

## Functional Comparison of Two Human Monocyte Chemotactic Protein-2 Isoforms, Role of the Amino-Terminal Pyroglutamic Acid and Processing by CD26/Dipeptidyl Peptidase IV<sup>†</sup>

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**ABSTRACT:** Human Monocyte Chemotactic Protein (MCP)-2 has originally been isolated from stimulated osteosarcoma cells as a chemokine coproduced with MCP-1 and MCP-3. Here, a 5'-end extended MCP-2 cDNA was cloned from a human testis cDNA library. It encoded a 76 residue MCP-2 protein, but differed from the reported bone marrow-derived MCP-2 cDNA sequence in codon 46, which coded for a Lys instead of a Gln. This MCP-2Lys<sub>46</sub> variant, caused by a single nucleotide polymorphism (SNP), was biologically compared with MCP-2Gln<sub>46</sub>. The coding regions were subcloned into the bacterial expression vector pHEN1, and after transformation of *Escherichia coli*, the two MCP-2 protein variants were recovered from the periplasm. The recombinant proteins were purified to homogeneity by heparin–Sephacryl affinity chromatography and reversed-phase HPLC. Edman degradation revealed a Gln residue at the NH<sub>2</sub> terminus instead of a pGlu. To evaluate the influence of the cyclization, this Gln was chemically converted into pGlu in both MCP-2 variants. The conversion was confirmed by electrospray mass spectrometry. rMCP-2Gln<sub>46</sub> and rMCP-2Lys<sub>46</sub> and the NH<sub>2</sub>-terminal cyclic counterparts were tested on monocytic cells in calcium mobilization and chemotaxis assays. No significant difference in biological activity was observed between the rMCP-2Gln<sub>46</sub> and rMCP-2Lys<sub>46</sub> isoforms. However, for both MCP-2 variants the NH<sub>2</sub>-terminal pyroglutamate was shown to be essential for chemotaxis, but not for calcium mobilization. NH<sub>2</sub>-terminal truncation of rMCP-2Lys<sub>46</sub> by the serine protease CD26/dipeptidyl peptidase IV (CD26/DPP IV) resulted in the cleavage of the NH<sub>2</sub>-terminal Gln-Pro dipeptide, whereas synthetic MCP-2 with an amino-terminal pGlu remained unaffected. CD26/DPP IV-clipped rMCP-2Lys<sub>46</sub>(3–76) was almost completely inactive in both chemotaxis and signaling assays. These observations indicate that the NH<sub>2</sub>-terminal pGlu in MCP-2 is necessary for chemotactic activity but also that it protects the protein against degradation by CD26/DPP IV.

Chemokines constitute a heterogeneous group of small proinflammatory cytokines, which induce the migration and activation of leukocytes. These recently discovered cytokines are now key targets of biomedical research, as some of these possess anti-HIV activities and certain receptors for chemokines were shown to serve as fusion cofactors for HIV infection (1–3). At present, chemokines are structurally classified in two main families: CXC and CC chemokines

(4–6). More recently, the C chemokine lymphotactin (7) and the CX<sub>3</sub>C chemokine fractalkine/neurotactin (8, 9) were identified as chemoattractants.

The monocyte chemotactic proteins (MCPs)<sup>1</sup> may be considered as a subfamily of the CC chemokines (10, 11). From the chromosomal region 17q11.2, a YAC contig that contained most of the CC chemokine genes was isolated (11). From the latter YAC contig, the gene for monocyte chemotactic protein-2 was cloned and the existence of two polymorphic MCP-2 gene variants (encoding a Gln<sub>46</sub> or a Lys<sub>46</sub>) was established (12). Natural MCP-2 protein was previously purified from tumor cell-derived conditioned medium and characterized as a chemotactic factor for

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<sup>‡</sup> The testis MCP-2 cDNA sequence has been deposited at the EMBL Nucleotide Sequence Database under the Accession Number Y16645.

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<sup>1</sup> Abbreviations: cDNA, complementary DNA; DPP IV, dipeptidyl peptidase IV; EST, expressed sequence tag; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; HPLC, high-performance liquid chromatography; MCP, monocyte chemotactic protein; pGlu, pyroglutamic acid; PCR, polymerase chain reaction; rMCP, recombinant MCP; SNP, single nucleotide polymorphism; YAC, yeast artificial chromosome.

monocytes (13). From a human bone marrow cDNA library, a full-size MCP-2 cDNA of ~900 bp was cloned, which encoded a Gln residue at position 46 in the mature protein. The occurrence of other longer MCP-2 mRNA transcripts in testis and small intestinal tissue was inferred from Northern blot analysis (12, 14), but the biochemical nature of these longer transcripts was not yet studied.

Structurally, MCPs are characterized by the presence of an amino-terminal pyroglutamic acid residue, which precludes these for Edman degradation (10). It is not yet clear whether this pyroglutamate is essential for biological activity or not. In mammalian species, pyroglutamic acid as the amino-terminal residue in proteins is most probably formed after cyclization of glutamine prior to the secretion of the folded protein (15). It is speculated that this post-translational modification is an enzymatic reaction mediated by glutaminy cyclase (16–18). It has also been observed that amino-terminal glutaminy residues can be nonenzymatically converted to pyroglutamyl residues under various physicochemical conditions (19, 20). In the case of specific peptide hormones, the role of this pyroglutamyl residue for biological activity has been shown (21–23).

Recently, it has been demonstrated that the serine protease CD26/dipeptidyl peptidase IV (CD26/DPP IV; EC 3.4.14.5) cleaves specific chemokines (24, 25). CD26/DPP IV occurs as a membrane-bound or secreted enzyme that hydrolyzes the first two amino acids from peptides with a Pro, Hyp, or Ala in the second position (26, 27). The CD26-truncated form of RANTES, a variant that has been purified from leukocytes or tumor cells, showed reduced chemotactic activity but increased antiviral potency (25).

Here, we document that the apparent chemokine heterogeneity at the genetic level is complemented by considerable post-translational structural and functional variation of an individual chemokine. We describe the cloning of a 5'-end extended MCP-2 cDNA variant from a human testis cDNA library that encodes Lys<sub>46</sub>. The two polymorphic MCP-2 forms (MCP-2Gln<sub>46</sub> and MCP-2Lys<sub>46</sub>) were expressed in *Escherichia coli*, and their biological activities were compared. The amino-terminal glutamine residue was chemically converted into a pyroglutamic acid, and the biological role of this conversion was analyzed. Furthermore, it was shown that rMCP-2Lys<sub>46</sub> with an amino-terminal Gln, in contrast to synthetic MCP-2 with an NH<sub>2</sub>-terminal pGlu, is enzymatically clipped at the amino terminus by CD26/DPP IV. These observations indicate that the pyroglutamic acid plays a dual role: it is necessary to induce chemotactic activity and it serves as a protection against degradation by CD26/DPP IV.

