The Human CC Chemokine MIP-1β Dimer Is Not Competent to Bind to the CCR5 Receptor

Hongjun Jin, Xiaohong Shen, Brandi Renee Baggett, Xiangming Kong, and Patricia J. LiWang

From the Department of Biochemistry and Biophysics, Texas A & M University, College Station, Texas 77843

Chemokine dimerization has been the subject of much interest in recent years as evidence has accumulated that different quaternary states of chemokines play different biological roles; the monomer is believed to be the receptor-binding unit, whereas the dimer has been implicated in binding cell surface glycosaminoglycans. However, although several studies have provided evidence for this paradigm by making monomeric chemokine variants or dimer-impaired chemokines, few have provided direct evidence of the receptor function of a chemokine dimer. We have produced a covalent dimer of the CC chemokine macrophage inflammatory protein-1β (MIP-1β) by placing a disulfide bond at the center of its dimer interface through a single amino acid substitution (MIP-1β-A10C). This variant was shown to be a nondissociating dimer by SDS-PAGE and analytical ultracentrifugation. NMR reveals a structure largely the same as the wild type protein. In studies of glycosaminoglycan binding, MIP-1β-A10C binds to a heparin-Sepharose column as tightly as the wild type protein and more tightly than monomeric variants. However, MIP-1β-A10C neither binds nor activates the MIP-1β receptor CCR5. It was found that the ability to activate CCR5 was recovered upon reduction of the intermolecular disulfide cross-link by incubation with 1 mM dithiothreitol. This work provides the first definitive evidence that the CC chemokine MIP-1β dimer is not able to bind or activate its receptor and implicates the CC chemokine monomer as the sole receptor-interacting unit.

Chemokines (chemotactic cytokines) are a group of small (7–14 kDa) structurally related proteins that regulate cell trafficking of various types of leukocytes through interactions with a subset of seven-transmembrane, G protein-coupled receptors and cell surface glycosaminoglycans (GAGs). There are two major chemokine subfamilies, CC and CXC, named for the placement of conserved Cys residues near the N terminus. The critical role played by chemokines in recruiting leukocytes and inhibiting human immunodeficiency virus entry has led to a great deal of interest in their structural biology. Many chemokine structures have been solved, both by NMR and x-ray crystallography (1–9). These structures reveal that all chemokines share a common fold, composed of three β-strands in a Greek key arrangement, followed by a C-terminal α-helix. Many chemokines form dimers with affinities generally in the high nanomolar or low micromolar range, leading several groups to study the biological role of the chemokine quaternary state. An early study in this area showed that the obligate monomer of the CXC chemokine IL-8 did retain activity on neutrophils in vitro (10), and later work by our group and others showed that monomeric variants of the CC chemokines MIP-1β and MCP-1 were also able to bind and activate their respective receptors (11, 12).

However, a growing body of evidence suggests a biological role for the dimer. An obligate dimer of the CXC chemokine IL-8 was shown to be able to bind its receptor (13–15) as was the CC chemokine MCP-1 (12, 16), although in the latter case it is not clear whether the cross-linked dimers that were reported had a similar dimer structure to the wild type protein. More recently, it was shown that monomeric variants of some chemokines were unable to recruit leukocytes in vivo, despite having receptor activity in vitro (17). Further indication of the role of the chemokine dimer includes studies in which GAGs have been shown to mediate chemokine oligomerization (12), including playing a direct role in tightening the CC chemokine dimer (18).

Despite the accumulation of data regarding the role of the quaternary state in chemokine function, and the clear evidence that at least some chemokine monomers are competent to activate cognate receptors in vitro, no direct evidence has yet been reported on whether a CC chemokine dimer can or cannot bind to its receptor. In particular, it has never been shown definitively if a chemokine dimer is able to bind the CCR5 receptor or if the dimer has some role in receptor function, in part because it is not possible to observe the quaternary state of a chemokine in standard receptor experiments. Chemokine receptors have been shown to dimerize (19, 20), leading to the possibility that multimerization of chemokine ligand on the receptor could lead to multimerization of the receptor. This question is particularly intriguing because the two chemokine subfamilies have completely different dimer structures, leading to the possibility of different results for different subfamilies (21). In the present study, a covalent dimer of the CC chemokine MIP-1β was pro-
duced by a single amino acid substitution (MIP-1β-A10C). This variant was shown to be a nondissociating dimer that forms a disulfide bond at the center of the dimer interface, and its structure is largely the same as the wild type protein. It has been determined that MIP-1β-A10C neither binds nor activates the MIP-1β receptor CCR5. However, the receptor activity was recovered upon reduction of the intermolecular disulfide bond. To the best of our knowledge, results reported here represent the first direct evidence that the CC chemokine dimer does not bind to its receptor and thus offers new implications for chemokine-related drug development.

