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A Dominant Negative Inhibitor Indicates that Monocyte Chemoattractant Protein 1 Functions as a Dimer

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Monocyte chemoattractant protein 1 (MCP-1) is a member of the chemokine family of proinflammatory cytokines, all of which share a high degree of amino acid sequence similarity. Aberrant expression of chemokines occurs in a variety of diseases that have an inflammatory component, such as atherosclerosis. Although structural analyses indicate that chemokines form homodimers, there is controversy about whether dimerization is necessary for activity. To address this question for MCP-1, we obtained evidence in four steps. First, coprecipitation experiments demonstrated that MCP-1 forms dimers at physiological concentrations. Second, chemically cross-linked MCP-1 dimers attract monocytes in vitro with a 50% effective concentration of 400 pM, identical to the activity of non-cross-linked MCP-1. Third, an N-terminal deletion variant of MCP-1 (called 7ND) that inhibits MCP-1-mediated monocyte chemotaxis specifically forms heterodimers with wild-type MCP-1. Finally, although 7ND inhibits wild-type MCP-1 activity, it has no effect on cross-linked MCP-1. These results indicate that 7ND is a dominant negative inhibitor, implying that MCP-1 activates its receptor as a dimer. In addition, chemical cross-linking restores activity to an inactive N-terminal insertional variant of MCP-1, further supporting the need for dimerization. Since the reported K_d for MCP-1 monomer dissociation is much higher than its 50% effective concentration or K_d for receptor binding, active dimer formation may require high local concentrations of MCP-1. Our data further suggest that the dimer interface can be a target for MCP-1 inhibitory drugs.

Monocyte chemoattractant protein 1 (MCP-1) is a potent chemoattractant for monocytes and T lymphocytes (5, 14, 28) and induces histamine release from basophils (3, 11). MCP-1 is a member of a family of proinflammatory cytokines called chemokines, all of which share a high degree of amino acid sequence similarity (16, 19, 22). All chemokines have four cysteines in conserved positions, but two subfamilies can be distinguished on the basis of the absence or presence of an intervening amino acid between the two cysteines nearest the N termini of the proteins. The former subfamily is denoted C-C and includes MCP-1; the latter subfamily is denoted C-X-C and includes the neutrophil chemoattractant interleukin 8 (IL-8). The structures of some chemokines (including IL-8 [1, 7], MIP-1β [12], platelet factor 4 [9, 25], and NAP-2 [13]) have been solved, and as expected, the tertiary structures of chemokine monomers are considerably similar. However, under conditions required for crystallization or nuclear magnetic resonance analysis, all chemokines form dimers, and despite the nearly identical structures of their monomeric subunits, the structures of the dimers differ appreciably. The IL-8 dimer is compact (1, 7), while the MIP-1β dimer (another C-C chemokine) is elongated and cylindrical (12).

Although there are no data directly demonstrating a pathogenic role for MCP-1, its aberrant expression occurs in a variety of diseases that feature a monocyte-rich inflammatory component, such as atherosclerosis (15, 27) and rheumatoid arthritis (10). Rational design of MCP-1 inhibitors that might treat these diseases depends on understanding how MCP-1 interacts with its receptor, a 7-transmembrane-spanning (7-TMS), G protein-coupled receptor (6). A critical and contentious issue about chemokine-receptor interaction is whether the ligands bind to their receptors as dimers. Published estimates for the affinity of chemokine dimerization place the K_d for subunit dissociation several log units above the 50% effective concentration for biological activity and the K_d for receptor binding (4, 17, 18). In addition, variant IL-8 proteins that are unable to dimerize in solution have full biological activity as neutrophil chemoattractants (18). Although these results imply that dimerization may not be a prerequisite for activity, there is contrary evidence that IL-8 binds to its receptors as dimers even at low concentrations (2, 23).

