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1: Title page

**Identification of the extracellular loop 2 as the point of  
interaction between the N-terminal of the chemokine  
MIP-1 $\alpha$  and its receptor CCR1**

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**2: Running title page**

**2a): Running title**

Identification of MIP-1 $\alpha$  interaction on CCR1

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aa: amino acid; BP: Benzophenone; CCL: CC-chemokine receptor Ligand; CCR: CC-chemokine receptor; CHO: Chinese Hamster Ovary cell derived cell line; CNBr: cyanogen bromide; DMSO: dimethylsulfoxide; DTT: dithiothreitol; E1, E2, and E3: extracellular loops; GPCR: G-protein coupled receptor; GRO: Growth regulated protein; HA: Hemagglutinin tag; HEK293: Human Embryonic Kidney-cells 293; HPLC: high performance liquid chromatography; IL8: Interleukin 8; IP10: Interferon inducible protein 10; LD78b: Tonsillar Lymphocyte LD78  $\beta$  protein; MCP: Monocyte Chemotactic Protein; MGSA: Melanoma growth stimulatory activity; MIP: Macrophage Inflammatory Protein; MPIF: Myeloid Progenitor Inhibitory Factor; PBS: phosphate buffered Saline; PCR: polymerase chain reaction; RANTES: Regulated on Activation Normal T Expressed and Secreted; SDF: Stromal cell Derived Factor; SDS-PAGE: sodium dodecyl sulfate - polyacrylamide gel electrophoresis; TFA: trifluoro acetic acid; THP-1: stable cell-line; TM: transmembrane domain; Glu-C: Staphylococcus aureus endopeptidase V8; Lys-C: Lysobacter enzymogenes endopeptidase; Arg-C: Clostridium histolyticum endoproteinase.

### **3: Abstract**

#### **Abstract**

Macrophage Inflammatory Peptide-1 $\alpha$  (MIP-1 $\alpha$ )/CCL3 is an 8kDa peptide that induces chemotaxis of various lymphocytes to sites of inflammation through interaction with the G protein-coupled chemokine receptors CCR1 and CCR5. We recently described the preparation of a photoactivatable derivative of MIP-1 $\alpha$  labeled with a benzophenone group at the extreme N-terminal end which is a determinant for the agonist character of chemokines. Benzophenone-MIP-1 $\alpha$  is a full agonist that specifically and covalently labels CCR1 and CCR5 receptors upon irradiation. In the present study we use enzymatic and chemical cleavage methods on wild type and mutated CCR1 receptors to show that the N-terminus of the chemokine MIP-1 $\alpha$  interacts in a specific manner with the second extracellular loop of the CCR1 receptor, within a segment comprising amino acids 178 to 194. This is the first report on the direct identification of a contact point between the N-terminal of a chemokine and its membrane-bound receptor. The work shows that the part of chemokines that is endowed with agonist properties interactw with extracellular parts of the receptor rather than the transmembrane core of the protein.

#### 4: Introduction

Chemokines constitute a large family of 8-12 kDa small proteins, characterized by a well-conserved three-dimensional structure involving two highly conserved cysteine bridges (Rollins, 1997). Chemokines are responsible for the orchestration of leukocyte recruitment to sites of inflammation or lymphoid tissue (Rollins, 1997). Their receptors belong to the rhodopsin-like group of G-protein coupled receptors. So far approximately 20 chemokine receptors and 50 chemokines have been identified. They can be subdivided into four groups, based on position of cysteine residues (Murphy et al., 2000).

The human homolog of the CC-chemokine Macrophage Inflammatory Protein 1 $\alpha$  (Nakao et al., 1990) (MIP-1 $\alpha$ ), also known as LD78 $\alpha$  or CCL3 (Murphy, et al., 2000), belongs to the largest group of chemokines, characterized by two adjacent cysteines in the N-terminus.

MIP-1 $\alpha$  expression has been identified in Peripheral Blood monocytes and different lymphocyte derived cell-lines (Menten et al., 1999; Nakao et al., 1990). It is found at sites of inflammation such as wounded tissue (DiPietro et al., 1998). The folding of the peptide backbone determined by NMR is very similar to that of other chemokines (Czaplewski et al., 1999), such as MIP-1 $\beta$ , RANTES and MCP-1: A relatively flexible N-terminal sequence of 10 amino acids followed by the so-called N-loop, a helical turn, a three-stranded  $\beta$ -sheet and a C-terminal  $\alpha$ -helix. The whole structure is stabilized by the two disulfide bridges linking the N-terminus and the N-loop to the  $\beta$ -sheet at  $\beta$ -strand 3 and to the loop connecting  $\beta$ -strand 1 and 2 (Czaplewski et al., 1999).

MIP-1 $\alpha$  mediates its effects through binding to CCR1 and CCR5 receptors.

CCR1 receptors are expressed on lymphocytes such as monocytes and peripheral blood monocytes (Neote et al., 1993; Su et al., 1996). Apart from MIP-1 $\alpha$ , this receptor interacts with

several other CC-chemokines such as RANTES, leukotactin, MCP1-4, MIP1-1 and LD78 $\beta$  (Murphy et al., 2000; Menten et al., 1999).

Independent approaches using specific antibodies and CCR1 antagonist have proven that the interaction between MIP-1 $\alpha$  and CCR1 is involved in chemotaxis of peripheral blood monocytes in vitro (Hesselgesser et al., 1998; Su et al., 1996).

Knockout mice have been generated for both MIP-1 $\alpha$  (Cook et al., 1995) and the CCR1 receptor (Gao et al., 1997). In both cases gene disruption is non-lethal, but a reduced immunological response by cell infiltration is in general observed when the two strains of mice are challenged in different ways. Better understanding of MIP-1 $\alpha$  - CCR1 receptor interactions may thus be used as a basis for development of new agents for the treatment of a variety of inflammatory diseases such as asthma, arthritis or multiple sclerosis.

Very little is known about the topology of the complex formed between MIP-1 $\alpha$  and the CCR1 receptor. The effect of a few structural changes, such as point mutagenesis of MIP-1 $\alpha$ , on the interaction with CCR1 have been investigated (Czaplewski et al., 1999; Koopmann and Krangel, 1997).

One powerful approach to study receptor-ligand interactions is affinity- or photoaffinity-labeling. By analysis of the covalently labeled receptor, it has been possible to identify interaction regions or even amino acids for various peptide ligands such as substance P (Girault et al., 1996), angiotensin (Boucard et al., 2000), cholecystokinin (Dong et al., 1999) and vasopressin (Cotte et al., 1998). In most cases the preferred photoactivatable group was benzophenone. Benzophenone incorporation can take place at many different types of amino acids (Kotzyba-Hibert et al., 1995). The reaction involves a diradical intermediate, that is not deactivated by water and that reacts with the target protein within approximately 3 Å distance (Dorman and Prestwich, 1994; Kotzyba-Hibert et al., 1995).

We previously described the preparation and pharmacological characterization of MIP-1 $\alpha$  derivatized with benzophenone (BP- MIP-1 $\alpha$ ) and fluorescein at the N-terminal position (Zoffmann et al., 2001). We showed that BP- MIP-1 $\alpha$  is specifically cross-linked to either CCR1 or CCR5 receptor upon irradiation (Zoffmann et al., 2001). In the present work, we use enzymatic and chemical methods to identify the site of covalent incorporation of BP- MIP-1 $\alpha$  in the CCR1 receptor.