EXPERIMENTAL PROCEDURES

Production and Purification of MIP-1β Variants—The genes for MIP-1β-A10C and A10S were produced using the QuikChange™ procedure (Stratagene, La Jolla, CA) in a variant of pET32-Xa/LIC (Novagen, Madison, WI). Mutations were confirmed through DNA sequencing. These MIP-1β variants were produced in Escherichia coli strain BL321(ΔE3) and purified as follows. The cells were grown in 1 liter of minimal medium containing $^{15}$NH$_4$Cl as the sole nitrogen source (when preparing protein for NMR assignment experiments, [13C₆]glucose was the sole carbon source). Protein production was induced by addition of isopropyl β-D-thiogalactopyranoside to 1 mM in 37 °C culture for 7 h, and the cells were harvested by centrifugation at 6,000 g for 30 min and lyophilized. The C4 column was found to effectively separate protein with correctly formed disulfide bonds from protein that was observed to be unfolded, presumably because of incorrect disulfide bond formation. To remove the fusion tag, protein was solubilized into 1% Triton X-100, 20 mM Tris (pH 8), 50 mM NaCl, and 2 mM CaCl$_2$. Factor Xa (Novagen) was used for the proteolytic cleavage, which typically took 2 weeks at room temperature. SDS-PAGE was used to monitor the cutting reaction. Finally, the cut MIP-1β variants were purified over a C4 reversed phase chromatography column and lyophilized.

Nonreducing SDS-PAGE—Equal amounts of MIP-1β variant samples (~10 µg) were resuspended in 10 µl of 20 mM Tris-HCl (pH 8.0) buffer and mixed with 10 µl of 2X loading sample buffer (20 mM Tris-HCl (pH 6.8), 4% SDS, 0.2% bromphenol blue, 20% glycerol) with or without 50 mM β-mercaptoethanol (reducing agent), boiled, and electrophoresed on a 17% SDS-polyacrylamide gel.

Analytical Ultracentrifugation—Sedimentation equilibrium experiments were carried out on a Beckman XL-A analytical ultracentrifuge using rotor An-60 Ti at 25 °C. For MIP-1β-A10C under nonreducing conditions, the speeds used were 25,000, 38,000, and 45,000 rpm. Samples were composed of 10 µM protein in 20 mM (pH 2.5) sodium phosphate buffer containing 150 mM NaCl. For MIP-1β-A10C under reducing conditions, the protein was dissolved into 200 µl of 20 mM (pH 2.5) sodium phosphate buffer containing 150 mM NaCl to a concentration of 10 µM. The pH was raised to 7.4 by addition of 2 µl of 1 M NaOH; the solution was made to 1 mM DTT and incubated overnight. Then 2 µl of 50% phosphoric acid was added to lower the pH to 2.5 for analytical ultracentrifugation experiments. The rotor speeds for this reduced sample were 25,000, 35,000, and 45,000 rpm. During each experiment, samples were monitored by absorbance at 280 nm. The solvent density (ρ) and partial specific volume of the protein (v) were calculated from the amino acid compositions using the program Sednterp (obtained from the Boston Biomedical Research Institute RASMB web site). The data were processed using the program Origin (Beckman) for detecting multiple equilibria and estimating the value of the equilibrium constants from the absorbance data (11, 22). Each set of experimental data was fit to an ideal model or monomer-dimer equilibrium model using a nonlinear least squares fit.

Selective Reduction of MIP-1β-A10C—To reduce the intramolecular disulfide bond formed by the substitution of Cys for Ala in MIP-1β, the purified MIP-1β-A10C powder was dissolved in 20 mM phosphate buffer saline (pH 7.4), 1 mM DTT (Sigma) was added, and the solution was incubated at room temperature for 16 h and then quenched by adding 1 M phosphoric acid to lower the pH to 2.5. Trifluoroacetic acid was added to 0.1%; acetonitrile was added to 10%, and the reduced protein was purified on a C4 column and lyophilized into dried powder.

NMR Spectroscopy—All NMR spectra were acquired at 25 °C on a Varian INOVA 600-MHz spectrometer using protein samples at ~1 mM concentration in 20 mM phosphate, 10 mM NaCl buffer (pH 2.5). Chemical shifts were referenced to 4,4-dimethyl-4-silapentane-1-sulfonate by the method of Wishart et al. (23).

