Antagonists of Monocyte Chemoattractant Protein 1 Identified by Modification of Functionally Critical NH₂-terminal Residues

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Summary

Monocyte chemoattractant protein (MCP)-1 analogues were designed to determine the role of the NH₂-terminal region in structure and function. The NH₂-terminal residue was important for function and receptor binding, as it could not be deleted or extended. However the NH₂-terminal pyroglutamate residue of the wild type was not essential as it could be replaced by several other noncyclic amino acids without loss of activity. Residues 7–10 were essential for receptor desensitization, but were not sufficient for function, and the integrity of residues 1–6 were required for functional activity. A peptide corresponding to MCP-1, 1-10 lacked detectable receptor-binding activities, indicating that residues 1–10 are essential for MCP-1 function, but that other residues are also involved. Several truncated analogues, including 8-76, 9-76, and 10-76, desensitized MCP-1–induced Ca^{2+} induction, but were not significantly active. These analogues were antagonists of MCP-1 activity with the most potent being the 9-76 analogue (IC_{50} = 20 nM). The 9-76 specifically bound to MCP-1 receptors with a K_d of 8.3 nM, which was three-fold higher than MCP-1 (K_d 2.8 nM). The 9-76 analogue desensitized the Ca^{2+} response to MCP-1 and MCP-3, but not to other CC chemokines, suggesting that it is MCP receptor specific. The availability of these compounds will be helpful in evaluating MCP receptor antagonists as anti-inflammatory therapeutics.

Monocyte chemoattractant protein (MCP)[1]-1 is a member of the chemotactic cytokine (chemokine) superfamily of inflammatory mediators (1, 2). The human chemokines can be divided into two families based on sequence similarity: the CC family, which includes MCP-1, for which the first two cysteines are adjacent; and the CXC family, e.g., IL-8, for which the first two cysteines are separated by one residue (1, 2). Besides MCP-1 (3), the human CC family also includes: MCP-2 (4) (also termed HC14) (5); MCP-3 (6); a protein that is regulated on activation, and normal T cell expressed and secreted (RANTES) (7); macrophage inhibitory protein (MIP)-1α (8) (first identified in humans as LD78) (9); MIP-1β (8) (human ACT-2) (10); and I-309 (11). Whereas all these CC chemokines have been described as monocyte chemoattractants with varying potencies (4, 12, 13, 14), they diverge in their functional activities on other cell types, such as basophils (15), eosinophils (15, 16), T lymphocytes (17), and hemopoietic cells (18). The receptor interactions of the

CC chemokines are also complex. Two receptors have been sequenced: one for MIP-1α, which also cross-reacts with RANTES (19–21), and recently a receptor for MCP-1 (22). However, binding and cross-desensitization studies suggest that additional receptors also exist for RANTES (23) (which may cross-react with MCP-3) (15), and a promiscuous receptor that binds MCP-1, MIP-1α, and RANTES (23). Cellular expression and functional activities that are mediated by the various receptors are still unclear.

MCP-1 has been implicated in a number of allergic and chronic inflammatory diseases (24), such as arthritis (25, 26), arteriosclerosis (27, 28), and various lung diseases (24, 29). In these conditions, monocyte infiltration may be a key early event in disease progression. Experimentally, MCP-1–neutralizing antibodies inhibited immune complex alveolitis in the rat (29). Receptor antagonists for MCP-1 and other chemokine receptors are an alternative approach to blocking MCP-1 actions and could potentially provide novel anti-inflammatory therapeutics. Development of such molecules requires knowledge of the residues that are involved in binding as well as those required for receptor functions. However, as yet there is little information regarding the structural requirements for function of MCP-1 or other CC chemokines. In this study we examine structure–activity relationships of MCP-1 focusing particularly on the NH₂-terminal region which, by analogy

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[1] Abbreviations used in this paper: Aba, α aminobutyric acid; Ac, acetyl; nVal, norvaline; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; RANTES, regulated on activation, normal T cell expressed and secreted.

with IL-8 (30–32), is a candidate receptor binding determinant. The results demonstrate the importance of the integrity of residues 1–10, for MCP-1 receptor binding and activity. Furthermore, function and receptor binding is dissociated in several truncated analogues (8-76, 9-76, 10-76, and 11-76), which are receptor antagonists.

Materials and Methods

Synthetic Proteins. The human chemokines and their analogues were synthesized using tertiary N^α-butyloxycarbonyl amino acid chemistry on an automated peptide synthesizer (model 430A; Applied Biosystems, Foster City, CA) using methods described in detail elsewhere (33). After hydrogen fluoride deprotection, the polypeptides were folded using air oxidation and purified by reverse phase HPLC as described (33). The molecular weights of the synthetic proteins were determined by electrospray mass spectrometry (AP-III, PE-SCIEX; Thornhill, ON) (33). Acetylation of the NH_2 terminus was carried out immediately before hydrogen fluoride deprotection using acetic anhydride (10%) in dimethylformamide. Chemokines and peptides with NH_2-terminal glutamine were converted to pyroglutamate by treatment for three days with 1% acetic acid in H_2O. Conversion was readily monitored because of the longer HPLC retention time of the pyroglutamate form and confirmed by the 17-Da difference in molecular mass.

Cell Preparations. Human monocytes were isolated from buffy coats of normal donor blood. The cell suspension was loaded on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) and centrifuged at 400 g for 25 min, followed by density centrifugation on a discontinuous Percoll (Pharmacia) gradient at 500 g for 30 min. Cells with a density of 1.051–1.053 (g/ml) were >70% monocytes by morphology and were used for the chemotaxis assay. The monocytic cell line THP-1 was obtained from the American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 medium supplemented with 10% FCS.

