinase-sensitive manner and that these bound chemokines are then capable of stimulating leukocyte adhesion.

To begin to understand the role of GAG association in the function of chemokines, we have sought to identify GAG binding sites in the β-chemokine MIP-1α (13). Interactions between the acidic GAGs and proteins are largely electrostatic (14) and thus require basic amino acids. Often, these interactions involve α-helical structures with regularly spaced basic residues (15). The structures of several chemokines, both α- and β- have been solved, and all include three antiparallel α-strands with an overlying α-helix at the C terminus of the protein (16–21). In the α-chemokines, the C-terminal helix tends to be highly basic and has been shown to mediate GAG association for PF-4 and IL-8 (22, 23). However, the C-terminal helices of at least some β-chemokines tend to have a lower concentration of basic residues. Since all of the α- and β-chemokines examined to date bind to GAGs, and since basic residues often contribute to GAG binding of proteins, we sought to delineate additional basic structural motifs conserved among β-chemokines. By aligning the amino acid sequences of a number of β-chemokines, we identified four conserved basic amino acids. In MIP-1α, two of these are found at positions 18 and 48, while two are adjacent at positions 45 and 46 and are predicted to lie in a turn between

1 The abbreviations used are: IL-8, interleukin-8; GAG, glycosaminoglycan; MIP, macrophage inflammatory protein; PF-4, platelet factor 4; sulfo-EGS, ethylene glycol bis(sulfosuccinimidyl succinate); CCR1, CC-chemokine receptor 1; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary; RANTES, regulated on activation normal T cell expressed and secreted.
β-strands. These residues, as well as the single basic residue in the MIP-1α C-terminal helix, were individually changed to alanines by site-directed mutagenesis. GAG binding of the wild-type and mutant MIP-1α proteins was evaluated by heparin-Sepharose chromatography. The results demonstrate that three charged residues that are predicted to form a cleft on one face of the molecule are all essential for heparin binding of MIP-1α. Thus, these studies define a novel GAG-binding structure that is different from the amphipathic helix defined for some α-chemokines. We also examined receptor binding and cell activation by wild-type and non-heparin-binding mutant MIP-1α. The data support the conclusion that GAG-binding capability is not a prerequisite for the biological activity of MIP-1α in solution and allow the design of experiments to test the importance of GAG binding for chemokine activity in vivo.

EXPERIMENTAL PROCEDURES

Cloning of Wild-type MIP-1α—Human MIP-1α full-length coding region cDNA was cloned from phytohemagglutinin/phorbol 12-myristate 13-acetate-stimulated HMC-1 human mast cells (24, 25) by reverse transcriptase-polymerase chain reaction using a primer pair derived from the 5′ and 3′ ends of the published MIP-1α sequence (26); primers included EcoRI sites to facilitate cloning. The sequences of the primers used are 5′-GGGGATTCAGAATCTGACGGTCTTC-3′ (sense) and 5′-GGGAAATCAGCTCAGCTCGG-3′ (antisense). The cDNA, which included 8 base pairs of 5′-untranslated sequence, was subcloned into the mammalian expression vector pCAGGS (27, 28) (the kind gift of T. Yoshimura, NCI-Frederick Cancer Research and Development Center, Frederick, MD) and sequenced in its entirety using Sequenase (U.S. Biochemical Corp.). The sequence obtained was identical to the published human MIP-1α sequence AT464.2 (26).