Backbone $^{13}$C, $^{15}$N, and $^1$H assignment of MIP-1β-A10C and reduced MIP-1β-A10C were assigned by using the triple resonance experiments HN(CA)CB (24, 25), CBCA(CO)NH (26), and HBHA(CO)NH (27). Side chain assignments of MIP-1β-A10C were determined by using C(CO)NH-TOCSY (28, 29), HC(CO)NH-TOCSY (30) (in H$_2$O), and HCC(CO)-S Y (31) in D$_2$O. Proline assignment was aided by a home-written proline-edited $^{13}$C-HSQC experiment. Distance constraints were obtained from three-dimensional $^{13}$N- and $^{13}$C-edited NOEY.
and four-dimensional $^{13}$C/$^{13}$C-edited NOESY (32) experiments with a mixing time of 150 ms.

**CCR5-expressing Cell Lines**—A CHO-K1 cell line coexpressing CCR5, G616, and apo-aequorin was a kind gift from Dr. Marc Parmentier from the Institute of Interdisciplinary Research of the Free University of Brussels (ULB) Medical School, Brussels, Belgium. This cell line was described previously (33) and is used for binding and functional assays. Briefly, cells were cultured in Ham's F-12 medium supplemented with 10% fetal calf serum (Invitrogen), 100 units/ml penicillin, and 100 $\mu$/ml streptomycin (Invitrogen). Following selection with 400 $\mu$/ml G418 (Invitrogen) for 14 days, the population of mixed cell clones was used in binding and functional studies.

A TZM-bl HeLa cell line stably expressing a large amount of human CCR5 was obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health; TZM-bl was from Dr. John C. Kappes, Dr. Xiaoyuan Wu, and Tranzyme Inc. The details of this cell line were described previously (34, 35).

**Binding Assays**—CHO-K1 cells expressing wild type CCR5 were collected from plates with Ca$^{2+}$- and Mg$^{2+}$-free phosphate-buffered saline supplemented with 5 mM EDTA, gently pelleted for 2 min at 1000 × g, and resuspended in binding buffer (50 mM Hepes (pH 7.4), 1 mM CaCl$_2$, 5 mM MgCl$_2$, and 0.5% bovine serum albumin). Competition binding assays were performed in Minisorb tubes (Nunc), using 0.08 nM radiolabeled $^{125}$I-MIP-1β (2000 Ci/mmol; Amersham Biosciences) as a tracer, variable concentrations of MIP-1β or its mutants, and 40,000 cells in a final volume of 0.1 ml. The level of total binding was measured in the absence of competitor, and the level of nonspecific binding was measured with a 100-fold excess of unlabeled ligand. Samples were incubated for 90 min at 25 °C, and then the bound tracer was separated by filtration through GF/B filters presoaked in 0.5% polyethyleneimine. Filters were counted for 1 min in the Beckman Coulter Gamma LS 5000TA counter.

Binding assays utilizing HeLa cells were carried out in the TZM-bl cell line by fixing the cells onto the 24-well culture plate following the protocol mentioned previously (36). Briefly, TZM-bl cells were cultured in 75-cm$^2$ flask in DMEM with 10% FBS, 100 units of penicillin, and 0.1 mg/ml streptomycin until 30% confluency. Then the cells were detached and seeded onto 24-well culture plate overnight at 10$^4$ cells per well. The next day, cells were washed twice in cold phosphate-buffered saline and then were overlaid with 150 $\mu$l of the cold binding buffer. Cells were incubated for 2 h at 4 °C with 0.05 nm $^{125}$I-labeled human MIP-1β (Amersham Biosciences) in the presence of various concentrations of chemokine mutants. The reactions were stopped by washing wells four times with the cold binding buffer plus 0.5 M NaCl. Cells were lysed by the addition of 0.5 ml of 1% SDS. Lysates were transferred to a counting vial, and bound radioactivity was counted for 1 min in the Beckman Coulter Gamma LS 5000TA counter.

All determinations were performed in duplicate and repeated at least three times. Binding parameters (IC$_{50}$) were determined with KaleidaGraph version 3.6 (Synergy Software) using nonlinear regression applied to a one-site competition model. Representative data are shown in Fig. 4, B and C. The results are reported as fitted IC$_{50}$ mean value ± S.D. nM).

**Functional Assays**—The functional response of CCR5-expressing cells to chemokines was analyzed by measuring the luminescence of aequorin as described previously (37). CHO-K1 cells (described above) were collected from plates with Ca$^{2+}$- and Mg$^{2+}$-free DMEM (Invitrogen) supplemented with 5 mM EDTA, pelleted for 2 min at 1000 × g, resuspended in DMEM at a density of 5 × 10$^5$ cells/ml, and incubated for 2 h in the dark in the presence of 5 $\mu$m coelenterazine H (Promega). Cells were diluted 5-fold before being used. Agonists in 50 $\mu$l of DMEM were added to 50 $\mu$l of a cell suspension (50,000 cells), and luminescence was measured for 30 s in an Orion II microplate luminometer (Berthold Techniques, Germany). Determinations were performed in triplicate and repeated at least three times. The receptor activation EC$_{50}$ values were determined with KaleidaGraph using nonlinear regression applied to a one-site ligand binding model. Representative data is shown in Fig. 4A. The results are reported as fitted EC$_{50}$ mean value ± S.D. nM.

