Monomeric Monocyte Chemoattractant Protein-1 (MCP-1) Binds and Activates the MCP-1 Receptor CCR2B*

(Received for publication, June 11, 1998, and in revised form, September 10, 1998)

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To address the role of dimerization in the function of the monocyte chemoattractant protein-1, MCP-1, we mutated residues that comprise the core of the dimerization interface and characterized the ability of these mutants to dimerize and to bind and activate the MCP-1 receptor, CCR2b. One mutant, P8A*, does not dimerize. However, it has wild type binding affinity, stimulates chemotaxis, inhibits adenylate cyclase, and stimulates calcium influx with wild type potency and efficacy. These data suggest that MCP-1 binds and activates its receptor as a monomer. In contrast, Y13A*, another monomeric mutant, has a 100-fold weaker binding affinity, is a much less potent inhibitor of adenylate cyclase and stimulator of calcium influx, and is unable to stimulate chemotaxis. Thus Tyr13 may make important contacts with the receptor that are required for high affinity binding and signal transduction. We also explored whether a mutant, [1+9–76]MCP-1 (MCP-1 lacking residues 2–8), antagonizes wild type MCP-1 by competitive inhibition, or by a dominant negative mechanism wherein heterodimers of MCP-1 and [1+9–76]MCP-1 bind to the receptor but are signaling incompetent. Consistent with the finding that MCP-1 can bind and activate the receptor as a monomer, we demonstrate that binding of MCP-1 in the presence of [1+9–76]MCP-1 over a range of concentrations of both ligands fits well to a simple model in which monomeric [1+9–76]MCP-1 functions as a competitive inhibitor of monomeric MCP-1. These results are crucial for elucidating the molecular details of receptor binding and activation, for interpreting mutagenesis data, for understanding how antagonistic chemokine variants function, and for the design of receptor antagonists.

Chemokines are small secreted proteins that function as intercellular messengers to control migration and activation of specific subsets of leukocytes (1–3). This process is mediated by chemokine receptor complexes on the surface of target cells. Interest in these proteins was first stimulated by the observation of elevated levels in a number of inflammatory diseases (4, 5) including rheumatoid arthritis (6, 7), arteriosclerosis (8, 9), and asthma (10). Although it is not clear whether excessive production is the cause or consequence of these diseases, the demonstration that neutralizing antibodies (11–13) reduced symptoms in a number of animal models generated optimism that receptor antagonists may have therapeutic benefit (14). Recently, it has been shown that certain chemokine receptors also serve as obligate coreceptors for entry of the human immunodeficiency virus into CD4+ cells (15–17) and that viral replication can be inhibited by the ligands of the coreceptors (18–21). Thus a wide range of clinically important diseases are associated with chemokines and their receptors, motivating many studies to understand the molecular details of chemokine function.

Chemokines have been classified into two major families based on their pattern of cysteine residues, their chromosomal location, and their cell specificities (22). α-C Chemokines such as IL-8\(^1\) have a conserved CXC cysteine motif and act predominantly on neutrophils, whereas β-chemokines have a CC signature and attract monocytes and T-cells. The recently discovered chemokines lymphotactin (23) and fractalkine/neurotactin (24, 25) are characterized by C and CX\(_C\) motifs, respectively, and chemoattract T-cells and NK cells. Mutagenesis studies, particularly of α- and β-chemokines, have provided some insight into the structural determinants of receptor binding and the specificity of these proteins, but many details have yet to emerge.

Considerable effort has been devoted to characterizing the stoichiometry of chemokine-receptor complexes because most chemokines oligomerize to an extent that depends on concentration and pH (26–30). Above micromolar concentrations many form homodimers, whereas at nanomolar concentrations the monomeric form predominates in solution. High resolution structures of IL-8 (31), MGS/A/GRO (32), MCP-1 (33, 34), RANTES (35, 36), and MIP-1β (37) have also revealed a striking correlation between chemokine class and mode of dimerization (38), suggesting that dimerization may play an important role in chemokine function. A key question, however, is whether dimerization is required for receptor binding and activation or whether it plays a more subtle role in protein stability, regul-
lution, surface presentation and retention, formation of the chemotactic gradient, or other processes unrelated to chemotaxis.

