Design and Receptor Interactions of Obligate Dimeric Mutant of Chemokine Monocyte Chemoattractant Protein-1 (MCP-1)*

Background: Pro-inflammatory CC chemokines form conserved dimeric structures.

Results: An obligate dimeric form of MCP-1 retains the wild type dimer structure but cannot bind or activate receptor CCR2.

Conclusion: CC chemokine dimers cannot bind to their receptors at affinities approaching those of the chemokine monomers.

Significance: Chemokine monomer-dimer equilibria are critical in regulating leukocyte recruitment during inflammation.

Chemokine-receptor interactions regulate leukocyte trafficking during inflammation. CC chemokines exist in equilibrium between monomeric and dimeric forms. Although the monomers can activate chemokine receptors, dimerization is required for leukocyte recruitment in vivo, and it remains controversial whether dimeric CC chemokines can bind and activate their receptors. We have developed an obligate dimeric mutant of the chemokine monocyte chemoattractant protein-1 (MCP-1) by substituting Thr10 at the dimer interface with Cys. Biophysical analysis showed that MCP-1(T10C) forms a covalent dimer with similar structure to the wild type MCP-1 dimer. Initial cell-based assays indicated that MCP-1(T10C) could activate chemokine receptor CCR2 with potency reduced 1 to 2 orders of magnitude relative to wild type MCP-1. However, analysis of size exclusion chromatography fractions demonstrated that the observed activity was due to a small proportion of MCP-1(T10C) being monomeric and highly potent, whereas the majority dimeric form could neither bind nor activate CCR2 at concentrations up to 1 µM. These observations help to reconcile previous conflicting results and indicate that dimeric CC chemokines do not bind to their receptors with affinities approaching those of the corresponding monomeric chemokines.

A hallmark of the immune system is its ability to recruit large numbers of leukocytes to sites of infection or injury. This recruitment is mediated by a group of ~50 small, secreted proteins called chemokines, which act as chemoattractant signals to direct leukocyte migration. Chemokine function is mediated by high affinity interactions with seven-transmembrane G protein-coupled receptors on leukocyte membranes. During inflammatory responses, the array of leukocytes recruited is dependent on the chemokines expressed in the inflamed tissue, the selectivity of those chemokines for chemokine receptors, and the expression of those receptors on various types of leukocytes (1).

Chemokines are classified into two major families (CC and CXC) and two minor families (C and CX3C), depending on the spacing of conserved cysteine residues near the protein N terminus. The monomeric structure of all chemokines is highly conserved, consisting of a disordered N terminus, an irregularly structured loop (N-loop) ending with a turn of a 310-helix, a three-stranded antiparallel β-sheet, and a C-terminal α-helix (2–5). The monomeric unit is sufficient to induce receptor activation in vitro, as demonstrated for constitutively monomeric forms of the CXC chemokine interleukin-8 (IL-8) and the CC chemokines monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1β (MIP-1β) (6–8). According to the widely accepted two-site model of receptor activation (9), chemokine monomers activate their receptors in two stages. First, the chemokine uses core (non-N-terminal) residues to bind with high affinity to the receptor N terminus. Subsequently, the chemokine N terminus activates the receptor by binding to its transmembrane helices and/or extracellular loops.
Although monomeric chemokines are active in vitro, most chemokines are capable of forming dimers or higher order oligomers and can exist in equilibrium between different oligomeric states in solution. Members of the two major chemokine families display different modes of dimerization. CC chemokines use their N-terminal regions to form a new antiparallel β-sheet, resulting in an elongated dimer structure, whereas CXC chemokines dimerize through the existing β1-strands of the monomers, resulting in a more compact structure. Initially, it was suggested that dimeric chemokines are not biologically relevant because the systemic physiological concentrations of chemokines are in the nanomolar range, whereas chemokines typically dimerize with dissociation constants in the micromolar range (10). However, subsequent studies have shown that the ability of monomeric chemokines to recruit leukocytes in vitro cannot be replicated in vivo. A seminal study by Proudfoot et al. (11) demonstrated that constitutively monomeric MCP-1, MIP-1β, and RANTES7 could not recruit leukocytes in vivo when injected into the peritoneal cavity of mice. Furthermore, monomeric IL-8 had weaker leukocyte-recruiting potencies compared with its wild type counterpart (12). These observations suggested that, although the monomeric state is likely to be important for receptor binding and activation, dimerization is also necessary for physiological function.

The requirement of oligomerization for the physiological function of chemokines is believed to be due, at least in part, to the ability of chemokine oligomers to bind with high avidity to glycosaminoglycans on endothelial cell surfaces (1). These interactions are likely to modulate local chemokine concentrations as well as the rates of chemokine clearance from inflammatory loci. However, Rajarathnam and co-workers (12–15) have recently reported that a disulfide-trapped dimer of the CXC chemokine IL-8 can bind and activate the receptors CXCR1 and CXCR2 and elicit neutrophil recruitment in vivo, albeit more weakly than the IL-8 monomer. In contrast, a disulfide-trapped dimer of the CC chemokine MIP-1β is unable to activate the receptor CCR5, and dimerization of the CC chemokine RANTES prevents binding to the N-terminal region of CCR5 (16, 17). The observations that CXC chemokine dimers are active whereas CC chemokine dimers are not can be rationalized based on the different dimer structures; CXC chemokine dimerization leaves the receptor-binding and activation regions exposed, whereas CC chemokine dimerization involves the N-terminal region, which is critical for receptor activation. In this light, it is surprising that two independent studies of the CC chemokine MCP-1 found that cross-linked dimeric forms of MCP-1 could bind and activate its receptor CCR2 with near wild type potency (7, 18). Unfortunately, it is uncertain whether the dimers characterized in those studies resembled the wild type dimer or had alternative structures in which the receptor binding regions were more exposed than in the native dimer. Thus, it remains uncertain whether the wild type MCP-1 dimer acts differently from the other chemokines within the CC chemokine family and whether it is able to bind and activate its receptor.

