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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.

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 cytokines 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-1α 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 $CD4^-/CD8^-$ T cells. The activity of the chemokines for specific subsets of leukocytes is maintained within each subfamily and the fact that each subfamily maps to separate chromosomes [14] suggests that chemokine structure and genetics may contribute to their leukocyte selectivity.

Although the chromosomal location of human MCP-1 on chromosome 17 [15] has not yet been correlated with any genetic diseases, the activity of MCP-1 in inducing monocyte infiltration implies its role in several inflammatory diseases such as rheumatoid arthritis [16,17], atherosclerosis [18–20], and inflammatory bowel disease [21]. Its potential role in these disease processes has led several laboratories to study MCP-1 structure-function relationships with the hope that this information may lead to the development of therapeutic modulators of inflammatory diseases. The three-dimensional structure of MCP-1 by NMR analysis and its structural homology to MIP-1β [22,23] indicate that MCP-1 contains a long, flexible N-terminus followed by three antiparallel β sheets over which the C-terminal α-helix rests. Deletion or mutational analysis of MCP-1 suggests that the N-terminal residues of this chemokine are important for receptor binding and chemotaxis (Table 1). This region, however, does not appear to be sufficient for activity since N-terminal peptides containing residues 1–10 are also inactive [24,25]. Additionally, the β sheet region has been reported to be important for MCP-1 activity since site-directed mutagenesis of residues Tyr^{28} and Arg^{30} results in a loss of monocyte chemoattractant activity, while point mutations in Thr^{10} and Tyr^{13} have greatly lowered activity [25–27]. Further support for the importance of the N-terminal region includes the finding that high concentrations of synthetic peptides encompassing amino acids 13–35 of MCP-1 stimulate monocyte migration and competitively inhibit the

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Abbreviations: MCP, monocyte chemoattractant protein; IC_{50}, inhibitory concentration required for 50% reduction in activity; CCR2, CC chemokine receptor 2; MIP, monocyte inflammatory protein; C5a, complement factor 5a; RANTES, regulated on activation, normal T cell expressed, and secreted

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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% CO_2 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 $CaCl_2$, 5 mM $MgCl_2$, 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 1×10^7 cells/ml. For peptide antagonist studies, 1.5×10^5 THP-1 cells were incubated with the indicated concentrations of peptides 5 min prior to transfer of cells to the transwell plates. Either 6 nM recombinant human MCP-1 or MCP-3 was added to the bottom of the filter plate, and migration was allowed to proceed for 2 h at 37°C. After this time, the number of labeled cells which had migrated into the lower compartment was measured on a Wallac Microbeta plate reader. For MCP-1 mutant analysis, purified FLAG-tagged MCP-1 proteins replaced the commercial MCP-1/MCP-3.

2.5. Peptide synthesis

Amino acid precursors were purchased from Bachem (Torrance, CA). Synthetic peptides were prepared on a System 990 automated peptide synthesizer (Beckman Instruments, Inc., Palo Alto, CA) using *t*-butoxycarbonyl methodology following the standardized cycle [29]. Cyclized peptides were formed by the iodine method [30]. Peptides were purified on a Waters Delta Prep 3000 high performance liquid chromatography system with a C18 column (RPC18 Ultrasphere, 5 mm, 4.6×150 mm inner diameter) and sent to Beckman Research Institute of the City of Hope (Duarte, CA) for FAB mass spectral analysis. Tables 3 and 4 list the peptides used in these studies. Abbreviations have been used to replace the actual amino acid sequences in Fig. 2 and are as follows:

C-first loop: AC*YNFTNRKISVQRLASYRRITSSKC*-NH2
second loop: PKEAVIFKTIVAKEI

2.6. MCP-1 cloning

Total RNA was isolated from human umbilical vein endothelial cells using RNAzol B following the manufacturer instructions (Bio-Tec, Houston, TX). 1 μg of total RNA was used to prepare MCP-1 cDNA by reverse transcriptase and polymerase chain reaction using the GeneAmp RNA PCR kit (Perkin-Elmer, Norwalk, CT) with the following primers: 5′-GGATCCATGAAAGTCTCTGCCGCCCTTCTGT-3′; 5′-GGATCCTCAAGTCTTCGGAGTTTGGGTTTGC-3′. The resultant PCR fragment was subcloned into the plasmid pCR 2.1 using the TA Cloning Kit (Invitrogen, San Diego, CA) to create pTAMCP-1. FLAG peptide fusion subclones were created by performing PCR using Pfu and pTAMCP-1 following the instructions of the manufacturer (Stratagene, La Jolla, CA) with the following primers (FLAG peptide sequence is underlined): 5′-AGCGGATCCTCACTTGTCATCGTCGTCCTTGTAGTCCCCTCCAGTCTTCGGAGTTTGGGTTTGCTT-3′; 5′-GGATCCATGAAAGTCTCTGCCGCCCTTCTGT-3′. The resulting products were cloned into pCRScript (Stratagene). Restriction fragments were subcloned into pBlueBac III (Invitrogen, San Diego, CA) yielding the plasmids pBB3MCP1FLAG, pBB3Y13AFLAG, and pBB3R18AFLAG. All clones were verified by DNA sequence analysis using Sequenase (Amersham/USB, Cleveland, OH).