In a previous study, we demonstrated that an N-terminal deletion variant of MCP-1 inhibited monocyte chemotaxis in response to wild-type MCP-1 (29). Although this variant only bound to a small proportion of MCP-1 receptors on monocytes, it was capable of preventing MCP-1 from binding to its receptor, suggesting that it could be a dominant negative inhibitor. In the present study, we show that this variant has the properties of a dominant negative inhibitor, which implies that MCP-1 activates its receptor as a dimer. If the in vitro estimates for monomer affinities accurately reflect in vivo affinities, these results have important implications for chemokine physiology.

MATERIALS AND METHODS

Chemokines. Recombinant human MCP-1 was purchased from Genzyme Corp. (Boston, Mass.); ^{125}I-radiolabeled MCP-1, IL-8, and RANTES were purchased from NEN/DuPont (Boston, Mass.). All had specific activities of 2,200 Ci/mmol. FX2 (C-terminal epitope-tagged MCP-1), an N-terminal epitope-tagged MCP-1, and 7ND (N-terminal MCP-1 deletion variant) were prepared by expression of their cDNAs in Chinese hamster ovary cells (20) and were purified by FLAG affinity purification as described elsewhere (29).

Immune precipitations. ^{125}I-radiolabeled chemokines were mixed with epitope-tagged chemokines in phosphate-buffered saline (PBS) and incubated at 4°C for 1 h, and then 10 μg of anti-FLAG M2 antibody (International Biotechnologies, Inc., New Haven, Conn.) or murine immunoglobulin G (Sigma, St. Louis, Mo.) was added. The mixture was incubated at 4°C for 1 h with gentle

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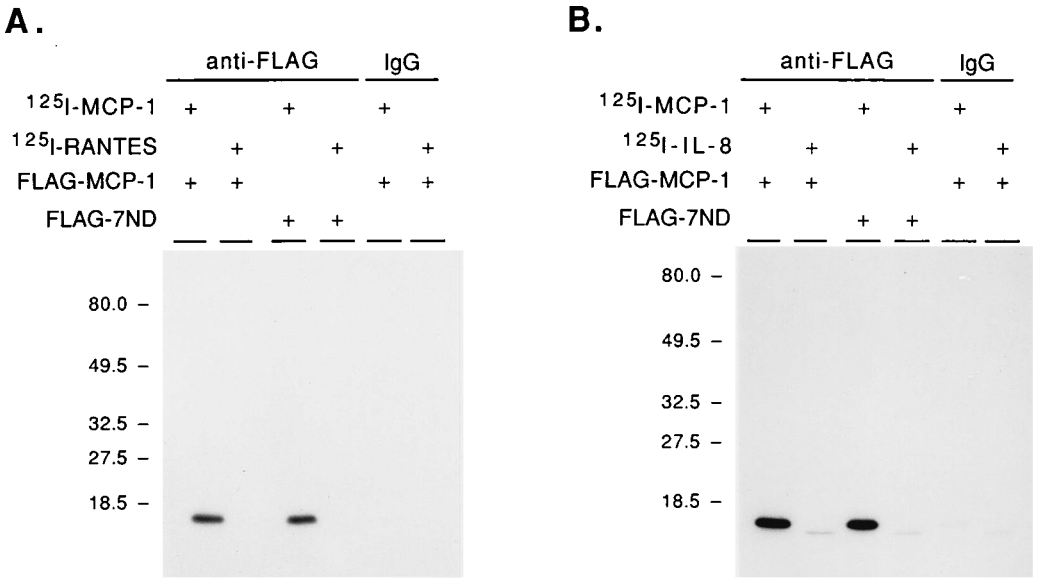

FIG. 1. (A) A 1-ng amount of ^{125}I-radiolabeled MCP-1 or RANTES (at the same specific activity) was mixed with 10 ng of nonradiolabeled FX2 (C-terminal FLAG epitope-tagged MCP-1) or 7ND (C-terminal FLAG epitope-tagged mutant MCP-1) as indicated in a total volume of 0.5 ml. Mixtures were subjected to immunoprecipitation with anti-FLAG antibody or murine immunoglobulin G (IgG) and analyzed by SDS-PAGE. (B) Same as panel A, except that ^{125}I-radiolabeled IL-8 was used instead of RANTES and 600 ng of FX2 or 7ND was used. Numbers on the left indicate molecular mass in kilodaltons.