**Heparin-Sepharose Chromatography of GAG Binding Studies**—GAG binding capacity of chemokine mutants was studied by using the heparin-Sepharose chromatography as mentioned in previous publications (38, 39). Briefly, equal amounts of MIP-1β-A10C, MIP (9), MIP-1β-F13L, and MIP-1β-L34W (~10 $\mu$g of lyophilized protein) were taken up in 0.5 ml of 50 mM Tris (pH 7.4) and injected onto a 1-ml Hi-Trap heparin column (GE Healthcare) using the AKTA FPLC system (GE Healthcare). The column was equilibrated with 5 ml of the same buffer followed by a gradient of 0–1.0 M NaCl in 50 mM Tris (pH 7.4) at a rate of 0.5 ml/min for 60 min. The elution profile was monitored by UV absorbance at 280 nm. The salt concentration corresponding to the center of each eluted peak is a relative determinant of the GAG binding ability of that mutant.

**RESULTS**

**MIP-1β-A10C Is a Covalent Nondissociating Dimer**—To obtain an obligate MIP-1β dimer, a single substitution from Ala to Cys at the 10th position, which is in the center of dimer interface, was made (Fig. 1A). To determine the oligomerization state of MIP-1β-A10C, SDS-PAGE experiments were carried out. In the presence of reducing agent β-mercaptoethanol, MIP-1β-A10C migrates nearly identically to wild type MIP-1β on the gel. In the absence of β-mercaptoethanol, MIP-1β-A10C migrates more slowly than the wild type MIP-1β (Fig. 1B) at a position indicative of a covalent dimer. To further confirm this, we carried out analytical ultracentrifugation equilibrium experiments on the purified protein. The ultracentrifugation data did not fit to either a dissociating dimer model or to a monomer model, but rather the best fit of the data was to a single species of 15.5 kDa (twice the size of the calculated the monomer molecular weight), indicating MIP-1β-A10C is indeed a non-dissociating covalent dimer (Fig. 1C).

**MIP-1β-A10C Is Similar in Structure to the Wild Type MIP-1β Dimer**—The A10C mutation results in three contiguous cysteines (Cys$^{10}$, Cys$^{11}$, and Cys$^{12}$), each of which must form a correct disulfide bond to result in correctly folded protein. Although Cys$^{10}$ is involved in an intermolecular cross-link,
Cys\textsuperscript{11} and Cys\textsuperscript{12} form internal disulfide links (to Cys\textsuperscript{35} and Cys\textsuperscript{51}, respectively) as a critical component of the wild type protein. Binding assays measure the level of chemokine stores after productive engagement of the chemokine receptor by its ligand. Binding assays measure the level of chemokine activity.

**FIGURE 1.** MIP-1\textbeta\textbeta-A10C is a covalent dimer. A, ribbon diagram of the homodimer structure of MIP-1\textbeta (PDB code 1hum). Ala\textsuperscript{10} (red sphere) is in the center of the dimer interface. B, results of nonreducing and reducing 17% SDS-PAGE. Lanes from left to right: molecular weight marker (units in kDa), MIP-1\textbeta-A10C without BME; MIP-1\textbeta-A10C with BME; MIP-1\textbeta WT without BME; MIP-1\textbeta WT with BME; MIP-1\textbeta-A10S without BME; MIP-1\textbeta-A10S with BME. Only the sample MIP-1\textbeta-A10C without BME shows a molecular weight (\approx 16 kDa) approximately twice that of the other samples (\approx 8 kDa) indicating MIP-1\textbeta-A10C behaves as a single species having the size of a MIP-1\textbeta dimer.

**FIGURE 2.** MIP-1\textbeta-A10C has a nearly identical structure to wild type MIP-1\textbeta. \(^{13}\text{C}_{\alpha}\) (filled circles) and \(^{13}\text{C}_{\beta}\) (open circles) chemical shift plot of wild type MIP-1\textbeta versus MIP-1\textbeta-A10C is shown. The chemical shift values of \(^{13}\text{C}_{\alpha}\) and \(^{13}\text{C}_{\beta}\) for most residues between the two proteins are essentially identical except at the site of mutation (Ala\textsuperscript{10} on MIP-1\textbeta, Cys\textsuperscript{10} on MIP-1\textbeta-A10C).