## 5: Materials and Methods

### **Materials and methods**

The N-terminal photoactivatable chemokine  $^{125}\text{I}$ -BP-MIP-1 $\alpha$  was prepared as described (Zoffmann et al., 2001). The Chinese hamster ovary (CHO) cell line, stably expressing human CCR1 with a hemagglutinin tag (HA-sequence: YPYDVPYASLRS) introduced at the N-terminus was prepared by Christine Power (Solari et al., 1997).

Reagents and materials was purchased from: Clontech, BD (France), Le pont de Claix France: pIRES-Neo plasmid. Stratagene, Amsterdam Netherlands: QuickChange Site-Directed Mutagenesis Kit. Invitrogen, Cergy Pontoise France: Solutions for cell-culture. Kinematica, Lucerne Switzerland: Polytron PT1200 sonicator. Roche diagnostics, Meylan France: Trypsin, Glu-C, Lys-C, Arg-C, all sequencing grade, protease inhibitor cocktail, bacitracin, chymostatin, leupeptin. ICN biochemicals, Aurora Ohio: Phosphoramidon, bestatin. Philips AG lightning, Zurich Switzerland: Philips HPR 125W lamp. Pall life sciences, Bioblock Scientific, Illkirch France: Microsep concentrators. Novex, ICT AG, Basel Switzerland: Discontinuous polyacrylamide gels. Spectrum, Struers Kebolab, Albertslund Denmark: Spectra/Por Cellulose Ester DispoDialyzer.

#### *Introduction of point mutations in the CCR1 receptor:*

The CCR1 gene was inserted in the KS-bluescript vector by use of the sites BamH1 and Xho1, and the mutations Phe149 to asparagine (TM4) and Leu205 to glycine (TM5) were introduced by use of the QuickChange Site-Directed Mutagenesis Kit. The vector was multiplied in the dam-methylation positive E.Coli strain XL1-Blue. For each mutation two complementary mutated oligo-nucleotides were designed. The whole plasmid was replicated by PCR with the

pfu-turbo DNA polymerase. The WT template DNA was removed by digestion with the dam-methylation recognizing endopeptidase DpnI and the nicked mutated plasmids were transfected into the E.Coli strain XL1-Blue. The genes for CCR1, CCR1[F149N] and CCR1[L205G] were excised with BamHI and NotI and introduced in the pIRES-Neo plasmid, opened with EcoRV and NotI.

### *Cell membrane preparation*

A 60-liter suspension culture of CCR1 expressing CHO cells was grown to an approximate density of  $10^6$  cells/ml in Dulbecco's modified Eagles F12-medium supplied with 10% Foetal Calf Serum, 2 mM glutamine and 100 units/ml penicillin/streptomycin. Cell membranes were prepared by sonication with a Polytron PT1200 sonicator in a small volume of lysis buffer (HEPES pH 7.4, 1 mM EDTA, 10 mM  $MgCl_2$  supplied with protease inhibitor cocktail), followed by centrifugation at 500g for 30 min to remove cell debris. Membranes were recovered from the supernatant by centrifugation at 48000g for 30 min. The resulting pellets were reconstituted in a small volume of breaking buffer, aliquoted and stored at  $-80^\circ C$  until use. Stored under these conditions, the receptors retained their binding activity for several months. The protein content was estimated by use of a colometric assay.

Human embryonic kidney 293 (HEK293) cells were transfected with the calcium phosphate precipitation procedure and stable cell lines established by selection for neomycin resistance. HEK293 cells stably expressing CCR1, were grown in Dulbecco's minimal growth medium supplied with 10% foetal calf serum, 2 mM glutamine, 100 units/ml penicillin/streptomycin and 800  $\mu g/ml$  neomycin. For CCR1, CCR1[F149N] or CCR1[L205G] expressing cells-lines, cells from five 225  $cm^2$  cell culture bottles were detached with 5 mM EDTA in PBS (135 mM NaCl, 4.5 mM KCl, 10 mM  $NaH_2PO_4$ , pH 7.8), resuspended in 3 ml TE buffer (50 mM Tris, 1 mM

EDTA, pH 7.4 supplied with protease inhibitors: bacitracin, bestatin, chymostatin, leupeptin, phosphoramidon and the cells broken by 25 passes in a glass-Teflon potter. Cell membranes were isolated, stored and protein content measured as for the CHO cell-membranes.

#### *Photoaffinity labeling of CCR1 with $^{125}\text{I}$ -BP- MIP-1 $\alpha$*

For photoaffinity labeling of receptors, 1 mg membrane protein (30  $\mu\text{g}/\mu\text{l}$ ) was diluted in 2 ml Rosen A buffer (50 mM HEPES, 1 mM  $\text{CaCl}_2$ , 5 mM  $\text{MgCl}_2$ , 0.5% BSA pH 7.2).  $^{125}\text{I}$ -BP-MIP-1 $\alpha$  was added to a final concentration of 2 nM and the mixture incubated at room temperature for 2 h. Membranes were irradiated at 365 nm with a Philips HPR 125W lamp at 6 cm distance while kept on ice and with continuous stirring. After 30 min irradiation, the membrane fraction was separated from unbound ligand by centrifugation at 20,000g for 25 min at 4 °C and washed once in 2 ml ice-cold Rosen A buffer.

#### *Alkylation and purification of CCR1- $^{125}\text{I}$ -BP- MIP-1 $\alpha$ complex*

For reduction and alkylation of the free cysteines, the membrane pellet was resuspended in 200  $\mu\text{l}$  0.1 M Tris pH 8.5 containing 8 M urea, sonicated and flushed with nitrogen. 10  $\mu\text{l}$   $\beta$ -mercapto-ethanol was added and the reaction incubated at 40 °C for 16 h under nitrogen atmosphere. A 200  $\mu\text{l}$  solution of 24 mg iodoacetamide and 30 mg Tris-base was added, pH adjusted to 9 with HCl, the reaction incubated at room temperature for 15 min in the dark and a further 10  $\mu\text{l}$   $\beta$ -mercapto ethanol was added to quench unreacted iodoacetamide. Afterwards the reaction mixture was desalted by two consecutive dilutions and concentration steps using Microsep concentrators (MW>10 kDa cut-off). Finally the sample was separated on polyacrylamide gel. A small part of the undried gel was autoradiographed at –80 °C for two

hours and the position of the labeled receptor used to identify the position of the complex in the rest of the undried and unstained gel. For endopeptidase and  $\text{NH}_4\text{OH}$  cleavage, the peptide was extracted by passive elution at room temperature for 24-48 h in 1.5 ml 0.1 M  $\text{NH}_4\text{HCO}_3$ -buffer pH 8.5. To remove salt and SDS traces from the gel and to exchange buffer, the extract was dialyzed in a DispoDialyzer with a molecular cut-off at 25 kDa against 10 mM  $\text{NH}_4\text{HCO}_3$  or 10 mM Tris-buffer, and the peptide concentrated 10 times under vacuum or in a Microsep concentrator.

