THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 7, Issue of February 14, pp. 5179–5187, 2003

Printed in U.S.A.

The Core Domain of Chemokines Binds CCR5 Extracellular Domains while Their Amino Terminus Interacts with the Transmembrane Helix Bundle*

Received for publication, June 8, 2002, and in revised form, November 21, 2002
Published, JBC Papers in Press, December 3, 2002, DOI 10.1074/jbc.M205684200

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CCR5 is a functional receptor for various inflammatory CC-chemokines, including macrophage inflammatory protein (MIP)-1α and RANTES (regulated on activation normal T cell expressed and secreted), and is the main coreceptor of human immunodeficiency viruses. The second extracellular loop and amino-terminal domain of CCR5 are critical for chemokine binding, whereas the transmembrane helix bundle is involved in receptor activation. Chemokine domains and residues important for CCR5 binding and/or activation have also been identified. However, the precise way by which chemokines interact with and activate CCR5 is presently unknown. In this study, we have compared the binding and functional properties of chemokine variants onto wild-type CCR5 and CCR5 point mutants. Several mutations in CCR5 extracellular domains (E172A, R168A, K191A, and D276A) strongly affected MIP-1α binding but had little effect on RANTES binding. However, a MIP/RANTES chimera, containing the MIP-1α N terminus and the RANTES core, bound to these mutants with an affinity similar to that of RANTES. Several CCR5 mutants affecting transmembrane helices 2 and 3 (L104F, L104F/F109H/F112Y, F85L/L104F) reduced the potency of MIP-1α by 10–100 fold with little effect on activation by RANTES. However, the MIP/RANTES chimera activated these mutants with a potency similar to that of MIP-1α. In contrast, LD78β, a natural MIP-1α variant, which, like RANTES, contains a proline at position 2, activated these mutants as well as RANTES. Altogether, these results suggest that the core domains of MIP-1α and RANTES bind distinct residues in CCR5 extracellular domains, whereas the N terminus of chemokines mediates receptor activation by interacting with the transmembrane helix bundle.

Chemokines are a family of small proteins (8–12 kDa) that play a crucial role in the development of immune response by organizing the recruitment and the trafficking of immune cell populations throughout the body, under both physiological and pathological conditions (1, 2). They mediate their biological activities by signaling through G protein-coupled receptors (2). CCR5 is a functional receptor for the CC-chemokines MIP-1α[1] (CCL3), MIP-1β (CCL4), RANTES (CCL5), MCP-2 (CCL8), LD78β, and HCC-1-(9–78) (CCL14-(9–78)) and is expressed on memory T cells, macrophages, dendritic cells, and migroglia (2–6). CCR5 is also the principal coreceptor of human immunodeficiency viruses that, in concert with CD4, mediates the binding of the viral envelope protein to the cell surface, allowing subsequent entry into target cells (7). The key role played by CCR5 in human immunodeficiency virus pathogenesis has been demonstrated by the almost complete resistance to human immunodeficiency virus-1 infection of individuals homozygous for a 32-base pair deletion in the coding sequence of the receptor, which results in the absence of functional coreceptor at the cell surface (8, 9). The absence of pathological phenotype in individuals lacking functional CCR5, together with the potent human immunodeficiency virus-suppressive activity of CCR5 antagonists, makes this receptor an attractive candidate for pharmacological intervention (10). Moreover, CCR5 appears to be involved in a much broader range of human immune diseases, including multiple sclerosis, rheumatoid arthritis, and renal allograft rejection, suggesting that blocking CCR5 function might be beneficial in these diseases as well (11, 12). For these reasons, understanding at the molecular level how CCR5 interacts with chemokines and gp120 and how CCR5 activates G protein signaling as a consequence of chemokine binding might help in the rational design of CCR5 blocking agents.