Site-directed Mutagenesis—Mutagenesis was performed by extension overlap amplification (29) using 100 ng of pCAGGS-MIP1α as template. The sequences of the mutagenic primers used are as follows: MIP-1α R18A (sense), 5′-TGGAGATTCAGAATCTGACGGTGTTAG-3′; MIP-1α K45A (sense), 5′-CCCTTAAACCGGGAGGCGGCCG-3′; MIP-1α R46A (sense), 5′-CTGCGGCCGCTTCAGCTGGTTAG-3′; MIP-1α R46A (antisense), 5′-CTGCGGCCGCTTCAGCTGGTTAG-3′; MIP-1α R18A (antisense), 5′-TGGAGATTCAGAATCTGACGGTGTTAG-3′; MIP-1α K45A (antisense), 5′-CCCTTAAACCGGGAGGCGGCCG-3′; MIP-1α R18A (antisense), 5′-TGGAGATTCAGAATCTGACGGTGTTAG-3′; MIP-1α K45A (antisense), 5′-CCCTTAAACCGGGAGGCGGCCG-3′; MIP-1α R46A (sense), 5′-CTGCGGCCGCTTCAGCTGGTTAG-3′; MIP-1α R46A (antisense), 5′-CTGCGGCCGCTTCAGCTGGTTAG-3′; MIP-1α R46A (antisense), 5′-CTGCGGCCGCTTCAGCTGGTTAG-3′; MIP-1α K45A (antisense), 5′-CTGCGGCCGCTTCAGCTGGTTAG-3′; MIP-1α K45A (antisense), 5′-CTGCGGCCGCTTCAGCTGGTTAG-3′.

Polymerase chain reaction products were subcloned into pCAGGS and sequenced in their entirety using either Sequenase or TaqSeq (U.S. Biochemical). Transient Transfection and Metabolic Labeling—COS-7 fibroblasts, the kind gift of Dr. M. Caron (Duke University), were maintained at 37 °C/5% CO2 in Dulbecco’s modified Eagle’s medium (Mediatech, Herndon, VA) supplemented with 10% fetal calf serum and transiently transfected with wild-type or mutant pCAGGS-MIP1α constructs with DEAE-dextran (30) (M, 500,000; Pharmacia Biotech Inc.) in 100-mm plates. Forty-eight hours after transfection, plates were washed twice with PBS, and the medium was replaced with 10 ml of methionine-free, cysteine-free Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 0.5 mM of Tran32Slabeled ICN, Costa Mesa, CA). Following an additional 24 h of culture, 32P-labeled supernatants were collected.

Heparin-Sepharose Chromatography—Metabolically labeled supernatants containing wild-type or mutant MIP-1α were diluted 4-fold into buffer A (20 mM Tris-HCl, pH 8.0) and applied to 1-ml Hi-trap Heparin columns (Pharmacia), previously equilibrated in buffer A, at a flow rate of 0.5 ml/min. Equivalent amounts of labeled chemokines, normalized by SDS-PAGE (31) and fluorography (32) of unfractionated supernatants, were loaded onto the columns. Columns were washed with 20 column volumes of buffer A and developed with a 15 ml gradient of 0–400 mM NaCl in buffer A at a flow rate of 0.5 ml/min. Control experiments confirmed that no additional chemokine eluted from the heparin-Sepharose columns at NaCl concentrations up to 1 M. Column chromatography was carried out at room temperature.

Purification of Wild-type and Mutant MIP-1α Proteins by Ion Exchange Chromatography—Unlabeled wild-type and mutant MIP-1α proteins were partially purified from supernatants of transiently transfected COS-7 cells by chromatography on Q-Sepharose. Following transfection, cells were cultured for 48 h as described above. The plates were then washed twice with PBS, and the medium was replaced with phenol red-free Dulbecco’s modified Eagle’s medium (Life Technologies). Cells were cultured for an additional 24 h, and the supernatants were collected. Supernatants were applied to 5-ml Hi-trap Q anion exchange columns (Pharmacia) previously equilibrated in buffer B (50 mM Tris-HCl, pH 8.0, 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 400 mM NaCl in buffer B; control experiments confirmed that no additional chemokines eluted from the heparin-Sepharose columns at NaCl concentrations up to 1 M. The partially purified chemokines were dialyzed extensively against PBS at 4 °C (molecular weight cut-off, 3000), aliquoted, and stored at −70 °C. Chemokine concentrations were determined by resolving samples of Q-Sepharose-purified chemokines (see above) alongside a series of 2-fold serial dilutions of a commercial MIP-1α standard (Peprotech, Rocky Hill, NJ) on 15% SDS-PAGE gels and comparing band intensities following silver staining (33). We estimate that the determined protein concentrations must be within half a dilution of the actual concentration. This level of uncertainty has no impact on any of the conclusions drawn.

