

# Identification of the Extracellular Loop 2 as the Point of Interaction between the N Terminus of the Chemokine MIP-1 $\alpha$ and Its CCR1 Receptor

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Received March 4, 2002; accepted June 17, 2002

This article is available online at <http://molpharm.aspetjournals.org>

## ABSTRACT

Macrophage inflammatory peptide-1 $\alpha$  (MIP-1 $\alpha$ )/CC-chemokine receptor ligand 3 is an 8-kDa 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 photo-activatable 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 terminus of a chemokine and its membrane-bound receptor. The work shows that the part of chemokines that is endowed with agonist properties interacts with extracellular parts of the receptor rather than the transmembrane core of the protein.

Chemokines constitute a large family of 8- to 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 $\alpha$  (MIP-1 $\alpha$ ) (Nakao et al., 1990), also known as LD78 $\alpha$  or CC-chemokine receptor ligand 3 (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 (Nakao et al., 1990; Menten et al., 1999). 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  $\beta$ -strands 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, MCP-1, myeloid progenitor inhibitory factor-1, and LD78 $\beta$  (Menten et al., 1999; Murphy et al., 2000).

This work was supported by GlaxoWellcome (fellowship to S.Z.), the Ph.D. study council, Faculty of Health, Copenhagen University (S.Z.), the European Molecular Biology Organization (Fellowship to S.Z.), the Centre National de la Recherche Scientifique, the Association pour la Recherche sur le Cancer, the Agence Nationale pour la Recherche sur le SIDA, and the Université Louis Pasteur.

**ABBREVIATIONS:** aa, amino acid; Arg-C, *Clostridium histolyticum* endoprotease; BP, benzophenone; CCR, CC-chemokine receptor; CHO, Chinese hamster ovary; E1, E2, and E3, extracellular loops 1, 2, and 3; Glu-C, *Staphylococcus aureus* endopeptidase V8; HA, hemagglutinin tag; HEK, human embryonic kidney; LD78 $\beta$ , tonsillar lymphocyte LD78  $\beta$  protein; Lys-C, *Lysobacter enzymogenes* endopeptidase; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; PAGE, polyacrylamide gel electrophoresis; RANTES, regulated on activation normal T-cell expressed and secreted; TM, transmembrane domain; WT, wild-type.

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

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 nonlethal, but a reduced immunological response by cell infiltration is observed in general 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 (Koopmann and Krangel, 1997; Czaplewski et al., 1999).

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  $\sim 3$ -Å distance (Dorman and Prestwich, 1994; Kotzyba-Hibert et al., 1995).

We described previously 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.

## Materials and Methods

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

**Reagents and Materials.** pIRES-Neo plasmid was purchased from BD Biosciences Clontech (Le pont de Claix, France); QuikChange site-directed mutagenesis kit was purchased from Stratagene (Amsterdam Netherlands); solutions for cell-culture were purchased from Invitrogen (Cergy Pontoise France). The Polytron PT1200 sonicator was from Kinematica (Lucerne, Switzerland). Trypsin, Glu-C, Lys-C, Arg-C (all sequencing grade), protease inhibitor cocktail, bacitracin, chymostatin, leupeptin were purchased from Roche Diagnostics (Meylan France); phosphoramidon and bestatin were purchased from ICN Biochemicals (Aurora OH). The Philips HPR 125-W lamp was from Philips AG lightning (Zurich, Switzerland), and Microsep concentrators were from Pall Life Sciences, Bioblock Scientific (Illkirch, France). Discontinuous polyacrylamide gels were from Novex, ICT AG (Basel, Switzerland), and the Spectra/

Por Cellulose Ester DispoDialyzer was from Spectrum, Struers Kjolbolab (Albertslund, Denmark).

