Identification of CC Chemokine Receptor 7 Residues Important for Receptor Activation*

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The binding pocket of family A GPCRs that bind small biogenic amines is well characterized. In this study we identify residues on CC chemokine receptor 7 (CCR-7) that are involved in agonist-mediated receptor activation but not in high affinity ligand binding. The mutations also affect the ability of the ligands to induce chemotaxis. Two of the residues, Lys3.33 (137) and Glu5.42 (227), are consistent with the binding pocket described for biogenic amines, while Lys3.26 (130) and Asn7.32 (305), are found at, or close to, the cell surface. Our observations are in agreement with findings from other peptide and chemokine receptors, which indicate that receptors that bind larger ligands contain contact sites closer to the cell surface in addition to the conventional transmembrane binding pocket. These findings also support the theory that chemokine receptors require different sets of interactions for high affinity ligand binding and receptor activation.

CC chemokine receptor 7 (CCR-7) is a GPCR expressed on mature dendritic cells (DCs) and naive T-cells (1–3). Its ligands, CC chemokine ligand 19 (CCL-19) and CCL-21, belong to the family of chemokines that act as chemotactants and play a fundamental role as activators of leukocytes in the acute and chronic inflammatory response of the immune system (4, 5). Depending on the relative position of the first two conserved cysteines, two main families of chemokines have been identified (CC and CXC chemokines) (4, 5). Chemokines act by signaling through their cognate G protein-coupled receptors (4, 5). Most chemokine receptors couple to members of the G family of heteromeric G proteins, because both signaling and chemotactic properties of cells expressing chemokine receptors are pertussis toxin-sensitive (6, 7). It is, however, possible that Gβγ and other downstream effectors like RGS (regulators of G protein signaling) and MAP (mitogen-activated protein) kinases, also play a major role in the process of signal transduction of chemokine receptors (7). Some chemokine receptors have also been shown to act as HIV co-receptors (4, 5).

CCL-19 and CCL-21 play an important role in the migration of naive T-lymphocytes and antigen-presenting DCs to secondary lymphoid organs where these ligands are predominantly expressed (8–12). Circulating CCR-7 naive T-cells and lymph-derived antigen-loaded mature DCs enter lymph nodes, Peyer’s Patches and the spleen by recognizing CCL-21 expressed on the high endothelial venule interface and in the afferent lymphatic vessels, respectively (13). Upon entry these cells migrate to the paracortical T-cell rich areas where CCL-19 is expressed, and therefore both ligands facilitate the encounter of antigen-loaded DC with naive T-cells. Additionally, it has been shown that CCR-7-deficient mice have defective T-cell and DC migration and are therefore unable to lodge antigenic T-cell responses (14). Elucidating mechanisms underlying ligand binding and receptor activation of CCR-7 could therefore help in designing drugs for the treatment of autoimmune diseases and transplantation.

Even though the primary structure of chemokines can be highly variable, NMR and x-ray crystallography studies have shown that the overall tertiary structure of chemokines is highly conserved (4, 5). Preceding the conserved cysteines is a disordered N terminus that has been shown to be involved in stabilizing the active conformation of a number of chemokines. Following the conserved cysteines is an N-loop, which ends in one turn of a 3₁₀ helix, three antiparallel strands of a β-sheet and an α-helix (4, 5, 15, 16). Both the N terminus and N-loop are tethered to the β-sheet through disulfide bonds with the conserved cysteines, while the α-helix is also in close proximity (4, 5, 15).

The action of chemokines is thought to occur through two separate sets of interactions: interactions responsible for high affinity binding and interactions involved in stabilizing the active conformation of the receptor (17–19). On the chemokine, the N-loop and the loops connecting the β-sheet strands are thought to be important for high affinity binding (4, 5). It is believed that these regions interact with extracellular domains of the receptor. Other low energy interactions between the N-terminal domain and the helix bundle of the receptor are thought to stabilize the active conformation of the receptor (17–19). The above mechanisms, however, can vary between different chemokines, as e.g. CCL-19 (MIP-3β) (20) and CXCL-12 (SDF-1) (19) contain residues important for high affinity binding in the N terminus, while CCL-11 (Eotaxin-1) contains residues important for receptor activation in the N-loop (21).

In this study, we investigate residues of the integral membrane protein, CCR-7, that potentially interact with CCL-19 and CCL-21 and play a role in signal transduction. Residues toward the extracellular surface that are predicted to face into the binding pocket were mutated to establish their role in

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‡ The abbreviations used are: CCR, CC chemokine receptor; CCL, CC chemokine ligand; CXCL, CXC chemokine ligand; CXC, CXC chemokine receptor; DC, dendritic cells; GPCR, G protein-coupled receptor; GAGs, glycosaminoglycans; TMD, transmembrane domain; ELD, extracellular loop domain; GTPγS, guanosine 5′-3-O-(thiotriphosphate); PBS, phosphate-buffered saline; BSA, bovine serum albumin.

§ This is also known as macrophage inflammatory protein 3β (MIP-3β), EBI1 ligand chemokine (ELC), chemokine-11 (CΚβ-11), eotaxin-3, or small inducible cytokine A19 (SCYA19).

¶ This is also known as secondary lymphoid organ chemokine (SLC), 6-Ckine, Exodus-2, TCA-4 or small inducible cytokine A2 (SCYA2).