rotation, after which 50 μl of staphylococcal protein A beads (Bio-Rad, Richmond, Calif.) was added and the incubation was continued for an additional hour. Beads were collected by centrifugation and then washed four times with RIPA buffer (50 mM Tris hydrochloride [pH 7.5], 150 mM NaCl, 0.1% sodium dodecyl sulfate [SDS], 1% Triton X-100, 0.5% deoxycholate, 1 mM phenylmethylsulfonyl fluoride). Beads were resuspended in sample buffer containing SDS and β-mercaptoethanol, and then the mixture was boiled. Released proteins were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) through 15% polyacrylamide, and dried gels were analyzed by autoradiography.

Gel filtration chromatography. Seven to 9 μg of MCP-1 or its variants was loaded on a 25-ml Superdex-75 column (Pharmacia, Piscataway, N.J.) which was developed in 50 mM sodium phosphate (pH 7.4)–150 mM NaCl at a flow rate of 0.5 ml/min. The column was calibrated in the same buffer by using standards obtained from Bio-Rad.

Cross-linking. Five micrograms of chemokine was resuspended in 445 μl of PBS at 4°C, and 5 μl of a 20 mM solution of DSS (disuccinimidyl suberate) (Pierce, Rockford, Ill.) was added. The mixture was incubated at 4°C for 45 min and then concentrated and cleared of cross-linker by centrifugation by using a Centricon-10 device (Amicon, Danvers, Mass.). Cross-linked products were fractionated by SDS-PAGE on 15% polyacrylamide gels. After silver staining of the gels, the proportions of monomers and multimeric species were determined by laser densitometry (Pharmacia).

Monocyte chemoattraction assays. Monocyte chemoattraction assays were performed with human peripheral blood mononuclear cells by using a multiwell modified Boyden chamber device as described elsewhere (29).

RESULTS

Specific MCP-1 dimerization in solution. Our first experiments indicated that MCP-1 monomers interact specifically to form dimers at physiological concentrations. These studies used a C-terminal FLAG epitope-tagged variant of MCP-1 (called FX2) that has full biological activity (29). When FX2 was mixed with ^{125}I-MCP-1 at a final chemokine concentration of 2 nM, an anti-FLAG antibody coprecipitated radiolabeled MCP-1 (Fig. 1). FX2's apparent molecular mass on gel chromatography is 30,600 kDa (Fig. 2A), similar to that of unmodified MCP-1 (21) and double its apparent molecular mass on SDS-PAGE (29). FX2's molecular mass is also double the apparent molecular mass of 7ND, an N-terminal deletion variant that does not form dimers (see below) and whose predicted molecular mass is only 680 Da less than that of monomeric FX2 (Fig. 2B). These observations indicate that MCP-1 forms dimers at physiologically relevant concentrations. Serial immune precipitations showed that approximately 65% of radiolabeled MCP-1 was complexed with FX2 at a concentration of 2 nM. (This interaction was not due to the FLAG epitope, since anti-FLAG immune precipitation of a mixture of 80 nM