In the course of studying the interactions between MCP-1 and an N-terminal peptide derived from CCR2, we observed that both monomeric and dimeric forms of MCP-1 could bind to this fragment of the receptor. To enable detailed characterization of the dimer interactions, we have now designed a new covalent MCP-1 dimer, MCP-1(T10C), which forms an intermolecular disulfide bond in the center of the dimerization interface and therefore should not be capable of dissociating into monomeric units. Biophysical and structural characterization showed that MCP-1(T10C) is a nondissociating disulfide-linked dimer that faithfully mimics the wild type structure. Although initial functional studies suggested that the MCP-1(T10C) dimer could bind and activate CCR2, detailed analysis indicated that the apparent activity was attributable to a low level contaminant of monomer. These results have allowed us to reconcile the previously conflicting data on the functional properties of CC chemokine dimers.

**EXPERIMENTAL PROCEDURES**

Expression and Purification of Wild Type and Mutant MCP-1—MCP-1 was expressed as a His₆-tagged fusion protein in *Escherichia coli* BL21(DE3) cells. Gene and protein sequences of expression constructs are given in supplemental Fig. S1. Expressed proteins consisted of an N-terminal His₆ tag followed by a modified thrombin cleavage site (Leu-Val-Pro-Arg*-Gln¹-Pro²) such that thrombin cleavage after the Arg* residue released the Gln¹-Pro² N terminus of MCP-1. Cells transformed with the MCP-1 construct were grown in 2 liters of LB medium for unlabeled protein or 2 liters of M9 minimal media supplemented with 1.5 g/liter ¹⁵NH₄Cl (Novachem Pty Ltd., Melbourne, Australia) for ¹⁵N-labeled protein. Cells were cultured at 37 °C to an A₆₀₀ of ~0.6, induced with 1 mM isopropyl β-D-thiogalactopyranoside (final concentration), and grown overnight at 30 °C. The cells were centrifuged at 10,400 × g for 4 °C for 10 min, and the cell pellets were resuspended in 30 ml of lysis buffer (20 mM Tris, 500 mM NaCl, 5 mM imidazole, pH 8.0). Hen egg white lysozyme (1 ml of 10 mg/ml) was added, and the cells were incubated at room temperature for 30 min and then sonicated (eight times at 30-s bursts) at 10 A. The lysate was centrifuged at 43,000 × g, 4 °C for 20 min; the supernatant was discarded, and the inclusion bodies were resuspended in 30 ml of lysis buffer containing several grains of DNase I. After 30 min of incubation at room temperature, the resuspended inclusion bodies were centrifuged at 43,000 × g at 4 °C for 20 min. The supernatant was discarded, and the inclusion bodies were resuspended in 30 ml of lysis buffer containing several grains of DNase I. After 30 min of incubation at room temperature, the resuspended inclusion bodies were centrifuged at 43,000 × g at 4 °C for 20 min. The supernatant was discarded, and the inclusion bodies were resuspended in 30 ml of denaturing buffer (6 M guanidine hydrochloride, 20 mM Tris, 20 mM β-mercaptoethanol, 20 mM imidazole, pH 8.0) by thorough, regular vortexing. The inclusion body solution was stored for 16–60 h at 4 °C.

The dissolved inclusion bodies were centrifuged at 39,000 × g at 4 °C for 20 min, and the supernatant was loaded on a 5-ml
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nickel–nitrilotriacetic acid affinity column (Qiagen, Hilden, Germany). The column was washed with the denaturing buffer, and the denatured protein was eluted using the same buffer containing 200 mM instead of 20 mM imidazole. Elution fractions containing the protein were pooled and refolded by drop-wise addition to 1 liter of refolding buffer (20 mM Tris, 400 mM NaCl, 2 mM reduced glutathione, 0.5 mM oxidized glutathione, pH 8.0, filtered, and degassed) supplemented with two complete EDTA-free protease inhibitor mixture tablets (Roche Applied Science). The refolded protein was filtered and loaded onto a 5-ml HisTrap nickel affinity column (GE Healthcare) pre-equilibrated with HisTrap buffer A (20 mM Tris, 500 mM NaCl, 20 mM imidazole, pH 8.0). The protein was eluted at 5 ml/min using a stepwise isocratic elution from 0 to 100% His-Trap buffer B (20 mM Tris, 500 mM NaCl, 200 mM imidazole, pH 8.0), with an intermediate 10% HisTrap buffer B wash step. The eluted protein was dialyzed against thrombin cleavage buffer (20 mM Tris, 400 mM NaCl, 2.5 mM CaCl$_2$, pH 8.0) at 4 °C three times for 1 h. The His$_6$ tag was cleaved from the fusion protein by incubation with thrombin (Sigma) at a final concentration of 10 units/mg protein at 37 °C for ~20 h. Phenylmethylsulfonyl fluoride (PMSF) was added to quench the thrombin cleavage reaction.

