Identification of Surface Residues of the Monocyte Chemotactic Protein 1 That Affect Signaling through the Receptor CCR2[†]

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ABSTRACT: The CC chemokine, monocyte chemotactic protein, 1 (MCP-1) functions as a major chemoattractant for T-cells and monocytes by interacting with the seven-transmembrane G protein-coupled receptor CCR2. To identify which residues of MCP-1 contribute to signaling though CCR2, we mutated all the surface-exposed residues to alanine and other amino acids and made some selective large changes at the amino terminus. We then characterized the impact of these mutations on three postreceptor pathways involving inhibition of cAMP synthesis, stimulation of cytosolic calcium influx, and chemotaxis. The results highlight several important features of the signaling process and the correlation between binding and signaling: The amino terminus of MCP-1 is essential as truncation of residues 2−8 ([1+9−76]-hMCP-1) results in a protein that cannot stimulate chemotaxis. However, the exact peptide sequence may be unimportant as individual alanine mutations or simultaneous replacement of residues 3−6 with alanine had little effect. Y13 is also important and must be a large nonpolar residue for chemotaxis to occur. Interestingly, both Y13 and [1+9−76]hMCP-1 are high-affinity binders and thus affinity of these mutants is not correlated with ability to promote chemotaxis. For the other surface residues there is a strong correlation between binding affinity and agonist potency in all three signaling pathways. Perhaps the most interesting observation is that although Y13A and [1+9−76]hMCP are *antagonists* of chemotaxis, they are *agonists* of pathways involving inhibition of cAMP synthesis and, in the case of Y13A, calcium influx. These results demonstrate that these two well-known signaling events are not sufficient to drive chemotaxis. Furthermore, it suggests that specific molecular features of MCP-1 induce different conformations in CCR2 that are coupled to separate postreceptor pathways. Therefore, by judicious design of antagonists, it should be possible to trap CCR2 in conformational states that are unable to stimulate all of the pathways required for chemotaxis.

Chemokines are a family of proteins whose prototypic function is to promote the extravasation of leukocytes from the blood to surrounding tissues in response to inflammatory signals (*1*−*5*). This occurs upon binding of the chemokines to specific seven transmembrane G protein-coupled receptors. The binding event is transduced into a variety of intracellular changes involved in cell migration, including upregulation or activation of adhesion proteins (*6*−*8*), receptor desensitization and internalization (*9*−*12*), and cytoskeletal rearrangement and coupling to the cell motility machinery (*13*). In the case of macrophages and neutrophils, chemokine binding also triggers cellular activation, resulting in lysozomal enzyme release and generation of toxic products from the respiratory burst (*13*−*15*). Unfortunately, the signal transduction pathways that link binding to these physiological changes in the cell are poorly understood. Likewise, the molecular details of the ligand−receptor interactions responsible for inducing signal transduction are also unclear.

Of the more than 30 human chemokines presently known, the monocyte chemotactic protein 1 (MCP-1)[1] has received a great deal of attention because of its elevated expression in a number of chronic inflammatory diseases (*4*, *16*) including rheumatoid arthritis (*17*−*21*), asthma (*22*−*25*), and atherosclerosis (*26*, *27*). Importantly, treatment with MCP-1

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[1] Abbreviations: hMCP-1, human monocyte chemotactic protein 1; mMCP-1, mouse monocyte chemotactic protein 1; [1+9−76]hMCP-1, human monocyte chemotactic protein 1 with residues 2−8 deleted; [1+10−76,7/9]hMCP-1, human monocyte chemotactic protein 1 with residues 2−9 deleted and seven of nine lysines changed to arginine (K75 and K69 are retained); WT*, wild-type monocyte chemotactic protin 1 with a mutation of M64I (all point mutations in the M64I background are denoted by an asterisk, e.g., Y13A*); cAMP, cyclic adenosine monophosphate; LDL, low-density lipoprotein.

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neutralizing antibodies or other biological antagonists can reduce inflammation in a number of animal models (*28*−*31*). Knockout mice that lack MCP-1 or its receptor, CCR2, are also deficient in their ability to recruit monocytes and T-cells to several types of inflammatory lesions (*32*−*35*). Finally, LDL[1]-receptor/MCP-1-deficient and apoB-transgenic/MCP-1-deficient mice show significantly less lipid deposition and macrophage accumulation throughout their aortas compared to the WT MCP-1 strains (*36*, *37*). These findings suggest that the development of reagents that block MCP-1 activity would be beneficial in treating inflammatory diseases. To aid such an endeavor, we became interested in understanding the molecular basis of how MCP-1 binds CCR2 and induces receptor signaling.

Previously, several groups demonstrated that deletion of residues at the amino terminus of MCP-1 yields a truncated protein that acts as an antagonist of chemotaxis (*38*−*40*). Other studies identified additional residues that play a role in stimulating chemotaxis or cytosolic calcium influx (*40*−*42*). These studies provided important insight into some of the features of MCP-1-induced signal transduction. However, a complete examination of the surface-exposed residues that are involved in chemotaxis and other upstream signaling pathways has not yet been reported.

We set out to measure the contribution of each surface-exposed residue to binding (*82*) and signal transduction (this work) by deletion, mutation to alanine, and in some cases mutation to other residues. Our intent was to identify and differentiate key residues that contribute to signaling via "active triggering" versus residues that contribute to signaling by virtue of binding affinity. Active triggering residues are those that significantly or completely cripple receptor signaling while maintaining high-affinity binding. Such mutations change agonist potency to a degree that is disproportionate to their effect on binding affinity. Drawing upon the idea that seven-transmembrane proteins may adopt multiple conformational states that are coupled to different postreceptor pathways, we also investigated whether some of the mutants could selectively stimulate some signaling pathways but not others. This would imply that it is possible to design ligands that lock the receptor in particular conformations that either activate or inhibit specific signaling pathways. We believe these studies provide new, fundamental information regarding chemokine signal transduction and should contribute to the design of novel chemokine antagonists.

MATERIALS AND METHODS

Choice of Residues for Mutagenesis. Residues were targeted for mutation if the fraction of solvent-accessible surface area exceeded 33%. Calculations were performed within the program GRASP 1.1 (*43*) using a 1.4 Å probe radius. The maximum solvent-accessible surface area for each amino acid was estimated by utilizing GGXGG model peptides. The C-terminal residues from 59 to 76 were not mutated because others have shown that they are not involved in interactions with the receptor (*41*).

Preparation of MCP-1 Mutants. Gene construction, expression, and purification of WT and mutant MCP-1 was done as previously reported (*44*). The purity and composition of all proteins was carefully characterized. Molecular masses were verified by electrospray mass spectrometry and differed by no more than 1 Da from the expected value. Protein purity was analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC) and averaged 95% ± 5%. Protein concentrations were measured by use of an extinction coefficient at 280 nm calculated from the amino acid composition (*45*).