In the case of the CXC chemokine IL-8, monomeric mutants were shown to recruit and activate neutrophils in vitro as efficiently as wild type (26, 30), consistent with the view that it interacts with its receptor as a monomer. For the CC chemokine, MCP-1, some data suggest that a dimer may be the receptor-bound form of the protein (29). In these studies, a heterogeneous mixture of chemically cross-linked MCP-1 was shown to be active in chemotaxis assays. In addition, a deletion mutant lacking residues 2–8 ([1–9–76]MCP-1), which acts as a receptor antagonist, was also shown to inhibit chemotaxis by wild type but not by chemically cross-linked MCP-1. Finally, [1–9–76]MCP-1 containing a C-terminal FLAG epitope tag coprecipitated iodinated MCP-1 in an immunoprecipitation assay. Based on these results, it was postulated that MCP-1 interacts as a dimer and that [1–9–76]MCP-1 acts as a dominant negative antagonist by formation of inactive heterodimers with the wild type protein. Although this conclusion is consistent with the data, the heterogeneous nature of the cross-linked MCP-1 species allows alternative interpretations. Other data including the function of IL-8 obligate monomers, geometric considerations based on the three-dimensional structure of MCP-1, and the best data and models regarding G-protein-coupled receptor structure and function (39–42) also seem difficult to reconcile with a heterodimer model. Because resolving this issue is necessary for a complete understanding of the molecular mechanisms of leukocyte migration and for the modeling and utilization of structural data for the design of receptor antagonists, we felt compelled to investigate further whether MCP-1 can bind and activate its receptor as a monomer.

To explore the requirement of dimerization in the interaction of MCP-1 with its receptor, we mutated residues that contribute significantly to the dimer interface and assessed the effect of these mutations on receptor binding, on activation, and on dimerization. We also characterized the aggregation state of [1–9–76]MCP-1 at concentrations up to the millimolar range to assess whether it can form a homodimer. Finally, to elucidate the mechanism by which [1–9–76]MCP-1 acts as a receptor antagonist, we carried out binding competition experiments to address whether it functions as a dominant negative or classic competitive inhibitor.

MATERIALS AND METHODS

Gene Construction of Human MCP-1 Variants for Expression in Escherichia coli—With the exception of [1–9–76]MCP-1, all mutants were made in the context of MCP-1 M64I, referred to as WT*, and expressed in E. coli. We have demonstrated that WT* behaves identically to WT in binding and activity assays (see Table I). This alteration in the primary structure improves the purity and homogeneity of the mutants by eliminating the formation of species containing methionine-sulfoxide at position 64.

The gene for WT* MCP-1 was constructed by standard gene synthesis techniques with optimal codon usage for expression in E. coli (43). Mutant constructs were made by polymerase chain reaction mutagenesis of the WT* template and cloned into a pET3 based plasmid, pBAD-4 (44). All sequences were confirmed by double-stranded DNA sequencing. Plasmids were then transformed into TAP302 cells, which are BL21 pLYS S cells engineered with a thioredoxin reductase knockdown strain, 2% peptone, 100 mM K2PO4, pH 6.0, 0.08 mM MnSO4, 0.43 mM methionine, and either BMMY (Invitrogen) containing buffered complex medium or the cell growth medium but without the aminopeptidase step.

Analytical Characterization—The molecular masses of all proteins were characterized by electrospray mass spectrometry and differed by no more than 1 Da from the expected value. Protein purity was analyzed using reversed-phase HPLC; the average purity was 95 ± 5%. All protein concentrations were determined using an η90% extinction coefficient calculated from the amino acid composition at 80–90% power.

Binding Assay—A complete description of our binding assay can be

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aS. Lichter, T. M. Handel, H. J. George, and T. Patterson, unpublished results.

