THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 269, No. 22, Issue of June 3, pp. 15918-15924, 1994
Printed in U.S.A.

Structure/Activity Analysis of Human Monocyte Chemoattractant Protein-1 (MCP-1) by Mutagenesis

IDENTIFICATION OF A MUTATED PROTEIN THAT INHIBITS MCP-1-MEDIATED MONOCYTE CHEMOTAXIS*

(Received for publication, October 27, 1993, and in revised form, March 1, 1994)

Yu Jun Zhang, Barbara J. Rutledge, and Barrett J. Rollins‡

From the Department of Medicine, Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115

Monocyte chemoattractant protein-1 (MCP-1) is a monocyte-specific chemoattractant and activator and is a member of the chemokine-β family of cytokines. To identify regions of MCP-1 which are required for its biological activity, we constructed human MCP-1 mutants that were expressed in eukaryotic cells and tested for their ability to attract monocytes *in vitro*. Deletion of amino acids 2–8 destroyed activity, suggesting that the amino-terminal region is necessary for activity. Within the deleted region, mutation of aspartate 3 to alanine produced a protein with 9% of wild-type activity, whereas mutation of asparagine 6 to alanine produced a protein with 52.9% of wild-type activity. Mutation of amino acids within the first intercysteine loop yielded variable results. Changing tyrosine 28 to aspartate or arginine 30 to leucine each produced proteins with essentially no monocyte chemoattractant activity. The side chains of these amino acids are predicted to point into a putative receptor binding cleft, and these loss-of-function mutations are consistent with this model. Also consistent is the retention of 60% of wild-type activity after mutation of serine 27 to glutamine, since the side chain of serine 27 is predicted to point away from the binding cleft. However, mutation of arginine 24, which lies outside of this area, to phenylalanine produced a protein with only 5% of wild-type activity, suggesting more complex interactions. Truncations of the carboxyl terminus, as well as mutation of aspartate 68 to leucine, generated proteins with 10–20% of wild-type activity. (Another carboxyl-terminal insertional mutation demonstrated that *O*-linked carbohydrate in MCP-1α may be added to a threonine in the carboxyl-terminal region.) These findings are consistent with a structural model of dimeric MCP-1 which is similar to interleukin-8, in which amino acids that point into a cleft between the two carboxyl-terminal α-helices of the subunits are important for receptor binding. In addition, however, amino acids at the amino terminus and others outside of the interhelical cleft are also essential for activity. The carboxyl-terminal α-helix is not required for signaling *per se* but is required for maximal specific activity. Finally, four mutant proteins partially inhibited the ability of wild-type MCP-1 to attract monocytes *in vitro*. In particular, mutant 7ND (deletion of amino acids 2–8) inhibited MCP-1 activity by 50% at a molar ratio of 75:1, displaced MCP-1 from its receptor on monocytes at a similar ratio, and bound to a single class of receptors on human monocytes with a K_d of 2.6 nM. However, 7ND bound only to 10% of the number of receptors to which MCP-1 bound, suggesting that part of its inhibitory activity may be due to binding to MCP-1 itself.

Chemokines are proinflammatory cytokines that attract and activate specific types of leukocytes (for review see Refs. 1 and 2). Members of this protein family share common structural motifs, in particular the positions of 4 cysteines, as well as other highly conserved regions of primary structure. Despite their structural similarities, most chemokines have nonoverlapping target cell specificities. Chemokines can be grouped into two subfamilies based on structural and genetic criteria. In the chemokine-α proteins, a single amino acid is interposed between the 2 cysteines nearest the amino terminus (for this reason they are also known as C-*X*-C proteins), and their genes cluster on the long arm of chromosome 4. In the chemokine-β proteins, the 2 cysteines nearest the amino terminus are adjacent to each other (thus C-C proteins), and their genes cluster on the long arm of chromosome 17. In terms of secondary or tertiary structure, it is unlikely that the intervening amino acid in the chemokine-α proteins has much significance (3).

For the most part, chemokine-α family members attract and activate neutrophils, although other target cells have been identified. A great deal of structural data has been generated for two chemokine-α proteins, namely platelet factor 4 and IL-8,[1] the latter of which is a neutrophil-specific chemoattractant. Crystal and solution structures for IL-8 have been solved, and both suggest that IL-8 is a homodimer in which the carboxyl-terminal α-helices of each subunit overlie two three-stranded anti-parallel β-sheets (4–6). This provides a cleft between the two α-helices which is predicted, by analogy to other structures, to be an IL-8 receptor binding region. However, mutational analysis has demonstrated an absolute requirement for the amino terminus of IL-8, in particular amino acids 4–6 (glutamate-leucine-arginine (ELR)), for neutrophil chemotaxis and activation (7, 8).

In contrast to the chemokine-α proteins, chemokine-β proteins tend to attract and activate monocytes. In particular, monocyte chemoattractant protein-1 (MCP-1) attracts monocytes *in vitro* at subnanomolar concentrations (9, 10). MCP-1 expression has been detected in a variety of pathologic conditions that involve monocyte accumulation and activation, including atherosclerosis. Much less is known about the structure of MCP-1 than the structures of IL-8 or platelet factor 4. For example, there is no direct evidence that MCP-1 is active as a dimer. By analogy to IL-8, however, proper disulfide bridging

* This work was supported in part by United States Public Health Service Grant CA53091 and by a grant from the Sandoz/DFCI Drug Development Program. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of a Junior Faculty Research Award from the American Cancer Society. To whom correspondence should be addressed: D930, Dana-Farber Cancer Institute, 44 Binney St., Boston, MA 02115.

[1] The abbreviations used are: IL-8, interleukin 8; MCP-1, monocyte chemoattractant protein-1.

is probably necessary for activity (11). Because of their similar primary structures, MCP-1 is predicted to have a higher order structure like that of IL-8 (3). In fact, it has been shown that mutation of 2 amino acids in the putative receptor binding domain between the two α-helices of the presumed MCP-1 dimer destroys its ability to attract monocytes (12).