Chemotaxis. Cell migration was assayed using 48-well microchemotaxis chambers (Neuroprobe, Cabin John, MD). Peptides were dissolved in RPMI containing 0.5 mg/ml BSA, diluted in the same medium and 26-μl aliquots were added to the lower chamber. A 5-μm pore size polycarbonate filter membrane (Nucleopore, Pleasanton, CA) was sandwiched between the lower and upper chamber. Cells, 50 μl of 10^7/ml suspension, were added to the upper chamber. After incubation for 2 h at 37°C in 5% CO_2 in air, the filter was removed, fixed, and stained with Canco Quik Stain II (Baxter, McGaw Park, IL). The migrated cells were counted and the chemotactic index determined as the ratio of the migrated cells in the presence of sample, to the control migration in the absence of sample.

Analysis of Cytosolic-free Calcium. THP-1 cells (4 × 10^5) were loaded with 12.5 μg/ml Fluo-3AM in PBS saline with 0.38 mg/ml Pluronic F127 (Molecular Probes, Eugene, OR) at 37°C for 30 min. After washing with PBS, the cells were resuspended in 25 mM Hepes, 140 mM NaCl, 10 mM glucose, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 3 mM KCl, pH 7.3. The fluorescence was monitored at 7-s intervals over 150 s, after addition of test sample. Maximum Ca^{2+} levels were established using Fluo-3AM (designated 100% saturation) for each set of measurements by addition of 5 μM Ionomycin (Sigma Chemical Co., St. Louis, MO). Ca^{2+} desensitization was performed by addition of one ligand, and then after 150 s the cells were treated again with either the same or a different ligand.

Receptor Binding. MCP-1 (10 ug) was labeled with monoiodinated Bolton-Hunter reagent (specific activity 2,200 Ci/mmol; DuPont, Wilmington, DE) at 4°C for 30 min. The specific activity of ^{125}I-labeled MCP-1 was 130 Ci/mmol. To determine the binding kinetics, THP-1 cells (5 × 10^6) in 200 ul of binding buffer (RPMI 1640, 0.5 mg/ml BSA, 50 mM Hepes, and 0.1% NaN_3) were incubated with varying concentrations of ^{125}I-MCP-1 at 4°C for 30 min. The cells were pelleted through a mixture of diacetylphthalate and dibutylphthalate and radioactivity that was cell associated was counted (total binding). Nonspecific binding was determined in the presence of a 100-fold concentration of unlabeled ligand and was subtracted from the total binding. Kinetic parameters (K_d and receptor number) were determined by Scatchard analysis. The competition assay was performed with 4 nM of ^{125}I-MCP-1 in the presence or absence of varying concentrations of unlabeled ligand as described above.
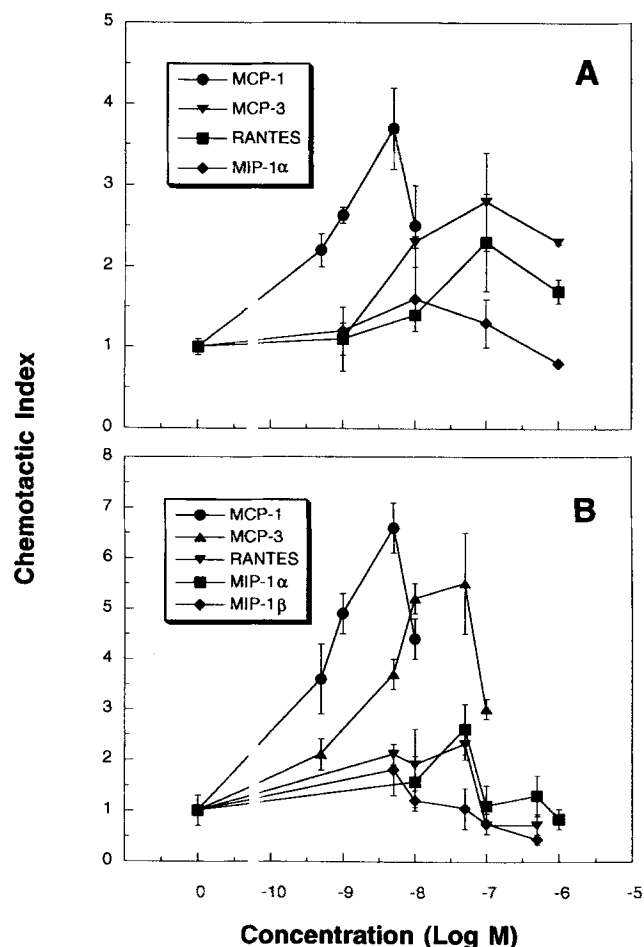

Figure 1. Chemotactic activity of CC chemokines. Migration of monocytes (*A*) and THP-1 cells (*B*) at the indicated chemokine concentrations are shown as the mean ± SD of triplicate determinations. All the chemokines had significant activity and MCP-1 was significantly different from the others (p <0.05). Results presented are representative of three experiments.

Results

Synthesis and Characterization of Analogues. CC chemokines were synthesized according to the primary structures of the secreted forms. These were MCP-1 (34), MCP-3 (4), MIP-1α (35), MIP-1β (10), and RANTES (7). The average measured synthetic yields were 99.3% per residue. Overall yields of pure folded protein were 20–50 mg. The synthetic products, including all of the MCP-1 analogues, were found to fold spontaneously as indicated by the absence of free thiols and the characteristic shift in HPLC retention time that is observed on folding of chemokines (33, 36). The 11-76 analogue was acetylated as other studies had indicated that this was required for folding of analogues with NH_2-terminal cysteine (30). For MCP-1, it was found that this was not required, and 11-76 and Ac 11-76 were equivalent. The full-length MCP-1 and MCP-3 proteins were converted to the NH_2-terminal pyroglutamate form before folding and this form was then isolated as a homogenous product. The products eluted as a single peak on reverse phase HPLC and gave a single band on analytical isoelectric focusing (30, 33, data not shown). The measured average molecular masses of the proteins were within experimental error (0.9 amu average) of the mass calculated from their target folded covalent structures (not shown).