Chemical Cross-linking—Concentration dependence of chemokine dimerization was assessed as described by Fuolini et al. (34). Briefly, radiolabeled chemokines in PBS, which had been partially purified by Q-Sepharose chromatography, were combined and incubated with 5 mM sulfo-EGS (Pierce) for 1 h at room temperature prior to quenching with SDS-PAGE sample buffer (31). Reactions were boiled for 5 min and analyzed by 15% SDS-PAGE. Gels were fluorographed with 2,5-diphenyloxazol/Me2SO and exposed to BioMax MR or X-Omat AR (Eastman Kodak Co.) film at −70 °C.

Selection of Stably Transfected Fibroblast Cell Lines Expressing the MIP-1α/RANTES Receptor CC-CR1—CDNA for the MIP-1α/RANTES receptor CR1 (35, 36) was cloned into the expression vector pRc/CMV (Invitrogen, San Diego, CA). CHO-K1 fibroblasts (the kind gift of Dr. J. Esko, UAB), were maintained in Ham’s F12 medium supplemented with 10% fetal calf serum and transfected with the pRe-CMV-CR1 construct using DOTAP (Boehringer Mannheim) according to the manufacturer’s instructions. Transfectants were selected by growth in G418 (Life Technologies) (0.6 mg/ml), and drug-resistant cells were subcultured by limiting dilution (0.3 cells/well) in 96-well flat-bottomed plates. Plates expressing high levels of CR1 mRNA were identified by Northern analysis, and one clone, CHO-K1/CR1.12, was selected for further studies. Human fibroblast kidney cells were maintained in Eagle’s minimal essential medium (Life Technologies) supplemented with 10% fetal calf serum and were transfected, selected, and cloned as described above. One clone, HEK-293/CR1.10F6, was selected for further analysis.

Measurement of Elevations in Intracellular Free Calcium Concentrations in Response to Chemokines—Human peripheral blood mononuclear cell, normal donor (Kennon Technika, Durham, NC), washed three times with serum-free RPMI 1640 (Mediatech), and resuspended at a concentration of 1 × 10^6 cells/ml in serum-free RPMI 1640. Stably transfected HEK-293/CR1.10F6 cells expressing the MIP-1α/RANTES receptor CR1 (see above) were briefly trypsinized, washed twice in serum-free RPMI 1640, and resuspended at 1 × 10^6 cells/ml in serum-free RPMI 1640; labeling conditions were identical for monocytes and HEK-293 cells. Indo-1/AM (Molecular Probes, Eugene, OR) was added to a final concentration of 2 μg/ml from a freshly prepared 1 mg/ml stock in Me2SO, 2% pluronic F-127 (Molecular Probes). The cells were incubated for 30 min at 37 °C in the dark, washed twice in serum-free RPMI 1640, and resuspended at a concentration of 2 × 10^6/ml in Hanks’ balanced salt solution (with divalent cations; Life Technologies), 0.1% (w/v) BSA (Boehringer Mannheim) that had been prewarmed to 37 °C. Cells were equilibrated to 37 °C and were analyzed for chemokine-stimulated elevations in intracellular free calcium concentrations using a FACStar Plus (Becton Dickenson, Mountainview, CA) at the Duke University Comprehensive Cancer Center Flow Cytometry Shared Resource. After a base-line violet/blue fluorescence ratio was established, chemokines in PBS were added to Indo-1/AM-labeled cells at NaCl concentrations up to 0.5 M. Changes in fluorescence ratio over time. Calcium mobilization was quantified in terms of the percentage of total cells responding to chemokines. This was accomplished by generating histograms plotting cell number versus fluorescence ratio before the addition of chemokine and at the peak of chemokine-stimulated increase in fluorescence ratio and determining the net increase in the percentage of cells that had fluorescence ratios above baseline at

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the time of peak response. In experiments with peripheral blood mono-
nuclear cells, analysis was restricted to monocytes by gating by forward
and side scatter with the FACStar. Control experiments showed that
nontransfected HEK-293 cells did not mobilize calcium in response to
chemokines (data not shown).

