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Synthetic, site-specific biotinylated analogs of human MCP-1

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Abstract: Human monocyte chemoattractant protein 1 (MCP-1, CCL2) is a 8.6-kDa protein that has been implicated in a number of diseases including atherosclerosis, rheumatoid arthritis, chronic obstructive pulmonary disease and cancer. As part of a program to identify antibodies against MCP-1, we synthesized site-specific, biotinylated human MCP-1 analogs to be used for panning of an antibody phage display library. In contrast to material obtained from random biotinylation, the site-specific biotinylated analogs were homogeneous and retained full activity. Copyright © 2005 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: peptide synthesis; solid-phase peptide synthesis; MCP-1; CCL2; biotinylation; monoclonal antibodies (mAbs); calcium mobilization; receptor binding

INTRODUCTION

Chemical modification of proteins is complicated by the limited functional groups in native amino acids that can be derivatized, their usual occurrence at multiple positions in a given protein and the fact that derivatization of certain amino acids necessary for activity may compromise the activity of the modified protein. One approach to bypass these issues is the total chemical synthesis of proteins using modified amino acids. We have used this approach to prepare site-specific biotinylated human monocyte chemoattractant protein 1 (MCP-1, also known as CC chemokine ligand 2, CCL2). MCP-1 is a member of the CC family of chemoattractant cytokines that binds to CCR2, a G-protein-coupled receptor. It has been implicated in a number of diseases including atherosclerosis, rheumatoid arthritis and cancer and may also function as an important proinflammatory mediator and immune regulator. Previously, we described a synthetic strategy for the preparation of human MCP-1 and variants [1] for use in the identification of neutralizing monoclonal antibodies targeted against specific regions of MCP-1. As part of that strategy, both synthetic and recombinant human MCP-1 proteins were chemically biotinylated to facilitate selection and screening of antibodies generated by phage display. However, this simple process led to considerable loss of binding and functional activity, despite evaluation of a range of conditions. Here, we described the chemical synthesis and characterization of site-specific biotinylated analogs of MCP-1. In contrast to postsynthesis, nonspecific biotinylation, this approach gave fully active protein.

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MATERIALS AND METHODS

Materials

Peptide synthesis grade *N*-methylpyrrolidinone (NMP), DMF, DCM, TFA, MeOH, 1 M HOBt/NMP, and 1 M DCC/NMP were purchased from Applied Biosystems (Foster City, CA). Piperidine, thioanisole, phenol, and triisopropylsilane (TIS) were purchased from Aldrich (Milwaukee, WI). EDTA, BSA, 1, 2-ethanedithiol, reduced and oxidized glutathione, iodoacetamide, dithiothreitol (DTT), sinapinic acid, α-cyano-4-hydroxycinnamic acid (CHCA), and 2-(4-hydroxyphenylazo) benzoic acid (HPBA) were purchased from Sigma (St Louis, MO). Sodium phosphate monobasic, monohydrate and dibasic, anhydrous, guanidine hydrochloride, Tris HCl, acetic acid (AcOH), and acetonitrile were purchased from J.T. Baker (Phillipsburg, NJ). HBTU was purchased from NovaBiochem (San Diego, CA). Phosphate buffered saline (PBS) and pepsin were from Princeton Separations, Inc. (Adelphia, NJ). The endopeptidase Lys-C was purchased from Wako (Richmond, VA). Amino acids were purchased from Applied Biosystems and Bachem (King of Prussia, PA). Fmoc-Lys(Aloc) was purchased from AnaSpec (San Jose, CA). Fmoc-Lys(Mtt) was from NovaBiochem. Boc-Glp and Fmoc-Thr(Bu^t)-Wang resin were purchased from Bachem. ImmunoPure® Immobilized Monomeric Avidin Kit and the EZ-Link® Sulfo-NHS-LC-biotin were purchased from Pierce (Rockford, IL). The NHS-dPEG$_4$-biotin was purchased from Quanta BioDesign (Powell, OH).