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ligand binding, receptor activation, and chemotaxis (Fig. 1). We identify sites that are involved in CCL-19 and CCL-21 activation as well as a site that distinguishes between CCL-19 and CCL-21 activation. Results from chemotaxis assays mirror those from GTP-γS binding assays indicating that mutational effects on G protein activation are predictive of effects on the more complex cellular behavior of chemotaxis.

**EXPERIMENTAL PROCEDURES**

**Point Mutations**—Since GPCRs differ in length, a common numbering system is used that allows comparison of transmembrane domain (TMD) residues from different receptors more easily (22). Residues are given a number x.y, where x represents the TMD and y the number of the residue in relation to the most conserved residue in the helix, which is arbitrarily given the number 50. Mutations were performed on CCR-7 that contained a V5-His tag on the C terminus (Invitrogen Life Technologies, Baltimore, MD). Initially the wild-type receptors and mutants were transiently transfected into COS-7 cells. It was shown that the V5-His-tagged receptor behaved like the wild-type receptor in assay systems used for characterization in this article (Table 1). Point mutations on the human CCR-7 were produced using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. Sense and antisense primers were constructed containing the mutations flanked by at least 12 bases of the wild-type sequence on either side. Mutant receptors were subcloned into pcDNA5/FRT/V5-His (Invitrogen Life Technologies). All sequences were confirmed by DNA sequencing.

**Cell Culture**—All cell culture media and solutions were purchased from Cellgro (Fisher Scientific). FLP-In CHO-K cells were obtained from Invitrogen Life Technologies. A stable FLP-In CHO-K cell line expressing CCR-7 (GenBank accession number XM_0499599) or CCR-7 mutants was established according to the manufacturer’s instructions. COS-7 (ATCC) and CHO-K cell lines were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 10 mM HEPES, 2 mM l-glutamine, 1 mM sodium pyruvate, 50 units/ml penicillin, 50 μg/ml streptomycin with an additional supplementation of 200 μg/ml Hygromycin (Invitrogen) for stably transfected CHO-K cell lines, while media for the acute T-cell leukemia Jurkat cell lines (ATCC) was supplemented with 1 x non-essential amino acids (Sigma), 0.65 μM β-mercaptoethanol (Sigma, Dallas) and 200 μg/ml geneticin (Invitrogen Life Technologies, Inc.). CHO-K cells were seeded in 200-cm² dishes and grown to 90–100% confluency prior to membrane preparation.

**Protoporation of COS-7 Cells**—50 million COS-7 cells were electro-ported with 50 μg of DNA according to BTX (Genetronics, San Diego, CA) protocols (in Dulbecco's phosphate-buffered saline, 1000 microfarad capacitance, 48 Ω resistance, 750 Vcm charging voltage) using a BTX ECM 830 expression electroporator. Transfected cells were grown for 48 h in Dulbecco's modified Eagle's medium supplemented as above prior to membrane preparations.

**Membrane Preparations**—COS-7-expressing cells were scraped in membrane buffer (20 mM HEPES, 6 mM MgCl₂, 1 mM EDTA, pH 7.2). Cell lysis was performed using a pressure of 900 bar for 30 min in a nitrogen cavitation chamber. The homogenate was spun at 1,000 × g for 10 min at 4 °C to remove nuclei and cellular debris. The supernatant was taken and spun at 45,000 × g for 45 min at 4 °C to collect the membrane fraction. Membranes were resuspended in membrane buffer to a final concentration of 1 mg/ml, snap frozen in liquid nitrogen in aliquots, and stored at −80 °C.

**Determination of Cell Surface Expression**—CHO-K cells were detached with 10 mM EDTA. 1 × 10⁶ cells were washed with PBS and resuspended in 0.2 ml of FACS buffer (0.01% sodium azide, 0.1% BSA in PBS). 50 μl of the cell suspension were incubated with PE-conjugated anti-human CCR-7 antibodies (BD Biosciences Clontech, Palo Alto, CA) in the dark on ice. PE rat IgG₂a-κ (BD Biosciences Clontech) was used as an isotype-matched control antibody. After a 30-min incubation, cells were spun down at 1,000 × g for 5 min, washed twice, and resuspended in 200 μl of FACS buffer. Cell surface expression was determined by flow cytometry using a FACSscan (BD Biosciences Clontech). The mean fluorescence obtained in the isotype control was subtracted from the mean fluorescence obtained by CCR-7 antibodies to calculate relative expression levels in relation to wild-type CCR-7 expression. Data presented are a mean of three independent experiments.

**GTP-γS Binding Assays**—CCL-19 and CCL-21 were obtained from ID Labs (London, ON). 5 μg of membranes were incubated at 37 °C for 30 min in assay buffer (50 mM HEPES, 100 mM NaCl, 10 mM MgCl₂, 1 mM EDTA, 0.1% BSA, pH 7.2) in the presence of 10 μM GDP, 0.5 mM [γ³²P]GTP-γS (PE Life Sciences) and varying concentrations of agonist (total volume, 100 μl in a 96-well plate). Membranes were filtered onto a 96-well GPC filter plate (Packard Bioscience, Hartford CT) and washed with 500 ml of wash buffer (50 mM Tris-HCl, 5 mM MgCl₂) using a Filtermate 196 Harvester (Packard Instruments, Downers Grove, IL). The filter plates were dried under a heat lamp before addition of 50 μl of scintillation fluid to each well (Scintisafe, 30% mixture, Fisher Scientific, Tucson, CA) and counted on a Topcount NXT (Packard, Downers Grove, IL).