2.7. Site-directed mutagenesis

Site-specific mutagenesis was performed according to the methods of Kunkel et al. [31]. Codon Y13 of human MCP-1 was mutated to encode alanine using the following oligonucleotide (underlined bases indicate change from wild type): 5′-GGTGAAGTTAGCGCAGCAGGT-3′. Codon R18 was mutated to encode alanine using the following oligonucleotide (underlined bases indicated change from wild type): 5′-TGAGATCTTCGCATTGGTGAA-3′. Oligonucleotides were synthesized on an ABI 381A DNA synthesizer (Applied Biosystems, Foster City, CA). Clone identification was verified by restriction digest and DNA sequence analysis using Sequenase (Amersham/USB, Cleveland, OH).

2.8. Baculovirus and protein production

Baculovirus were generated in Sf9 cells using the FLAG fusion plasmid constructs described previously with the Bac-N-Blue transfection kit (Invitrogen). Viral isolates were plaque purified and amplified according to the manufacturer's instructions. MCP-1 producing isolates were identified by performing ELISA on culture supernatants with the Quantikine Human MCP-1 Immunoassay (R&D Systems, Minneapolis, MN).

Spinner cultures of log phase Sf9 cells were infected with virus stock for 10 days. Cell debris and virus particles were removed by centrifugation at $67\,000\times g$ for 1 h, and MCP-1 wild type and mutant FLAG-tagged recombinant proteins were isolated from Sf9 cell supernatants as follows. 250 ml of baculovirus supernatant containing between 0.4 and 2 μg/ml FLAG fusion protein were affinity purified over a 5 ml Anti-FLAG M2 Affinity Gel (Eastman Kodak Company, New Haven, CT) column as recommended by Kodak. Fractions were assayed for MCP-1 content by the Quantikine Human MCP-1 Immunoassay, and positive fractions were pooled. The pooled fractions were then quantitated again and analyzed for purity by Coomassie protein staining and MCP-1 immunoblotting.

2.9. MCP-1 immunoblots

To verify that MCP-1 quantitation was accurate, commercial and FLAG-tagged MCP-1 recombinant proteins were analyzed by immunoblotting with MCP-1-specific antibodies. Equal amounts of commercial and FLAG-tagged MCP-1 proteins were resolved through a 14% SDS-polyacrylamide gel and transferred to nitrocellulose (Micron

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].

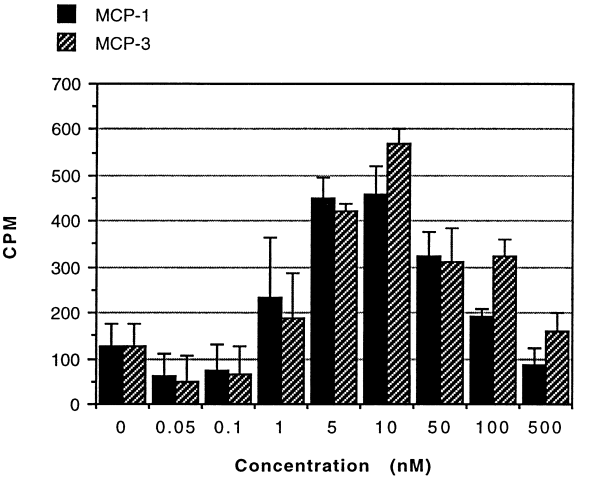

Fig. 1. Chemotactic activity of MCP-1 and MCP-3. The ability of MCP-1 and MCP-3 to induce migration of THP-1 monocytic cells was measured as described in Section 2. 1.5×10^5 [^{3}H]thymidine-labeled THP-1 cells were placed in the upper compartment of a 5 μm transwell plate, and allowed to migrate for 2 h at 37°C in response to the indicated concentrations of either MCP-1 or MCP-3. This figure represents one of a minimum of two independent experiments (mean CPM ± S.D. are shown, $n=4$).

