

Mapping of MCP-1 functional domains by peptide analysis and site-directed mutagenesis

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Abstract Monocyte chemoattractant protein-1 (MCP-1) is a member of the β chemokine family which acts through specific seven transmembrane receptors to recruit monocytes, basophils, and T lymphocytes to sites of inflammation. To identify regions of the human MCP-1 protein which are important for its biological activity, we have synthesized domain-specific peptides and tested their ability to antagonize MCP-1 binding and chemotaxis in THP-1 cells. We have found that an intercysteine first loop peptide encompassing amino acids 13–35 inhibits MCP-1 binding and chemotactic activity, while peptides representing the amino-terminus (amino acids 1–10), second loop (amino acids 37–51), and carboxy-terminus (amino acids 56–71) of MCP-1 have no effect. In addition, we have found that cyclization of the first loop peptide by disulfide linkage and blocking the C-terminus of the peptide by amidation increases the activity of this peptide to block MCP-1 binding and chemotaxis. In order to specifically identify amino acid residues within the first loop that are crucial for MCP-1 functional activity, we have substituted alanine for tyrosine (Y13A) or arginine (R18A) in MCP-1 recombinant proteins. While baculovirus produced wild type and R18A MCP-1 proteins are indistinguishable in their ability to induce THP-1 chemotaxis and show modest effects in binding activity compared to commercially available recombinant MCP-1 protein, the Y13A point mutation causes a dramatic loss in function. The identification of functional domains of MCP-1 will assist in the design of MCP-1 receptor antagonists which may be clinically beneficial in a number of inflammatory diseases.

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Key words: Monocyte chemoattractant protein; CC chemokine receptor 2; Chemokine

1. Introduction

Monocyte chemoattractant protein-1 (MCP-1) belongs to a family of low molecular weight proteins collectively called chemokines, which mediate the recruitment of specific subsets of leukocytes including monocytes and lymphocytes into inflammatory sites [1–3]. Chemokines are chemoattractant cyto-

kines which are separated into four subfamilies based on the arrangement of two N-terminal conserved cysteines (for reviews, see [4–11]). The α or CXC subfamily includes such proteins as IL-8 and NAP-2 and is characterized by a single residue separating the two N-terminal conserved cysteines. The β or CC chemokine subfamily includes MCP-1, -2, -3, -4, and -5, eotaxin, RANTES, and MIP-1 α and β which have their N-terminal cysteines located adjacent to each other. The third, γ or C subfamily has a single cysteine at this position and is represented by lymphotactin as its sole member [12]. The most recently identified subfamily is a class of membrane bound chemokines which express a CX3C motif [13]. In addition to structural differences, chemokines subfamilies display differential activities for leukocyte subsets. The CXC chemokines preferentially attract neutrophils while the CC chemokines are chemotactic for monocytes, memory T cells, and basophils. Lymphotactin, on the other hand, is a chemoattractant for CD4

binding of native MCP-1 to peripheral blood monocytes [28]. The functional role of the carboxy-terminal tail of MCP-1 remains uncertain though it has been proposed that this region may be required for full chemoattractant potency of the protein [25,27,28].

In this study we determined the activity of MCP-1 domains using a combination of domain-specific peptides and site-directed mutagenesis. We have analyzed the ability of synthetic peptides to compete with radiolabeled MCP-1 for binding to its receptor on monomyelocytic THP-1 cell, and correlated these activities to effects on MCP-1-mediated THP-1 cell migration. These studies allow us to conclude that the first loop of MCP-1 is necessary for receptor binding and migratory activity but it alone is not sufficient for full agonist activity that is seen in the native chemokine.

2. Materials and methods

2.1. Reagents

MCP-1 and MCP-3 were purchased from R&D Systems (Minneapolis, MN). 125 I-labeled (2200 Ci/mmol) recombinant human MCP-1 and [3 H]thymidine (6.7 Ci/mmol) were from Dupont NEN (Wilmington, DE), and chemicals were obtained from Sigma (St. Louis, MO).