**Radioligand Binding Assays**—1 μg of membranes (10 μg for membranes expressing the triple mutant) were incubated with 80,000 cpm (0.1 μCi) ¹²⁵I-CCL-19 (PE Life Sciences) in binding buffer (50 mM HEPES, 5 mM MgCl₂, 1 mM CaCl₂, 0.1% BSA, pH 7.2) in the presence/absence of unlabelled ligand or guanine nucleotide at room temperature for 1 h (100 μl in a 96-well plate). Membranes were filtered onto a 96-well GFC plate (Packard Bioscience, Hartford, CT) that had been presoaked for a minimum of 1 h in a 0.25% polyethyleneimine/0.5% BSA/0.5 × PBS and washed with 500 ml of wash buffer (50 mM HEPES, 0.5 mM NaCl, 0.1% BSA) using a Filtermate 196 Harvester (Packard Instruments, Downers Grove, IL). The filter plates were dried under a heat lamp before addition of 50 μl of scintillation fluid to each well (Scintisafe, 30% mixture, Fisher Scientific, Tucson, CA) to each well, and counted on a Topcount NXT (Packard, Downers Grove, IL). Nonspecific binding was determined by performing the assay on membranes from untransfected FLP-In CHO-K cells and was found to be not significantly different from binding in the presence of high amounts of cold ligand (data not shown).
RESULTS

To identify residues important for receptor activation, the protein sequence of CCR-7 was aligned to the protein sequence of other chemokine receptors as well as other GPCRs (Fig. 2). Residues in extracellular domains and residues in TMDs close to the cell surface were identified that were (a) highly conserved among chemokine receptors or (b) highly conserved among chemokine receptors and non-conserved at CCR-7. A literature search was done to identify residues that are important for agonist binding and receptor activation in other family A GPCRs. Residues fitting these criteria were then checked in a model of CCR-7 that was built on the crystal structure of rhodopsin (24) to confirm that the side chains were likely to face into the potential binding pocket. Residues fulfilling the above criteria were considered potentially capable of playing a role in the activation of CCR-7 and were mutated to alanines.

Cell Surface Expression of Wild-type and Mutant CCR-7—In order to assess the function of mutant receptors, they were either expressed transiently in COS-7 or stably in CHO-K1 FL-P-In and Jurkat cells. Cell surface expression of wild type and mutant CCR-7 on stably transfected CHO-K1 and Jurkat cells was determined by flow cytometry. In stably transfected FL-P-In CHO-K1 cells, expression levels of the Tyr3.32 (136) mutant and all other mutant receptors were found to be slightly elevated but similar to expression levels of the wild-type receptor (Table I), while clones of stably transfected Jurkat cells were selected to have similar expression levels for all mutant and wild-type receptors (data not shown).

Chemotaxis of Jurkat Cells—In order to establish whether receptor mutations had effects on more complex cellular behaviors we established an assay to determine chemotactic abilities of cells transfected with wild-type and mutant CCR-7. As determined by FACS and quantitative RT-PCR, the Jurkat T leukemia cell-line expresses a number of chemokine receptors on the cell surface, yet no detectable levels of CCR-7 were found (data not shown). We therefore stably transfected this cell line with wild-type and several mutant CCR-7s. To show that chemotaxis of the transfected cell lines in response to CCL-19 and CCL-21 was mediated through the transfected CCR-7, we performed a chemotaxis assay on untransfected Jurkat cells and Jurkat cells transfected with wild type CCR-7. Untransfected cells did not chemotax in response to either CCL-19 or CCL-21, but CCR-7 transfected cells showed a robust chemotactic re-
pIC\textsubscript{50} and pEC\textsubscript{50} were determined as described under "Experimental Procedures." Data presented are the mean ± S.E. of at least four independent experiments. p values were calculated on the pIC\textsubscript{50} and pEC\textsubscript{50} by a paired two-tailed Student’s t test where the wild-type receptor is the point of reference using Prism.

| Receptor | pIC\textsubscript{50} | pEC\textsubscript{50} | pIC\textsubscript{50} | pEC\textsubscript{50} | Expression levels\textsuperscript{b} |
|----------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Wild type | 8.67 ± 0.14 (2.1) | 8.74 ± 0.08 (1.8) | 8.25 ± 0.08 (5.7) | 8.51 ± 0.10 (3.1) | 335 ± 10 (100%) |
| Tyr\textsuperscript{3.32 (136)} → Ala | 8.64 ± 0.09 (2.3) | 8.81 ± 0.06 (1.6) | 8.37 ± 0.17 (4.3) | 8.73 ± 0.11 (1.9) | 479 ± 55 (156%) |
| Lys\textsuperscript{3.31 (137)} → Ala | 8.51 ± 0.18 (3.1) | 8.58 ± 0.04 (2.7) | 7.69 ± 0.14 (20) | 7.83 ± 0.04 (15) | 656 ± 17 (224%) |
| Lys\textsuperscript{3.32 (130)} → Ala | 8.57 ± 0.27 (2.7) | 8.21 ± 0.08 (6.1) | 8.50 ± 0.16 (3.2) | 8.07 ± 0.06 (8.4) | 653 ± 77 (222%) |
| Gln\textsuperscript{5.42 (227)} → Ala | 8.60 ± 0.11 (2.5) | 7.87 ± 0.08 (131) | 7.92 ± 0.14 (12) | 7.28 ± 0.04 (53) | 656 ± 85 (225%) |
| Asn\textsuperscript{3.32 (305)} → Ala | 8.46 ± 0.15 (3.5) | 8.31 ± 0.06 (4.9) | 8.40 ± 0.17 (4.0) | 8.16 ± 0.06 (7.0) | 692 ± 58 (238%) |
| Triple mutant\textsuperscript{c} | 8.48 ± 0.27 (3.3) | 8.66 ± 0.10 (138) | 7.56 ± 0.14 (28) | 6.71 ± 0.08 (197) | 518 ± 68 (172%) |