Binding Assay. Binding was measured with membranes prepared from two cell lines, THP-1 and CCR2-CHL cells. Each assay was composed of membranes, 50 pM ^{125}I-MCP, MCP buffer, protease inhibitors, and test protein. Equilibrium was achieved by incubation at 28 °C for 90 min. Membrane-bound ^{125}I-MCP was collected by filtration through GF/B filters presoaked in poly(ethylenimine) and bovine serum albumin (BSA), followed by four rapid washes with approximately 0.5 mL of ice-cold buffer containing 0.5 M NaCl and 10 mM HEPES, pH 7.4. MCP buffer consists of 50 mM HEPES, pH 7.2, 1 mM $CaCl_2$, 5 mM $MgCl_2$, and 0.1% BSA. Protease inhibitors include 0.1 mM phenylmethanesulfonyl fluoride (PMSF), 1 μM leupeptin, and 0.35 mg/mL pepstatin.

THP-1 cells are a human monocyte cell line (ATCC TIP-202) that express both CCR1 and CCR2. CCR2-CHL cells are Chinese hamster lung cells (ATCC CRL-1657) that have been stably transformed with an expression vector, pSW104, bearing the human CCR2b receptor and a neomycin resistance marker plasmid as previously described (*46*).

Calcium Influx Assay. MCP-1-stimulated cytosolic calcium concentration was measured with fura-2 AM- (Molecular Probes, Eugene, OR) loaded THP-1 cells (*47*). The cells were cultured as for chemotaxis (described below) and were in log-phase growth, $(0.6-1) \times 10^6$ cells/mL. The cells were washed in loading buffer and resuspended in loading buffer [$(2-4) \times 10^6$ cells/mL)] containing 2 μM fura-2 AM and incubated at 37 °C with occasional mixing. After 40 min, cells were pelleted, washed with loading solution, and resuspended in Ringers solution at 1×10^6 cells/mL. Loading buffer consisted of Hanks' balanced salts solution containing 10 mM HEPES, pH 7.0, and 3 mM dextrose. Ringers solution contains 154 mM NaCl, 5.5 mM KCl, 2 mM $CaCl_2$, 0.1 mM $MgCl_2$, 100 mM HEPES, pH 7.3, and 2.5 μM sulfinpyrazone.

Individual aliquots of 1×10^6 cells in 2.0 mL were prewarmed at 37 °C and then transferred to a quartz cuvette in a 37 °C water-jacketed cuvette holder with a magnetic stirrer. Proteins to be tested were premixed and added to the cuvette after a stable baseline was obtained. The samples were excited at 340 and 380 nm, and 505 nm fluorescence was monitored in a CM1T11I Spex spectrofluorometer. The data were collected at 0.5 s intervals. Maximal fluorescence was obtained by lysing the cells with 0.03% Triton X-100, and minimal fluorescence was obtained after addition of 6 mM EGTA and 100 mM Tris base. The ratio of the fluorescence excited at 340 nm versus that at 380 nm was used to calculate the calcium concentration (*48*). The integrated total calcium content of the cells 82 s after addition of the test protein was used as a measure of protein efficacy.

Receptor Signaling Measured by Inhibition of Adenylate Cyclase: Direct Determination of [cAMP]. Direct determination of [cAMP] was done in HEK-293 cells stably expressing CCR2 prepared as described above for the CCR2-CHL cells. HEK-293-CCR2 cells were grown to confluence in Ham's F-12 medium containing 10% fetal bovine serum

(FBS), 30 μg/mL penicillin, 30 μg/mL streptomycin, and 250 μg/mL G-418. The cells were washed with phosphate-buffered saline (PBS) and incubated for 45 min at 37 °C with Ham's F-12 medium containing 0.1 μM adenine and 50 μCi of [2,8-^{3}H]adenine (740 GBq−1.48 TBq/mmol, New England Life Science, Bedford, MA). The cells were harvested by treatment with trypsin−EDTA (0.01 mg/L and 0.011 mM) in Ca^{2+}/Mg^{2+}-free PBS for 5 min at 37 °C and centrifugation at 840*g*. The cells were washed once and resuspended at 1×10^6 cells/mL in prewarmed 37 °C Krebs buffer containing 1 mM isobutyl methylxanthine (IBMX). The assay was performed in 96-well 1.2 mL polypropylene round-bottom plates by combining the following to a final volume of 0.55 mL: 35 000 cells, 0.5 mM forskolin, MCP or mutant MCP, and Krebs buffer containing 1 mM IBMX. Samples were incubated for 15 min at 37 °C. The reactions were terminated by adding 50 μL of cold 20% perchloric acid, incubating at 4 °C for 10 min, and neutralizing with 185 μL of 1 M KOH. The protein precipitate was cleared by centrifugation at 850*g* for 15 min. The supernatant (500 μL) was then transferred to a dry 1.3 g acidic alumina (activity grade I) column. The columns were washed twice with 4 mL of 0.005 M HCl followed by 2 mL of 0.1 M ammonium acetate. The cAMP was eluted with 3 mL of 0.1 M ammonium acetate into scintillation vials containing 20 mL of Beckman Ready Gel scintillation fluid (Beckman, Palo Alto, CA) and the radioactive content was measured.

Measurement of the Inhibition of Adenylate Cyclase by cAMP-Dependent Luciferase Expression. To create CHO-K1-CCR2b-cAMP-Luc-neo-22, CHO−K1 cells were simultaneously cotransfected with a reporter plasmid, the CCR2b receptor-encoding plasmid MCP1b-7 consisting of the CCR2b receptor cDNA in pSW104 (*46*, *49*), and pcDNA3.1+ neo vector (Invitrogen, Carlsbad, CA). In the transfection, a 5:5:1 ratio of reporter, receptor, and neo marker plasmids was used. The reporter plasmid was derived from pXP1 (*50*) and consists of a cAMP response element enhancer, a synthetic pentameric CREB binding enhancer from VIP, and a human glycoprotein hormone α-subunit promoter attached 5′ to luciferase cDNA (*51*, *52*). The sequence of the enhancer and promoter region have been deposited in GenBank under accession number AF047543 (A.P.-T. and A.K., unpublished results). The enhancer−promoter DNA was inserted in pXP1 between the *Bam*HI and *Hin*dIII sites. After the triple transfection and clone isolation, a single clone, neo-22, was selected. Selection was based on strong stimulation of luciferase activity by forskolin, low unstimulated luciferase expression, and strong inhibition of forskolin-stimulated luciferase activity by treatment with MCP-1. These cells were routinely passaged in Ham's F12 medium containing 10% FBS and 250 μg/mL G-418. Sixteen to twenty hours prior to an experiment, the cells were split into 96-well white tissue culture plates with 100 μL of Ham's F12 medium with phenol red omitted (Gibco/BRL, Gaithersburg, MD) and 10% FBS with G418 omitted. Dynatech flat-bottom W MicroliFB plates were used since they provide lower room light-stimulated autofluorescence than similar plates from other manufacturers. The plates were seeded at $(35-50) \times 10^3$ cells/well and incubated overnight at 37 °C. To begin an experiment, the medium was replaced with 80 μL of medium containing the test protein, 0.5 μM forskolin, 1% DMSO, and Ham's F12 medium (no phenol red) containing 5% FBS. The cells were incubated for 6 h at 37 °C. Subsequently the cells were washed with PBS. Luclite luciferase substrate mixture (Packard Instruments, Downers Grove, IL) diluted 1:1 with PBS was added to each well and the plates were read immediately in a 96-well format scintillation counter (Top Count, Packard Instruments, Downers Grove, IL) set for the detection of Luclite as per the manufacturer's instructions. A typical dilution curve consisted of 16 points, each in triplicate. The standard deviation of each data point is about 10%.