## EXPERIMENTAL PROCEDURES

**Isolation of a Human Testis MCP-2 cDNA Clone and Sequence Analysis.** A human testis-derived cDNA library in pCDM8 (Clontech Laboratories, Palo Alto, CA) was screened with an MCP-2 cDNA probe of 685 bp (14). The probe was radiolabeled by random priming with [ $\alpha$ -<sup>32</sup>P]dCTP (Amersham, Buckinghamshire, U.K.). After two screening rounds, a single colony was isolated, and plasmid DNA was purified. The insert DNA was excised with *Xho*I and subcloned in pBluescript (pBMCP2TA). The construct was sequenced by the dideoxynucleotide termination method with the use of thermosequase (Amersham) and 5'-Cy5-labeled

universal forward and reverse M13 primers. The reaction products were analyzed on an Automated Laser Fluorescent DNA sequencer (ALF Pharmacia, Uppsala, Sweden).

**Construction of the MCP-2 Bacterial Expression Vectors.** Two different 231 bp cDNA fragments, encoding the human MCP-2 mature proteins, were amplified from the plasmid p $\lambda$ 1MCP2A (containing a Gln codon at residue position 46) (14) and from the plasmid pBMCP2TA (containing a Lys<sub>46</sub>) by polymerase chain reaction (PCR) with two specific primers (cMCP2NCO1 = 5'-TACCATGGCCCAGCCAGATTCAGTTTCCATTCCAATC-3' and cMCP2XHO1 = 5'-TACTCGAGTTATCATGGCTTCAGATTTTGA-AATATTTGG-3', respective *Nco*I and *Xho*I sites are underlined). The MCP-2-specific PCR products were eluted from the gel, restriction-digested with *Nco*I and *Xho*I, and ligated into the corresponding sites of the pHEN1 expression vector (28). These constructs (pHENGln and pHENLys) were transformed into JM83 *E. coli* cells. Verification of the sequences of the constructs was done by the dideoxynucleotide termination method as described above.

**Expression of MCP-2 in *Escherichia coli*.** The JM83 bacterial clones containing the pHENGln and pHENLys plasmids were inoculated in LB medium with 50  $\mu$ g/mL ampicillin and 1% glucose and grown overnight at 37 °C. The cells were diluted 1:20 in LB, supplemented with 50  $\mu$ g/mL ampicillin and 0.1% glucose, and grown for 2 h at 37 °C. The *lacZ* promoter was then induced by the addition of 1 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG). Induction was done in Erlenmeyer flasks for 3 h at 37 °C in 400 mL cultures. The periplasmic fractions were isolated by an osmotic shock (29).

**Purification of rMCP-2 and Amino Acid Sequencing.** A 200 mL periplasmic fraction from 8  $\times$  400 mL bacterial culture was dialyzed against equilibration buffer (50 mM NaCl, 50 mM Tris/HCl pH 7.4). The sample was loaded on a heparin–Sephacrose (CL-6B; Pharmacia) column, and the column was washed with equilibration buffer. Different fractions were eluted with a linear NaCl gradient (0.05–2 M) in 50 mM Tris/HCl pH 7.4 and analyzed by SDS–PAGE. The stacking, spacer, and separating gels contained 5% T (total percentage concentration of acrylamide and bisacrylamide) and 5% C (percentage concentration of cross-linker relative to T), 10% T and 3.3% C, and 13% T and 5% C, respectively (30). After electrophoretic separation, the proteins were silver-stained. The following relative molecular mass ( $M_r$ ) markers were used: ovalbumin ( $M_r$  45 000), carbonic anhydrase ( $M_r$  31 000), soybean trypsin inhibitor ( $M_r$  21 500), lysozyme ( $M_r$  14 400), and aprotinin ( $M_r$  6500). The fractions that contained the highest amount of  $\pm$ 8.0 kDa protein were further purified by reversed-phase HPLC on a C-8 Aquapore RP-300 column (4.6  $\times$  220 mm) (Applied Biosystems/Perkin-Elmer, Foster City, CA) and eluted with an acetonitrile gradient (0–80% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid (TFA)). The purity of the different fractions was examined by SDS–PAGE and silver staining. Amino-terminal protein sequence analysis was done on a pulsed liquid (477A/120A) amino acid sequencer (Applied Biosystems/Perkin-Elmer).

**Cyclization of the NH<sub>2</sub>-Terminal Glutamine Residue.** For the cyclization of the NH<sub>2</sub>-terminal Gln residue, HPLC-purified recombinant MCP-2 was lyophilized, resuspended

Table 1: Biochemical Characterization of Recombinant MCP-2 Forms: NH<sub>2</sub>-Terminal Amino Acid Analysis and *M<sub>r</sub>* Determination of rMCP-2 Forms

rMCP-2 form	HPLC fraction	NH <sub>2</sub> -terminal sequence		observed mass (Da) <sup>a</sup>	corrected mass (Da) <sup>b</sup>	theoretical mass (Da)
rMCP-2Gln <sub>46</sub>	56	QPDSVSIP...	major peak <sup>c</sup>	(A): 8903.7 ± 0.9	8912.7 ± 2.5	8910.4
rMCP-2Gln <sub>46</sub>	51 <sup>d</sup>	QPDSVSIP...	single peak	(B): 8926.8 ± 0.8	8926.8 ± 0.8	8910.4
rMCP-2pGlu <sub>1</sub> Gln <sub>46</sub>	51	blocked	single peak	(A): 8884.9 ± 1.2	8893.9 ± 2.8	8893.3
rMCP-2Lys <sub>46</sub>	53–54	QPDSVSIP...	major peak <sup>c</sup>	(A): 8902.8 ± 1.4	8911.8 ± 3.0	8910.4
rMCP-2Lys <sub>46</sub>	47 <sup>d</sup>	QPDSVSIP...	single peak	(B): 8926.9 ± 0.3	8926.9 ± 0.3	8910.4
rMCP-2pGlu <sub>1</sub> Lys <sub>46</sub>	49	blocked	single peak	(A): 8884.5 ± 1.2	8893.5 ± 2.8	8893.4
				(B): 8893.5 ± 0.4	8893.5 ± 0.4	8893.4
rMCP-2Lys <sub>46</sub> (3-76)	50	DSVSIPIT...	ND			

<sup>a</sup> (A) Molecular mass ±SEM determined with a Micromass Autospec mass spectrometer. (B) Molecular mass ±SEM determined with a Sciex single quadrupole LC/MS mass spectrometer. ND: not determined. <sup>b</sup> Various mass spectrometers yielded different mass data with the same standard calibration preparation (rMCP-2pGlu<sub>1</sub>Lys<sub>46</sub>). The original values, obtained on the Micromass instrument (A), were corrected on the basis of the data of the Sciex mass spectrometer (B) that yielded mass data with a smaller range (SEM ≤ 0.8 Da) and with the expected theoretical mass. <sup>c</sup> Major peak represented at least 80% of the sample. <sup>d</sup> These variants, with an amino-terminal glutamine, did not possess the expected mass and were excluded from further biological tests.

in 0.01 M Na<sub>2</sub>HPO<sub>4</sub> pH 8.0, and incubated for 24 h at 37 °C (20). Then, the sample was separated by reversed-phase HPLC on a C-8 Aquapore RP-300 column (1 × 50 mm) (Applied Biosystems/Perkin-Elmer) equilibrated with 0.1% TFA. Elution was done in an 0–100% acetonitrile gradient. Different fractions were analyzed by SDS–PAGE and silver-stained as described above.

