Lysine 58 and Histidine 66 at the C-terminal α-Helix of Monocyte Chemoattractant Protein-1 Are Essential for Glycosaminoglycan Binding

(Received for publication, June 8, 1998, and in revised form, August 6, 1998)

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Monocytes rolling on the endothelial cell layer interact with monocyte chemoattractant protein-1 (MCP-1) that is tethered to the proteoglycans on the luminal side of the endothelial cells and consequently initiate adhesion of monocytes in the early phase of immune response. The amino acid residues in MCP-1 involved in tethering to the proteoglycans have not been elucidated. MCP-1 showed binding to [3H]heparin with a \( K_D \) of 1.5 \( \mu \text{M} \). We substituted lysine or histidine residues at the C-terminal end of MCP-1 with alanine residues and tested these mutants for their ability to bind heparin, heparan sulfate, hyaluronic acid, and chondroitin sulfate-C. Substitution of Lys-58 or His-66 drastically reduced glycosaminoglycan binding. Substitution of Lys-56 or deletion of the five amino acid residues at the C-terminus, including Lys-75, did not alter the heparin binding ability, suggesting that the other lysine residues at the C terminus are not involved in glycosaminoglycan binding. MCP-1 and its mutants did not bind hyaluronic acid as strongly as the other subunits of the GAGs. Substitution of Lys-58 or His-66 by alanine that prevented glycosaminoglycan binding did not affect Ca\(^{2+}\) influx, receptor binding, or chemotactic activity elicited by the chemokine on monocytes THP-1 cells. Therefore, we conclude that the Lys-58 and His-66 residues in the C-terminal α-helix of MCP-1 are essential for glycosaminoglycan binding and probably for the binding to the endothelial surface proteoglycans.

Chemokines are small chemotactic proteins, which mediate directional migration of leukocytes from the blood to the site of injury. Usually the chemokines, produced at the site of injury, cause leukocyte migration (1–5). Monocyte chemoattractant protein-1 (MCP-1), \(^1\) a member of the CC chemokine family, promotes recruitment of monocytes and basophil in response to injury and infection signals in various inflammatory diseases (6–8), different types of tumors (9–13), cardiac allograft (14), injury and infection. The amino acid residues in MCP-1 involved in the heparin binding of the members of CXC chemokine family, were found to be responsible for heparin binding (27). Progressive C-terminal truncation of the α-helix of interleukin-8 inhibited heparin binding (28). Amino acid residues involved in the heparin binding of the members of CC chemokine family are not well understood. In MIP-1α, one study reported that two basic residues were involved in binding heparin as well as the receptor (29). Another report indicated that three noncontiguous basic residues were responsible for binding GAG without affecting interaction with MIP-1α receptor (30). Residues involved in the proteoglycan binding of MCP-1 have not been elucidated. Here we report that substitution of Lys-59 and His-66 with alanine in the C-terminal α-helix of MCP-1, but not the other basic residues at the C-terminal end, prevents GAG binding without affecting receptor binding, Ca\(^{2+}\) influx, or chemotactic activity.

MATERIALS AND METHODS

Construction and Expression of MCP-1 Mutant—A human MCP-1 cDNA (hMCP-1) clone (31) was used as a template to produce single and double mutations at residues 55, 58, 66, 69, and 75, deletion of C-terminal five amino acids (residues 72–76), and a double mutation at 58 and 60. For amino acid mutations, a pair of primers was designed from the 5′ and 3′ ends of MCP-1 sequence, which included BamHI and EcoRI sites at the 5′ and 3′ ends, respectively, to facilitate cloning. The sequences of the primers were 5′-GCGCGAGGATCCATAGAAGTCTCCTGCGCCGCCG-3′ (sense) and 5′-GCGGGGATCCATGGATGTTGTGGG-3′ (antisense). For each specific mutagenesis, two sense and antisense overlapping primers were designed as follows: K56A (sense), 5′-GACCCCGCGAAGG-3′; K56A (antisense), 5′-CCACTTCTGCGGGGTTTGGC-3′; K58A (sense), 5′-CCAAGCGAAGGTTGTTCACTGAC-3′; K58A (antisense), 5′-GAAATCTCTGCTTCTTCTG-3′; H66A (sense), 5′-CCAT-