Chemotaxis. The THP-1-4X line was selected for these studies by repetitive culture and reselection of cells that passed through chemotaxis filters with MCP-1 as the chemoattractant. Thus, THP-1-4X have enhanced chemotaxis toward MCP-1 as compared to their ATCC provided parent (ATCC TIB-202). The cells were grown in RPMI1640 medium (Gibco/BRL, Gaithersburg, MD) containing 10% fetal calf serum and 5.5 μM 2-mercaptoethanol. Immediately prior to the assay, the cells were harvested by centrifugation, washed twice in RBH, and resuspended in RBH at 4×10^6 cells/mL. RBH is RPMI1640 with 1 mg/mL BSA and 10 mM HEPES, pH 7.4. The assay was performed in a chemotaxis apparatus (Neuro Probe, Inc, Cabin John, MD) equipped with a 96-well fluorescence view plate in the bottom chamber. To each well, 400 μL of RBH containing various concentrations of chemokine was added, and the view plate was fitted with a polycarbonate filter (pore size and thickness = 8 μm). A suspension of THP-1-4X cells (100 μL, 4×10^5/well) was then added to the top chamber. The chemotaxis apparatus was incubated at 37 °C for 1 h. Cells remaining in the top chamber were then discarded, the top surface of the filter was washed free of cells, and the plate and filter were centrifuged at 500*g* for 1 min. A 200 μL aliquot of liquid was removed from each well, followed by the addition of 150 μL of 4 mM propidium iodide in a 0.1% aqueous solution of Triton X-100. After incubation for at least 1 h, plate fluorescence was read in a Millipore Cyto23 reader with excitation and emission wavelengths of 530 and 590 nm, respectively.

Statistical Significance of Data. The data were analyzed for statistical significance by the use of mean pK_d or pIC_{50}. Significance is indicated in Table 1 by footnotes *b* ($p < 0.01$, highly signicant) and *c* ($p < 0.001$, significant). The use of pK_d and pIC_{50} is justified by our examination of the distribution of 441 determinations of K_d for the WT molecule. The data are log-normally distributed, as expected from the ratio definition of K_d. Although we have reported the data in a traditional format (nanomolar plus or minus a standard deviation), the readers should evaluate the significance of the data by recognizing that this format represents binding and function parameters as nonlogarithmic numbers. Since equilibrium constants and related parameters are continuous numbers with no meaning for zero, conversion of K_d's to logarithms (pK_d or pIC_{50}, etc.) would more accurately represent the accuracy of the data. For example, the reporting of data in molar format suggests that a K_d of 1 nM ± 1 nM has zero as part of its range. In fact if the data were reported in their more natural logarithmic form the value would be accurately reported as pK_d = 9.0 ± 0.43 (*53*).

Complete Database of Results. This paper describes the affinity and signaling ability of 58 point mutants as assessed in two different binding assays, two assays of cAMP

Table 1: Summary of the Binding and Activation of CCR2 by MCP-1 and MCP-1 Mutants[a]

	binding to CCR2			inhibition of cAMP synthesis		blockade of cAMP-induced gene synthesis		stimulation of Ca^{2+} influx		stimulation of chemotaxis	
mutation	*x*-fold (mut/wt) THP-1	*x*-fold (mut/wt) CHLV	K_d (nM) THP-1	*x*-fold (mut/wt)	IC_{50} (nM)	*x*-fold (mut/wt)	IC_{50} (nM)	*x*-fold (mut/wt)	EC_{50} (nM)	*x*-fold (mut/wt)	EC_{50} (nM)
[1−76] (wild type)	1.0	1.0	0.035 ± 0.021 (441)	1.0	0.069 ± 0.091 (25)	1.0	0.31 ± 0.22 (14)	1.0	3.39 ± 2.3 (19)	1.0	0.360 ± 0.315 (16)
[1−76, M64I] (wild type*)	0.9	0.9	0.031 ± 0.018 (5)	0.8	0.054 ± 0.022 (4)					0.5	0.170 ± 0.085 (2)
[Y13A]*	95[b]	160[b]	3.32 ± 1.43 (20)	740[b]	50.8 ± 36.5 (11)	300[b]	95.10 ± 22.9 (4)	45	153 ± 109 (2)	antagonist	> 100000[d] (4)
[R24A]*	35[b]	28[b]	1.23 ± 0.46 (4)	27[b]	1.88 ± 0.90 (5)	12[c]	3.64 ± 0.35 (3)	14	47.3 ± 11.6 (3)	40[b]	14.50 ± 3.54 (2)
[K49A]*	15[b]	14[b]	0.54 ± 0.10 (3)	28[b]	1.95 ± 1.35 (4)	7.5[c]	2.36 ± 0.71 (2)	9.3	31.7 ± 16.8 (3)	10[b]	3.7 ± 2.12 (2)
[3−7A]*	2.4[b]	4.9[b]	0.085 ± 0.042 (6)	4.5[b]	0.31 ± 0.12 (4)	2.3	0.72 ± 0.25 (4)	4.1	14.0 ± 4.24 (2)	2.4	0.87 ± 0.12 (2)
[1+9−76]	6.8[b]	7.2[b]	0.24 ± 0.15 (5)	74[b]	5.2 ± 4.3 (5)	55[b]	17.1 ± 16.3 (3)	antagonist	> 200 000[e] (2)	antagonist	> 10 000[f] (4)

[a] The columns labeled *x*-fold (mut/wt) are the ratios of the measured parameter for the mutants versus WT. The error statistic shown is the standard deviation for the number of replicate tests given in parentheses. Blank spaces represent unmeasured parameters. The *x*-fold change, Mut K_d/WT K_d, is reported for binding to THP-1 4X and CCR2-CHL cells. The reported K_d is for THP-1 binding only. CHLV represents CCR2-CHL Chinese hamster lung cells that have been stably transformed with the human CCR2b. Significance is noted by footnotes *b* (highly significant, $p < 0.001$) and *c* (significant, $p < 0.01$). [d] No chemotaxis observed to 100 μM Y13A. [e] No calcium influx observed to 200 μM [1+9−76]. [f] No chemotaxis observed to 10 μM [1+9−76].

synthesis, cytosolic calcium influx, and chemotaxis. Clearly, presentation of this volume of data (1386 concentration−response curves) is beyond the capacity of a single journal page. Thus, the data have been presented graphically in Figure 3. Representative examples of the concentration response curves are presented in Figure 1. A complete data table with replicate statistics and potencies is available from the corresponding authors by E-mail.