Because regions outside the interhelical cleft of IL-8 are clearly necessary for activity, we hypothesized that a similar situation would apply to MCP-1. We have constructed a series of mutants involving the amino terminus, the first intercysteine loop, and the carboxyl-terminal α-helix of MCP-1 and tested them for their ability to attract monocytes *in vitro*. We have also examined these mutants for their ability to block the activity of wild-type MCP-1. Our results point to the amino terminus and specific areas of the first intercysteine loop as being necessary for monocyte chemoattraction and to the carboxyl-terminal α-helix as a region required for maximal potency.

MATERIALS AND METHODS

Construction of Epitope-tagged MCP-1 and MCP-1 Mutations—Starting with pGEM-hJE34 (human MCP-1 cDNA in pGEM-7 (Promega, Madison, WI)) (13) as template, recombinant polymerase chain reaction was used to insert 30 nucleotides immediately 5′ to the termination codon (position 366) encoding the amino acid sequence GGDYKDDDDK. The last 8 amino acids of this insert represent the so-called FLAG epitope (14), and the first 2 glycines were included to provide a "spacer" between MCP-1 sequences and the epitope. Sequence analysis of the resulting cDNA revealed no other base alterations, and the entire cDNA was cloned into the expression vector pmt21 (a derivative of p91023(B) (15)) to yield a plasmid designated pFX2. Similar techniques were used to insert the FLAG epitope and spacer immediately 3′ to the codon for aspartate 3 in processed MCP-1 (nucleotide position 146); this expression plasmid was designated pFX3. (The amino terminus of proteolytically processed MCP-1 is glutamine and is designated as position 1 in this study.) With pFX2 as template, recombinant polymerase chain reaction was used to insert termination codons or single amino acid changes as listed in Fig. 4. All mutations were confirmed by sequence analysis of both DNA strands. (Sequences for oligonucleotides used in generating FLAG epitope-tagged MCP-1 or its mutations are available on request.) Wild-type MCP-1 was purchased from Genzyme Corp. (Cambridge, MA).

Expression of MCP-1 and Its Derivatives—COS cells were suspended in serum-free Dulbecco's modified Eagle's medium at 5×10^6 cells/ml. Four $\times 10^6$ cells (0.8 ml) were placed in a cooled electroporation cuvette with a 0.4-cm gap, 10 μg of plasmid DNA was added, and the cells were electroporated at 0.36 kV, 960 microfarads (yielding a time constant of 16–17 ms). Cells were allowed to recover in the cuvette at room temperature for 10 min and were then plated in a 100-mm dish in Dulbecco's modified Eagle's medium with 10% bovine calf serum. After 24 h, the medium was changed to serum-free Dulbecco's modified Eagle's medium. After an additional 48 h, conditioned medium was collected, cells and debris were removed by centrifugation, and the medium was stored at −20 °C.

Immunoblotting—Conditioned medium from transfected COS cells was concentrated ≈ 50-fold using a Centricon-10 device (Amicon, Danvers, MA), boiled in sample buffer, and subjected to electrophoresis through a 12% polyacrylamide gel in SDS. Proteins were electrophoretically transferred to nitrocellulose and probed either with an anti-FLAG M2 monoclonal antibody (International Biotechnologies, Inc., New Haven, CT) or with rabbit anti-MCP-1 antiserum (13). Blots were developed using the appropriate horseradish peroxidase-conjugated secondary antibody and substrate solution (Vector Laboratories, Burlingame, CA).

Quantitation of MCP-1 and Its Derivatives—MCP-1 was expressed as a FLAG fusion protein in *Escherichia coli* using the FLAG biosystem and purified using anti-FLAG M2 affinity gel (International Biotechnologies, Inc.). Known amounts of pure FLAG-MCP-1 fusion protein were included in every immunoblot for MCP-1 produced by COS cells. Immunoblots were then analyzed by laser densitometry (Pharmacia Biotech Inc.), and the mass of each COS cell-produced protein was determined by reference to the FLAG-MCP-1 fusion standard. The amount of COS cell supernatant loaded in each well generated an immunoblot signal within the linear response range of the laser densitometer. When possible, samples were quantitated using both anti-FLAG and anti-MCP-1 antibodies. There were no disparities between the amounts of protein determined with the two antibodies except when one of the epitopes was absent (see "Results").

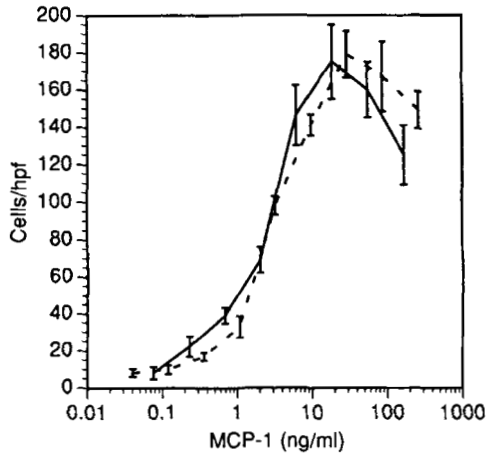

FIG. 1. Wild-type MCP-1 and carboxyl-terminal epitope tagged MCP-1 (FX2) were produced by transient transfection of COS cells and quantitated by Western blotting as described under "Materials and Methods." Varying amounts of both proteins were used in monocyte chemotaxis assays, and the number of monocytes per high powered field (*hpf*) which migrated in response to each protein was counted. *Solid line*, wild-type MCP-1; *dotted line*, FX2.

Monocyte Chemotaxis—Human peripheral blood mononuclear cells were prepared from volunteers as described (16). Chemotaxis assays were performed using a multiwell chamber fitted with a polycarbonate filter having 5-μm pores, as described. Each COS cell supernatant was tested over a wide range of dilutions, and the concentration of monocyte chemoattractant activity in each supernatant was defined as the inverse of the dilution producing half-maximal chemotactic response (17).

Purification of 7ND—Mutant 7ND was expressed in stably transfected Chinese hamster ovary cells as described (18, 19). Conditioned medium was loaded onto an anti-FLAG M2 affinity gel (International Biotechnologies, Inc.), the column was washed with phosphate-buffered saline, and protein was eluted using 0.1 M glycine hydrochloride (pH 3.0) followed by neutralization using Tris base. Protein was quantified by Bradford assay (30) and its purity assessed by SDS-polyacrylamide gel electrophoresis followed by staining with Coomassie Blue.

