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Synthesis and biological characterization of human monocyte chemoattractant protein 1 (MCP-1) and its analogs

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Abstract: Novel analogs of human monocyte chemoattractant protein 1 (MCP-1) were designed, synthesized and characterized to be used as tools to generate monoclonal antibodies as potential human therapeutics. MCP-1 and three analogs were synthesized by step-wise Fmoc solid phase synthesis. After oxidation to form the two-disulfide bonds, affinity chromatography using an immobilized mouse anti-human MCP-1 monoclonal antibody (mAb) was utilized for a simple and highly effective purification procedure for the proteins. The final products were extensively characterized and compared with recombinant human MCP-1 (rhMCP-1). All proteins showed identical binding with mouse anti-human MCP-1 mAbs as measured by surface plasmon resonance. Synthetic MCP-1 and the analogs were comparable to recombinant MCP-1 in competition radio-ligand binding to CCR2 receptors on THP-1 cells, and MCP-1-induced, calcium mobilization and chemotaxis assays.

Keywords: peptide synthesis; solid phase synthesis; MCP-1; analogs; monoclonal antibodies (mAbs); BIAcore; chemotaxis; calcium mobilization

INTRODUCTION

Human monocyte chemoattractant protein 1 (MCP-1; also known as CC chemokine ligand 2, CCL2) is a member of the CC family of chemoattractant cytokines, chemokines. MCP-1 elicits its biological functions by binding and signaling through CC chemokine receptor 2 (CCR2), a seven transmembrane G-protein-coupled receptor. MCP-1 has been implicated in a number of diseases such as atherosclerosis, rheumatoid arthritis and cancer, and may function as an important pro-inflammatory mediator and immune regulator. The cDNA of the MCP-1 gene [1] codes for a 99 amino acid protein with a 23 amino acid signal sequence and a 76 amino acid mature protein. The mature protein has a *N*-terminal glutamine which can cyclize to form a pyroglutamyl residue [2]. To develop antibodies to human MCP-1, native and single amino acid variants of MCP-1 were synthesized that may be used as antigens for the generation of antibodies. Using the crystal structure of MCP-1 [3], amino acids were selected for substitution that were not involved in dimerization or in the putative receptor-binding domain. The criteria for substitution were that neither backbone structure nor surface topology would be significantly altered. Using Sybyl (Tripos), proposed amino acid substitutions were made and the resulting structures subjected to a cycle of molecular dynamics and minimization. The three MCP-1 analogs chosen for synthesis were those that most closely fit the criteria.

The stepwise solid phase synthesis using both the Boc [4–12] and Fmoc strategies [13–18] of several chemokines, analogs and small proteins has been described. In several previous syntheses of chemokines, obtaining a well characterized, high purity product free of deletions, truncations or incorrect disulfide patterns, or with full biological activity has been difficult. Our objective was to develop a synthetic strategy that could be used readily to prepare fully active human MCP-1 as well as single point mutants that can be used for the generation of monoclonal antibodies (mAbs) against specific regions of MCP-1. To overcome the purification issues seen by previous investigators, an affinity column prepared with a mouse anti-human MCP-1 mAb was used as the final purification step. By this approach, native and variant synthetic MCP-1 with binding and biological activities comparable to recombinant human MCP-1 (rhMCP-1) were obtained.

MATERIALS AND METHODS

Materials

Peptide synthesis grade *N*-methylpyrrolidinone (NMP), DMF, DCM, TFA, methanol, acetic anhydride, 1 M HOBt/NMP and 1 M DCC/NMP were purchased from Applied Biosystems. Piperidine, thioanisole, phenol and triisopropylsilane (TIS) were purchased from Aldrich. EDTA and reduced and oxidized glutathione were purchased from Sigma. Sodium

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phosphate and guanidine hydrochloride were purchased from J. T. Baker. EDT was purchased from Fluka. HBTU was purchased from NovaBiochem. Amino acids and resins were from Applied Biosystems (Foster City, CA) or from Bachem (King of Prussia, PA). The anti-human MCP-1/CCL2 purified mouse monoclonal antibody (IgG_{2B}) was purchased from R&D Systems and coupled to CNBr-activated Sepharose 4 Fast Flow (Amersham Biosciences). The endopeptidase Lys-C was purchased from Wako (Richmond, VA) and pepsin from Princeton Separations (Adelphia, NJ). Protein A was purchased from Pierce Chemicals, Rockville, IL.