Analytical HPLC

Analytical HPLC was performed using a Waters 600E solvent delivery system equipped with a Waters 486 tunable absorbance detector equipped with a Vydac C-18 column, 10 μm (0.46 × 25 cm), and eluted 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) was done on a Beckman Coulter P/ACE™ MDQ instrument with photodiode array

(PDA) detector and Version 5.0 of the 32 Karat™ Software. Bare fused-silica capillary (75 μm i.d. × 50 cm) and 50 mM phosphate buffer, pH 2.5 were used. The normal polarity voltage (25 kV) 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 with standard Fmoc protocols for HBTU coupling. The following side chain–protecting groups were used: Arg(Pbf), Asp(OBu^t), Asn(Trt), Gln(Trt), Glu(OBu^t), Cys(Trt), His(Trt), Lys(Boc), Lys(Aloc), Lys(Mtt), Ser(Bu^t), Thr(Bu^t), Trp(Boc), and Tyr(Bu^t). Boc-Glp was coupled as the *N*-terminal amino acid.

Cleavage and Deprotection

The protein–resin (1.41 g, 0.06 mmol) was stirred with a mixture of trifluoroacetic acid (40 ml), 1, 2-ethanedithiol (8 ml), phenol (3 g), thioanisole (2 ml), water (2 ml), and TIS (2 ml) for 4 h at ambient temperature. 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 under reduced pressure.

Preparative HPLC

Preparative HPLC purification was done using two tandem 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 after cleavage from the resins, the linear proteins were dissolved in 20% acetic acid for injection onto the columns. After oxidation, proteins 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 proteins were concentrated on the column, while the salts and acetic acid were washed out by elution with 0.1% TFA/H_2O. Proteins 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 monitoring at 220 nm. Column fractions were analyzed by HPLC and mass spectrometry (MS), and appropriate fractions were 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 72 h. The oxidized protein was purified by reversed-phase (RP) HPLC as described above and by affinity chromatography.

Affinity Chromatography Purification

The HPLC purified protein was repurified using an affinity column with immobilized monomeric avidin. The protein (1.5 mg) was dissolved in PBS (2 ml) and added onto the column (2 ml). The column was washed with PBS (6 × 2 ml) and the bound biotinylated protein was eluted using biotin-containing buffer (2 mM D-biotin in PBS) (6 × 2 ml). The collected fractions were analyzed by HPLC and MS, and selected fractions were pooled, concentrated, and dialyzed into PBS.

Mass Spectrometry

Molecular weight determination. Proteins were analyzed using electrospray (ESI) MS to obtain accurate intact molecular masses. Before analysis, the samples were desalted using C18 ZipTips with the proteins eluted into 50 : 50 : 0.1 H_2O/acetonitrile/TFA. The eluate was further diluted with 50 : 50 : 2 MeOH/H_2O/AcOH. The analyses were performed using ESI MS and a Waters Q-TOF API US (Beverly, MA). The data were deconvoluted using MaxEnt 1 provided with MassLynx software. For SELDI MS, the samples were analyzed using a Protein Biological System II protein chip reader (Ciphergen Biosystems, Freemont CA). Two microliters of each sample was spotted on an 8-spot H4 (C18 surface) protein chip array (Ciphergen Biosystems, Freemont CA) and allowed to dry at room temperature. Then, 1 μl of saturated sinapinic acid was applied to each spot and allowed to dry at room temperature.

Amino acid sequencing. After intact mass analysis, the samples were reduced, alkylated with iodoacetamide, and digested with pepsin. Five microliters of each protein solution in PBS (0.5 mg/ml) 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. Five microliters 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 eluted with 50 : 50 : 0.1 acetonitrile/H_2O/TFA. The samples were spotted onto a MALDI (matrix-assisted laser desorption/ionization) plate with CHCA as a matrix. The digests were analyzed using the 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA). Each peptide sequence was confirmed using tandem TOF–TOF MS.

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 protein solution in PBS (0.5 mg/ml; 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 analyzed using a Voyager DE-STR MALDI TOF (time-of-flight) MS (Applied Biosystems, Foster City, CA). Two matrices were used to analyze the Lys-C digestions: a saturated solution of CHCA or a 1 : 10 mix of HPBA/HCCA. Comparison of the data from the mixed matrix with the data from the straight CHCA matrix showed which disulfide-bonded peptides were occurring naturally and which ones were potentially formed during MALDI ionization.