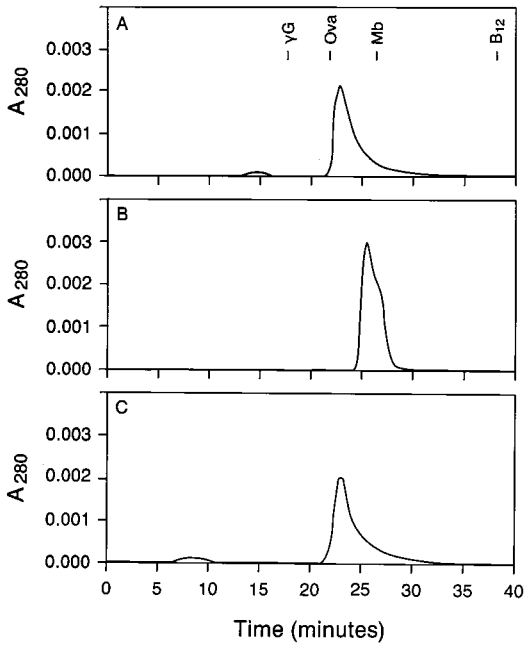

FIG. 2. Gel filtration chromatography of MCP-1 variants on a Superdex-75 column. The column was calibrated with standard proteins as described in Materials and Methods, and M_rs were determined for each species. (A) FX2 (M_r, 30,600); (B) 7ND (M_r, 15,500); (C) N-terminal FLAG epitope-tagged MCP-1 (M_r, 30,600). Molecular weight markers include bovine gamma globulin (γG; M_r, 158,000), chicken ovalbumin (Ova; M_r, 44,000), equine myoglobin (Mb; M_r, 17,000), and vitamin B_{12} (B_{12}; M_r, 1,350).

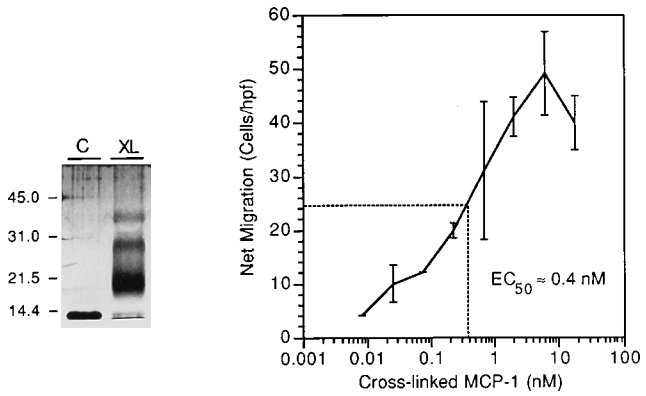

FIG. 3. (Left) SDS-PAGE analysis of non-cross-linked (C) and cross-linked (XL) MCP-1. Samples were fractionated by electrophoresis through 15% polyacrylamide gels, which were then silver stained and analyzed by laser densitometry. The relative contribution of each molecular species is described in the text. Numbers on the left indicate molecular mass in kilodaltons. (Right) Cross-linked MCP-1 was tested in an in vitro monocyte chemoattraction assay. Molar concentrations were determined by assuming an average molecular weight based on the proportional contribution of each molecular species to the cross-linked preparation. Error bars indicate the standard errors of measurements in duplicate wells; this experiment is representative of three. hpf, high-power field; EC_{50}, 50% effective concentration.

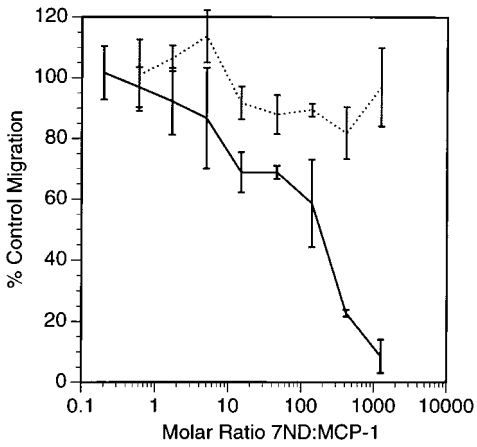

FIG. 4. Increasing amounts of purified 7ND were added to constant amounts of non-cross-linked MCP-1 (10 ng/ml; 0.9 nM; solid line) or cross-linked MCP-1 (19 ng/ml; 0.7 nM; dotted line). The mixtures were then tested for their ability to attract monocytes in vitro relative to their chemoattractant activity in the absence of 7ND. Error bars indicate the standard errors of measurements in duplicate wells; this experiment is representative of three. For cross-linked MCP-1, 100% migration was 65 cells per high-power field (hpf); for non-cross-linked MCP-1, 100% migration was 64 cells per hpf.

FLAG epitope-tagged C5a [a gift of C. Gerard, Children's Hospital, Boston, Mass.] with ^{125}I-MCP-1 did not precipitate ^{125}I-radiolabeled material.)