For wild type MCP-1 and MCP-1(P8A), the protein was then loaded onto a 5-ml HisTrap column to remove uncleaved protein and residual His$_6$ tag. The cleaved protein was collected from the flow-through, diluted 5-fold to reduce the NaCl concentration to <100 mM, and loaded onto a 5-ml HiTrap SP cation exchange column (GE Healthcare) pre-equilibrated with ion exchange buffer A (20 mM Tris, pH 8.0). The protein was eluted using a linear gradient from 0 to 100% ion exchange buffer B (20 mM Tris, 2 mM NaCl, pH 8.0) at 3 ml/min. Wild type MCP-1 and MCP-1(P8A) eluted at ~950 mM NaCl. For MCP-1(T10C), after PMSF was added, the protein was immediately diluted 5-fold with 20 mM Tris, pH 8.0, and loaded onto a 5-ml HisTrap column pre-equilibrated with buffer A (20 mM Tris, pH 8.0). MCP-1(T10C) bound to the column by nonspecific electrostatic interactions and was eluted using a linear gradient from 0 to 100% of 20 mM Tris, 500 mM NaCl, 20 mM imidazole, pH 8.0, at 3 ml/min. Thrombin-cleaved MCP-1(T10C) eluted at ~440 mM NaCl, whereas uncleaved MCP-1(T10C) remained on the column and was eluted subsequently using a higher concentration of imidazole.

**Analytical Size Exclusion Chromatography**—Size exclusion chromatography was carried out using a BioSEF SEC S2000 4.6 × 300 mm column (Phenomenex) attached to an Agilent 1200 Series quaternary pump HPLC system, with detection conducted at 280 nm. The samples were run at 0.35 ml/min in 0.1 M sodium phosphate, 0.15 mM NaCl, 0.025% w/v NaN$_3$, pH 6.8.

**SDS-PAGE**—MCP-1 samples (10 μM in NMR buffer) were mixed with equal volumes of SDS-PAGE reducing and nonreducing buffer (62.5 mM Tris, pH 6.8, 25% glycerol, 0.01% w/v bromophenol blue, 2% w/v SDS, with and without 70 mM β-mercaptoethanol, respectively). The samples were boiled and run on a 15% SDS-polyacrylamide gel at 100 V for ~12 min and then at 120 V for ~65 min. The protein bands were visualized by silver staining.

**Mass Spectrometry**—Liquid chromatography coupled to electrospray-mass spectrometry (LC-MS) was carried out by the Monash Biomedical Proteomics Facility; details are given in supplemental Fig. S2.

**NMR Spectroscopy and Assignments**—All NMR experiments were conducted at 25 °C on a Bruker Avance 600 MHz NMR spectrometer equipped with a triple-resonance cryoprobe. NMR samples for $^1$H–$^{15}$N heteronuclear single quantum correlation (HSQC) experiments contained 50 μM to 1 mM $^{15}$N-labeled MCP-1 in 20 mM sodium acetate-$d_4$, 5% D$_2$O, 0.02% NaN$_3$, pH 7.0, whereas samples for three-dimensional $^{15}$N NOESY-HSQC and three-dimensional $^{15}$N TOCSY-HSQC experiments contained 0.3–1.0 mM $^{15}$N-labeled MCP-1 in the same buffer. Chemical shifts were referenced to internal or external 4,4-dimethyl-4-silapentane-1-sulfonic acid as described (19). NMR data were processed using Bruker TopSpin and analyzed using Sparky (T. D. Goddard and D. G. Kneller, University of California, San Francisco). Tentative assignments for many wild type MCP-1 amide resonances were made by reference to previous data (3) obtained under different conditions. These assignments were confirmed and extended using the three-dimensional $^{15}$N NOESY-HSQC and three-dimensional $^{15}$N TOCSY-HSQC data. Assignments for MCP-1(P8A) and MCP-1(T10C) resonances were based on the wild type MCP-1 assignments and were verified using three-dimensional $^{15}$N NOESY-HSQC and three-dimensional $^{15}$N TOCSY-HSQC experiments. For each form of MCP-1, amide resonance assignments were more than 85% complete. Several residues could not be assigned as they had weak signals due to their location in regions such as the 30-s loop or the N-loop, which have high conformational heterogeneity. For MCP-1(T10C), the mutated residue Cys$^{10}$ could not be assigned. Amide chemical shift assignments are listed in the supplemental Table S1.

**NMR Titration with CCR2 N-terminal Receptor Peptide**—A peptide containing residues 18–31 of CCR2 (primary sequence EYVTFTFDYDYGAP) was made by solid-phase synthesis as described (20) and quantified using absorbance measurements at 280 nm (molar extinction coefficient, $\epsilon_{280} = 2980$ cm$^{-1}$ M$^{-1}$). The titration of each form of MCP-1 with the peptide was performed using an initial MCP-1 concentration of 50 μM in NMR buffer (20 mM sodium acetate-$d_4$, 5% D$_2$O, 0.02% NaN$_3$, pH 7.0). The peptide was added from a 1 mM stock solution (in NMR buffer) in aliquots such that the final peptide/protein molar ratios were 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 2.00, 3.50, and 5.00 respectively. The initial volume of MCP-1 was 500 μl, and the volume after the final peptide addition was 625 μl. For the initial sample and each titration point, a two-dimensional $^{15}$N–$^1$H HSQC was run at 25 °C using 96 and 1024 complex points and spectral widths of 24.0 and 12.0 ppm in the $^{15}$N and $^1$H dimensions, respectively.