2S. Lichter, T. M. Handel, H. J. George, and T. Patterson, unpublished results.
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Statistical Analysis of Binding and Signaling—All 441 determinations of the \( K_D \) for the WT protein were tested for statistical normality; the data are log-normally distributed as would be expected from the ratio definition of \( K_D \). Similarly, the WT tests of CCR2-CHL binding, inhibition of CAMP synthesis, calcium flux, and chemotaxis were found to log-normally distributed. Thus all of the statistical tests on the \( K_D \) values and IC\(_{50}\) values were performed as pK\(_a\) values and pIC\(_{50}\) values. Significance is noted in Table I as explained in the footnotes to the experiment.

Fitting the Binding of WT MCP-1 in the Presence of [1–9–76]MCP-1 to a Competitive Inhibition Model—The competitive inhibition data of Fig. 8 were fit to the following equation.

\[
\frac{1}{M} = \frac{1}{F} + \frac{1}{R_T} + \frac{1}{R_T M_D} + \frac{1}{M_D} \quad (E)\]

where \( M_D \) and \( M \) are the bound and free concentrations of MCP-1, \( R_T \) is the ratio of receptor, \( K_D \) is the dissociation constant for WT binding, \( K_I \) is the dissociation constant for binding of [1–9–76]MCP-1, and \( I \) is the concentration of [1–9–76]MCP-1. It was assumed that the concentration of I is much greater than the concentration of receptor; this is a reasonable assumption because even at the lowest concentration of [1–9–76]MCP-1 used in these experiments, it exceeds the concentration of receptor by a factor of 10.

RESULTS

Y13A* Affects Receptor Binding and Activation—To investigate the role of dimerization on binding and activity we designed mutants that potentially disrupt or weaken the ability of MCP-1 to self-associate. Based on the structure (33, 34) and calculations of the difference in solvent accessibility between monomer and dimer, we identified several candidates for mutation including Tyr\(^{13}\), Thr\(^{10}\), Val\(^{9}\), and Pro\(^{8}\) (Fig. 1). These residues form a short stretch of \( \beta \)-sheet at the interface between the two subunits. Tyr\(^{13}\) and Tyr\(^{13}\) are oriented toward each other and in the crystal structure (34) are hydrogen bonded through an intervening water molecule. They also make hydrophobic contacts that stabilize the dimer by packing onto Val\(^{9}\) of the opposing subunit. Thr\(^{10}\) and Thr\(^{10}\) are packed against each other on the concave face of the dimer and interact through their hydroxyl groups. In addition to these side chain interactions, there are also four hydrogen bonds stabilizing the \( \beta \)-sheet.

\( ^{4} \) K. Jarnagin, D. Grunberger, M. Mulkins, B. Wong, S. Hemmerich, C. Paavola, A. Bloom, S. Bhakta, R. Freedman, D. McCarley, I. Polsky, A. Ping-Tsou, and T. M. Handel, manuscript in preparation.

\( ^{5} \) W. Boucher, unpublished results.
We also observed that [1+9–76]MCP-1 did not induce chemotaxis (Fig. 4a).

These results suggest that some of the N-terminal residues and particularly Tyr13 in the dimerization interface are important for binding and activation. However, from these data alone it is not possible to conclude whether the effects are due to the fact that these residues directly contact the receptor or whether dimerization is required and the mutations destabilize the dimer structure. To explore these possibilities, we characterized the ability of each of the mutants to form homodimers.

**Characterization of Aggregation States; Y13A*, [1+9–76]MCP-1, and P8A* are Monomeric—**To evaluate the impact of the above mutations on dimerization, equilibrium sedimentation measurements were carried out on each variant. Representative sedimentation profiles for WT*, [1+9–76]MCP-1, and P8A* are illustrated in Fig. 5. Also shown are theoretical curves for a monomer versus dimer. As previously reported, WT* forms a homodimer (27, 33) with a $K_d$ of approximately 0.5 $\mu M$. The two mutants V9E* and T10E* also formed dimers but were slightly less stable than WT*, with $K_d$ values of 8 and 12 $\mu M$, respectively, whereas the dimerization of V9A* was not significantly altered. As expected from the structure, dimerization of the Y13A* mutant was severely destabilized, and the protein remained monomeric up to 100 $\mu M$, the maximum concentration of the experiment (data not shown). In light of the fact that V9A*, V9E*, and T10E* maintained the ability to dimerize, the most surprising result was that P8A* was monomeric (Fig. 5c). From inspection of the structure (Fig. 1), we anticipated that all of the mutated residues, Pro$^8$ would contribute least to the stability of the dimerization interface. [1+9–76]MCP-1 was also monomeric (Fig. 5c), a result that is consistent with the P8A* data, because Pro$^8$ is replaced by glutamine in [1+9–76]MCP-1.