**Mass Spectrometry.** Electrospray mass spectra were recorded with a Micromass AutoSpec mass spectrometer fitted with a nanoflow sample introduction probe (Micromass Ltd. Wythenshawe, Manchester, U.K.). The flow rate was 40–50 nL/min, and spectra were acquired at a scan speed of 10 s/decade. The instrument was calibrated with myoglobin. Spectra were processed by the Micromass deconvolution software (giving the molecular weights, method A in Table 1). Additional electrospray mass spectra were determined with a Single Quadrupole LC/MS Mass Spectrometer (API 150EX) (Perkin-Elmer Sciex Instruments, Concord, Ontario, Canada). The spectra were processed with the LC2Tune 1.3 software (giving the molecular weights, method B in Table 1). One sample, rMCP-2pGlu<sub>1</sub>Lys<sub>46</sub> (fraction 49), was used as an internal control and yielded an absolute mass difference of 9.0 ± 1.6 Da compared to the mass obtained with the Micromass Autospec mass spectrometer.

**Incubation of MCP-2 with CD26/DPP IV and Detection of Proteolytic Processing.** Human CD26/DPP IV was obtained from prostasomes (prostate-derived organelles), which occur freely in seminal plasma. The enzyme was purified to homogeneity as described before using ion exchange followed by affinity chromatography onto adenosine deaminase (31).

A 1000-fold molar excess of synthetic MCP-2 (2 μg) with an NH<sub>2</sub>-terminal pyroglutamic acid (32) or of RP-HPLC-purified recombinant MCP-2 (2 μg) with an NH<sub>2</sub>-terminal glutamine was incubated overnight at 37 °C with CD26/DPP IV in 100 mM Tris/HCl pH 7.7. As a positive control for CD26/DPP IV activity, a similar digestion was performed with 2 μg of recombinant RANTES (PeproTech, Rocky Hill, NJ) plus 2 μg of synthetic MCP-2. In all instances, MCP-2 was separated from CD26/DPP IV by SDS–PAGE as described above. Proteins were electroblotted on a Problott membrane (Applied Biosystems/Perkin-Elmer) and stained with Coomassie brilliant blue R250. After destaining, the

membrane was rinsed at least 5 times with ultrapure water (Milli Q; Millipore, Bedford, MA). CD26/DPP IV-treated chemokines excised from PVDF blots were NH<sub>2</sub>-terminally sequenced by Edman degradation as described above.

To obtain sufficient amounts of truncated recombinant MCP-2 for functional assays, 30 μg of RP-HPLC-purified recombinant MCP-2 with an NH<sub>2</sub>-terminal Gln was treated with CD26/DPP IV at the same enzyme/substrate ratio of 1:1000, and the cleavage product was acidified with 0.1% TFA. Tween 20 (0.01%) was added to prevent adsorption of the protein to the test tube. The chemokine was separated from CD26/DPP IV by reversed-phase HPLC on a C-8 Aquapore RP-300 column (1 × 50 mm) (Applied Biosystems/Perkin-Elmer) and eluted in an acetonitrile gradient. Protein fractions were analyzed by SDS–PAGE and silver staining and NH<sub>2</sub>-terminally sequenced by Edman degradation.

**Chemotaxis Assays.** Chemotaxis tests with myelomonocytic cells (THP-1 cell line) were performed as described (33). Briefly, in the lower compartments, dilutions of test samples were pipetted (27 μL). The upper compartments of the microchambers (Neuroprobe Inc., Cabin John, MC) were filled with a THP-1 cell suspension (50 μL) at a concentration of 0.5 × 10<sup>6</sup> cells/mL. The cells were allowed to migrate through a 5-μm-pore poly(vinylpyrrolidone)-treated polycarbonate membrane for 2 h. After fixation and staining, migrated cells were counted and the chemotactic index was calculated as the number of cells migrated to the test sample divided by the number of cells migrated to the control medium.

**Detection of Intracellular Ca<sup>2+</sup> Concentrations.** The intracellular calcium concentration [Ca<sup>2+</sup>]<sub>i</sub> was measured with the use of the fluorescent indicator fura-2. THP-1 cells were grown in RPMI 1640 (GIBCO BRL, Paisley, Scotland) supplemented with 10% FCS (GIBCO BRL). Two days after subcultivation, the cells were harvested and incubated (10<sup>7</sup> cells/mL) with the fluorescent indicator fura-2 (fura-2/AM 2.5 μM; Molecular Probes Europe BV, Leiden, The Netherlands) and 0.01% Pluronic F-127 (Sigma, St. Louis, MO) in Eagle's minimum essential medium (EMEM; GIBCO BRL) supplemented with 0.5% FCS. After 30 min at 37 °C, the cells were washed twice and resuspended at 10<sup>6</sup> cells/mL in HBSS with 1 mM Ca<sup>2+</sup> and 0.1% FCS (buffered at pH 7.4 with 10 mM Hepes/NaOH). The cells



were incubated for 10 min at 37 °C before fura-2 fluorescence was measured in an LS50B luminescence spectrophotometer (Applied Biosystems/Perkin-Elmer). Upon excitation at 340 and 380 nm, fluorescence was detected at 510 nm. The  $[Ca^{2+}]_i$  was calculated using the Grynkiewicz equation (34). To determine  $R_{max}$ , the cells were lysed with 50  $\mu$ M digitonin. Subsequently, the pH was adjusted to 8.5 with 20 mM Tris and  $R_{min}$  was obtained by addition of 10 mM EGTA to the lysed cells. The  $K_d$  used was 224 nM.

Desensitization experiments were performed with murine 3T3 cells, transfected with CD4 and CCR5 (35), as responsive cells. These cells (CD4/CCR5/3T3) were grown to confluency in Dulbecco's minimum essential medium (DMEM, GIBCO BRL) supplemented with 10% FCS, 4.5 g/L glucose and 584 mg/L L-glutamine (Bio Whittaker, Boehringer Ingelheim Bioproducts Partnership, Verviers, Belgium). Puromycin (1  $\mu\text{g/mL}$ ; Sigma) was added to the medium as a selection agent. Trypsinized cells ( $10^7$  cells/mL) were incubated in the culture medium with 2.5  $\mu\text{M}$  fura-2 and 0.01% Pluronic F-127, and the  $[\text{Ca}^{2+}]_i$  was determined as described above. The cells were first stimulated with buffer or recombinant MCP-2 at different concentrations. As a second stimulus, recombinant RANTES (PeproTech) was used at a concentration of 100 ng/mL, inducing a significant increase in the  $[\text{Ca}^{2+}]_i$  after prestimulation with buffer. The second stimulus was applied 100 s after the first one.