RESULTS

Assays of Receptor Activation. In the present study we used four assays of receptor activation: chemotaxis, calcium influx, and two assays of adenylate cyclase inhibition. Typical dose−response curves for each assay as well as for binding are shown in Figure 1. One adenylate cyclase inhibition assay involved the direct measurement of intracellular cAMP levels 15 min after stimulation of cells with WT and mutant MCP-1. The second method indirectly measures the levels of cAMP over 6 h with a cAMP response element coupled to the firefly luciferase reporter gene. The first method, while direct, is very laborious and difficult to automate. The second method is less labor-intensive and easily automated. Inhibition potency of cAMP synthesis, measured for 21 MCP-1 mutants, was well correlated between both assays as shown in Figure 2 (correlation coefficient = 0.958). Inhibition efficacy for the mutants was similar to that for WT. Thus the reporter assay is as good a method for monitoring inhibition of adenylate cyclase as direct measurement of cAMP levels.

The N-Terminus of MCP-1 Actively Triggers Receptor Signaling. In two separate deletion studies, residues at the amino terminus of MCP-1 were shown to contribute significantly to signaling because removal of residues 1−8 or 2−8 resulted in proteins that could not induce chemotaxis or calcium influx (*40*, *41*). We reproduced some of these mutants and observed that [1+9−76]hMCP-1[1] has a surprisingly high affinity for CCR2. It binds THP-1 cells and CCR2-CHL cells with an affinity that is reduced by a factor of only 6.8−7.2 compared to WT (Table 1). Similar reductions in binding affinity, ranging from 3- to 7-fold, are seen for [1+9−77]mMCP-1,[1] [9−77]mMCP-1, and [1+10−76]-hMCP-1. Nevertheless, [1+9−76]hMCP-1 is unable to stimulate chemotaxis even at concentrations of 10 μM or calcium influx at 200 μM (Table 1, Figure 1C, D). These concentrations are 3500- and 10 000-fold higher than the EC_{50} predicted from a correlation of binding affinity with chemotaxis and calcium (Figure 4C, D), respectively. Thus the amino terminus appears to be a region of the protein involved in actively triggering the receptor.

[1+9−76]hMCP-1 Is an Agonist of cAMP Inhibition and an Antagonist of Chemotaxis and Calcium Influx. [1+9−76]hMCP-1 can also antagonize cytosolic calcium influx driven by 10 nM MCP-1 with an IC_{50} of 97 nM (Figure 5A) and it can block THP-1 chemotaxis driven by 3 nM MCP-1 with an IC_{50} of 60 nM (*41*; Figure 5B). However, it is able to inhibit synthesis of cAMP, with reasonable potency, at concentrations 55−75-fold lower than WT (Table 1, Figure 1A,B). Thus [1+9−76]hMCP-1 is an antagonist of chemotaxis and an antagonist of calcium influx but, surprisingly, an agonist of cAMP synthesis inhibition.

The Exact Chemical Nature of the N-Terminal Residues Is Not Critical for Signaling. Previous studies of IL-8

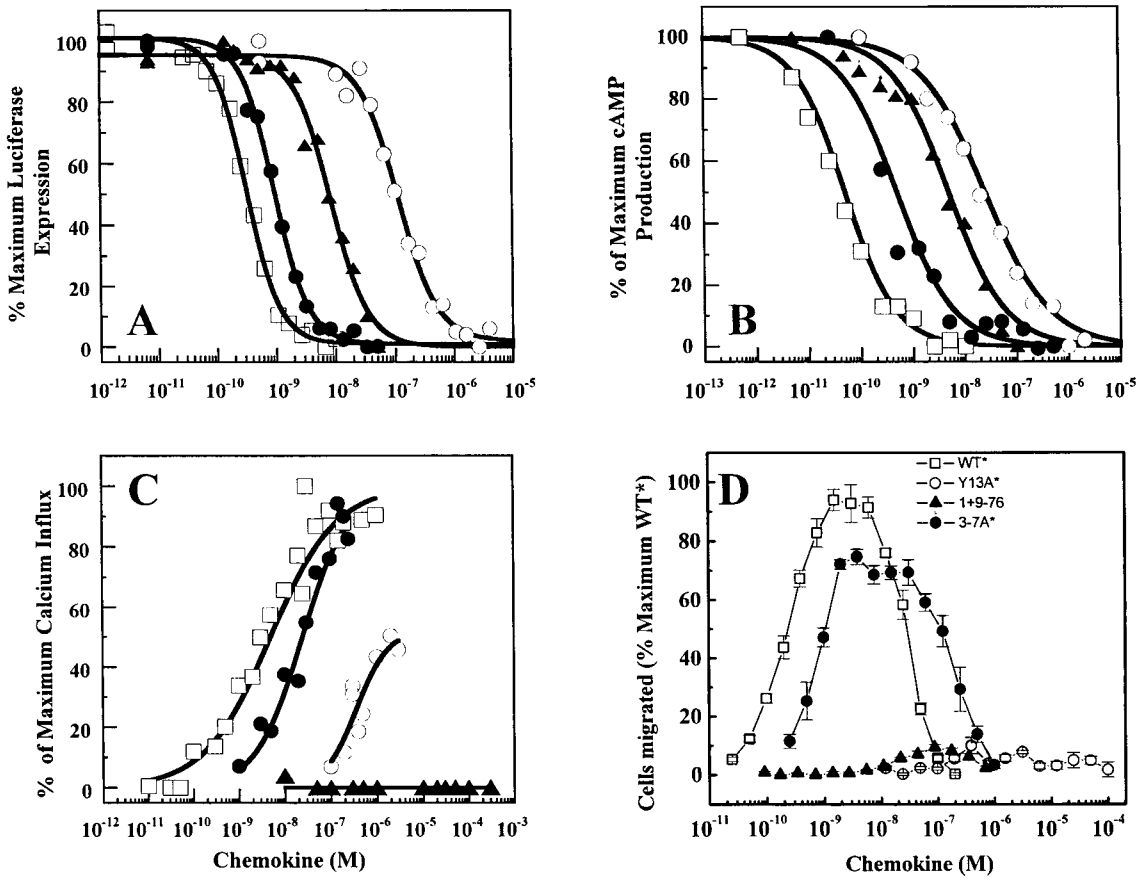

FIGURE 1: Representative dose−response curves for the four assays of receptor signaling. (A) Inhibition of forskolin-stimulated cAMP synthesis as measured by the cAMP responsive luciferase reporter assay. (B) Inhibition of forskolin-stimulated cAMP synthesis as measured by direct cAMP quantitation. (C) Stimulation of cytosolic calcium influx. (D) Stimulation of chemotaxis. For the cAMP assays each curve is representative of at least two and up to 441 curves performed on separate occasions; each data point is the mean of triplicate measurements with a standard deviation of 5−10%. The cytosolic calcium data points are the mean of up to 21 repetitions for WT MCP-1 and two for Y13A*. A single concentration−response curve was constructed for 3−7A. The standard deviation of each data point is approximately 30%. The chemotaxis curves are representative single curves of data collected on 2−16 occasions; each data point is the mean of quadruplicate data points with the standard deviation as shown.