\(^*\) This work was supported by National Institutes of Health Grant HL48916. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; GAG, glycosaminoglycan; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.
GAGCCGCTGGACAAG-3', H66A (antisense), 5'-TTTTGCCGGC-GTCATG-3'. For deletion of five amino acids from C terminus designated CΔ5, a pair of primers incorporating EcoRI sites were used: CA5 (sense), 5'-CACCCTGAGAAGCGACCTG-3', and C5 (antisense), 5'-GGGGCCGAATTTGCTGTTGCTGAGC-3'. Each subsequent mutagenesis was carried out by a two-step polymerase chain reaction procedure (32). The products were purified from agarose gel through Ultrafree- MC centrifugal filter units (Millipore Corp.) and used as template for the second step of amplification using the primers designed from either end of the MCP-1 coding region. The polymerase chain reaction products were cloned into pFastBac vector (33). Sequence was confirmed to confirm the targeted mutation and the absence of any other mutation. The recombinant baculoviral bacmid constructs were generated by following the protocol described in the BAC-TO-BAC baculovirus expression system manual (Life Technologies, Inc.).

Cell Culture—Sf21 insect cells were maintained in serum-free SF-900 II medium (Life Technologies, Inc.) at 28 °C until the cell density was 9 × 10^7 cells/ml. Monolayers were grown in 150-cm^2 flasks, and suspension cultures were maintained on a roller shaker at 100–120 rpm.

Expression and Purification of Recombinant MCP-1 and Its Mutants—The insect cell culture was transfected with the recombinant bacmids (BAC-TO-BAC baculovirus expression system instruction manual) in a monolayer and were incubated for 6–7 days. For larger scale production, Sf21 cells were grown in 250 ml of SF-900 H medium inoculated with 3 × 10^8 cells/ml and incubated at 28 °C until cell density was 9 × 10^9/ml. The cultures were transfected with recombinant virus stock and incubated for 3–4 days for protein expression. The supernatants were recovered from the cultures by centrifugation at 100,000 × g for 45 min. The recombinant proteins were purified by a two-step FPLC protocol using Mono S HR 5/5 and Superdex-75 HR 10/30 columns (Amersham Pharmacia Biotech), as described earlier (31). The recombinant proteins were subjected to SDS-PAGE and Western blot analysis to verify their identity and purity.

Measurement of Heparin Binding to MCP-1 and Its Mutants—[^3H]Heparin (NEN Life Science Products) was purified by passing 150 μl of solution through a Sephadex G100 column (0.8 × 29 cm) in phosphate-buffered saline (pH 7.4). Heparin binding to MCP-1 and its mutants was measured using a procedure similar to that used to measure binding of heparin to N-CAM (34).[^3H]Heparin (10,000 cpm) and varying amounts of protein and PBS buffer (pH 7.4) were mixed in a total volume of 25 μl in a 1.5-ml microcentrifuge tube, incubated at 37 °C for 1 h, and then passed through a 0.45-μm Schleicher & Schuell NCTM nitrocellulose membrane. Unbound heparin passed through the filter whereas heparin bound to MCP-1 was retained on the membrane. The membrane-bound[^3H]heparin was measured by liquid scintillation spectrometry in a Beckman LS-3801 scintillation counter.

In competition assays, microcentrifuge tubes containing[^3H]heparin, 3 μg (14 μM) of protein, and different concentrations of unlabeled GAGs (chondroitin sulfate C, heparan sulfate, hyaluronic acid, and heparin) in a total volume of 25 μl of PBS buffer were incubated for 1 h at 37 °C. The membrane-bound[^3H]heparin on the nitrocellulose was measured by scintillation spectrometry. The K_B values were determined using the KAleidograp program.

Measurement of Ca^{2+} Influx Induced by MCP-1 and Its Mutants—Intracellular Ca^{2+} influx was monitored in THP-1 monocytic leukemia cells as reported previously (35). THP-1 cells (10^6/ml) were incubated with 2 μM fura-2 acetoxymethyl ester, washed in PBS buffer, and resuspended in Hepes-Tyrode buffer in the presence of 1 mM CaCl_2. Fluorescence was measured in the absence and presence of the chemokine in a Perkin-Elmer LS-3B fluorescence spectrometer with constant stirring. The samples were excited at 340 nm, and emission was recorded at 500 nm. The saturation fluorescence was measured after treating the cells with 50 μM digitonin, and the values are expressed as percentage of saturation (maximum) fluorescence.

Monocyte Chemotaxis—Chemotaxis of THP-1 cells was measured as described previously (31). THP-1 cells (10^6/ml) were suspended in Gey’s balanced salt solution containing 0.2% bovine serum albumin, incubated with 2 μM calcine acetoxymethyl ester (Molecular Probes, Eugene, OR), and after 30 min, washed with PBS buffer, and resuspended in Gey’s bovine serum albumin at 1 × 10^6 cells/ml. Different amounts of MCP-1 protein were placed in a 96-well Polytronics view plate (Neuprobeh, Cabin John, MD), and 200 μl of cells were added to the top wells of the chamber. Following a 1-h incubation at 37 °C, the number of migrating cells were determined by measuring the fluorescence with a Cytofluor 2300 plate reader (Millipore, Bedford, MA).