To confirm these results and evaluate aggregation states at even higher concentrations, we also carried out translational diffusion measurements by NMR. In these experiments, attenuation of the signal intensity as a function of gradient strength increases with the diffusion rate, $D_t$ (Fig. 6). For the dimeric WT protein, the measured $D_t$ was $0.931 \times 10^{-6}$ cm$^2$/s. For P8A*, Y13A*, and [1+9–76]MCP-1 the values range from $1.44 \times 10^{-6}$ to $1.60 \times 10^{-6}$ cm$^2$/s (see the legend to Fig. 5). By comparison with the value of $1.62 \times 10^{-6}$ cm$^2$/s for ubiquitin, a model protein of approximately the same size as an MCP-1 monomer, we conclude that these three mutants are monomeric at concentrations in excess of 1 mM. Thus dimerization in these mutants is destabilized by at least 3 orders of magnitude relative to WT*.

**NMR of MCP-1 Variants Indicate They Are Properly Folded**—To rule out the possibility that the mutations cause major structural perturbations, we recorded two-dimensional $^1$H–$^{15}$N HSQC spectra on $^{15}$N-labeled WT*, Y13A*, P8A*, and [1+9–76]MCP-1 and one-dimensional $^1$H spectra on unlabeled proteins. In all cases, the spectra were well dispersed, indicating that the mutations do not cause misfolding of the protein. In the one-dimensional $^1$H spectra, all variants had two isolated upfield shifted resonances at chemical shifts similar to those observed in WT, which were previously assigned to $\gamma$-methyl protons of Val$^{52}$ and Val$^{54}$ (33) (data not shown). These residues cluster together with Phe$^{91}$ in the core of the protein, and preservation of the shifted methyl protons in the mutants suggests that their core structures are similar to WT. The HSQC spectra (Fig. 7) are also well dispersed and contain the expected number of cross-peaks, but there are significant chemical shift changes between WT* compared with Y13A*, P8A*, and [1+9–76]MCP-1. This is not surprising because the dimerization interface in MCP-1 is extensive, and loss of dimer contacts would be expected to affect residues from the N ter-

![Fig. 1. Molscript diagrams (71) of an MCP-1 monomer (a) and dimer (b). The $\alpha$-carbons of residues mutated in this study (Pro$^8$, Val$^9$, Thr$^{10}$, and Tyr$^{13}$) are highlighted.](http://www.jbc.org/content/271/20/13316/fig/1)
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The columns labeled Fold (Mut/Wt) are the ratio of the measured parameter for the mutant MCP-1 to WT MCP-1. The error statistic is the standard deviation for the number of replicate experiments shown in parentheses. Blank cells represent unmeasured parameters.

WT activity but does not dimerize. This leads to the conclusion that MCP-1 can interact and activate CCR2b as a monomer. The fact that the Hill coefficient for the binding of WT MCP-1 is 0.97 ± 0.17 (average of 441 measurements) corroborates this conclusion. Consequently we suggest that the Y13A* mutation causes a loss of function because Tyr13 interacts directly with the receptor and not because the mutation impairs dimerization or folding.