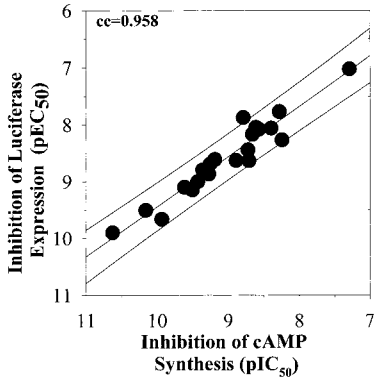

FIGURE 2: Correlation between ability of various mutants to inhibit forskolin stimulated adenylate cyclase as measured by two different assays: direct cAMP quantitation and luciferase expression.

demonstrated the importance of three N-terminal residues, collectively referred to as the ELR motif, on binding and signaling. To determine if specific residues in the N-terminus of MCP-1 were also crucial for signaling, we individually mutated residues 3−10 to alanine. Surprisingly, none of these mutations, D3A, I5A, N6A, P8A, V9A, and V9A+T10A, had more than a 2.5-fold reduction in binding affinity. Likewise, they showed no more than a 7.7- and 4-fold reduction in the potency of cAMP inhibition and chemotaxis, respectively (Figure 3). Even a triple mutant of D3A+I5A+N6A (making residues 3−7 alanine) showed only a 2.4−4.9-fold loss in affinity and a 2.4−4.5-fold reduction in Ca^{2+} influx, cAMP inhibition, and chemotaxis (Figure 3, Table 1). Efficacy in these assays was also similar to WT except for a slight decrease relative to WT in the chemotaxis assay (Figure 1D). Thus the changes in signaling for these mutants were not statistically different from changes in binding affinity (Figure 4, Table 1). This contrasts with [1+9−76]hMCP-1, where binding and signaling are completely uncorrelated (Figure 4). Thus, although the N-terminus plays a critical role in receptor activation, the presence of polypeptide chain but not the exact chemical nature of the side chains appears to be most important. Exceptions to this are mutants with more extreme changes of Val and Thr to Glu. These mutants, V9E and T10E, have lower affinities than the corresponding alanine mutations and lower potency in the cyclic AMP and chemotaxis assays (Figure 3). However, reduction in signaling still correlates with the changes in binding affinity, similar to the N-terminal alanine mutants (Figure 4).

Helical Secondary Structure in the N-Terminus Is Not Critical for Receptor Signaling. The crystal structure of MCP-1 (*54*) revealed a single turn of 3_{10} helix from P2 to N6 in the N-terminus of MCP-1. In the solution structure determined by NMR, this helix was only transiently formed