Cell Culture of THP-1 Cells

THP-1 cells (ATCC, Manassas, VA) are a monocytic cell line derived from a patient with acute monocytic leukemia [2]

and express 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 × 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 recombinant human (rh) MCP-1 (279-MC, R&D Systems, Minneapolis, MN) or synthetic proteins. All reagents were diluted in binding buffer consisting of RPMI Medium 1640 (Invitrogen Corp., Grand Island, NY) and 0.1% BSA. 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 on 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 nonadherent cells and a FLEXstation™ (Molecular Devices, Sunnyvale, CA). The peak RFU values were imported into Graphpad Prism for analysis.

RESULTS AND DISCUSSION

We previously described novel synthetic analogs of hMCP-1: [Ser^{40}], [Ile^{41}] and [Tyr^{43}], with biological activities comparable to rhMCP-1 [1]. Random biotinylation of MCP-1[Ile^{41}] using an EZ-Link® Sulfo-NHS-LC-biotin reagent resulted in biotinylated preparations with a high degree of variability in the number of biotin per protein molecule. The SELDI MS data of two such preparations showed the presence of 0–4 biotins per molecule of MCP-1 (Figure 1). This heterogeneous, randomly biotinylated MCP-1[Ile^{41}] was only partially active in a calcium mobilization assay, as shown in Figure 2.

Moreover, the loss of activity appears disproportionate to the degree of biotinylation. Although purification of monobiotinylated material might be possible, the yield would be reduced substantially, and the preparation would still consist of a mixture of multiple biotinylated species. Batch-to-batch variability of such biotinylated preparations can make it difficult to reproduce results. Thus, the random biotinylation of MCP-1[Ile^{41}] resulted in both heterogeneous biotinylation and, most likely, the lysine residues involved in biological function, with the resulting loss of activity.

To overcome the problems with random biotinylation, we prepared site-specific biotinylated MCP-1 analogs by total chemical synthesis. The human MCP-1 is a 76 amino acid protein [3] containing nine lysine

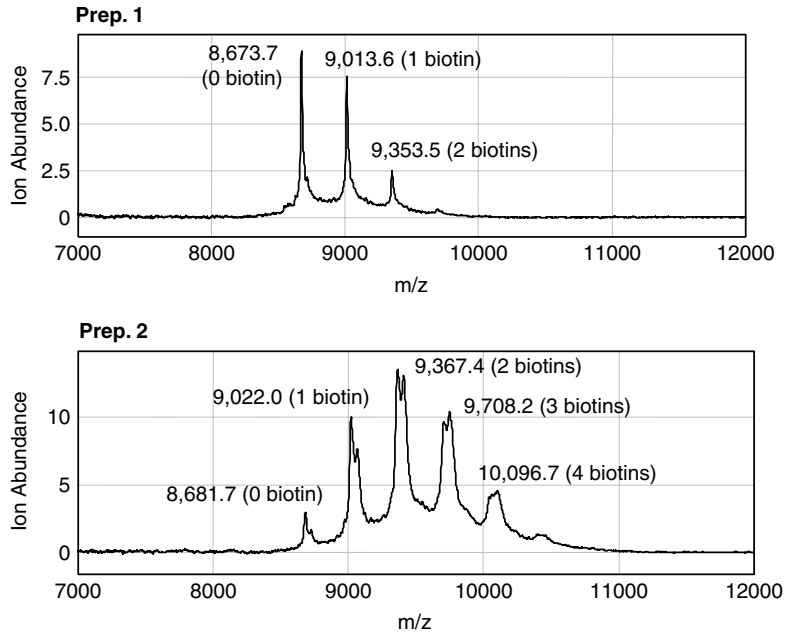

Figure 1 SELDI MS of randomly biotinylated variants of MCP-1 [Ile^{41}] shows the presence of 1–4 biotin molecules coupled to protein as well as unmodified MCP-1[Ile^{41}]. Calculated molecular weights: 0 biotin (MCP-1[Ile^{41}]): 8,678.0 Da; 1 biotin: 9,017.5 Da; 2 biotins: 9,356.9 Da; 3 biotins: 9,696.4 Da; 4 biotins: 10,035.8 Da (mass accuracy: 0.1–0.5%). Data varied among experiments.