Chemokines share a similar monomeric fold, characterized by a disordered amino-terminal domain, followed by a conserved core region, consisting of the so called "N-loop," three anti-parallel β-strands, and a carboxyl-terminal α-helix (13). The currently prevailing model for chemokine-chemokine receptor interaction postulates a two-step mechanism, in which the core of the chemokine interacts first with a binding site formed by the extracellular domains of the receptor, while another interaction is required between the chemokine N terminus and a second binding site on the receptor in order to

* This work was supported by the Actions de Recherche Concertées of the Communauté Française de Belgique; the French Agence Nationale de Recherche sur le Syndrome d'Immunodéficience Acquise; the Centre de Recherche Inter-universitaire en Vaccinologie; the Belgian program on Interuniversity Poles of Attraction initiated by the Belgian State, Prime Minister's Office, Science Policy Programming; the Fonds de la Recherche Scientifique Médicale of Belgium, Télévie; and the Fondation Médicale Reine Elisabeth (to M. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: MIP, macrophage inflammatory protein; RANTES, regulated on activation normal T cell expressed and secreted; mAb, monoclonal antibody; PBS, phosphate-buffered saline; wtCCR5, wild type CCR5; TM, transmembrane.

trigger receptor activation. Such a two-step model is analogous to the interaction of C5a, a chemoattractant protein with a size similar to that of chemokines, with its cognate G protein-coupled receptor (14). In agreement with this model, amino-terminal truncations of various CC-chemokines, including CCR5 ligands such as MIP-1α and RANTES, result in a profound reduction of their biological activity, although they retain most of their binding capability (13, 15–17). Amino-terminally truncated CC-chemokines act therefore as partial agonists or full antagonists. On the other hand, we and others have recently identified various residues located in the core domain of CC-chemokines that contribute to their high affinity binding to receptors (17–22). There is, however, no direct experimental demonstration that the N-terminal and core domains of chemokines interact with structurally and functionally independent sites on their cognate receptors.

In this study, we have determined the binding and functional properties of chemokines and chemokine variants onto CCR5 point mutants. We have found that several residues in CCR5 extracellular domains are able to discriminate between the core region of MIP-1α and RANTES. We have also identified several residues in CCR5 transmembrane helices 2 and 3 that are crucial for chemokine-induced receptor activation. Their substitution affects differentially the functional response of MIP-1α and RANTES, and we show that the N-terminal domain of chemokines is involved in this specificity.

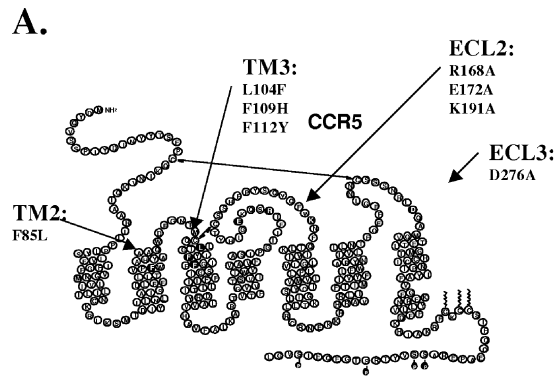
MATERIALS AND METHODS

CCR5 Mutants—Plasmids encoding the CCR5 mutants were constructed by site-directed mutagenesis, using the QuikChange method (Stratagene). Following sequencing of the constructs, the mutated coding sequences were subcloned into the bicistronic expression vector pEFIN3 as previously described (23). All constructs were verified by sequencing before transfection.

Chemokines—RANTES, the MIP/RANTES chimera, and RANTES-(8–68) were produced as previously described (16, 24). The proteins were subjected to Edman degradation and electrospray mass spectroscopy for sequence verification. MIP-1α, LD-78β, and MCP-2 were purchased from R&D Systems.

Expression of Mutant Receptors in CHO-K1 Cells—CHO-K1 cells were cultured in Ham's F-12 medium supplemented with 10% fetal calf serum (Invitrogen), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Constructs encoding wild-type or mutant CCR5 in the pEFIN3 bicistronic vector were transfected using Fugene 6 in a CHO-K1 cell line expressing an apoaequorin variant targeted to mitochondria, as previously described (25). Selection of transfected cells was made for 14 days with 400 μg/ml G418 (Invitrogen), and the population of mixed cell clones expressing wild-type or mutant receptors was used for binding and functional studies. Cell surface expression of the receptor variants was measured by flow cytometry using mAbs recognizing distinct extracellular epitopes of the receptor. The phycoerythrin-conjugated 2D7 and 3A9 mAbs were purchased from Pharmingen. mAbs 531, 523, and CTC5 were purchased from R&D Systems. mAbs MC-1, MC-4, MC-5, and MC-6 were kindly provided by Mathias Mack (University of Munich, Munich, Germany). The epitope mapping of these mAbs has been described previously (26, 27).