For each MCP-1 mutant, the weighted changes in chemical shift ($\Delta \delta_{\text{NH}} = [\Delta \delta_{\text{NH}}] + 0.2[\Delta \delta_{\text{C}}]$) were fit simultaneously for all residues exhibiting final $\Delta \delta_{\text{NH}}$ values above a threshold of 0.02 ppm. Fitting was performed using GraphPad Prism to a 1:1 binding equation (Equation 1) to give a single dissociation constant ($K_d$) value for the binding between the peptide and MCP-1 variant of interest.
\[ \Delta \delta_{NH} = \left( \frac{\Delta \delta_{NH}}{2} \right) \left( 1 + r_m + a \right) - \sqrt{(1 + r_m + a)^2 - 4r_m} \]

(Eq. 1)

In which \( a = K_d (L_0 + P_r) r_m/(P_r L_0) \); \( P_r \) is the initial concentration of protein; \( L_0 \) is the stock concentration of ligand; and \( r_m \) is the molar ratio ([peptide]/[protein]).

**Preparative Size Exclusion Chromatography of MCP-1(T10C)—**

Preparative size exclusion chromatography of the MCP-1(T10C) sample was conducted using a Superdex™ 75 5/150 GL column (GE Healthcare), with detection at 280 nm. MCP-1(T10C) (100 μL of 500 μM) was loaded onto the column, which was run at 0.2 ml/min in 0.1 M sodium phosphate, pH 5.5. Fractions (0.1 ml) were collected over a volume range of 0.5–6 ml. The fractions that contained protein were analyzed by reducing and nonreducing SDS-PAGE. The protein concentration in each sample was determined using \( A_{280} \) readings. A fraction with no detectable monomer contamination (fraction 16) and three fractions with increasing amounts of monomer (fractions 17, 18, and 20) were tested for receptor activation ability using the Ca²⁺ mobilization assay as described below.

**Cell-based Assays—**Cell-based assays were performed in inducible FlpIn TRex 293 cells expressing FLAG-CCR2. Briefly, for stable inducible expression FlpIn TRex 293 cells were transfected with pCDNA5/FRT/TO-FLAG-CCR2 (21, 22) using Lipofectamine 2000 (Invitrogen) and maintained in high glucose DMEM containing 10% Tet-free fetal bovine serum (FBS), 5 μg/ml blasticidin, and 200 μg/ml hygromycin B at 37 °C in 5% CO₂. For induction of FLAG-CCR2 expression, cells were incubated overnight with 1 μg/μl tetracycline.

For whole cell ¹²⁵I-MCP-1 binding competition assays, cells were plated in a poly-β-Lys-coated 96-well plate (50,000 cells/well) and grown overnight in the presence of 1 μg/μl tetracycline. The fractions that contained protein were analyzed by reducing and nonreducing SDS-PAGE. The protein concentration in each sample was determined using \( A_{280} \) readings. A fraction with no detectable monomer contamination (fraction 16) and three fractions with increasing amounts of monomer (fractions 17, 18, and 20) were tested for receptor activation ability using the Ca²⁺ mobilization assay as described below.

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**Activation of CCR2 by the MCP-1 variants was tested using an extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation assay and an intracellular Ca²⁺ mobilization assay. For ERK1/2 phosphorylation, cells were seeded at 50,000 cells/well into a poly-β-Lys-coated 96-well plate and grown overnight in the presence of 1 μg/μl tetracycline. Initial time course experiments were used to determine the time required to stimulate maximum ERK1/2 phosphorylation by each ligand, and the subsequent concentration-response experiments were conducted by stimulating cells with agonist for 2.5 min, which represented the time at which maximal ERK1/2 phosphorylation was achieved. The reaction was terminated by removal of media and drugs, and sample processing using the AlphaScreen SureFire pERK1/2 kit was performed following the manufacturer’s instructions (PerkinElmer Life Sciences). Data were normalized to the response of FBS and analyzed in GraphPad Prism 5.02. For intracellular Ca²⁺ mobilization experiments, cells were seeded at 50,000 cells/well into a poly-β-Lys-coated 96-well plate and grown overnight in the presence of 1 μg/μl tetracycline. Cells were washed twice in Ca²⁺ assay buffer (150 mM NaCl, 2.6 mM KCl, 1.2 mM MgCl₂, 10 mM d-glucose, 10 mM Hepes, 2.2 mM CaCl₂, 0.5% (w/v) BSA, and 4 mM probenecid), then replaced with assay buffer containing 1 μM Fluo-4-AM (Invitrogen), and incubated for 1 h at 37 °C in 5% CO₂. Cells were washed twice more and replaced with warm assay buffer. Addition of the drugs and fluorescence measurements were performed in a Flexstation™ (Molecular Devices) using 485 nm excitation and 520 nm emission wavelengths. Peak fluorescence was measured as a marker for Ca²⁺ mobilization and used in further analyses. In experiments using the SEC fractions of MCP-1, the pH of the Ca²⁺ assay buffer was reduced to 5.5. Data were normalized to the response of 1 μM ionomycin and analyzed in GraphPad Prism 5.02. All experiments were performed at least three times in duplicate.