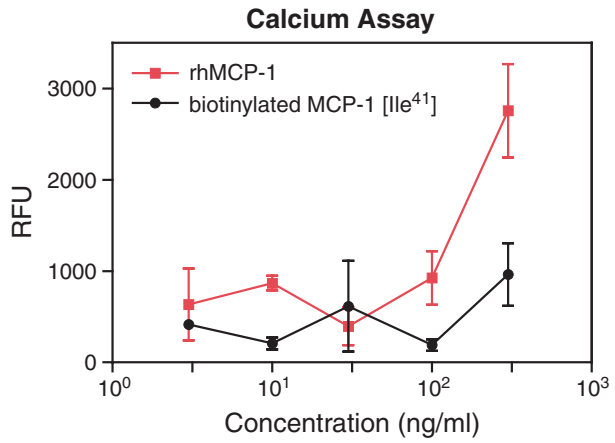

Figure 2 Calcium mobilization assay of randomly biotinylated MCP-1 [Ile^{41}] shows reduced activity in comparison to rhMCP-1. Overbiotinylation, or biotinylation of Lys molecules involved in biological function, resulted in loss of protein activity.

residues that were potential sites for biotinylation. Lysine residues that are appropriate for site-specific biotinylation are those that are surface exposed and distant from the active surface. Figure 3 shows the side chains of MCP-1 residues that have been determined to be important for function as well as the exposed Lys^{69} that is not required for biological activity.

Lys^{75} is also not required for activity but is in a section of the protein that is disordered in the crystal structure. Lysines at positions 35, 38, and 49 were excluded because of their importance for MCP-1 bioactivity [4–8]. Lysines at 19, 44, 56, and 58 are in the vicinity of the active surface and were not considered as potential sites for biotinylation to minimize potential disruption of MCP-1 function. Thus, lysines 69 and 75 were selected as sites for biotinylation. A hydrophilic spacer of four ethyleneoxy units (PEG_4) was inserted between the biotin and the ε-amino group of lysine residue. The chain length from biotin amide to terminal carbonyl is 19.2 Å. The spacer was chosen to increase solubility and provide sufficient spacer length for binding streptavidin conjugates.

Human MCP-1(Ile^{41}, Lys(biotin-PEG_4)^{69}) (I)

The protected octapeptide–resin, Fmoc-Lys(Aloc)-Gln(Trt)-Thr(Bu^t)-Gln(Trt)-Thr(Bu^t)-Pro-Lys(Boc)-Thr (Bu^t)–resin (0.1 mM), was assembled on *p*-alkoxybenzyl alcohol resin using an ABI 431A peptide synthesizer and the Fmoc protocol. All amino acids were coupled as the HOBt-active esters using 1 mmol of activated amino acid per coupling. A low substituted (0.12 mmol/g), preloaded resin was used. The peptide–resin was transferred into a manual shaker, and the Aloc group was selectively removed by treating the peptide–resin with palladium tetrakis(triphenylphosphine), acetic acid, and *N*-methylmorpholine [9]. The NHS-dPEG_4-biotin was coupled to ε-amino group of Lys^{69} in NMP/HOBt solution. The final (1–68) amino acids were added to approximately a half of octapeptide–resin (0.05 mM) in two runs using a Rainin multiple peptide synthesizer (Symphony, Model SMPS-110). For this synthesis, Fmoc amino acids (0.5 mM) were activated by HBTU/HOBt. The mature hMCP-1 has a *N*-terminal glutamine which can cyclize to form a pyroglutamyl residue [10]. Therefore, Boc-L-pyroglutamic acid (Boc-Glp) was used for coupling as the *N*-terminal amino acid. After assembly, the proteins were simultaneously deprotected and removed from the resin by acidolysis using TFA in the presence of a scavenger cocktail.