Binding Assays—CHO-K1 cells expressing wild-type or mutant CCR5 were collected from plates with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline supplemented with 5 mM EDTA, gently pelleted for 2 min at 1000 $\times$ *g*, and resuspended in binding buffer (50 mM Hepes, pH 7.4, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 0.5% bovine serum albumin). Competition binding assays were performed in Minisorb tubes (Nunc), using 0.1 nM [^{125}I]MIP-1β or 0.05 nM [^{125}I]RANTES (2000 Ci/mmol; Amersham Biosciences) as tracer, variable concentrations of competitors, and 40,000 cells in a final volume of 0.1 ml. Total binding was measured in the absence of competitor, and nonspecific binding was measured with a 100-fold excess of unlabeled ligand. Samples were incubated for 90 min at 27 °C, and then bound tracer was separated by filtration through GF/B filters presoaked in 1% bovine serum albumin for [^{125}I]MIP-1β or 0.5% polyethylenimine (Sigma) for [^{125}I]RANTES. Filters were counted in a β-scintillation counter. Binding parameters were determined with the Prism software (GraphPad Software) using nonlinear regression applied to a one-site competition model.



B.

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                    10                  30
                    ?                   ?
MIP-1α :        ASLAADTPTACCFSYTSRQIPQNFIADYFETSSQCSKP
LD-78β :        APLAADTPTACCFSYTSRQIPQNFIADYFETSSQCSKP
RANTES :        SPYSSDT TPCCFAYIARPLPRAHIKEYFYTSGKCSNP
MIP/RANTES :    ASLAADTPTACCFAYIARPLPRAHIKEYFYTSGKCSNP


MIP-1α :        GVIFLTKRSRQVCADPSEEWVQKYVSDLELSA
LD-78β :        SVIFLTKRGRQVCADPSEEWVQKYVSDLELSA
RANTES :        AVVFVTRKNRQVCANPEKKWVREYINSLEMS
MIP/RANTES :    AVVFVTRKNRQVCANPEKKWVREYINSLEMS
                 ?                   ?
                40                  60
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FIG. 1. **CCR5 mutants and chemokine variants.** *A*, CCR5 mutants. The putative transmembrane organization of CCR5 is represented, as well as the location and the nature of the amino acid substitutions (in *black*) analyzed in this study. Extracellular loops (*ECL*), intracellular loops (*ICL*), and transmembrane domains (*TM*) are numbered. Disulfide bonds linking together CCR5 extracellular domains are shown (Cys^{20}–Cys^{269} and Cys^{101}–Cys^{178}). *B*, sequence alignment of the various CCR5 agonists used in this study.

Functional Assays—The functional response to chemokines was analyzed by measuring the luminescence of aequorin as described (28, 29). Briefly, cells were collected from plates in Ca^{2+}- and Mg^{2+}-free Dulbecco's modified Eagle's medium supplemented with 5 mM EDTA, pelleted for 2 min at 1000 $\times$ *g*, resuspended in Dulbecco's modified Eagle's medium at a density of 5 $\times$ 10^6 cells/ml, and incubated for 2 h in the dark in the presence of 5 μM coelenterazine H (Molecular Probes). Cells were diluted 5-fold before use. The cell suspension (50,000 cells in 50 μl of Dulbecco's modified Eagle's medium) was added to agonists placed in the wells or microtiter plates in 50 μl of the same medium, and luminescence was recorded for 30 s in a Berthold luminometer.

