The Viral CC Chemokine-binding Protein vCCI Inhibits Monocyte Chemoattractant Protein-1 Activity by Masking Its CCR2B-binding Site

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Monocyte chemoattractant protein-1 (MCP-1) is a chemotactic cytokine mainly acting on monocytes and T cells that elicits its biological effects by interacting with the seven-transmembrane helix receptor CCR2B. The vaccinia virus strain Lister and many other poxviruses express soluble proteins (vCKBs) that bind MCP-1 and other CC chemokines and inhibit their function. In order to define the interaction site of MCP-1 with vCKBs from vaccinia, surface exposed residues of MCP-1 were identified and mutated to alanine. The MCP-1 variants were expressed, purified, and their interaction with vCKBs was characterized. The site on MCP-1 for vCKB binding is dominated by arginine 18 with important additional contributions from tyrosine 13 and arginine 24. These residues define a binding site that largely overlaps with the CCR2B receptor interaction site. The viral chemokine-binding protein vCCI thus inhibits the biological function of MCP-1 by directly masking its CCR2B receptor-binding site.

Chemokines are small (8–14 kDa) structurally related proteins that regulate cell trafficking of various leukocyte subtypes through interaction with a set of G protein-coupled receptors. The two major subfamilies are the CXC chemokines which act on neutrophils and non-hemopoietic cells and the CC chemokines which bind to receptors mainly expressed on monocytes, T cells, eosinophils, and basophils. Additional members of the chemokine family are the C chemokine lymphotactin and the CX3C chemokines which bind to a receptor mainly expressed on monocytes, T cells, and basophils. Additional members of the chemokine family are the C chemokine lymphotactin and the CX3C chemokines which bind to a receptor mainly expressed on monocytes, T cells, and basophils.

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The abbreviations used are: vCKBP, virus-encoded chemokine-binding protein; CHO, Chinese hamster ovary; ELISA, enzyme-linked immunosorbent assay; GAG, glycosaminoglycan; HEK.EBNA, human embryonic kidney-Epstein-Barr nuclear antigen; MCP-1, monocyte chemoattractant protein-1; SPR, surface plasmon resonance; vCKB, virus-encoded chemokine-binding protein-2 from vaccinia strain Lister; PBS, phosphate-buffered saline.

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EXPERIMENTAL PROCEDURES

Cloning and Mutagenesis of Human MCP-1—The human MCP-1 gene was cloned from a yeast two-hybrid cDNA library which was synthesized from activated human leukocytes (CLONTECH). The oligonucleotides used to amplify the human MCP-1 gene were caggtcaattctagaTGCCCTCTAGACATGAAAGTTTCGCGC and caggtcaattctagaTGCGAGAATGATGATGATGAGATGATGCTCGAGTTTTTGTTTG (the start and the stop codon are shown in bold, lowercase nucleotides indicate restriction sites and cleavable extensions). The oligonucleotides incorporated SalI and XhoI sites (underlined), respectively, for cloning into the mammalian expression vector pRS5a (a modification of pRK5 (Pharmingen) provided by S. Geisse, Novartis Pharma AG).
Moreover, the 3'-amplification oligonucleotide incorporated a C-terminal (His)6 tag for detection and purification purposes.

In the polymerase chain reaction, 0.4 μg of the cDNA library were amplified with Pfu polymerase (Promega) using the following protocol: 2 min at 95°C, 20 cycles: 30 s at 94°C, 30 s at 55°C, 1 min at 72°C, 5 min at 72°C. The PCR products were purified from 3 μl of mixture by using the Ampure Magnetic Beads Kit (Agencourt Bioscience Corporation) and cloned into pBS5a. Mutations were introduced into the MCP-1 cDNA by using the QuickChange Site-directed Mutagenesis kit (Stratagene) and high performance liquid chromatography purified oligonucleotides carrying the desired mutations.

Expression and Purification of MCP-1 Mutants—The day prior to the transformation, 2 × 10^6 WEHI 231 cells were seeded into 10 cm plates. Two micrograms of pBS5a expression plasmids carrying the MCP-1 sequences and 10 μl of Geneporter transfection reagent (Gene Therapy Systems) were each separately diluted in 500 μl of serum-free Dulbecco’s modified Eagle’s medium. The diluted DNA was added to the diluted transfection reagent and incubated at room temperature for 45 min. The culture medium was aspirated from the cells and the DNA/transfection reagent mixture was carefully added to the cells and incubated for 5 h at 37°C. After the incubation, 2 ml of Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum were added and the cells were incubated for 3 days at 37°C. The supernatants were then collected, centrifuged for 5 min, and subjected to purification prior to storage at −20°C.