**RESULTS**

**Binding of MCP-1 to an N-terminal Peptide from CCR2—**Numerous previous studies have found that peptides derived from the N-terminal regions of chemokine receptors bind weakly to the cognate chemokines of those receptors, apparently mimicking the first step in the two-step model of receptor activation. We have used NMR spectroscopy to investigate binding of MCP-1 to the peptide R2A, composed of residues 18–31 of CCR2. At the MCP-1 concentration used in this study (50 μM), two-dimensional spectra contain two sets of peaks corresponding to the monomeric and dimeric forms of the chemokine in slow exchange with each other on the NMR chemical shift time scale. Upon addition of peptide R2A, we observed chemical shift changes for both monomer and dimer peaks (Fig. 1A), indicating that both forms of the chemokine can bind to the N-terminal region of the receptor. These data appeared to support the previous indications (7, 18) that dimeric MCP-1 can bind to CCR2.

**Design of Obligate Dimeric MCP-1—**An examination of the dimer interface in the wild type MCP-1 structure reveals that residues Thr¹⁰ of the two monomer subunits are located adjacent to the 2-fold symmetry axis and on the same side of the intermolecular β-sheet (Fig. 2A), with their Oγ atoms separated by only 3.1 Å. Thr¹⁰ is unlikely to be specifically important for MCP-1 structure or function, as simultaneous mutation of Val¹⁰ and Thr¹⁰ to Ala in previous experiments did not affect binding or signaling properties (23, 24). Therefore, we reasoned that substitution of Thr¹⁰ by Cys might enable the formation of a disulfide bond across the dimerization interface without compromising wild type structure. This mutant protein, MCP-1(T10C), was expressed in E. coli as inclusion bodies, refolded and purified to homogeneity. To allow comparative evaluation of the structural and functional properties of this mutant, we also prepared wild type MCP-1 and a previously characterized obligate monomeric mutant, MCP-1(P8A) (7).
MCP-1(T10C) Is an Obligate Disulfide-bonded Dimer—To determine whether MCP-1(T10C) behaves as a dimeric species in solution, we analyzed wild type and mutant MCP-1 by analytical size exclusion chromatography (Fig. 2B). MCP-1(P8A) eluted as a single peak with a retention time of 11.21 min. However, wild type MCP-1 eluted as a relatively broad peak whose retention time varied from 11.21 to 10.97 min with increasing concentrations of the injected sample from 10 to 62 μM. These results are consistent with the expected concentration-dependent equilibrium of monomeric and dimeric species. MCP-1(T10C) injected at the lowest concentration (10 μM) eluted as a single peak with a retention time of 10.85 min. This is consistent with MCP-1(T10C) behaving as an obligate dimer.

To determine whether the MCP-1(T10C) dimer contains an intermolecular disulfide bond, we compared wild type MCP-1, MCP-1(P8A), and MCP-1(T10C) by SDS-PAGE (Fig. 2C). As expected, wild type MCP-1 and MCP-1(P8A) migrated as monomeric species under both reducing and nonreducing conditions. In contrast, MCP-1(T10C) migrated as a single dimeric species under nonreducing conditions, indicating the presence of an intermolecular disulfide bond.

To obtain independent confirmation that MCP-1(T10C) is an obligate dimer, the MCP-1 variants were subjected to liquid chromatography coupled to electrospray mass spectrometry (LC-MS). The molecular weights obtained by LC-MS indicated that both MCP-1(P8A) and wild type MCP-1 are monomeric (data not shown), suggesting that noncovalent dimers of the wild type protein were disrupted during either chromatography or ionization. However, MCP-1(T10C) yielded ions corresponding to both dimer and monomer, with ion current integrals in the ratio ~9:1 (supplemental Fig. S2). Although these integrals cannot be directly interpreted in terms of the concentrations of the two species due to possible differences in ionization efficiencies, these data indicated that MCP-1(T10C) samples may contain a small fraction of contaminating monomer, with the potential to complicate analysis of functional assays.

MCP-1(T10C) Maintains Wild Type Dimer Structure—The T10C mutation results in MCP-1(T10C) possessing three adjacent cysteine residues (Cys10, Cys11, and Cys12), increasing the likelihood of aberrant disulfide bond formation and protein misfolding. Therefore, NMR experiments were conducted to verify that the MCP-1(T10C) dimer is correctly disulfide-linked.
and maintains wild type dimer structure. The $^{15}$N-1H HSQC spectrum of MCP-1(T10C) is well dispersed and overlays well with the spectrum of the wild type MCP-1 dimer, indicating that the mutant protein is correctly folded (Fig. 3A). As shown in Fig. 3B, the few resonances that shift substantially between the wild type and mutant spectra correspond to residues sequentially adjacent to the site of mutation (e.g. Val9 and Cys11) or linked to the mutated region by the Cys11 to Cys36 disulfide bond (Cys36 and Lys38). All of these residues are spatially close to the mutation site (Fig. 3C), indicating that the T10C mutation only results in slight local structural perturbation but does not affect the overall structure of dimeric MCP-1.

As confirmation that the spectrum of MCP-1(T10C) is indicative of dimeric structure, we compared the $^{15}$N-1H HSQC spectra of both T10C and P8A mutants to the spectrum of dilute (50 μM) wild type MCP-1 (Fig. 3D). The wild type MCP-1 spectrum (Fig. 3D, red peaks) contained two sets of peaks corresponding to the monomer and dimer, respectively, as both species were present in detectable proportions at the lower protein concentration. As expected, the MCP-1(P8A) spectrum (Fig. 3D, blue peaks) overlaid well with wild type monomer peaks, confirming that this mutant is an obligate monomer (7). Importantly, peaks associated with the monomeric state of wild type MCP-1 were not observed in the spectrum of MCP-1(T10C) (Fig. 3D, green peaks), indicating that the level of monomer contamination is probably lower than the ~5% suggested by mass spectrometry (see above).