## RESULTS

(1) *Isolation of a Human Testis MCP-2 cDNA Clone and Sequence Analysis.* In most tissues the major MCP-2 transcript is ~900 bases long. The presence of an extended MCP-2-mRNA transcript (~1.5 kb) in human testis tissue was previously documented by Northern blot analysis (12). In accordance with this, a human testis cDNA library was screened and this longer transcript was isolated. After two selection rounds of colony hybridizations with a radioactively labeled human MCP-2 cDNA probe (14), several positive clones were isolated. The clones were tested for insert length, and the longest insert was subcloned into pBluescript (pBMCP2TA). With the use of M13 fluorescent labeled primers, the sequence was determined (Figure 1). The testis MCP-2 cDNA was 1368 bp in length and contained a Lys codon at position 46 instead of Gln<sub>46</sub> in the shorter cDNA from bone marrow. Furthermore, the size difference of 416 nucleotides (between the short transcript and the testis transcript) was due to 5'-end extension. Both transcripts encoded mature MCP-2 proteins of 76 residues.

(2) *Cloning of the Coding Regions of Two Allelic Variants of MCP-2 cDNA in pHEN1 and Bacterial Expression.* To compare the two polymorphic MCP-2 forms, these two variants were expressed in *E. coli*. The construction of the bacterial MCP-2 expression vectors (pHENGln and pHENLys) is detailed in the Materials and Methods section and illustrated in Figure 2. The 231 bp PCR fragments coding for the rMCP-2Gln<sub>46</sub> and rMCP-2Lys<sub>46</sub> were directly cloned in the pHEN1 vector and confirmed by sequence analysis to exclude PCR-mediated mutations. The DNA sequences were identical to the published bone marrow MCP-2 cDNA sequence and the testis MCP-2 cDNA sequence, respectively. Bacterial cells containing the pHENGln and pHENLys

ATCATTTGTGCTCTAAAGTGATGGAGAGCACCAGCAAGCCTTAGGGGCCATCCCTGGCCCTCTTGATCCACAGAGGGG	80
TAGGCCCTTGGCTCTCTCCATATGACGTACGTTCCATTCTTCTTTCTATAGACAATTTCCATTTCAAGGAAATC	160
AGAGCCCTTAATAGTTTCAGTGAGGTCACTTTGTGAGACAATCCATCAACTCTTCAGCCTCTGCTCCACAGAGCCTTAAGC	240
AAAGATAGAAACAACAATCTCTGTTTGTTCATTTGGAATATCCAGGACTGTGGCTTAACGATCAGATATTCAG	320
GAGGCTTTACTTCTTCTTCTGTTTGTGACCATCCGAGGCTCTGTGCTCCCTATAGAGCAGAGGACGAGCA	400
GGAGCAGAGAGGTTGAGAACACCCAGAAACCTTCACCTCTCATGCTGAAGCTCACACCTTGCCCTCCAAGTGAAGGT	480
MetLysVal -23	
TTCTGCAGCGCTTCTGTGCTGCTGCTCATGGCAGCACTTTCAGCCCTCAGGGAAGCTTGCACGCCAGATTCA	560
SerAlaAlaLeuLeuLysLeuLeuLeuMetAlaAlaThrPheSerProGlnGlyLeuAlaGlnProAspSerValSer	
-20 -10 1	
TTCCAATCAGCTGCTGTTTAAAGTGATCAATAGGAAATTCCTATCCAGAGGCTGGAGAGCTACACAAGATCACCAC	640
LeProIleThrCysCysPheAsnValIleAsnArgLysIleProIleGlnArgLeuGluSerThrYrrThrIleThrAsn	
10 20 30	
ATCCAATGCTCCAAGGAAGCTGTGATCTTCAAGACAACAGGGGCAAGGAGGCTGTGCTGACCCCAAGGAGAGATGGGT	720
IleGlnCysProLysGluAlaValIlePheLeuThrArgGlnGlyLysGluValCysAlaAspProLysGluArgTrpVal	
40 50 60	
CAGGAGATCCATGAGCATCTGGACAAATATTTCAAATTCAGGACCTGAGGCTTCATCATGGAATGAGAGTCAGAG	800
ArgAspSerMetLysHisLeuAspGlnIlePheGlnAsnLeuLysPro	
70 76	
CTTGAAGAAAAGCTATTATTTATTTCCCAACCTCCCCAGGTGCAGTGTGACATATTTTATATAACATCCACAAGAG	880
ATTATTTTAAATAATTAAGACATAATATCTCTAAAAAGATTATATATATTAAGTGTGTGATGTTTAACTCTAT	960
CTGTCTATACATCTAGTGAATGTAAATGCAAAATCTGGTGATGTGTTTGTGTTTCTGTGAGCTCAACTA	1040
AGTTTCAGGCAAAATGTCAATTGCTCTCTTACCCTGCTGTGATGTTGTGGGGTCCCTCCATGGATCATCAAGGTAA	1120
ACATCTTGGTATCTTGGCAATCAGTGCTCTGTGAAGTCAATGTGTGCTTGTACTGCTGTGTGTTGAATGTATGTTA	1200
CTGTATATAACATAGTGAATTTAAAAAAAATTTCAAAAAAGAAAATATATATATATATTTAAACCT (A) <sub>102</sub>	1360

FIGURE 1: Human testis MCP-2 cDNA sequence. The insert DNA of the pCDM8 MCP-2 cDNA clone was sequenced after subcloning in pBluescript. The cDNA and the derived amino acid sequences are shown. The protein coding sequence is in bold, and the conserved cysteine residues and the substitution resulting in a protein polymorphism at aa position 46 are underlined. In the 3'-end region, mRNA-destabilizing signals are underlined. The protein numbering is based on the mature protein.

