Monocyte chemoattractant protein-1 (MCP-1) mediates monocyte migration into tissues in inflammatory diseases and atherosclerosis. We have investigated structure–activity relationships for human MCP-1. Mutations were introduced based upon differences between MCP-1 and the structurally related but functionally distinct molecule interleukin-8 (IL-8). Mutant proteins produced using the baculovirus/insect cell expression system were purified and their ability to stimulate monocyte chemotaxis and elevation of intracellular calcium in THP-1 monocytes were measured. Two regions in MCP-1 were identified as important for its biological activity. One region consists of the sequence Thr-Cys-Cys-Tyr (amino acids 10–13). Point mutations of Thr-10 to Arg and Tyr-13 to Ile greatly lowered MCP-1 activity. The second functionally important region is formed by Ser-34 and Lys-35. Insertion of a Pro between these two residues, or their substitution by the sequence Gly-Pro-His, caused nearly complete loss of MCP-1 activity. Competition binding experiments showed that the mutations that affected activity also lowered the ability to compete with wild-type MCP-1 for receptors on THP-1 cells. Point mutations at positions 8, 15, 30, 37, 38 and 68 had little effect on MCP-1 activity. The important regions that we have identified in MCP-1 correspond with previously identified functionally important regions of IL-8, suggesting that the two molecules bind to their respective receptors by similar contacts.

INTRODUCTION

The migration of leucocytes from the circulation into tissues is a key event in many normal and pathophysiological processes. In many cases such migration involves chemotaxis mediated by members of a family of related small proteins known as chemokines. Most chemokines have two cysteine residues that are either adjacent to each other or separated by a single amino acid. These two residues are involved in disulphide bridges with two other cysteines. Chemokines with two adjacent Cys residues are called CC or β chemokines, and those with an amino acid between the two Cys residues are called CXC or α chemokines. Monocyte chemoattractant protein-1 (MCP-1) is a member of the CC chemokine family that attracts and activates monocytes and basophils, whereas interleukin-8 (IL-8), a CXC chemokine, is a chemoattractant for neutrophils that shows no monocyte chemotactic activity. Both MCP-1 and IL-8 exert their effects through seven-transmembrane-segment receptors [1–4]. The three-dimensional structure of MCP-1 has not been published. However, the structures of the CXC chemokines IL-8 [5,6], platelet factor-4 [7] and growth-related protein/melanoma growth-stimulatory activity (GRO/MGSA) [8,9] and the CC chemokines macrophage inflammatory protein-1/β (MIP-1/β) [10] and reduced on activation normal T-cell expressed and secreted (RANTES) [11] have been reported. The structures of monomers of each of these chemokines are fairly similar, consisting of an N-terminal loop and a β sheet with a C-terminal α helix lying across it. Surprisingly, though, the quaternary structures of the three proteins are different. In the IL-8 structure, a continuous β sheet is formed between two monomers to produce a dimer. In platelet factor-4, similar dimers to IL-8 are further packed into tetramers. MIP-1/β and RANTES also form dimers, but along a completely different interface, leading to much more elongated structures. It has been suggested that MCP-1 may resemble MIP-1/β in structure [10], in disagreement with a previously published model for MCP-1 based on IL-8 [12]. However, even though chemokine dimers are found in crystals and in solution at high concentrations, several lines of evidence strongly suggest that at physiological concentrations chemokines are monomers and that monomers have full biological activity [13–15].

The relationships between chemokine structures and their functions have been examined in several studies. IL-8 has been the most extensively studied in this regard. Several studies have shown that mutations or deletions of the three amino acids (Glu-Leu-Arg) at positions 4, 5 and 6 of the 72-amino-acid form of IL-8 cause defects or alterations in biological activity and receptor binding [16–19]. It has been shown that other structural residues are involved in IL-8 receptor binding and activity, including residues Tyr-13, Lys-15, Leu-25, Gly-31, Pro-32 and Leu-49 [20–23]. Positions 4–6 of IL-8 correspond to positions 8–10 of MCP-1, and residues 31 and 32 of IL-8 correspond to residues 34 and 35 of MCP-1.