HPLC Analysis

Analytical HPLC was on a Vydac C-18 column, 10 μm, (0.46 × 25 cm) using a Waters 600E solvent delivery system equipped with a Waters 486 tunable absorbance detector and eluting with a 15 min linear gradient of 0–80% acetonitrile in 0.1% aqueous TFA at a flow rate of 2 ml/min.

Capillary Electrophoresis

Capillary electrophoresis (CE) of the synthetic MCP-1 and analogs was performed by the Beckman Coulter P/ACE™ MDQ instrument using a photodiode array (PDA) detector and Version 5.0 of the 32 Karat™ Software. Bare fused-silica capillary (75 μm ID × 50 cm) and 50 mM phosphate buffer, pH 2.5 were used. The 25 kV, normal polarity voltage was applied for over 30 min at 25 °C.

Solid Phase Synthesis

Peptides were synthesized on an Applied Biosystems (ABI) 431A peptide synthesizer or a Rainin Instrument LLC Symphony/Multiplex multiple peptide synthesizer SMPS-110 using Fmoc-Thr(Bu^t) 4-alkoxybenzylalcohol resin and standard Fmoc protocols for HBTU coupling. The following side-chain protecting groups were used: Arg(Pmc), Arg(Pbf), Asp(OBu^t), Asn(Trt), Gln(Trt), Glu(OBu^t), Cys(Trt), His(Trt), Lys(Boc), Ser(Bu^t), Thr(Bu^t), Trp(Boc) and Tyr(Bu^t). Either Fmoc-Gln(Trt), followed by terminal Fmoc removal, or Boc-L-pyroglutamic acid (Boc-Glp) was coupled as the *N*-terminal amino acid. Where capping was done, 10% acetic anhydride with 5% DIEA in DMF were used.

Cleavage and Deprotection

The resin-protein (0.02 mM – 0.04 mM) was stirred with a mixture of trifluoroacetic acid (20 ml), 1,2-ethanedithiol (4 ml), phenol (1.5 g), thioanisole (1 ml), water (1 ml) and triisopropylsilane (1 ml) for 4 h at ambient temperature (RT). The resin was removed by filtration and diethyl ether (400 ml) was added to the filtrate. The precipitate was collected by filtration, washed with diethyl ether (3 × 30 ml) and dried in vacuum.

HPLC Purification

Preparative HPLC purification was done using two Vydac C-18 columns, 10 μm, 2.2 × 25 cm (Nest Group) on a Waters 600 controller equipped with a Waters 486 tunable absorbance detector and a Waters 717 autosampler. The system was equilibrated with 0.1% TFA and the linear peptides were dissolved in 20% acetic acid for injection onto the columns. The solutions of oxidized peptides at a concentration of 20 μg/ml were adjusted to pH 3.5 with acetic acid, filtered through a 0.2 μm cellulose acetate filter, and loaded directly onto the columns. The oxidized peptides were concentrated onto the columns while the salts and acetic acid were washed out by pumping 0.1% TFA/water. Peptides were eluted from the columns using a linear gradient of 0.1% TFA to 0.1% TFA in 80% acetonitrile at a flow rate of 6 ml/min. The eluate was monitored at 220 nm. Column fractions were analysed, pooled and lyophilized.

Oxidation

The purified linear proteins were dissolved in 100 mM Tris HCl, pH 8.6 containing 1 M guanidine hydrochloride, 1 mM EDTA, 0.3 mM oxidized glutathione and 3 mM reduced glutathione, and stirred at ambient temperature for 70–100 h. The oxidized protein was purified by reversed-phase HPLC as described above and/or by affinity chromatography.

Affinity Chromatography Purification

Anti-human MCP-1/CCL2 monoclonal antibody (15 mg) was immobilized on CNBr-activated Sepharose-4B (1.5 g, gel volume: 4.5 ml). The HPLC purified proteins (1.5 mg) were dissolved in PBS and loaded onto the affinity column (1.3 × 5 cm). The column was eluted with 100 mM glycine buffer pH 2.5 and 1.5 ml fractions were collected. The fractions were analysed by reversed-phase HPLC, and MALDI-MS and the fractions containing pure protein were pooled, concentrated and dialysed into PBS. The crude and purified protein yields are given in Table 1.

Molecular Weight Determination using Mass Spectrometry

The analogs were analysed using the Qq/TOF MS to obtain accurate intact molecular masses. Before analysis, the samples were desalted using C18 ZipTips with the proteins eluting into 50 : 50 : 0.1 H_2O/AcCN/TFA and the eluate further diluted with 50 : 50 : 2 MeOH/H_2O/HOAc. The analyses were performed using electrospray (ESI) MS and a Waters Q-TOF API US (Beverly, MA). The data were deconvoluted using MaxEnt 1 provided with MassLynx.