To further characterize the structural details at the dimer interface of MCP-1(T10C), we analyzed its three-dimensional $^{15}$N NOESY-HSQC spectrum to identify unambiguous intermolecular NOE contacts involving amide hydrogens. As shown in Fig. 3E, several NOEs were identified between nuclei on opposite strands of the intermolecular β-sheet, and long range NOEs were identified between residue Ala1’, near the N terminus of one monomer, and residues Ile51 and Cys52, within the third β-strand of the other monomer. All of these intermolecular NOEs correspond to distances of ~5 Å in the dimer structure of wild type MCP-1. These results clearly indicate that the dimerization interface of MCP-1(T10C) closely resembles that of the wild type protein.

**Obligate Dimeric Chemokine MCP-1**

To validate our initial conclusion that both monomeric and dimeric forms of MCP-1 bind to the N-terminal CCR2 peptide R2A, we titrated R2A into NMR samples of both MCP-1(P8A) and MCP-1(T10C). In both cases, we observed concentration-dependent changes in chemical shifts indicative of peptide binding (Fig. 1, B and C). Moreover, there was an excellent correspondence between the resonance changes observed for wild type MCP-1 and for the two mutants. Fitting the titration data for wild type MCP-1 is difficult due to overlap of dimer and monomer peaks and the existence of coupled binding and dimerization equilibria. However, the titration data for MCP-1(P8A) and MCP-1(T10C) could be readily fit to simple 1:1 binding isotherms (Fig. 1, D and E), in which the dimer was treated as two independent monomer units. This analysis yielded $K_d$ values of 396 ± 66 and 198 ± 44 μM for MCP-1(P8A) and MCP-1(T10C), respectively. Thus, these titrations confirmed that both monomeric and dimeric forms of MCP-1 can bind weakly to the N-terminal region of CCR2. The resonances of MCP-1(P8A) and MCP-1(T10C) that shift in response to binding of peptide R2A are plotted in Fig. 1F and color-mapped onto the MCP-1 structure in the supplemental Fig. S3.

**CCR2 Binding and Activation by MCP-1(T10C)**—The ability of MCP-1(T10C) to interact with CCR2 was investigated in cell-based binding and activation assays. We tested receptor binding using a radiolabeled ligand competition assay, in which FlpIn Trex 293 cells expressing CCR2 were incubated with $^{125}$I-labeled wild type MCP-1 in the presence of increasing concentrations of each form of MCP-1 (Fig. 4A). Wild type MCP-1 and MCP-1(P8A) bound with similarly high affinities to CCR2 ($pK_a = 8.19 ± 0.27$ and $8.36 ± 0.19$, respectively; where $pK_a$ is negative log of concentration required for 50% binding inhibition) (Table 1), in agreement with previous data (7). In contrast to these two forms of MCP-1, much higher concentrations of MCP-1(T10C) were required to displace the radiolabeled ligand (apparent $pK_a = 6.71 ± 0.43$). Activation of CCR2 by the different ligands was tested using two independent assays, a Ca$^{2+}$ mobilization assay and an ERK1/2 phosphorylation assay. Concentration-response curves are shown in Fig. 4, B and C, and the corresponding potency ($pEC_{50}$) values are given in Table 1. Both assays yielded results consistent with the binding data. Wild type MCP-1 and MCP-1(P8A) stimulated Ca$^{2+}$ mobilization and ERK1/2 phosphorylation pathways with high potencies ($pEC_{50} ~8–9$). Indeed, MCP-1(P8A) showed higher potency than wild type MCP-1 in the Ca$^{2+}$ mobilization assay, consistent with previously reported findings on the obligate monomer (7). In contrast, MCP-1(T10C) stimulated both pathways with 30–65-fold weaker potency.

Although the initial binding and activation data appeared to confirm the previous indications that dimeric MCP-1 can bind
FIGURE 3. MCP-1(T10C) maintains wild type dimeric structure. A, overlaid HSQC spectra of MCP-1(T10C) (green) and the wild type MCP-1 dimer (red). At the wild type sample concentration (1 mM), the dimer peaks dominate the spectrum, and the monomer is not observable. Peaks labeled with residue numbers have differences in weighted shift ($\Delta \delta_{\text{H}}$) above 0.15 ppm. B, bar graph of weighted changes in chemical shift ($\Delta \delta_{\text{H}}$) of MCP-1(T10C) relative to the wild type dimer. Positions of intra-monomer disulfide bonds and secondary structure elements are indicated schematically. Black dots represent residues that were not assigned (including the N-terminal residue and five proline residues), and the red dot highlights the residue at the mutation site, which was also unassigned. C, ribbon representation of the MCP-1 dimer color-coded according to weighted changes in chemical shift. The position of the T10C mutation is shown in blue. Unassigned residues are in black. D, expanded region of overlaid HSQC spectra of wild type MCP-1 (red), MCP-1(P8A) (blue), and MCP-1(T10C) (green). At the wild type sample concentration used (50 mM), both dimer and monomer peaks are observable, with dimer peaks having higher intensity. E, schematic diagram of the MCP-1 dimer interface showing intermolecular NOEs detected in the three-dimensional $^1$H NOESY-HSQC spectrum of MCP-1(T10C). Red arrows show unambiguous intermolecular NOEs, with correlated protons also colored red. The dotted green lines indicate intermolecular hydrogen bonds in the wild type dimer. NOEs are shown in only one direction (chain A to chain B) and side chains are shown in chain B only if they exhibit intermolecular NOEs. The Cys$^{12}$-Cys$^{52}$ disulfide bond is indicated schematically.
Obligate Dimeric Chemokine MCP-1