Ligand Binding Assay—MCP-1 (wild type) was iodinated by the chloramine-T method described previously (36) with minor modifications. MCP-1 (1.5 μg) protein was mixed with 250 μCi of Na^125I (NEN Life Science Products) in 100 μl of 0.1 M sodium phosphate buffer (pH 7.2) and 26.4 μg of chloramine-T. After a 30-s incubation at room temperature, the reaction was stopped by the addition of 100 μg of sodium metabisulfite. Iodinated MCP-1 protein was isolated by elution through a QAE-Sephadex column and quantitated as described previously (31).

MCP-1 binding to THP1 cells, which express CC-CKR2B receptor, was measured as described previously (31, 37). The final assay volume (250 μl) contained 5 × 10^6 THP-1 cells, different amounts of mutant chemokine, and 0.02 μl of iodinated MCP-1 protein. Following incubation, the mixture was passed through a Whatman GFC filter and radioactivity on the filters was measured in a Packard COBRA γ-radioactivity counter. The dissociation constant (K_D) value for each ligand protein was evaluated using the LIGAND program (38).

RESULTS
Since positively charged residues in the C terminus, located away from the receptor-binding N-terminal region, appeared to be likely sites for binding negatively charged proteoglycan, the effect of removal of such charges on proteoglycan binding was tested. Constructs for deletion of five amino acids including Lys-75 (CΔ5) and substitution of several residues (K56A, H66A, V58A, K56A) and K56A were analyzed on a 16.5% SDS-PAGE, and Table I shows the results. SDS-PAGE of human MCP-1 and its mutants produced in insect cells and purified by FPLC, FPLC-purified recombinant mutants CΔ5, H66A, K56A, and K56A were analyzed on an 16.5% SDS-PAGE, and the gel was stained with Coomassie Blue. Low molecular weight protein marker (Life Technologies, Inc.) was used as standards.

![FIG 1. SDS-PAGE of human MCP-1 and its mutants produced in insect cells and purified by FPLC.](image-url)

By competition assays, the binding affinities of the wild type MCP-1 to THP1 cells, which express CC-CKR2B receptor, were measured as described previously (31, 37). The final assay volume (250 μl) contained 5 × 10^5 THP-1 cells, different amounts of mutant chemokine, and 0.02 μl of iodinated MCP-1 protein. Following incubation, the mixture was passed through a Whatman GFC filter and radioactivity on the filters was measured in a Packard COBRA γ-radioactivity counter. The dissociation constant (K_D) value for each ligand protein was evaluated using the LIGAND program (38).

Interaction of MCP-1 with[^3H]heparin was measured. We analyzed the results by non-linear curve fitting and found that heparin bound to MCP-1 with a K_D of 1.5 μM (Fig. 2A). K56A and H66A mutants of MCP-1-1 showed drastically reduced binding compared with that of the wild type MCP-1 protein (Fig. 2A and Table I). On the other hand, K56A and CΔ5 mutants bound[^3H]heparin just as well as the wild type MCP-1 with similar K_D (Fig. 2A, B and C, and Table I). By competition assays, the binding affinities of the wild type MCP-1 was tested with various GAGs, such as heparan sulfate, chondroitin sulfate-C, and hyaluronic acid. Based on the satu-
For both K56A and C50, HS, 16, 000; HA, 757, 000. The values for chemotaxis were obtained at 10 nM chemokine.

The involvement of histidine in GAG binding is unusual, as residues 58 and 66 did not affect the biological response as far as their poor heparin binding ability. In the competition assays, chondroitin-C, heparin, or heparan sulfate competed with [3H]heparin with similar affinities to bind MCP-1. The relative Kd values for heparan sulfate, chondroitin sulfate-C, and heparin were close to each other (Table I). Since GAGs contain a range of molecular weights, the Kd values for competition assays were calculated using the average molecular weights indicated in Table I. Binding affinity of wild type MCP-1 for hyaluronic acid was lower compared with the affinity for the rest of the GAGs. The competition for mutants K58A and H66A with the same pH range from 5 nM at pH 5.8 to 16 nM at pH 6.8 and to 29 nM at pH 7.8.