Amino Acid Sequencing using Mass Spectrometry

After intact mass analysis, the samples were reduced, alkylated with iodoacetamide, and digested with pepsin. 5 μl

Table 1 Crude and Purified Yields of Synthetic Proteins

Product	MCP-1	Ser^{40} mg (%)	Ile^{41}	Tyr^{43}
Crude linear	316 (91)	165 (93)	156 (90)	166 (95)
Purified linear	57 (30[a])	45 (27)	42 (27)	56 (34)
Purified oxidized	7 (13)	6 (13)	11 (26)	13 (23)

[a] 190 mg of crude peptide was used for purification.

of each protein was mixed with 4 μl of 45 mM DTT and 45 μl of PBS, PH 7.0. The solutions were incubated at 60 °C for 20 min. Then, 5 μl of 100 mM iodoacetamide was added and the solutions incubated in the dark at room temperature for 20 min. After alkylation, 1 μl of pepsin (0.5 μg/μl) was added and the reaction mixture incubated at 37 °C for 1 h. Before analysis, the samples were desalted using C18 ZipTips with the digested peptides eluting into 50 : 50 : 0.1 AcCN/H_2O/TFA. The samples were spotted onto a MALDI plate with α-cyano-4-hydroxycinnamic acid (CHCA) as a matrix. The digests were analysed using the 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA). Each peptide sequence was confirmed using tandem TOF-TOF mass spectrometry.

Disulfide Mapping

The disulfide-bond structure was studied after digestion of each protein construct with endopeptidase Lys-C. The digests were performed by mixing 50 μl peptide solution in PBS (pH 7.0) with 1 μl of endopeptidase Lys-C at a concentration of 1.3 μg/μl, and incubating the mix for 2 h at 37 °C. After incubation, the samples were desalted using C18 ZipTips (Millipore Corp., Bedford, MA). The samples were analysed using a Voyager DE-STR MALDI TOF MS (Applied Biosystems, Foster City, CA). Two matrices were used to analyse the Lys-C digestions: a saturated solution of CHCA or a 1 : 10 mix of 2-(4-hydroxyphenylazo)benzoic acid (HPBA)/HCCA. Comparison of the data from the mixed matrix to the data from the straight CHCA matrix showed which disulfide-bonded peptides were occurring naturally, and which were potentially formed during MALDI ionization.

BIAcore Analysis

A BIAcore 3000 (Biacore Inc. Piscataway, NJ) was used to determine the binding constants of the interaction between anti-MCP-1 antibodies and the recombinant and synthetic proteins. A capture sensor surface was prepared by covalently immobilizing Protein A (Pierce Chemicals, Rockville, IL) onto a CM-5 chip using a NHS/EDC amine coupling kit (Biacore Inc. Piscataway, NJ). Approximately 3000 RU of Protein A were immobilized. Anti-MCP-1 antibody C 775 was captured onto the Protein A modified sensor surface by passing a 5 μg/ml solution of the monoclonal antibody diluted into phosphate buffered saline (PBS, 10 mM sodium phosphate, 150 mM sodium chloride, pH 7.4). Approximately 160 response units (RU) of C 775 were captured by the surface during the experiment cycle. Binding studies were performed by equilibrating the instrument and sensor surface using a running buffer of PBS buffered saline containing 3 mM EDTA and 0.005% Tween-20. Samples consisting of 25 nM of protein were then passed over this surface at a flow rate of 30 μl/min for 4 min using the KINJECT command. Following the sample injection, the buffer was flowed over the surface so that 1200 s of dissociation data could be collected. After each sample, the Protein A treated surface was regenerated using a 30 s injection of 100 mM phosphoric acid. The binding data for each sample was analysed using the BIAevaluation 3.2 software using the simple 1 : 1 model. An equilibrium dissociation constant for each sample was determined by the ratio of the dissociation rate and association rate constants.

Cell Culture of THP-1 Cells

THP-1 cells (ATCC, Manassas, VA) is a monocytic cell line derived from a patient with acute monocytic leukemia [19] and expresses CCR2. Cells were maintained in culture in RPMI 1640 medium containing 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES and 1.0 mM sodium pyruvate, 90%; 10% fetal bovine serum (FBS; Vitacell RPMI 20–2001, ATCC, Manassas, VA) at 37 °C and 5% CO_2 at a density of $4–8 \times 10^5$ cells/ml.