1.9–76]MCP-1 Is a Competitive Inhibitor—If MCP-1 interacts with CCR2b as a monomer, this suggests that receptor antagonism by [1–9–76]MCP-1 probably does not occur by a dominant negative mechanism involving heterodimer formation. To explore this, we carried out equilibrium binding displacement measurements of MCP-1 in the presence of various amounts of the inhibitor. The data are shown in Fig. 8 as double-reciprocal (Benesi-Hildebrand) plots. Using a simple model of competitive inhibition, the data (including binding at additional concentrations not included in the figure) were simultaneously fit to estimate the dissociation constants for binding of WT MCP-1 (Kd), [1–9–76]MCP-1 (Kd), and the receptor concentration (R). The Kd, Kd, and R calculated from the fit are 439, 12.3, and 9.3 pm, respectively, in good agreement with the independently measured values reported in Table I. This supports the conclusion that antagonism by [1–9–76]MCP-1 occurs through a competitive inhibition mechanism in which monomeric WT MCP-1 is displaced from the receptor by monomeric [1–9–76]MCP-1.

Demonstrating direct binding of [1–9–76]MCP-1 to the receptor would provide additional evidence in favor of competitive inhibition. However, we have determined that for the concentrations of receptor achievable in our assay, the antagonist affinity and the level of nonspecific binding, specific antagonist binding is not measurable.

Disulfide Cross-linked WT*(Cys77) and [1–9–76](C77) Bind to CCR2b—We made specific disulfide cross-linked MCP-1 dimers by adding a cysteine residue to the C terminus of WT* and [1–9–76]MCP-1. These proteins, WT*(Cys77) and [1–9–76](C77), were expressed and purified as monomers and then dimerized by air oxidation. The homogeneity and purity of the dimers was confirmed by mass spectrometry and reversed-phase HPLC. The WT*(Cys77) dimer interacts with CCR2b with near wild type affinity on THP-1 and CCR2-CHL.

Fig. 2. Displacement of [125I]MCP-1 from CCR2 expressed on THP-1 cells by unlabeled WT* and the mutants P8A*, T10E*, Y13A*, V9A+T10A*, and [1–9–76]MCP-1. The data shown are representative of experiments done in triplicate and repeated on a minimum of 4 and a maximum of 441 separate days. Within each day standard deviations for each point are 10–15%.

* p < 0.001, highly significant.
† p < 0.01, significant.
‡ No calcium influx was observed at 200 μM, the highest concentration tested.
§ No chemotaxis was observed at 100 μM, the highest concentration tested.

a No chemotaxis was observed at 10 μM, the highest concentration tested.

L. S. Mizoue, F. Bazan, E. C. Johnson, and T. M. Handel, Biochemistry, in press.

Table I

| Mutation | Binding to THP-1 cells | Binding to CCR2-CHL cells | Inhibition of cAMP synthesis | Stimulation Ca2+ influx | Stimulation of chemotaxis |
|----------|------------------------|---------------------------|-----------------------------|------------------------|--------------------------|
|          | Fold | Kd (nM) | Fold | Kd (nM) | Fold | IC50 (nM) | Fold | EC50 (nM) | Fold | EC50 (nM) |
| [1–76] (Wild type) | 1.0 | 0.035 ± 0.021 (441) | 1.0 | 0.030 ± 0.017 (46) | 1.0 | 0.048 ± 0.017 (21) | 1.0 | 3.39 ± 2.3 (19) | 1.0 | 0.360 ± 0.315 (16) |
| [1–76, M641] (Wild Type*) | 0.9 | 0.031 ± 0.018 (5) | 0.9 | 0.027 ± 0.016 (6) | 1.1 | 0.054 ± 0.022 (4) | 0.5 | 0.170 ± 0.085 (2) |
| P8A* | 1.0 | 0.035 ± 0.015 (4) | 0.7 | 0.020 ± 0.012 (4) | 0.5* | 0.024 ± 0.009 (4) | 0.4 | 1.49 ± 0.80 (3) | 0.2 | 0.089 ± 0.003 (2) |
| V9A* | 1.3 | 0.046 ± 0.016 (5) | 1.4 | 0.041 ± 0.012 (5) | 2.1 | 0.088 ± 0.068 (3) | 0.5 | 0.175 ± 0.021 (2) |
| V9E* | 6.2* | 0.217 ± 0.015 (4) | 7.6* | 0.229 ± 0.009 (2) | 13.4* | 0.641 ± 0.339 (4) | 6.3* | 2.27 ± 0.55 (3) |
| V9A + T10A* | 2.3 | 0.080 ± 0.027 (2) | 2.8* | 0.083 ± 0.020 (2) | 8.3* | 0.388 ± 0.018 (2) | 0.5 | 0.175 ± 0.021 (2) |
| T10E* | 20* | 0.704 ± 0.298 (7) | 27* | 0.801 ± 0.510 (8) | 118* | 5.654 ± 3.906 (5) | 64* | 23.0 ± 14.1 (2) |
| Y13A* | 95* | 3.319 ± 1.433 (20) | 160* | 4.768 ± 2.973 (6) | 1065* | 50.82 ± 36.48 (11) | 45* | 153 ± 109 (2) | 2 Antagonist | >100,000* (2) |