*Protease digestion, CNBr and  $\text{NH}_4\text{OH}$  fragmentation of the  $\text{CCR1-}^{125}\text{I-BP- MIP-1}\alpha$  complex*

For the Glu-C cleavage, the receptor-ligand complex was incubated at 25 °C for 18 h in 20  $\mu\text{l}$  25-50 mM  $\text{NH}_4\text{HCO}_3$  pH 7.8 with 2  $\mu\text{g}$  Glu-C. For trypsin cleavage,  $\text{CCR1-}^{125}\text{I-BP- MIP-1}\alpha$  complex was incubated in 20  $\mu\text{l}$  100 mM Tris pH 8.5 containing for 18 h at 37 °C containing 2  $\mu\text{g}$  trypsin. After reaction the samples were analyzed by SDS PAGE on a 12% Tris-tricine gel or a Novex 10-20% Nupage gel, followed by autoradiography.

The  $\text{CCR1-}^{125}\text{I-BP- MIP-1}\alpha$  covalent complex was cleaved in gel with CNBr (Grutter et al., 2000). The isolated gel fragment containing the ligand receptor complex was cut into small pieces, washed twice 20 min in a 1:1 mixture of water and acetonitrile and dried to approximately half the initial size. 0.7 ml or 150 mM CNBr solution in 70% formic acid, was added in several steps to allow the gel to absorb the reagent and finally cover the reconstituted gel pieces. The reaction was allowed to proceed in the dark for 3 days under argon atmosphere. To eliminate the CNBr and acid, the samples were repeatedly dried under vacuum and reconstituted 7 times in 1 ml of water. Finally peptides were extracted from gel-pieces in 2 ml 100 mM  $\text{NH}_4\text{HCO}_3$  solution for 24 h at room temperature. The recovery of radioactivity was

80-90%. Extract was concentrated on a Microsep concentrator (3 kDa cut-off). After reaction the samples were analyzed by SDS-PAGE on a 12% Tris-tricine gel, followed by autoradiography.

For cleavage with  $\text{NH}_4\text{OH}$ , the receptor ligand complex was prepared as for protease digest. A hydroxylamine solution at pH 9 in 8 M urea was prepared according to Allen (Allen, 1989), with urea replacing 6 M guanidinium chloride. 25  $\mu\text{l}$   $\text{NH}_4\text{OH}$  was added to the labelled receptor and the reaction was incubated at 45°C overnight. The content of salt was reduced 20-fold by use of a Microsep concentrator (10 kDa cut-off). The reaction products were analyzed by SDS-PAGE on a 10% Tris-glycine gel, followed by autoradiography.

*Protease digestion, CNBr and  $\text{NH}_4\text{OH}$  fragmentation of  $^{125}\text{I}$ -BP- MIP-1 $\alpha$ .*

For protease digestion and CNBr cleavage of  $^{125}\text{I}$ -BP- MIP-1 $\alpha$  the ligand was reduced, alkylated, purified by gel electrophoresis and the gel containing the radioactivity isolated as described for the complex. The ligand was extracted in  $\text{NH}_4\text{HCO}_3$  buffer, concentrated under vacuum and digested with Glu-C and trypsin, as described for the receptor ligand complex. For Lys-C the incubation condition was respectively 1  $\mu\text{g}$  enzyme in 20  $\mu\text{l}$  25 mM Tris-HCl, 1 mM EDTA, pH 8.5 for 18 h. The conditions used for fragmentation with Arg-C was 1  $\mu\text{g}$  enzyme in total 100  $\mu\text{l}$  100 mM Tris-HCl, 10 mM  $\text{CaCl}_2$ , 5 mM DTT, 0.5 mM DTT supplied with 10  $\mu\text{l}$  Arg-C activation solution and incubation for 18 h at 37 °C.

CNBr cleavage of MIP-1 $\alpha$  ligand was performed in gel following the same procedure as for the photoaffinity labeled receptor. For the cleavage with  $\text{NH}_4\text{OH}$ , a sample of  $^{125}\text{I}$ -BP-MIP-1 $\alpha$  was incubated with  $\text{NH}_4\text{OH}$  using the same protocol as for the complex.

The resulting fragments from CNBr cleavage,  $\text{NH}_4\text{OH}$  cleavage and protease digest were separated by SDS-PAGE and the gels analyzed by autoradiography.

## 6: Results

### **Results**

#### *Photoaffinity labeling and partial purification of CCR1*

Photoaffinity labeling of the recombinant CCR1 receptor is carried out with a radioactive photoactivatable benzophenone derivative of the chemokine MIP-1 $\alpha$ ,  $^{125}\text{I}$ -BP-MIP-1 $\alpha$  on membrane preparations. This high affinity agonist ligand covalently binds in a specific manner to CCR1 to form a complex with an apparent molecular weight (54 kDa), which represents the addition of 8 kDa of one MIP-1 $\alpha$  into CCR1 receptor (46 kDa). The ratio between unincorporated ligand and crosslinked complex is approximately 10 to 1 for receptors in membrane preparations, which is comparable to the values found in assays with intact cells (Zoffmann et al., 2001).

#### *Experimental strategy used to identify the site of MIP-1 $\alpha$ incorporation*

The general approach used here to determine the site of MIP-1 $\alpha$  incorporation consists in identifying the smallest sizable radioactive fragment which corresponds to a plausible subfragment of the CCR1-MIP-1 $\alpha$  complex, but not to a subfragment of MIP-1 $\alpha$  alone. The size of the fragment is then compared to the size of all expected digestion products. A combination of cleavage methods yielding receptor fragments of different sizes is then used to reduce the number of possibilities to a single domain of the receptor.

$^{125}\text{I}$ -BP- MIP-1 $\alpha$  is a 69 amino acid photoactivatable ligand into which no modifications were made to remove existing enzymatic or chemical cleavage sites. Thus is the number of putative fragmentation approaches reduced, since only enzymatic or chemical methods that do not cleave MIP-1 $\alpha$  between the BP group and the radioiodinated tyrosine(s) could be used for this approach.

*Pattern of enzymatic and chemical cleavage of MIP-1 $\alpha$  ligand with Glu-C, trypsin, Lys-C, Arg-C proteases and CNBr.*

To analyze the fragmentation pattern of the reduced and alkylated BP-MIP-1 $\alpha$ , the peptide was submitted to cleavage with Glu-C, trypsin, Lys-C and Arg-C endopeptidases or with CNBr.

Migration of the cleavage products is shown in Figure 1. Using separation on SDS-PAGE it is possible to identify peptides with molecular weights as low as approximately 2 kDa.

Assuming that all three tyrosine residues from MIP-1 $\alpha$  are radiolabeled upon radioiodination, the expected size of radioactive fragments obtained after Glu-C fragmentation is 3.6 kDa (Y15 and Y28) and 1.5 kDa (Y62). We observe only one band at 3.4 kDa (Figure 1, panel A).

The Lys-C digestion is expected to result in two radiolabeled peptides with weight at 4.4 (Y15 and Y28) and 1 kDa (Y62) (cleavage at positions 37, 45 and 61). A unique band at 4 kDa is observed (Figure 1, panel B).

The Glu-C and Lys-C indicate that  $^{125}\text{I}$  is present at least on one of the two tyrosines Y15 and Y28 at the N-terminal fragment.