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 μM. Additionally, the first loop peptide weakly inhibited MCP-1-induced cell migration with an IC_{50} of 633 μM. In contrast, no inhibitory activity was observed from peptides derived from the N- or C-terminus or the second loop. To determine if potency of the peptides could be enhanced, two first loop peptides were synthesized and cyclized by disulfide linkage via the cysteine residues. Table 4 shows that the activity of the first loop peptides in these assays was increased 53–71% by cyclization and amidation.

As demonstrated in Fig. 2A, first loop cyclic peptides blocked the binding of ^{125}I-MCP-1 to THP-1 cells in a dose-dependent manner while a peptide derived from the second loop did not. The estimated IC_{50} of the cyclic peptide is 15 μM. Next we evaluated whether the cyclic peptide could inhibit MCP-1-directed migration. In this experiment, labeled THP-1 cells were preincubated with or without peptide antagonists for 5 min and then placed in the upper compartment of a transwell filter plate. 6 nM MCP-1 was placed in the lower compartment and the ability of MCP-1 to mediate the migra-

Table 1
MCP-1 sequence

N-terminus	First loop	Second loop
Q^1PDAINAPVT^{10}	CCYNFTNRKISVQRLASYRRITSSK^{35}	CPKEAVIFKTIVAKEI^{51}C
C-terminus		
A^{53}DPKQKWVQDSMDHLDKQTQTPKT^{76}		

The human MCP-1 sequence as described by Yoshimura et al. [33]. The tertiary structure indicated is based on structural homology of MCP-1 with MIP-1β [23].

Table 2
THP-1 cell migration in response to MCP-1 is dependent on a concentration gradient

Lower chamber (ng/ml)	Upper chamber (ng/ml)			
	0 CPM	10 CPM	20 CPM	50 CPM
0	265 ± 46	179 ± 41	142 ± 23	133 ± 60
10	710 ± 159	311 ± 64	151 ± 15	191 ± 67
20	877 ± 141	448 ± 58	220 ± 79	132 ± 31
50	638 ± 163	598 ± 62	334 ± 58	86 ± 15

Indicated concentrations of MCP-1 were placed in the lower and upper compartments of a 96 well transwell plate and migration of 1.5×10^5 THP-1 cells was evaluated following a 2 h, 37°C incubation. This table represents one of a minimum of two independent experiments. Mean CPM ± S.D. are shown ($n=4$).

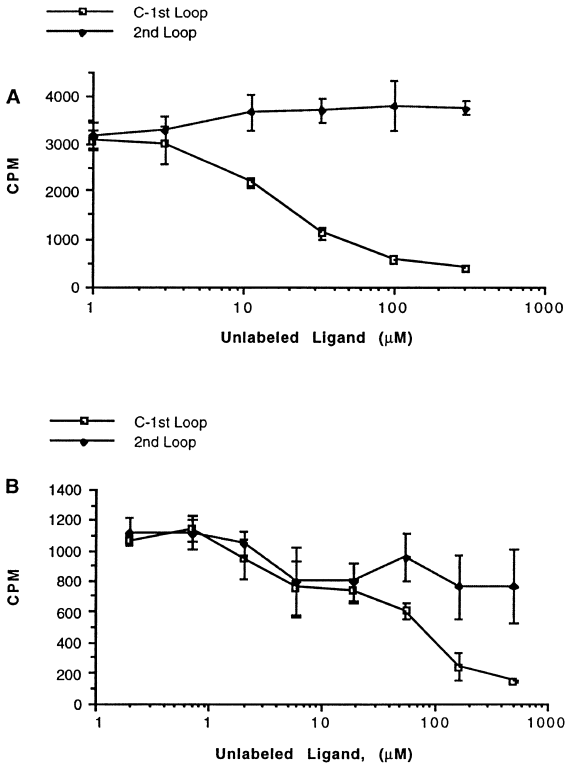

Fig. 2. Inhibition of MCP-1 binding and chemotaxis by a cyclized first loop peptide. Titration of cyclized first (C-1st Loop) versus the second (2nd Loop) loop peptides in inhibiting ^{125}I-MCP-1 binding to THP-1 cells (A) or MCP-1-directed migration (B) was done using methods described in Section 2 and Table 3. Each data point represents the mean CPM ± 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 migration, whereas the second loop peptide had little effect. These results suggest that binding and migration inhibitory activity is localized to the first loop of MCP-1. However, it is clear that the activity of the peptide is significantly reduced compared to native MCP-1 (see Fig. 4). In addition, the first loop of MCP-1 does not appear to be sufficient for migration activity since the cyclic peptide alone fails to promote THP-1 chemotaxis (data not shown).