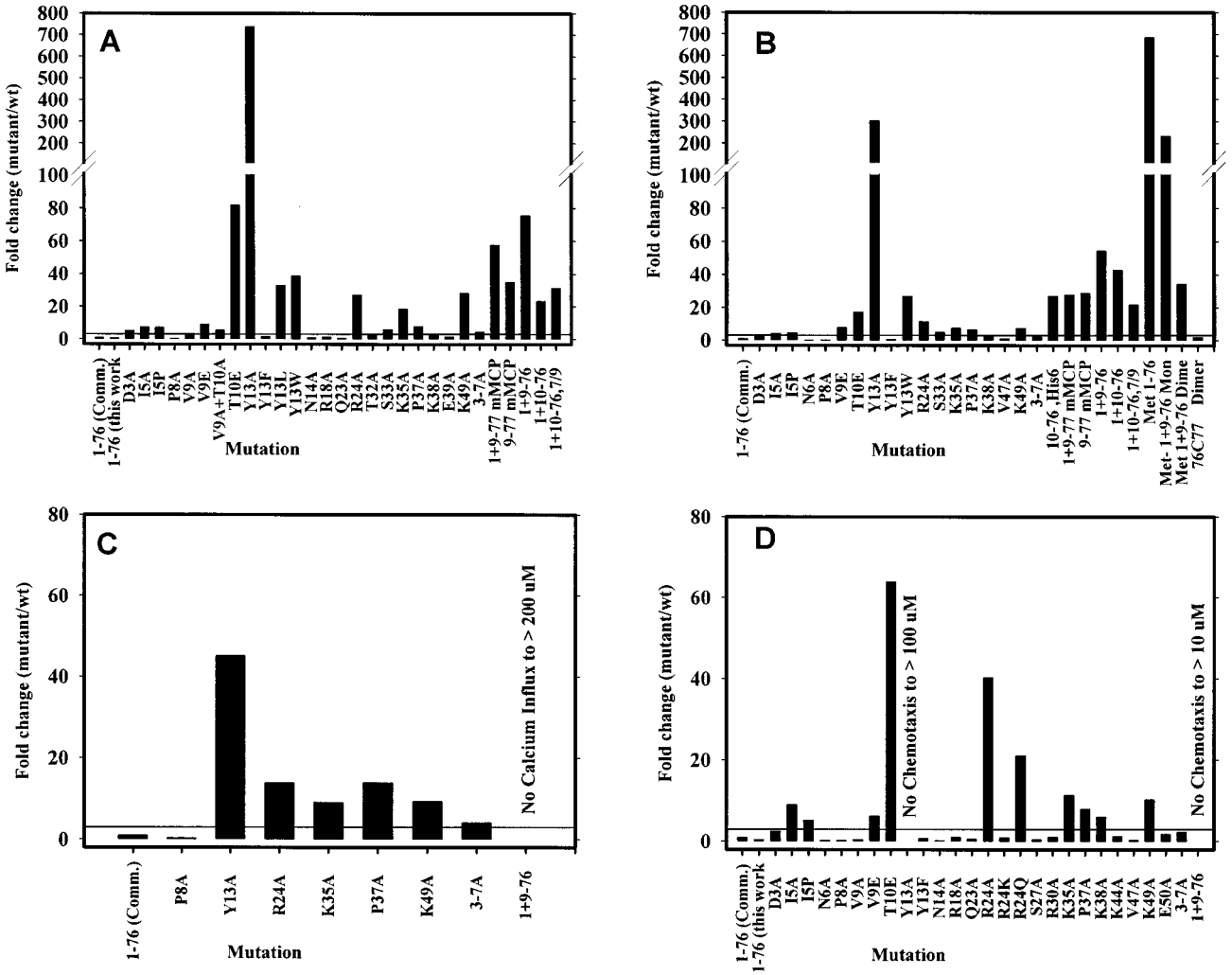

FIGURE 3: Stimulation of various postreceptor pathways by MCP-1 mutants. Shown is the ratio of mutant K_d, EC_{50}, or IC_{50} versus the value for WT. (A) Inhibition of forskolin-stimulated cAMP synthesis as measured by direct cAMP quantitation. The data shown are mean ratios of experiments performed on from 2 to 25 different days. (B) Inhibition of forskolin-stimulated cAMP synthesis as measured by a cAMP-responsive luciferase reporter assay. The data shown are the mean ratios of experiments performed on from 2 to 14 different days. (C) Stimulation of cytosolic calcium influx in THP-1−4X cells. The data are the mean ratios of experiments performed on from 2 to 19 different days. Note that [1+9−76] did not stimulate calcium influx even at 200 μM. (D) Stimulation of chemotaxis of THP-1-4X cells. The data are the mean ratios of experiments performed on from 2 to 16 different days. Note that Y13A* and [1+9−76] did not stimulate chemotaxis at 100 μM and 10 μM, respectively.

(*55*). To probe the importance of secondary structure in this region of the protein, an I5P mutant was constructed with the expectation that it would disrupt helix formation. This mutation reduced binding affinity by only a factor of 1.8 (Hemmerich et al., submitted for publication). It also reduced potency in the cAMP assays by 4.7−7.4-fold and in the chemotaxis assay by 5.3-fold (Figure 3), levels that are statistically similar to the changes in binding (Figure 4). This suggests that helical secondary structure in the N-terminus, like residue identity, is not likely to be important for binding or signaling.

Y13 Is an Agonist of cAMP Inhibition and Calcium Influx but an Antagonist of Chemotaxis. Of all the mutants, Y13A showed the largest reduction in binding: 95-fold on THP-1 cells and 160-fold on CCR2-CHL cells (Table 1). In addition, Y13A reduced cAMP inhibition potency by a factor of 300−740 (Table 1, Figure 1A,B). These alterations in ability to inhibit adenylate cyclase are contained within the 95% confidence interval correlating binding affinity with adenylate cyclase activity (Figure 4A,B). Thus loss of binding affinity adequately explains reduction in adenylate cyclase inhibition. In addition to being an agonist of adenylate cyclase, Y13A also stimulates cytosolic calcium influx with an EC_{50} of 153 nM, 45-fold less than WT (Table 1, Figure 1C). We note that Y13A is either 40−50% less efficacious than WT or the estimate of EC_{50} could be up to 5-fold lower than the actual value because of our inability to reach saturation. However, even with this caveat, the reduction in binding affinity also seems to fully account for the reduction in calcium flux potency (Figure 4C).

In contrast to the agonist effects on cAMP and calcium, Y13A does not stimulate chemotaxis even at concentrations of 100 μM (Figure 1D, Table 1). This concentration is 30 000 times the binding affinity of Y13A for CCR2 on THP-1 cells and 1300 times the EC_{50} predicted from the correlation shown in Figure 4D. The observation that Y13A is a potent agonist of at least two other postreceptor signaling pathways makes the lack of chemotaxis quite surprising.

Y13 can be changed to leucine (Y13L), tryptophan (Y13W), and phenylalanine (Y13F) with no alteration in ability to inhibit adenylate cyclase or stimulate chemotaxis that are disproportionate to the loss in binding affinity (Figure

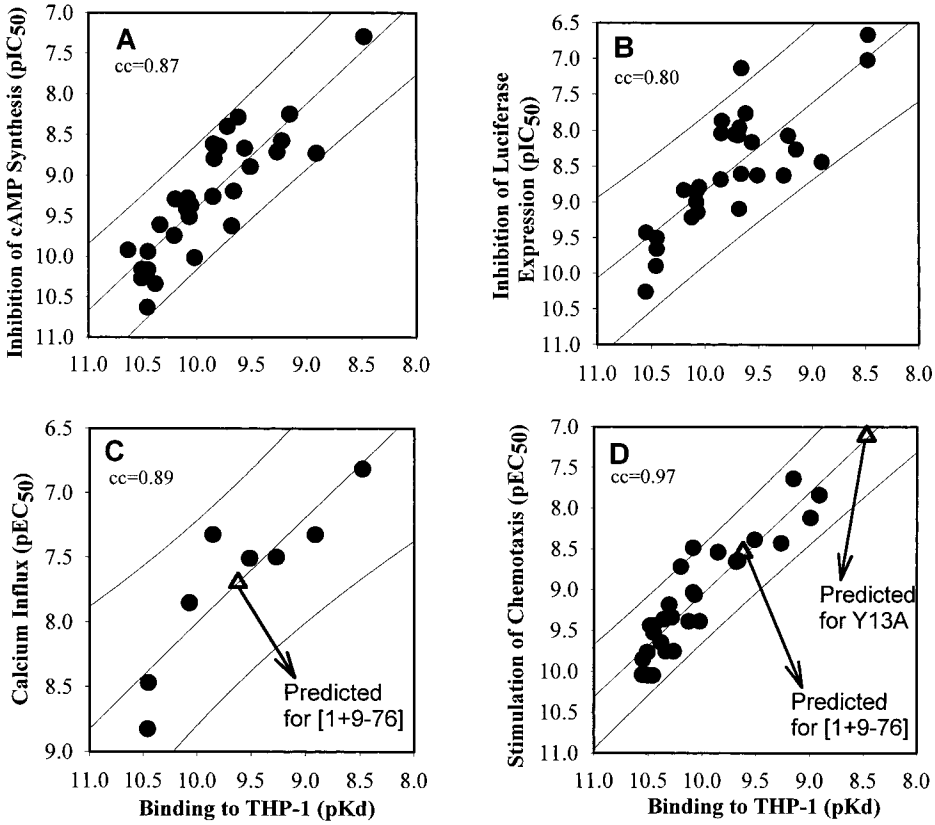

FIGURE 4: Correlation between binding affinity and agonistic potency. Shown is THP-1 pK_d plotted versus the pIC_{50} (adenylate cyclase assays) or pEC_{50} (calcium influx and chemotaxis) for the mutants shown in Figure 3. For all panels the outer pair of lines represents the 95% confidence interval for prediction and the middle line represents the linear regression. (A) Correlation between binding and inhibition of cAMP synthesis measured by direct cAMP quantitation. (B) Correlation between binding and inhibition of cAMP synthesis measured by the cAMP-responsive luciferase reporter assay. (C) Correlation between binding and stimulation of cytosolic calcium influx. (D) Correlation between binding and chemotaxis of THP-1 cells. The open triangles in panels C and D are the predicted positions for [1+9−76] and Y13A*.