Radioligand Binding Assay

Competition assays were performed in Millipore filter plates (Millipore, Bedford, MA). 1×10^6 THP-1 cells/well were incubated with ^{125}I-MCP-1 (1 ng/ml; Perkin Elmer Life Science, Boston, MA) together with different concentrations of rhMCP-1 (279-MC, R&D Systems, Minneapolis, MN) or synthetic MCP-1. All reagents were diluted in binding buffer consisting of RPMI medium 1640 (Invitrogen Corp., Grand Island, NY) and 0.1% BSA (Sigma, St Louis, MO). The competition was allowed to proceed for 1 h at RT and the wells were washed three times with 150 μl/well wash buffer (binding buffer +1 M NaCl). The radioactivity of the filters was counted using the Wallac Wizard 1470 Automatic Gamma Counter (Perkin Elmer Life Sciences Inc., Boston, MA). Percent inhibitions of the binding of ^{125}I-MCP-1 to CCR2 by the varying doses of either recombinant or synthetic MCP-1 were calculated. The percent inhibition values were then imported into the Graphpad Prism program and plotted using a sigmoid dose-response curve with a variable slope and constants of bottom = 0 and top = 100.

Calcium Mobilization Assay

The Ca^{2+} mobilization assay was performed in a 96-well format, using the FLEXstation™ Ca^{2+} Plus Assay Kit (Molecular Devices, Sunnyvale, CA) following the manufacturer's protocol for non-adherent cells and a FLEXstation™ (Molecular Devices, Sunnyvale, CA). The peak RFU values were imported into GraphPad Prism and plotted.

Chemotaxis Assay

Chemotaxis was performed using the 96-well disposable chemotaxis apparatus (8 μm pore size; Neuro Probe Inc., Gaithersburg, MD). For calcein AM incorporation, THP-1 cells were resuspended in 10 ml of growth media with 2.5 μg/ml calcein AM (Molecular Probes, Eugene, OR) and incubated for 1 h at 37 °C and 5% CO_2. The bottom chamber contained 315 μl of varying concentrations of either recombinant or synthetic protein. Calcein-loaded cells were washed and resuspended in assay buffer comprising PBS (Invitrogen Corp., Grand Island, NY) and 2% FBS (Invitrogen Corp., Grand) at a density of 2×10^6 cells/ml. Fifty μl of the cell suspension was added to the top of the membrane filter. The chamber was incubated for 1 h at 37 °C and 5% CO_2. Unmigrated cells were rinsed off the chamber with water and blotted dry. The chamber was centrifuged for 30 s at 1200 rpm and then read in the Tecan Fluorometer, (535 nm, bottom read). Data were saved as RFU values in the Microsoft Excel program and plotted using XY line plots using GraphPad Prism.

RESULTS AND DISCUSSION

Protected human MCP-1 (Figure 1) was assembled on a p-alkoxybenzyl alcohol resin using an Applied Biosystems 431A synthesizer using the standard Fmoc protocol. The synthesis of human MCP-1 was initially investigated using two different coupling protocols and two different synthesizers. Using an Applied Biosystems 431A synthesizer, all amino acids were coupled as the HOBt active esters using 1 mmol of activated amino acid per coupling. Both single and double coupling protocols were evaluated, where 0.02–0.04 mM of a low substituted (0.12 mmol/g), preloaded resin was used. In those syntheses where double coupling was done, unreacted amino groups were capped with acetic anhydride after each coupling step before removal of the *N*-terminal Fmoc group. In those syntheses where only a single coupling was done, no capping was performed. In both coupling protocols, resin samples were removed for analysis after cycles 30, 40, 50, 60 and 70. These resin-peptide samples were cleaved, and the intermediate peptides were analysed by HPLC and MALDI-MS. All intermediate peptides showed material corresponding to the expected molecular weights. MCP-1 was also prepared using a Symphony Multiple Peptide Synthesizer SMPS-110, utilizing a single coupling protocol. For this synthesis, Fmoc amino acids (0.5 mmol) were activated by HBTU/HOBt. After assembly, the peptides were simultaneously deprotected and removed from the resin by acidolysis using trifluoroacetic acid (TFA) in the presence of a scavenger cocktail.