The 3 ml of culture supernatant containing the secreted MCP-1 protein, imidazole was added to a final concentration of 10 mM. To this solution 100 μl of Ni-NTA Magnetic Agarose Beads (Qiagen) were added and the suspension was incubated for 2 h at 4°C on a spinning wheel. The beads were separated from the solution with a 12-tube magnet (Qiagen) and washed three times with wash buffer (50 mM Na2HPO4, 300 mM NaCl, 10 mM imidazole, 0.05% Tween 20, pH 8.0). The bound MCP-1 was eluted with 200 μl of elution buffer (50 mM Na2HPO4, 300 mM NaCl, 250 mM imidazole, 0.05% Tween 20, pH 8.0) by resuspending the beads and incubation at room temperature for 1 min. The concentration of purified MCP-1 was measured with a Protein Assay (Bio-Rad) or by anti-MCP-1 ELISA (Biomol). Mutant MCP-1 concentrations were determined using the Protein Assay (Bio-Rad) with purified MCP-1 as standard. Typically 40 μg of purified human MCP-1 or MCP-1 mutants were obtained from 3 ml of culture.

The purity of expressed MCP-1 proteins was assessed by SDS-PAGE on 18% pre-cast gels (Novagen). The gels were stained with GelCode Blue Stain (Pierce).

Binding of MCP-1 to vCCI by ELISA—An ELISA was developed to measure binding affinities of MCP-1 and mutant MCP-1 to the vCCI protein, which was fused to the Fc portion of human IgG (R & D Systems). 96-well Maxisorp plates (Nunc) were coated with 100 μl of goat F(ab)2-anti-human IgG Fc (Cappel) at a concentration of 3.2 μg/ml and the suspension was incubated for 2 h at room temperature and washed. Bound antibody was detected with mouse antibodies (Santa Cruz Biotechnologies, 30 min at room temperature). The plates were washed and blocked buffer and incubated for 2 h at room temperature. The plates were washed and serial dilutions of MCP-1 (0–2700 ng/ml) or MCP-1 mutants (0–8100 ng/ml) in blocking buffer were added and incubated for 2 h at room temperature with constant agitation. After washing, 100 μl/well of a biotinylated polyclonal anti-human anti-MCP-1 antibody (R & D Systems) in blocking buffer was added at a concentration of 100 ng/ml, incubated for 2 h at room temperature and constant agitation. The plates were washed and serial dilutions of MCP-1 (0–2700 ng/ml) or MCP-1 mutants (0–8100 ng/ml) in blocking buffer were added and incubated for 2 h at room temperature with constant agitation. After washing, 100 μl/well of a biotinylated polyclonal anti-human MCP-1 antibody (R & D Systems) in blocking buffer was added at a concentration of 100 ng/ml, incubated for 2 h at room temperature and washed. Bound antibody was detected by incubation with alkaline phosphatase conjugated to streptavidin (1:3000, Immuno Research) for 30 min at room temperature. The color was developed for 30 min with 1.5 μl of 5% NaOH and the absorption was measured at 405 and 490 nm with a Spectra Max 250 reader ( Molecular Devices). Dose-response curves were fitted to a four-parameter logistic function. EC50 values of MCP-1 mutants were normalized to the EC50 observed with MCP-1 (24.9 ng/ml).

Kinetic Characterization of the vCCI/MCP-1 Interaction by Surface Plasmon Resonance (SPR)—Certified BIAcore sensorchips CM-5 (BIAcore, Uppsala, Sweden) were utilized throughout this study. For activation of the carboxymethylated dextran layer, equal volumes of 0.2 μl of 0.9 mmol/L N-ethyl-N-(3-diethylaminopropyl)-carbodiimide and 0.05 μl of 0.1 mmol/L hydroxysuccinimide (BIAcore “Amine coupling kit” were preincubated for 15 min, washed fully applied to define protein-protein interfaces of growth hormone/growth hormone receptor (24), neurotrophin/receptor (Jackson ImmunoResearch), prepared by diluting the stock solution to 10 mM acetic acid, pH 4.5, was then injected at 5 μl/min for 3 min to yield an increase in resonance units of about 10,000. Remaining activated N-hydroxysuccinimide groups were quenched during a pulse of 1% ethanolamine, pH 8.5, and the surface was exposed to a regeneration solution of 2 M HCl. Next, vCCI was accumulated on the surface by injecting 5 μl of a 5 μg/ml solution, at a flow rate of 5 μl/min. This procedure yielded about 200 to 300 resonance units (statistics of a representative experiment: 268 ± 2 resonance units; mean ± S.E.; n = 35), and was repeated each time after a regeneration and before the injection of chemokine. Dilutions of MCP-1 or MCP-1 mutants were prepared in BiAAssay buffer (200 μM HEPES, pH 7.4, 150 mM NaCl, 0.05% (v/v) to yield final concentrations of 20 μM HCl. Next, vCCI was injected on the sensor chip and the ratio of green and red fluorescence was measured at a flow rate of 5 μl/min. Association was followed for 12.5 min, dissociation for 5 min, and the flow rate was kept at 20 μl/min. Each cycle was finished by two 30-s pulses of 100 μM HCl at 20 μl/min. After subtraction of the sensorgram obtained by injecting BiAbuffer, the titration series was analyzed using the BiAevaluation 3.0 software (BIAcore). The KD values for the mutants are the average of Kd values determined from individual injections and are not calculated from the average Kd and koff values shown in Table III.