\textsuperscript{a} Mean IC\textsubscript{50} and EC\textsubscript{50} values are shown in parentheses in nM concentrations.

\textsuperscript{b} Expression levels were determined by flow cytometry; Expression levels are expressed as mean fluorescent shift calculated as described under "Experimental Procedures."

\textsuperscript{c} No significant difference from the wild type.

\textsuperscript{d} p < 0.05.

\textsuperscript{e} p < 0.01.

\textsuperscript{f} Tyr\textsuperscript{3.32 (136)} → Ala/Asn\textsuperscript{3.32 (305)} → Ala.

Table I
Comparison of binding affinity (pIC\textsubscript{50}) and potency (pEC\textsubscript{50}) of CCL-19 and CCL-21, and expression levels of wild-type and mutant CCR-7s in CHO-K cells

Fig. 3. Chemotactic abilities of untransfected and stably transfected Jurkat cells. Jurkat cells were stably transfected with wild-type and mutant CCR-7, and single cell clones were picked that had similar expression of CCR-7 as outlined under "Experimental Procedures." Panel A compares the chemotactic abilities of untransfected (open symbols) and wild-type CCR-7 transfected (closed symbols) Jurkat cells in response to CCL-19 (□ and ■) and CCL-21 (◇ and ▼). To show that the transfected cell lines still had normal chemotactic properties, chemotaxis was measured in response to an endogenously expressed receptor (CXCR-4) (B). Chemotaxis assays were performed in response to CXCL-12 on untransfected Jurkat cells (□), wild-type (■), Lys\textsuperscript{3.33 (137)} → Ala (▲), Asn\textsuperscript{3.32 (305)} → Ala (▼), and the Lys\textsuperscript{3.32 (130)} → Ala, Gln\textsuperscript{5.42 (227)} → Ala, Asn\textsuperscript{3.32 (305)} → Ala triple mutant (●) CCR-7. Data shown are mean ± S.E. from three independent experiments.

II and Fig. 4). The ability of CCL-21 to stimulate GTP-γS binding, however, was decreased by 22- and 4.8-fold in transiently transfected COS-7 and stably transfected CHO-K cells respectively. The affinity of CCL-21, however, was unchanged at the mutant receptor in transiently transfected COS-7 cells.

Response to both CCL-19 and CCL-21 (Fig. 3A), indicating that the chemotactic ability of transfected cells is mediated through the transfected receptor. To determine whether the transfection had nonspecific effects on the ability of Jurkat cells to chemotax, we measured the chemotactic response to CXCL-12, which activates CXCR-4 that is endogenously expressed on Jurkat cells, and compared it to the chemotaxis of untransfected Jurkat cells. For all transfected cell lines, chemotaxis in response to CCL-19 and CCL-21 (Fig. 3A) was similar to that of the untransfected Jurkat cells (Fig. 3B), indicating that the ability of transfected cell lines to migrate was not affected by the expression of CCR-7, and that any modulation in chemotaxis assays in response to CCL-19 and CCL-21 would be due to the mutations of CCR-7.

Tyr\textsuperscript{3.32 (136)} Is Not Involved in Ligand Binding or Receptor Activation—The region toward the extracellular surface of TMD-III has been identified to be involved in ligand binding to family A GPCRs. Asp\textsuperscript{3.32} of the β-adrenergic (25), M\textsubscript{1}-muscarinic (26), histamine H\textsubscript{2} receptors (27) and Lys\textsuperscript{3.32} of the GnRH receptor (28) have been shown to be involved in agonist binding. Furthermore, Tyr\textsuperscript{3.33} and Asn\textsuperscript{3.37} of the TRH (29, 30), as well as Asn\textsuperscript{3.35} of the angiotensin II (31) and Gln\textsuperscript{3.26} and Gln\textsuperscript{3.36} of rhodopsin (32) have been shown to be crucial for agonist interactions. On CCR-7, Tyr\textsuperscript{3.32 (136)} is found in an equivalent position as the residues that act as a counterion for agonists at the small biogenic amine receptors. This residue is completely conserved among CC chemokine receptors (Fig. 2A) and might therefore be of functional importance. On CCR-7, Tyr\textsuperscript{3.32 (136)} is followed by Lys\textsuperscript{3.33 (137)} , which is only shared with CCR-9, with most other CC chemokine receptors carrying an uncharged side-chain in this position (Fig. 2A). We therefore mutated both Tyr\textsuperscript{3.32 (136)} and Lys\textsuperscript{3.33 (137)} to Ala to investigate their involvement in agonist binding and receptor activation.