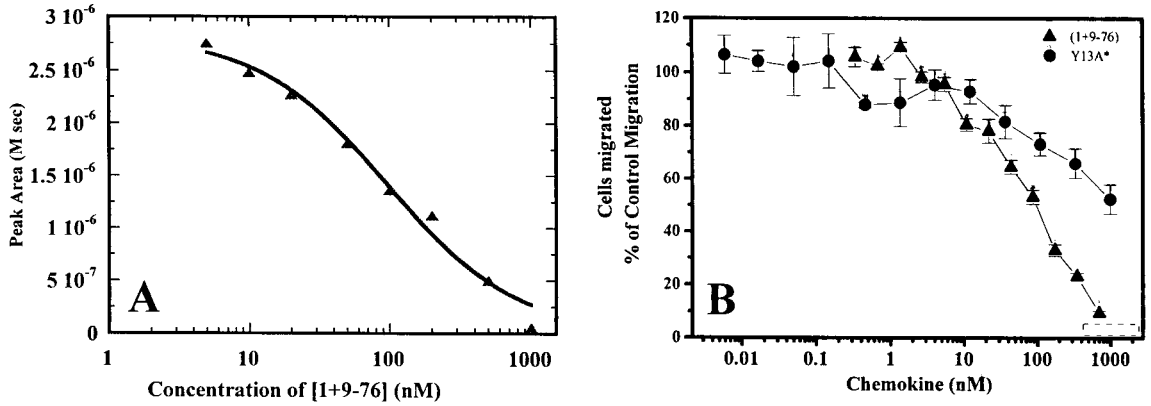

FIGURE 5: Antagonism of MCP-1-stimulated calcium influx by [1+9−76] and MCP-1-stimulated chemotaxis by [1+9−76] and Y13A*. (A) Inhibition by [1+9−76] of calcium influx into THP-1 cells stimulated by 10 nM MCP-1. Shown is a representative curve of data repeated 3 times. (B) Inhibition by [1+9−76] and Y13A* of chemotaxis stimulated by 3 nM MCP-1. Shown is the mean of quadruplicate measurements repeated on 2 separate days.

3). These data, combined with results from Beall et al. (*42*) that show small reductions in binding affinity for a Y13I mutant, indicate that any large hydrophobic residue can be substituted at this position, but small hydrophobics residues cause a significant loss of signaling ability.

Additional Important Residues Are in the Core Domain of MCP-1. Other residues also showed effects on signaling; however, they are all proportional to changes in binding affinity (Figure 3). This includes K35, P37, and K38, which are held in close proximity to the N-terminus by virtue of a disulfide bond between C11 and C36. Of these, K35A showed the largest effects on binding and signaling. This was significantly amplified by mutation to Glu, indicating that K35 may interact with a negatively charge residue in the receptor (*82*). Mutation of K38 to Glu only reduced binding by an additional factor of 2−3, indicating it is probably not involved in a salt bridge.

Two additional charged residues also affected binding and signaling. R24A has the second largest effect on binding affinity (28−35-fold), only surpassed by Y13A (Figure 3,

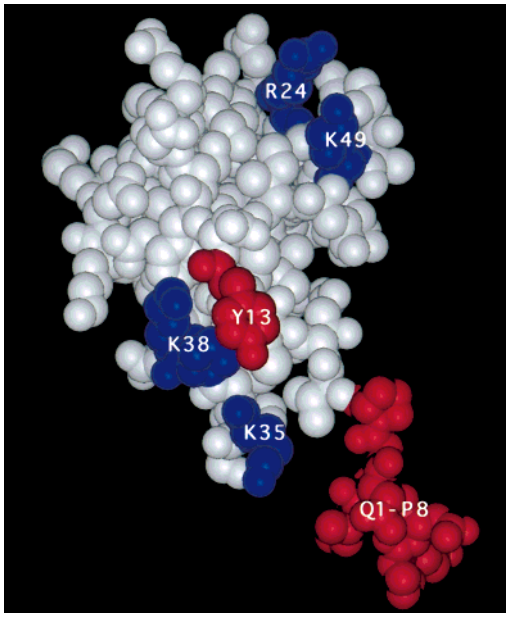

FIGURE 6: CPK representation of the solution structure of MCP-1 (*55*) showing the location of residues that affect signaling. Mutations that cripple signaling are shown in red: Y13A* and [1+9−76]. Mutations that affect signaling to an extent correlated with binding affinity are shown in blue: R24, K35A, P37A, K38A, and K49.

Table 1). Similar reductions in activity were observed in all signaling assays. Like K35, this residue may make an important ion-pair interaction with CCR2 since mutation to Glu caused a 30−50-fold additional loss of affinity (*82*). K49 also provides a critical contact with the receptor since mutation caused a 14−15-fold loss in binding and similar losses in signaling potency (Figure 3, Table 1).

In summary, these studies have identified two classes of MCP-1 mutants. Members of one class, which contains the majority of mutants, have altered signaling capacity to an extent commensurate with alterations in binding affinity. Members of the second group, which includes [1+9−76]-MCP and Y13A, have reduced but nonetheless high affinity for CCR2 but are completely crippled in their ability to stimulate one or more signaling pathways (thereby defining them as active triggering residues). Figure 6 summarizes many of these observations by mapping the location of the two residue classes onto the three-dimensional structure of MCP-1. The striking feature of this figure is the clear segregation of residues involved in active triggering to the N-terminus (residues 1−13), the clustering of the rest of the mutants to the core domain of the protein, and the presence of two distinct regions separated by 30−35 Å that are necessary for binding and signaling. These results further support and refine the concept of a two-site model for the interaction of chemokines with their receptors.

DISCUSSION

To understand how MCP-1 binds and activates its receptor, we initiated a mutagenesis study of all surface-exposed residues. In previous work, we identified a long hydrophobic groove on the surface of MCP-1, bounded by five key residues that are responsible for approximately 50% of the 14.3 kcal mol^{-1} binding affinity (*82*). These residues, Y13, R24, K49, K35, and K38, define two primarily basic patches at the extremes of the 35 Å groove. We also examined, by replacement and truncation, the contribution of the amino terminus to binding since previous work had suggested that this region was critical for agonism (*38*, *40*, *41*). Importantly, we demonstrated by NMR that none of the mutations perturbed the structure of the protein, so we could confidently conclude from the data that these residues are involved in direct interactions with the receptor. In the present study, we analyzed the effect of these mutations on receptor signaling.

Sequence Dependence of Signaling. Most of mutations that affect binding showed correlated changes in receptor activation as measured in all three signaling pathways, i.e., signaling-competent mutations. These include mutations of R24, K49, K35, and K38 and individual mutations of residues in the N-terminus. Very different behavior was observed with two other mutants (Y13A and [1+9−76] MCP-1). These proteins (signaling-crippled mutants) efficiently bind CCR2 but are unable to stimulate some of the signaling pathways.