Radioiodination of 7ND—Pure 7ND was radioiodinated using Bolton-Hunter reagent (DuPont NEN) and separated from unincorporated reagent by gel filtration chromatography. Analysis by SDS-polyacrylamide gel electrophoresis showed a single radioactive species of appropriate M_r. Specific activity was in the range of 120–204 μCi/μg.

Receptor Binding—Human monocytes were purified from Leukopaks obtained from the Blood Component Laboratory at the Dana-Farber Cancer Institute. Peripheral blood mononuclear cells were isolated by Ficoll-Hypaque density centrifugation, and monocytes were purified by centrifugal counterflow elutriation as described (20). Binding experiments using radioiodinated 7ND or MCP-1 (DuPont NEN) were performed as described (20).

RESULTS

Epitope-tagged MCP-1—Using polymerase chain reaction, we modified wild-type MCP-1 cDNA so that it encoded a protein with a FLAG epitope tag. In the tagged protein, the amino acid sequence GGDYKDDDDK appears after the carboxyl-terminal threonine of processed MCP-1. This protein, designated FX2, was expressed by transient transfection in COS cells and quantitated by densitometric analysis of an immunoblot using an anti-FLAG antibody and a standard amount of FX2 purified from a bacterial expression system. Serial dilutions of conditioned medium from COS cells transfected with FX2 were used in a monocyte chemotaxis assay as shown in Fig. 1. At the same time, wild-type MCP-1 was also produced by COS cell transfection (16) and quantitated by densitometric analysis of a Western blot using an anti-MCP-1 antiserum and a standard amount of FX2. This material was also tested in a monocyte

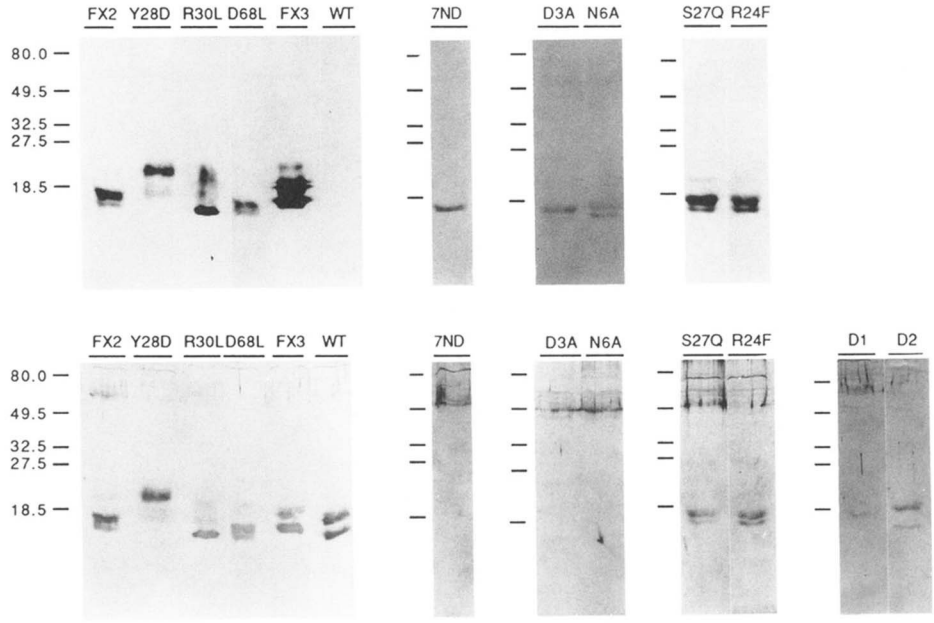

FIG. 2. Wild-type (*WT*) and mutant MCP-1 proteins were produced by transient transfection of COS cells. Conditioned medium from transfections was boiled in reducing SDS-containing sample buffer and fractionated by electrophoresis through SDS-containing 15% polyacrylamide gels. Proteins were blotted onto nitrocellulose, and immunoblots were developed using either an anti-FLAG monoclonal antibody (*upper panel*) or a rabbit anti-MCP-1 antiserum (*lower panel*). Protein designations correspond to those listed in Fig. 4. Molecular size markers are indicated for each separately processed gel.

chemotaxis assay. Fig. 1 shows that both proteins had similar dose-response characteristics. Using the quantitation results from immunoblotting, analysis of the dose-response curves showed that the specific activity of FX2 is 408,000 units/ml, and that of wild-type MCP-1 is 442,000 units/ml. Both values compare favorably with the specific activity determined for purified, eukaryotically produced, recombinant MCP-1, suggesting that the epitope tag in this position does not interfere with MCP-1's ability to attract monocytes *in vitro* (16, 21).

Construction and Expression of MCP-1 Mutants—FX2 was used as a template for the introduction of a variety of mutations. To quantify each mutant protein, conditioned medium from transfected COS cells was analyzed by immunoblotting. Representative examples are shown in Fig. 2. Although most mutants were produced in high quantity, the carboxyl-terminal deletion mutations, D1 (consisting of amino acids 1–62 of processed MCP-1) and D2 (amino acids 1–68), were either unstable or inefficiently produced. Sufficient D1 and D2 could be produced for analysis, but detection by immunoblot using anti-MCP-1 required concentrating the conditioned medium. Since their carboxyl termini were deleted, the anti-FLAG antibody could not be used.

It is possible that the apparently low levels of D1 and D2, as determined by immunoblotting, might be due to loss of antigenic determinants in the carboxyl-terminal domain which are normally detected by the anti-MCP-1 antiserum. To rule this out, we engineered a FLAG epitope tag near the amino terminus of both deletion mutants as well as wild-type MCP-1 (the amino-terminal FLAG-tagged full-length protein is denoted FX3). Immunoblotting detected large amounts of FX3, suggesting that insertion of the FLAG epitope in this position did not effect secretion or stability. However, even with the anti-FLAG antibody, only low levels of carboxyl-terminal deletion proteins carrying the amino-terminal tag could be detected. Thus both D1 and D2 are either unstable or inefficiently secreted. By performing several transfections and pooling media, enough D1 and D2 were produced for further analysis.