Trypsin digestion results in a broad band positioned between 3.5 kDa and 2.5 kDa. For this digest three peptides with the respective sizes of 2.2 (Y15), 2.3 (Y28) and at 1.0 kDa (Y62) were expected (Figure 1, panel C). Also the Arg-C digest was predicted to result in three bands, at 2.2 kDa (Y15), 3.3 kDa (Y28) and 2.6 kDa (Y62) respectively. Only a band with a size

around 3.0 kDa is seen (Figure 1, panel D). Both trypsin and Arg-C are expected to cleave at position 18 between the two tyrosines Y15 and Y28. The detectable bands show similar apparent size for the two digests. The apparent molecular weight is slightly larger than expected for all labeled bands from trypsin, and for the N-terminal part of Arg-C, where the size of the observed fragment better corresponds to the size of the Y28 radiolabeled fragment. The size of each fragment is however close to the detection limit and slight changes in migration may occur. From the fragmentation of BP-MIP-1 $\alpha$  with Arg-C and trypsin it is not possible to conclude whether iodine is present at each of the tyrosines Y15 and Y28 or only at one of them. CNBr treatment under acidic conditions does, as expected, result in no cleavage, since no methionines are present in BP-MIP-1 $\alpha$  (panel E). Thus, from all digestion analyses, it can be concluded that Y15 and/or Y28 are labeled by  $^{125}\text{I}$ . Iodination of Y62 is not crucial for the analysis and it is difficult to conclude whether it is present or not.

#### *Fragmentation of the CCR1-BP-MIP-1 $\alpha$ complex*

The Glu-C endopeptidase (*Staphylococcus aureus* endopeptidase V8) hydrolyses peptide bonds after glutamate residues. As can be seen in figure 2, digestion of the receptor-ligand complex is not complete, such pattern has been observed in four independent cleavage experiments with this enzyme. The smallest clearly detectable peptide on the autoradiography of Glu-C digest has an apparent molecular weight of 3.5-4.5 kDa. As compared to digestion of the ligand alone, the smallest band detected here is larger by up to 1.5 kDa indicating that a small fragment of the receptor protein is covalently attached to the radioactive MIP-1 $\alpha$  fragment. Assuming that BP-MIP-1 $\alpha$  does not link to intracellular parts of the receptor, putative small-sized receptor fragments that may correspond to the 3.5-4.5 kDa band which results from Glu-C cleavage, are:



-13 to 29 (N-term), 178-194 (E2) or 273-287 (E3). Their position in the receptor structure is illustrated in Figure 4.

*Trypsin* cleaves after the basic residues arginine and lysine. The smallest peptide resulting from trypsin treatment (n=4), migrates with an apparent molecular weight of 3.5-4.5 kDa (Figure 2, lane 2). This size is about 0.5-1.5 kDa larger than that seen in trypsin fragmentation of the ligand alone. Putative receptor fragments that could account for the increase in weight are the N-terminal amino acids (aa -13 to -2 in the HA sequence); aa 27-30 (N-term), 94-106 (E1) and 174-196 (E2). Their position in the expected fragmentation pattern for the cleavage of CCR1 with trypsin is shown in Figure 4.

*CNBr cleavage.* Due to the acid labile nature of the covalent bond between benzophenone and MIP-1 $\alpha$ , the conditions required to cleave with CNBr were optimized, by adjusting the incubation time and pH of the reaction, in order to selectively cleave methionine peptide bonds before releasing the BP group. Figure 3 shows the result of incubation in 70% formic acid for 24 h, in the presence (lane 1) and absence (lane 2) of CNBr. The amount of free ligand released by acid treatment is high, but weak bands with apparent weights of 14, 16 and 20 kDa, which are larger than the ligand alone, are clearly detected in three independent experiments. These bands are not seen when the sample is incubated in acid without CNBr. The observed size and relative intensity of these fragments was very reproducible. Furthermore, bands around this size are well separated on a 12% Tricine gel. The use of molecular weight markers at 10 kDa, 14.4 kDa, 17.0 kDa and 20 kDa allow accurate estimate of apparent molecular weights. The contribution from the receptor to the 14 kDa band is thus expected to be about 6 kDa. This fits with the expected fragment 106-166 (TM3-4) or the fragment 167-215 (E2 + TM5), as indicated in Figure 4.

Eventhough cleavage with CNBr is reproducible, it may not necessarily be complete (Grutter et al., 2000). Therefore the weak bands with apparent sizes of 17 kDa and 20 kDa (figure 3) could result from partial cleavage. In particular if the 14 kDa band corresponds to segment 167-215, the size of higher molecular weight bands could be accounted for by partial cleavage at position 166 leading to a 20.4kDa fragment (106-215) and by partial cleavage at position 215 leading to a 17 kDa fragment (167-245). In contrast, a similar reasoning for cross-linking to the fragment containing E1 + TM2 (106-166) would result in partial cleavage bands at 20.4 kDa (aa 106-215) and 18.9 kDa (aa 69-166) respectively, which do not correspond to the experimentally detected fragment sizes. Our results thus support cross-linking of BP-MIP-1 $\alpha$  to fragment 167-215.

The N-terminal parts of the receptor that include aa 27-29, and -13 to -2 (in HA-tag), two potential segments from the trypsin and Glu-C cleavages, have calculated weights at 16.5 kDa, and 23 kDa for a non-complete cleavage at the C-terminal which do not fit with the CNBr cleavage pattern.

Despite purification attempts carried out to get mass spectrometry analysis, the amount of available material was too low to get a detectable signal that could lead to a direct confirmation of a part of the receptor as being photoaffinity labelled. We therefore used a site directed mutagenesis approach to support one receptor sequence as interacting with the N-terminal of BP-MIP-1 $\alpha$ .

#### *Cleavage with NH<sub>4</sub>OH of WT and point mutated receptors*

Two mutant receptors were designed, each with a unique Asn-Gly cleavage site for hydroxylamine introduced after position 150 (TM4) and 205 (TM5), (Figure 6). Both receptors (F149N and L205G) are expressed in HEK293 cells at a level comparable to wild type CCR1 (data not shown). The mutations did not significantly change the BP-MIP-1 $\alpha$  binding affinity

and BP-MIP-1 $\alpha$  was incorporated in the mutants at a level similar to that of wild type receptor (data not shown). Migration on a gel was identical for the mutant and wildtype receptors expressed in HEK293 cells and the wildtype receptor expressed in CHO cells used for the Glu-C, trypsin and CNBr cleavages. Both L205G and F149N are cleaved by NH<sub>4</sub>OH, albeit incompletely (Figure 5). However for both mutated receptor- BP-MIP-1 $\alpha$  complexes, a band migrating at an apparent molecular weight of 33 kDa was detected. This band was not present in the WT samples. Cleavage of the mutated receptors by NH<sub>4</sub>OH is expected to produce two fragments. In the F149N mutant, the calculated molecular weights expected are respectively 25.5 kDa for the N-terminal part (aa 1-150) and 32.5kDa for the C-terminal part (aa 151-354) when the weight of MIP-1 $\alpha$  is included. The experimental weight fits with the size of the C-terminal fragment. In the L205G mutant, the similarly calculated molecular weights of the two receptor fragments after cleavage are respectively 32 kDa for the N-terminal part (aa 1-205) and 26 kDa for the C-terminal part (aa 206-354). For this mutant the experimental weight fits with the N-terminal part. The overlap between the identified regions for the two mutants extends from the beginning of TM4 to the middle of TM5 (aa 151-205), as illustrated in figure 4.