**Introduction of Point Mutations in the CCR1 Receptor.** The CCR1 gene was inserted in the KS-Bluescript vector by use of the sites *Bam*HI and *Xho*I, and the mutations Phe149 to asparagine (TM4) and Leu205 to glycine (TM5) were introduced by use of the QuikChange site-directed mutagenesis kit. The vector was multiplied in the dam-methylation positive *Escherichia coli* strain XL1-Blue. For each mutation, two complementary mutated oligonucleotides were designed. The whole plasmid was replicated by polymerase chain reaction with the *Pfu*-turbo DNA polymerase. The WT template DNA was removed by digestion with the dam-methylation recognizing endopeptidase *Dpn*I 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 *Bam*HI and *Not*I and introduced in the pIRES-Neo plasmid, opened with *Eco*RV and *Not*I.

**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 Eagle's/Ham's F-12 medium supplied with 10% fetal 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<sub>2</sub> 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 48,000g for 30 min. The resulting pellets were reconstituted in a small volume of breaking buffer, aliquoted, and stored at  $-80^\circ\text{C}$  until use. Stored under these conditions, the receptors retained their binding activity for several months. The protein content was estimated by use of a colorimetric assay.

Human embryonic kidney (HEK) 293 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% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin/streptomycin, and 800  $\mu\text{g}/\text{ml}$  neomycin. For CCR1, CCR1[F149N] or CCR1[L205G] expressing cell-lines, cells from five 225-cm<sup>2</sup> cell culture bottles were detached with 5 mM EDTA in phosphate-buffered saline (135 mM NaCl, 4.5 mM KCl, and 10 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.8), resuspended in 3 ml of Tris/EDTA buffer (50 mM Tris and 1 mM EDTA, pH 7.4) supplied with protease inhibitors: bacitracin, bestatin, chymostatin, leupeptin, phosphoramidon and the cells broken by 25 passes in a cell homogenizer (glass tube, teflon pestle). Cell membranes were isolated and stored, and protein content was 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 of Rosen A buffer (50 mM HEPES, 1 mM CaCl<sub>2</sub>, 5 mM MgCl<sub>2</sub>, 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 125-W lamp at 6-cm distance while kept on ice with continuous stirring. After 30 min irradiation, the membrane fraction was separated from unbound ligand by centrifugation at 20,000g for 25 min at  $4^\circ\text{C}$  and washed once in 2 ml of 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  $\mu\text{l}$  of 0.1 M Tris, pH 8.5, containing 8 M urea, sonicated, and flushed with nitrogen.  $\beta$ -mercaptoethanol (10  $\mu\text{l}$ ) was added and the reaction incubated at  $40^\circ\text{C}$  for 16 h under nitrogen atmosphere. A 200- $\mu\text{l}$  solution of 24 mg of iodoacetamide and 30 mg of 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  $\mu\text{l}$  of  $\beta$ -mercaptoethanol was added to quench unreacted iodoacetamide. Afterward, the reaction mixture was desalted by two consecutive dilutions and concentration steps

using Microsep concentrators (molecular mass >10-kDa cut-off). Finally, the sample was separated on polyacrylamide gel. A small part of the undried gel was autoradiographed at  $-80^{\circ}\text{C}$  for 2 h 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 to 48 h in 1.5 ml of 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 mass 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 CCR1- $^{125}\text{I}$ -BP-MIP-1 $\alpha$  Complex.** For the Glu-C cleavage, the receptor-ligand complex was incubated at  $25^{\circ}\text{C}$  for 18 h in 20  $\mu\text{l}$  25–50 mM  $\text{NH}_4\text{HCO}_3$ , pH 7.8, with 2  $\mu\text{g}$  of Glu-C. For trypsin cleavage, CCR1- $^{125}\text{I}$ -BP-MIP-1 $\alpha$  complex was incubated in 20  $\mu\text{l}$  of 100 mM Tris, pH 8.5, containing 2  $\mu\text{g}$  trypsin for 18 h at  $37^{\circ}\text{C}$ . After reaction, the samples were analyzed by SDS-PAGE on a 12% Tris-tricine gel or a Novex 10 to 20% Nupage gel, followed by autoradiography.