Competition Radioligand Binding Studies—
The ability of wild-type
and mutant MIP-1α to compete for binding of 125I-MIP-1α to CCR1
expressed in CHO-K1 cells was evaluated by incubating 2
310⁶ cells,
harvested with 20 mM EDTA in PBS, with 0.5 nM 125I-MIP-1α (DuPont
NEN; specific activity 2200 Ci/mmol) in binding buffer (phenol red-free
RPMI 1640, 1% BSA (w/v), 25 mM Na-HEPES, pH 7.4) and various
concentrations of unlabeled competitor; the volume of unlabeled com-
petitor added did not exceed 10% of the final assay volume (200
mL). The
reactions were rotated end over end in 1.5-ml microcentrifuge tubes for
2 h at 4 °C and filtered through Whatman GF/C filters. The filters were
washed twice with 5 ml of ice-cold binding buffer, twice with 5 ml of
ice-cold competitor protein was commercially available pure MIP-1α
(Peprotech).

RESULTS

Identification of MIP-1α Amino Acids Involved in Heparin
Binding—Alignment of the amino acid sequences of several
β-chemokines revealed the presence of conserved basic amino
cids at positions 18, 45, 46, and 48 (MIP-1α numbering, Fig.
1A). The sequence at positions 45–48 constitutes an example of the
consensus GAG-binding sequence BB
XBB, where B repre-
sents a basic amino acid and X represents any amino acid (15).
Mapping the amino acid sequence of MIP-1α onto the coordi-
nates of the closely related chemokine MIP-1β (19) revealed that residues 45 and 46 define a turn between adjacent
β-strands (Fig. 1A) and that the side chains of residues 18, 46, and 48 are in close proximity to each other (Fig. 1B). We
constructed a set of point mutants in which each of these
residues was mutatged to alanine. We also mutated the single
basic residue in the MIP-1α C-terminal helix, Lys61, to alanine; residue 61 was chosen for mutagenesis because the only two
chemokines for which GAG-binding motifs have been identi-
fied, PF-4 and IL-8, have both been shown to bind via highly
basic helices (22, 23).

In initial experiments, we expressed wild-type human
MIP-1α in a COS cell transient expression system and evalu-
ated the binding of metabolically labeled protein to heparin-
Sepharose. SDS-PAGE analysis of unfractionated superna-
tants from transiently transfected and metabolically labeled
COS cells revealed a major 8-kDa species, MIP-1α, that was not
present in supernatants of mock-transfected cells (Fig. 2
A). Heparin-Sepharose chromatography of supernatants of MIP-
1α-transfected cells revealed a single peak of radioactivity elut-
ing at 250 mM NaCl, whereas no radioactivity eluted during
similar chromatographic analysis of supernatants of mock-
transfected cells (Fig. 2A). SDS-PAGE analysis of column frac-
tions revealed that the peak of radioactivity obtained in chro-
mnography of supernatants of MIP-1α-transfected cells
(fraction 12) is almost entirely MIP-1α (Peprotech).

FIG. 1. Basic motifs in β-chemokines. A, alignment and conservation of basic residues in β-chemokines. Numbering is for MIP-1α (26). Vertical lines denote lysine or arginine residues. The positions of the β-strands and the C-terminal α-helix are indicated. B, a space-filling model created by modeling the amino acid sequence of MIP-1α on the coordinates of MIP-1β (19) using Swiss-Model (46, 47). The model was visualized with the program WebLab Viewer (Molecular Simulations). Side chains of residues Arg18 (R18), Lys45 (K45), Arg46 (R46), and Arg48 (R48) are indicated. The carboxyl terminus and the side chain of residue Lys61 are not visible in this view.
identified by measurement of radioactivity in the eluate fractions, as described above. Chemokines in the load, flow-through, and wash fractions were identified by SDS-PAGE analysis, since these fractions contained high levels of contaminating unincorporated [35S]methionine and [35S]cysteine. Wild-type MIP-1α bound quantitatively to the column (Fig. 3B) and eluted at 250 mM NaCl (Fig. 3A). However, mutants R18A, R46A, and R48A all failed to stably bind to and elute from heparin-Sepharose (Fig. 3A). Mutant R46A was recovered quantitatively in the column flow-through (Fig. 3B), indicating that interaction with heparin was completely abolished. Mutant R48A was recovered in both the column flow-through and wash fractions (Fig. 3C), and mutant R18A was recovered exclusively in the wash fractions (Fig. 3D), suggesting very weak interactions between these molecules and the heparin matrix. In contrast, the K45A mutant bound stably to heparin-Sepharose but eluted from the column at lower concentrations of NaCl than the wild-type protein (Fig. 3A and B). The R61A mutant bound to heparin-Sepharose and required the same concentration of NaCl as the wild-type protein for elution (Fig. 3, A and B). Thus, our data identify three amino acids, Arg18, Arg46, and Arg48, that are absolutely required for heparin binding of MIP-1α. The side chains of these residues fold together to form a basic cleft on one face of the molecule (Fig. 1B), defining a novel chemokine heparin-binding motif.