Intracellular Ca2+ Mobilization Assay—Chinese hamster ovary (CHO) cells, stable transfected with the human CCR2B variant V64I (19) (CHO/CCR2B), were trypsinized, washed, and resuspended in Hanks’ balanced salt solution buffer (Life Technologies) containing 20 mM HEPES, 0.5% bovine serum albumin and 0.5% bovine serum albumin (Sigma). The cells were loaded with 3 μl Fura Red (Molecular Probes, emission at 660 nm) and 1.5 μl Fluoro3 (Molecular Probes, emission at 530 nm) in the presence of 0.04% pluronic acid (Molecular Probes) for 1 h at room temperature. After two washes with Hanks’ balanced salt solution, HEPES, 0.5% bovine serum albumum, the cells were ready for stimulation with MCP-1 or MCP-1 mutants. Fluorescence emission in response to MCP-1 was measured at room temperature with a FACSscan (Becton Dickinson) and the ratio of green and red fluorescence was calculated (20). Data were analyzed with the FlowJo software (TreeStar Inc.). The values measured with MCP-1 were normalized to 100% and the signals obtained with MCP-1 mutants were expressed relative to this. This results shown are the mean of two independent experiments with triplicate values in each measurement.

Binding of MCP-1 to Monoclonal Anti-MCP-1 Antibodies—in order to assess the structural integrity of MCP-1 mutants, their ability to interact with a panel of five monoclonal antibodies directed against human MCP-1 (Anogen: S8, S14, S101, S382, and 9G10) was measured in ELISAs using Ni-NTA HisSorb strips (Qiagen). MCP-1 mutants were bound via their C-terminal (His)6 tag to the Ni-NTA on the solid support. Bound MCP-1 mutants were detected with the monoclonal antibodies and the relative binding compared with MCP-1 was calculated. MCP-1 or mutants were added to the wells at 2700 ng/ml in 50 μl of blocking buffer and incubated for 2 h at room temperature. The plates were washed and monoclonal antibodies S8 or S101 were added at 500 ng/ml. For the monoclonal antibodies S14, S392, and 9G10 a concentration of 2000 ng/ml was used. The plates were kept for 2 h at room temperature and then washed. Bound monoclonal antibodies were detected with 1:7500 diluted horseradish peroxidase-coupled goat anti-mouse antibodies (Santa Cruz Biotechnologies, 30 min at room temperature). The color was developed by addition of 50 μl/well of BM blue horseradish peroxidase substrate (Roche Molecular Biochemicals) and incubation for the Fe up to 15 min. The enzymatic reaction was stopped by addition of 50 μl/well of 1 N H2SO4 and the absorption was measured at 450 and 690 nm on a Spectra Max 250 (Molecular Devices). Mutant MCP-1 binding to the five monoclonal antibodies was determined twice independently with triplicate values in each experiment and normalized to the values obtained for MCP-1 binding. Prior to normalization, the values were corrected for the absorption measured with buffer alone which accounted always for less than 5% of total binding.

RESULTS

Design of MCP-1 Mutants—the three-dimensional structure of MCP-1 was determined by NMR (21) and x-ray crystallography (22). We identified surface-exposed residues by visual inspection of the MCP-1 structure and probed a total of 57 residues that could potentially interact with vCCI by alanine-scanning mutagenesis (23). This approach has been successfully applied to define protein-protein interfaces of growth hormone/growth hormone receptor (24), neurotrophin/receptor...
interactions (25) and other ligand/receptor pairs (26). Alanine can adopt the dihedral angles of all secondary structural elements in proteins and mutation to alanine is thus not expected to perturb the structure of the mutated MCP-1 variants. Residues that are likely involved in maintaining the overall integrity of the MCP-1 structure were not analyzed. These residues included amino acids forming disulfides, Cys-11, Cys-12, Cys-36, and Cys-52 as well as amino acids involved in hydrophobic core formation, Leu-25, Val-41, Phe-43, Trp-59, Val-60, and Leu-67. Finally, proline and alanine residues were generally not probed (Pro-2, Ala-4, Ala-26, Pro-37, Ala-40, Ala-53, Pro-55, and Pro-74).

**Expression, Purification, and Analysis of MCP-1 Variants**—MCP-1 and MCP-1 mutants were transiently expressed in HEK.EBNA cells and proteins were purified from conditioned medium using Ni-NTA magnetic beads. Peptide mapping of the purified protein followed by N-terminal sequencing of the fragments yielded the expected sequence (data not shown). Expressed and purified MCP-1 induced a similar dose response as *Escherichia coli*-derived commercially available MCP-1 (R+D Systems) in Ca^{2+}-mobilization assays using CHO/CCR2B cells (data not shown). The affinity of MCP-1 to vCCI was determined by SPR. The $K_D$ for this interaction was 294 ± 22 pm with $k_{on} = 2.47 ± 0.14 \text{ M}^{-1} \text{s}^{-1}$ and $k_{off} = 6.79 ± 0.33 \text{ s}^{-1} \times 10^{-4}$.