Electrophoretic Analysis of MCP-1 Mutants—The electrophoretic mobility of MCP-1 mutations in SDS-containing polyacrylamide gels was not predictable. The largest alterations in mobility were associated with the least conservative amino acid substitutions, *e.g.* Y28D. A number of mutations showed more than one band, which is most likely due to post-translational modification by COS cells. For example, wild-type MCP-1 is often secreted by eukaryotic cells in α and β forms which differ because of the presence of *O*-linked carbohydrate in the α form (22). (Glycosylation is not necessary for its *in vitro* chemoattractant activity (16).) In the course of other experiments, we inserted a 5-amino acid substrate for cAMP-dependent protein kinase between lysine 75 and threonine 76, the 2 amino acids at the carboxyl terminus of wild-type MCP-1. Expression of this mutation in COS cells (Fig. 3) shows that the carbohydrate-containing α form is not detected. This suggests that the inserted sequences have altered the substrate recognition site for a glycosyl transferase and that the *O*-linked carbohydrate may be added to a threonine in this region, *i.e.* threonine 76, or perhaps threonine 73. However, then one would then expect that the carboxyl-terminal deletion mutants, D1 and D2, should show only a single band, and although this is true for preparations of D1, preparations of D2 show a second, lower molecular weight band. D2 still has a serine (serine 63) near its caraboxyl terminus, whereas D1 does not. Whether D2's second species is due to utilization of this potential glycosylation site or to proteolytic degradation is currently unknown.

It is possible that lower molecular weight bands of some of the other, non-carboxyl-terminal truncation transfectants shown in Fig. 2 might be due to proteolytic degradation. This is unlikely for two reasons. First, the anti-FLAG antibody detected its epitope in all proteins in which it was expressed. Since the epitope was placed at the carboxyl terminus, carboxypeptidase activity would have been revealed as loss of the FLAG epitope in one of the bands still recognized by anti-MCP-1. This was not observed. Second, the rabbit anti-MCP-1 serum detected all forms of MCP-1 except for the amino-terminal deletion and amino-terminal point mutations (Fig. 2). Thus the predominant epitopes detected by this serum are at the amino terminus of MCP-1, and aminopeptidase activity would have been revealed as loss of anti-MCP-1 reactivity in one of the bands still recognized by anti-FLAG. This was only observed for the amino-terminal deletion and point mutants.

Activity of MCP-1 Mutations—Based on modeling considerations as well as structure/activity data from other chemokines (in particular IL-8), we constructed MCP-1 mutations in three regions: the amino terminus, the first intercysteine loop, and the carboxyl-terminal predicted α-helix (Figs. 4 and 7). At the amino terminus, insertion of the FLAG epitope after aspartate

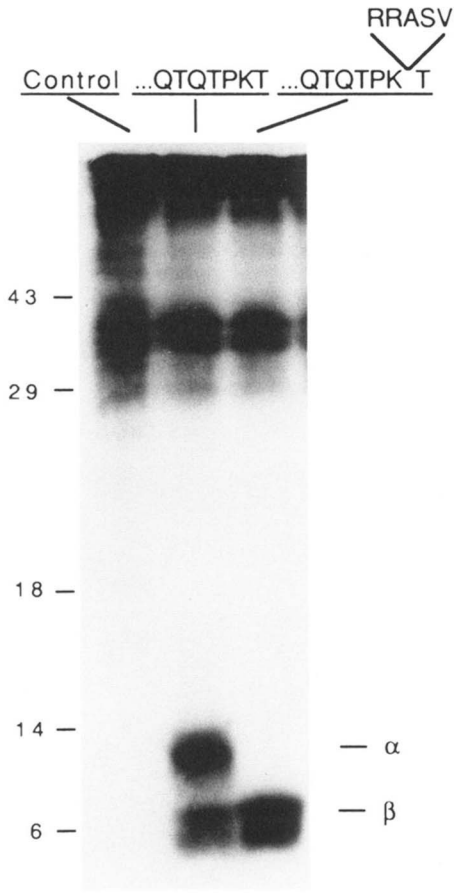

FIG. 3. Using recombinant polymerase chain reaction, nucleotide sequences encoding the amino acid sequence RRASV were introduced near the end of the MCP-1 cDNA coding region as indicated. After transient transfection of indicated expression vectors into COS cells, cells were radiolabeled with [^{35}S]methionine, and conditioned medium was subjected to immune precipitation using anti-MCP-1 antiserum. Immune precipitates were electrophoresed through a 15% polyacrylamide gel. From *left* to *right*: *first lane*, vector alone; *second lane*, wild-type MCP-1; *third lane*, MCP-1 with RRASV insert.

3 in wild-type MCP-1 destroyed the protein's monocyte chemoattractant activity (FX3). Consistent with that finding was the fact that deletion of amino acids 2–8 also yielded an inactive protein. In an attempt to accomplish finer mapping, we changed the 2 charged amino acids in this region (aspartate 3 and asparagine 6) to alanine. Changing aspartate 3 significantly reduced the activity of the protein, whereas changing asparagine 6 yielded a protein that retained 52.9% of wild-type MCP-1's activity.

Next, we constructed four point mutations in the first intercysteine loop. Mutations of arginine 24 to phenylalanine (R24F), tyrosine 28 to aspartate (Y28D), and arginine 30 to leucine (R30L) all produced proteins with activities that were only a fraction of wild-type. However, mutation of another polar amino acid in the same region, namely serine 27 to glutamine (S27Q), produced a protein with 60% of wild-type activity.

Finally, manipulations of the carboxyl-terminal α-helix produced proteins that were still able to signal but had reduced potency compared with wild-type. Deletions of half (D2) or all (D1) of the α-helix yielded proteins with 17 and 11.3% of wild-type activity, respectively. In the predicted model structure of dimeric MCP-1, aspartate 68 projects into the cleft between the two α-helices (3). Mutation of this amino acid to leucine (D68L) had the same effect as deletion of the entire helix.