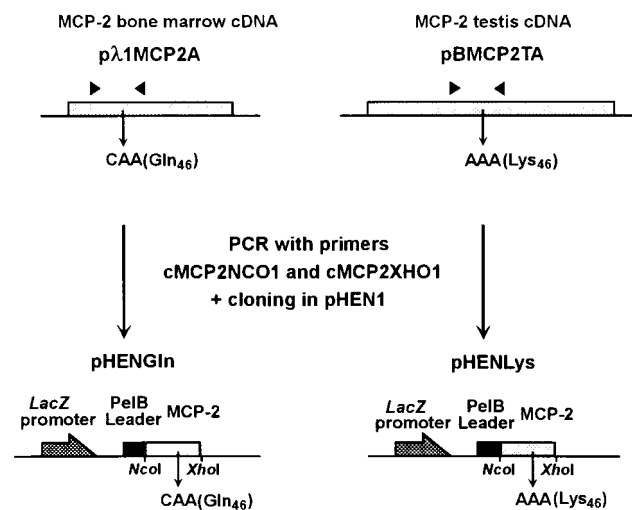


FIGURE 2: Construction of the vectors for expression of MCP-2 in bacteria. With the clones p1MCP2A (a human bone marrow-derived MCP-2 cDNA clone, containing a Gln<sub>46</sub> codon) and pBMCPTA (a human testis-derived MCP-2 cDNA clone, containing a Lys<sub>46</sub> codon) as templates for PCR, respectively, two fragments were amplified. These fragments, provided with *Nco*I and *Xho*I restriction sites by the amplification primers, were directly cloned in the expression vector pHEN1 to generate the expression constructs pHENGln and pHENLys. The expression is under the control of the *LacZ* promoter, and the expression product is secreted into the periplasm through the PelB signal peptide.

expression constructs were induced with 1 mM IPTG. The presence of recombinant protein in the periplasm and supernatant fractions was analyzed with SDS-PAGE followed by immunodetection with a specific antiserum against synthetic MCP-2 (data not shown).

(3) *Purification of Recombinant MCP-2Gln<sub>46</sub> and MCP-2Lys<sub>46</sub> and Characterization.* The periplasmic fractions of the pHENGln and pHENLys cultures were dialyzed and

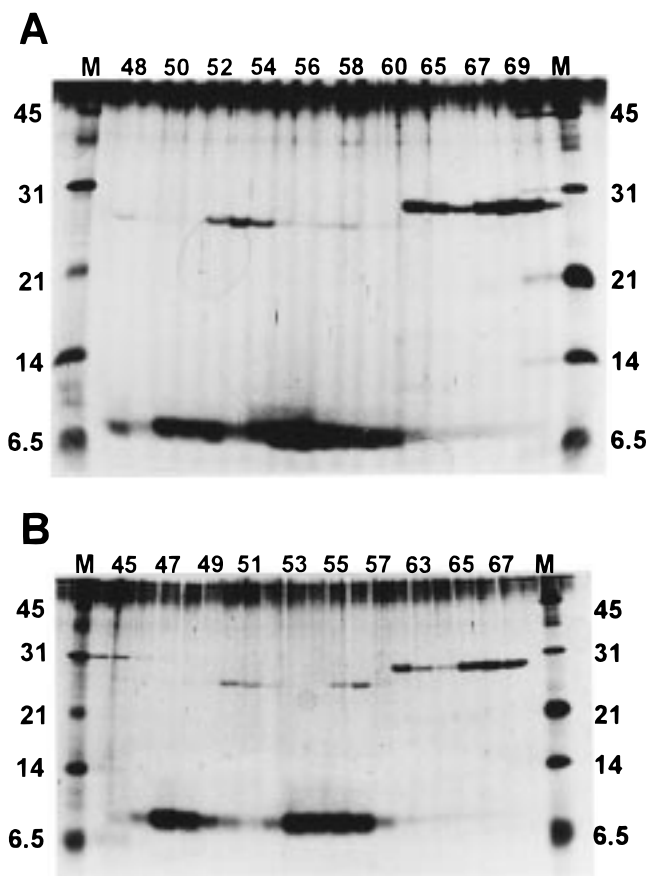


FIGURE 3: Purification of rMCP-2Gln<sub>46</sub> and rMCP-2Lys<sub>46</sub>. rMCP-2Gln<sub>46</sub> and rMCP-2Lys<sub>46</sub> from heparin–Sephacryl affinity chromatography were loaded on a reversed-phase HPLC column. Proteins were eluted with a linear acetonitrile gradient. Silver-stained SDS–PAGE analysis of RP–HPLC fractions 48–69 of rMCP-2Gln<sub>46</sub> (A) and fractions 45–67 of rMCP-2Lys<sub>46</sub> (B) are shown. Relative molecular mass standards are indicated (kDa).

purified by heparin–Sephacryl affinity chromatography, and the fractions were analyzed by SDS–PAGE. In both cases, a 6.5 kDa protein was eluted between 0.6 and 1.2 M NaCl. Fractions 11–15, containing this 6.5 kDa protein, were further purified to homogeneity by reversed-phase HPLC (Figure 3). The total amount of purified recombinant chemokine recovered from the 3.2 L culture was estimated to be  $\pm 500 \mu\text{g}$  for rMCP-2Gln<sub>46</sub> and  $\pm 200 \mu\text{g}$  for rMCP-2Lys<sub>46</sub>, corresponding to  $\pm 150$  and  $\pm 60 \mu\text{g/L}$ , respectively. For both MCP-2 variants two  $A_{220}$  absorbency peaks were observed after RP–HPLC separation corresponding to two zones of 6–7 kDa proteins upon SDS–PAGE analysis. In contrast to natural human MCP-2, the NH<sub>2</sub>-terminal residue of the recombinant proteins was a Gln instead of a pyroglutamic acid (pGlu). This enabled us to sequence the recombinant product by Edman degradation and to analyze the chemical conversion of the NH<sub>2</sub>-terminal glutamine into a pyroglutamate. Except for this NH<sub>2</sub>-terminal amino acid, the resulting sequence (Gln–Pro–Asp–Ser–Val–Ser–Ile–...) corresponded to the NH<sub>2</sub>-terminal sequence of mature natural human MCP-2 (Table 1).

(4) *Conversion of the NH<sub>2</sub>-Terminal Gln into pGlu.* To evaluate the influence of the cyclization of NH<sub>2</sub>-terminal Gln into pGlu on the biological activity, we converted the recombinant MCP-2 isoforms to NH<sub>2</sub>-terminally blocked proteins and confirmed the chemical cyclization by mass