2.2. Cells

The THP-1 monocytic cell line was purchased from American Type Tissue Culture (Rockville, MD) and grown in RPMI medium (JRH Biosciences, Lenexa, KS) containing 10% fetal calf serum (FCS; Gemini Products Inc., Calabasas, CA), 2 mM glutamine (BioWhittaker, Walkersville, MD), 100 units/ml penicillin and 100 units/ml streptomycin (Irvine Scientific, Santa Ana, CA). Cells were incubated at 37°C, 5% CO₂ under humid conditions.

2.3. MCP-1 binding assay

To measure the ability of MCP-1, MCP-3, and FLAG-tagged MCP fusion proteins to bind THP-1 cell surface receptors, 1 µm glass fiber filter plates (Millipore, Bedford, MA) were coated overnight at 4°C with 0.1% polyethylamine, 2% bovine serum albumin (Intergen, Purchase, NY) w/v in water. Peptides or FLAG-tagged proteins were added to each well at the indicated concentrations. 125 I-MCP-1 and THP-1 cells diluted in 50 mM HEPES, pH 7.2, 1 mM CaCl₂, 5 mM MgCl₂, 0.5% ovalbumin were then added to a final concentration of 0.25 nM and 1.3×10^6 cells per well, respectively. After 45 min incubation at room temperature, the plates were washed with 10 mM HEPES, pH 7.2, 0.5 M NaCl, and 0.5% ovalbumin and counted on a Wallac Microbeta plate reader (Gaithersburg, MD).

2.4. MCP-1-mediated migration assay

The ability of wild type and mutant MCP-1 to mediate the migration of monocytic THP-1 cells through a filter was measured with and without the addition of potential peptide antagonists. 96-transwell 5 µm filter plates (Neuroprobe, Cabin John, MD) were coated overnight at 4°C with 0.12 µg/well human fibronectin derived from whole plasma in phosphate buffered saline (PBS). THP-1 cells were labeled with 1 mCi/ml [3 H]thymidine in RPMI-10% FCS at 37°C overnight. After labeling, the cells were washed in human serum albumin (Intergen)-DME (Irvine Scientific) and adjusted to a density of <

Separations, Westboro, MA) using standard procedures. The nitrocellulose was then blocked with 5% non-fat milk in PBS for 1 hour followed by incubation with horseradish peroxidase-conjugated MCP-1 antibodies (R&D Systems, Minneapolis, MN). After washing with PBS, blots were developed using the enhanced chemiluminescent system (ECL; Amersham, Arlington Heights, IL) and autoradiography.

3. Results

3.1. MCP-1-mediated chemotaxis

To determine the activity of wild type or mutant MCP-1 constructs, a 96 well transwell migration assay was established. The activity of MCP-1 and MCP-3, two chemokines known to bind to the β chemokine receptor CCR2b [32], was measured. Under the conditions described in Section 2, we have demonstrated that MCP-1 or MCP-3 chemokines can equally promote the chemotaxis of monocytic THP-1 cells (Fig. 1). As reported [24], THP-1 cell migration mediated by these chemokines is concentration-dependent such that it reaches a maximum response at 10^{-7} nM then declines at higher concentrations. To determine if the assay measured chemotaxis, we evaluated whether migration was dependent on a concentration gradient by carrying out a checkerboard analysis. Table 2 demonstrates that fewer cells migrated as the concentration of MCP-1 was increased in the upper compartment. In contrast, when equal concentrations of MCP-1 were generated in both the upper and lower compartment there was no net movement of cells. Taken together, these results establish that our experimental conditions are reflective of MCP-1-mediated activities as previously cited [25].