Cys\textsuperscript{11} and Cys\textsuperscript{12} are critical components of the wild type tertiary structure. A detailed NMR investigation was carried out to determine whether the structure of the covalent dimer MIP-1\textbeta-A10C was identical to that of the wild type MIP-1\textbeta dimer. Complete backbone (\(^{15}\text{N}, {^1}\text{H}_{\text{N}}, {^{13}}\text{C}_{\alpha}, {^{13}}\text{C}_{\beta}\)) chemical shifts were determined for MIP-1\textbeta-A10C and compared with the wild type values. \(^{13}\text{C}_{\alpha}\) and \(^{13}\text{C}_{\beta}\) chemical shifts are sensitive to secondary structure and overall fold of the protein, and Fig. 2 shows that these values are essentially identical between the two proteins except at the site of mutation. This indicates that the A10C variant has a typical chemokine fold that is likely very similar to wild type MIP-1\textbeta. Furthermore, these data in addition to intramolecular NOE distance contacts (data not shown) indicate that the native disulfide bonds (Cys\textsuperscript{11}—Cys\textsuperscript{35} and Cys\textsuperscript{12}—Cys\textsuperscript{51}) have not been perturbed.

To determine whether the interaction of the two monomeric subunits in MIP-1\textbeta-A10C is the same as in the wild type dimer, we carried out three-dimensional \(^{15}\text{N}-\) and \(^{13}\text{C}\)-edited NOESY and four-dimensional \(^{13}\text{C}_{\alpha}\)/\(^{13}\text{C}_{\beta}\)-edited NOESY experiments on MIP-1\textbeta-A10C. These data show that the MIP-1\textbeta-A10C dimer makes almost all of the same inter-subunit contacts as the wild type dimer, including contacts between Gly\textsuperscript{4}, Ser\textsuperscript{5}, Asp\textsuperscript{6}, and Pro\textsuperscript{7} to Val\textsuperscript{50}; Asp\textsuperscript{6} to Cys\textsuperscript{51}; Pro\textsuperscript{8}, Thr\textsuperscript{9} to Cys\textsuperscript{12}; Pro\textsuperscript{8} to Val\textsuperscript{51}; and numerous contacts between Phe\textsuperscript{13} and Thr\textsuperscript{19}, and Leu\textsuperscript{34}, which can only be made across the dimer and not intramolecularly (Fig. 3, supplemental Fig. 2, and supplemental Table 1).

Not every contact reported for the wild type dimer was able to be distinguished for MIP-1\textbeta-A10C, although in many cases NOE peaks may be present but were too overlapped or of low intensity to be assigned unambiguously. For example, the description of the wild type MIP-1\textbeta dimer mentions contact between Ser\textsuperscript{47} and both Gly\textsuperscript{9} and Ser\textsuperscript{5} (1), but because of extensive overlap we could not unambiguously assign an NOE peak for these contacts. In addition, a few contacts are present in MIP-1\textbeta-A10C that indicate closer intersubunit contacts for certain atoms than were seen in the wild type dimer structure, including NOE contact between Ser\textsuperscript{5} and Thr\textsuperscript{19} (Hy), and between Phe\textsuperscript{13} (H\textalpha) (as opposed to the side chain) and Leu\textsuperscript{34}. This could indicate an altered structure compared with the wild type protein or could be explained by spin diffusion during the NMR experiment. However, overall the structural results suggest that MIP-1\textbeta-A10C is nearly identical in structure to the wild type dimer, and so our functional results do indeed reflect the function of the chemokine dimer and are not an artifact of altered conformation.

**The MIP-1\textbeta-A10C Dimer Is Not Active on the Receptor CCR5**—Two measures of chemokine receptor function are typically carried out, activity assays and binding assays. The activity assays generally measure the release of intracellular calcium stores after productive engagement of the chemokine receptor by its ligand. Binding assays measure the level of chemokine...
binding to the receptor, regardless of whether an intracellular signal is able to be transmitted. To measure the ability of MIP-1β-A10C to activate the CCR5 receptor, activity assays were carried out on CHO-K1 cells bearing human CCR5. As shown in Fig. 4A, MIP-1β-A10C is unable to elicit a response even at micromolar concentrations. Similarly, binding assays reveal that MIP-1β-A10C is unable to bind CCR5 (Fig. 4B). To demonstrate that mutation to the 10th position is functionally tolerant and that the lack of activity in the A10C variant is because of the obligate dimer, the A10S mutation was made in MIP-1β. This variant was shown to be nearly fully active, as expected (Fig. 4, A and B). Because NMR analysis indicates that the obligate MIP-1β-A10C dimer has the same structure as wild type MIP-1β, our data suggest that the lack of ability to bind CCR5 is indeed because of the quaternary state of the molecule (i.e. the dimer) rather than because of any disruption caused by an amino acid change at the 10th position.