and activate CCR2, it is important to consider the alternative possibility that the weak CCR2 binding and activation displayed by MCP-1(T10C) samples could be due to the small contaminant of monomeric MCP-1(T10C) having near wild type receptor affinity and potency. To distinguish the activity of the majority dimeric and minority monomeric forms of MCP-1(T10C), we fractionated the MCP-1(T10C) sample by preparative size exclusion chromatography using conditions (pH 5.5) under which formation and breakage of disulfide bonds is expected to be extremely slow (Fig. 5A). SDS-PAGE (Fig. 5B) indicated that fraction 16 contained no observable monomer, whereas fractions 17–20 contained monomer in increasing proportions. Subsequently, Ca\(^{2+}\) mobilization concentration-response curves were recorded for fractions 16–18 and 20, as well as for unfraccionated MCP-1(T10C) and wild type MCP-1 at pH 5.5 (Fig. 5C). Fraction 16 gave no detectable activity, whereas fractions 17, 18, and 20 displayed increasing levels of activity in parallel with the proportion of monomer in each sample; the most active fraction (fraction 20) eluted with the most activity in parallel with the proportion of monomer in each sample. The most active fraction (fraction 20) eluted with approximately the same retention time as the obligate monomeric mutant MCP-1(P8A) (Fig. 5A) and had potency similar to that of the MCP-1(T10C) sample prior to fractionation. These data strongly indicate that the activity observed for MCP-1(T10C) can be completely accounted for by the small proportion of monomer within the predominantly dimeric sample. If the dimer has any residual activity, it appears to be at least ~1000-fold lower than that of wild type MCP-1. Finally, we evaluated the ability of purified dimeric MCP-1(T10C) to bind to CCR2. Size exclusion fraction 16 at 1 \(\mu M\) concentration failed to displace \(^{125}\)I-MCP-1 from CCR2 (Fig. 5D) and failed to inhibit the calcium mobilization induced by an \(EC_{50}\) concentration of wild type MCP-1 (Fig. 5E). Thus, dimeric MCP-1(T10C) was unable to bind to CCR2 significantly at the concentrations tested.

DISCUSSION

Previous structural and biophysical studies have shown that many CC chemokines exist in equilibrium between monomeric and dimeric forms in solution. The dimeric forms are required for physiological function as obligate monomers are insufficient for leukocyte recruitment in vivo (11). Nevertheless, attempts to test the specific roles of the dimers have been impeded by the monomer-dimer equilibrium, which makes it difficult to separate the function of the dimer from that of the monomer. Three previous reports have described the receptor binding and activation properties of cross-linked CC-chemokine dimers. Jin et al. (17) introduced the A10C mutation into MIP-1\(\beta\), resulting in a disulfide cross-linked dimer similar to the MCP-1(T10C) mutant described herein. This obligate MIP-1\(\beta\) dimer was unable to activate the MIP-1\(\beta\) receptor CCR5 and did not significantly displace radiolabeled MIP-1\(\beta\) from CCR5, although interestingly, an ~30% reduction of bound radioligand was observed in the presence of 1 \(\mu M\) MIP-1\(\beta\)(A10C), suggesting potentially weak binding. In contrast, two previous reports (7, 18) have described different cross-linked forms of MCP-1, both of which exhibited substantial CCR2 activation ability.

The data presented here clearly indicate that the MCP-1(T10C) mutant is correctly folded, retains wild type dimer structure, and is unable to bind or activate CCR2 at concentrations up to 1 \(\mu M\). This mutant is structurally similar to MIP-1\(\beta\)(A10C) (17). Both of these mutants were purified and then characterized by biophysical methods and both displayed ~1000-fold or greater reduction in receptor binding and acti-

TABLE 1
Summary of cell-based receptor binding and activation data

| Chemokine         | Binding assay (pK\textsubscript{b}) | Activation assay (pEC\textsubscript{50}) |
|-------------------|--------------------------------------|----------------------------------------|
| Wild type MCP-1   | 8.19 ± 0.27                          | 7.78 ± 0.09                            |
| MCP-1(P8A)        | 8.36 ± 0.19                          | 8.02 ± 0.10\(^a\)                     |
| MCP-1(T10C)\(^b\) | 6.71 ± 0.43                          | 6.28 ± 0.07\(^c\)                     |

\(^a\) p < 0.05, compared with wild type MCP-1 (Student’s t test).

\(^b\) MCP-1(T10C) assays were performed prior to fractionation by preparative size exclusion chromatography.

\(^c\) p < 0.0001, compared with wild type MCP-1 (Student’s t test).