RESULTS

Effects of CCR5 Extracellular Loop Mutations on the Functional and Binding Properties of RANTES, MIP-1α, LD78β, and MIP/RANTES—We have previously shown that various CCR5 agonists display differential sensitivity to substitutions of residues located in the extracellular domains of the receptor, suggesting that the binding site of these agonists is partially nonoverlapping (30). We have therefore investigated mutants of all charged amino acids present in the CCR5 extracellular domain for their ability to bind [^{125}I]MIP-1α, MIP$-$1β, or RANTES (data not shown). Several mutants, affecting residues located in ECL2 (R168A, E172A, K191A) or ECL3 (D276A) (Fig. 1*A*), discriminated between MIP-1α and RANTES in a binding assay. These mutants and wild type CCR5 were stably expressed in CHO-K1 cell lines coexpressing apoaequorin, and cell surface expression of the receptor was assayed by flow cytometry using a panel of mAbs recognizing different linear as well as conformation-sensitive amino-terminal epitopes (MC-4, MC-5, CTC5), conformation-sensitive epitopes in ECL2 (MC-1, 2D7), or a multidomain epitope (MC-6) (26, 27).

All mutants were expressed at levels similar to that of wtCCR5 (Fig. 2*A*). For most mutants, there was a fairly good

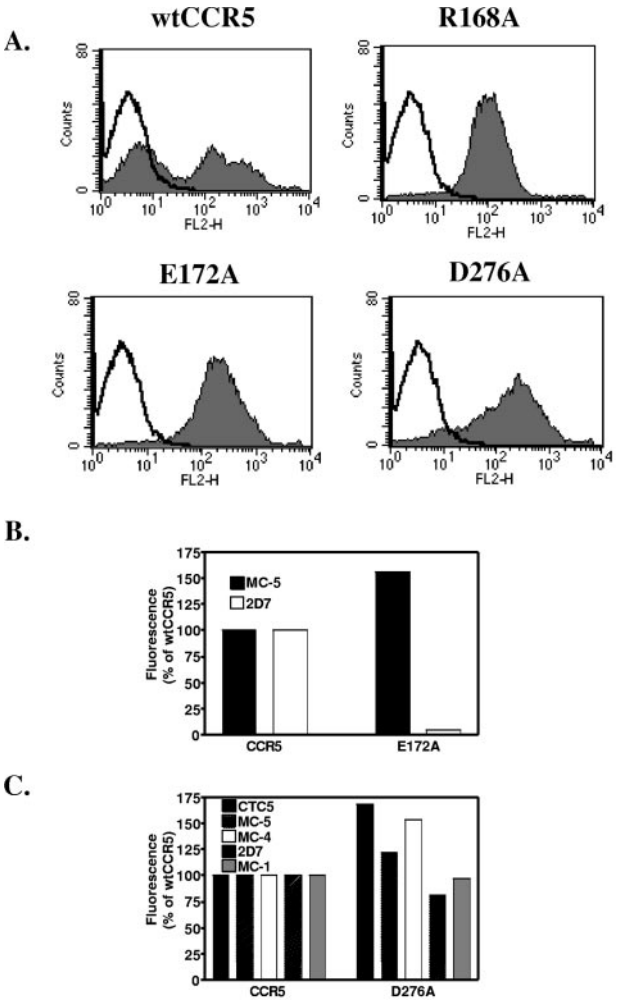

FIG. 2. **Surface expression and receptor conformation of CCR5 mutants.** Cell surface expression of wtCCR5 and the different mutants was analyzed by fluorescence-activated cell sorting using 2D7-PE and MC-5 monoclonal antibodies. Mean channel fluorescence was obtained for all mutants using the 2D7 and MC-5 mAb. A typical experiment out of three performed independently is represented. Staining of untransfected cells with mAb was used as a negative control. Receptor conformation of wtCCR5 and the different mutants was analyzed by fluorescence-activated cell sorting using mAbs of various classes.

correlation in the relative fluorescence obtained with the different mAbs. Differences in the staining pattern by the various mAbs were, however, found for mutants E172A and D276A. The substitution of Glu^{172} by Ala strongly affected the labeling by some ECL2 and multidomain mAbs (Fig. 2*B*), whereas recognition by other mAbs (MC-1, MC-4, MC-5, etc.) was not altered (see Fig. 2*B* and Refs. 26 and 27), suggesting that these mutations affected the epitopes recognized by some mAbs but did not alter receptor expression. A slight increase in relative staining was observed for D276A, using the antibodies directed at amino-terminal epitopes, such as MC-4, MC-5, or CTC5 (Fig. 2*C*).