When Tyr\textsuperscript{3.32 (136)} was mutated to Ala, we found no change in GTP-γS binding assays in response to CCL-19 and CCL-21 in both transiently transfected COS-7 and stably transfected CHO-K cells (Tables I and II and Fig. 4). The ability of the mutant receptor to bind both ligands was also not affected. (Tables I and II and Fig. 4).

Lys\textsuperscript{3.33 (137)} Affects CCL-21 but Not CCL-19 Binding and Receptor Activation—When Lys\textsuperscript{3.33 (137)} was mutated to Ala, we observed no significant change in ligand binding and receptor activation in response to CCL-19 in both transiently transfected COS-7 and stably transfected CHO-K cells (Tables I and
with a 3.5-fold loss in binding affinity observed in stably transfected CHO-K cells (Tables I and II and Fig. 4). Generally the effects of this and other mutations described below as measured by GTP-γ-S binding assays were more pronounced in transiently transfected COS-7 cells than in stably transfected CHO-K cells.

To investigate whether the Lys<sub>3.33</sub> (137)→Ala mutation affects chemotaxis we performed chemotaxis assays using Jurkat cells expressing wild-type and Lys<sub>3.33</sub> (137)→Ala CCR-7. The mutation had little effect on CCL-19-mediated chemotaxis (Fig. 5A) but significantly decreased chemotaxis in response to CCL-21 (Fig. 5B), which mirrors the effects seen in GTP-γ-S binding assays.

**Mutants Affecting CCL-19- and CCL-21-mediated Receptor Activation but Not Binding**—In TMD-III of CCR-7, two helical turns closer to the extracellular surface than residue 3.33, a highly conserved Lys<sub>3.26</sub> (130) can be found (Fig. 2A). As this residue potentially faces into the binding pocket, it might be involved in ligand binding or receptor activation as shown in CXCR-2 (33). We therefore mutated Lys<sub>3.26</sub> (130) to Ala and found that the ability of CCL-19 and CCL-21 to stimulate GTP-γ-S binding was decreased 9.3- and 12-fold, respectively in transiently transfected COS-7, whereas it was decreased 3.4- and 2.7-fold, respectively, in stably transfected CHO-K cells. The binding affinity of both ligands for the receptor remained unchanged (Tables I and II and Fig. 6).

In TMD-V, residue 5.42 has been identified in being involved in ligand binding at the histamine (27), acetylcholine (26), and angiotensin II (31) receptors. At CCR-7, Gln is found in the equivalent position. This residue is highly variable among CC chemokine receptors (Fig. 2B). To investigate the function of this side-chain, we mutated Gln<sub>5.42</sub> (227) to Ala. It was found

![Fig. 4](image)

**Fig. 4.** Lys<sub>3.33</sub> (137) affects CCL-21-mediated receptor activation without affecting CCL-19-mediated receptor activation or ligand binding. GTP-γ-S (A and C) and ligand binding (B and D) assays were performed in the presence of increasing amounts of CCL-19 (A and B) and CCL-21 (C and D) on CHO-K membranes expressing wild-type (■), Tyr<sub>3.32</sub> (136)→Ala (○), and Lys<sub>3.33</sub> (137)→Ala (▲) CCR-7 as described under “Experimental Procedures.” Data shown are mean ± S.E. from at least three independent experiments.

![Fig. 5](image)

**Fig. 5.** Lys<sub>3.33</sub> (137)→Ala selectively impairs CCL-21-mediated chemotaxis. Chemotaxis assays were performed as outlined under “Experimental Procedures” in response to CCL-19 (A) and CCL-21 (B) on Jurkat cells transfected with wild-type (■) and Lys<sub>3.33</sub> (137)→Ala (▲) CCR-7. Data shown are mean ± S.E. from three independent experiments.
that the mutant receptor was impaired in its ability to be activated by both CCL-19 (20-fold in transiently transfected COS-7 cells and 7.2-fold in stably transfected CHO-K cells) and CCL-21 (40-fold in transiently transfected COS-7 cells and 17-fold in stably transfected CHO-K cells). At the same time CCL-19 and CCL-21 binding to the mutant receptor appeared to be normal (Tables I and II and Fig. 6).

At the junction of ELD-3 and TMD-VII, residue 7.32 has been identified in being involved in ligand binding at the angiotensin II (31) and mammalian GnRH (28) receptors as well as CCR-2 (34) and CCR-5 (35). This residue is a highly conserved Asp in most CC chemokine receptors, but a unique Asn at CCR-7 (Fig. 2C). We therefore mutated Asn7.32 (305) to Ala to investigate its role in ligand binding and receptor activation. It was found that this mutant receptor showed an impaired ability to be activated by CCL-19 (3.8-fold in transiently transfected COS-7 cells and 2.7-fold in stably transfected CHO-K cells) and CCL-21 (5.5-fold in transiently transfected COS-7 cells and 2.3-fold in stably transfected CHO-K cells) while its ability to bind these ligands was not affected (Tables I and II and Fig. 6). The Asn7.32 (305) → Ala CCR-7 mutant also significantly impaired chemotaxis to both ligands when stably transfected into Jurkat cells (Fig. 7), demonstrating that the mutational effects on G protein activation were reflected in chemotaxis assays as well.