The specificity of homodimerization was reflected by the fact that FX2 did not form complexes with ^{125}I-RANTES (a C-C chemokine like MCP-1) or ^{125}I-IL-8 (a C-X-C chemokine), both radiolabeled to the same specific activity as was ^{125}I-MCP-1. In the experiment shown in Fig. 1A, no detectable interaction occurred between FX2 and ^{125}I-RANTES at a physiological chemokine concentration of 2 nM. In the experiment shown in Fig. 1B, we increased FX2's concentration 60-fold. Under these conditions, FX2 still preferentially complexed with ^{125}I-MCP-1, although a small amount of ^{125}I-IL-8 coprecipitated with FX2 when anti-FLAG antibody was used. However, control immunoglobulin G also precipitated a similar amount of ^{125}I-IL-8, suggesting that the specificity of homodimerization persisted at concentrations as high as 120 nM.

Activity of cross-linked MCP-1. Having determined that MCP-1 forms dimers, we next demonstrated that MCP-1 dimers are biologically active by using the bifunctional cross-linker DSS to construct stably dimerized molecules. Figure 3 shows an example of a cross-linked MCP-1 preparation in which the relative contributions of monomeric and multimeric species, as determined by laser densitometry, were 3% for monomers, 63% for dimers, 21% for trimers, and 13% for tetramers. (Independent cross-linking experiments performed under the same conditions routinely produced similar product distributions.) We tested this material for monocyte chemoattractant activity and, using a molecular weight based on the abundance of each species, found that its 50% effective concentration was approximately 400 pM, the same as the specific activity of non-cross-linked MCP-1 (Fig. 3) (14, 28). In addition, the maximal number of monocytes attracted by cross-linked material was the same as that attracted by non-cross-linked material, indicating that the efficacies of the two species were also identical.

Effect of 7ND on cross-linked MCP-1 activity. The data in Fig. 3 indicate that multimers of MCP-1, including dimers, are fully active as chemoattractants, but they do not show that dimerization is necessary for activity. To demonstrate this, we used an MCP-1 variant which lacks amino acids 2 to 8 of the processed protein. This variant, called 7ND, is a potent and specific inhibitor of monocyte chemotaxis in response to MCP-1 (29). (The inhibitory activity is not due to the epitope tag because other tagged variants had no inhibitory activity [29]). In an earlier study, we suggested that 7ND might be a dominant negative inhibitor, which would necessarily imply that the active form of MCP-1 is a dimer.

Two experiments indicated that 7ND is a dominant negative inhibitor. First, 7ND specifically formed heterodimers with wild-type MCP-1. Figure 1 shows that anti-FLAG antibodies coprecipitated radiolabeled MCP-1 (but not RANTES or IL-8) along with 7ND, which contains the FLAG epitope. Second, we reasoned that 7ND could inhibit wild-type MCP-1 activity by two possible mechanisms: either it is a competitive inhibitor for receptor binding or it is a dominant negative inhibitor. We used the cross-linked dimer to distinguish between these possibilities. Figure 4 shows that 7ND inhibited monocyte chemotaxis in response to non-cross-linked MCP-1 but had no effect on cross-linked MCP-1. This indicates that 7ND exerts its effects as a dominant negative inhibitor and further suggests that the biologically active form of MCP-1 is a dimer.

The most direct way to demonstrate 7ND's dominant suppression would have been a test of cross-linked 7ND/MCP-1 heterodimers. So far, however, we have been unable to cross-link 7ND to itself or to MCP-1 using a variety of cross-linkers with different reactive groups and spacer arm lengths. This suggests that 7ND cannot form homodimers (Fig. 2B). Figure 1 shows that 7ND can form heterodimers with MCP-1, but it appears that DSS requires N-terminal amino acids which are deleted from 7ND in order to cross-link 7ND/MCP-1 heterodimers. This is consistent with the proposed three-dimensional model of C-C homodimers (12).