We first measured the ability of the mutant receptors to activate intracellular cascades in response to MIP-1α, RANTES, LD78β (a natural variant of MIP-1α containing, like RANTES, a Pro in position 2), and a MIP/RANTES chimera, by using a calcium reporter assay based on the coexpression of apoaequorin (28). The MIP/RANTES chimera, which contains the amino-terminal domain of MIP-1α and the RANTES core, was designed to investigate which domain of the chemokines is involved in their receptor binding and activation properties. Earlier work has shown that the proline in position 2 of LD78β contributes greatly to the high affinity of this chemokine for CCR5 (5). As described previously (5, 24, 30), LD78β appeared as the most potent ligand for wtCCR5, with an EC_{50} of 0.71 nM, followed by RANTES (EC_{50} of 3.2 nM), MIP-1α (EC_{50} of 3.2 nM), and MIP/RANTES (EC_{50} of 6.7 nM; Fig. 3*A* and Table I). On R168A-expressing cells, LD78β, RANTES, and MIP/RANTES elicited a much stronger functional response (EC_{50} of 14, 14, and 45 nM, respectively) than MIP-1α, for which a minute signal could be detected only at the highest concentration tested ($EC_{50} \gg 300$ nM; Fig. 3*B*). The E172A mutant responded to RANTES with a potency similar to that of wtCCR5 (EC_{50} = 3.4 nM); its response to LD78β (EC_{50} = 4.1 nM) and MIP/RANTES (EC_{50} = 27 nM) was moderately affected, whereas that to MIP-1α was strongly reduced both in terms of potency and efficacy (Fig. 3*C*). K191A behaved grossly in a similar way (EC_{50} of 14 and 45 nM for RANTES and MIP/RANTES, respectively), except that no functional response was observed up to 300 nM of MIP-1α (Fig. 3*D*). The response of D276A (mutation located in ECL3) to the three agonists tested was affected (EC_{50} of 9.8, 72, and 104 nM for RANTES, MIP/RANTES, and MIP-1α, respectively) (Fig. 3*E*). Interestingly, whereas RANTES was usually more efficient than MIP-1β on the various mutants affecting CCR5 extracellular domains, MIP$-$1β elicited the strongest functional response in D276A-expressing cells (EC_{50} = 29 nM, E_{max} twice as high as for RANTES; data not shown).

To determine whether the reduction in the functional response of the mutants to some CCR5 ligands is the consequence of a reduced affinity for these chemokines, we performed competition binding assays on the mutants, using the best agonist as a tracer. In agreement with the functional assay, wtCCR5 displayed a higher binding affinity for LD78β than for RANTES, MIP-1α, or MIP/RANTES, both in homologous and heterologous assays (Fig. 4*A* and Table I). Using [^{125}I]RANTES as a tracer, LD78β, RANTES, and MIP/RANTES bound the R168A mutant with similar potency (IC_{50} of 0.46, 0.47, and 2.4 nM, respectively), whereas MIP-1α hardly competed for RANTES binding at the highest concentrations tested ($IC_{50} >$ 1 μM; Fig. 4*B*). E172A bound RANTES with an affinity similar to that of wtCCR5 (IC_{50} = 0.15 nM) but displayed a reduced affinity for LD78β (IC_{50} of 4.1 nM) and MIP/RANTES (IC_{50} of 25 nM) and, to a much greater extent, for MIP-1α ($IC_{50} > 1$ μM; Fig. 4*C*). As shown in Fig. 4*D*, RANTES and MIP/RANTES competed with a similar potency for binding to D276A, using [^{125}I]MIP-1β as a tracer (IC_{50} = 0.27 and 0.15 nM, respectively), whereas MIP-1α was 20-fold less potent (IC_{50} = 5.9 nM). Specific binding to K191A-expressing cells was below the limit of detection, even using higher concentrations of [^{125}I]RANTES (data not shown), making it impossible to determine chemokine binding affinities for this mutant.

From this series of experiments, we can conclude that mutants affecting charged residues in CCR5 extracellular domains (mostly in ECL2) affected differently the binding and, as a consequence, the functional properties of RANTES and MIP-1α. The MIP/RANTES chimera behaved essentially as RANTES, suggesting that these CCR5 extracellular residues constitute binding sites for the core domain of chemokines. The behavior of LD78β, which keeps a relatively high affinity for the mutant receptors, suggests a strong contribution of its N-terminal domain in the overall stability of the interaction with CCR5.