Human MCP-1(Ile^{41}, Lys(biotin-PEG_4)^{75}) (II)

The protected decapeptide–resin: Fmoc-Leu-Asp(OBu^t)-Lys(Boc)-Gln(Trt)-Thr(Bu^t)-Gln(Trt)-Thr(Bu^t)-Pro-Lys

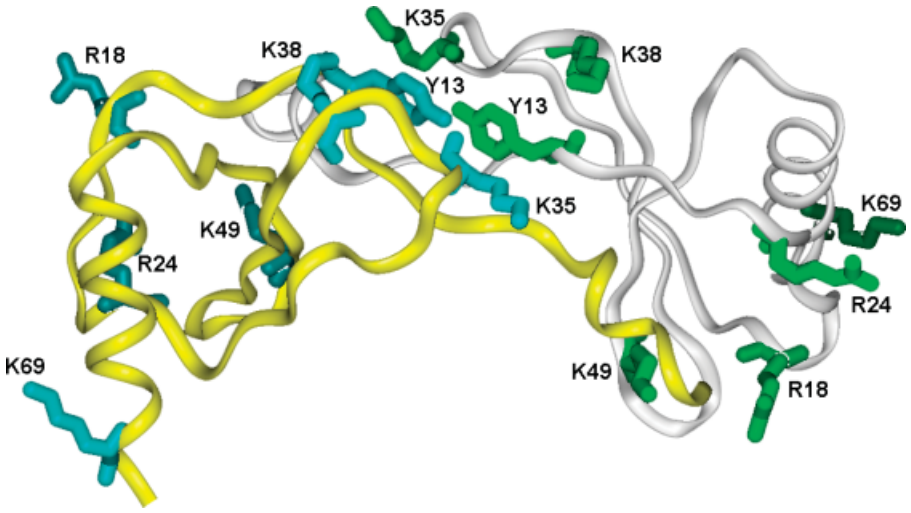

Figure 3 Ribbons representation of hMCP-1 dimer based upon IDOL.PDB. The side chains of the functionally important residues and the side chain of Lys^{69} are shown in sticks. Lys^{75} is disordered in the crystal structure and not shown.

(Mtt)-Thr(Bu^t)–resin (0.1 mM), was prepared on an ABI 431A peptide synthesizer as described for derivative **(I)**. The peptide–resin was transferred into a manual shaker and the Mtt group was selectively removed upon treatment with 1% TFA in dichloromethane [11] with 5% TIS. The NHS-dPEG$_4$-biotin was coupled to ε-amino group of Lys^{75} in NMP/HOBt solution. Approximately one half of protected decapeptide–resin (0.05 mM) was used to prepare the final (1–76) protein–resin in two runs on an ABI 431A peptide synthesizer. All amino acids were coupled as the HOBt-active esters using 1 mmol of activated amino acid per coupling. Boc-Glp was coupled as the *N*-terminal residue.

Deprotection of the Synthetic MCP-1 Proteins

The proteins were simultaneously deprotected and removed from the resin by acidolysis using TFA in the presence of a scavenger cocktail. The crude MCP-1 [Ile^{41}, Lys(biotin-PEG$_4$)^{69}] assembled on a Symphony SMPS-110 was comparable in both yield and purity with crude MCP-1 [Ile^{41}, Lys(biotin-PEG$_4$)^{75}] synthesized on an ABI 431A. The summary of the syntheses is presented in Table 1. From 1.4 g (0.06 mM) of resin, cleavage yielded 496 mg (90%) (**I**) and 463 mg (84%) (**II**) of crude protein. The proteins were purified by preparative RP HPLC. After purification, the linear MCP-1 analogs were oxidized using a glutathione redox system [12] at a protein concentration of 20 μg/ml with progress monitored by HPLC. The oxidized proteins were isolated by preparative RP HPLC and subsequently purified a second time using affinity column with immobilized monomeric avidin.

Characterization of the Biotinylated MCP-1 Proteins

Both purified proteins were characterized by a number of complementary analytical techniques. The HPLC chromatograms and CE of the final MCP-1 [Ile^{41}, Lys(biotin-PEG$_4$)^{69}] and MCP-1 [Ile^{41}, Lys(biotin-PEG$_4$)^{75}] presented as single peaks (data not shown). Initially, the proteins were screened using MALDI TOF MS for protein molecular weights and purity, after which, more accurate mass analyses were done using ESI Qq/TOF MS. The data were deconvoluted and the results are shown in Table 2. All of the analogs showed expected molecular weights. ESI MS of both the synthetic products are shown in Figure 4.