It was found that the crude MCP-1 from a single coupling protocol was of much higher quality than the product from double coupling/capping protocol based on both HPLC (Figure 2A and 2B) and mass spectrophotometric (not shown) data. The classical capping reagent, solution of acetic anhydride/tertiary base in DMF or DCM, has been shown to be extremely successful for the synthesis of short peptides [20,21] but its application to longer polypeptides has not been well documented. In our hands, the use of an exhaustive double coupling/capping procedure gave a product of significantly lower quality than the single coupling protocol. Other investigators have made similar observations [22]. No appreciable difference was seen between the Applied Biosystems protocol using 1 mmol amino acid and DCC/HOBt coupling and the

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Gln Pro Asp Ala Ile Asn Ala Pro Val Thr Cys Cys Tyr Asn Phe Thr Asn Arg Lys Ile
 1              5                  10                  15                  20

Ser Val Gln Arg Leu Ala Ser Tyr Arg Arg Ile Thr Ser Ser Lys Cys Pro Lys Glu Ala
 21             25                  30                  35                  40

Val Ile Phe Lys Thr Ile Val Ala Lys Glu Ile Cys Ala Asp Pro Lys Gln Lys Trp Val
 41             45                  50                  55                  60

Gln Asp Ser Met Asp His leu Asp Lys Gln Thr Gln Thr Pro Lys Thr
 61             65                  70                  75
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Figure 1 Amino acid sequence of human monocyte chemoattractant protein 1 (MCP-1, CCL2).

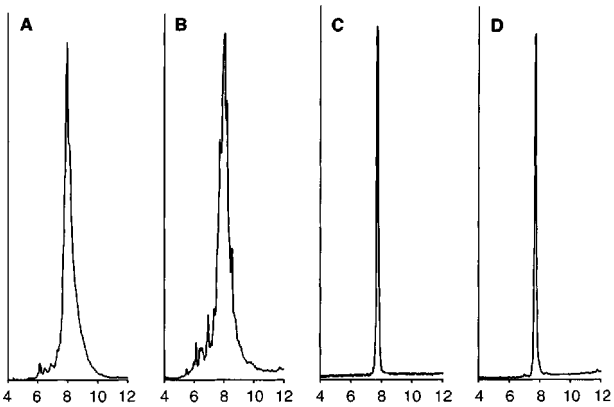

Figure 2 HPLC of crude, reduced MCP-1 from a single coupling protocol (A) and from double coupling-capping protocol (B). (C) shows HPLC of purified, linear MCP-1 and (D) represents HPLC of purified, folded synthetic MCP-1.

Symphony protocol using 0.5 mmol of amino acid with HBTU/HOBT coupling. The crude MCP-1 assembled on the Symphony SMPS-110 was comparable in both yield and purity to material synthesized on an ABI 431A using the single coupling protocol.

Three analogs of MCP-1 were assembled using a standard, small scale, single coupling Fmoc protocol without capping on an ABI 431A peptide synthesizer. The summary of the syntheses is presented in Table 1. From 0.02 mmol of resin used for coupling of analogs, the yields of crude peptides were in the range 156–166 mg (90%–95%). From 0.04 mmol of resin, the yield of crude MCP-1 was 316 mg (91%). MCP-1 and analogs were purified by preparative reversed-phase HPLC. After purification, the linear MCP-1 and analogs were oxidized using a glutathione redox system [23] at a protein concentration of 20 μg/ml. Progress was monitored by HPLC. The oxidized proteins were isolated by preparative reversed-phase HPLC and underwent subsequent purification using affinity column prepared with a mouse anti-human MCP-1 mAb.

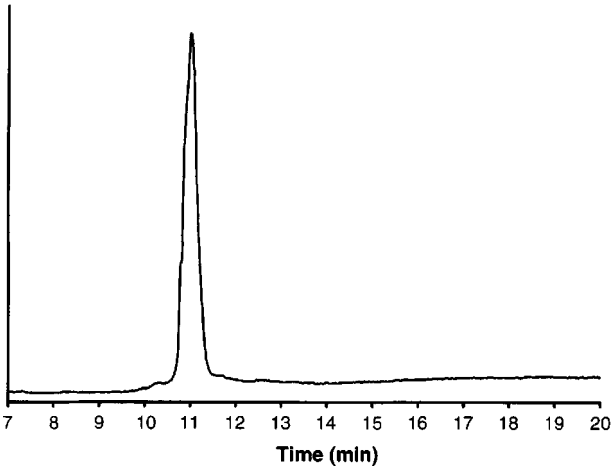

Figure 3 Capillary electrophoresis of purified, folded synthetic MCP-1.