Partial Rescue of Receptor Activity by Selective Reduction of the Intermolecular Disulfide Bond—To demonstrate that the dimer form of the chemokine is responsible for the loss of activity, MIP-1β-A10C was incubated in mild reducing conditions (1 mM DTT) to reduce the intermolecular disulfide bond, breaking the covalent bond between the monomeric subunits. Fig. 4A shows that some activity on CCR5 is observed for the reduced mutant.

To demonstrate that mild treatment with DTT does indeed break the disulfide bond of the covalent dimer, analytical ultracentrifugation was carried out on MIP-1β-A10C in the presence of DTT. Under these conditions the best fit to the data were to a dissociating dimer with a $K_d$ of 2.7 μM, indicating that the covalent link had successfully been reduced (supplementary Fig. 1). To eliminate the possibility that this treatment may also reduce the internal disulfide bonds of the protein, NMR experiments were carried out on the MIP-1β-A10C variant after treatment with DTT to determine whether these conditions disrupted the structure of the mutant. Carbon chemical shifts are not only powerful indicators of protein conformation but have been shown to be diagnostic of the participation of cysteine in a disulfide bond (40). The chemical shift assignments for the backbone atoms of MIP-1β-A10C and reduced MIP-1β-A10C were obtained using a series of three-dimensional experiments (24, 26) with $^{13}$C, $^{15}$N-labeled protein. The chemical shift values of $^{13}$C$_\beta$ for Cys$^{10}$, Cys$^{11}$, Cys$^{12}$, Cys$^{35}$, and Cys$^{51}$ for MIP-1β-A10C under oxidizing conditions were consistent with a fully disulfide cross-linked species, whereas under mild reducing conditions (1 mM DTT), the chemical shifts of MIP-1β-A10C were consistent with a reduced Cys$^{10}$, and oxidized Cys$^{11}$, Cys12, Cys$^{35}$, and Cys$^{51}$ (Fig. 5). This strongly indicates that under the mild reducing conditions used, the intermolecular cross-link is reduced, whereas the overall structure of the protein is intact and still contains the wild type disulfide bonds. Overall then, the results show that the rescue of activity upon reduction is because of the breaking of the dimer into monomeric units that are structurally intact. Therefore, the intermolecular disulfide bond of MIP-1β-A10C, and not any structural rearrangement, is the cause of the lack of receptor activity for the covalent dimer form of the mutant.

Because only a moderate level of rescue was observed for the MIP-1β-A10C variant in the presence of DTT, it is possible that variation in the 10th position does affect CCR5 activity of MIP-1β, or it is possible that DTT negatively affects the outcome of the activity assay. Therefore, activity assays with wild type MIP-1β in the presence of DTT were also carried out and showed a reduced activity for the wild type protein (Fig. 4, A and B). Because the above NMR data suggest that the structure of the chemokine remains intact in the presence of the reducing agent, these experiments suggest that the presence of DTT is harmful to the CHO cells, even in the brief period of an activity assay. Results were more dramatic for binding assays in the presence of DTT, where the several hours required for the experiment resulted in visibly unhealthy cells and poor results. Therefore, binding results were not able to be obtained for the MIP-1β-A10C variant in the presence of DTT on CHO cells. Binding experiments on CCR5-expressing TZM-bl HeLa cells consistently suggested that reduced MIP-1β-A10C could compete for CCR5 binding, but again the date were very poor, possibly because of the presence of DTT, even though the cells appeared healthy (data not shown).

The Covalent MIP-1β-A10C Dimer Retains Glycosaminoglycan Binding Ability—Heparin-Sepharose chromatography is a well established technique used to assess the ability of a protein
to bind to physiological GAGs (38, 39). Several chemokine mutants of varying quaternary state, including the monomeric variant MIP(9) (41), the weak dimers F13L (11) and L34W (18), and the covalent dimer MIP-1β/H9252-A10C, were tested for their ability to bind to a heparin sulfate column under identical conditions. Elution was carried out with a sodium chloride gradient. Although monomeric variant MIP (9) and weakly dimerizing variants F13L and L34W elute earlier than the wild type protein, the covalent dimer MIP-1β/H9252-A10C required at least as much salt as the wild type protein to elute from the column (Fig. 6) indicating that the dimer form of the protein is competent to bind GAGs even though it has no receptor binding capacity.