Comparison of CC Chemokine Activities. The chemotactic activities of the synthetic CC chemokines were compared using peripheral blood monocytes and THP-1 cells as targets

(Fig. 1). MCP-1 consistently gave the highest level of migration and was the most potent inducer with both cell sources. The related chemokine MCP-3 (71% identity to MCP-1) also gave significant chemotaxis, but was less potent. MCP-3 was around 10-fold less potent than MCP-1, but was active over the same concentration range. RANTES, MIP-1α, and MIP-1β had detectable but lower activity than MCP-1 or MCP-3. In general, results with THP-1 cells were found to parallel findings with monocytes and thus THP-1 cells were used in subsequent assays.

Role of the NH_2-terminal Residue. The natural forms of MCP-1 and MCP-3 are blocked at the NH_2 terminus (4, 34). This is because after removal of the signal peptide, the NH_2-terminal glutamine spontaneously converts to pyroglutamate (12) (Fig. 2). To test the role of the pyroglutamate in determining function, nine MCP-1 analogues varying in the NH_2-terminal residue were synthesized according to the sequences shown in Fig. 2. To prevent pyroglutamate formation, we acetylated the NH_2-terminus to form the MCP-1 (Ac) analogue, and found that the chemotactic potency was 300-fold reduced compared to the pyroglutamate form (Fig. 3 A). Addition of alanine, which corresponds to the last residue of the signal peptide (MCP, Ala^{-1}, Fig. 2), or deletion of the NH_2-terminal pyroglutamate (2-76 analogue) resulted a similar loss of activity.

Despite these results, replacement of the NH_2-terminal

		1	2	3	4	5	6	7	8	9	10	11	12
MCP-1		<Glu	Pro	Asp	Ala	Ile	Asn	Ala	Pro	Val	Thr	Cys	Cys
MCP-1 (Ac)		AcGln	Pro	Asp	Ala	Ile	Asn	Ala	Pro	Val	Thr	Cys	Cys
MCP-1 (Ala^{-1})	Ala	Gln	Pro	Asp	Ala	Ile	Asn	Ala	Pro	Val	Thr	Cys	Cys
MCP-1 (Asn^1)		Asn	Pro	Asp	Ala	Ile	Asn	Ala	Pro	Val	Thr	Cys	Cys
MCP-1 (AcAsn^1)		AcAsn	Pro	Asp	Ala	Ile	Asn	Ala	Pro	Val	Thr	Cys	Cys
MCP-1 (Asp^1)		Asp	Pro	Asp	Ala	Ile	Asn	Ala	Pro	Val	Thr	Cys	Cys
MCP-1 (Ala^1)		Ala	Pro	Asp	Ala	Ile	Asn	Ala	Pro	Val	Thr	Cys	Cys
MCP-1 (Aba^1)		Aba	Pro	Asp	Ala	Ile	Asn	Ala	Pro	Val	Thr	Cys	Cys
MCP-1 (nVal^1)		nVal	Pro	Asp	Ala	Ile	Asn	Ala	Pro	Val	Thr	Cys	Cys
2-76 analog			Pro	Asp	Ala	Ile	Asn	Ala	Pro	Val	Thr	Cys	Cys
3-76 analog				Asp	Ala	Ile	Asn	Ala	Pro	Val	Thr	Cys	Cys
4-76 analog					Ala	Ile	Asn	Ala	Pro	Val	Thr	Cys	Cys
5-76 analog						Ile	Asn	Ala	Pro	Val	Thr	Cys	Cys
6-76 analog							Asn	Ala	Pro	Val	Thr	Cys	Cys
7-76 analog								Ala	Pro	Val	Thr	Cys	Cys
8-76 analog									Pro	Val	Thr	Cys	Cys
9-76 analog										Val	Thr	Cys	Cys
10-76 analog											Thr	Cys	Cys
11-76 analog												Cys	Cys