**The Main Determinants for MCP-1 Binding to vCCI Are Arg-18, Tyr-13, and Arg-24**—The most important residue for the interaction of MCP-1 with vCCI was discovered by analysis of the mutation R18A. This mutation caused a more than 10-fold increase in the EC_{50} value in the ELISA (Table I). This result was confirmed by SPR measurements using the BIACore instrument where no specific binding could be detected for this mutant (Table II). The mutation R18A thus strongly affects binding to vCCI. In order to ensure that the overall structure of this mutation was not perturbed, we determined the functional response mediated through the human CCR2B receptor by measuring the Ca^{2+} mobilization in CHO/CCR2B cells. The CCR2B response was first titrated with MCP-1 and a half-maximal response was observed at 1 nM (data not shown). Subsequently, all mutants were assessed at 1 nM in order to ensure maximal sensitivity of the functional assay. R18A activates CCR2B with an efficacy of 76 ± 3% of the MCP-1 response at 1 nM, indicating that Arg-18 is crucially important for the MCP-1 interaction with CCR2B (Table III). This mutant has also been shown to have full binding affinity to THP-1 cells and to a recombinant cell line expressing the CCR2B receptor (28). A loss in activity of a protein upon mutation of a side chain can be attributed either to the importance of this side chain to the function of the protein or to an involvement in maintaining the structural integrity of the protein. An additional method to verify the structural integrity of mutants with reduced activity is to determine their reactivities against a panel of monoclonal antibodies (26). The interaction of the R18A mutant with a panel of five monoclonal anti-MCP-1 antibodies was assessed and it was found that the residue Arg-18 is important for the interaction with S8 but not S14 and S382 (Table III). Therefore, the strong activity of R18A on the CCR2B receptor and its interaction with two of the monoclonal antibodies suggests that the overall structure of R18A is intact and that the large decrease of affinity to vCCI is due to the removal of a functional group crucial for this particular interaction. Hence, Arg-18 is the major determinant on MCP-1 for binding to vCCI.

Two additional residues, Arg-24 and Tyr-13, displayed strongly reduced affinity to vCCI after mutation to alanine (Table I). Determination of the kinetic constants by SPR.
Mapping of MCP-1-binding Site for vCCI

Affinities of MCP-1 and mutants were determined by surface plasmon resonance as described under “Experimental Procedures.” Values for $k_{on}$, $k_{off}$ and $K_D$ are given as mean ± S.E. of at least two independent dose-response experiments. At least five measurements of all the kinetic constants per experiment were performed. $K_D$ was derived by fitting of experimental data to a 1:1 Langmuir association/dissociation model. Kinetic constants of mutants probing the main vCCI binding determinants Tyr-13, Arg-18, and Arg-24 as well as ∆MCP-1 are marked bold.

| MCP-1 variant | $k_{on}$ | $k_{off}$ | $K_D$ | $K_D$ (mut/∆K) (wt) |
|---------------|----------|-----------|-------|---------------------|
| MCP-1         | 2.47 ± 0.14 | 6.79 ± 0.33 | 294 ± 22 | 1.00 |
| P8A           | 1.32 ± 0.05 | 11.6 ± 0.89 | 875 ± 59 | 3.00 |
| V9A           | 2.92 ± 0.19 | 3.23 ± 0.29 | 119 ± 20 | 0.40 |
| T10A          | 4.23 ± 0.32 | 8.65 ± 0.52 | 221 ± 30 | 0.75 |
| Y13A          | 1.06 ± 0.09 | 40.2 ± 2.36 | 3810 ± 217 | 12.96 |
| N14A          | 1.89 ± 0.08 | 19.1 ± 1.67 | 1030 ± 101 | 2.50 |
| F15A          | 0.72 ± 0.08 | 7.37 ± 0.81 | 1040 ± 93 | 3.54 |
| T16A          | 2.55 ± 0.17 | 7.19 ± 0.33 | 293 ± 26 | 1.00 |
| N17A          | 1.60 ± 0.10 | 19.0 ± 0.78 | 1240 ± 121 | 4.22 |
| R18A          | ND        | ND        | >6000 | >20 |
| K19A          | 1.11 ± 0.09 | 15.1 ± 0.63 | 1410 ± 81 | 4.80 |
| I20A          | 1.54 ± 0.17 | 12.5 ± 0.50 | 895 ± 124 | 3.04 |
| R24A          | 0.71 ± 0.07 | 23.5 ± 1.13 | 3580 ± 387 | 12.18 |
| Y28A          | 1.05 ± 0.05 | 7.35 ± 0.82 | 728 ± 94 | 2.48 |
| I31A          | 1.68 ± 0.13 | 9.08 ± 0.23 | 567 ± 40 | 1.93 |
| K35A          | 3.15 ± 0.25 | 9.13 ± 0.48 | 313 ± 40 | 1.06 |
| K38A          | 1.19 ± 0.16 | 11.3 ± 0.04 | 1130 ± 184 | 3.84 |
| I42A          | 1.90 ± 0.10 | 14.0 ± 1.61 | 754 ± 87 | 2.56 |
| K49A          | 1.99 ± 0.17 | 15.8 ± 0.18 | 102 ± 26 | 0.35 |
| I51A          | 1.61 ± 0.17 | 11.9 ± 1.47 | 773 ± 118 | 2.63 |
| D65A          | 1.26 ± 0.04 | 8.62 ± 0.37 | 687 ± 28 | 2.34 |
| K56A          | 1.72 ± 0.09 | 9.34 ± 0.38 | 554 ± 39 | 1.58 |
| K57A          | 2.12 ± 0.09 | 6.45 ± 0.16 | 308 ± 16 | 1.05 |
| K58A          | 2.03 ± 0.09 | 15.1 ± 0.63 | 495 ± 18 | 1.68 |
| Q61A          | 1.94 ± 0.16 | 9.10 ± 0.30 | 491 ± 57 | 1.67 |
| M64A          | 1.36 ± 0.10 | 11.5 ± 0.35 | 877 ± 56 | 2.98 |
| D65A          | 1.98 ± 0.13 | 6.88 ± 0.77 | 347 ± 31 | 1.18 |
| ∆MCP-1        | 3.21 ± 0.21 | 1.31 ± 0.17 | 42 ± 6 | 0.14 |