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FIGURE 4. CCR2 binding and activation by wild type and mutant MCP-1. A, inhibition of equilibrium binding of radiolabeled \(^{125}\)I-MCP-1 in FlpIn TRex 293 CCR2 cells by wild type MCP-1, MCP-1(P8A), and MCP-1(T10C). B, intracellular calcium mobilization by wild type MCP-1, MCP-1(P8A), and MCP-1(T10C) in FlpIn TRex 293 CCR2 cells. C, ERK1/2 phosphorylation in response to CCR2 activation by wild type MCP-1, MCP-1(P8A), and MCP-1(T10C) in FlpIn TRex 293 CCR2 cells. Data symbols used are the same in all three panels: wild type MCP-1 (filled circles), MCP-1(P8A) (open circles), and MCP-1(T10C) (open squares). All data points represent mean ± S.E. of at least three independent experiments.
vation relative to the corresponding wild type chemokines. Thus, these two studies suggest that dimeric CC chemokines are indeed unable to bind to their receptors at affinities approaching those of their monomeric counterparts.

The current conclusion that MCP-1 dimers cannot bind and activate CCR2 is in stark contrast to the previous studies of cross-linked MCP-1 dimers. These differences can be reconciled by considering the different cross-linking methods used. Zhang and Rollins (18) used the cross-linking reagent disuccinimidyl suberate to chemically cross-link MCP-1 and obtained a mixture of oligomeric species. Disuccinimidyl suberate is a bifunctional cross-linker with amine-reactive functional groups spaced 11.4 Å apart. Although MCP-1 has 10 amine groups per monomer (9 lysine residues and the N terminus), most of them are located distant from the dimer interface, and the closest intermolecular pairs are barely within cross-linking distance; in the Protein Data Bank structure 1DOM, the closest pairs are N terminus to Lys49 (10.8 Å) and Lys38 to Lys49 (12.4 Å). Moreover, it is unlikely that cross-linking of any two amino groups would prevent transient dissociation of the dimerization interface to expose residues that could participate in receptor interactions. In the second study of cross-linked MCP-1 dimers, Paavola et al. (7) induced dimer cross-linking by adding a cysteine residue to the C terminus of MCP-1 (mutant designated WT*(Cys77)). Although the resulting obligate dimer had near wild type activity, it is noteworthy that the C termini of the two monomers within the MCP-1 dimer are ~50 Å apart. Thus, it would not be possible for WT*(Cys77) to form an intermolecular disulfide bond without completely disrupting the native dimer interface. Based on these considerations of the cross-linking strategies, we suggest that the previous cross-linked dimers displayed activity because the cross-linking approaches did not lock these molecules into native-like dimer structures, whereas the lack of activity for MCP-1(T10C) and MIP-1β(A10C) is a consequence of these mutants being successfully trapped in the native dimer conformation. Alternatively, it is possible that the activity observed for dimers in previous studies may have been due to low concentrations of noncross-linked monomeric contaminants.

This study was motivated, in part, by our observation that the wild type MCP-1 dimer binds to an N-terminal peptide from CCR2; in a separate study,8 we have found that this interaction is enhanced by sulfation of receptor tyrosine residues, a common post-translational modification of chemokine receptors. Our NMR titration data indicate that the residues of MCP-

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8 J. H. Y. Tan and M. J. Stone, unpublished results.
1(T10C) whose NH groups are sensitive to peptide binding are predominantly located in the N terminus, N-loop, 3i\textsubscript{10}-turn, and β\textsubscript{3}-strand (Fig. 1F and supplemental Fig. S3). These are the same regions that are sensitive to peptide binding in MCP-1(P8A) and also correspond to the chemokine regions found to interact with receptor peptides in previous studies of other chemokines (15, 25, 27–33). Thus, it appears likely that the observed interactions represent a conserved aspect of chemokine-receptor recognition and could occur in the context of the full-length receptor.

In light of the peptide binding results, as well as a previous report that the N terminus of CCR2 is necessary and sufficient for MCP-1 binding (26), it is therefore surprising that no significant CCR2 binding was observed for MCP-1(T10C) in cell-based assays. There are several possible explanations for this apparent inconsistency. First, the peptide binding is low affinity (K\textsubscript{D} values in the micromolar range), whereas the concentrations used in cell-based assays were sub-micromolar. Second, the receptor N terminus might be occluded or conformationally restrained in the cell surface receptor relative to more exposed and flexible peptide models. Third, binding of the dimer, but not the monomer, to the receptor N terminus may be hindered by steric overlap with the receptor extracellular loops. Finally, the signals in the cell-based assays are dependent on the kinetics as well as the thermodynamics of binding, whereas the peptide binding experiments are pure equilibrium assays. Thus, it remains possible that the dimeric chemokine binds transiently to the receptor N terminus, but dissociation is too rapid for the binding to be detected in the calcium mobilization and radioligand binding inhibition assays.

In summary, we have shown that introduction of a non-native cysteine residue in the center of the MCP-1 dimerization interface gives rise to an obligate covalent dimer whose structure closely resembles that of the native MCP-1 dimer and whose ability to bind or activate CCR2 is dramatically impaired (or completely eliminated) relative to the wild type chemokine. Comparison of these results to those for other cross-linked CC chemokine dimers helps to reconcile previous discrepancies and leads to the overall conclusion that CC-chemokine dimers are unable to bind or activate their receptors at sub-micromolar concentrations. Considering that chemokine dimerization is required for high affinity glycosaminoglycan binding and for in vivo activity, activation of receptors on rolling leukocytes is likely to require dissociation of chemokine dimers from the glycosaminoglycan surface and subsequent dissociation of dimers to their receptor-activating monomeric forms.

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