Figure 2. NH_2-terminal sequences of MCP-1 analogues with modifications to the NH_2-terminal ten residues. The wild-type MCP-1 sequence is listed on top.
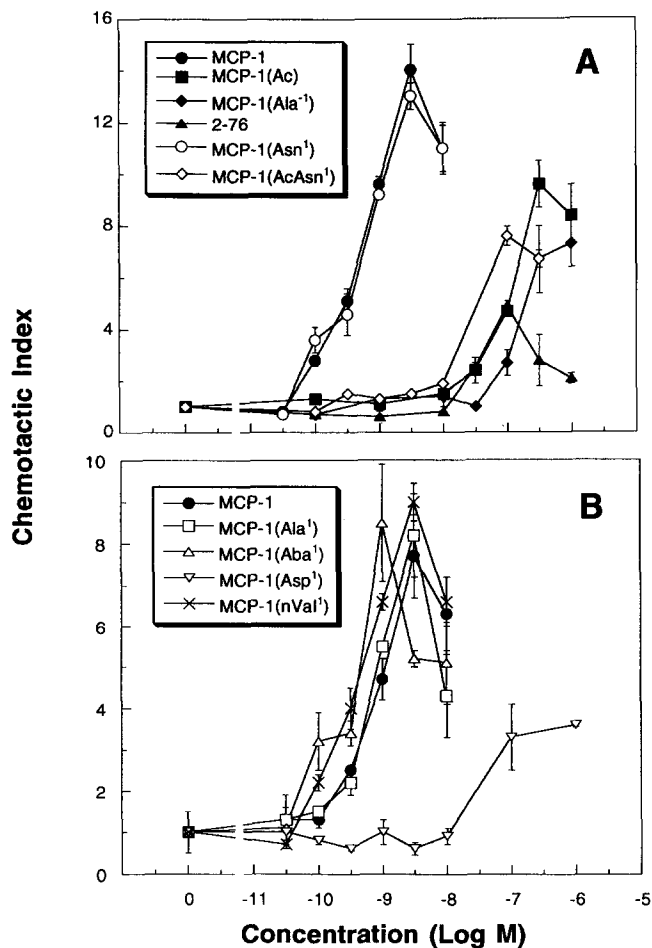
Figure 3. Chemotactic activity of MCP-1 analogues that are modified at the NH$_2$-terminal residue. The analogues (Fig. 2) were titrated in assays for THP-1 chemotaxis as described for Fig. 1. MCP-1, MCP-1 (*Asn¹*), MCP-1 (*Ala¹*), and MCP-1 (*nVal¹*) were not significantly different; however the remaining analogues were significantly different from MCP-1 (p <0.05). Results shown are representative of two experiments.
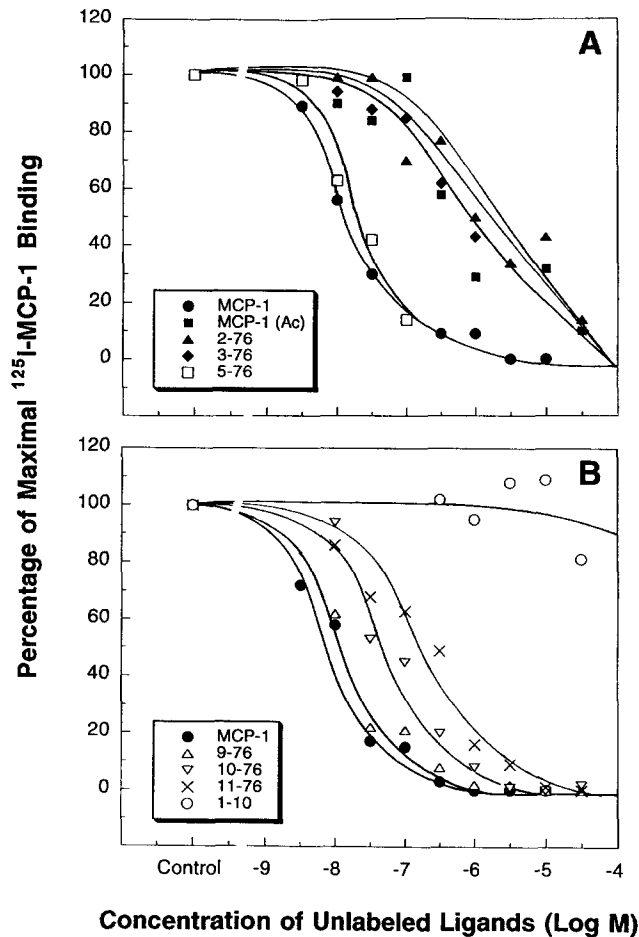

Figure 4. Receptor binding of MCP-1 analogues. The indicated concentrations of MCP-1 analogues (Fig. 2) were added to THP-1 cells in the presence of 4 nM ^{125}I-MCP-1. Nonspecific binding was subtracted from total binding, and the results expressed as % maximum specific binding. Results are representative of two experiments.

pyroglutamate with asparagine (MCP-1, Asn¹) resulted in an analogue with full activity (Fig. 3 *A*). As with the wild type, acetylation of the NH$_2$ terminus of MCP-1, Asn¹ considerably reduced activity, suggesting that the acetylation alone, rather than prevention of pyroglutamate formation, was responsible for the loss of potency. Asparagine and pyroglutamate are smaller than glutamine, so to test the effects of side chain size on function, the nonpolar residues Ala (-CH$_3$), Aba (-CH$_2$CH$_3$), and nVal (-CH$_2$CH$_2$CH$_3$) were substituted. All three were highly active and similar in potency to MCP-1 (Fig. 3 *B*). Interestingly, the MCP-1 (Aba¹) was threefold more active than MCP-1. In contrast, aspartic acid was not tolerated at the NH$_2$-terminal position (Fig. 3 *B*). Induction of cytoplasmic free Ca^{2+} generally correlated with the chemotaxis results (not shown). The 2-76 analogue and MCP-1 (Ac) were assayed for binding to the MCP-1 receptor(s) by competition with labeled MCP-1. From the binding curves shown in Fig. 4 *A*, MCP-1, the 2-76 and MCP-1 (Ac) ana-

logues had K_ds of 2.8, 385, and 333 nM, respectively. For these two analogues, this corresponds to greater than 100-fold loss in binding affinity, which correlates with the decrease in functional activity relative to MCP-1. These results indicate that the integrity of a residue at the NH$_2$ terminus of MCP-1 is critical for binding and function, but that the wild-type pyroglutamate is not essential.