Receptor Binding of a Non-heparin-binding MIP-1α Point Mutant—The chemokine presentation hypothesis proposes that chemokines are presented to leukocytes in the form of a solid-phase gradient while bound to GAGs. It is possible that chemokine-GAG interactions are important not only for presentation but also for chemokine activation of target cells in vivo. The generation of MIP-1α point mutants in which heparin binding was abolished allowed us to design experiments to test whether the capacities of MIP-1α to bind to glycosaminoglycans and to activate cells were causally linked. We initially addressed this issue by developing a stably transected CHO cell line expressing the MIP-1α/RANTES receptor CCR1 (35, 36). Competition radioligand binding analysis of wild-type and R46A mutant MIP-1α was performed by incubating the CCR1-expressing cells with purified iodinated wild-type MIP-1α together with increasing concentrations of partially purified, unlabeled, COS cell-derived wild-type and mutant MIP-1α (Fig. 4). Competitor chemokine concentrations were determined as described under “Experimental Procedures.” Mutant MIP-1α competed as effectively as wild-type MIP-1α in this assay, indicating that the mutant protein binds to the receptor with properties that are indistinguishable from wild type (Fig. 5A).

To ensure that the competition we observed in this assay was due to the MIP-1α in the COS cell supernatants and that no other proteins in these partially purified preparations interfered with the competition assay, we also tested the effects of preparations of mock-transfected supernatants. These were prepared by mock-transfecting COS-P cells and subjecting the supernatants to the same protein purification procedures used for the preparation of unlabeled chemokines. Mock-transfected cell supernatants were added to binding assays to a final concentration of 10% (v/v), since this was the maximum volume contributed to the binding assay by unlabeled chemokine preparations in the competition binding assays. Such preparations did not compete for the binding of 125I-MIP-1α and did not affect competition by pure unlabeled MIP-1α (Fig. 5B). Therefore, the results of the competition binding experiments establish that the capacity to bind GAGs is not a prerequisite for receptor binding. However, they do not address a possible relationship between GAG-binding capacity and cell activation.

Signal Transduction in Response to a Non-heparin-binding MIP-1α Point Mutant—To investigate a possible linkage between GAG-binding capacity and cell activation, partially purified MIP-1α wild-type and R46A mutant proteins were tested for their ability to stimulate increases in intracellular free calcium concentrations in human monocytes in a dose-response experiment. Indo-1-loaded peripheral blood mononuclear cells were incubated with varying concentrations of chemokine, and the fraction of monocytes displaying elevations in intracellular free calcium concentrations was determined. We noted no significant differences between the mutant and wild-type MIP-1α (Fig. 6, top panel). Since human monocytes may bear a number of chemokine receptors capable of transducing signals by MIP-1α (37), we also examined signaling via a single receptor by using stably transfected HEK-293 cells expressing the MIP-1α/RANTES receptor CCR1. The stably transfected cells also responded equivalently to wild-type MIP-1α and the R46A mutant (Fig. 6, bottom panel). Taken together, the receptor binding and calcium mobilization experiments indicate that the capacity to bind GAG is a prerequisite for neither receptor binding nor signaling by MIP-1α in solution.