The 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 for 20 min in a 1:1 mixture of water and acetonitrile and dried to approximately half the initial size. CNBr solution (0.7 ml or 150 mM) 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 seven times in 1 ml of water. Finally, peptides were extracted from gel pieces in 2 ml of 100 mM  $\text{NH}_4\text{HCO}_3$  solution for 24 h at room temperature. The recovery of radioactivity was 80 to 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 the method of Allen (1989), with urea replacing 6 M guanidinium chloride.  $\text{NH}_4\text{OH}$  (25  $\mu\text{l}$ ) was added to the labeled receptor and the reaction was incubated at  $45^{\circ}\text{C}$  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 conditions were 1  $\mu\text{g}$  of enzyme in 20  $\mu\text{l}$  of 25 mM Tris-HCl and 1 mM EDTA, pH 8.5, for 18 h. The conditions used for

fragmentation with Arg-C were 1  $\mu\text{g}$  of enzyme in a total of 100  $\mu\text{l}$  of 100 mM Tris-HCl, 10 mM  $\text{CaCl}_2$ , 5 mM dithiothreitol, and 0.5 mM EDTA supplied with 10  $\mu\text{l}$  of Arg-C activation solution and incubation for 18 h at  $37^{\circ}\text{C}$ .

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 were analyzed by autoradiography.

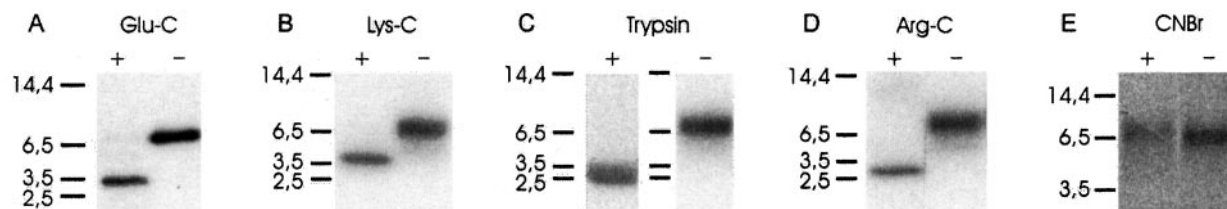
## Results

**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 covalently binds in a specific manner to CCR1 to form a complex with an apparent molecular mass of 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 cross-linked complex is approximately 10 to 1 for receptors in membrane preparations, which is comparable with 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 of identifying the smallest sizeable radioactive fragment that 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 with 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 to which no modifications were made to remove existing enzymatic or chemical cleavage sites. Thus, 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 CNBr. Migration of the cleavage products is shown in Fig. 1. Using separation on SDS-PAGE, it is



**Fig. 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. A,  $^{125}\text{I}$ -BP-MIP-1 $\alpha$  incubated with (+) and without (-) the presence of Glu-C for 24 h. B, Lys-C digest of  $^{125}\text{I}$ -BP-MIP-1 $\alpha$  for 24 h. C, Trypsin digest of  $^{125}\text{I}$ -BP-MIP-1 $\alpha$  for 24 h. D, Arg-C digest of  $^{125}\text{I}$ -BP-MIP-1 $\alpha$  for 24 h. E, CNBr cleavage of  $^{125}\text{I}$ -BP-MIP-1 $\alpha$  for 72 h



possible to identify peptides with molecular masses as low as ~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 (Y15 and Y28) and 1.5 kDa (Y62). We observe only one band at 3.4 kDa (Fig. 1A).

The Lys-C digestion is expected to result in two radiolabeled peptides with molecular masses 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 (Fig. 1B). The Glu-C and Lys-C indicate that  $^{125}$ I is present on at least one of the two tyrosines Y15 and Y28 at the N-terminal fragment.