showed that the mutation Y13A affected mainly the off-rate of the kinetics (6-fold increase) with a small effect on the on-rate (2-fold decrease) (Table II). Both effects together led to an almost 13-fold decrease of affinity to vCCI. The mutation R24A affected both the on- and off-rate component of the kinetics and led to a 12-fold decrease in affinity to vCCI (Table II).

The two mutations Y13A and R24A resulted in an almost complete loss of functional activity on the CCR2B receptor (Table III). An earlier study in which the binding of these mutations to CCR2B was analyzed came to similar conclusions (28). However, Y13A bound equally well as MCP-1 or even slightly better to all monoclonal antibodies in the panel suggesting that its overall structure remained intact. R24A strongly affected binding to two (S14 and 9G10) of the five antibodies tested, but displayed a strong interaction with the remaining three (S101, S8, and S5S2). Moreover, the structure of the R24A mutant was probed by NMR and a similar pattern of cross-peaks was found as in MCP-1, reflecting the structural integrity of this MCP-1 variant (28). Hence, Y13A and R24A are likely folded correctly. These observations, taken together with the strong decrease of affinity to vCCI, let us conclude that Tyr-13 and Arg-24 are important vCCI binding determinants on MCP-1. Hence, the residues Arg-18, Tyr-13, and Arg-24 define the surface on MCP-1 required for its interaction with vCCI.

Additional Residues of Importance for MCP-1 Binding to vCCI—In addition to the three main determinants, residues of lower importance for the MCP-1 interaction with vCCI were identified. The mutations P8A, N14A, F15A, N17A, K19A, I20A, and K38A all led to a 3-fold or larger decrease of affinity to vCCI (Table II). All mutants either displayed strong activity on CCR2B (P8A, F15A, K19A, I20A, and K38A) and/or bound well to all (P8A, N14A, F15A, N17A, I20A, and K38A) or a subset (K19A) of monoclonal antibodies (Table III), suggesting that none of these mutations led to important structural changes. These residues thus constitute the binding site on MCP-1 in addition to the three key residues.

Two point mutations (V9A and K49A) and a variant, ∆MCP-1, where the first eight amino acids were removed (29), displayed an increased affinity to vCCI. The SPR experiments yielded values of 119 ± 20, 102 ± 26, and 42 ± 6 pm for V9A, K49A, and ∆MCP-1, respectively. Val-9 is located in direct contact to Tyr-13 and removal of this side chain may either change the conformation of Tyr-13 or lead to a better access of vCCI to this key residue on MCP-1. Removal of the first eight amino acids may similarly facilitate access to Tyr-13 or interfere with the monomer/dimer equilibrium and thereby modulate the interaction with vCCI. Finally, Lys-49 is located close to Arg-24 and could increase the affinity of vCCI to MCP-1 by analogous mechanisms as suggested for the V9A mutant.

Two additional residues, Tyr-28 and Met-64 displayed small but significant effects upon mutation to alanine. The interactions of these mutants with monoclonal antibodies and their activity on the CCR2B receptor suggest that they are correctly folded. These two residues form a small surface-exposed patch that is located opposite to the main vCCI interaction site formed by Tyr-13, Arg-18, and Arg-24.