Activity of NH$_2$-terminally Truncated Analogues. To further examine the role of the NH$_2$-terminal region in determining function, we designed a series of analogues that were shortened at the NH$_2$ terminus (Fig. 2). When they were examined for THP-1 chemotaxis, the natural 1-76 form (MCP-1) had the highest activity, the 2-76 analogue was 300-fold lower whereas the 3-76 and 4-76 analogues had only marginal activity (Fig. 5 *A*). Surprisingly, the 5-76 analogue had readily detectable activity (2/3 the level of migration of MCP-1), and was only fourfold less potent than MCP-1 (Fig. 5 *B*). The 6-76 analogue had lower, but significant chemotactic activity. The remaining shortened analogues, 7-76, 8-76, 9-76,
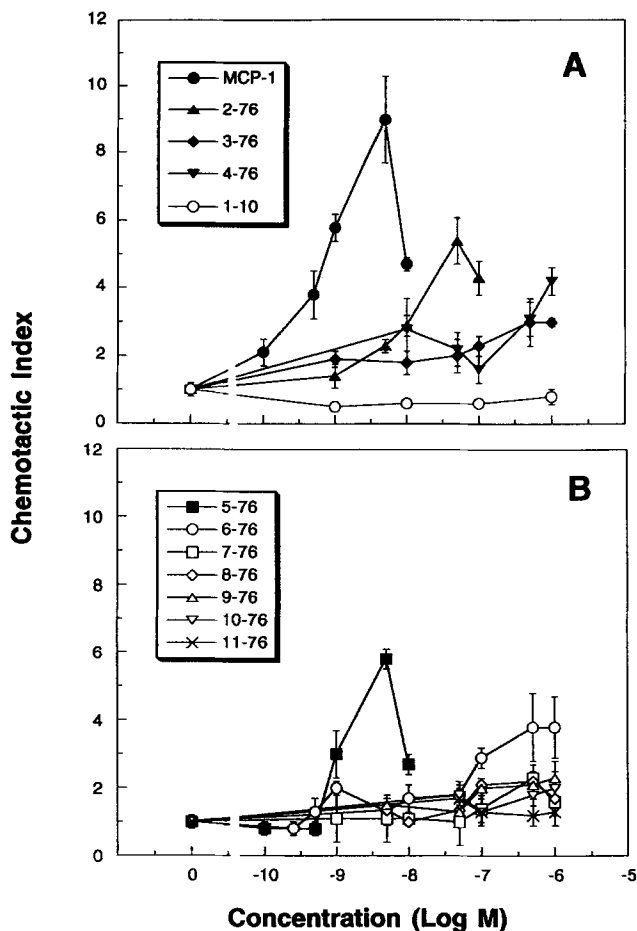
Figure 5. Chemotactic activity of NH_2-terminal truncated analogues. The indicated analogues (Fig. 2) were titrated in the THP-1 cell chemotaxis assay as described for Fig. 1. MCP-1 and 5-76 were significantly different from each other and from the remaining analogues ($p < 0.05$). Except for the 1-10 peptide, MCP-1, 2-76, 3-76, 4-76, 5-76, and 6-76 were all significantly different than the negative control ($p < 0.05$). Results shown are representative of three experiments.

10-76, and 11-76, lacked detectable chemotactic activity (Fig. 5 B). A peptide corresponding to residues 1–10 (Fig. 2) also lacked detectable activity (Fig. 5 A). Similar results were obtained with monocytes as the target cells (data not shown).

Ca^{2+} Mobilization and Desensitization by Truncation Analogues. Transient intracellular Ca^{2+} mobilization was assayed as a second measure of function. The native MCP-1 was the most efficient in inducing cytosolic Ca^{2+} mobilization of the analogues tested (Figs. 6 and 7 A). However, consistent with its chemotactic activity, the 5-76 analogue also induced a Ca^{2+} rise in THP-1 cells (Figs. 6 and 7 A). As summarized in Fig. 7 A, the 2-76, 4-76, and 6-76 analogues induced a lower response. The 3-76, 7-76, 8-76, 9-76, 10-76, and 11-76 analogues (Figs. 6 and 7 A) or the 1-10 peptide (not shown) did not induce significant cytosolic Ca^{2+} at levels up to 1,000 nM.

After treatment with an MCP-1 receptor ligand, the calcium response is temporally desensitized to treatment with a second MCP-1 receptor ligand. When the analogues were examined for their ability to desensitize the subsequent Ca^{2+} response to MCP-1, there was little correspondence with the Ca^{2+} induction results (Figs. 6 and 7 B). All truncated analogues desensitized THP-1 cells to a subsequent MCP-1 challenge, although the 11-76 analogue required a higher concentration 1,000 nM for a detectable response. The MCP-1, 1-10 peptide did not attenuate the Ca^{2+} response (not shown). Most significantly, the 3-76, 7-76, 8-76, 9-76, and 10-76 analogues desensitized but did not induce Ca^{2+} (compare Fig. 7, A and B). Of the noninducing analogues, 9-76 was consistently the most effective at desensitization. The results indicate that several truncated analogues desensitize MCP-1 receptors, but do not activate Ca^{2+}-dependent signaling.

Receptor Binding of Truncated Analogues. Several of the shortened analogues were tested for MCP-1 receptor binding by competition for ^{125}I-MCP-1 (Fig. 4). The 2-76 and 3-76 analogues had K_ds (385 and 487 nM, respectively) ~150-fold higher than MCP-1 (K_d 2.8 nM) when analyzed for binding on THP-1 cells (Fig. 4 A). In keeping with its functional activity, the 5-76 analogue had only an eightfold higher K_d (23 nM) than MCP-1. The inactive 9-76 analogue had threefold higher K_d than MCP-1, whereas the K_ds of 10-76 and 11-76 analogues were 13- and 48-fold higher, respectively. The MCP-1 (1-10) peptide did not bind (Fig. 4 B). The 9-76 and 11-76 analogues were labeled, and direct binding assays were performed (not shown). The binding results correlated well with the Ca^{2+} desensitization results.

Inhibition of MCP-1 Chemotaxis Activity. The ability of the non-chemotactic analogues to desensitize calcium mobilization of MCP-1 suggested that they were binding to MCP-1 receptors but not activating the signaling pathways of the receptor(s). If this hypothesis is correct, then it would be expected that they would block the biological response induced by MCP-1 through a competitive binding mechanism. To test this, the 8-76, 9-76, 10-76, and 11-76 analogues were titrated in the presence of 5 nM MCP-1 (Fig. 8). The four analogues inhibited MCP-1–stimulated chemotaxis in a dose-dependent manner. The 9-76 analogue was the most potent (IC_{50} = 20 nM), the 8-76 analogue was threefold less potent (IC_{50} = 60 nM), and the 10-76 and 11-76 analogues were much less potent (IC_{50} = 0.6 and 1 μM, respectively). The 1-10 peptide did not significantly inhibit chemotaxis (not shown).