Dimerization of Wild-type and R46A Mutant MIP-1α Proteins—Although the reported structures of the two CC chemokines for which primary data are available, RANTES and MIP-1β, are dimeric (19–21), the association states in which CC chemokines exist in vivo are not known. We reasoned that if MIP-1α binds heparin as a dimer, mutations that interfere

**Fig. 2.** Expression of human MIP-1α in COS-P cells and evaluation of MIP-1α glycosaminoglycan binding by heparin-Sepharose chromatography. A, analysis of metabolically labeled proteins in supernatants of mock-transfected COS-P cells (mock) and COS-P cells transiently transfected with pCAGGS-MIP-1α (MIP-1α) by 15% SDS-PAGE and fluorography. B, heparin-Sepharose chromatography of metabolically labeled proteins in supernatants of mock-transfected COS-P cells (open circles) and COS-P cells transiently transfected with pCAGGS-MIP-1α (closed circles). Only eluate (not flow-through and wash) fractions are shown; radioactivity was determined by scintillation counting. C, analysis of heparin-Sepharose column eluate fractions from MIP-1α-transfected COS-P cells shown in B by 15% SDS-PAGE and fluorography.
with dimerization could inhibit heparin binding, due to the loss of half of the heparin binding sites per molecule. The predicted structure of MIP-1α reveals that two of the residues implicated as critical for heparin binding, Arg\textsuperscript{18} and Arg\textsuperscript{48}, are unlikely to be involved in dimer formation. However, the basic turn region of one monomer is in close contact with the amino terminus of the other. Residue Arg\textsuperscript{46} may, in fact, form part of the dimer interface. To address the possibility that the R46A mutation has a direct effect on dimerization, we assessed the fraction of MIP-1α in monomeric and dimeric form as a function of MIP-1α concentration (Fig. 7). To do so, a constant amount of radiolabeled wild-type or R46A mutant MIP-1α was mixed with graded amounts of unlabeled wild-type or R46A mutant, respectively, and radiolabeled dimeric species were detected by trapping with the chemical cross-linker sulfo-EGS. The results indicate that R46A dimerizes with a concentration dependence similar to that of wild-type MIP-1α. Although the proportion of MIP-1α in dimers appears higher for wild-type than the R46A mutant at the highest concentration tested, this difference is not apparent at lower concentrations. More importantly, this partial effect is unlikely to account for the complete abolition of heparin binding by the R46A mutation. While we cannot exclude the possibility that there may be differences in the association states of wild-type and R46A mutant MIP-1α that are indistinguishable by the cross-linking technique used, we sug-

![Diagram](image-url)

**Fig. 3.** Heparin-Sepharose chromatography of wild-type and mutant MIP-1α proteins. A, heparin-Sepharose elution profiles. Equivalent amounts of labeled chemokines were applied to heparin-Sepharose columns, and chromatography was performed as described under “Experimental Procedures.” Radioactivity in elute fractions was determined by scintillation counting. Heparin-Sepharose chromatography was performed at least three times with essentially identical elution profiles for each chemokine. B, analysis of radiolabeled proteins in supernatants (Load) and heparin-Sepharose flow-through fractions (F/T) for wild-type MIP-1α and mutants K45A, R46A, and K61A by 15% SDS-PAGE and fluorography. C, analysis of radiolabeled proteins in supernatants (Load) and heparin-Sepharose flow-through and wash fractions (F/T, Wash fractions) for MIP-1α mutant R48A by 15% SDS-PAGE and fluorography. D, analysis of MIP-1α mutant R18A as in C.

**Fig. 4.** Wild-type and mutant MIP-1α protein preparations. Partial purification of unlabeled wild-type and R46A mutant MIP-1α proteins from transiently transfected COS cell supernatants was performed by chromatography on Q-Sepharose. Partially purified chemokines (200 ng of chemokine/lane) were resolved by 15% SDS-PAGE and visualized by silver staining. Supernatants from mock-transfected COS-P cells were subjected to the same protein purification procedures and were analyzed along with the chemokine preparations as a control.
Numerous studies have focused on the role of charged residues within \( \alpha \)-helical domains in protein-GAG interactions (for review, see Ref. 15), but there is evidence for involvement of nonhelical basic motifs in such interactions as well. As one specific example, recent basic fibroblast growth factor-heparin co-crystallization experiments (40) have demonstrated the importance of a series of residues in loop regions connecting \( \beta \)-strands for heparin binding. These residues are widely separated in primary structure but fold together to form a functional heparin-binding site. Similarly, a series of six basic residues in lactoferrin fold together to form a structure, denoted a cationic cradle, that has been shown to make up a functional heparin-binding site. Similarly, a series of six basic residues in lactoferrin fold together to form a functional heparin-binding site.