MCP-1 has not been as extensively characterized as IL-8 up to now. Our earlier work had found that residues 28 and 30 of MCP-1 are involved in the cell-type specificity of MCP-1 [24]. The importance of these residues for monocyte activity was confirmed in another study [25]. Two recent studies [25,26] showed that deletions of residues at the N-terminus of MCP-1 abolished activity. It was found that some N-terminally deleted derivatives were antagonists of MCP-1. We present here a

Abbreviations used: MCP-1, monocyte chemoattractant protein-1; IL-8, interleukin-8; GROα/MGSA, growth-related protein/melanoma growth-stimulatory activity; MIP-1/β, macrophage inflammatory protein-1/β; RANTES, reduced on activation normal T-cell expressed and secreted; HBB, Hepes-containing binding buffer.

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mutational analysis of MCP-1 that identifies two regions of the primary structure that are critical for biological activity of MCP-1. The two regions are found at the base of the extended N-terminal region in the predicted three-dimensional structure.

EXPERIMENTAL

Construction of the expression clones

We used the baculovirus/insect cell expression system to produce wild-type and mutant MCP-1 proteins. A human MCP-1 cDNA clone which had been isolated from an endothelial cell cDNA library [27] was used to generate the expression constructs. We performed a PCR using the human cDNA as the template and primers complementary to the 3' and 5' ends of the MCP-1 coding sequence, that also included BamHI restriction sites (GGGATCCTCAAGTCTTCCGAGTTTT and GGGGATC-CAGCATTGAAAGTCTCCTG). The PCR product was purified, cut with BamHI and subcloned into the pBacPack 1 transfer vector downstream of the baculovirus polyhedrin promoter. We completely sequenced the insert of the clone to confirm its orientation and the absence of mutations. Recombinant baculoviruses were generated with the Bacpack system (Clontech, Palo Alto, CA, U.S.A.) according to the manufacturer's instructions and as described [28]. Plasmid DNA was isolated using Plasmid Select-10 columns (5 prime-3 prime Inc., Boulder, CO, U.S.A.) and co-transfected with Bsu36I-digested BacPack 6 viral DNA into Spodoptera frugiperda Sf21 cells. The supernatants of the co-transfections were used in a plaque assay to isolate recombinant viruses. The virus stocks were then amplified by two to three transfections were used in a plaque assay to isolate recombinant viruses. The medium was spun at low speed to remove the cells, and then centrifuged at 10000 g for 1 h to remove virus particles and debris. Recombinant MCP-1 was purified by a two-step protocol. First the supernatant was directly applied on to a Mono S HR 5/5 column (Pharmacia, Piscataway, NJ, U.S.A.). The bound protein was eluted with a 50 min linear gradient from 0 to 1 M NaCl in 20 mM Mops, pH 6.5. Fractions of 1 ml were collected and assayed by Western blots using anti-MCP-1 antibody. The MCP-1-containing fractions were pooled, and the protein was further purified by gel filtration on a Superdex-75 HR 10/30 column in 0.1 M ammonium acetate. The MCP-1-containing fractions were pooled, lyophilized and stored at −80 °C until use.

Characterization of the recombinant protein

Laser desorption time of flight MS analysis of the purified MCP-1 was carried out by the Bioanalytical Research Facility of the Department of Microbiology, University of Virginia, Charlottesville, VA, U.S.A. Horse heart cytochrome c was used as an internal standard with N,N,N,N'-tetramethyl-2,4,6-triazine-1,3,5-triuronium tetrafluoroborate as the matrix.

For protein sequencing, 5 µg of MCP-1 was run on a 16.5% Tris-Tricine/SDS gel [29], and transferred to Pro-Blott (Applied Biosystems, Foster City, CA, U.S.A.). Asp-N digestion used 5 µg of MCP-1 and 0.2 µg of endoproteinase Asp-N (Boehringer-Mannheim, Indianapolis, IN, U.S.A.) in 0.1 M NaPO₄ (pH 8.0) for 4 h at 37 °C. Peptide sequencing was performed at the Biotechnology Instrumentation Facility of the Department of Biochemistry, University of California at Riverside, CA, U.S.A.