## 7: Discussion

The recent elucidation of rhodopsin structure (Palczewski et al., 2000), a protein from the same group as chemokine, tachykinin, cholecystokinin and angiotensin receptors, sheds new light on N-terminal domain and extracellular loops folding. Indeed, most of these receptors contain extracellular domains of comparable length. In addition, they all contain a disulfide bridge linking E1 to E2. It is thus plausible that these receptors exhibit similar folding of their external parts, which, according to rhodopsin structure, would limit the access of large ligands to transmembrane segments.

As compared to most ligands interacting with G protein-coupled receptors, chemokines are characterized by a large size and a conserved three dimensional structure (Rollins, 1997). Their interactions with chemokine receptors therefore may take place at the level of a large area and involve multiple contacts between the ligand and the receptor, as was shown for the interaction of  $\alpha$ -bungarotoxin with the nicotinic acetylcholine receptor (Maelicke and Conti-Tronconi, 1989). Analyses of chemokine interactions with receptors included two separate aspects, namely chemical specificity of receptor recognition and receptor activation (Crump et al., 1997; Wells et al., 1996).

**Receptor recognition** is mainly driven by an extended hydrophobic groove delineated by the second and third  $\beta$ -sheet strands (Clubb et al., 1994; Mayer and Stone, 2000; Mizoue et al., 1999; Ye et al., 2000) as well as the N-terminal end and the N-loop (Lowman et al., 1996) of the chemokine. On the receptor side, the N-terminus has been identified as being counterpart in the interaction. The interaction is likely to involve other parts of the receptor as well, since the structure of rhodopsin demonstrates a three dimensional structure, where the extracellular loops of the receptor are in proximity of the N-terminal end. For the interaction between MIP-1 $\alpha$  and the CCR1 receptor all available data indicate that it involve several parts of the receptor,

including the N-terminal, and E3 loop (Monteclaro and Charo, 1996; Pease et al., 1998; Su et al., 1996).

**Receptor activation** mostly relies on residues situated in the N-terminus of the chemokines (Gong et al., 1996; Laurence et al., 2000; Solari et al., 1997; Townson et al., 2000; Zoffmann et al., 2001), while the corresponding part of the receptor that interact with the chemokine are poorly identified.

Based on the structure-activity relationship analyses carried out on chemokines, a two-domain model of ligand interactions with the receptor has been proposed (Crump et al., 1997; Wells et al., 1996; Hesselgesser et al., 1998). According to this model, the residues of the chemokine which confer binding selectivity or specificity may be distinguished from those which confer agonist versus antagonist potency.

In this work we use a photoactivatable chemokine carrying the photochemically reactive group at the N-terminus, within a domain of the ligand that is involved in receptor activation rather than receptor recognition. From separate cleavages carried out with Glu-C, trypsin or CNBr of covalently labelled CCR1, we obtain data that support more than one candidate for interaction with the N-terminal of the chemokine as presented in figure 4. However, when combined, the cleavage data converge towards labeling occurring in the extracellular loop 2 of CCR1, within a 17 amino acid sequence comprising amino acids 178 to 194. To confirm this potential incorporation site, we further analyse the cleavage pattern of mutant receptors containing hydroxylamine sites in TM4 or TM5 (Figure 4). This approach, which consists in adding new sites rather than removing existing ones, is based on the following arguments: **i) Since chemical or enzymatic cleavage reactions may not be quantitative, removal of existing sites may not be conclusive. In contrast, introduction of new cleavage sites is expected to lead to positive**

cleavage results, in particular for hydroxylamine which has no consensus cleavage site on the WT CCR1 receptor ii) the mutations requested to create hydroxylamine sites are minor and located at some distance from the site of chemokine labeling. They are less likely to modify ligand-receptor interaction, in contrast to mutations removing Glu-C, or trypsin sites which are located close to MIP-1 $\alpha$  incorporation site, iii) removal or introduction of CNBr sites was ruled out due to the poor efficacy of CNBr cleavage on the WT receptor.

The hydroxylamine cleavage data on the mutant receptors, in combination with the chemical and enzymatic cleavage data obtained on WT receptor, unambiguously establish that the N-terminus of MIP-1 $\alpha$  interacts within a segment comprising residues 178 to 194. The corresponding sequence in rhodopsin is entirely situated in the E2, likely reflecting the situation in the CCR1 since the length of the extracellular domains is highly conserved between the two receptors.

In association with previous work describing that fluoresceine, incorporated at the same N-terminal position as benzophenone in MIP-1 $\alpha$ , is fully accessible to buffer-soluble fluorescence quenchers, such as iodine (Zoffmann et al., 2001), the present data support the view that the N-terminal of receptor-bound MIP-1 $\alpha$ , as well as the the labeled part of the E2 loop of the receptor, are located at the exterior of the receptor.

According to the location of the photoactivatable group on the chemokine, the E2 loop would contribute with interactions that are responsible for the agonist activity of chemokines, while other interactions, in particular with the N-terminus and the E3 loop of the chemokine receptor, would contribute with residues participating to the chemical specificity of receptor recognition, i.e. that are specific to chemokine recognition independently of their agonist or antagonist character.

In conclusion, the present data pinpoint the interaction between the second extracellular loop of CCR1, and a portion of MIP-1 $\alpha$  which determines the agonist character of the ligand rather than its recognition specificity. This result does not provide support to the idea that agonists must have access to the transmembrane core of the protein. Rather, the possibility that the whole receptor molecule undergoes structural changes during the transition leading to receptor activation, should be considered. The conformational transition would be global and would imply all intracellular, transmembrane as well as extracellular domains of the protein. Accordingly, agonists would be able to selectively recognize the active state of the receptor by interacting with any of these domains, depending on their size, on the structure of their binding site and on accessibility of these regulatory sites.

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## 9: References

Allen G (1989) *Sequencing of proteins and peptides*. Elsevier, Amsterdam, London, New York, Tokyo.

Boucard AA, Wilkes BC, Laporte SA, Escher E, Guillemette G and Leduc R (2000) Photolabeling identifies position 172 of the human AT(1) receptor as a ligand contact point: receptor-bound angiotensin II adopts an extended structure. *Biochemistry* **39**:9662-70.

Clubb TR, Omichinski JG, Clore GM and Gronenborn AM (1994) Mapping the binding surface of interleukin-8 complexed with an N-terminal fragment of the Type 1 human interleukin-8 receptor. *FEBS letters* **338**:93-97.

Cook DN, Beck MA, Coffman TM, Kirby SL, Sheridan JF, Pragnell IB and Smithies O (1995) Requirement of MIP-1 $\alpha$  for an inflammatory response to viral infection. *Science* **269**:1583-5.

Cotte N, Balestre MN, Phalipou S, Hibert M, Manning M, Barberis C and Mouillac B (1998) Identification of residues responsible for the selective binding of peptide antagonists and agonists in the V2 vasopressin receptor. *J Biol Chem* **273**:29462-8.