The Effects of Mutating Lys3.26 (130), Gln5.42 (227), and Asn7.32 (305) Are Additive—As the Lys3.26 (130) → Ala, Gln5.42 (227) → Ala, and Asn7.32 (305) → Ala mutations affected both CCL-19- and CCL-21-mediated receptor activation, we wanted to investigate whether the effects observed are additive. The triple mutant exhibited a highly impaired ability to activate G proteins in response to both CCL-19 (77-fold) and CCL-21 (64-fold) in stably transfected CHO-K cells (Table II and Fig. 8), and the chemotaxis response of stably transfected Jurkat cells to either ligand was completely abolished (Fig. 7).

Although the receptor appeared to be expressed at similar levels as the wild-type receptor based on CCR-7 immunostaining in stably transfected CHO-K cells, the specific binding of the mutant receptor was 10-fold lower compared with expression levels of the wild-type receptor, which translated into \( B_{\text{max}} \) values of 113 pmol/mg and 12 pmol/mg for wild-type and mutant receptors. To compensate for this reduced amount of specific binding we used 10 µg of membranes in radioligand binding assays of the triple mutant, compared with 1 µg for all other receptors. Binding affinities for CCL-19 remained unchanged while the ability of CCL-21 to inhibit radioligand binding was reduced 4.9-fold (Table II and Fig. 8).
pIC$_{50}$ and pEC$_{50}$ were determined as described under "Experimental Procedures." Data presented are the mean $\pm$ S.E. of at least three independent experiments unless indicated otherwise. $p$ values were calculated on the pIC$_{50}$ and pEC$_{50}$ by a paired two-tailed Student’s $t$ test where the tagged wild-type receptor is the point of reference using Prism.

### Table II
Comparison of binding affinity (pIC$_{50}$) and potency (pEC$_{50}$) of CCL-19 and CCL-21 for wild-type and mutant CCR-7s in transiently transfected COS-7 cells

| Receptor          | CCL-19$^a$ | CCL-19$^b$ |
|-------------------|------------|------------|
|                   | pIC$_{50}$ | pEC$_{50}$ | pIC$_{50}$ | pEC$_{50}$ |
| Wild type         | 8.60 $\pm$ 0.13 (2.5) | 8.72 $\pm$ 0.26 (1.9) | 8.02 $\pm$ 0.13 (9.5) | 8.31 $\pm$ 0.08 (4.9) |
| Tagged            | 8.52 $\pm$ 0.11 (3.0) | 8.83 $\pm$ 0.10 (1.5) | 8.39 $\pm$ 0.09 (4.1) | 8.66 $\pm$ 0.20 (2.2) |
| Tyr$^{32}$ (138) $\rightarrow$ Ala | 8.47 $\pm$ 0.53 (3.4) | 9.16 $\pm$ 0.21 (6.7) | 8.19 $\pm$ 0.32 (6.5) | 8.04 $\pm$ 0.23 (9.1) |
| Lys$^{8}$ (137) $\rightarrow$ Ala | 8.74 $\pm$ 0.17 (1.8) | 8.43 $\pm$ 0.20 (3.7) | 8.38 $\pm$ 0.10 (4.2) | 7.31 $\pm$ 0.11 (49) |
| Lys$^{26}$ (130) $\rightarrow$ Ala | 8.78 $\pm$ 0.22 (1.7) | 7.85 $\pm$ 0.30 (14) | 8.32 $\pm$ 0.17 (4.7) | 7.50 $\pm$ 0.04 (31) |
| Gln$^{42}$ (227) $\rightarrow$ Ala | 8.40 $\pm$ 0.21 (4.0) | 7.52 $\pm$ 0.07 (30) | 8.04 $\pm$ 0.12 (9.1) | 7.06 $\pm$ 0.11 (88) |
| Asn$^{7,32}$ (305) $\rightarrow$ Ala | 8.83 $\pm$ 0.21 (1.5) | 8.24 $\pm$ 0.23 (5.7) | 8.41 $\pm$ 0.20 (3.9) | 7.92 $\pm$ 0.13 (12) |

$^a$ Mean IC$_{50}$ and EC$_{50}$ values are shown in parentheses in nM concentrations.

**Fig. 8.** Lys$^{3,26}$ (130) $\rightarrow$ Ala, Gln$^{5,42}$ (227) $\rightarrow$ Ala, and Asn$^{7,32}$ (305) $\rightarrow$ Ala mutations are additive. GTP-$\gamma$S (A and C) and ligand binding (B and D) assays were performed in the presence of increasing amounts of CCL-19 (A and B) and CCL-21 (C and D) on CHO-K membranes expressing wild-type (●) and Lys$^{8}$ (137) $\rightarrow$ Ala/ Lys$^{26}$ (130) $\rightarrow$ Ala/ Gln$^{42}$ (227) $\rightarrow$ Ala/ Asn$^{7,32}$ (305) $\rightarrow$ Ala (●) CCR-7 as described under "Experimental Procedures." Data shown are mean $\pm$ S.E. from at least three independent experiments.