Monocyte and neutrophil chemotaxis

Mononuclear cells and neutrophils were isolated from fresh blood donated by healthy adults. The involvement of human subjects was in accordance with the Declaration of Helsinki, was approved by the O.S.U. Human Subjects Committee, and all subjects gave their informed consent. Mononuclear cells were obtained by centrifugation using Accuspin tubes (Sigma, St. Louis, MO, U.S.A.), and neutrophils were isolated by centrifugation on Polymorphprep (Gibco/BRL, Grand Island, NY, U.S.A.), both by following the manufacturers’ instructions. For chemotaxis measurements, we used a modification of the fluorescence method described by Barker et al. [30]. Briefly, the cells were suspended in Gey’s balanced salt solution containing 0.2% BSA (Gey’s-BSA) at a concentration of 1 × 10⁶ cells/ml and labelled with 2 µM calcium acetoxymethyl ester (Molecular Probes, Eugene, OR, U.S.A.) at 37 °C for 30 min. The labelled cells were washed with PBS and resuspended in Gey’s-BSA at 1 × 10⁶ cells/ml. Variable concentrations of the proteins were placed in a 96-well Polystere viewplate inside a 96-well chamber (Neuroprobe, Cabin John, MD, U.S.A.). The plate was overlaid with a polycarbonate polycyndro-free framed filter of either 3 µm (for neutrophils) or 5 µm (for monocytes) pore size. Cell samples of 200 µl were added to the top wells of the chamber. The chamber was incubated for 30 min for neutrophils and 90 min for monocytes at 37 °C in humidified air with 5% CO₂. After the incubation the membrane was removed and the non-migrating cells from the top of the filter were wiped off. The filter was air-dried and the number of cells migrating was measured by their fluorescence in a CytoFluor 2300 plate reader.
Measurement of intracellular Ca\(^{2+}\) concentration

Changes in the intracellular Ca\(^{2+}\) concentration were monitored using THP-1 cells labelled with the fluorescent probe fura-2 as described [31]. Briefly, THP-1 cells were washed in PBS and resuspended in Hepes-Tyrode buffer (129 mM NaCl, 8.9 mM NaHCO\(_3\), 2.8 mM KCl, 0.8 mM KH\(_2\)PO\(_4\), 5.6 mM dextrose, 0.8 mM MgCl\(_2\), 10 mM Hepes, pH 7.4). The cells (10\(^5\)/ml) were incubated with 2 \(\mu\)M fura-2 acetoxymethyl ester (Molecular Probes) at 37 °C for 30 min. After labelling, the cells were washed twice with PBS and resuspended in Hepes-Tyrode buffer in the presence of 1 mM CaCl\(_2\). Fluorescence was measured at 5 \(\times\) 10\(^5\) cells/ml in a Perkin-Elmer LS-3B fluorescence spectrophotometer (Perkin-Elmer Corp., Norwalk, NJ, U.S.A.) with constant stirring. The samples were excited at 340 nm and emission was recorded at 500 nm. The saturation fluorescence was measured after treating the cells with 50 \(\mu\)M digitonin.

Iodination of MCP-1

MCP-1 was iodinated by a modification of the chloramine-\(\tau\) method described by Hunter and Greenwood [32]. To 1.5 \(\mu\)g of recombinant protein and 250 \(\mu\)Ci of Na\(^{131}\)I (Dupont-NEN, Boston, MA, U.S.A.) in 100 \(\mu\)l of 0.1 M sodium phosphate buffer (pH 7.2) was added 26.4 \(\mu\)g of chloramine-\(\tau\); the mixture was then left at room temperature for 30 s and the reaction was stopped by addition of 100 \(\mu\)g of sodium metabisulphite. Iodinated protein was separated from free iodine on a 1.5 ml QAE-Sephadex column using an elution solution of 5 \(\%\) sucrose and 0.25 \(\%\) BSA. The estimated specific radioactivity of MCP-1 was 40–90 Ci/\(\mu\)g. The labelled protein fraction contained less than 3 \(\%\) free iodine as assessed by TLC on Silica G plates using 0.9 \(\%\) NaCl as the developing solvent.

MCP-1 binding assay

MCP-1 binding to THP-1 cells was performed essentially as described by Van Riper et al. [33]. Exponentially growing THP-1 cells were washed twice at room temperature with Hepes binding buffer (HBB) composed of 50 mM Hepes (pH 7.2), 1 mM CaCl\(_2\), 5 mM MgCl\(_2\) and 0.5 \(\%\) BSA. The final assay volume was 250 \(\mu\)l of HBB and contained 5 \(\times\) 10\(^6\) cells, 0.02 pmol of \(^{131}\)I-MCP-1 and various amounts of unlabelled wild-type or mutant MCP-1. Following incubation at room temperature for 2 h, 1 ml of HBB containing 0.5 M NaCl was added and the mixture was immediately passed through Whatman GFC filters (pre-wetted with 0.03 \(\%\), polyethyleneimine). The filters were then washed with 2 ml of HBB containing 0.5 M NaCl and radioactivity was measured in a Packard COBRA \(\gamma\)-radiation counter. Binding data were analysed and \(K_d\) values determined using the LIGAND program [34].