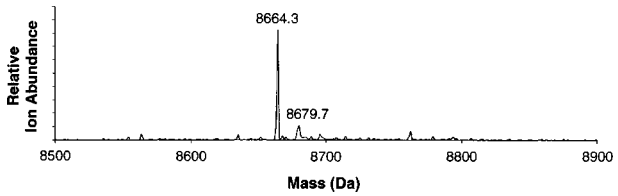

Figure 4 ESI-MS of synthetic MCP-1. Observed: 8664.3 (theoretical molecular weight: 8664.0); 8679.7 (MCP-1[Met(O)^{64}, theoretical molecular weight: 8680.0).

The progression from crude to purified protein is illustrated in Figure 2; the HPLC of the crude MCP-1 (Figure 2A), purified by preparative HPLC linear MCP-1 (Figure 2C), and, purified by HPLC and affinity column, oxidized MCP-1 (Figure 2D). Capillary electrophoresis indicated a high degree of homogeneity, as illustrated in Figure 3 for MCP-1; and ESI MS of the synthetic proteins gave the expected molecular weights. Figure 4 shows the ESI mass spectrum for synthetic MCP-1.

All the purified proteins were characterized by a number of highly discriminating, complementary analytical techniques. Initially the proteins were screened using MALDI TOF MS for protein molecular weights and purity. After which, accurate mass analyses were done using ESI Qq/TOF mass spectrometry. The data were deconvoluted using MaxEnt 1 (packaged with MassLynx), providing the results shown in Table 2. All the analogs showed expected molecular weights. In the initial synthesis, the solution of crude, oxidized MCP-1 was concentrated, dialysed into PBS, and purified by affinity column as described for the HPLC purified proteins. This batch of synthetic MCP-1 was equally potent in bioassays as the recombinant MCP-1; however, Met^{64} was partially oxidized as determined by ESI-MS and MALDI-MS. After 18 months storage in PBS at −50 °C, oxidation of Met^{64} in this batch was complete, as confirmed by tandem mass spectrometry. In bioassays and affinity measurements with mAbs, this material was equivalent to material free of Met(O)^{64}, suggesting that Met^{64} is not required for activity. Other batches of synthetic MCP-1 and analogs were purified, first by HPLC and then by affinity column. None of the purified proteins showed the presence of Met(O)^{64}.

For comparison, rhMCP-1 (279-MC-050/CF; R&D Systems) free of carrier protein was analysed. Analysis included ES-MS of the intact sample in its native and alkylated forms, and peptide mass fingerprinting of a tryptic digest of the native sample (data not shown). The rhMCP-1 showed the correct molecular weight, amino acid sequence and disulfide bonding. However, there was approximately 15% of Met(O)^{64}, 15% of Gln^{1} and about 5% of a putative des[pGlu-Pro] isoforms.

Table 2 The Results of ESI-MS Analyses of Synthetic MCP-1 and Analogs

Product	Molecular weight	
	Theoretical (Da)	Observed (Da)
rhMCP-1	8664.0	8663.9
MCP-1	8664.0	8664.3
MCP-1[Met(O)^{64}][a]	8680.0	8679.8
Ser^{40}	8680.0	8679.9
Ile^{41}	8678.0	8677.8
Tyr^{43}	8680.0	8679.7

[a] Single batch of MCP-1 with Met(O)^{64}.

Table 3 List of the Analysed Pepsin Peptides, Measured and Theoretical Molecular Masses, and Amino Acid Sequences from the MS/MS Experiment. Bold and Underlined Amino Acids indicate Mutations, while Bold M(O) indicates Methionine Sulfoxide. All Amino Acid Mutations were Confirmed via Tandem Mass Spectrometry

Product	Observed peptides	Molecular weight		Sequence
		Observed (Da)	Theoretical (Da)	
All	1–13	1490.37	1490.64	PQPDAINAPVTCCY
All	8–13	798.21	798.30	PVTCCY
All	16–23	944.41	944.54	TNRKISVQ
All	24–28	608.26	608.33	RLASY
MCP-1	29–43	1791.74	1790.98	RRITSSKCPKEAVIF
Ser^{40}	29–43	1806.80	1806.98	RRITSSKCPKE**S**VIF
Ile^{41}	29–43	1804.75	1805.00	RRITSSKCPKEA**I**IF
Tyr^{43}	29–43	1806.74	1806.98	RRITSSKCPKEAVI**Y**
All	44–59	1914.77	1914.04	KTIVAKEICADPKQKW
All	60–76	1970.66	1970.94	VQDSMDHLDKQTQTPKT
MCP-1[a]	60–76	1986.64	1986.94	VQDS**M(O)**DHLDKQTQTPKT

[a] Single batch of MCP-1 with Met(O)^{64}.