Crump MP, Gong JH, Loetscher P, Rajarathnam K, Amara A, Arenzana-Seisdedos F, Virelizier JL, Baggiolini M, Sykes BD and Clark-Lewis I (1997) Solution structure and basis for functional activity of stromal cell- derived factor-1; dissociation of CXCR4 activation from binding and inhibition of HIV-1. *Embo J* **16**:6996-7007.

Czaplewski LG, McKeating J, Craven CJ, Higgins LD, Appay V, Brown A, Dudgeon T, Howard LA, Meyers T, Owen J, Palan SR, Tan P, Wilson G, Woods NR, Heyworth CM, Lord BI, Brotherton D, Christison R, Craig S, Cribbes S, Edwards RM, Evans SJ, Gilbert R, Morgan P, Hunter MG and et al. (1999) Identification of amino acid residues critical for aggregation of human CC chemokines macrophage inflammatory protein MIP-1 $\alpha$ , MIP-1 $\beta$  and RANTES. Characterization of active disaggregated chemokine variants. *J Biol Chem* **274**:16077-84.

DiPietro LA, Burdick M, Low QE, Kunkel SL and Strieter RM (1998) MIP-1 $\alpha$  as a critical macrophage chemoattractant in murine wound repair. *J Clin Invest* **101**:1693-8.

Dong M, Ding XQ, Pinon DI, Hadac EM, Oda RP, Landers JP and Miller LJ (1999) Structurally related peptide agonist, partial agonist and antagonist occupy a similar binding pocket within the cholecystokinin receptor. Rapid analysis using fluorescent photoaffinity labeling probes and capillary electrophoresis. *J Biol Chem* **274**:4778-85.

Dorman G and Prestwich GD (1994) Benzophenone photophores in biochemistry. *Biochemistry* **33**:5661-73.

Gao JL, Wynn TA, Chang Y, Lee EJ, Broxmeyer HE, Cooper S, Tiffany HL, Westphal H, Kwon-Chung J and Murphy PM (1997) Impaired host defense, hematopoiesis, granulomatous inflammation and type 1-type 2 cytokine balance in mice lacking CC chemokine receptor 1. *J Exp Med* **185**:1959-68.

Girault S, Sagan S, Bolbach G, Lavielle S and Chassaing G (1996) The use of photolabelled peptides to localize the substance-P-binding site in the human neurokinin-1 tachykinin receptor. *Eur J Biochem* **240**:215-22.

Gong JH, Uguccioni M, Dewald B, Baggiolini M and Clark-Lewis I (1996) RANTES and MCP-3 antagonists bind multiple chemokine receptors. *J Biol Chem* **271**:10521-7.

Grutter T, Ehret-Sabatier L, Kotzyba-Hibert F and Goeldner M (2000) Photoaffinity labeling of Torpedo nicotinic receptor with the agonist [3H]DCTA: identification of amino acid residues which contribute to the binding of the ester moiety of acetylcholine. *Biochemistry* **39**:3034-43.

Hesselgesser J, Ng HP, Liang M, Zheng W, May K, Bauman JG, Monahan S, Islam I, Wei GP, Ghannam A, Taub DD, Rosser M, Snider RM, Morrissey MM, Perez HD and Horuk R (1998) Identification and characterization of small molecule functional antagonists of the CCR1 chemokine receptor. *J Biol Chem* **273**:15687-92.

Koopmann W and Krangel MS (1997) Identification of a glycosaminoglycan-binding site in chemokine macrophage inflammatory protein-1 $\alpha$ . *J Biol Chem* **272**:10103-9.

Kotzyba-Hibert F, Kapfer I and Goeldner M (1995) Recent trends in photoaffinity labeling. *Angew Chem Int Ed Engl* **34**:1296-1312.

Laurence JS, Blanpain C, Burgner JW, Parmentier M and LiWang PJ (2000) CC chemokine MIP-1 $\beta$  can function as a monomer and depends on Phe13 for receptor binding. *Biochemistry* **39**:3401-9.

Lowman HB, Slagle PH, DeForge LE, Wirth CM, Gillece-Castro BL, Bourell JH and Fairbrother WJ (1996) Exchanging interleukin-8 and melanoma growth-stimulating activity receptor binding specificities. *J Biol Chem* **271**:14344-52.

Maelicke A and Conti-Tronconi BM (1989) Multipoint attachment of ligands to the nicotinic acetylcholine receptor from torpedo electric organ. *J Protein Chem* **8**:326-7.

Mayer KL and Stone MJ (2000) NMR solution structure and receptor peptide binding of the CC chemokine eotaxin-2. *Biochemistry* **39**:8382-95.

Menten P, Struyf S, Schutyser E, Wuyts A, De Clercq E, Schols D, Proost P and Van Damme J (1999) The LD78 $\beta$  isoform of MIP-1 $\alpha$  is the most potent CCR5 agonist and HIV-1-inhibiting chemokine. *J Clin Invest* **104**:R1-5.

Mizoue LS, Bazan JF, Johnson EC and Handel TM (1999) Solution structure and dynamics of the CX3C chemokine domain of fractalkine and its interaction with an N-terminal fragment of CX3CR1. *Biochemistry* **38**:1402-14.

Montecarlo FS and Charo IF (1996) The amino-terminal extracellular domain of the MCP-1 receptor, but not the RANTES/MIP-1 $\alpha$  receptor, confers chemokine selectivity. Evidence for a

two-step mechanism for MCP-1 receptor activation. *J Biol Chem* **271**:19084-92.

Murphy PM, Baggiolini M, Charo IF, Hebert CA, Horuk R, Matsushima K, Miller LH, Oppenheim JJ and Power CA (2000) International union of pharmacology. XXII. Nomenclature for chemokine receptors. *Pharmacol Rev* **52**:145-76.

Nakao M, Nomiyama H and Shimada K (1990) Structures of human genes coding for cytokine LD78 and their expression. *Mol Cell Biol* **10**:3646-58.

Neote K, DiGregorio D, Mak JY, Horuk R and Schall TJ (1993) Molecular cloning, functional expression and signaling characteristics of a C-C chemokine receptor. *Cell* **72**:415-25.

Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, Yamamoto M and Miyano M (2000) Crystal structure of rhodopsin: A G protein-coupled receptor. *Science* **289**:739-45.

Pease JE, Wang J, Ponath PD and Murphy PM (1998) The N-terminal extracellular segments of the chemokine receptors CCR1 and CCR3 are determinants for MIP-1 $\alpha$  and eotaxin binding, respectively, but a second domain is essential for efficient receptor activation. *J Biol Chem* **273**:19972-6.

Rollins BJ (1997) Chemokines. *Blood* **90**:909-28.

Solari R, Offord RE, Remy S, Aubry JP, Wells TN, Whitehorn E, Oung T and Proudfoot AE (1997) Receptor-mediated endocytosis of CC-chemokines. *J Biol Chem* **272**:9617-20.

Su SB, Mukaida N, Wang J, Nomura H and Matsushima K (1996) Preparation of specific polyclonal antibodies to a C-C chemokine receptor, CCR1 and determination of CCR1 expression on various types of leukocytes. *J Leukoc Biol* **60**:658-66.