Since substitution of amino acids might perturb the structure of the chemokine and thus affect its binding to GAGs and biological function, we tested whether the mutations in the C-terminal regions affected the receptor binding, elicitation of Ca2+ influx, and chemotaxis, using the monocytic THP1 cell line, which expresses CCR-2B receptor of MCP-1. Receptor binding was tested in THP1 cells in presence of different concentrations of the wild type MCP-1 and four mutants with THP-1 cells (Fig. 5, A and B). At 2 nM concentration of the wild type MCP-1, the peak fluorescence was 49% of the saturation fluorescence (caused by digitonin). Similar pattern of fluorescence peaks were obtained by the mutants (Fig. 5A). At 4 nM concentration of MCP-1 and mutants, the fluorescence peak was similar to that observed at 2 nM concentration. Thus, the results indicated that the amino acid substitution at residues 58 and 66 did not affect the biological response as far as Ca2+ influx was concerned.

Chemotaxis of monocytes was tested at various concentrations of MCP-1 and K58A and H66A mutants. The mutants binding was determined (Fig. 4). Changes in GAG binding with pH showed the involvement of a residue with a pKa of 6.8, strongly supporting the proposed role of a histidine residue of MCP-1 in heparin binding. Kd for heparin binding increased with pH from 5 nM at pH 5.8 to 16 nM at pH 6.8 and to 29 nM at pH 7.8.

Table I. Binding affinity of wild type MCP-1 for hyaluronic acid

| Chemokine | Chemokine (Kd, nM) | Competitive binding of GAGs (Kd) | Intracellular Ca2+ elevation (% of saturation) | Receptor binding (Kd, nM) | Chemotaxis (% migration) |
|-----------|---------------------|--------------------------------|-----------------------------------------------|--------------------------|--------------------------|
| Hep | CSC | HS | HA | Hep | CSC | HS | HA |
| MCP-1 | 1.5 | 29 | 27 | 15 | 211 | 49.5 | 1.12 × 10^-10 | 100 |
| K56A | 1.55 | 14 | 5 | 29 | 125 | 50 | 1.5 × 10^-10 | 93 |
| C5 | 1.55 | NA | NA | 48 | 3.8 × 10^-10 | 98 |
| K58A | NA | NA | 48 | 3.8 × 10^-10 | 98 |
| H66A | NA | NA | 50 | 3.8 × 10^-10 | 98 |

The membrane-bound [3H]heparin was measured by liquid scintillation. The error bars show the standard deviations of multiple measurements (minimum three) carried out for each data point. Bovine serum albumin (open triangle) was used as a negative control.

**Heparin binding and Ca2+ mobilization by MCP-1 and its mutants**

Hep., CSC, HS, and HA are abbreviations of heparin, chondroitin sulfate-C, heparan sulfate, and hyaluronic acid, respectively. NA indicates not applicable because they did not bind under the experimental conditions. Average molecular weights were used to calculate KD values of MCP-1, K56A, and C50 confirmed that the experimentally determined KD values for heparan sulfate, chondroitin sulfate-C, and heparin were close to each other (Table I). Since GAGs contain a range of molecular weights, the KD values for competition assays were calculated using the average molecular weights indicated in Table I. Binding affinity of wild type MCP-1 for hyaluronic acid was much lower compared with the affinity for heparan sulfate, and chondroitin sulfate-C (Fig. 3A). K56A and C5Δ mutants bound to [3H]heparin just like wild type (Fig. 2A), 14 μM chemokine was added for competition assays. Chondroitin sulfate-C, heparan sulfate, and heparin competed with similar affinities to bind MCP-1. The relative KD values for heparan sulfate, chondroitin sulfate-C, and heparin were close to each other (Table I). Since GAGs contain a range of molecular weights, the KD values for competition assays were calculated using the average molecular weights indicated in Table I. Binding affinity of wild type MCP-1 for hyaluronic acid was much lower compared with the affinity for heparan sulfate, and chondroitin sulfate-C (Fig. 3A).

In the competition assays, chondroitin-C, heparan, or heparan sulfate competed with [3H]heparin with similar affinities for binding the mutant K56A (Fig. 3B and Table I). Similar to the wild type MCP-1, binding affinity of the mutant K56A for hyaluronic acid was lower compared with the affinity for the rest of the GAGs. The competition for mutants K58A and H66A could not be tested due to their poor heparin binding ability.

The involvement of histidine in GAG binding is unusual, as lysines and arginines are the commonly involved residues in other GAG-binding proteins. To further test for the role of histidine in GAG binding of MCP, pH dependence of heparin binding was determined (Fig. 4). Changes in GAG binding with pH showed the involvement of a residue with a pKa of 6.8, strongly supporting the proposed role of a histidine residue of MCP-1 in heparin binding. Kd for heparin binding increased with pH from 5 nM at pH 5.8 to 16 nM at pH 6.8 and to 29 nM at pH 7.8.