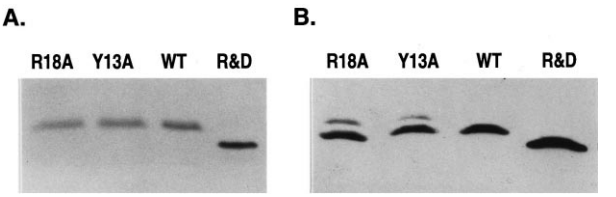

Fig. 3. Recombinant protein analysis of affinity purified FLAG-tagged MCP-1 recombinant proteins. FLAG-tagged mutant and wild type proteins were expressed using the baculovirus system and affinity purified over an anti-FLAG antibody column. Pooled wild type (WT), mutant (R18A, Y13A), or commercial (R&D) recombinant protein were resolved through a 14% polyacrylamide gel and either stained with Coomassie blue (A) or immunoblotted with MCP-1-specific antibodies (B).

3.3. Site-directed mutagenesis of tyrosine 13 and arginine 18 of MCP-1

Since the activities of the MCP-1 peptides suggest that the chemotactic activity of native MCP-1 resides primarily in the first loop, amino acids within this region, which are predicted to be solvent exposed and therefore potentially available for receptor interactions, were chosen for mutagenesis [22]. Residues tyrosine 13 and arginine 18 were converted to alanine by site-directed mutagenesis and full length mutant recombinant MCP-1 proteins were generated.

Previous reports have demonstrated that the addition of a FLAG epitope to the carboxy-terminus of MCP-1 would not significantly alter its activity [25]. We chose to fuse the FLAG

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
PKEAVIFKTIVAKEI	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 ± SD ($n=4$). IC_{50}s 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

Sequence	Binding IC_{50} (μM)	Migration IC_{50} (μM)
AYNFTNRKISVQRLASYRRITSSK	32	633
AC*YNFTNRKISVQRLASYRRITSSKC*	16	401
AC*YNFTNRKISVQRLASYRRITSSKC*-NH2	15	186

The effect of peptide cyclization was measured as described in Section 2and Table 3. The IC_{50}s shown represent an average of a minimum of two independent experiments ($n=4$). For chemical stability, an alanine was used in replacement of C11 and C12.

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 disease processes including inflammation and human immunodeficiency virus-1 cell entry and replication [34,35]. Since an inhibitor of these molecules may be clinically important, we are investigating which amino acid residues or domains are required for receptor binding and cell signaling. In this study we have determined the activity of MCP-1 domains using a combination of domain-specific peptides and site-directed mutagenesis. We have shown that synthetic peptides from the first loop of MCP-1, and not the second loop, N-terminus, or C-terminus, could compete with radiolabeled MCP-1 for binding to its receptor on THP-1 cells. This activity correlated with a parallel loss in MCP-1-mediated THP-1 cell migration. To substantiate our findings, we chose specific amino acids within the first loop for site-directed mutagenesis, and found that a substitution of tyrosine 13 with an alanine significantly altered MCP-1 receptor binding and chemotactic activity while a substitution of arginine 18 for alanine had no effect.

NMR analysis of MCP-1 reveals high structural homology to MIP-1β [23,36]. The three dimensional conformation includes a long, flexible N-terminus leading to three anti-parallel β sheets followed by a carboxy-terminal α helix. Previous reports have provided evidence for a role of the N-terminal tail in proper signaling activity [24]. However, the mechanism by which N-terminal deletions or mutations alter MCP-1 activity is still controversial. Some groups propose that the N-terminus is essential for MCP-1 dimer formation, and it is the homodimer which binds and activates the MCP-1 cell surface

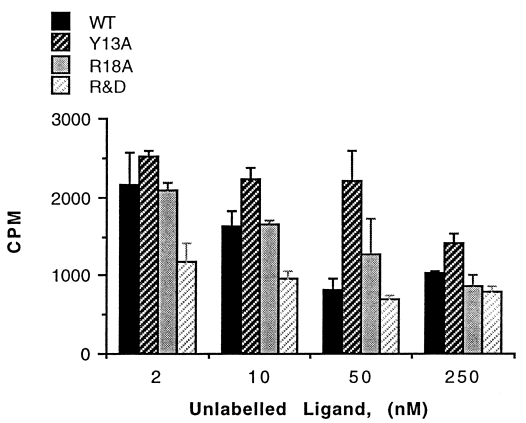

Fig. 4. FLAG-tagged wild type and mutant MCP-1 binding to THP-1 cells. Affinity purified FLAG-tagged wild type (WT), mutant (R18A, Y13A), and commercial (R&D) recombinant proteins were tested for their ability to compete with ^{125}I-MCP-1 binding to THP-1 cells. The indicated concentrations of recombinant 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 ± S.D. ($n=4$). This figure is a representative experiment from three independent experiments.