* [1–9–76]MCP-1 occurs through a competitive inhibition mechanism.
cells; the $K_d = 0.075 \pm 0.040$ and $0.037 \pm 0.016$ nM, respectively (Table I). It also drives chemotaxis ($EC_{50} = 0.41 \pm 0.19$ nM) and stimulates luciferase expression (not shown) with wild type efficacy. By comparison, dimers of $[119–76](C77)$ have an affinity nearly equivalent to monomeric $[119–76]MCP-1$; the $K_d = 0.343 \pm 0.209$ and $0.246 \pm 0.123$ nM on THP-1 and CCR2-CHL cells, respectively (Table I). Similarly, $[119–76](C77)$ dimers inhibit cAMP signaling to an extent comparable with $[119–76]MCP-1$ as measured in the luciferase assay; $IC_{50} = 10.8 \pm 1.2$ and $17.1 \pm 16.3$ nM, respectively (Table I). These data suggest that cross-linking in this manner does not alter the ability of these proteins to bind and activate the receptor.

**DISCUSSION**

We have presented several pieces of evidence that support a model in which MCP-1 binds and activates CCR2b as a monomer. The most conclusive data are based on P8A* which is fully functional in binding and activity assays yet does not dimerize even at very high concentrations. We have also shown that $[1+9–76]MCP-1$ does not efficiently homodimerize, suggesting that it also probably binds to the

![Image](http://www.jbc.org/content/33162/Fig3a.png)  
**Fig. 3.** Receptor activation by WT* and the mutants P8A*, V9E*, T10E*, Y13A*, and [1+9–76]MCP-1 (symbols are the same for all panels and are shown in the inset of panel a). a, inhibition of forskolin-stimulated cAMP synthesis in HEK-293-CCR2b cells by the MCP-1 mutants. Shown is a representative experiment done in triplicate on from 2 to 25 separate days. Within each day the standard deviation of each concentration is 5–15%. b, inhibition of forskolin-stimulated luciferase expression in CHO-K1-CCR2b-neo-22 cells, which are stably transformed with a DNA reporter. Shown is a representative experiment done in quadruplicate on from 2–12 days. Within each day standard deviations for each point are 10%. c, increase in cytosolic calcium. Shown is the mean cytosolic calcium concentration measured on 21 days for WT MCP-1, 3 separate days for P8A*, 2 separate days for Y13A*, and 3 days for [1+9–76]MCP-1. The standard deviation of each data point is approximately 30%.

![Image](http://www.jbc.org/content/33162/Fig4a.png)  
**Fig. 4.** Chemotaxis of THP-1 cells by MCP-1 mutants. The indicated concentrations are the starting (bottom well) concentrations of each protein. a, chemotaxis of WT MCP-1, P8A*, and [1+9–76]MCP-1. b, chemotaxis of WT, WT*, V9A*, V9E*, T10E*, and Y13A*. The error bars shown are the standard deviations of sextuplicate measurements. The data shown are representative of experiments repeated on a minimum of 2 and a maximum of 16 separate days.