3.2. Functional activity of MCP-1 domains

To determine which domains of MCP-1 were important for receptor binding and chemotactic activity, synthetic peptides derived from the amino-terminus, first loop, second loop, and carboxy-terminus of MCP-1 were tested for their ability to inhibit 125 I-MCP-1 binding and chemotaxis. As presented in Table 3, peptides derived from the first loop blocked the binding of 125 I MCP-1 to THP-1 cells with an estimated IC_{50} of 32 μ M. Additionally, the first loop peptide weakly inhibited MCP-1-induced cell migration with an IC_{50} of 633 μ M. In contrast, no inhibitory activity was observed from peptides

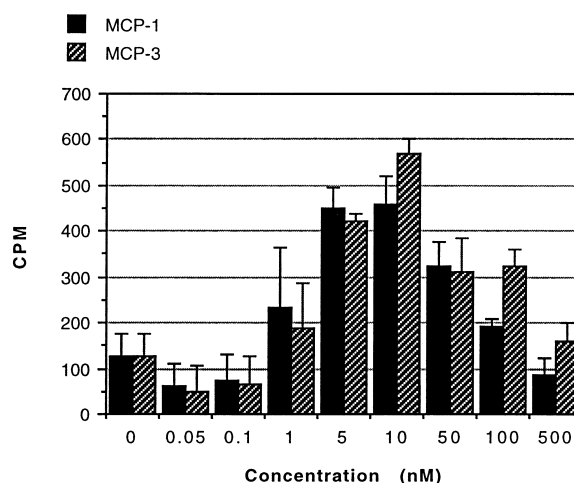


Fig. 1. Chemotactic activity of MCP-1 and MCP-3. The ability of MCP-1 and MCP-3 to induce migration of THP-1 monocytic cells was measured as described in Section 2. 1.5×10^5 [3

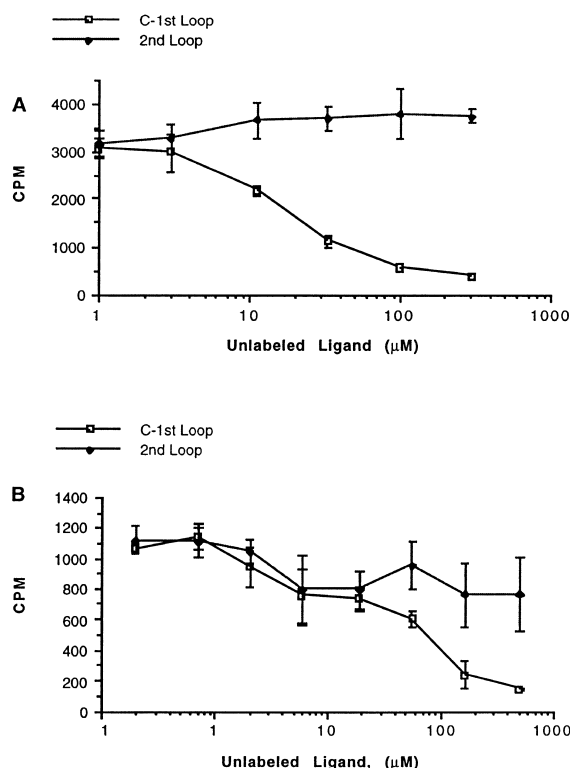


Fig. 2. Inhibition of MCP-1 binding and chemotaxis by a cyclized first loop peptide. Titration of cyclized first (C-1st Loop) versus the second (2nd Loop) loop peptides in inhibiting 125 I-MCP-1 binding to THP-1 cells (A) or MCP-1-directed migration (B) was done using methods described in Section 2 and Table 3. Each data point represents the mean CPM $\pm$ S.D. of four replicates from one of a minimum of two independent experiments.

tion of the labeled THP-1 cells in the presence of these peptides was measured. The first loop cyclic peptide demonstrated a dose-dependent inhibition of MCP-1-mediated THP-1 cell

Table 3
Binding and migration inhibitory activity is present in the first loop

Sequence	Domain	Binding IC ₅₀ (μM)	Migration IC ₅₀ (μM)
QPDAINAPVTA	N-terminus	> 300	> 1000
AYNFTNRKISVQRLASYRRITSSK	First loop	32	633
PKEAVIFKTIYAKEI	Second loop	> 300	> 1000
KQKWVQDSMDHLDKQT	C-terminus	> 300	> 1000