RESULTS

In the present study we have expressed wild-type MCP-1 and mutant forms in the baculovirus/insect cell system. We made a construct by putting the MCP-1 coding region under the control of the polyhedrin promoter, and produced recombinant virus. Immunodetectable MCP-1 was secreted into the medium of infected cells. The level of expression was about 5–10 \(\mu\)g of protein per ml of culture, according to immunoblot measurements (results not shown). We utilized a two-step method of purification for the recombinant protein. Typical elution profiles for the two chromatographic steps employed are shown in Figure 1.

SDS/PAGE of the MCP-1-containing fractions from the Superdex 75 column revealed a major band that co-migrated with recombinant MCP-1 isolated from E. coli, and sometimes one or two weak bands that migrated slightly more slowly (Figure 2). The basis of this heterogeneity was examined by laser desorption MS (results not shown). This technique gave a major peak with a molecular mass of 8664 Da, very close to the calculated molecular mass of 8658 Da for MCP-1 (with an N-terminal pyroglutamic acid as is found in the natural protein [35]). On the mass spectrum we detected two minor forms at slightly higher molecular mass (about 8850 Da and 9050 Da on MS). We assume that these minor forms are the same as the minor forms observed on SDS/PAGE, because we did not see any other peaks by MS. We showed that the minor forms are due to modifications of MCP-1 as follows. We performed MS on a purified mutant protein (threonine-10 to arginine) and found that the major peak had shifted to a higher mass of 8725 Da (compared with 8713 Da calculated). Significantly, the mutant sample had minor peaks that were shifted to higher molecular mass by the same amount as the major peak, showing that the minor peaks must represent modified forms of MCP-1. Another study of MCP-1 expression using a baculovirus [36] found
and the stimulation of the elevation of intracellular Ca

similar heterogeneity in the expressed protein, which was shown to be probably due to O-linked glycosylation. The differences in mass that we observed for the minor products could correspond to the addition of one or two hexose residues.

MCP-1 produced by insect cells was originally reported to be missing the N-terminal 6 amino acids compared with native human MCP-1 [36]. We performed N-terminal sequencing of the purified protein, but obtained less than 1 pmol yield from 5 µg of protein, indicating a blocked N-terminus. We then digested our protein with endoproteinase Asp-N, which cleaves peptides N-terminal to aspartate residues. We electrophoresed the protein, transferred it and sequenced it. The sequence derived was Asp-Ala-Ile-Asn-Ala-Asp, with the expected yield of 290 pmol. This sequence corresponds to a product that would be derived by cleavage between Pro-2 and Asp-3 in the mature MCP-1 sequence. This result and the results of MS show that our recombinant protein did not have a cleaved N-terminus, as does the natural protein. Another recent study also reported the isolation of intact MCP-1 from insect cells [37]. The MCP-1 in the earlier report may have been subject to proteolysis at some stage in its isolation. We found that the biological activity of our recombinant material was indistinguishable from that of commercial MCP-1 in both the induction of monocyte chemotaxis and the stimulation of the elevation of intracellular Ca\(^{2+}\) in THP-1 cells.

To identify residues of MCP-1 that are important to its biological activity, we produced two classes of mutations. One class comprised point mutations in amino acids that are conserved differently between MCP-1 and IL-8 from different species, and were predicted to be solvent-exposed by a structural model [12]. We mutated each residue in MCP-1 to the corresponding residue in IL-8. The following mutations were made: proline-8 to glutamic acid (P8E), threonine-10 to arginine (T10R), tyrosine-13 to isoleucine (Y13I), phenylalanine-15 to threonine (F15T), arginine-30 to valine (R30V), proline-37 to alanine (P37A), lysine-38 to asparagine (K38N) and aspartic acid-68 to leucine (D68L).