As previously reported and reproduced by us, [1+9-76]-MCP-1 has mildly reduced binding affinity but no capacity to stimulate calcium influx or chemotaxis. Thus the presence of the amino terminus appears to be an active trigger for these downstream signaling events. Our data also reveal additional details about the role of these residues: Mutation rather than truncation of the amino terminus (*40*; this work) indicates that the nature of the protein sequence in positions 3−10 has little influence on the ability to signal through the three pathways investigated here. Gong and Clark-Lewis (*40*) also showed that residue 1 (pyroglutamate in WT) can be substituted with any of five different acidic and hydrophobic amino acids with little effect on signal transduction. These results are surprising, given that removal of the N-terminus yields a protein that is unable to stimulate chemotaxis and calcium. The requirement for the 3_{10} helix, observed in the crystal structure of MCP-1, also does not seem to be crucial since introduction of a proline in the middle of the helix does not impair binding or signaling. The low stringency in the sequence requirements for binding and signaling of MCP-1 suggests that interactions between the amino terminus of MCP-1 and the receptor may involve primarily backbone atoms. It also contrasts with other chemokine receptor systems that have strong sequence requirements. For example, in interleukin IL-8, replacement of E4, L5, or R6 by Ala results in a large reduction in both binding (30−1000-fold for individual replacements) and loss of calcium signaling (*56*). Likewise alanine replacement of P2 in RANTES results in a 5000-fold change in binding to CCR5 and loss of ability to stimulate calcium influx (*57*).

Since the length of the N-terminus is important, it was not surprising to find that the addition of extra residues also affects binding and signaling. Extension by an alanine ([Ala]-MCP-1) (*40*) or methionine ([Met]-MCP-1) (*82*) reduces the binding affinity for CCR2 on THP-1 cells by 119-fold and 95-fold, respectively. These mutants and acetyl-MCP-1 also show an approximately 300-fold loss of chemotactic potency (*40*; this work). However, in contrast to [1+9−76] MCP-1, the reduction in agonist potency correlates well with reduction in binding affinity. Again, this is unlike results reported for other chemokines. For RANTES, extension by Met (Met-RANTES) (*58*) or chemical modification of the N-terminus

to form (aminooxy)pentane RANTES (AOP-RANTES) (*59*) results in receptor antagonists that bind with high affinity but do not induce chemotaxis.

In summary, the data suggest that affinity alone is insufficient for signal transduction and that the length of the amino terminus is the most important structural feature for agonism of calcium and chemotaxis of MCP-1. We also note that truncation variants of many chemokines have been isolated from natural sources (*60*, *61*), suggesting that the sensitivity to the length of the N-terminus may be of general importance for regulation. For some chemokines, truncation increases activity; for others, it decreases activity (*62*) or changes cell specificity (*63*). Viruses also produce truncation variants (*64*), presumably to alter or inhibit signaling and promote viral pathogenesis (*65*).

Implications for CCR2 Signaling. One of the most interesting findings about [1+9−76] MCP-1 is that although it functions as an antagonist of chemotaxis and calcium, it is able to stimulate inhibition of adenylate cyclase. By comparison, Y13A is also unable to stimulate chemotaxis, but it can promote both calcium influx and adenylate cyclase inhibition. The question then remains, what G-proteins and signaling pathways are necessary and sufficient for chemotaxis to occur? Adenylate cyclase inhibition and calcium influx are abolished by pertussis toxin (PTX) implicating the $G_{\alpha i}$ class of G proteins (*66*−*68*). Since PTX treatment also inhibits chemotaxis, this suggests that G_i activation is required for chemotaxis. However, the data on Y13A and [1+9−76] suggest that it is not sufficient. In addition, since [1+9−76] inhibits cyclase but does not stimulate calcium influx, calcium must require coupling to something in addition to G_i or the released $\beta\gamma$ subunits. Thus, other G-proteins and downstream signaling molecules must be involved. In transfected COS cells, CCR2 couples to G_q and G_{16} as well as G_i (*69*). In primary monocytes and transfected CHO cells, MCP-1 binding stimulates a MAP kinase pathway, probably through G_q (*70*). Since MAP kinase activation and chemotaxis are blocked by the inhibitor PD98059, stimulation of this PTX-resistant pathway may be required for migration (*70*) (note, however, that in THP-1 cells PD98059 does not block chemotaxis even at 100 μM; Mulkins and Jarnagin, unpublished results). In THP-1 cells, primary monocytes, and T-cells, chemotaxis is also blocked by the phosphatidylinositol-3-kinase inhibitor wortmanin (*70*, *71*).

In addition to these pathways, which have been correlated with chemotaxis, MCP-1 has been shown to regulate phospholipase A2 and thus arachidonic acid release, possibly via phosphorylation (*72*). We also note a recent publication (*73*) which demonstrates that G-protein coupled receptors containing the sequence AsnProXXTyr in their seventh transmembrane region bind and stimulate the small G-proteins Arf and RhoA. Interestingly, all known chemokine receptors, including CCR2, have this motif. Rho in particular has been shown to be involved in triggering the rapid adhesion of leukocytes through integrin receptors (*74*). This integrin-mediated adhesion is blocked by cAMP and is PTX-sensitive, thereby linking it to G_i activation (*75*). Thus multiple signaling molecules have been shown to be activated by MCP-1 binding to CCR2, and simultaneous stimulation of several pathways seems to be required for chemotaxis to occur.

How does this happen? It has been suggested that G protein-coupled receptors can assume several different "active" conformations depending on the ligand, and this determines in part which particular pathways they activate (*76*−*79*). This multiconformation hypothesis offers a plausible explanation for our data. We suggest that specific molecular features of MCP-1 are required to induce different conformations that couple into distinct postreceptor pathways. The core domain alone (i.e., [1+9−76]MCP) is able to activate G_i, liberating $\beta\gamma$ subunits from the heterotrimeric complex; however, without the N-terminal trigger it cannot drive the formation of a conformation that activates calcium influx. Y13A, on the other hand, is able to induce a conformation that activates both G_i and the calcium-related pathways. However, like [1+9−76]MCP, it cannot induce a conformation that stimulates all the components necessary for chemotaxis. Amino-terminal derivatives of RANTES must selectively activate some pathways and not others. AOP-RANTES, for example, is unable to stimulate chemotaxis but is able to promote agonist-dependent receptor internalization (*80*). Finally, it is also possible that certain viral chemokines (*81*) have minor sequence modifications such as Y13A that eliminate their ability to signal through some of the pathways required for chemotaxis, since many do not have obvious N-terminal truncations to explain their antagonism. One could even imagine a related scenario where they activate some signaling pathways that enhance viral propagation but not others that would lead to chemotaxis and stimulation of the immune response. It is interesting to contrast these signaling-crippled mutants with the other class, which in some cases (R24A) have substantially reduced affinity but can still couple into the full repertoire of signaling pathways required for chemotaxis. The nature of the conformational changes that stimulate particular pathways will surely be the subject of further investigation, and our MCP-1 mutants provide important reagents for such studies.

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