### DISCUSSION

Chemokines are thought to bind to their cognate receptors mainly through their N-loop, while the main function of the N terminus of the ligand is believed to be in receptor activation. It is likely that the high affinity interactions between the N-loop of the ligand, and the N terminus and/or extracellular loop domains of the receptor position the ligand in an appropriate orientation, so that crucial contacts can be made with residues in the helix bundle of the receptors, which stabilize the receptor in the active conformation (17–19). We have previously shown in CCR-7 that the N-terminal domain of CCL-19 is involved in both high affinity ligand binding and receptor activation (20). With the aid of sequence alignments we identified five residues near the extracellular surface of CCR-7 that appeared likely to be involved in stabilizing the active conformation. Four of these residues were shown to be important for
The involvement of TMD-III in ligand binding and receptor activation in class A GPCRs has been well documented. (See for example Refs. 36 and 37). Residues 3.32 and 3.33 have been shown to be involved in agonist binding for receptors that bind ligands as varied as biogenic amines and peptides (25–31), while residues 3.31, 3.36, and 3.37 have been shown to be important for activation but not high-affinity ligand binding at CCR-5 (35). When Tyr3.32 (136) was mutated in CCR-7, no change in function was observed for the mutant receptor compared with the wild-type receptor in radioligand binding and GTP-γS binding assays. This finding is somewhat surprising as Tyr3.32 is completely conserved among CC chemokine receptors.

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On the other hand, when the adjacent Lys3.33 (137) was mutated to Ala in CCR-7, CCL-21 but not CCL-19 mediated receptor activation was impaired 5- to 22-fold, with similar effects being observed in chemotaxis assays. This indicates that CCL-19 and CCL-21 require at least one different interaction for activating CCR-7. Agonist binding of both ligands at the Lys3.33 (137) → Ala mutant was found to be similar to that at the wild-type receptor in transiently transfected COS-7 cells, whereas in stably transfected CHO-K cells, CCL-21 exhibited a 3.9-fold decreased binding affinity. However, the 22-fold drop in activity of CCL-19 at the Lys3.33 (137) → Ala mutant receptor in transiently transfected COS-7 cells cannot be attributed to this drop in binding affinity, implying that mutating Lys3.33 (137) to Ala uncouples CCL-19 binding and receptor activation.

Two helical turns closer to the extracellular surface than Lys3.33 (137) is a highly conserved Lys3.26 (130) is found in CC chemokine receptors. When this residue was mutated, both CCL-19 and CCL-21 mediated receptor activation was impaired 3 to 9-fold and 3 to 12-fold, respectively. Ligand binding affinities were not affected. These findings strengthen the hypothesis that receptor activation requires different interactions between the agonist and the receptor than those needed for high-affinity ligand binding. The equivalent residue, Lys3.26 (130), and other residues in ELD-1 of CXCR-2 have also been shown to be involved in receptor activation (33).

CCL-19 and CCL-21 are highly positively charged proteins, however, in the N-terminal domain a cluster of negatively charged residues are found. We previously reported that the N terminus of CCL-19 plays a central role in high affinity binding and receptor activation (20) and while CCL-19 and CCL-21 share little sequence homology, a conserved Asp is found immediately preceding the CC motif in both chemokines, which was shown to be crucial for receptor activation (20). Because Lys3.26 (130) Ala affects both CCL-19- and CCL-21-mediated receptor activation, this might suggest that Lys3.26 (130) interacts with Asp7 of the ligands (38, 39). However, when Asp7 in CCL-19 was mutated to Ala, binding affinity to the wild-type receptor was reduced 70-fold and potency for G protein activation was reduced 250-fold (20), which is inconsistent with the present result. Truncation of the N terminus of CCL-19 showed that it is likely that there is an interaction between the backbone of Glu6 or Asp7 of CCL-19 and CCR-7, required for activation (20), and it is possible that Lys3.26 (130) interacts with the backbone of one of these residues. Alternatively, Lys3.26 (130) might only be indirectly involved in receptor activation.

Residue 5.42 has been shown to be involved in ligand interactions at the histamine (27), acetylcholine (26), and angiotensin II (31) receptors. Similarly, residue 7.32 at the border of ELD-3 and TMD-VII has been shown to be important for receptor function of CCR-2 (34) as well as mammalian GnRH (28) and angiotensin II (31) receptors. Mutating Glu5.42 (227) and Asn7.32 (305) to Ala affected both CCL-19 (7 to 20-fold and 3 to 4-fold respectively) and CCL-21 (17 to 40-fold and 2 to 6-fold respectively) mediated receptor activation. As seen with the Lys3.26 (130) → Ala and Lys3.33 (137) → Ala mutations, ligand binding affinities for CCL-19 and CCL-21 remained unchanged at the Glu5.42 (227) → Ala and Asn7.32 (305) → Ala mutant receptors. Residue 5.42 is in close proximity to residues 5.39 and 5.35, which are one and two turns closer to the cell surface, and have been shown to be involved in agonist binding in CXCR-1 (40, 41). It is interesting to note that the reduction in functional potency of CCL-19 and CCL-21 is very similar for both mutations, suggesting that Glu5.42 (227) and Asn7.32 (305) either interact with conserved features of the ligands or through destabilization of the active state of the receptor. As earlier findings showed that truncations, but not Ala substitutions, of Glu6 or Asp7 affected CCL-19-mediated receptor activation (20), it is possible that Glu5.42 (227) and/or Asn7.32 (305) interact with the backbone of these residues through side-chain amide to backbone amide hydrogen bonding. Alternatively the role of Glu5.42 (227) and Asn7.32 (305) might be more structural, i.e. in forming interactions with other residues in the helix bundle that are important for stabilizing the active conformation of the receptor. This loss in activity without loss in high affinity binding further strengthens the hypothesis that receptor activation and high affinity ligand binding require different sets of interactions.
The effects of removing the side chains of Lys^3.26 (130), Gln^4.22 (227), and Asn^7.32 (300) appear to be additive, as the ability of the combined triple mutant CCR-7 to be activated was highly impaired (EC_{50} = 138 nM for CCL-19 and 197 nM for CCL-21), while chemotaxis of Jurkat cells stably expressing the triple mutant receptor was also severely affected. At the same time the affinity of CCL-19 at the mutant receptor remained unchanged, while CCL-21 showed only a 4.9-fold reduced potency to inhibit radioligand binding. The loss in functional activity of CCL-21 therefore cannot be explained by the loss in binding affinity indicating that both CCL-19 and CCL-21-mediated receptor activation is uncoupled with the loss of these side chains. As the effects of the single mutants are additive, it is likely that the side chains of Lys^3.26 (130), Gln^4.22 (227), and Asn^7.32 (300) contribute independently to ligand activation of CCR-7.