We also carried out mutations of tyrosine-28 to leucine, and the double mutation of tyrosine-28 to leucine and arginine-30 to valine. The latter mutant protein was previously made in E. coli [24]. However, we found that these two mutant proteins were retained in the cell pellet rather than being secreted into the medium, and appeared to be only partially processed based upon the appearance of higher-molecular-mass immunoreactive species (Figure 3). Because these mutations interfered with processing and secretion by the insect cells, we were unable to purify enough of these proteins for analysis.

In a further attempt to construct MCP-1 mutants that incorporate structural features found in IL-8, we made an insertion mutant of proline between serine-34 and lysine-35 (34P35). This is a position where an insertion is found in IL-8 relative to MCP-1 [12]. We also made a protein with replacement of residues 34 and 35 with the three residues glycine-proline-histidine (34,35[GPH]), a combined insertion and double substitution. These also were expressed and purified (Figure 2).

We measured the biological activity of the mutant MCP-1 forms by two assays: monocyte chemotaxis and elevation of intracellular calcium in THP-1 cells. The effects of point mutations on chemotaxis are shown in Figures 4(A) and 4(C). In these experiments, wild-type MCP-1 showed the most stimulation of chemotaxis at a concentration of 10\(^{-8}\) M; chemotactic activity decreased at 10\(^{-9}\) and 10\(^{-10}\) M. Mutation of Thr-10 to Arg caused a decrease of 50% in chemotaxis at 10\(^{-9}\) M, while the Y13I mutant showed a decrease of 70% in chemotaxis at 10\(^{-9}\) M. Although the potency of the mutants is difficult to estimate accurately, it appears that the Thr-10 mutation decreased the chemotactic activity about 10-fold, while it was decreased by as
Functionally important regions of monocyte chemoattractant protein-1

Figure 4  Bioactivity measurements of MCP-1 wild-type and mutant forms

Panels (A), (C) and (E) present monocyte chemotactic assays. Fluorescently labelled monocytes were assayed in a chemotaxis chamber with the indicated concentrations of ligands. Fluorescence was normalized to a percentage value. Panels (B), (D) and (F) present internal calcium measurements on THP-1 cells performed as described in the text. (A) and (B) show results for substitution mutations of residues 8, 10, 13 and 15. (C) and (D) show results for substitution mutations of residues 30, 37, 38 and 68. (E) and (F) show results for the insertion mutation and the combined insertion/double substitution. Note that the chemotaxis values for the 34P35 mutant were less than 1% at every concentration and hence cannot be seen on the graph. The error bars show the S.E.M. from multiple measurements, carried out on at least two cell preparations.

much as 100-fold in Y13I. The proteins containing the other point mutations showed relatively high activities, greater than 50% of wild type. Intracellular calcium measurements (Figures 4B and 4D) basically mirrored the chemotaxis measurements. Both T10R and Y13I had decreased activity, with Y13I being lower, while the other single substitution mutants showed substantial activity.

Both the insertion mutant 34P35 and the combined insertion/substitution mutant 34,35[GPH] caused chemotactic activity to be basically absent, with calcium elevation activity detected only at the highest concentrations (Figures 4E and 4F). In order to more accurately determine the reduction in potency caused by these mutations, we performed internal calcium measurements with a broader range of concentrations of the two mutants (Figure 5). The highest stimulation that was obtained with the wild-type MCP-1 gave a peak fluorescence that was 38% of the saturation fluorescence caused by digitonin. We found that both of the mutant proteins could stimulate to this level when sufficiently high protein concentrations were used. A comparison of the dose–response curves reveals that the 34P35 mutant decreased the potency in this assay by about 100-fold, while the 34,35[GPH] mutant decreased it by about 50-fold.

Because the mutations that we had made introduced amino acids found in the corresponding positions of the neutrophil
Figure 5  Elevation of internal calcium in THP-1 cells stimulated by wild-type (wt) MCP-1 and 34–35 region mutants

Fura-2-loaded THP-1 cells were stimulated with the indicated concentrations of ligand and the responses were recorded. The peak responses were quantified as percentages of the fluorescence measured following digitonin treatment (saturation value). The error bars show the S.D.s of multiple measurements carried out on at least two cell preparations.