The synthetic proteins were sequenced by mass spectroscopy. After intact mass analysis, the samples were reduced, alkylated and digested with pepsin. The digests were analysed using the 4700 Proteomics Analyzer. Each peptide sequence was confirmed using tandem mass spectrometry. Table 3 provides a summary of the sequenced peptides from the experiments. These experiments confirmed all the amino acid substitutions. When all the data were compiled, 97% sequence confirmation was achieved across the entire protein. There was one dipeptide (Asn^{14}-Phe^{15}) that was unaccounted for due to loss during desalting.

The disulfide-bond structure for the synthetic MCP-1 and analogs was studied after digestion of each protein construct with Lys-C. Each digest was analysed using two different matrixes: CHCA and 1 : 10 HPBA/CHCA. The mixed matrix suppresses disulfide-bond cleavage during MALDI ionization. The samples were analysed using a DE-STR MALDI TOF MS. The data from this experiment were used to determine if there were alternate disulfide bond linkages present in the samples. A list of the observed peptides from the Lys-C digestions of the MCP-1 proteins is not shown. In all cases, the disulfide-bonded species L3-L1-L6 (3255.7 Da) was observed, where L3 peptide corresponds to CPK (36–38; observed 345.4 Da); L1 to <EPDAINAPVTCCYNFTNRK (1–19; observed 2136.4 Da) and L6 to EICADPK (50–56; observed 773.9 Da). The Cys^{11} and Cys^{12} in peptide L1 are adjacent to each other, preventing specific linkage assignment; in other words, it was not possible to distinguish which peptides are bound to which cysteines in peptide L1. Minor levels of either partially or fully reduced cysteines were detected in Ser^{40}, Ile^{41} and Tyr^{43} analogs of MCP-1. The peptide fragments produced by the enzymatic digest of the synthetic proteins and rhMCP-1 (279-MC-050/CF; R&D Systems) were analysed, and found to be identical.

Biological Characterization

The binding to anti-MCP-1 mAb C775 was studied using surface plasmon resonance (BIAcore) to confirm that the proteins had the correct tertiary structure. The data are shown in Table 4. The association and dissociation rate constants of the synthetic proteins were comparable to those observed with rhMCP-1. In addition to mAb C775, six additional mouse anti-human MCP-1 mAbs were shown to have comparable binding kinetics to synthetic and recombinant MCP-1 (data not shown).

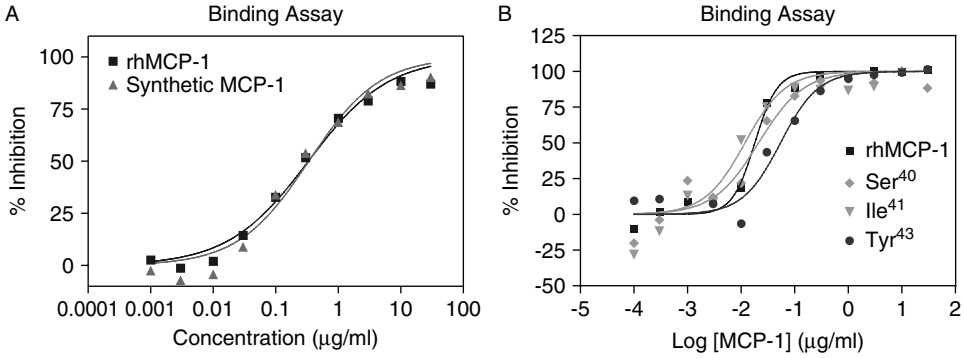

Figure 5 Synthetic and recombinant MCP-1 proteins were equivalent in competing with the binding of ^{125}I-rhMCP-1 to CCR2 receptors on THP-1 cells (A); the three synthetic analogs and rhMCP-1 competed with similar potency for CCR2 on THP-1 cells in the ^{125}I-MCP-1 competitive binding assay (B).

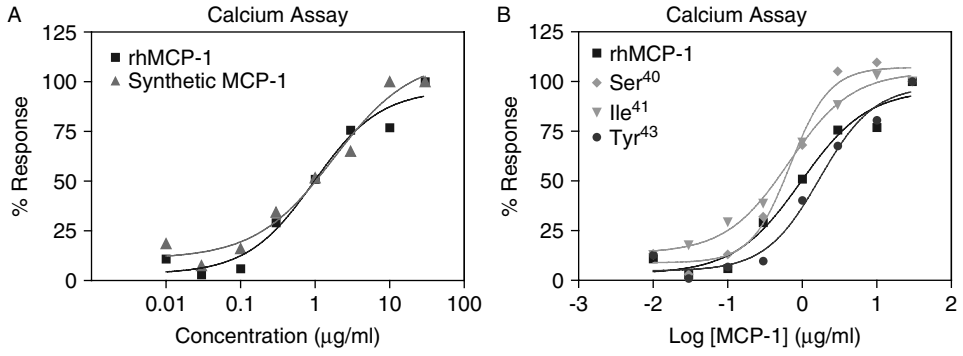

Figure 6 The synthetic and recombinant MCP-1 proteins were equivalent in inducing mobilization of calcium from internal stores as a result of CCR2 activation on THP-1 cells (A); comparable intracellular Ca^{2+} mobilization in response to the three synthetic analogs and rhMCP-1 was observed (B).