Binding and Functional Properties of MIP/RANTES and LD78β onto Mutants Affecting CCR5 Transmembrane Segments—We have recently observed that mutations in CCR5 transmembrane (TM) helix 2 differentially affected the functional response to various high affinity ligands without significantly affecting their binding affinity (32). In our effort to

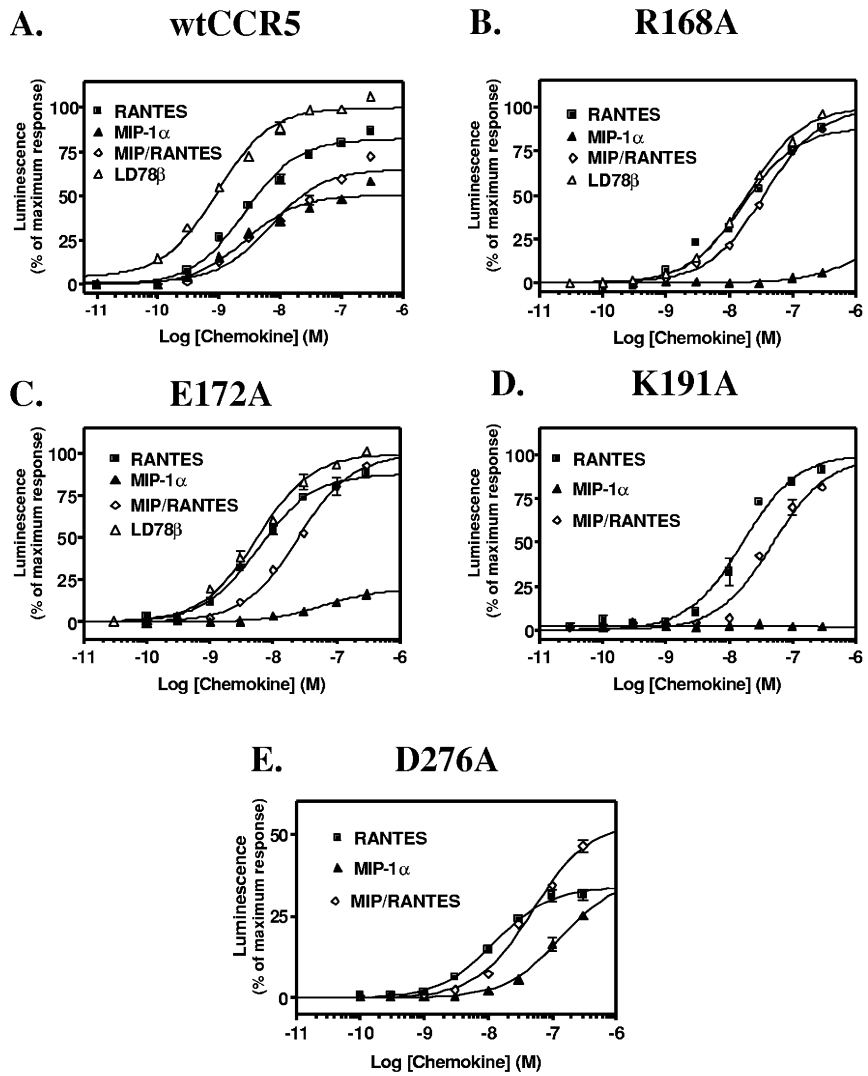

FIG. 3. **Functional response of CCR5 extracellular domain mutants.** The functional response of the cell lines co-expressing apoaequorin and CCR5 extracellular domain mutants was tested following the addition of MIP-1α, RANTES, LD78β, and the MIP/RANTES chimera. *A*, wtCCR5; *B*, R168A; *C*, E172A; *D*, K191A, *E*, D276A. The luminescent signal resulting from the activation of the apoaequorin-coelenterazine complex was recorded for 30 s in a luminometer. Results were analyzed by nonlinear regression using the Graphpad Prism software. The data were normalized for basal (0%) and maximal luminescence (100%). All points were run in triplicate (*error bars* represent S.E.). The displayed curves represent a typical experiment out of three performed independently.