The synthetic proteins were sequenced by MS to confirm the primary sequence and substitution sites. The samples were reduced, alkylated, and digested with pepsin. The digests were analyzed using the 4700 Proteomics Analyzer and sequenced using tandem MS. Table 3 provides a summary of the sequenced peptides from the experiments and the data confirm the expected structures for each protein. When all of the data were compiled, 97% sequence confirmation was achieved across the 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 determined after digestion of each protein construct with Lys-C. Each digest was analyzed using two different matrixes – CHCA and 1 : 10 HPBA/CHCA. The mixed matrix suppresses disulfide-bond cleavage during MALDI ionization. The samples

Table 1 Crude and Purified Yields of Synthetic MCP-1 [Ile^{41}] Analogs

Product	Lys(biotin-PEG$_4$)^{69}	Lys(biotin-PEG$_4$)^{75}
	mg (%)	
Crude linear	496 (90)	463 (84)
Purified linear	101 (20)	83 (18)
Oxidized		
HPLC purified	33 (33)	14 (17)
Affinity purified	2.9 (9)	1.7 (12)

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

Product	Molecular weight	
	Calculated (Da)	Found (Da)
rhMCP-1[a]	8,664.0	8,663.9
MCP-1[b]	8,664.0	8,664.3
Ile^{41}[,b]	8,678.0	8,677.8
Ile^{41}, Lys(biotin-PEG$_4$)^{69}	9,150.7	9,151.0
Ile^{41}, Lys(biotin-PEG$_4$)^{75}	9,150.7	9,151.4

[a] R&D Systems.
[b] Synthesized as described in Ref. 1.

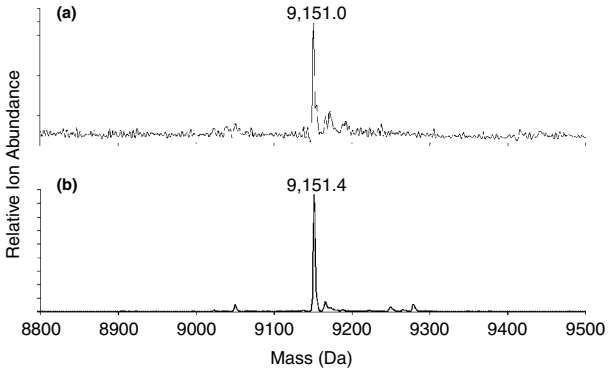

Figure 4 (a) ESI MS of the synthetic MCP-1 [Ile^{41}, Lys(biotin-PEG$_4$)^{69}] and (b) MCP-1 [Ile^{41}, Lys(biotin-PEG$_4$)^{75}]. Found: 9,151.0 Da (a) and 9,151.4 Da (b) (calculated molecular weight: 9,150.7).

Table 3 List of the Analyzed Pepsin Peptides, Measured and Theoretical Molecular Masses, and Amino Acid Sequences from the MS/MS Experiment. Bold and Underlined Amino Acids indicate Site-specific Mutation Ile^{41}. Bold Amino Acids indicate biotin-PEG_4 Tags. All Amino Acid Mutations were Confirmed via Tandem Mass Spectrometry

Product	Observed peptides	Molecular weight		Sequence
		Found (Da)	Theoretical (Da)	
All	1–13	1490.37	1490.64	<EPDAINAPVTCCY
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
Ile^{41}	29–43	1804.75	1805.00	RRITSSKCPKEA**I**IF
Ile^{41}, $Lys(X)^{69}$	29–43	1804.75	1805.00	RRITSSKCPKEA**I**IF
Ile^{41}, $Lys(X)^{75}$	29–43	1804.75	1805.00	RRITSSKCPKEA**I**IF
All	44–59	1914.77	1914.04	KTIVAKEICADPKQKW
MCP-1, Ile^{41}	60–76	1970.66	1970.94	VQDSMDHLDKQTQTPKT
Ile^{41}, $Lys(X)^{69}$	60–76	2443.85	2443.64	VQDSMDHLD**K(X)**QTQTPKT
Ile^{41}, $Lys(X)^{75}$	60–76	2443.85	2443.64	VQDSMDHLDKQTQTP**K(X)**T

X, biotin-PEG_4; <E, pyroglutamyl.