Since substitution of amino acids might perturb the structure of the chemokine and thus affect its binding to GAGs and biological function, we tested whether the mutations in the C-terminal regions affected the receptor binding, elicitation of Ca2+ influx, and chemotaxis, using the monocytic THP1 cell line, which expresses CCR-2B receptor of MCP-1. Receptor binding was tested in THP1 cells in presence of different amounts of wild type MCP-1. The mutants bound the receptor with the same KD as the wild type (Table I). Thus, the mutations in the C terminus did not affect receptor binding.

Elevation of intracellular calcium was measured with a range of concentrations of the wild type MCP-1 and four mutants with THP-1 cells (Fig. 5, A and B). At 2 nM concentration of the wild type MCP-1, the peak fluorescence was 49% of the saturation fluorescence (caused by digitonin). Similar pattern of fluorescence peaks were obtained by the mutants (Fig. 5A). At 4 nM concentration of MCP-1 and mutants, the fluorescence peak was similar to that observed at 2 nM concentration. Thus, the results indicated that the amino acid substitution at residues 58 and 66 did not affect the biological response as far as Ca2+ influx was concerned.

Chemotaxis of monocytes was tested at various concentrations of MCP-1 and K58A and H66A mutants. The mutants...
showed chemotactic activity virtually identical to that observed with wild type MCP-1. Induction of chemotactic activity was measured at various concentrations of the chemokine and maximum induction was observed at $10^{-9}$ M and $10^{-10}$ M concentrations of MCP-1 and its mutants (Fig. 6).

**DISCUSSION**

The proteoglycans on the luminal surface of endothelial cells are thought to tether MCP-1 to present it to circulating monocytes without being carried away by the blood flow. To identify the specific residues of MCP-1 involved in proteoglycan binding, we tested the effect of substitution of several basic residues at the C terminus of MCP-1 with alanine on GAG binding. The work presented here identifies two amino acids at the C-terminal $\alpha$-helix of MCP-1 that probably play a critical role in the binding of MCP-1 with GAGs. The substitution of Lys-58 and His-66 with alanine abolished the heparin binding ability of MCP-1. However, substitution of a nearby lysine with alanine (K56A) did not affect binding. In addition, deletion of five amino acids including Lys-75 (CΔ5) from the C-terminal $\alpha$-helix of MCP-1 also did not affect the heparin binding ability. The $K_D$ values of heparin binding to MCP-1 and its mutants were extremely similar at around 1.5 $\mu$M. In the competition assays, the wild type MCP-1 or K56A bound to chondroitin sulfate-C and heparan sulfate much more strongly than to hyaluronic acid.

Affinity between the chemokine and GAG is most likely based on ionic interaction. The limited number of studies conducted on the binding of chemokines to GAGs have reached somewhat conflicting conclusions about the regions involved in proteoglycan binding. In CXC chemokine the C terminus is highly basic, and in the two cases of such chemokines that have been studied, this basic region was suggested to be involved in GAG association. Progressive reduction of heparin binding was caused by successive deletions of three to four residues from the C-terminal $\alpha$-helix of interleukin-8 (28). In PF4, another member of CXC chemokine family, substitution of two pairs of...
lysines at 61/62 and at 65/66 with Gln and Glu in the α-helix abolished heparin binding (27). However, in this case, substitution of the positive charges with negative charges complicated the interpretation concerning the role of the lysine residues in binding the negatively charged heparin. Another study indicated that substitution of Arg-20, Arg-22, and Arg-49 simultaneously with Gln decreased heparin binding even more than that caused by substitution of Lys-61, Lys-62, Lys-65, and Lys-66 simultaneously with Ala (39). In certain members of the CC chemokine family such as RANTES (regulated on activation normal T cell expressed and secreted), MIP-1α, MIP-1β, and MCP-2, the conserved Arg residues found at positions 18, 46, and 48 are thought to be involved in heparin binding. Site-directed mutagenesis indicated that these residues were required for MIP-1α to bind heparin (29, 30). Based on the limited amount of information available on chemokine binding to heparin, the need to review the proposed primary structure for heparin binding motif (40) has been pointed out (39). Crystal structures of different heparin-binding proteins other than chemokines (41–43) strongly suggest that spatial proximity of positively charged amino acids in the three-dimensional structure probably makes the heparin binding site. According to the reported results pertaining to the GAG binding of MIP-1α and the present results on the amino acid residues of MCP-1 involved in GAG binding, it is clear that even within the CC chemokine family different structural features may be involved in this binding. In the present study, substitution for Lys-58 and His-66 in the C-terminal α-helix of MCP-1 virtually eliminated heparin binding. In three-dimensional structure, these residues are located in spatial proximity to play a significant role in heparin binding. (Fig. 7). The unusual involvement of a histidine, rather than the arginines and lysines that are usually found to be involved in GAG binding, was strongly supported by the pH dependence of GAG binding that showed a critical role for a residue with a pKₐ of 6.8. As expected from the involvement of histidine in heparin binding, KD was pH-dependent, increasing from 5 nM at pH 5.8 to 29 nM at pH 7.8. Substitution at position 56 or deletion of residues 72–76 (CD5) from the C terminus did not affect heparin binding, suggesting that the positively charged residues at 56 and 75 had no role in GAG binding. According to the crystal structure, these residues do not reside within the α-helix region (17).