**DISCUSSION**

Structural studies show that many chemokines form dimers, and biophysical investigations have revealed that the dimer dissociation constant of chemokines is generally in the low micro-molar range (9). Although this dimer affinity suggests a predominant monomeric form of the protein at the nanomolar concentrations presumed to be present biologically, numerous studies have shown that the dimer $K_d$ is very sensitive to solu-
tion conditions, so it is possible that dimers are present in significant concentrations in vivo (42, 43). In support of the chemokine dimer as a biologically functional entity, it has been shown that chemokines mutated to remove the ability to dimerize do not function in vivo, despite these variants having the ability to bind and activate their cognate receptor in vitro (17). Overall, although a great deal has been reported about the receptor function of chemokine variants (11, 42, 44), little evidence has been accumulated regarding the specific role of the dimer in receptor binding or activation, because it is difficult to determine which protein species is actually making contact with the receptor. A “monomeric” variant may actually dimerize on the receptor, or alternatively, even a high affinity chemokine dimer may dissociate before binding the receptor.

The only way to definitively test the affinity of a dimer for the chemokine receptor is to covalently join two monomeric subunits in a way that maintains the structure of the wild type dimer. This has been reported for the CXC chemokine IL-8, where several strategies have been utilized to make a covalent dimer (13–15, 45). In general, it was found that a covalent IL-8 dimer was able to bind both receptors CXC R1 and CXC R2, but with lower affinity than the wild type protein. Further work with peptides derived from the receptor CXC R1 indicate that the IL-8 monomer is the high affinity ligand for the receptor (14, 46). However, the CXC chemokine dimer has a completely different structure than the CC chemokine dimer and has many receptor binding residues still on the surface of the protein. In contrast, the CC chemokine dimer buries residues that have been shown to be critical for receptor binding (11, 44, 47). This suggests that a receptor-bound CC dimer would make different contacts than a receptor-bound CC chemokine monomer. Therefore, we have designed a trapped dimer of MIP-1β by replacing Ala10 at the center of the dimer interface with Cys, forming a disulfide bond between two monomeric subunits (Fig. 1A). This protein is demonstrably a nondissociating dimer and shows almost all of the intermolecular interactions typical of the MIP-1β dimer. Assays of both CCR5 binding and CCR5 activity reveal that this covalent dimer is not competent to bind its receptor (Fig. 4, A and B). Therefore, we conclude that the MIP-1β dimer is not competent to bind or activate its receptor and that this conclusion is very likely to be general for the CC chemokine subfamily.

What then is the biological role of the CC chemokine dimer? Evidence has accumulated that the ability of chemokines to bind cell surface GAGs is linked to the chemokine quaternary state. Some years ago it was shown that GAG binding causes aggregation of both CC and CXC chemokines (48). In more recent studies, it was demonstrated that the monomeric form of MIP-1β has a lower affinity for a variety of GAG disaccharides than the wild type (dimer) form (49). Furthermore, the binding of GAGs by MIP-1β was shown to increase the chemokine dimer affinity (18). Similarly, the CXC chemokine SDF-1 was shown to dimerize with higher affinity in the presence of several solutes, including GAGs (43). Although GAG-induced oligomerization may be a mechanism to reduce chemokine activity, the GAG binding surface and receptor binding surface of MIP-1β do overlap so lesser activity of chemokines in the presence of GAGs may also be a function of competition between GAG and receptor for chemokine binding (44).

These data support a model of chemokine action in which the chemokine is immobilized on the endothelial surface in the dimeric (or higher oligomeric) form by binding to cell surface GAGs. The ability to dimerize is likely critical for GAG affinity, for chemokine gradient formation, or for the ability of the chemokine to be presented appropriately to the receptor on the surface of a chemotaxing leukocyte that is passing nearby. Dissociation of the chemokine oligomer into monomeric subunits likely occurs as part of the process of transferring the chemokine from the GAG to the receptor. This dissociation from the dimer (or oligomer) form to the monomer form is evidently necessary to bind and activate the cognate receptor, at least in the case of CC chemokines, as shown by the present work on the CC chemokine MIP-1β.