The mutations K56A, Q57A, K58A, and Q61A affected the signal in the ELISA strongly (Table I) but when these mutations were analyzed with SPR their affinity to vCCI was found to be unchanged (Table II). These four residues form a contiguous patch on the surface of MCP-1 which is separate from the vCCI-binding site and it is likely that these mutations affected the epitope of the detection antibody and thereby led to the strong reduction of the signal observed in ELISA. In this respect it is interesting to note that the mutations of Lys-58 and Gln-61 to alanine resulted in a complete loss or strong reduction, respectively, of binding to three of the five antibodies in
Mutants probing the main vCCI binding determinants Tyr-13, Arg-18, and Arg-24 as well as the corresponding alanine reduced the functional signal to less than 5% of that described earlier (29, 30). Previous studies using site-directed mutagenesis of MCP-1 compared with CCR2B. Finally, the strongly decreased the signaling capacity of the resulting mutant MCP-1 variant.

The vCCI-binding Site on MCP-1

| MCP-1 variant | Ca²⁺ mobilization | S101 | S8 | S14 | S382 | 9G10 |
|---------------|-------------------|------|----|-----|------|------|
| MCP-1         | 100 ± 7           | 1.00 ± 0.04 | 1.00 ± 0.05 | 1.00 ± 0.04 | 1.00 ± 0.06 | 1.00 ± 0.02 |
| P8A           | 129 ± 8           | 1.25 ± 0.07 | 3.04 ± 0.42 | 1.62 ± 0.05 | 3.16 ± 0.27 | 1.03 ± 0.08 |
| V9A           | 90 ± 8            | 1.21 ± 0.02 | 1.82 ± 0.10 | 0.73 ± 0.04 | 1.95 ± 0.10 | 0.25 ± 0.02 |
| T10A          | 36 ± 14           | 1.01 ± 0.02 | 1.27 ± 0.05 | 0.61 ± 0.06 | 1.23 ± 0.10 | 0.42 ± 0.05 |
| Y13A          | 4 ± 1             | 1.19 ± 0.11 | 2.03 ± 0.24 | 1.03 ± 0.07 | 1.65 ± 0.26 | 0.94 ± 0.12 |
| N14A          | 94 ± 3            | 0.92 ± 0.03 | 0.81 ± 0.04 | 0.64 ± 0.08 | 0.84 ± 0.04 | 0.56 ± 0.05 |
| F15A          | 45 ± 9            | 1.22 ± 0.03 | 1.65 ± 0.12 | 0.74 ± 0.03 | 1.72 ± 0.05 | 0.42 ± 0.04 |
| T16A          | 95 ± 10           | 0.90 ± 0.04 | 0.80 ± 0.03 | 0.89 ± 0.07 | 0.74 ± 0.14 | 0.79 ± 0.07 |
| N17A          | 96 ± 6            | 0.93 ± 0.04 | 0.99 ± 0.03 | 0.96 ± 0.05 | 0.96 ± 0.03 | 0.97 ± 0.10 |
| R18A          | 78 ± 4            | 0.28 ± 0.03 | 0.10 ± 0.02 | 0.37 ± 0.04 | 0.40 ± 0.07 | 0.25 ± 0.03 |
| K19A          | 88 ± 4            | 0.45 ± 0.06 | 0.15 ± 0.02 | 0.72 ± 0.03 | 0.48 ± 0.03 | 0.69 ± 0.06 |
| I20A          | 79 ± 7            | 1.21 ± 0.15 | 1.58 ± 0.12 | 0.46 ± 0.02 | 2.25 ± 0.29 | 0.16 ± 0.12 |
| R24A          | 4 ± 1             | 0.68 ± 0.06 | 0.38 ± 0.06 | 0.08 ± 0.02 | 0.52 ± 0.18 | 0.01 ± 0.02 |
| Y25A          | 71 ± 11           | 1.22 ± 0.03 | 1.88 ± 0.16 | 0.83 ± 0.02 | 2.29 ± 0.09 | 0.46 ± 0.03 |
| I31A          | 7 ± 8             | 0.96 ± 0.10 | 1.08 ± 0.06 | 0.82 ± 0.05 | 1.00 ± 0.52 | 0.67 ± 0.04 |
| K35A          | 37 ± 8            | 0.89 ± 0.03 | 0.81 ± 0.02 | 0.79 ± 0.04 | 0.85 ± 0.04 | 0.54 ± 0.06 |
| K38A          | 45 ± 2            | 1.01 ± 0.17 | 1.08 ± 0.18 | 1.18 ± 0.05 | 1.28 ± 0.09 | 1.34 ± 0.14 |
| I42A          | 1 ± 1             | 1.34 ± 0.13 | 2.19 ± 0.13 | 0.62 ± 0.05 | 1.95 ± 0.12 | 0.24 ± 0.05 |
| K49A          | 4 ± 1             | 0.91 ± 0.02 | 0.78 ± 0.04 | 0.58 ± 0.04 | 0.85 ± 0.05 | 0.38 ± 0.06 |
| I51A          | 92 ± 4            | 1.09 ± 0.12 | 1.23 ± 0.08 | 0.57 ± 0.02 | 1.16 ± 0.25 | 0.28 ± 0.05 |
| D54A          | 86 ± 8            | 1.13 ± 0.03 | 1.18 ± 0.05 | 0.74 ± 0.03 | 1.36 ± 0.10 | 0.96 ± 0.12 |
| K56A          | 98 ± 11           | 0.90 ± 0.05 | 0.60 ± 0.05 | 0.91 ± 0.05 | 1.07 ± 0.23 | 0.89 ± 0.16 |
| Q57A          | 96 ± 3            | 0.97 ± 0.13 | 0.30 ± 0.19 | 0.93 ± 0.04 | 0.91 ± 0.12 | 0.83 ± 0.10 |
| K58A          | 99 ± 5            | 0.01 ± 0.01 | 0.02 ± 0.01 | 0.73 ± 0.05 | 0.00 ± 0.03 | 0.46 ± 0.04 |
| Q61A          | 90 ± 5            | 0.28 ± 0.05 | 0.12 ± 0.03 | 1.0 ± 0.08 | 0.23 ± 0.07 | 1.13 ± 0.15 |
| M64A          | 84 ± 6            | 0.35 ± 0.12 | 0.36 ± 0.02 | 1.26 ± 0.18 | 0.26 ± 0.10 | 1.81 ± 0.34 |
| D65A          | 99 ± 5            | 0.94 ± 0.07 | 1.48 ± 0.04 | 0.90 ± 0.12 | 1.20 ± 0.08 | 0.86 ± 0.05 |
| ΔMCP-1        | 2 ± 1             | 1.22 ± 0.06 | 2.72 ± 0.41 | 1.08 ± 0.03 | 2.48 ± 0.12 | 0.72 ± 0.18 |