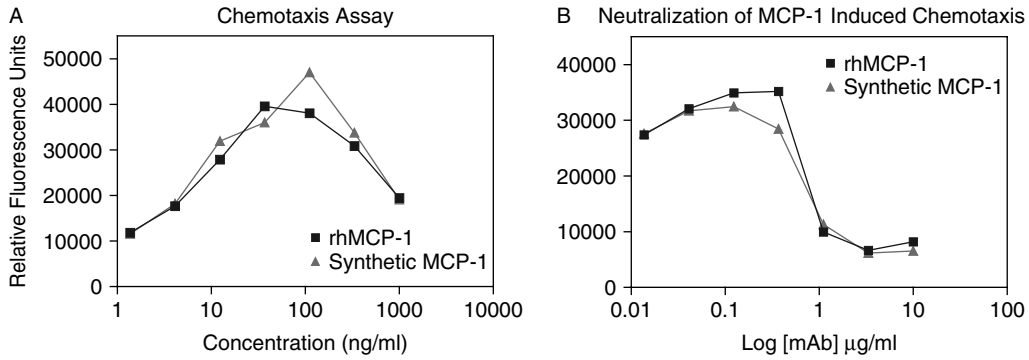

Figure 7 Comparable chemotactic activity in response to both, synthetic and rhMCP-1 was observed (A); anti MCP-1 mAb (R&D Systems) neutralized the chemotactic activity in response to both, synthetic and rhMCP-1 proteins, in a dose-dependent manner (B).

Table 4 BIAcore Analysis of Synthetic MCP-1 and its Analogs Binding to Anti-MCP-1 Monoclonal Antibody C775

Product	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	Calculated K_D (nM)
rhMCP-1	1.4×10^6	7.4×10^{-4}	0.5
MCP-1	0.9×10^6	9.9×10^{-4}	1.1
Ser^{40}	1.2×10^6	3.6×10^{-4}	0.3
Ile^{41}	1.2×10^6	7.8×10^{-4}	0.5
Tyr^{43}	0.6×10^6	9.9×10^{-4}	0.8

In addition to binding to anti-MCP-1 antibodies, all synthetic proteins showed similar inhibition curves to recombinant MCP-1 in a competition assay involving ^{125}I-MCP-1 binding to CCR2 on THP-1 cells (Figure 5). In a functional assay involving calcium mobilization in THP-1 cells, similar response curves were seen for rhMCP-1 and all synthetic proteins (Figure 6). The chemotactic effect of synthetic MCP-1 on THP-1 cells was studied. Both synthetic and rhMCP-1 had identical chemotactic activity (Figure 7A). This activity could be neutralized by an anti-MCP-1 mAb (R&D Systems) in a concentration-dependent manner (Figure 7B). Comparable chemotactic activity was seen for the three analogs (data not shown).

CONCLUSIONS

MCP-1 and its analogs can be readily synthesized to give material that is indistinguishable from material produced via recombinant expression in both physical and biological assays. Rather than improving the quality of the crude protein, the more rigorous double coupling-capping solid phase protocol gives material that is significantly more heterogeneous with the associated issues of more difficult purification and lower protein yields. As expected, there were no significant differences between material prepared using different solid phase synthesizers. The quality of the crude products was similar whether 0.5 mmol amino acid with HBTU/HOBt activation or 1.0 mmol amino acid with DCC/HOBt activation were used. Methionine-64 is particularly prone to oxidation, although the conversion to methionine sulfoxide does not alter the biological activity. Using a glutathione redox system, the correct disulfide pairing of the four cysteines is readily formed. The combination of reversed-phase HPLC and a monoclonal affinity chromatography provides a rapid and efficient method for purification of MCP-1 and its analogs. The methods described herein give ready access to a variety of MCP-1 analogs that can be used for structure-function studies and antibody generation as well as to analogs that cannot be prepared by recombinant methods.

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