spectrometry analysis. After chemical modification of  $\pm 20 \mu\text{g}$  of rMCP-2Gln<sub>46</sub> (fraction 56) and  $\pm 50 \mu\text{g}$  of rMCP-2Lys<sub>46</sub> (fractions 53–54), the variants were again purified by RP–HPLC. The obtained fractions were electrophoretically pure as analyzed by SDS–PAGE (not shown). Appropriate fractions from the RP–HPLC separation of chemically modified rMCP-2Gln<sub>46</sub> and rMCP-2Lys<sub>46</sub> (i.e., rMCP-2pGlu<sub>1</sub>Gln<sub>46</sub> and rMCP-2pGlu<sub>1</sub>Lys<sub>46</sub>) were subjected to Edman degradation. Although sufficient amounts of protein were loaded on the sequencer, no sequence was detected. This indicated that the fractions predominantly contained blocked protein and that the conversion of the NH<sub>2</sub>-terminal Gln into pGlu had occurred. To determine the molecular composition of the rMCP-2 variants, converted and nonconverted samples were analyzed by electrospray mass spectrometry. The results are shown in Table 1. In RP–HPLC samples of nonconverted rMCP-2Gln<sub>46</sub> (fraction 56) and rMCP-2Lys<sub>46</sub> (fractions 53–54), a major and a minor signal (not shown in Table 1) with a difference in molecular mass of 17 daltons were detected. The latter difference corresponded to the molecular mass of NH<sub>3</sub> and is probably due to spontaneous conversion of Gln into pGlu. After induced chemical cyclization, single molecular mass peaks were obtained that corresponded to that of blocked rMCP-2pGlu<sub>1</sub>Gln<sub>46</sub> and rMCP-2pGlu<sub>1</sub>Lys<sub>46</sub>, respectively. Additional sequencing and mass determination experiments were performed to analyze the rMCP-2 variants that are shown in Figure 3: rMCP-2Gln<sub>46</sub> (fraction 51) and rMCP-2Lys<sub>46</sub> (fraction 47). Edman degradation revealed an NH<sub>2</sub>-terminal Gln in both fractions. The amino-terminal sequences as well as the mass data are shown in Table 1. These fractions contained most probably oxidized forms of the respective MCP-2 variants and were not further analyzed. Samples of biochemically characterized MCP-2 variants, of which the corrected mass corresponded with the theoretical mass (Table 1), were biologically compared in *in vitro* assays.

(5) *Treatment of Synthetic MCP-2 and rMCP-2Lys<sub>46</sub> with CD26/DPP IV.* Synthetic MCP-2, with a Lys at position 46 and an NH<sub>2</sub>-terminal pGlu (32), was treated with CD26/DPP IV, blotted on a PVDF membrane, and stained with Coomassie blue. After Edman degradation, no NH<sub>2</sub>-terminal sequence was detected. This indicated that the NH<sub>2</sub>-terminal dipeptide of MCP-2, which is blocked by a pGlu, was not cleaved by CD26/DPP IV. To investigate whether the pGlu in MCP-2 prevents the cleavage by CD26/DPP IV, we treated the rMCP-2Lys<sub>46</sub> (with a Gln at position 1) in the same way. In this particular case, CD26/DPP IV removed the NH<sub>2</sub>-terminal dipeptide. This treatment yielded the truncated molecule rMCP-2Lys<sub>46</sub>(3–76). Truncated rMCP-2Lys<sub>46</sub>(3–76), separated from CD26/DPP IV by RP–HPLC, was sequenced by Edman degradation (Table 1) and further analyzed in calcium mobilization and chemotaxis assays.

(6) *Calcium Mobilization and Chemotaxis of THP-1 Monocytic Cells.* The different recombinant MCP-2 forms were tested for Ca<sup>2+</sup> mobilization on monocytic THP-1 cells at concentrations from 10 to 100 ng/mL. All the tested recombinant MCP-2 forms induced a transient rise in the [Ca<sup>2+</sup>]<sub>i</sub> in a dose-dependent manner (Figure 4). There was no difference in potency between MCP-2 isoforms with or without an amino-terminal pyroglutamate. In addition, rMCP-2Lys<sub>46</sub> and rMCP-2Gln<sub>46</sub> (both with an NH<sub>2</sub>-terminal Gln) were also equipotent in the calcium mobilization assay.

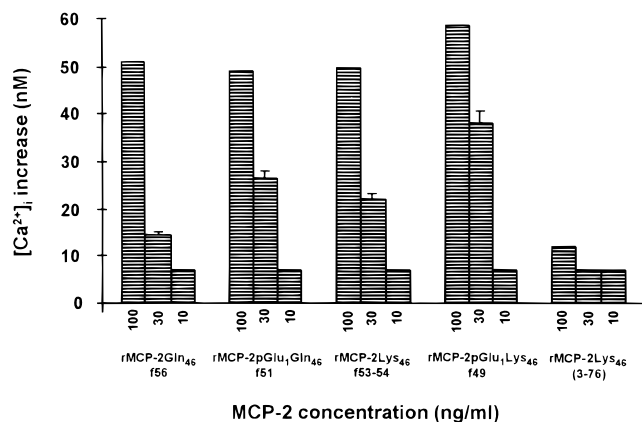


FIGURE 4: Induction of an increase in  $[Ca^{2+}]_i$  in THP-1 cells by rMCP-2 isoforms. THP-1 cells, loaded with fura-2/AM, were stimulated with different concentrations of rMCP-2Gln<sub>46</sub> (RP-HPLC fraction 56), rMCP-2pGlu<sub>1</sub>Gln<sub>46</sub> (RP-HPLC fraction 51), rMCP-2Lys<sub>46</sub> (RP-HPLC fractions 53–54), and rMCP-2pGlu<sub>1</sub>Lys<sub>46</sub> (RP-HPLC fraction 49). The  $[Ca^{2+}]_i$  (nM) was calculated by the method of Grynkiewicz et al. (34) as described in the Materials and Methods. Data shown for 30 ng/mL are the mean of two independent experiments. The detection limit for the increase in  $[Ca^{2+}]_i$  was 10 nM.

The minimal effective concentration was in all cases 30 ng/mL. However, when rMCP-2Lys<sub>46</sub> with an NH<sub>2</sub>-terminal Gln residue was cleaved by CD26/DPP IV into truncated rMCP-2Lys<sub>46</sub>(3-76), its ability to induce calcium mobilization was reduced 3–10 times (Figure 4).

The monocyte chemotactic potency of the different recombinant MCP-2 forms was compared in the microchamber chemotaxis assay with THP-1 cells. Synthetic MCP-2, with a Lys at position 46 and an NH<sub>2</sub>-terminal pGlu (32), was used as a positive control in these assays. The minimal effective dose for natural and synthetic MCP-2 in  $Ca^{2+}$  mobilization experiments is about 10 times higher than in chemotaxis assays (36). As shown in Figure 5A, rMCP-2pGlu<sub>1</sub>Gln<sub>46</sub> and rMCP-2pGlu<sub>1</sub>Lys<sub>46</sub> are still active at 5 ng/mL, whereas the minimal effective chemotactic concentration of rMCP-2Gln<sub>46</sub> and rMCP-2Lys<sub>46</sub> (both with an NH<sub>2</sub>-terminal Gln) is at least 10 times higher. As in the calcium assay, we did not observe a significant difference in activity between rMCP-2pGlu<sub>1</sub>Gln<sub>46</sub> and rMCP-2pGlu<sub>1</sub>Lys<sub>46</sub>. As can be seen in Figure 5B, the chemotactic activity of rMCP-2Lys<sub>46</sub>(3-76) was 20 times lower than that of synthetic MCP-2 (sMCP-2Lys<sub>46</sub>(1-76)), containing an NH<sub>2</sub>-terminal pyroglutamate.