Competition for Biological Effects—We next tested mutations for their ability to inhibit monocyte chemotaxis *in vitro* in response to nonmutated MCP-1. To a fixed concentration of FX2, we added increasing amounts of medium conditioned by COS cells expressing mutant proteins. We found that Y28D, R24F, 7ND, and D3A demonstrated an ability to inhibit wild-type MCP-1 to varying degrees (data not shown). 7ND appeared to be most potent and was examined in greater detail. Fig. 5 shows that pure 7ND inhibited MCP-1's ability to attract monocytes with an ID_{50} at a molar ratio of 75:1 or a concentration of 35 nM in the presence of 5 ng/ml MCP-1. This inhibition is specific for MCP-1, since 7ND did not inhibit monocyte chemotaxis in response to 10^{-7} M formyl-methionyl-leucyl-phenylalanine (data not shown).

To test 7ND's ability to bind to monocytes, we performed receptor binding assays on human monocytes using ^{125}I-7ND. In self-displacement assays (Fig. 6*A*), 7ND recognized a single class of receptor on human monocytes with a K_d of 2.6 ± 0.23 nM, which is approximately 3-fold lower than the affinity of wild-type MCP-1 for its receptor in similar experiments (Fig. 6*B*), *i.e.* 0.94 ± 0.36 nM. That this receptor is probably the MCP-1 receptor was demonstrated by displacement experiments in which excess unlabeled 7ND was able to displace ^{125}I-MCP-1 from monocytes (Fig. 6*B*). Similarly, excess unlabeled MCP-1 efficiently displaced ^{125}I-7ND (Fig. 6*A*). The derived K_d for ^{125}I-7ND was the same whether cold 7ND or MCP-1 was used for displacement. However, the K_d for ^{125}I-MCP-1 approached that of ^{125}I-7ND when cold 7ND was used for displacement. Interestingly, experiments using ^{125}I-MCP-1 identified 8,145 ± 1430 binding sites/cell (average of five experiments), whereas those using ^{125}I-7ND identified only 840 ± 563 sites/cell (average of six experiments).

Based on the observation of 7ND's ability to inhibit FX2 activity, we tested a peptide comprising the first 10 amino acids of processed MCP-1. This peptide neither had intrinsic monocyte chemoattractant activity, nor did it inhibit the activity of wild-type MCP-1.

DISCUSSION

In the absence of structural data for MCP-1, there is no complete context in which to interpret our results. The arguments that follow rely heavily on the structural modeling work of Gronenborn and Clore (3) and on biochemical and gel filtration results that predict the existence of MCP-1 dimers[2] (23). If the modeling is correct, MCP-1 should have the structure schematized in Fig. 7, in which we have indicated amino acids that were mutated in this study. Of course, this structure requires confirmation by crystal analysis or NMR-derived solution structure.

Our mutations focused on three regions of MCP-1. First, amino acids in the amino-terminal region appear to be absolutely required for MCP-1's monocyte chemoattractant activity. Both 7ND (amino-terminal deletion mutant) and FX3 (amino-terminal insertion mutant) had almost no activity compared with wild-type MCP-1. Furthermore, at least 1 charged amino acid in this region, aspartate 3, is necessary for activity, whereas the only other charged amino acid in this region, asparagine 6, is not. These results are reminiscent of work implicating the amino-terminal region of IL-8, in particular amino acids 4–6 (ELR), in neutrophil activation and receptor binding (7, 8). Although the importance of ELR might have been inferred by its conservation among the neutrophil-active chemokine-α proteins, there is no similar conservation of aspartate 3 or its neighboring amino acids among the monocyte-active chemokine-β proteins. Thus it remains to be determined whether this portion of MCP-1 contains a monocyte chemoattractant motif that would impart similar activity to another protein, as

[2] Y. J. Zhang and B. J. Rollins, manuscript in preparation.

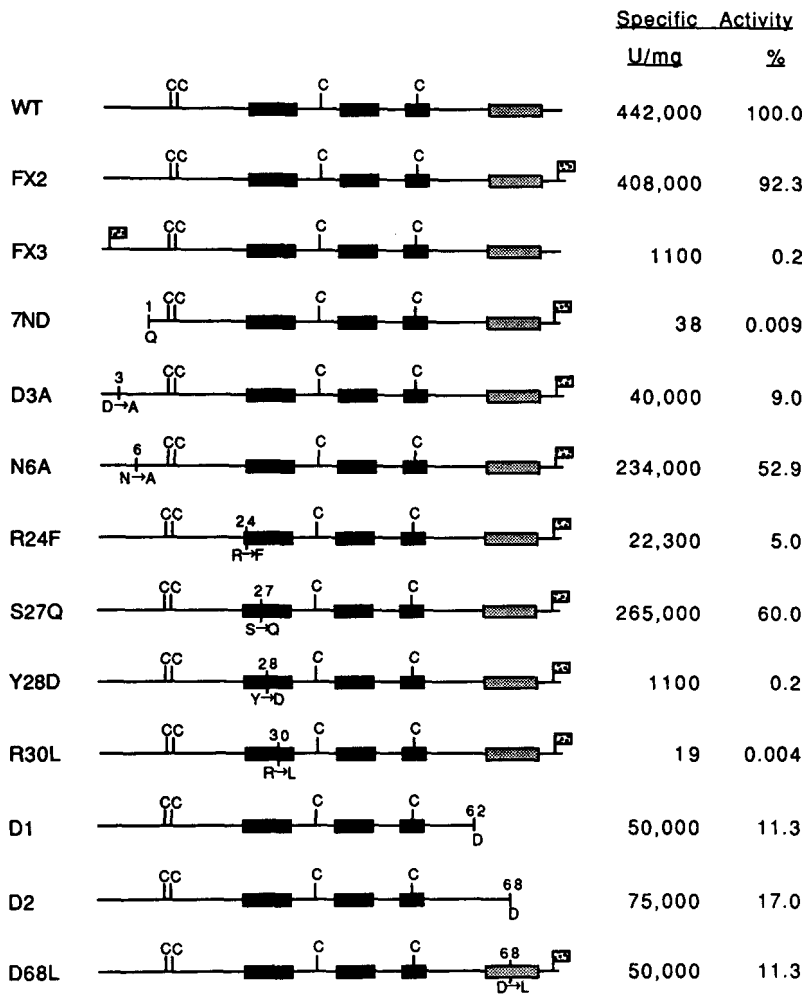

FIG. 4. **Schematic representation of MCP-1 mutations.** *WT* is wild-type, and other designations are described under "Results." Each mutation was expressed by transient transfection of COS cells. The concentration of monocyte chemoattractant activity in the conditioned medium of transfected COS cells was determined by *in vitro* monocyte chemotaxis assay as described under "Materials and Methods." The concentration of MCP-1 or mutated MCP-1 protein in conditioned medium was determined by densitometric analysis of immunoblots. Conditioned medium of COS cells transfected with a control vector contained 15 units/ml (of monocyte chemoattractant activity), whereas conditioned medium of COS cells transfected with vectors encoding FX2 or wild-type MCP-1 contained 400–600 units/ml.