Specificity of 9-76 Antagonists. The ligand-receptor specificity of the 9-76 analogue was tested by desensitization of Ca^{2+} induction. Chemokine ligands transiently desensitize their own receptor but not other receptors (15). As shown in Fig. 9, the 9-76 analogue desensitized the Ca^{2+} mobilization in response to MCP-1 and MCP-3, but did not significantly decrease the response to RANTES, MIP-1α, or MIP-1β. In addition, the 9-76 analogue was able to specifically block MCP-1–, MCP-2–, and MCP-3–induced cell migration, but not that induced by MIP-1α and RANTES, up to 1,000-fold molar excess (data not shown). Overall, the results suggest that the inhibition of MCP-1 and MCP-3 in-
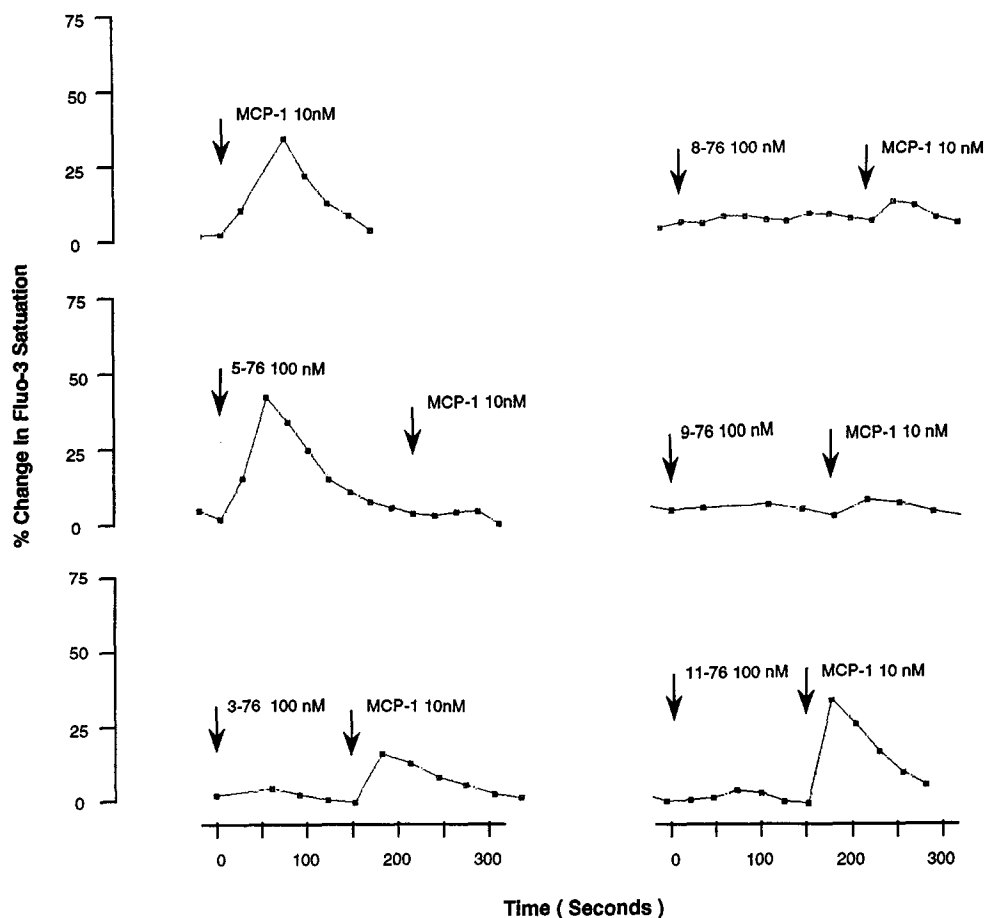
Figure 6. Ca^{2+} mobilization and desensitization of the indicated NH_2-terminal truncation analogues. The transient rise in fluorescence intensity was measured after initial treatment of Fluo-3AM loaded THP-1 cells with the indicated MCP-1 analogues and again on challenge with MCP-1 as indicated. Results are representative of at least two experiments. Over many experiments, the calcium response was found not to be significant at values under 5% fluorescence saturation. Therefore, the induction with the 3-76, 8-76, 9-76, and 11-76 analogues is not significant. The MCP-1 response after the 9-76 analogue treatment is also not significant.

duced chemotaxis is correlated with ability to desensitize the MCP-1 receptor without concomitant activation.

Discussion

Analogues of MCP-1 were analyzed for function and receptor interactions using monocytes and THP-1, a monocyte cell line. Our results indicate that the NH_2-terminal region is critical for receptor binding and function, and that some analogues, e.g., the 9-76 analogue, have receptor antagonist properties, suggesting that receptor binding can be dissociated from functional receptor activation.

Comparison of CC chemokines indicated that MCP-1 and MCP-3 stimulated chemotaxis via common receptors. MCP-2 also stimulated chemotaxis, but was the least potent of the three (data not shown). RANTES, MIP-1α, and MIP-1β stimulated a transient increase in cytosolic Ca^{2+} levels, but had only weak chemotaxis activity. The actions of these CC chemokines appeared to be mediated by distinct receptors (15, 19–23). We observed some desensitization of MIP-1α/RANTES receptors with high concentrations of MCP-1, but whether this cross-reaction has apparent physiological significance is uncertain. Overall, the results indicate that amongst the CC chemokines, MCP-1 is the most potent monocyte chemoattractant and is the ligand of choice for structure–activity relationships involving the MCP-1 receptor.

Residues within the NH_2-terminal region 1-10 of MCP-1 are essential for chemotaxis and receptor signaling, as demonstrated by the lack of chemotactic activity of the 11-76 analogue. The nature of the NH_2-terminal residue is critical as deletion or modification of the N^α group by acetylation resulted in substantial loss of activity. Nevertheless, asparagine or nonpolar residues of various sizes could fully substitute for pyroglutamate. Thus the integrity of the NH_2-terminal residue is essential for function but the side chain can be varied. This suggests that either it may form important secondary structure elements within MCP-1 rather than interacting directly with the receptor, or that the backbone component of the residue fits tightly into the binding pocket, such that an N-acetyl group cannot be accommodated, but its side chain can be varied.