(7) *Signaling of MCP-2 Forms via CCR5*. In view of the inactivity of rMCP-2Lys<sub>46</sub>(3-76) in monocyte chemotaxis and calcium mobilization experiments on THP-1 cells, we tested whether this truncated recombinant MCP-2 can still signal through CCR5, recently described as a functional receptor for intact MCP-2 (37). At a concentration of 300 ng/mL, rMCP-2pGlu<sub>1</sub>Lys<sub>46</sub> caused a strong calcium response in CCR5 transfectants and the subsequent  $[Ca^{2+}]_i$  increase in response to 100 ng/mL RANTES was strongly desensitized (Figure 6). Alternatively, pretreatment of the cells with 1000 ng/mL rMCP-2Lys<sub>46</sub>(3-76) induced only a weak response and minimally reduced the  $[Ca^{2+}]_i$  increase of RANTES (100 ng/mL). At 100 ng/mL, rMCP-2pGlu<sub>1</sub>Lys<sub>46</sub> induced a similar response pattern as 1000 ng/mL rMCP-2Lys<sub>46</sub>(3-76) (data not shown). In analogy with the calcium mobilizing data on THP-1 cells, the activity of truncated rMCP-2Lys<sub>46</sub>(3-76) on CCR5 transfected cells was reduced 10 times in comparison with that of rMCP-2pGlu<sub>1</sub>Lys<sub>46</sub>.

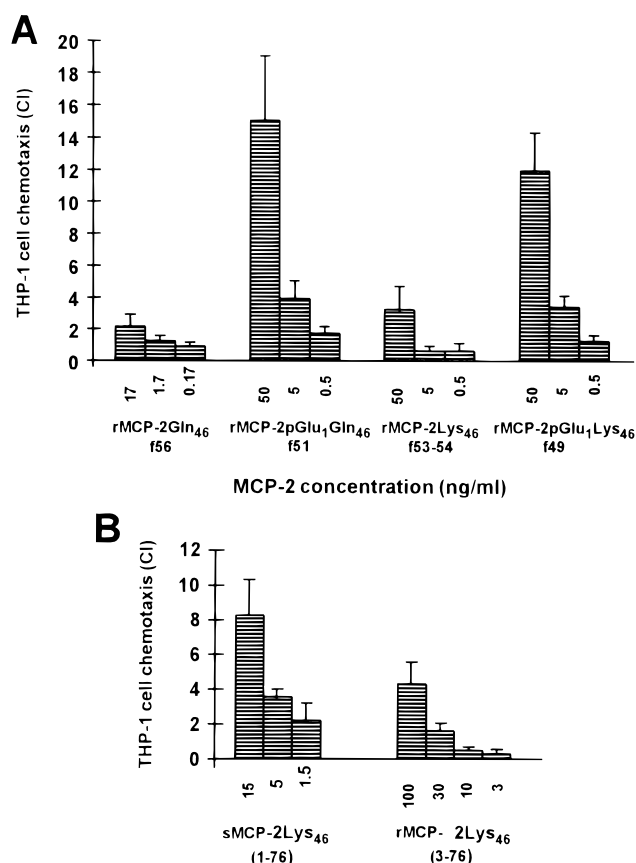


FIGURE 5: Comparison of the chemotactic activity of rMCP-2 isoforms on THP-1 cells. (A) rMCP-2Gln<sub>46</sub> (RP-HPLC fraction 56), rMCP-2pGlu<sub>1</sub>Gln<sub>46</sub> (RP-HPLC fraction 51), rMCP-2Lys<sub>46</sub> (RP-HPLC fractions 53–54), and rMCP-2pGlu<sub>1</sub>Lys<sub>46</sub> (RP-HPLC fraction 49) were compared in chemotaxis assays on THP-1 cells. The mean chemotactic index ( $\pm$ SEM) is derived from at least 5 different experiments (in duplicate). (B) The chemotactic activities of synthetic MCP-2 (sMCP-2Lys<sub>46</sub>(1-76)) and CD26/DPP IV-treated rMCP-2Lys<sub>46</sub>(3-76) were compared in the microchamber assay. The mean chemotactic index ( $\pm$ SEM) is derived from 7 experiments (in duplicate).

(3-76) on CCR5 transfected cells was reduced 10 times in comparison with that of rMCP-2pGlu<sub>1</sub>Lys<sub>46</sub>.

## DISCUSSION

Chemokines are small chemotactic proteins which play key roles in diverse mechanisms of immunity (4–6, 38). Recently, anti-HIV and hematopoietic activities were attributed to these proteins (1, 39, 40). Most of the known CC-chemokine genes are located at human chromosome 17q11.2 (11, 12, 41). Most probably, this gene cluster has originated by gene duplication. Recently, by bioinformatics, that is, EST library searches, several new CC chemokines have been discovered. Some of these are located at other chromosomal sites. For example, the genes for LARC (liver and activation-regulated chemokine) and TARC (thymus and activation-regulated chemokine) have been mapped at chromosomes 2q33–q37 and 16q13, respectively (42, 43). Furthermore, the genes for ELC (EBI1–ligand chemokine) and SLC (secondary lymphoid-tissue chemokine) have been localized at the chromosomal band 9p13 (44, 45).

In this study, we complement and extend the concept of heterogeneity of a single chemokine at several levels: single nucleotide polymorphism, tissue-specific transcript hetero-



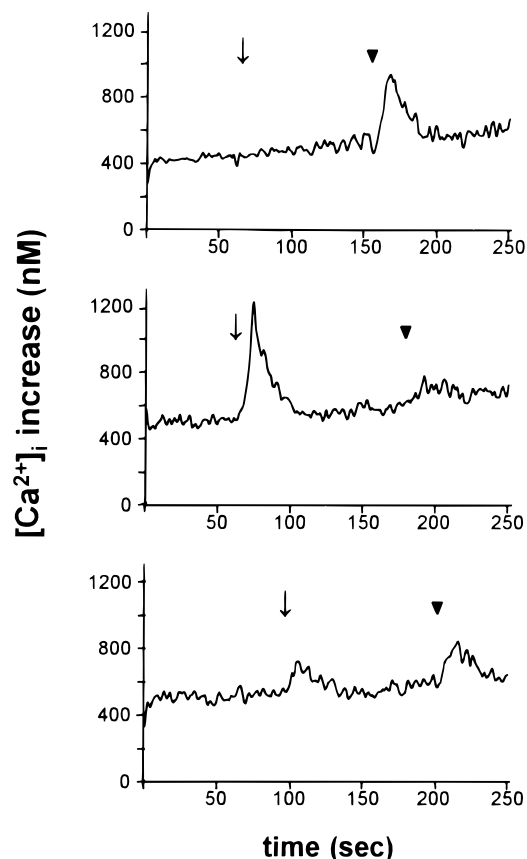


FIGURE 6: Desensitization of  $[Ca^{2+}]_i$  responses induced in CD4/CCR5/3T3 cells by rMCP-2pGlu<sub>1</sub>Lys<sub>46</sub> and rMCP-2Lys<sub>46</sub>(3-76). CD4/CCR5/3T3 cells were first (arrows) stimulated with buffer (upper panel), 300 ng/mL rMCP-2pGlu<sub>1</sub>Lys<sub>46</sub> (middle panel), or 1000 ng/mL rMCP-2Lys<sub>46</sub>(3-76) (lower panel). As a second stimulus (arrowheads), recombinant RANTES was added at a concentration of 100 ng/mL. The  $[Ca^{2+}]_i$  was calculated by the method of Grynkiewicz et al. (34). The results of a representative experiment are shown.