The relationship between the binding site of the chemokines for proteoglycan and the receptor is not clear. In MIP-1α, substitution of amino acid residues suspected to be involved in GAG binding was reported to eliminate also receptor binding (29). However, in another study, the three noncontiguous amino acid residues that define GAG binding site were not found to be a prerequisite for receptor binding and signaling by MIP-1α in vitro (30). A triple mutant in which the basic amino acids at 45, 46, and 48 were replaced with neutral amino acids did bind CC-CKR1, albeit less well than the wild type, and

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**Fig. 5.** Measurement of intracellular Ca²⁺ flux induced by MCP-1 and its mutants in THP-1 cells. A, peaks showing fluorescence induced by 2 nM concentration of MCP-1 or its mutants in THP-1 cells labeled with fura-2. B, concentration dependence of fluorescence enhancement caused by MCP-1 (open square), mutant K56A (open triangle), K58A (open diamond), H66A (open circle), and CD5 (solid triangle), which were mixed with 1 ml of fura-2-labeled THP-1 cells and the fluorescence was measured. The calcium flux is expressed as a percentage of the fluorescence elicited by treating the cells with digitonin.

**Fig. 6.** Chemotaxis in THP-1 cells induced by MCP-1 and its mutants. Chemotaxis was measured in THP-1 cells with MCP-1 (dotted bar), K58A (vertically striped bar), and H66A (solid black bar). To compare the data obtained by different mutants, we expressed the fluorescence values as the percentage of the migration to recombinant wild type MCP-1 at 0.1 nM concentration in each separate experiment. The error bars represent the standard deviation from the six measurements.
the results show that mutations in the (30, 44), indicating that the proteoglycan binding does not C-terminal chemokines increases the local concentration of the chemokine the number of GAG bound per mole of chemokine is very small (Tyr) is involved in receptor selectivity. The amino acid sequence of the red proteoglycan on the cell surface. (Fig. 7). According to the model, the C-terminal helix in the chemotaxis assay medium for MIP-1 may affect the receptor binding indirectly and such affects may decreased heparin binding had no effect on receptor binding, the fusion protein receptor (48, 49). The N-terminal rod of the chemokine would then interact with the membrane and trigger the G-protein-coupled signal transduction.

**Acknowledgments**—We thank Dr. Gregory J. Cole for providing us [3H]heparin and Jackie Crisman and Y.-K. Kim for technical assistance.