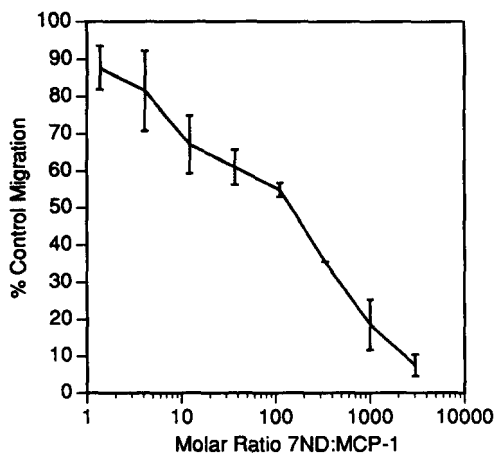

FIG. 5. 7ND was purified as described under "Materials and Methods," and increasing amounts were mixed with 5 ng/ml wild-type MCP-1. Mixtures were then tested in monocyte chemotaxis assays. *Error bars* represent the standard error for two separate wells in a single experiment, and this experiment is representative of two others.

the addition of ELR imparts neutrophil chemoattractant properties to platelet factor 4 (24).

Second, alteration of 2 amino acids in the first intercysteine loop, tyrosine 28 and arginine 30, almost completely destroyed monocyte chemoattractant activity. These results are similar, but not identical, to those of Beall *et al.* who demonstrated that a double tyrosine 28/arginine 30 mutation lost monocyte chemoattractant activity (12). In contrast to their results, our single Arg-30 → Leu (R30L) mutation had no activity, whereas their single Arg-30 → Val mutation retained activity. The disparity may be due to the different substitutions. Regardless of this difference, according to the predicted structural model of MCP-1, the side chains of tyrosine 28 and arginine 30 should point into the interhelical cleft, and loss of monocyte chemoattractant activity in Y28D and R30L is consistent with this region's putative role in receptor binding. Also consistent is the persistently high activity of S27Q, since the side chain of serine 27 is predicted to point away from the cleft (3). Valente *et al.* (25) also indicated the importance of this region in receptor binding by demonstrating that a peptide composed of amino acids 13–35 had chemoattractant activity and competed with MCP-1 for receptor binding. However, these activities required nearly millimolar concentrations of peptide.

Receptor interactions in this region are probably more complicated than this model suggests since mutation of arginine 24, which lies completely outside the interhelical cleft, to phenylalanine drastically reduced monocyte chemoattractant activity. IL-8's corresponding amino acid in this position is also