geneity, and post-translational modifications. We studied the role of the amino-terminal residue in MCP-2 by generating recombinant proteins as two polymorphic variants (rMCP-2Gln<sub>46</sub> and rMCP-2Lys<sub>46</sub>). By expression in *E. coli* we had the advantage to express MCP-2 variants with an ultimate glutamine instead of the naturally occurring pyroglutamate.

Human MCP-2 was discovered as a monocyte chemotactic factor coproduced with MCP-1 and MCP-3 (13). Later, the MCP-2 gene was cloned and the shortest (and most abundant) cDNA was isolated from a bone marrow-derived cDNA library (12, 14). In this study, a longer MCP-2 mRNA transcript was isolated from a human testis-derived cDNA library. Besides an extended 5'-untranslated region, its sequence differed from the bone marrow-derived MCP-2 cDNA at codon 46, which encodes a Lys instead of a Gln. These two polymorphic MCP-2 forms were expressed in *E. coli*, and the biological activities of the recombinant MCP-2 proteins were compared *in vitro* for their monocytic cell chemotactic activity and  $Ca^{2+}$  mobilization capacity. In addition, the effect of post-translational modification of the NH<sub>2</sub>-terminal Gln into a pGlu was evaluated on biological activity. Furthermore, synthetic MCP-2 (with a Lys at position 46 and an NH<sub>2</sub>-terminal pGlu) and rMCP-2Lys<sub>46</sub> (with an NH<sub>2</sub>-terminal Gln) were treated with CD26/

dipeptidyl peptidase IV to investigate the role of the NH<sub>2</sub>-terminal pGlu on protein processing by this aminopeptidase which is able to cleave Pro-Xaa bonds.

As demonstrated by the cloning and sequence analysis of two MCP-2 cDNAs, derived from different libraries, the MCP-2 gene contains a single nucleotide polymorphism (SNP). At position 46 in the mature protein, the sequence encodes either a Lys (AAA) or a Gln (CAA). Such sequence heterogeneity has also been observed for human eotaxin cDNA clones (46). In the case of MCP-2, the nonsynonymous mutation imposed by the SNP yielded two protein variants with a similar specific activity in calcium mobilization and chemotaxis assays, and this is irrespective of whether these MCP-2 variants have an NH<sub>2</sub>-terminal glutamine or a pyroglutamate. SNPs are, however, becoming increasingly important in terms of markers for human genomics (47). The MCP-2 gene contains such a reliable SNP marker on human chromosome 17q11.2.

For the monocyte chemotactic proteins (MCP-1, MCP-2, and MCP-3), transcripts of different sizes have been detected on Northern blot analysis (12, 14, 48). Here we show that for the testis MCP-2 transcript of 1.4 kb, this is due to the extension of the first exon at the 5'-end and not by specific splicing or polyadenylation. To document this, we took advantage of our earlier observation demonstrating differential expression of specific MCP-2 transcripts in various tissues. Indeed, it was shown that testis tissue predominantly expresses an MCP-2 transcript which is longer than the most abundant ~900 nucleotide MCP-2 mRNA formed in other tissues (14). The transcripts of 878 and 1368 bases encode proteins of exactly the same size. It is therefore concluded that the RNA polymerase II generates a level of chemokine gene transcript heterogeneity which is tissue-specific but without structural effect on the protein.

In several studies with CC chemokines, but not for MCP-2, it has been shown that the NH<sub>2</sub>-terminal residue is important for biological activity. For example, in MCP-1 it was found that this amino acid is essential for receptor binding and function, as it could not be deleted or extended (49). However, the latter authors suggested that the NH<sub>2</sub>-terminal pyroglutamate residue is not essential since it may be replaced by several noncyclic amino acids without loss of activity. The extension of MCP-3 with three amino acids at its NH<sub>2</sub> terminus resulted in a total loss of activity (50). RANTES(3-68), a naturally occurring variant of RANTES which arises by CD26/DPP IV cleavage, was inactive as a monocyte chemoattractant (25). Here, we show that the pGlu in human MCP-2 is essential for its chemotactic activity. The electrospray mass spectrometry results demonstrate that the initial samples (rMCP-2Gln<sub>46</sub> and rMCP-2Lys<sub>46</sub>) already contained a minor fraction with an amino-terminal pGlu. The presence of this minor fraction might explain the low biological activity detected with these samples. Partial spontaneous cyclization of amino-terminal Gln residues has been observed earlier (21, 22). Many proteins, for example, peptide hormones, have a pyroglutamic acid as an NH<sub>2</sub>-terminal residue. In thyrotropin-releasing hormone (21, 22) and luteinizing hormone-releasing hormone (23), the presence of an amino-terminal pGlu was shown to be necessary for the biological activity. In immunoglobulins, however, the functional activity of this residue was less clear (15). In contrast to its effect on monocyte chemotaxis, the NH<sub>2</sub>-

terminal pyroglutamate is not essential for calcium mobilization. Our study experimentally illustrates the dissociation between these two MCP-2 effects involving receptor interaction. It might well be that another level of receptor triggering is needed for calcium mobilization (e.g., less intrinsic but more generalized ligand receptor binding) than for chemotaxis. Alternatively, there might exist two MCP-2 receptors on THP-1 cells with different ligand interaction depending on the presence or absence of pyroglutamate.

This study further enabled us to demonstrate that the amino-terminal pyroglutamate in human MCP-2 is essential not only for chemotactic activity but also for the stability of the protein. Indeed, the NH<sub>2</sub>-terminal pyroglutamate protects MCP-2 against degradation by CD26/DPP IV and thus against complete loss of bioactivity including calcium mobilization. The latter exopeptidase that cleaves off dipeptides with a penultimate Pro, Hyp, or Ala from small substrates has recently been shown to act on RANTES (24, 25).

In conclusion, the heterogeneity of chemokines is considerable and exists at several levels. Multiple gene clusters are encoding numerous single molecular isoforms. Post-translational modification, yielding an amino-terminal pyroglutamate, is essential in the generation of efficient and stable natural chemokines. Our work also contributes to understand chemokine signal transduction.

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