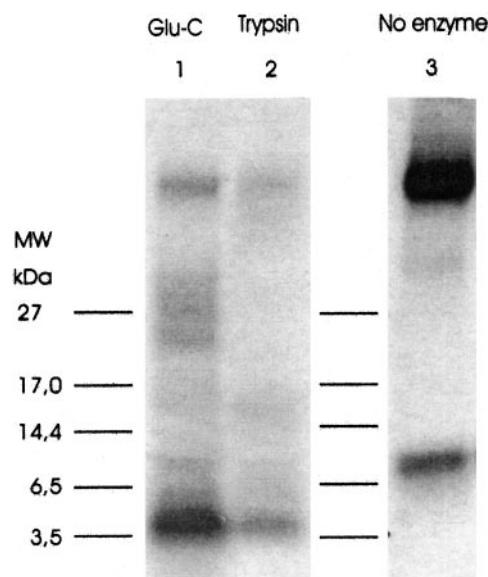
Trypsin digestion results in a broad band positioned between 3.5 and 2.5 kDa. For this digestion, three peptides with the respective sizes of 2.2 (Y15), 2.3 (Y28), and 1.0 kDa (Y62) were expected (Fig. 1C). In addition, the Arg-C digest was predicted to result in three bands at 2.2 (Y15), 3.3 (Y28), and 2.6 kDa (Y62). Only a band of ~3.0 kDa is seen (Fig. 1D). 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 mass 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, however, is near the detection limit, and slight changes in migration may occur. From the fragmentation of BP-MIP-1 $\alpha$  with Arg-C and trypsin, is it 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 result, as expected, in no cleavage, because no methionines are present in BP-MIP-1 $\alpha$  (Fig. 1E). Thus, from all digestion analyses, it can be concluded that  $^{125}$ I labels Y15 and/or Y28. 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) hydrolyzes peptide bonds after glutamate residues. As can be seen in Fig. 2, digestion of the receptor-ligand complex is not complete; such a 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 mass of 3.5 to 4.5 kDa. Compared with 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- to 4.5-kDa band that results from Glu-C cleavage are: -13 to 29 (N-terminal), 178-194 (E2), or 273-287 (E3). Their position in the receptor structure is illustrated in Fig. 4.

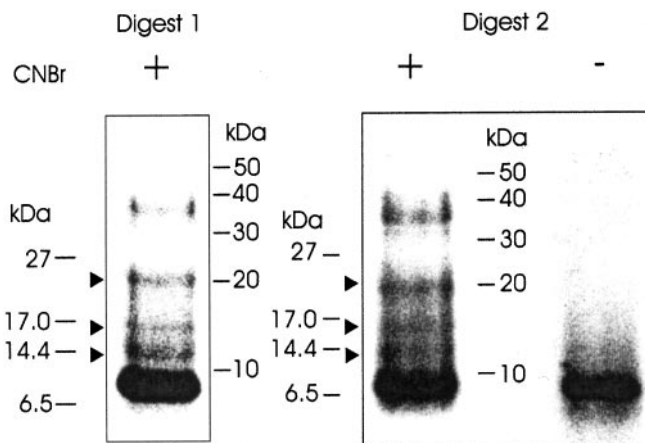
Trypsin cleaves after the basic residues arginine and lysine. The smallest peptide resulting from trypsin treatment ( $n = 4$ ) migrates with an apparent molecular mass of 3.5 to 4.5 kDa (Fig. 2, lane 2). This size is ~0.5 to 1.5 kDa larger than that seen in trypsin fragmentation of the ligand alone. Putative receptor fragments that could account for the increase in weight are the N-terminal amino acids (aa -13 to



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

-2 in the HA sequence) aa 27-30 (N-terminal), 94 to 106 (E1), and 174 to 196 (E2). Their position in the expected fragmentation pattern for the cleavage of CCR1 with trypsin is shown in Fig. 4.