**REFERENCES**

1. Baggiolini, M. (1998) Nature 392, 565–568
2. Aukrust, P., Ueland T., Muller, F., Andreassen, A. K. Nordoy, I., Aas, H., Kjekshus, J., Simonsen, S., Forland, S. S., and Gullestad, L. (1998) Circulation 12, 1196–1143
3. Forrester, J. S., Litvack, F., and Grundfest, W. (1991) Annu. Rev. Med. 42, 35–45
4. Vla-Hertuala, S., Lipton, B. A., Rosenberg, M. E., Sarksjo, T., Yoshimura, T., Leonard, E. J., Witzum, J. L., and Steinberg, D. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 5252–5256
5. Nelken, N. A., Coughlin, S. R., Gordon, D., and Wilcox, J. N. (1991) J. Clin. Invest. 88, 1121–1127
6. Molestaite, R. E., Miller, R. D., Ramirez, J. A., and Summersgill, J. T. (1998) Infect. Immun. 66, 1370–1376
7. Shibata, F. I., and Libby, P. (1998) Trends Cardiovasc. Med. 1, 216–223
8. Koch, A. E., Kunkel, S. L., Harlau, L. A., Johnson, B., Eavanoff, H. L., Haines, G. K., Burdick, M. D., Pope, R. M., and Strieter, R. M. (1992) J Clin. Invest. 90, 772–779
9. Jones, M. L., Mulligan, M. S., Flory, C. M., Ward, P. A., and Warren, J. S. (1992) J. Immunol. 149, 2147–2154
10. Takeya, M., Yoshimura, T., Leonard, E. J., Kato, T., Okada, H., and Takehashi, K. (1991) Exp. Mol. Pathol. 54, 61–71
11. Kuratsu, J., Yoshizato, K., Yoshimura, T., Leonard, E. J., Takedeh, H., and Ushio, Y. (1993) J. Natl Cancer Inst. 85, 1836–1839
12. Graves, D. T., Barnhill, R., Galanopoulos, T., and Antoniades, H. N. (1992) Am. J. Pathol. 140, 9–14
13. Negus, R. P. M., Stamp, G. W. H., Relf, M. G., Burker, F., Malik, S. T. A., Bernasconi, S., Alianova, P., Sossani, S., Mantovani, A., and Balk, P. (1990) J. Clin. Invest. 85, 2391–2396
14. Russel, M. E., Adams, D. H., Wyner, L. R., Yamashita, Y., Halnon, N. J., and Karnovsky, M. J. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 6036–6090
15. Conant, K., Gurrino-Demo, A., Nath, A., McArthur, J. C., Halliday, W., Power, C., Gallo, R. C., and Major, E. O. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 3117–3121
16. Lin, X., Gong, J., Zhang, M., Xue, W., and Barnes, P. F. (1998) Infect. Immun. 66, 2319–2322
17. Lukowski, J., Bujesz, G., Boc, L., Domaille, P. J., Handel, T. M., and Woldawer, A. (1997) Nat. Struct. Biol. 4, 64–69
18. Verheij, F. M., Torkelsson, R., and Lota, M. (1997) J. Immunol. 149, 722–727
19. Akahoshi, T., Wada, C., Endo, H., Hirota, K., Hosaka, S, Takagishi, K, Kondo, H., Kashiwazaki, S., and Matsushima, K. (1993) Arthritis Rheum. 36, 762–771
20. Shy, Y., Li, Y-S., and Kolattukudy, P. E. (1990) Biochem. Biophys. Res. Commun. 169, 346–351
21. Rovin, B. H., Rovin, M., Tann, L., and Dickerson, J. (1994) Lab. Invest. 71, 536–542
22. Wang, G. P., Deng, Z. D., Ni, J., and Qu, Z. L. (1998) Atherosclerosis 133, 31–36
23. Tanaka, Y., and Adams, D. H., and Shaw, S. (1993) Immunol. Today 14, 10–114
24. Tanaka, Y., Adams, D. H., Hubser, S., Hiranu, I., Siebenlist, U., and Shaw, S. (1993) Nature 361, 79–82
25. Geoghegert, A. J., Kusche, G. S. V., Proudfoot, A. E. I., Borlaf, F., Clark-Lewis, I., Power, C. A., and Wells, N. C. (1993) Biochemistry 36, 13570–13578
26. Kjellen, L., and Lindahl, U. (1991) Annu. Rev. Biochem. 60, 443–475
27. Maione, T. E., Gray, G. S., Hunt, A. J., and Sharpe, R. J. (1991) Cancer Res. 51, 2077–2083
28. Webb, L. M. C., Ehrengruber, M. U., Clark-Lewis I, Baggiolini, M., and Rot, A. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 7158–7162

55 58
K-Q-K-W-V-D-S-M-D-H-L-D-K-Q-T-Q-P-K-T

66

FIG. 7. Proposed model for MCP-1 interaction with its receptor while tethered to the endothelial cells by the interaction of the C-terminal lysine 58 and histidine 66 (arrows) residues with the proteoglycan on the cell surface. The N-terminal rod inserted into the membrane is involved in interaction with G-protein, the red residues were shown to be required for receptor binding, and the green (Tyr) is involved in receptor selectivity. The amino acid sequence of the C-terminal region of MCP is shown at the bottom with the residues in the α-helix in bold.

showed undiminished chemotactic activity (44). Substitution of the basic amino acid residues in the putative GAG binding sites may affect the receptor binding indirectly and such affects may not have physiological relevance. In fact, the presence of GAG in the chemotaxis assay medium for MIP-1α showed no effect (30, 44), indicating that the proteoglycan binding does not interfere with the receptor binding. In the case of MCP-1, our results show that mutations in the α-helix that drastically decreased heparin binding had no effect on receptor binding, calcium flux, and chemotaxis. These results clearly separate receptor binding of MCP-1 from proteoglycan binding and strongly suggest that the α-helix is involved only in the MCP-1 presentation to the monocyte. GAG binding can cause multimerezization of chemokines (45). As expected from this hypothesis, the number of GAG bound per mole of chemokine is very small in all of the binding experiments. The “polymerization” of the chemokines increases the local concentration of the chemokine and thus enhances the binding to the high affinity receptors within the local environment (45).