For binding studies, a total of 1.3×10^6 THP-1 cells were mixed with or without serial dilutions of synthetic peptides and 0.25 nM 125 I-MCP-1 for 45 min at room temperature. For MCP-1-mediated chemotaxis, 1.5×10^5 [3 H]thymidine-labeled THP-1 cells were preincubated with or without serial dilutions of peptide and placed in the upper chamber. Cells which had successfully migrated to the lower chamber were counted and expressed as mean CPM $\pm$ SD ($n=4$). IC₅₀s were calculated from peptide serial dilutions using the CurFit program (Interactive Microware, Inc., State College, PA) ($n=4$), and the data represent an average of a minimum of two independent experiments. For chemical stability, an alanine was used to replace residue C11.

Table 4
Activity of cyclized first loop peptides

epitope to MCP-1 wild type and mutant proteins for assistance in protein identification and purification. Since maintenance of biological activity of these proteins was crucial for our studies, the proteins were expressed in the eukaryotic baculovirus insect cell. FLAG-tagged mutants and wild type MCP-1 were purified from Sf9 cell supernatants using an anti-FLAG antibody column. Peak fractions were quantified for MCP-1 content using a MCP-1 immunoassay, then qualitatively evaluated by an MCP-1 immunoblot (Fig. 3A) and Coomassie stained polyacrylamide gel (Fig. 3B). A prominent band is observed with an estimated molecular mass of 10 kDa which is in close agreement with the predicted molecular weight of 8.7 kDa for MCP-1 plus the 1 kDa FLAG epitope [33].

The purified mutant and wild type proteins were then evaluated for their ability to compete for 125 I-MCP-1 binding to THP-1 cells (Fig. 4) and to mediate THP-1 chemotaxis in the transwell migration assay (Fig. 5). Both the wild type and R18A MCP-1 FLAG-tagged recombinant proteins demonstrated similar activity in the binding assay, although they varied slightly from the non-tagged commercial MCP-1 recombinant protein. Their similar activities in the binding assay were paralleled in the migration assay where only slight differences between commercial, wild type, and R18A mutant MCP-1 proteins were detected. In contrast, the Y13A mutant MCP-1 recombinant protein was less effective in competing with 125 I-MCP-1 binding to THP-1 cells. In agreement with the binding data, Y13A demonstrated no significant chemotactic activity in the migration assay. Thus, our data supports those of Beall et al. who reported that tyrosine 13 is a critical residue for MCP-1 biological activity [27].

4. Discussion

The study of β chemokines and their receptors has generated great interest due to their strong correlation with several

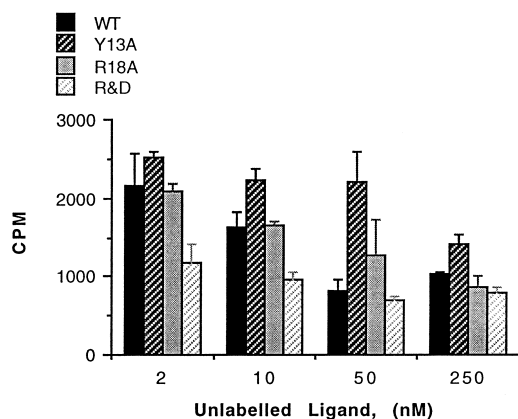


Fig. 4. FLAG-tagged wild type and mutant MCP-1 binding to THP-1 cells. Affinity purified FLAG-tagged wild type (WT), mutant (R18A, Y13A), and commercial (R&D) recombinant proteins were tested for their ability to compete with 125 I-MCP-1 binding to THP-1 cells. The indicated concentrations of recombinant protein were co-incubated with 0.25 nM 125 I-MCP-1 and 1.3×10^6 THP-1 cells for 45 min at room temperature. After washing the cells of unbound MCP-1, cell bound 125 I-MCP-1 was measured by counting on a Wallac Microbeta plate reader and expressed as the mean CPM $\pm$ S.D. (<