Residues Important for GAG Binding Are Not Involved in the Interaction of MCP-1 with vCCI—Chemokines interact with GAGs such as heparin and heparan sulfate. These highly charged polysaccharide chains are thought to tether MCP-1 secreted from endothelial cells. Substitutions of Lys-58 and His-66 in MCP-1 by alanine resulted in a loss of GAG binding (33) and it was concluded that these two residues are key determinants for the MCP-1/GAG interaction. In this study we demonstrated that the mutations K58A and H66A did not affect binding to vCCI (Table I). The K58A mutant displays an affinity of 495 ± 18 pM to vCCI which is only 1.7-fold lower than the K₅₀ of MCP-1 (Table II). Hence, residues involved in GAG binding are not important for the interaction of MCP-1 with vCCI. This finding is in agreement with the observation that heparin and heparan sulfate does not interfere with MIP-1α binding to vCCI (9).

**DISCUSSION**

The vCCI-binding Site on MCP-1—In the present study, site-directed mutagenesis and molecular modeling was employed to determine the interaction site of the C. caviae chemokine MCP-1 with the secreted chemokine-binding protein vCCI from vaccinia virus (strain Lister). Probing of all surface-exposed residues on MCP-1 for their importance to interact with vCCI revealed a functional binding site dominated by the three residues Tyr-13, Arg-18, and Arg-24 (Fig. 1A). Located between these three key amino acids are residues of significant but lower importance for the interaction with vCCI. These residues are Pro-8, Asn-14, Phe-15, Asn-17, Lys-19, Ile-20, Lys-38 and...
Asp-54. Residues that display an increased affinity to vCCI when mutated to alanine are Lys-49 and Val-9. The former is located adjacent to Arg-24 and removal of this side chain may change the conformation of Arg-24 to facilitate binding of vCCI to MCP-1. Finally, removal of the first eight amino acids in the ΔMCP-1 mutant increased binding to vCCI by 7-fold. The structural basis for this effect is unclear but it has been shown that ΔMCP-1 has lost its ability to homodimerize. Therefore, access to Tyr-13, which is located close to the dimer interface, could be facilitated in the ΔMCP-1 variant.

It is well established that the functional binding sites of protein-protein interactions are dominated by only few residues located in the interface (24). This has been demonstrated by structural and functional analyses for the interactions of human growth hormone (26, 34), vascular endothelial growth factor (35, 36), and neurotrophins (25, 37) with their respective receptors. However, these residues are usually localized close to each other forming a hydrophobic central patch (34) or providing a shielded hydrogen bond (37). The key residues on MCP-1 for its interaction with vCCI, however, are more than 10 Å apart. Although in their composition they consist of a hydrophobic residue (Tyr-13) and arginines (Arg-18 and Arg-24) which are both frequently found within protein-protein interfaces. While the presence of arginines and a tyrosine may be driven by the need to provide binding energy, their unusual arrangement may be a consequence of the fundamentally different evolutionary pressure applied on this viral chemokine inhibitor and on extracellular domains of growth factor receptors, respectively. Alternatively, vCCI may bind to the two sites sequentially by first docking to the Arg-18/Arg-24 area and only in a second step get tight binding by interacting with the more hydrophobic surface around Tyr-13.