The finding that the two positively charged residues in the C-terminal α-helix are involved in the tethering of the chemokine is consistent with a general model for the MCP-1 function (Fig. 7). According to the model, the C-terminal α-helix is used to tether the chemokine to the luminal side of the endothelial cells of the blood vessel via the ionic interaction between the two juxtapositioned basic amino acid residues (positions 58 and 66) (Fig. 7) with the negatively charged extracellular proteoglycan. This would leave the N-terminal extended rod and the base of this rod for binding to the receptor on the monocyte rolling on the endothelial layer. Based on the available information, it has been concluded that the residues Asp-3, Thr-10, Tyr-13, Ser-34, and Lys-35 at the N-terminal extended rod and Tyr-28 and Arg-30 at the globular region of MCP-1 interact with its receptor (31, 46, 47). Lys-58 and His-66 residues at the C-terminal α-helix of MCP-1 bind proteoglycans of endothelial layer without interfering with the receptor binding. This model will allow the interaction of N-terminal segment of the receptor with the globular body of the chemokine that might determine the chemokine selectivity of the receptor, as suggested by mutagenesis studies that indicated the importance of Tyr-28 in receptor selectivity (46, 47) and by the binding of chemokines to the fusion protein receptor (48, 49). The N-terminal rod of the chemokine would then interact with the membrane and trigger the G-protein-coupled signal transduction.
29. Graham, G. J., Wilkinson, P. C., Nibbs, R. J. B., Lowe, S., Kolset, S. O., Parker, A., Freshney, M. G., Tsang, L.-S., and Pragnell, I. B. (1996) EMBO J. 15, 6506–6515
30. Koopmann, W., and Krangel, M. S. (1997) J. Biol. Chem. 272, 10103–10109
31. Beall, J. C., Mahajan, S., Kuhn, E. D., and Kolattukudy, P. E. (1996) Biochem. J. 313, 633–640
32. Higuchi, R., Krummel, B., and Saiki, R. K. (1988) Nucleic Acids Res. 16, 7351–7367
33. Luckow, V. A., Lee, S. C., Barry, G. F., and Olins, P. O. (1993) J. Virol. 67, 4566–4579
34. Cole, G. J., and Akenson, R. (1989) Neuron 2, 1157–1165
35. Sozzani, S., Luini, W., Molino, M., Jilek, P., Bottazzi, B., Cerletti, C., Matsushima, K., and Mantovani, A. (1991) J. Immunol. 147, 2215–2221
36. Hunter, W. M., and Greenwood, F. C. (1962) Nature 194, 495–496
37. Van Riper, G, Siciliano, S., Fischer, P. A., Meurer, R., Springer, M. S., and Rosen, H. (1993) J. Exp. Med. 177, 851–856
38. Munsen, P. J., and Robard, D. (1988) Anal. Biochem. 170, 220–239
39. Maye, K. H., Ilyina, E., Roongta, V., Dundas, M., Joseph, J., Lai, C. K., Maione, T. E., and Daly, T. J. (1995) Biochem. J. 312, 357–365
40. Cardin, A. D., and Weintraub, H. J. R. (1989) Arteriosclerosis 9, 21–32
41. Mourey, L., Samama, J. P., Delarue, M., Pepitou, M., Chouy, J., and Moras, D. (1993) J. Mol. Biol. 232, 223–241
42. van Tilbeurgh, H., Roussel, A., Lalouel, J. M., and Cambillau, C. (1994) J. Biol. Chem. 269, 4626–4633
43. Eriksson, A. E., Cousens, L. S., and Matthews, B. W. (1993) Protein Sci. 2, 1274–1284
44. Crisman, J. M., Kolattukudy, P. E. (1998) Mol. Cell. Biochem., in press
45. Hoogewerf, A. J., Kuschert, G. S., Proudfoot, A. E., Borlat, F., Clark-Lewis, I., Power, C. A., and Wells, T. N. (1997) Biochemistry 36, 15570–15578
46. Beall, C. J., Mahajan, S., and Kolattukudy, P. E. (1992) J. Biol. Chem. 267, 3455–3459
47. Zhang, Y. J., Rutledge, B. J., and Rollins, B. J. (1994) J. Biol. Chem. 269, 15918–15924
48. Monteclaro, F. S., and Charo, I. F. (1996) J. Biol. Chem. 272, 23186–23190
49. Monteclaro, F. S., and Charo, I. F. (1996) J. Biol. Chem. 271, 19084–19092