We next examined the ability of the mutant proteins to compete with MCP-1 for binding to receptors on THP-1 cells. These cells were used because they were known from previous studies to express MCP-1 receptors [33,38]. We tested as competitors the wild-type protein, the R30V mutant (which showed activity comparable with that of the wild type) and all mutant proteins that had lowered activity in the two functional assays. We initially fitted the data for the wild-type self-competition with a single-site model, which gave an average \(K_D\) of 1.0 nM. The binding curves for each mutant were fitted with a single-site binding model that used the wild-type association constant as a fixed parameter. Representative data points and curves are shown in Figure 6 and the results of multiple experiments are summarized in Table 1. The R30V mutation, which had no effect on chemotaxis and \(Ca^{2+}\) elevation assays, competed with a \(K_D\) of 1.6 nM. The two substitution mutants that showed lowered activity, T10R and Y13I, had higher \(K_D\)s of 8.8 nM and 16.5 nM respectively. The results for these four mutants agreed well with the biological activity measurements, in that the rank order of both biological activity and binding affinity was found to be: R30V > T10R > Y13I. However, the insertion mutant 34P35 and the insertion/substitution mutant 34,35[GPH] did not fit well into this pattern. The average \(K_D\)s (19.3 nM and 8.8 nM respectively) for these two mutant proteins were in the same range as for the T10R and Y13I substitutions, despite the insertion mutations’ drastically lower biological activity. An additional notable point is that the computer-generated models for binding of T10R, Y13I, 34P35 and 34,35[GPH] consistently predicted lower receptor numbers than competition experiments with wild-type MCP-1 or R30V (Table 1).

Table 1  Binding competition studies

<table>
<thead>
<tr>
<th>Competing ligand</th>
<th>Mean (K_D) (nM)</th>
<th>Range of (K_D) values (nM)</th>
<th>Range of receptor no./cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCP-1 wild type</td>
<td>1.0 (6)</td>
<td>0.8–1.4</td>
<td>6300–9300 (3)</td>
</tr>
<tr>
<td>R30V</td>
<td>1.6 (2)</td>
<td>1.6–1.7</td>
<td>7200–9000 (2)</td>
</tr>
<tr>
<td>T10R</td>
<td>8.8 (4)</td>
<td>3.1–21</td>
<td>3900 (1)</td>
</tr>
<tr>
<td>Y13I</td>
<td>16 (3)</td>
<td>6.5–29</td>
<td>2800–3600 (3)</td>
</tr>
<tr>
<td>34P35</td>
<td>19 (3)</td>
<td>7.9–41</td>
<td>2600–3300 (3)</td>
</tr>
<tr>
<td>34,35[GPH]</td>
<td>8.8 (6)</td>
<td>4.1–17</td>
<td>3300–4200 (3)</td>
</tr>
</tbody>
</table>

Because the 34–35 region mutations appeared to affect the...
biological activity of MCP-1 much more than they affected receptor binding, we attempted to determine directly whether these mutants could have antagonistic effects on MCP-1 function. Mixtures of the 34P35 and 34,35[GPH] mutant proteins with wild-type MCP-1 were made and used in the calcium elevation and chemotaxis assays. We only observed small reductions of activity, averaging less than 20%, with a number of different wild-type and mutant mixtures (results not shown).

DISCUSSION

The results presented here identify two regions of the MCP-1 molecule that may be involved in MCP-1 receptor binding. The first region consists of the sequence from Thr-10 to Tyr-13. We found that mutation of either residue 10 or 13 caused a decrease in MCP-1 activity. This result agrees with the finding that mutations of the corresponding two residues in IL-8 cause large decreases in activity of this chemokine [16,17,21]. The findings that both chemokines have functionally important residues adjacent on either side of the first two cysteine residues suggest that the insertion that distinguishes the CC from the CXC chemokine subfamily may have functional significance. It is possible that the cysteines either are part of the receptor binding surface or somehow control the conformation relevant to receptor binding. It is noteworthy that a recently discovered novel chemokine, lymphotactin, has only one cysteine residue in this region [39]. Additional mutations could test some of these possibilities.