Deletion of the first two amino acids resulted in almost total loss of activity, but surprisingly, activity was regained on deletion of further amino acids. The 5-76 analogue had only fourfold lower potency than full-length MCP-1. Receptor binding affinities correlated with the functional activities. This suggests that the NH_2-terminal residue is not essential for signaling, but may be required for maximal binding (see
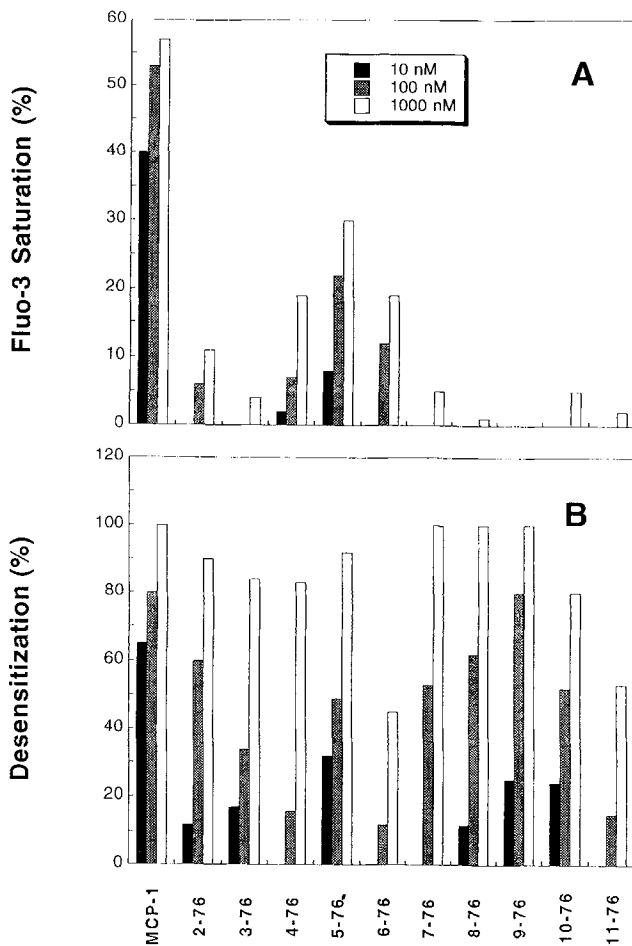
Figure 7. Summary of stimulation and desensitization of Ca^{2+} induction by truncated MCP-1 analogues. *A* shows the percent of Fluo-3 AM saturation (Materials and Methods) at 10 nM, 100 nM, and 1,000 nM analogue concentration. By the criteria outlined in Fig. 6, the response of the 3,76, 7-76, 8-76, 9-76, 10-76, and 11-76 analogues were not significant at any dose tested. *B* shows the percentage desensitization induced by the truncated analogues at 10 nM, 100 nM, or 1,000 nM, to a subsequent treatment by 10 nM MCP-1. Maximal desensitization of fluorescence obtained with MCP-1 was designated as 100% desensitization and the maximal fluorescence induced by 10 nM MCP-1 was designated as 0% desensitization. All the analogues gave significant desensitization for at least one of the doses tested.

above). With further deletions from the 5-76 analogue, biological activity was lost but receptor binding and desensitization was retained with the 8-76, 9-76, 10-76, and 11-76 analogues. Thus, receptor binding, within residues 7-10 is dissociated from receptor activation that requires residues 1-6. The loss regaining and subsequent loss of activity upon sequential truncation suggests that multiple NH_2-terminal region are required for full activity, and partial deletions can have a negative effect on receptor interactions. Consistent with these findings, residues 1, 2, 6, 9, and 10 are either identical or conservatively substituted when the sequences of MCP-1, MCP-2, and MCP-3 are compared. The fact that the NH_2-terminal peptide 1-10 did not bind or display agonist or an-
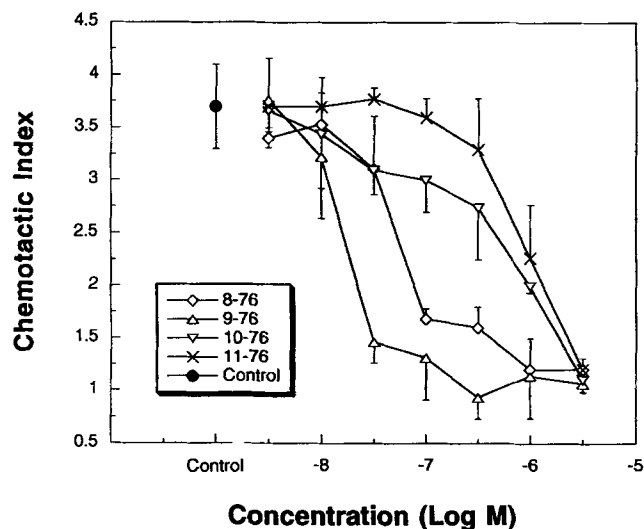

Figure 8. MCP-1 antagonist activity of truncated MCP-1 analogues. Constant MCP-1 (5 × 10^{-9} M) was added to each well and the indicated analogues were titrated in the THP-1 chemotaxis assay as described for Fig. 1. The 10-76 and 11-76 were not significantly different from each other. However, either 10-76 or 11-76, and all the other remaining analogues were significantly different from the MCP-1 control ($p < 0.05$). Results are representative of three experiments.

tagonist activity suggests that additional residues or regions within the 11-76 analogue are also required for function. Residual receptor binding was detected with the 11-76 analogue, indicating that receptor binding motifs are also contained in residues 11-76.