**CNBr Cleavage.** Because of 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 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 masses of 14, 16, and 20 kDa, which are larger than the ligand alone, are clearly detected in three independent experiments. These



**Fig. 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 3 days. Experiment 2, right: extract from gel incubated in 70% formic acid for 3 days. Arrows indicate the position of the three reproducible cleavage products in CNBr fragmentation of the CCR1- $^{125}$ I-BP-MIP-1 $\alpha$  complex.

bands are not seen when the sample is incubated in acid without CNBr. The observed size and relative intensity of these fragments were very reproducible. Furthermore, bands around this size are well separated on a 12% Tricine gel. The use of molecular mass markers at 10, 14.4, 17.0, and 20 kDa allow accurate estimate of apparent molecular masses. 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 to 166 (TM3–4) or the fragment 167 to 215 (E2 + TM5), as indicated in Fig. 4.

Even though cleavage with CNBr is reproducible, it may not necessarily be complete (Grutter et al., 2000). Therefore the weak bands with apparent sizes of 17 and 20 kDa (Fig. 3) could result from partial cleavage. In particular, if the 14-kDa band corresponds to segment 167 to 215, the size of higher molecular mass bands could be accounted for by partial cleavage at position 166, leading to a 20.4-kDa fragment (106–215), and 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 (aa 106–215) and 18.9 kDa (aa 69–166), 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 and 23 kDa for a noncomplete 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-labeled. We therefore used a site-directed mutagenesis approach to support one receptor sequence as interacting with the N-terminal of BP-MIP-1 $\alpha$ .

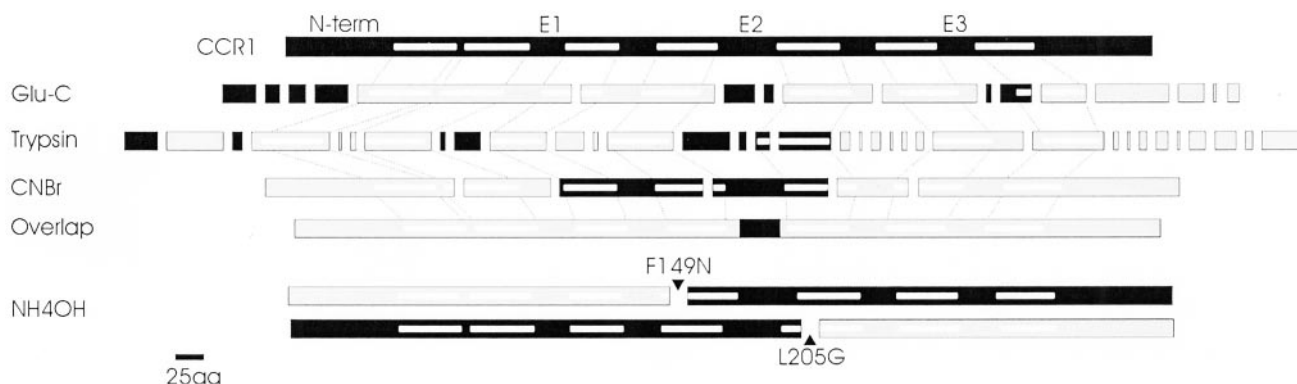
**Cleavage with NH<sub>4</sub>OH of WT and Point-Mutated Receptors.** Two mutant receptors were designed, each with a unique Asn-Gly cleavage site for hydroxylamine introduced after positions 150 (TM4) and 205 (TM5) (Fig. 6). Both receptors (F149N and L205G) are expressed in HEK293 cells at a level comparable with that of 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 wild-type receptors expressed in HEK293 cells and the wild-type 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 (Fig. 5). However, for both mutated receptor- BP-MIP-1 $\alpha$  complexes, a band migrating at an apparent molecular mass 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 masses expected are 25.5 kDa for the N-terminal part (aa 1–150) and 32.5 kDa for the C-terminal part (aa 151–354) when the mass of MIP-1 $\alpha$  is included. The experimental mass fits with the size of the C-terminal fragment. In the L205G mutant, the similarly calculated molecular masses of the two receptor fragments after cleavage are 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 Fig. 4.