Townson JR, Graham GJ, Landay NR, Rasala B and Nibbs RJB (2000) Aminooxypentane Addition to the Chemokine Macrophage Inflammatory Protein-1 $\alpha$  Increases Receptor Affinities and HIV Inhibition. *J Biol Chem* **275**:39254-61.

Wells TN, Power CA, Lusti-Narasimhan M, Hoogewerf AJ, Cooke RM, Chung CW, Peitsch MC and Proudfoot AE (1996) Selectivity and antagonism of chemokine receptors. *J Leukoc Biol* **59**:53-60.

Ye J, Kohli LL and Stone MJ (2000) Characterization of binding between the chemokine eotaxin and peptides derived from the chemokine receptor CCR3. *J Biol Chem* **275**:27250-7.

Zoffmann S, Turcatti G, Galzi J, Dahl M and Chollet A (2001) Synthesis and characterization of fluorescent and photoactivatable MIP-1 $\alpha$  ligands and interactions with chemokine receptors CCR1 and CCR5. *J Med Chem* **44**:215-22.

## 10: Footnotes

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#### b) Reprint request

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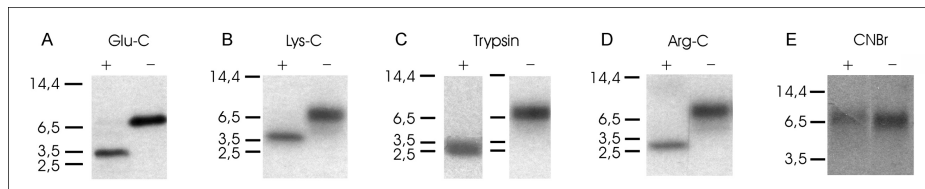
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## 12: Legends for figures

### Figure 1

#### *Fragmentation of $^{125}\text{I}$ -BP-MIP-1 $\alpha$ .*

Autoradiography of a polyacrylamide gel with  $^{125}\text{I}$ -BP-MIP-1 $\alpha$  cleaved with different amino acid specific cleavage techniques. *Panel A:*  $^{125}\text{I}$ -BP-MIP-1 $\alpha$  incubated with (+) and without (-) the presence of Glu-C for 24 hours. *B:* Lys-C digest of  $^{125}\text{I}$ -BP-MIP-1 $\alpha$  for 24 hours. *C:* Trypsin digest of  $^{125}\text{I}$ -BP-MIP-1 $\alpha$  for 24 hours. *D:* Arg-C digest of  $^{125}\text{I}$ -BP-MIP-1 $\alpha$  for 24 hours. *E:* CNBr cleavage of  $^{125}\text{I}$ -BP-MIP-1 $\alpha$  for 72 hours

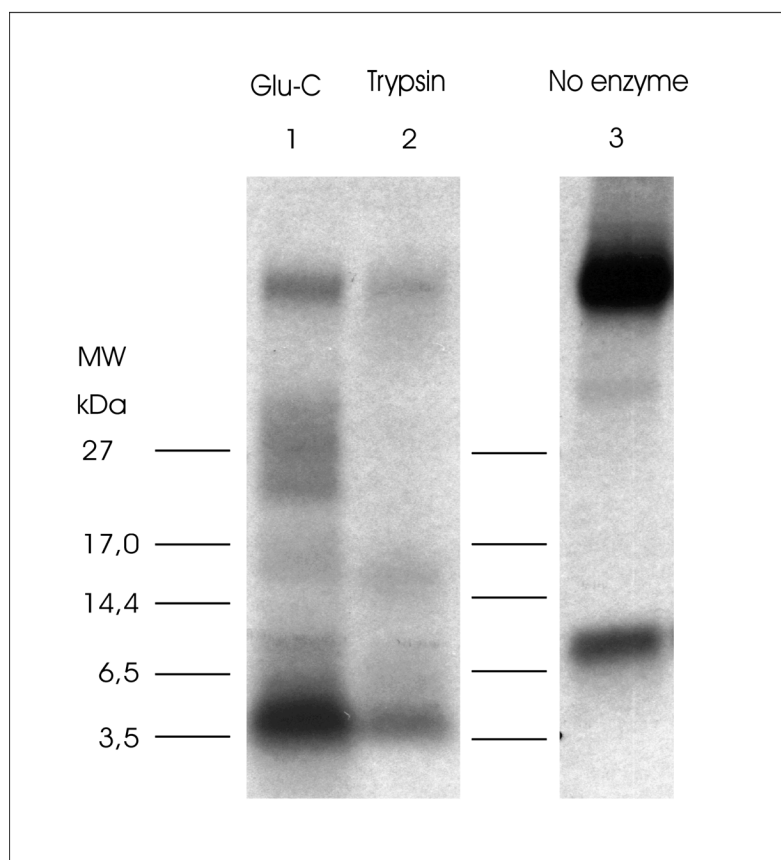


### Figure 2

#### *Enzymatic digest of CCR1- $^{125}\text{I}$ -BP-MIP-1 $\alpha$ , with Glu-C and trypsin*

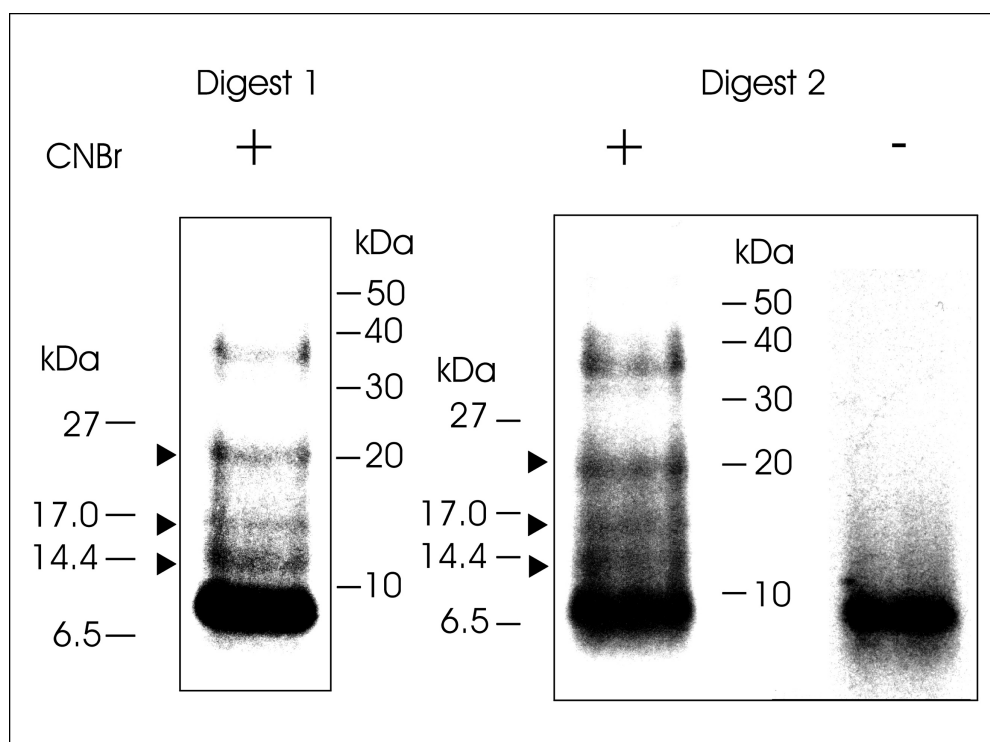
Glu-C and trypsin digests were separated SDS-PAGE on a 10-20% Nupage gel and the dried gel exposed to film for 24-48 hours. Lane 1: The CCR1- $^{125}\text{I}$ -BP-MIP-1 $\alpha$  complex incubated in the presence of Glu-C 24 h. 2: Complex incubated in the presence of trypsin 24 h. 3: Complex incubated in Glu-C cleavage buffer without enzyme for 24 h.