The observations with MCP-1 have parallels with IL-8, a member of the structurally related CXC chemokine family. In the case of IL-8, the NH_2-terminal residues 4, 5, and 6 are essential for receptor binding and triggering function (30–32). Interestingly, the three residues which in the aligned sequences correspond to IL-8, 4-6 are MCP-1, 8-10. These three residues are part of an MCP-1 receptor-binding site. In addition, the MCP-1 situation is more complex than that of IL-8, in that the functionally critical NH_2-terminal region extends over 10 residues and in addition to 8, 9, and 10, residues 1-7 are also required for activity. The three-dimensional structure of MCP-1 may assist in determining the roles of residues 1-10 but the NH_2-terminal regions of chemokines are often partially disordered (37, 38), making it difficult to suggest functional roles. As proposed here for MCP-1, additional downstream residues are also required for IL-8 structure–function (39).

Two truncated analogues, MCP-1, 8-76 and 9-76 were potent inhibitors of MCP-1 function. The 3-76, 7-76 (data not shown), 10-76, and 11-76 analogues were less potent. The inhibition potency correlated with their receptor affinity. The ability of these analogues to desensitize the calcium response induced by MCP-1 and MCP-3, coupled with their inability to induce Ca^{2+} mobilization, suggests that they bind to, but cannot activate MCP-receptors. The mechanism of transient chemotactic receptor desensitization is not known, but our
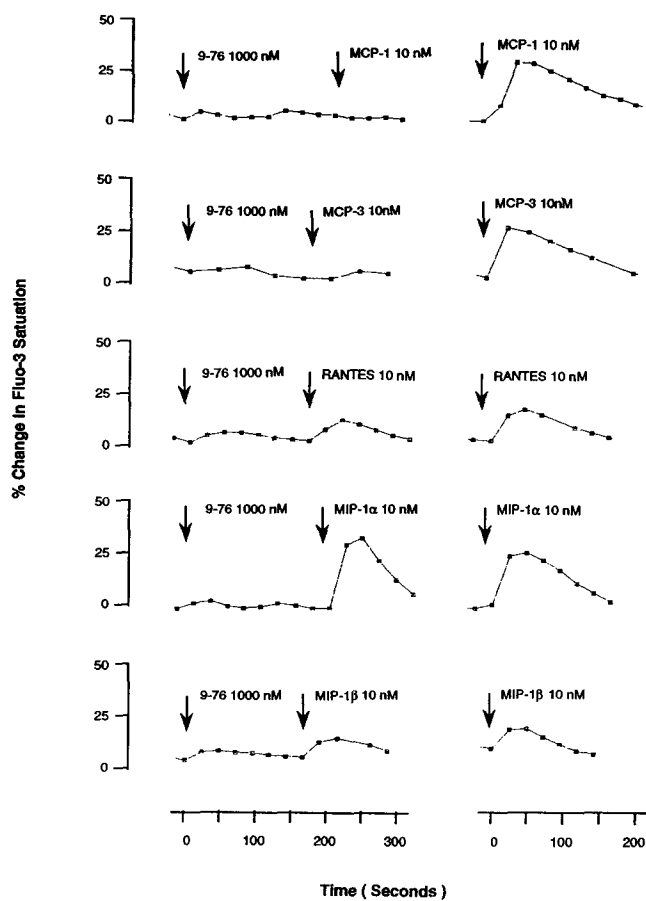
Figure 9. Specificity of the 9-76 antagonist. The ability of the 9-76 analogue to desensitize the subsequent Ca^{2+} response by the indicated CC chemokines was measured. By the criteria described for Fig. 6, the response of 9-76 was not significant, nor was the MCP-1 or MCP-3 response after 9-76 treatment. Results are representative of three experiments.

results suggest that receptor activation is not necessary. Receptor binding is likely to be a requirement for desensitization, as demonstrated by mechanistic studies suggesting receptor occupancy (40) and internalization (41) are involved

for the related FMLP receptor. Thus, our desensitization results reflect receptor occupancy.

The 9-76 analogue inhibited THP-1 cell migration induced by MCP-1 (Fig. 8), and MCP-3, but not by RANTES, MIP-1α, or MIP-1β (data not shown), further supporting the desensitization data indicating that the 9-76 analogue is specific for the MCP receptors and not RANTES or MIP-1α receptors. The 9-76 analogue was the most potent antagonist of chemotaxis tested: 20 nM inhibited 5 nM of MCP-1 by 50% (IC_{50} = 20 nM). This correlated with the receptor binding studies showing that 17 nM 9-76 analogue displaced 4 nM of MCP-1 by 50%. The binding affinity calculated for 9-76 was threefold lower than MCP-1 (K_d 8.3 nM and 2.8 nM, respectively). By comparison, the 50% of maximal effective dose (ED_{50}) for MCP-1 chemotaxis activity was around 10^{-9} nM. The potency of the MCP-1 antagonist was 15-fold higher than that of the truncated IL-8 antagonists previously described (32). NH$_2$-terminal deletion of chemokines may be a general method of dissociation of receptor binding from function, thus generating antagonists.

We predict that the 9-76 antagonist will be in the therapeutic range of effectiveness and will be useful for evaluating MCP-1 antagonists in animal models, such as the rat alveolitis system where anti–MCP-1 antibodies have been reported to inhibit in vivo function (29). As excess antagonist is always required to completely block a response by a competitive mechanism, higher affinity antagonists will be even more effective. Furthermore, the 9-76 analogue is not expected to be orally active, and although pharmakokinetic studies have not yet been done, it may be readily adsorbed, excreted and degraded.

The discovery of the 9-76 antagonist provides a lead for development of further MCP-receptor antagonists with high potency. Future studies that include more detailed analysis of the NH$_2$-terminal region and determination of the roles of other regions of MCP-1 in structure–function could help provide improved antagonists. The combination of in vivo studies with further structure–function analysis should provide second generation antagonists that may have therapeutic value.

Note added in proof: After submission of this manuscript, a report appeared describing partial inhibition of MCP-1 activity on human peripheral-blood monocytes by MCP-1 deletion mutants (Zhang, Y. J., B. T. Rutledge, and B. J. Rollins. Structure/activity analysis of human monocyte chemoattractant protein-1 (MCP-1) by mutagenesis. *J. Biol. Chem.* 269:15918–15924).

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