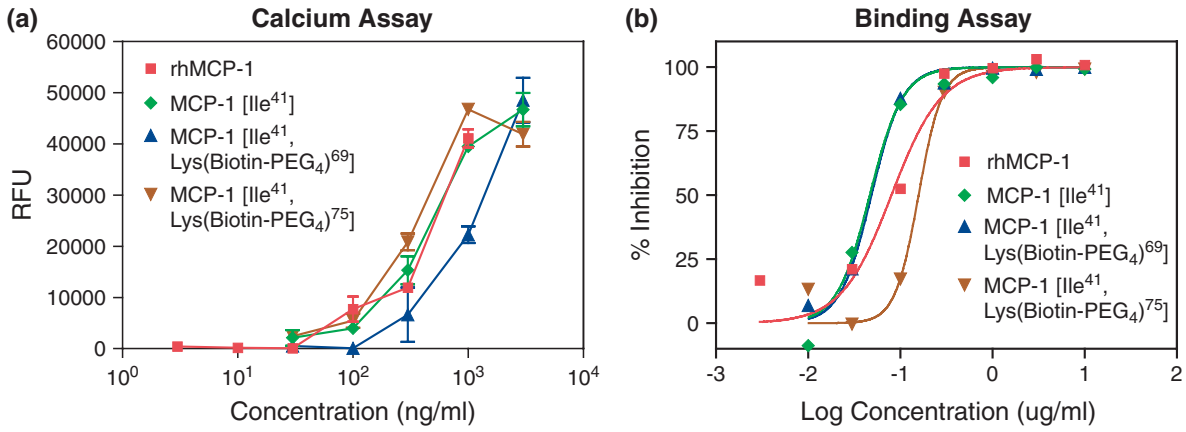

Figure 5 The synthetic analogs and rhMCP-1 were equivalent in inducing mobilization of calcium from internal stores as a result of CCR2 activation on THP-1 cells (a); the synthetic analogs and rhMCP-1 were equivalent in competing with the binding of ^{125}I-rhMCP-1 to CCR2 receptors on THP-1 cells (b). Data is expressed as the percent inhibition of ^{125}I-rhMCP-1 binding at each concentration of synthetic analog and results from the mean of two data points.

were analyzed using a Voyoger DE-STR MALDI TOF MS. The data from this experiment was used to determine whether there were alternate disulfide-bond linkages present in the samples. A list of the observed peptides from the Lys-C digestions of the 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; 345.4 Da); L1 to <EPDAINAPVTCCYNFTNRK (1–19; 2136.4 Da) (<E: pyroglutamyl) and L6 to EICADPK (50–56; 773.9 Da). Since Cys^{11} and Cys^{12} in peptide L1 are adjacent to each other, it was not possible to make a definitive linkage assignment as to which peptides are bound to which cysteines in peptide L1. The peptide fragments produced by the enzymatic digest of the synthetic proteins and rhMCP-1 (279-MC-050/CF; R&D Systems) were analyzed and found to be identical.

The synthetic MCP-1[Ile^{41}] analogs with biotin-PEG_4 attached to lysine residues at positions 69 or 75 showed bioactivity similar to rhMCP-1. As shown in Figure 5a, the MCP-1 [Ile^{41}], [Ile^{41}, $Lys(biotin\text{-}PEG_4)^{69}$], [$Ile^{41}$, $Lys(biotin\text{-}PEG_4)^{75}$], and rhMCP-1 were equivalent in inducing intracellular Ca^{2+} mobilization on THP-1 cells as a result of CCR2 activation. Figure 5b shows the synthetic MCP-1 [Ile^{41}], [Ile^{41}, $Lys(biotin\text{-}PEG_4)^{69}$], [$Ile^{41}$, $Lys(biotin\text{-}PEG_4)^{75}$], and rhMCP-1 competed with

similar potency in an assay of ^{125}I-rhMCP-1 binding to CCR2 receptors on THP-1 cells. These data confirm that site-specific biotinylation of Lys^{69} and Lys^{75} did not alter the biological activity.

CONCLUSIONS

Human MCP-1 [Ile^{41}] and its two site-specific biotinylated analogs, [Ile^{41}, Lys(biotin-PEG$_4$)^{69}] and [Ile^{41}, Lys(biotin-PEG$_4$)^{75}], were chemically synthesized and found to have comparable activities to rhMCP-1, demonstrating the feasibility of chemical synthesis for production of small proteins with site-specific modifications that cannot be achieved by recombinant expression systems.

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