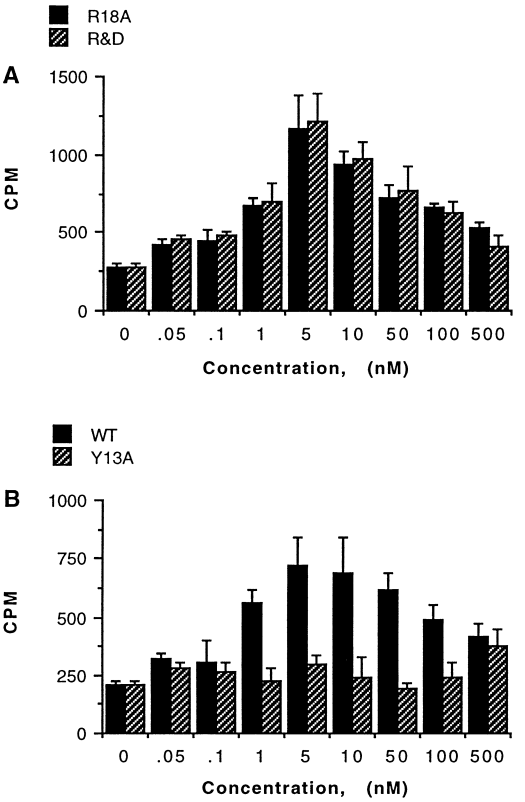

Fig. 5. FLAG-tagged wild type and mutant MCP-1 mediated migration of THP-1 cells. Affinity purified FLAG-tagged R18A mutant and commercial (R&D) (A), and wild type (WT), and Y13A mutant (B) were evaluated for their ability to promote THP-1 migration as described in Section 2 and Fig. 1. This figure is a single representative experiment from three independent experiments ($n=4$).

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 intercysteine 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 ~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 β1) 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 β1 strand at positions Y28, and R30 have greatly reduced or completely absent chemotactic activity [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-1's ability to promote monocyte chemotaxis and raise intracellular calcium levels [27]. This loss of chemotactic activity is correlated with a decrease in the ability of the protein to compete with native MCP-1 for receptor binding. The reduction of MCP-1 functional activity may be due to a conformational change in the MCP-1 protein such that the binding site, whether it be at position 13 or elsewhere, is not available to interact with CCR2b. Conservation of the aromatic side chain at position 13 by substitution of tyrosine to phenylalanine does not reduce MCP-1 activity [39] suggesting aromatic side chains may be essential for activity. NMR analysis of these mutants would assist our understanding of whether a conformational change has occurred.

NMR data predict that the dimer interface of CC chemokines is formed by the N-terminal β-sheet of the protein, whereas with CXC chemokines the second beta strand is involved in dimer formation [23,40]. Therefore, if MCP-1 dimers are required for binding and the Tyr^{13} residue is involved in dimer formation, substitution of the Tyr^{13} could lead to a loss of dimer formation and subsequent binding. Alternatively, the tyrosine 13 residue may be part of the binding site such that it is an absolute requirement for receptor binding.

Two major MCP-1 receptors have been identified (CCR2a and CCR2b) which differ in their cytoplasmic tails [41]. Like C5a, it has been proposed that MCP-1 binds to CCR2b through a two step mechanism [42,43]. In this two step model, MCP-1 first binds to the amino-terminal of the CCR2b receptor via a high affinity binding site. This interaction induces a conformational change in the receptor, such that a flexible region of MCP-1 binds to the lower affinity signaling site which is thought to be contained within the extracellular loops of the receptor. If this hypothesis is correct, then it is logical that there are several sites on MCP-1 which are critical for receptor activation. Since inhibition of low affinity interactions may not be measurable in our experimental conditions, we propose that the intercysteine first loop is critical for receptor interactions at the high affinity site. Preliminary data from our laboratory suggests that the cyclic first loop peptide can bind directly to the N-terminus of CCR2b (data not shown). If the high affinity interaction is the critical first step in MCP-1-CCR2b interactions, then it is tempting to speculate that potent antagonists to this site would be novel therapeutic agents for a variety of diseases characterized by lymphocyte and monocyte infiltration. In summary, we provide evidence using two distinct experimental approaches that

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

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