It is also notable that effects on GTP-γS binding assays are mirrored in chemotaxis assays for all mutants tested. This suggests that GTP-γS binding assays can be used as a good indicator for the effects of receptor mutations on chemotaxis, although the possibility that other mutations might be capable of dissociating aspects of G protein activation and chemotaxis cannot be ruled out.

CCR-7 is the second chemokine receptor where residue 3.26 has been shown to be important for agonist activity. Previously, it has been shown that residues in the N terminus of CXCR-1 (40), CXCR-2 (42), CXCR-4 (43, 44), CCR-2 (45) and CCR-5 (46–48), in or close to ELD-1 of CCR-2 (42), CCR-2 (49) and CCR-5 (35, 50), in or close to ELD-2 of CXCR-1 (40, 41), CXCR-4 (43) and CCR-5 (35, 51), and in or close to ELD-3 of CXCR-1 (40, 41), CCR-2 (34), and CCR-5 (35) are important for agonist binding and/or receptor activation. Therefore, chemokine receptors appear to have evolved to bind ligands at sites in the extracellular domains and close to the extracellular surface. This is also seen at the angiotensin II and GnRH receptors, which bind smaller peptide ligands with ELD-3/TMD-VII interactions (28, 31).

High affinity agonist binding mainly occurs at the G protein coupled form of GPCRs as demonstrated by guanine nucleotide sensitivity in radioligand binding assays (38). A degree of nucleotide-insensitive agonist binding is common and represents some labeling of the uncoupled form of the receptor (38, 39). As shown by flow cytometry, receptor protein expression for the triple mutant was found to be similar to wild-type levels even though B_{max} values measured by agonist radioligand binding for the wild-type and triple mutant receptors were 113 and 12 pmol/mg, respectively. This suggests that despite the triple mutant being expressed at normal levels, it is ineffective in forming the configuration that binds the G protein, and as this conformation is required for high affinity agonist binding, a reduced amount of specific binding is observed, even when receptor cell surface protein expression is normal. Further, agonist binding to both the Lys^3.33 (137) → Ala and triple mutant receptor showed a higher percentage of guanine nucleotide-insensitive agonist binding, which highlights the inability of both mutant receptors to form the G protein coupled conformation. This finding is somewhat surprising for the Lys^3.33 (137) → Ala mutant as it showed no difference in CCL-19-mediated receptor activation as measured by GTP-γS binding assays. The fact that no difference in specific radioligand binding was observed for the Lys^3.33 (137) → Ala mutant in the presence of GTP-γS might indicate that the difference in radioligand binding sensitivity to guanine nucleotides is too small to affect functional potency. Inhibition of agonist binding by guanine nucleotides has previously been used to show coupling of receptors to different G proteins and receptors that are poorly activated by demonstrating a shift in the apparent IC_{50} values (39). Here, the apparent IC_{50} values of the guanine nucleotides for inhibiting agonist binding at the wild-type and mutant receptors are similar, indicating that the low percentage of mutant receptors that are able to couple to G proteins are activated by the same mechanism as the wild-type receptor.

In summary, four residues in CCR-7 were demonstrated to be involved in receptor activation with little role in determining agonist binding affinity. Results of GTP-γS binding assays correlated with observations in chemotaxis assays, suggesting that chemotactic activity is mediated through pathways that are triggered through G protein activation and that GTP-γS binding assays may serve as a good indicator of biological activity. Our results fit the model proposed for other chemokine receptors where activation is thought to be mediated by two sets of independent interactions, as none of the mutants that impaired receptor activation showed impaired binding. Furthermore, residues shown for other family A GPCRs to be required for interactions with agonists are also involved in agonist mediated activation of CCR-7. The mutant receptors were found to be less efficient in coupling although it is likely that they assume the normal G protein-coupled conformation once they are activated. The above findings are consistent with observations from other peptide receptors, where residues close to the cell surface have been shown to play an important role in receptor activation. It is possible that residues near or at the top of TMD-III interact with agonists initiating rotation of TMD-III, which in turn leads to conformational changes of the interior regions of the receptor resulting in G protein activation. Understanding mechanisms underlying this process might help to design molecules that can regulate the activity of CCR-7. These molecules might be used in model systems investigating biological processes of a number of autoimmune diseases such as multiple sclerosis and rheumatoid arthritis, which can ultimately lead to the development of new drugs that can be used as treatments for these diseases.

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