**FIG. 5.** Equilibrium competition binding analysis. A, competition binding of wild-type and R46A mutant MIP-1\( \alpha \) proteins to CCR1. CHO fibroblasts stably transfected with CCR1 were used in equilibrium competition binding experiments as described under “Experimental Procedures.” Total binding was 4500 cpm. The ranges of duplicate determinations were 15% or less of total binding. Circles, wild-type competitor; triangles, R46A competitor. Experiments were repeated three times in duplicate with similar results, and representative experiments are shown. B, competition binding of MIP-1\( \alpha \) (Peprotech) to stably transfected CHO fibroblasts expressing CCR1 in the presence or absence of supernatant from mock-transfected cells (10%, v/v). Shaded bars, binding in the absence of supernatant. Mean values and ranges of duplicate determinations are shown. Total binding was 20,000 cpm.

**DISCUSSION**

The work presented here implicates basic amino acids in a conserved three-dimensional motif as critical for the association of the CC-chemokine MIP-1\( \alpha \) with glycosaminoglycans. Mutation of any one of the three residues in the motif (Arg\(^{18}\), Arg\(^{46}\), or Arg\(^{50}\)) to alanine resulted in a complete loss of heparin binding. Our data suggest that residue Lys\(^{45}\) plays a lesser role in heparin binding and that the single basic residue in the MIP-1\( \alpha \) helix, Lys\(^{41}\), plays no detectable role. The R46A mutant bound as efficiently as wild-type MIP-1\( \alpha \) to CCR1 stably expressed in CHO-K1 cells and exhibited signaling activity in solution that was indistinguishable from wild-type MIP-1\( \alpha \) as measured by calcium mobilization assays on both human monocytes and stably transfected cells expressing CCR1. Thus, our results define a novel heparin-binding motif in MIP-1\( \alpha \) and indicate that the capacity to bind GAGs is essential for neither receptor binding nor signaling by MIP-1\( \alpha \).

![MIP-1α](image)

**FIG. 6.** Calcium mobilization by wild-type and R46A mutant MIP-1\( \alpha \) proteins. Indo-1-loaded human monocytes (top) and stably transfected HEK-293 cells expressing the MIP-1\( \alpha \)/RANTES receptor CC-CR1 (bottom) were analyzed for chemokine-stimulated increases in intracellular free calcium concentrations as described under “Experimental Procedures.” Circles, wild-type; triangles, R46A. Experiments were repeated at least three times with similar results, and representative experiments are shown.

**FIG. 7.** Dimerization of wild-type and R46A mutant MIP-1\( \alpha \) proteins. Unlabeled, Q-Sepharose-enriched wild-type or R46A MIP-1\( \alpha \) proteins were serially diluted in 3-fold increments into PBS. Q-Sepharose-enriched wild-type or mutant MIP-1\( \alpha \) proteins (10,000 cpm) were added to each aliquot; the mass of chemokine contributed by the radiolabeled material, estimated by SDS-PAGE and silver staining, was not sufficient to significantly alter the total concentration of chemokine in each tube. Final chemokine concentrations ranged from 3 \( \mu \)M to 10 nM. Chemical cross-linking with sulfo-EGS was carried out as described under “Experimental Procedures.” Reaction products were resolved by 15% SDS-PAGE and visualized by fluorography. The experiment was repeated three times with similar results, and a representative experiment is shown.
In conclusion, the data presented here identify a novel three-amino acid motif, composed of residues Arg^{18}, Arg^{46}, and Arg^{48}, required for the binding of MIP-1α to GAGs. Since the R46A mutant is active in receptor binding and cell activation experiments in vitro, it may be an appropriate reagent to test the presentation hypothesis for MIP-1α and evaluate the importance of MIP-1α-GAG association for biological activity in vivo.

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