Restoring activity of N-terminal FLAG MCP-1 by cross-linking. Finally, we demonstrated the need for dimerization in a different way by examining an inactive MCP-1 variant in which the FLAG epitope was inserted immediately after the N-terminal glutamine. Although this variant appears to form

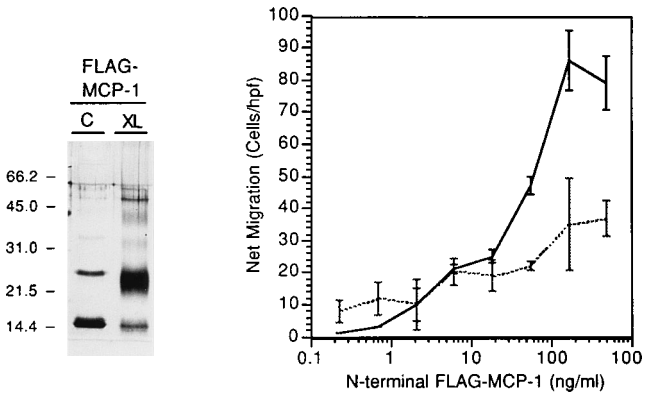

FIG. 5. (Left) SDS-PAGE analysis of non-cross-linked (C) and cross-linked (XL) N-terminal FLAG-MCP-1 by silver staining. Numbers on the left indicate molecular mass in kilodaltons. (Right) Non-cross-linked (dotted line) and cross-linked (solid line) N-terminal FLAG-MCP-1 species were tested in monocyte chemoattraction assays. Error bars indicate the standard errors of measurements in duplicate wells; this experiment is representative of two.

homodimers by the criteria of its size on gel filtration chromatography (Fig. 2C) and its ability to coprecipitate with MCP-1 (data not shown), it has only 0.3% of the wild-type chemoattractant activity (29). However, because it has a full-length N terminus, DSS can cross-link these homodimers, and cross-linking restores its ability to attract monocytes (Fig. 5). Thus, physically constraining the altered N termini of N-terminal FLAG MCP-1 dimers produces monocyte chemoattractant activity, again indicating that dimeric MCP-1 activates its receptor.

DISCUSSION

Using coprecipitation, we have demonstrated that MCP-1 specifically forms homodimers at physiological concentrations. Dimeric MCP-1 is fully active as a monocyte chemoattractant, because the specific activity and efficacy of chemically cross-linked MCP-1 were indistinguishable from those of non-cross-linked MCP-1. We examined the need for dimerization by using 7ND, an N-terminal deletion variant of MCP-1 that inhibits the monocyte chemoattractant activity of non-cross-linked MCP-1. Although 7ND inhibited non-cross-linked MCP-1 activity, its inability to inhibit monocyte chemotaxis in response to cross-linked MCP-1 led us to conclude that 7ND acts as a dominant negative inhibitor of MCP-1, which means, in turn, that MCP-1 activates its receptor as a dimer. Finally, supportive evidence for dimerization came from the observation that cross-linking an inactive N-terminal FLAG insertional mutant restores its activity.

Taken together, our results suggest a model in which dimeric MCP-1 binds to its receptor. Earlier work from our laboratory (29), as well as others (8), demonstrated that the N terminus and one face of the first predicted β sheet are essential for MCP-1's chemoattractant activity. These observations can be combined with our current results in three different models. First, altering the N terminus and first β sheet may destroy activity simply by preventing dimerization, and dimerization per se may be essential for activity because apposing noncontiguous regions of the proteins (not at the N terminus) creates the active ligand. However, this model would not explain why 7ND/MCP-1 heterodimers are inactive. Alternatively, the N terminus and first β sheet may be essential because they interact directly with the receptor, and dimerization is necessary because two of these areas must bind to one receptor in order to generate a signal. This might explain restoration of N-terminal FLAG MCP-1's activity by cross-linking. The N-terminal FLAG insertion would prevent two N termini from interacting with the receptor because of steric hindrance or charge repulsion (the FLAG epitope is essentially a polyanion of aspartic acids). Cross-linking, which occurs at the N termini, would then constrain the N termini to fit into their binding region in the receptor. This model would also explain 7ND's dominant negative activity: 7ND/MCP-1 heterodimers could dock in the receptor but could not activate it because one of the two required N termini would be missing. (There is a precedent for peptide ligands that interact with 7-TMS receptors at multiple sites [24]). In a third model, MCP-1 dimers might bring two receptors together for their activation, in a manner analogous to that of tyrosine kinase receptors. Our data are consistent with this model, but there is no evidence yet to support 7-TMS receptor interaction.