## 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 loop 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.

Compared with 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



**Fig. 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. Three middle rows, cleavage with Glu-C, trypsin, and CNBr of wild-type CCR1. In gray are indicated the parts of the receptor found not to be labeled by 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.

was shown for the interaction of  $\alpha$ -bungarotoxin with the nicotinic acetylcholine receptor (Maelicke and Conti-Tronconi, 1989).

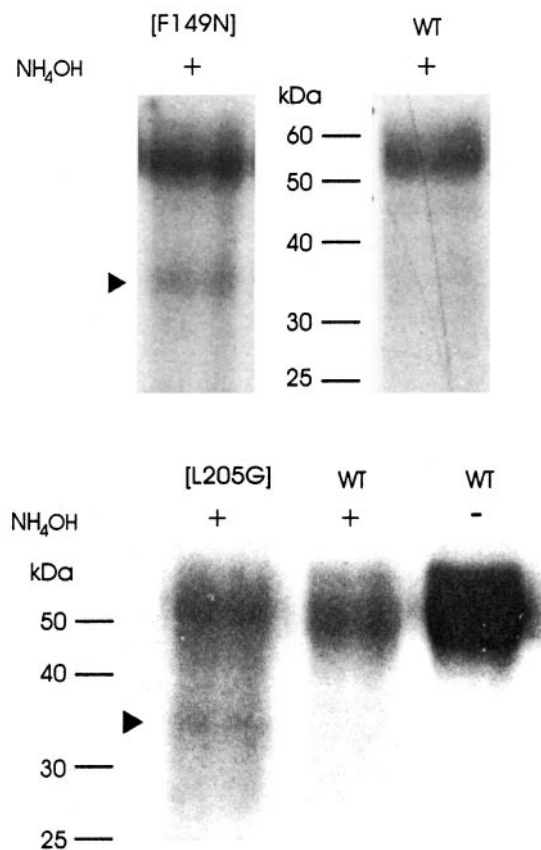
Analyses of chemokine interactions with receptors included two separate aspects, namely chemical specificity of receptor recognition and receptor activation (Wells et al., 1996; Crump et al., 1997). Receptor recognition is mainly driven by an extended hydrophobic groove delineated by the second and third  $\beta$ -sheet strands (Clubb et al., 1994; Mizoue et al., 1999; Mayer and Stone, 2000; 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, because the structure of rhodopsin demonstrates a three dimensional structure, whereas the extracellular loops of the receptor are near the N-terminal end. For the interaction between MIP-1 $\alpha$  and the CCR1 receptor, all available data indicate that it involves several parts of the receptor, including the N terminus and E3 loop (Montecarlo and Charo, 1996; Su et al., 1996; Pease et al., 1998).

Receptor activation mostly relies on residues situated in the N terminus of the chemokines (Gong et al., 1996; Solari et