![Image](http://www.jbc.org/content/33162/Fig5a.png)  
**Fig. 5.** Receptor activation by WT* and the mutants P8A*, V9E*, T10E*, Y13A*, and [1+9–76]MCP-1 (symbols are the same for all panels and are shown in the inset of panel a). a, inhibition of forskolin-stimulated cAMP synthesis in HEK-293-CCR2b cells by the MCP-1 mutants. Shown is a representative experiment done in triplicate on from 2 to 25 separate days. Within each day the standard deviation of each concentration is 5–15%. b, inhibition of forskolin-stimulated luciferase expression in CHO-K1-CCR2b-neo-22 cells, which are stably transformed with a DNA reporter. Shown is a representative experiment done in quadruplicate on from 2–12 days. Within each day standard deviations for each point are 10%. c, increase in cytosolic calcium. Shown is the mean cytosolic calcium concentration measured on 21 days for WT MCP-1, 3 separate days for P8A*, 2 separate days for Y13A*, and 3 days for [1+9–76]MCP-1. The standard deviation of each data point is approximately 30%.
curves for monomer and dimer, respectively.

The calculated value of the diffusion coefficient, $D$, for each of these proteins is $0.931 \times 10^{-6}$, $1.58 \times 10^{-6}$, $1.61 \times 10^{-6}$, $1.44 \times 10^{-6}$, and $1.62 \times 10^{-6}$ cm$^2$/s, respectively.

receptor as a monomer rather than an oligomer. Although it is possible that dimer formation may be more favorable on the receptor than in solution, the receptor binding data of Fig. 8 fits well to a model in which MCP-1 binds as a monomer and is competitively inhibited by monomeric [1-9-76]MCP-1. Therefore the highly conserved dimerization motif observed in most CC chemokines does not appear to be an integral part of receptor binding and activation. In fact, the increased potency of P8A compared with WT MCP-1 in the activity assays may reflect a negative influence of dimer formation on function; this is best rationalized by the fact that residues involved in stabilizing the dimer (e.g. Tyr13) are also involved in interactions with the receptor. 3,4

Even so, dimerization is probably not irrelevant to the biological function of MCP-1. It seems unlikely that & alpha; and $\beta$ chemokines would have evolved such strictly conserved modes of dimerization for no reason. Although we were surprised by the magnitude of the effect on dimerization of our proline mutation, the frequent occurrence of prolines at the boundaries of dimerization interfaces has been documented (58). It has been suggested that they impose constraints on the conformation and dynamics of the polypeptide chain in a way that favors oligomerization, often through a mechanism described as “arm exchange” (58). Importantly, the equivalent residue of Pro$^9$ of MCP-1 is conserved in most well characterized CC chemokines, again suggesting that dimerization is there by design and plays a functional role. An exception to this is MCP-3, which possesses a serine at the corresponding position and is monomeric (59). The viral CC chemokines, MC148R1 and MC148R2 (60), are also missing this proline, which may point to a function of oligomerization that is required for certain host chemokines but not for these viral chemokines.

Can MCP-1 bind as a dimer? Although this has been demonstrated by chemical cross-linking studies (29), it is not clear that the cross-linked dimer is the same type of dimer as occurs naturally (Fig. 1b) or whether it involves two monomers linked in locations that do not interfere with binding to the receptor. We have shown that specific C-terminal disulfide-linked dimers, WT*(C77) and [1-9-76](C77), bind and activate the receptor as effectively as monomeric MCP-1 and [1-9-76]-MCP-1. Thus in a nonspecifically cross-linked mixture, those species that are cross-linked near the C terminus would bind and activate the receptor like WT and [1-9-76]-MCP-1. We have also demonstrated that mutation of Tyr$^{13}$ to alanine impairs binding and receptor activation to the extent that there is no measurable chemotaxis, suggesting that this residue makes important contacts with the receptor. Because Tyr$^{13}$ probably cannot simultaneously stabilize the dimer and interact with the receptor, we speculate that a true dimer would bind more weakly than a monomer, would be impaired in its ability to induce intracellular signaling, and would also be unable to induce chemotaxis.