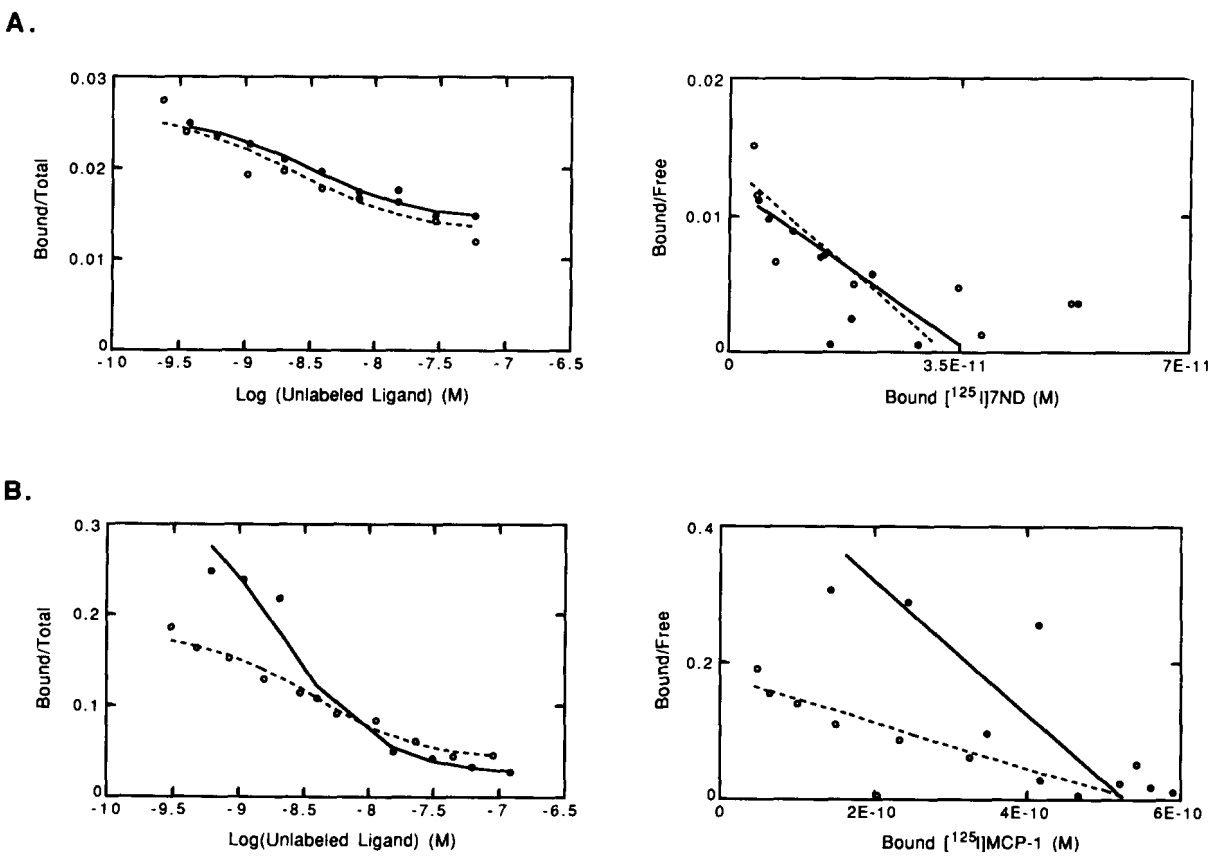

FIG. 6. *Panel A*, ^{125}I-7ND (0.12 nM) was added to 10^7 elutriated monocytes in the presence of increasing amounts of unlabeled MCP-1 (*closed circles, solid lines*) or 7ND (*open circles, dotted lines*). Binding was determined as described (20), and data were analyzed using the Ligand program (29). *Left*, displacement curves; *right*, Scatchard transformation. *Panel B*, ^{125}I-MCP-1 (0.12 nM) was added to 10^7 elutriated monocytes in the presence of increasing amounts of unlabeled FX2 (*closed circles, solid lines*) or 7ND (*open circles, dotted lines*), and binding and data analysis were determined as in *panel A*. *Left*, displacement curves; *right*, Scatchard transformation.

phenylalanine, but phenylalanine is not conserved among other neutrophil-active chemokine-α proteins. In fact, a number of chemokine-β proteins also have phenylalanine in this position. In IL-8, this amino acid is involved in hydrogen bond interactions that stabilize the loop between the second and third β-sheets (6). Although this region is not predicted to be involved in similar interactions in MCP-1, nonetheless alteration of arginine 24 may have disrupted other intrachain stabilization interactions. It is certainly possible that some mutants, such as R24F, may have lost activity because the introduced mutations severely disturbed the three-dimensional structure of the proteins. However, our extensive study of 7ND showed that it bound with high affinity to specific receptors on monocytes (most likely the MCP-1 receptor itself, based on displacement data), suggesting that this mutant, at least, had sufficient conservation of its three-dimensional structure to permit receptor binding.

Third, mutation and deletion in the carboxyl-terminal region of MCP-1 yielded proteins that were still capable of eliciting a monocyte chemotactic response, but with much lower potency. This is similar to carboxyl-terminal truncation variants of IL-8 which also displayed 10–20-fold lower activities (8). In IL-8, some of this reduction may be because the α-helix binds heparin, which enhances the activity of IL-8 (26). Preliminary data suggest that heparin also enhances the activity of MCP-1,[3] and it will be of interest to determine if MCP-1's α-helix is responsible for this enhancement.

Taken together, these results imply that like IL-8 (27) and C5a (28), MCP-1 contacts its receptor at multiple sites. The structurally compact region of C5a is believed to contact the extracellular amino terminus of its receptor, and the more disordered carboxyl terminus is believed to contact regions within the receptor's 7-transmembrane core at a point below the plane of the cell surface (28). It is tempting to ascribe analogous binding functions to domains of MCP-1 identified in the present work, *e.g.* MCP-1's interhelical cleft might bind to extracellular portions of the MCP-1 receptor, whereas the amino terminus might penetrate the receptor's 7-transmembrane core. The properties of mutant 7ND are consistent with this model: 7ND binds to specific receptors on monocytes and displaces wild-type MCP-1 from its receptor, suggesting that 7ND binds to the MCP-1 receptor without activating the signaling cascade. The absence of signaling by 7ND might be due to the absence of contacts with the receptor's 7-transmembrane core which are ordinarily supplied by the amino-terminal region of MCP-1. However, there is currently no direct evidence for such interactions.

Interestingly, some mutations, such as 7ND, R24F, Y28D, and D3A, were able to inhibit wild-type MCP-1's chemoattractant properties. As noted above, 7ND appears to be a competitive inhibitor of MCP-1, perhaps by blocking wild-type MCP-1's amino terminus from interacting with the receptor. However, the number of binding sites/monocyte for ^{125}I-7ND was only 10% of the number of binding sites for ^{125}I-MCP-1, and the K_d for ^{125}I-MCP-1 in the presence of excess cold 7ND was 3-fold lower than its K_d in the presence of excess cold MCP-1 (see Fig. 6). These confusing results might be explained by invoking a model that relies on two assumptions: (i) that MCP-1 forms dimers and that 7ND can bind and inactivate wild-type monomeric subunits, and (ii) that MCP-1 has a similar affinity for

[3] Y. J. Zhang and B. J. Rollins, unpublished observations.

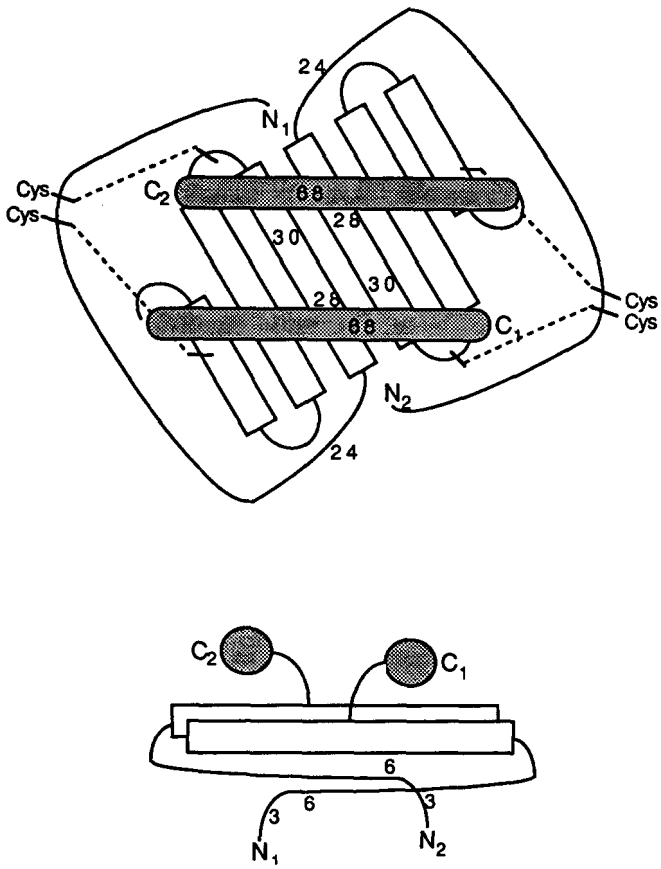

FIG. 7. **Hypothetical structure of MCP-1 dimer, by analogy to IL-8's structure, based on work of Gronenborn and Clore (3).** *Rectangles* indicate β-pleated sheets, *stippled cylinders* indicate α-helices, and *dotted lines* indicate disulfide bonds. Amino termini are depicted as extending below the plane defined by the β-sheets, but their positioning is arbitrary because these regions are disordered in IL-8 structures. Single amino acids substituted in the present study are indicated by their numbered positions.