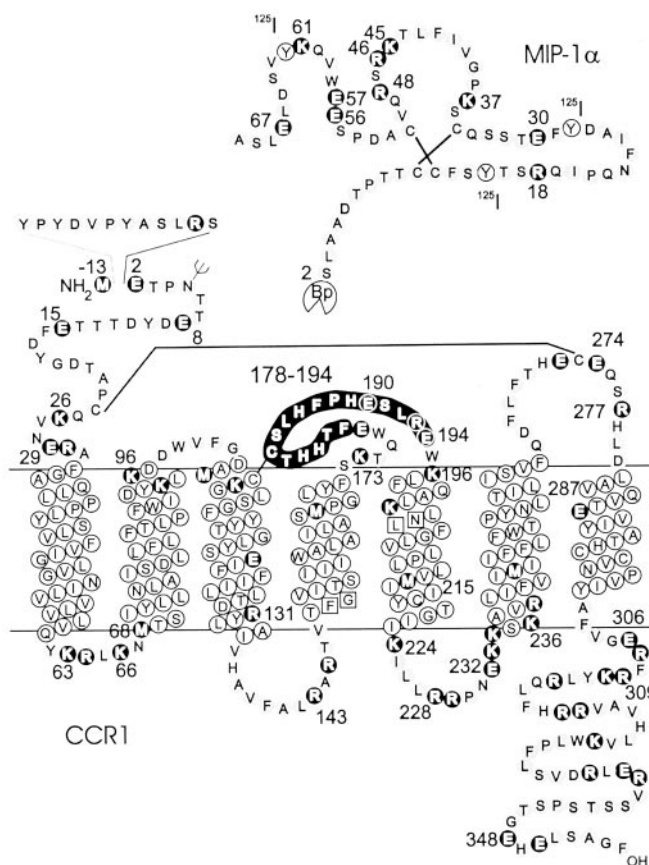
al., 1997; Laurence et al., 2000; Townson et al., 2000; Zoffmann et al., 2001), whereas the corresponding parts of the receptor that interact with the chemokine are poorly identified.

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



**Fig. 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 h. Top, the CCR1[F149N]- $^{125}\text{I}$ -BP-MIP-1 $\alpha$  and the CCR1- $^{125}\text{I}$ -BP-MIP-1 $\alpha$  complex incubated with  $\text{NH}_4\text{OH}$ . Bottom, 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  $\text{NH}_4\text{OH}$ .



**Fig. 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, an HA-epitope is inserted. In the upper part of the figure is indicated the photoactivatable analog of the chemokine MIP-1 $\alpha$  (Nakao et al., 1990; Menten et al., 1999; Zoffmann et al., 2001). Cysteine bridges are indicated as lines connecting two cysteine residues. Indicated with black background are amino acids that have not been excluded as interaction points by the different fragmentation analysis. Tyrosines shown as white circles with black text are putative points of iodination. BP, benzophenone group. Residues with black circles and white letters indicate 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 introduced.



valently labeled CCR1, we obtain data that support more than one candidate for interaction with the N terminus of the chemokine as presented in Fig. 4. However, when combined, the cleavage data converge toward 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 analyze the cleavage pattern of mutant receptors containing hydroxylamine sites in TM4 or TM5 (Fig. 4). This approach, which consists of adding new sites rather than removing existing ones, is based on the following arguments: 1) because 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, for hydroxylamine in particular, which has no consensus cleavage site on the WT CCR1 receptor; 2) 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 that are located near the MIP-1 $\alpha$  incorporation site; and 3) removal or introduction of CNBr sites was ruled out because of 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 receptors, 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 situated entirely in the E2 (Palczewski et al., 2000), probably reflecting the situation in the CCR1 because the length of the extracellular domains is highly conserved between the two receptors.

In association with previous work describing that fluorescein, 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 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 interaction points that are responsible for the agonist activity of chemokines, whereas other interactions, in particular those with the N terminus and the E3 loop of the chemokine receptor, would contribute 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 $\alpha$  that 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. An alternative activation mechanism, where the whole receptor molecule undergoes structural changes during the transition leading to receptor activation, should be considered. According to this hypothesis the conformational transition would be global and would imply all intracellular, transmembrane, and 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.

## Acknowledgments

We thank Amanda Proudfoot (Serono Pharmaceutical Research Institute) for the kind provision of MIP-1 $\alpha$ , Christine Power for advice in construction of the CCR1-mutants and Manuela Lima for molecular biology and cell-culture. We also thank Professor Thue Schwartz for his support, and Gerardo Turcatti and Tim Wells for stimulating discussion.

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