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MIP-1B Dimer Can Not Bind CCR5

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19. Mellado, M., Rodriguez-Frade, J. M., Manes, S., and Martinez, A. C. (2001) Annu. Rev. Immunol. 19, 397–421

20. Rodriguez-Frade, J. M., Vila-Coro, A. J., Martin de Ana, A., Albar, J. P., Martinez-A, C., and Mellado, M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 3628–3633

21. Jin, H., Hayes, G. L., Darbha, N. S., Meyer, E., and LiWang, P. J. (2005) Biochem. Biophys. Res. Commun. 338, 987–999

22. Johnson, M. L., and Correia, J. J. (1981) Biophys. J. 36, 575–588

23. Wishart, D. S., Bigam, C. G., Yao, J., Abildgaard, F., Dyson, H. J., Oldfield, E., Markley, J. L., and Sykes, B. D. (1995) J. Biomol. NMR 6, 135–140

24. Grzesiek, S., and Bax, A. (1992) J. Magn. Reson. 99, 201–207

25. Wittekind, M., and Mueller, L. (1993) J. Magn. Reson. 101, 201–205

26. Grzesiek, S., and Bax, A. (1992) J. Am. Chem. Soc. 114, 6291–6293

27. Grzesiek, S., and Bax, A. (1993) J. Biomol. NMR 3, 185–204

28. Grzesiek, S., Anglister, J., and Bax, A. (1993) J. Magn. Reson. 101, 114–119

29. Muhandiram, D. R., and Kay, L. E. (1994) J. Magn. Reson. 103, 203–216

30. Logan, T. M., Olejniczak, E. T., Xu, R. X., and Fesik, S. W. (1993) J. Biomol. NMR 3, 225–231

31. Ikura, M., Kay, L. E., and Bax, A. (1991) J. Biomol. NMR 1, 299–304

32. Vuister, G. W., Clore, G. M., Gronenborn, A. M., Powers, R., Garrett, D. S., Tschudin, R., and Bax, A. (1993) J. Magn. Reson. 101, 210–213

33. Blanpain, C., Lee, B., Vakili, J., Doranz, B. J., Govaerts, C., Migeotte, I., Sharron, M., Dupriez, V., Vassart, G., Doms, R. W., and Parmentier, M. (1999) J. Biol. Chem. 274, 18902–18908

34. Derdeyn, C. A., Decker, J. M., Sfakianos, J. N., Wu, X., O’Brien, W. A., Ratner, L., Kappes, J. C., Shaw, G. M., and Hunter, E. (2000) J. Virol. 74, 8358–8367

35. Wei, X., Decker, J. M., Liu, H., Zhang, Z., Arani, R. B., Kilby, J. M., Saag, M. S., Wu, X., Shaw, G. M., and Kappes, J. C. (2002) Antimicrob. Agents Chemother. 46, 1896–1905

36. Chackerian, B., Lowy, D. R., and Schiller, J. T. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 2373–2378

37. Blanpain, C., Doranz, B. J., Vakili, J., Rucker, J., Govaerts, C., Baik, S. S. W., Lorthioir, O., Migeotte, I., Libert, F., Baleux, F., Vassart, G., Doms, R. W., and Parmentier, M. (1999) J. Biol. Chem. 274, 34719–34727

38. Koopmann, W., Ediriwickrema, C., and Krangel, M. S. (1999) J. Immunol. 163, 2120–2127

39. Kuschert, G. S., Coulin, F., Power, C. A., Proudfoot, A. E., Hubbard, R. E., Hoogewerf, A. J., and Wells, T. N. (1999) Biochemistry 38, 12959–12968

40. Sharma, D., and Rajarathnam, K. (2000) J. Biomol. NMR 18, 165–171

41. Laurence, J. S., LiWang, A. C., and LiWang, P. J. (1998) Biochemistry 37, 9346–9354

42. Lowman, H. B., Fairbrother, W. J., Slagle, P. H., Kabakoff, R., Liu, J., Shire, S., and Hebert, C. A. (1997) Protein Sci. 6, 598–608

43. Veldkamp, C. T., Peterson, F. C., Pelzek, A. J., and Volkman, B. F. (2005) Protein Sci. 14, 1071–1081

44. Laurence, J. S., Blanpain, C., De Leener, A., Parmentier, M., and LiWang, P. J. (2001) Biochemistry 40, 4990–4999

45. Leong, S. R., Lowman, H. B., Liu, J., Shire, S., Deforge, L. E., Gilliece-Castro, B. L., McDowell, R., and Hebert, C. A. (1997) Protein Sci. 6, 609–617

46. Fernando, H., Chin, C., Rosgen, J., and Rajarathnam, K. (2004) J. Biol. Chem. 279, 36175–36178

47. Bondue, A., Jao, S. C., Blanpain, C., Parmentier, M., and LiWang, P. J. (2002) Biochemistry 41, 13548–13555

48. Hoogewerf, A. J., Kuschert, G. S. V., Proudfoot, A. E. I., Borlath, F., Clarke-Lewis, I., Power, C. A., and Wells, T. N. C. (1997) Biochemistry 36, 13570–13578

49. McCormack, M. A., Cassidy, C. K., and LiWang, P. J. (2003) J. Biol. Chem. 278, 1946–1956