Comparison of the CCR2B- and vCCI-binding Sites and the Mechanism of Action of vCCI—Various structure/function analyses of MCP-1 have been performed to determine its interaction site with the CCR2B receptor or to identify residues important for chemotaxis (27, 28, 30–32). The key amino acids Arg-24 and Tyr-13 are each located at the center of two clusters of important residues which are separated by a hydrophobic groove (28). The side chains of the N-terminal residues do not contribute significantly to binding but are crucial for transmitting the signal. In the present study we could confirm these findings (Fig. 1B, Table III). Although the most important residue for binding to vCCI, Arg-18, is not of crucial importance for the recognition of CCR2B, the vCCI interaction site on MCP-1 otherwise largely overlaps with the CCR2B-binding region (Fig. 1). The residues Tyr-13 and Arg-24 are used by both MCP-1 interacting proteins and seem to provide hot-spots of binding energy (24) that are recognized by completely unrelated proteins (i.e. vCCI and CCR2B). The intrinsic physicochemical properties of these two surfaces may facilitate these interactions. In addition to these dominant residues, amino acids located in the hydrophobic groove between Arg-24 and Tyr-13 make various contacts with the CCR2B receptor and vCCI. Finally, deletion of the N-terminal eight residues resulted in an enhanced affinity to vCCI mainly through a decrease in the off-rate while the same residues were shown to interact with CCR2B to induce signaling.

Taken these results together, we suggest that vCCI inhibits MCP-1 activity by effectively masking important residues required for its interaction with the CCR2B receptor. Complexed soluble MCP-1 will thus not be able to elicit a signal through CCR2B. Moreover, MCP-1 bound to GAGs can likely still interact with vCCI since the GAG-binding residues are not involved in vCCI binding. Therefore, even MCP-1 displayed on GAGs can potentially be inhibited by vCCI, making this viral antagonist of CC chemokines even more effective.

The vCCI-binding Site on MCP-1 in Relation to the Structure of vCCI—The three-dimensional structure of vCCI from cowpox, which is highly homologous to the vaccinia protein, was...
determined at a resolution of 1.85 Å (16). The vCCI protein adopts a β-sandwich topology not previously described which is very likely distinct from the CCR2B structure. A patch of conserved residues on the exposed face of a β-sheet that is strongly negatively charged was suggested to interact with CC chemokines. We have identified the MCP-1 residues Arg-18 and Arg-24 as important contributors to binding energy and suggest that these residues could indeed be the counterparts of the negatively charged amino acids on vCCI.

vCCI interacts with most if not all CC chemokines with remarkably high affinity (9). CC chemokines are related in their protein sequences but nevertheless display a large degree of sequence variation. The question arises thus, how vCCI can recognize such a diverse set of surfaces. It is possible that there are physicochemical features on the surface of chemokines that are conserved in the absence of a strict amino acid identity and that vCCI evolved to retain a certain flexibility to accommodate slightly different arrangements of key determinants in different ligands. In an analogous case, the hinge region of the Fe fragment of human immunoglobulin G has been shown to interact with completely unrelated proteins with high affinity using a common binding site (38). This site was highly accessible, adaptive, and hydrophobic. In contrast to the presumed vCCI surface it also contains few sites for polar interactions.

**vCCI Recognizes a Conserved Surface on Various CC Chemokines**—The interaction of five CC chemokines MCP-1, Eotaxin-1, RANTES, MIP-1α, and I-309 with vCCI was characterized by displacement binding assays with radiolabeled MIP-1α (9). The strength of the interaction of these chemokines with vCCI was determined and the observed rank order was MIP-1α > eotaxin-1 > RANTES > MCP-1 >> I-309 (no binding). The amino acids at the key positions 13, 18, and 24 were compared in an alignment of the protein sequences of these chemokines (Fig. 2). Arg-18 was identified as the key MCP-1 residue for binding to vCCI and consequently the multiple sequence alignment reveals that all vCCI-binding CC chemokines carry an arginine at this position. The only non-binding protein, I-309, carries a glutamine. Therefore, an arginine at this position in all other sequences suggests a requirement for the amino acid differences account for the different affinities of the CC chemokines for vCCI.

It was suggested that vCCI could be useful for the treatment of inflammatory conditions due to its broad neutralizing activity against CC chemokines (8). Indeed, administration of vCCI was determined and the observed rank order was MIP-1α > eotaxin-1 > RANTES > MCP-1 >> I-309 (no binding). The amino acids at the key positions 13, 18, and 24 were compared in an alignment of the protein sequences of these chemokines (Fig. 2). Arg-18 was identified as the key MCP-1 residue for binding to vCCI and consequently the multiple sequence alignment reveals that all vCCI-binding CC chemokines carry an arginine at this position. The only non-binding protein, I-309, carries a glutamine. Therefore, an arginine at this position in all other sequences suggests a requirement for the amino acid differences account for the different affinities of the CC chemokines for vCCI.

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