Not unexpectedly, 7ND's inhibitory properties are similar to those of a variant lacking amino acids 1 to 8, called "9-76" by Gong and Clark-Lewis (8). However, those authors suggested that 9-76 inhibited monocyte chemotaxis in response to MCP-1 by desensitizing the MCP-1 receptor. Neither they nor we have detected a calcium flux in monocytes or THP-1 cells in response to 9-76 or 7ND, which brings into question the notion that these ligands interact sufficiently with the MCP-1 receptor to desensitize it. Instead, their data are also consistent with a dominant negative mechanism of action. Confirmation will require testing 9-76's effects on cross-linked MCP-1.

Our data indicating the need for MCP-1 dimerization differ somewhat from those of Rajarathnam and colleagues, who demonstrated that IL-8 derivatives that cannot form dimers are nonetheless fully active (18). There are at least two possible explanations for this discrepancy. First, the experiments of Rajarathnam et al. do not rule out the possibility that two monomers bind to a single receptor without forming stable dimers. Consistent with this possibility are the findings that cross-linking IL-8 to its neutrophil receptor produces cross-linked IL-8 dimers and that the maximal amount of IL-8 bound to its receptors was greater than the amount of NAP-2 bound to the same receptors (2, 23). Second, the strikingly different quaternary structures of C-X-C chemokines (including IL-8) and C-C chemokines (including MCP-1) may produce alternative ways of binding and activating 7-TMS receptors (1, 7, 12).

Direct measurements of MCP-1 monomer-dimer equilibria suggest that the K_d for monomeric subunits is several log units above MCP-1's 50% effective concentration (17). These observations do not contradict our findings or our model. First, the equilibrium in solution may not reflect the equilibrium at the receptor, since the binding site for MCP-1 may have a conformation that greatly favors dimerization. Second, local concentrations of MCP-1 may be influenced by other molecules at the cell surface. For example, chemokines interact with glycosaminoglycans which cover the surface of monocytes and endothelial cells (26). As chemokines are added to monocytes in vitro or as they appear at the endothelial cell surface in vivo, their binding to glycosaminoglycans could create extremely high local concentrations that might exceed the K_d for dimerization. Finally, values for monomer affinities may depend on the techniques used for their measurement. For example, biospecific interaction analysis, in which one ligand would be immobilized to a sensor chip, might provide estimates for the K_d that are different from those obtained by sedimentation equilibrium analysis, in which both ligands are soluble.

If the reported K_d for monomer dissociation is a true estimate for in vivo affinities, this begs the question of why chemokines would evolve a system in which their active forms are

dimers whose subunits have relatively low affinities for each other. One reason may involve modulation of their leukocyte chemoattractant activity. If the K_d for MCP-1 dimerization is higher than the K_d for receptor binding, then the appearance of active MCP-1 depends on the achievement of high local concentrations. This effectively changes the shape of the chemoattractant gradient to one that is much steeper near its source and falls off more rapidly with distance than it otherwise would. In fact, MCP-1 may dimerize in the Golgi apparatus and be secreted in dimeric form. Self-inactivation would then occur rapidly with increasing distance from the secreting cell. The extremely steep gradient may prevent aberrant leukocyte migration and may enable cells to be precisely directed over extremely short distances, providing greater control and specificity for leukocyte trafficking. However, it is also possible that the true K_d for monomer dissociation is less than 2 nM, as our immune precipitation and depletion experiments imply. Either way, our data identify a new drug target for inhibiting MCP-1 activity, namely, the dimer interface.

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