If dimerization is not required for interaction with the receptor, then an important question is, why does it occur? One suggestion is that it serves a regulatory role to inhibit chemokine action at high chemokine concentrations, thus explaining the bell shape profiles observed in chemotaxis versus concentration experiments. However, all known chemotactic agents in both mammalian and nonmammalian cells elicit chemotaxis profiles that are bell shaped (61). Furthermore, in the elegant work of Lauffenburger and Zigmond and their co-workers (62, 63), it was demonstrated that bell shaped profiles are a result of the receptor number, equilibrium, and concentration gradient present during the chemotaxis experiment (62). Thus dimerization does not account for the inhibitory phase of chemotaxis curves. This is clearly demonstrated by the similarity of the profiles for P8A$^*$ and WT MCP-1 (Fig. 4a) despite the completely different dimer forming potential of these two proteins.

A more likely possibility is that oligomerization may be important for retention and presentation of chemokines by surface glycosaminoglycans (64–66). Mechanistically, this might occur through an avidity effect whereby oligomerization brings multiple chemokine heparin-binding domains together, increasing the affinity for glycosaminoglycans and facilitating the formation of surface concentration gradients necessary for

Fig. 5. Sedimentation equilibrium profiles for WT$^*$ (a), [1+9–76]MCP-1 (b), and P8A$^*$ (c). The solid and dashed lines are the theoretical curves for monomer and dimer, respectively.

Fig. 6. Gradient diffusion measurements for WT (17.3-kDa dimer); [1+9–76]MCP-1 (8.0-kDa monomer); and P8A$^*$, Y13A$^*$, and ubiquitin (8.5-kDa monomer). The calculated value of the diffusion coefficient, $D$, for each of these proteins is $0.931 \times 10^{-6}$, $1.58 \times 10^{-6}$, $1.61 \times 10^{-6}$, $1.44 \times 10^{-6}$, and $1.62 \times 10^{-6}$ cm$^2$/s, respectively.
haptotaxis. Consistent with this view, glycosaminoglycan-induced oligomerization does not occur for a monomeric form of IL-8 (66). Moreover, an analog of IL-8 lacking the heparin-binding domain was shown to have decreased in vivo promigratory activity, which correlated with its compromised ability to bind heparin sulfate and endothelial cell surfaces (67). In contrast, a different mechanism is used for presentation of the CX3C chemokine, fractalkine. This protein does not oligomerize but instead has a membrane anchored mucin-like stalk that tethers the chemokine domain to the cell surface. Together these domains appear to directly promote cell-cell adhesion as well as standard chemotactic responses (68).

Other roles for oligomerization are certainly possible and may be relevant to additional processes triggered by chemokine binding such as lysozomal enzyme release and generation of toxic products from the respiratory burst (69, 70). Alternatively, oligomerization may be involved in as yet unidentified functions unrelated to chemotaxis. Either way, the mutants generated in this work should provide important reagents for pursuing these questions.

In conclusion we have demonstrated that a monomeric form of MCP-1 is sufficient for receptor binding and activation. However, we support the involvement of oligomerization in some function of the protein, most likely in surface display and marking of surfaces as eligible for diapedesis. We have also shown that \([1^{1–76}]MCP-1\) functions as an antagonist via competitive inhibition of the WT protein. This suggests that virally encoded receptor antagonists that have natural N-terminal deletions, such as MC148R (60), may function in a similar manner. We believe these results provide new molecular details and insight into the mechanism of leukocyte chemotaxis.

Acknowledgment—We thank Sunil Bhakta for continued help and advice regarding many of the reported assays.

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7 L. S. Mizoue and T. M. Handel, submitted for publication.
Monomeric Monocyte Chemoattractant Protein-1 (MCP-1) Binds and Activates the MCP-1 Receptor CCR2B

Chad D. Paavola, Stefan Hemmerich, Dorit Grunberger, Irene Polsky, Adam Bloom, Richard Freedman, Mary Mulkins, Sunhil Bhakta, Debbie McCarley, Ludwig Wiesent, Belinda Wong, Kurt Jarnagin and Tracy M. Handel

J. Biol. Chem. 1998, 273:33157-33165.
doi: 10.1074/jbc.273.50.33157

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