The second region that appears to be important consists of residues 34 and 35 of the MCP-1 sequence. This conclusion is based upon the results with two mutations, one introducing a proline between Ser-34 and Lys-35, and the other a replacement of those two residues with the sequence Gly-Pro-His. Either mutation caused very severe decreases in MCP-1 activity. This finding again agrees with a study on IL-8, which indicated a functional role for the corresponding Gly-Pro sequence [21]. However, there is the possible caveat in our study, in that the insertion of a residue might cause conformational changes outside the mutated region, which could be leading to the observed loss of activity. The insertions that we made were designed to mimic the IL-8 structure. We are now in the process of producing amino acid substitutions at the two positions. Resolution of these questions will require structural analysis of the wild-type and mutant proteins.

We have examined the location of the two regions in a three-dimensional model of MCP-1 (Figure 7). We constructed a homology model of an MCP-1 monomer based on the published structures of IL-8 [5], GRO/MGSA [8] and MIP-1β [10]. The final structure was very close to that of MIP-1β, as determined by visual comparison of the two backbones. This is expected because MCP-1 is most closely related to MIP-1β of the three proteins. We show the predicted location of residues 10, 13 and 34–35 on the solvent-accessible surface of the monomer. We also find that the two regions are located at one end of the monomer, near the base of the extended rod formed by the N-terminus.

The 34P35 and 34,35[GPH] mutants showed interesting behaviour in that they competed for binding of the wild-type MCP-1 to THP-1 cells 8.8–19-fold less well than the wild-type, while their biological activity was 50–100-fold lower. Despite these differences, the two proteins did not act as effective antagonists in mixing experiments. One possible explanation is that binding parameters other than affinity, such as kinetic factors, may influence the observed biological activity. Another possibility is that the two mutant proteins bind most avidly to certain subclasses of MCP-1 receptor, and that stimulation of these subclasses is insufficient to cause chemotaxis or elevation of intracellular calcium. This possibility is consistent with observations that multiple subclasses of CC chemokine receptor exist [1,33,40], and that the two mutant proteins apparently competed for smaller numbers of receptors per cell than the wild-type or R30V mutant proteins. The existence of the two recently cloned MCP-1 receptors [1,41] may allow future testing of these possibilities.

The role of the region around residues tyrosine-28 and arginine-30 in MCP-1–receptor binding is somewhat unclear. Our previous results obtained with protein produced in E. coli showed that mutation of these two residues, to leucine and valine respectively, caused the partial loss of monocyte chemotactic activity and a gain of neutrophil chemotactic activity [24]. A recent report showed that mutation of the corresponding residue of IL-8 gave it CC-chemokine-like activity [23]. Other workers also found that mutation of residue 28 eliminated MCP-1 activity [25]. Our present finding is that either the double mutation or a single mutation of residue 28 blocked secretion of the protein from insect cells and apparently even affected processing of the signal peptide. These observations strongly suggest that tyrosine-28 has a role in maintaining the normal conformation of the protein that is required for processing and secretion. Therefore, although the mutant protein produced in E. coli used in the earlier study was expressed intracellularly, it may have had some sort of global alteration in its three-dimensional structure. If this were the case, it could argue against the simple interpretation of our earlier result that residue 28 is involved in a direct receptor contact. Instead, a more drastic change in three-dimensional structure could have given rise to the effects we observed on neutrophils. These possibilities may be further clarified by studying the biological activities of proteins containing other mutations of residue 28 that might allow secretion of the protein in a normal conformation.

We found that mutations of arginine-30 to valine and aspartate-68 to leucine had little measurable effect on MCP-1 activity. According to the model of MCP-1 based upon the IL-8 structure [12], arginine-30 was predicted to lie alongside tyrosine-28 in a groove between the two α helices, while aspartate-68 extended from the helix above the groove. Based on the
hypothesis that the groove might be central to receptor binding, we had earlier speculated that aspartate-68 might interact with the MCP-1 receptor [24]. With the more recent evidence that a monomer is likely to be the active form, such a groove could not be involved as the receptor binding site, and this is consistent with our present observation that the aspartate-68 mutation had little or no effect on MCP-1 activity. Our finding that mutation of either arginine-30 or aspartate-68 had little effect is slightly at variance with the results of Zhang et al. [25]. These workers found that mutation of arginine-30 to leucine nearly eliminated activity, while mutation of aspartate-68 to leucine reduced activity. These differences may be due to differences in the mutated residue in the case of residue 30, or to differences in expression systems or bioassays.

In summary, we have identified two regions in MCP-1 that are important for the function of this cytokine. Our observations should help in future efforts to modulate MCP-1 action in inflammatory disorders.

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