receptor CCR2b while opposing reports suggest dimerization does not occur at physiological concentrations and is thus not a component of receptor activation, leading one to question whether the N-terminal domain directly binds to CCR2b [37,38]. Studies performed by Gong and Clark-Lewis [24] identified critical N-terminal residues through synthetic modification or deletion. In these studies, the backbone structure of the N-terminal amino acid located at position 1, or chemical modification of this residue results in disruption of receptor binding and activity. Furthermore, most N-terminal deletion mutants lack monocyte chemoattractant activity as well as THP-1 calcium mobilization [24,39]. In agreement with published reports [24,39], we have found that peptides containing residues 1–10 from the amino-terminus of MCP-1 lack biological activity.

The C-terminal α -helix has not been convincingly shown to play a role in MCP-1 receptor binding and chemotactic activity. As presented in this study, C-terminal peptides do not inhibit binding or migration. Similarly, Beall et al. found that C-terminal point mutations had no effect on MCP-1 chemotactic activity. Alternatively, Zhang et al. [25] report that this region may not be a direct regulator of signaling, but suggest that modification of this site decreases MCP-1's chemoattractant potency. In all, these results do not support a functional requirement for the C-terminal alpha helix of MCP-1 although extensive deletions or alterations in this region may reduce the potency of MCP-1.

In contrast, the first loop of MCP-1 is gaining increased attention. For example, early work by Valente et al. [28] showed that synthetic peptides encompassing the first inter-cysteine loop amino acids 13–35 stimulated monocyte migration and competed with native MCP-1 for binding, suggesting this region may contain the most critical residues for MCP-1 activity. In this report we provide data that peptides from the first loop (amino acids 13–35) of human MCP-1 can compete for THP-1 receptor binding and chemotactic activity, while the amino-terminal, second loop (amino acids 37–51) and carboxy-terminal (amino acids 56–73) domains alone are not sufficient for biological activity. Furthermore, the potency of the first loop peptide can be increased by cyclization and amidation. It should be noted that intact MCP-1 binds and promotes migration with low nanomolar potency while micromolar concentrations of the cyclic first loop peptides are required to block binding and migration, suggesting that other regions of the molecule contribute to its full activity. Currently, we do not fully understand why the concentration of peptide required to block 125 I-MCP-1 binding to cells differs from the concentration required for blocking MCP-1 induced migration. However, it should be noted that the concentration of MCP-1 used in the two assays differ by ~ 25 -fold, that is, 0.25 nM and 6 nM were used for the binding and migration assays, respectively. Therefore the molar ratio of peptide to ligand is $\sim 115\,000:1$ for both assays. The specificity of the peptides was demonstrated by the fact that THP-1 cell migration in response to RANTES or neutrophil migration in response to FMLP was not blocked by the peptides (data not shown).

Residues from the second β strand (termed $\beta 1$) have been implicated as important regions for MCP-1 activity. Beall et al. have presented evidence that MCP-1 proteins with two point mutations in the $\beta 1$ strand at positions Y28, and R30 have greatly reduced or completely absent chemotactic activ-

ity [26,27]. However, Zhang et al. have shown that single amino acid changes in R24, Y28, and R30 resulted in MCP-1 proteins without chemoattractant activity, while point mutations at position 27 had only a slight effect [25].

To specifically identify critical amino acid residues, FLAG-tagged first loop MCP-1 mutants were made and expressed in a eukaryotic insect cell system. We have shown that wild type and R18A MCP-1 FLAG-tagged proteins mediate the migration of THP-1 cells at similar concentrations as commercial recombinant MCP-1 protein. In contrast, the substitution of tyrosine 13 with an alanine (Y13A) caused almost complete loss of MCP-1 activity even at the highest concentrations tested. The importance of tyrosine 13 in MCP-1 biological activity has recently been reported by Beall et al. in which replacement of tyrosine 13 with an isoleucine diminished MCP

the first loop of MCP-1 is important for binding and chemotactic activity.

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