two distinct receptors, whereas 7ND recognizes only one of these which represents 10% of the total receptor number for MCP-1. Our preliminary data suggest that MCP-1 does form dimers under physiologic conditions and that 7ND can bind to wild-type MCP-1.[2] According to this model, 7ND would efficiently displace ^{125}I-MCP-1 by a combination of mechanisms, namely competition at one set of receptors (10%) and dominant suppression at the other (90%). This model is highly speculative, and experiments are now under way to determine directly if 7ND acts as a dominant suppressor and if there are two classes of MCP-1 receptor. Regardless of their mechanisms of action, these mutants are interesting structures on which to base further work on MCP-1 inhibitors.

Acknowledgments—We acknowledge the technical assistance of Catherine Ernst and Christian Edwards and the secretarial assistance of Bethany Osborne.

REFERENCES

1. Oppenheim, J. J., Zachariae, C. O. C., Mukaida, N., and Matsushima, K. (1991) *Annu. Rev. Immunol.* **9,** 617–648
2. Schall, T. J. (1991) *Cytokine* **3,** 165–183
3. Gronenborn, A. M., and Clore, G. M. (1991) *Protein Eng.* **4,** 263–269
4. Baldwin, E. T., Weber, I. T., St. Charles, R., Xuan, J. C., Appella, E., Yamada, M., Matsushima, K., Edwards, B. F., Clore, G. M., and Gronenborn, A. M. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 502–506
5. Clore, G. M., Appella, E., Yamada, M., Matsushima, K., and Gronenborn, A. M. (1990) *Biochemistry* **29,** 1689–1696
6. Clore, G. M., and Gronenborn, A. M. (1992) *Cytokines* **4,** 18–40
7. Hebert, C. A., Vitangcol, R. V., and Baker, J. B. (1991) *J. Biol. Chem.* **266,** 18989–18994
8. Clark-Lewis, I., Schumacher, S., Baggiolini, M., and Moser, B. (1991) *J. Biol. Chem.* **266,** 23128–23134
9. Leonard, E. J., and Yoshimura, T. (1990) *Immunol. Today* **11,** 97–101
10. Rollins, B. J. (1991) *Cancer Cells* **3,** 517–524
11. Tanaka, S., Robinson, E. A., Yoshimura, T., Matsushima, K., Leonard, E. J., and Appella, E. (1988) *FEBS Lett.* **236,** 467–470
12. Beall, C. J., Mahajan, S., and Kolattukudy, P. E. (1992) *J. Biol. Chem.* **267,** 3455–3459
13. Rollins, B. J., Stier, P., Ernst, T. E., and Wong, G. G. (1989) *Mol. Cell. Biol.* **9,** 4687–4695
14. Prickett, K. S., Amberg, D. C., and Hopp, T. P. (1989) *Biotechniques* **7,** 580–589
15. Wong, G. G., Witek, J. S., Temple, P. A., Wilkens, K. M., Leary, A. C., Luxenberg, D. P., Jones, S. S., Brown, E. L., Kay, R. M., Orr, E. C., Shoemaker, C., Golde, D. W., Kaufman, R. J., Hewick, R. M., Wang, E. A., and Clark, S. C. (1985) *Science* **228,** 810–815
16. Rollins, B. J., Walz, A., and Baggiolini, M. (1991) *Blood* **78,** 1112–1116
17. Falk, W., Goodwin, R. H., Jr., and Leonard, E. J. (1980) *J. Immunol. Methods* **33,** 239–247
18. Kaufman, R. J., Murtha, P., and Davies, M. (1987) *EMBO J.* **6,** 187–193
19. Rollins, B. J., and Sunday, M. E. (1991) *Mol. Cell. Biol.* **11,** 3125–3131
20. Ernst, C. A., Zhang, Y. J., Hancock, P. R., Rutledge, B. J., Corless, C. L., and Rollins, B. J. (1994) *J. Immunol.* **152,** 3541–3549
21. Yoshimura, T., Robinson, E. A., Tanaka, S., Appella, E., and Leonard, E. J. (1989) *J. Immunol.* **142,** 1956–1962
22. Jiang, Y., Valente, A. J., Williamson, M. J., Zhang, L., and Graves, D. T. (1990) *J. Biol. Chem.* **265,** 18318–18321
23. Rollins, B. J., Yoshimura, T., Leonard, E. J., and Pober, J. S. (1990) *Am. J. Pathol.* **136,** 1229–1233
24. Clark-Lewis, I., Dewald, B., Geiser, T., Moser, B., and Baggiolini, M. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 3574–3577
25. Valente, A. J., Rozek, M. M., Schwartz, C. J., and Graves, D. T. (1991) *Biochem. Biophys. Res. Commun.* **176,** 309–314
26. Webb, L. M. C., Ehrengruber, M. U., Clark-Lewis, I., Baggiolini, M., and Rot, A. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 7158–7162
27. Schraufstatter, I. U., Barritt, D. S., Ma, M., Oades, Z. G., and Cochrane, C. G. (1993) *J. Immunol.* **151,** 6418–6428
28. Siciliano, S. J., Rollins, T. E., DeMartino, J., Konteatis, Z., Malkowitz, L., van Riper, G., Bondy, S., Rosen, H., and Springer, M. S. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 1214–1218
29. Munson, P. J. (1992) *MacLigand*, version 4.93. Analytical Biostatistical Section, Division of Computer Research and Technology, National Institutes of Health, Bethesda, MD
30. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254