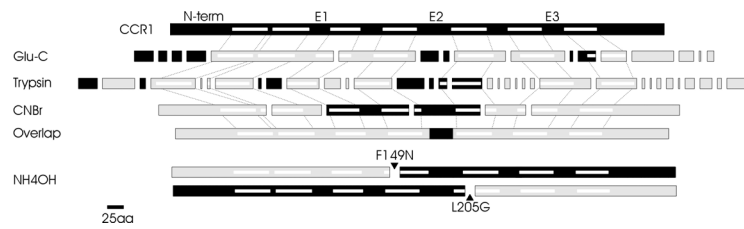
**Figure 3**

*Cyanogen bromide cleavage* of the photoaffinity-labeled human CCR1 chemokine receptor  
 Experiment 1 and experiment 2, left lane: Extract from gel incubated with 150 mM CNBr in 70% formic acid for three days. Experiment 2, right: Extract from gel incubated in 70% formic acid for three days. Arrows indicate the position of the three reproducible cleavage products in CNBr fragmentation of the CCR1-<sup>125</sup>I-BP-MIP-1 $\alpha$  complex.



**Figure 4**

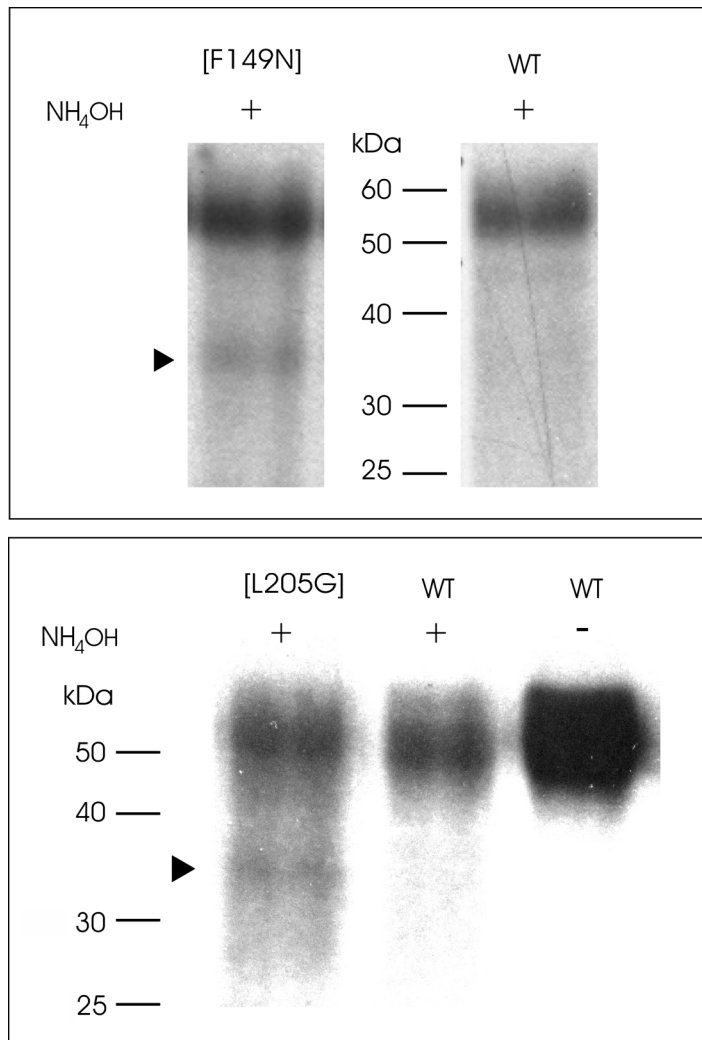
*Summary of the fragmentation analysis.* The sequence of CCR1 is presented as a bar; small white bars indicate the predicted position of transmembrane helices. Breaks in the bar correspond to the position of potential cleavage sites; lengths of the fragments are directly proportional to the number of amino acids. Top: Uncleaved receptor. The three middle rows: Cleavage with Glu-C, trypsin and CNBr of wild type CCR1. In gray is indicated the parts of the receptor found not to be interacting with the ligand, either because of size or intracellular position. Fifth row: Overlap of the result from the three different fragmentations of the wild type receptor. Bottom two rows: Fragmentation of the point-mutated receptors CCR1[F149N] and [L205G] with hydroxylamine.



**Figure 5**

*Hydroxylamine cleavage of the photoaffinity-labeled human CCR1 chemokine receptor*

Membranes from HEK-cells expressing the human CCR1 WT chemokine receptor, the point mutated receptors CCR1[F149N] or CCR1[L205G] were photoaffinity labeled and the photoaffinity labeled complex isolated. Receptor-ligand complex was then incubated in parallel with or without  $\text{NH}_4\text{OH}$  for 16 hours. Upper panel: The CCR1[F149N]- $^{125}\text{I}$ -BP-MIP-1 $\alpha$  and the CCR1 $^{125}\text{I}$ -BP-MIP-1 $\alpha$  complex incubated with  $\text{NH}_4\text{OH}$ . Lower panel: The CCR1[L205G]- $^{125}\text{I}$ -BP-MIP-1 $\alpha$  and the CCR1 $^{125}\text{I}$ -BP-MIP-1 $\alpha$  complex incubated in the presence (+) or absence (-) of  $\text{NH}_4\text{OH}$ .



**Figure 6**

*Schematic presentation of BP-MIP-1 $\alpha$  and the CCR1 receptor.* In the lower part of the figure is represented the human CCR1 chemokine receptor (Neote et al., 1993). The membrane region is indicated by two horizontal lines, with the intracellular part of the membrane as the lower and the seven predicted transmembrane regions of the receptor between the horizontal lines. Their position in the primary sequence corresponds to the one found for rhodopsin (Palczewski et al., 2000). After the first methionine is inserted a HA-epitope. In the upper part of the figure is indicated the photoactivatable analog of the chemokine MIP-1 $\alpha$  (Menten et al., 1999; Nakao et al., 1990; Zoffmann et al., 2001). Cystine bridges are indicated as lines connecting two cysteine

residues. Indicated with black background: The amino acids that has not been excluded as interaction points by the different fragmentation analysis. Tyrosines as white circles with black text: Putative points of iodination, BP: Benzophenone group. Residues with black circles and white letters indicates potential sites of cleavage by Glu-C, trypsin and CNBr, numbers indicate their position in the wild type sequence of the MIP-1 $\alpha$  and CCR1, squares indicate the positions where sites for cleavage with hydroxylamine were