understand the molecular mechanisms involved in CCR5 activation, we have investigated further the role played in this process by aromatic residues located in TM2 and TM3. As a result, we found that a number of additional amino acid substitutions within TM2 and TM3 also differentially affected the functional response to CCR5 ligands (33). We have selected here three mutants that do not affect the binding affinity to MIP-1α and RANTES (L104F, L104F/F109H/F112Y, and F85L-L104F) while exhibiting different patterns of functional responses to these chemokines (Fig. 1*A*). Combining a set of different chemokines with these mutants allowed us to determine which part of the chemokines account for this selective reduction of agonist potency.

The abilities of MIP-1α and RANTES to activate the mutant receptors were compared with that of MIP/RANTES (which contains MIP-1α amino terminus), LD78β, and RANTES-(8–68) (a variant lacking the first seven amino acids). All transmembrane mutant receptors were expressed in apoaequorin-expressing CHO-K1 cell lines. Their apparent expression level at the cell surface, as determined by fluorescence-activated cell sorting analysis using mAbs directed either at conformational or linear epitopes, was similar to that of wt CCR5 (data not shown). As described above (Fig. 5*A* and Table II), LD78β was the most potent agonist for wtCCR5 (EC_{50} of 0.71 nM). RANTES-(8–68) resulted only in a partial activation of CCR5 at high concentrations ($EC_{50} > 300$ nM). Some CCR5 mutants affecting aromatic residues in TM2 and/or TM3 (L104F, L104F/F109H/F112Y, F85L/L104F) were characterized by a general reduction of their functional response to chemokines, but the potency and efficacy of RANTES were generally less affected than those of MIP-1α (Fig. 5, *A–D*, and Table II). For some mutants, such as F85L/L104F, RANTES potency was decreased by half a log, whereas MIP-1α potency was decreased by more than 1 order of magnitude (Fig. 5*C*). For other mutants, such as L104F/F109H/F112Y, RANTES potency was not affected (EC_{50} of 2.3 nM), whereas MIP-1α potency was reduced by about 1 order of magnitude (Fig. 5*D*). For all of these mutants, the functional response to LD78β was similar to that of RANTES, whereas the MIP/RANTES chimera behaved similarly to MIP-1α (Fig. 5, *A–D*, and Table II).

The contribution of binding parameters to the observed reduction of functional response to some chemokine variants was investigated whenever possible by competition binding assays, using either [^{125}I]RANTES or [^{125}I]MIP-1β as tracers. As previously described, LD78β appeared as the natural ligand displaying the highest affinity for wtCCR5 (IC_{50}; 0.067 nM using [^{125}I]MIP-1β as tracer), followed by RANTES (IC_{50} = 0.23 nM), MIP/RANTES (IC_{50} = 0.36 nM) and MIP-1α (IC_{50} = 0.38 nM). In agreement with previous reports, amino-terminal truncation of RANTES resulted in a moderate decrease of its affinity for wtCCR5 (IC_{50} = 0.94 nM; Fig. 6*A*). For all three transmembrane CCR5 mutants, the affinity for the various ligands, including RANTES-(8–68) did not change significantly as compared with wtCCR5 (Fig. 6, *A–D*, and Table II).

From this second set of data, we can conclude that most mutations of aromatic residues located in TM2 and TM3 of CCR5 differentially affect the functional response to MIP-1α and RANTES, without significantly altering their binding affinity. A functional defect on these mutants was observed only for chemokine variants containing the N terminus of MIP-1α (*i.e.* MIP-1α and MIP/RANTES). The Pro in position 2 of LD-78β was sufficient to recover loss of activity of MIP-1α. These observations suggest strongly that the N terminus of chemokines triggers receptor activation through an interaction with the transmembrane helix bundle.

DISCUSSION

A number of studies have identified residues in chemokines or chemokine receptors that are important for binding and receptor activation, although the precise molecular mechanisms by which chemokines interact with their receptors are largely unknown. By using chimeras between CCR5 and its closest homologue, CCR2b, we have previously identified the second extracellular loop (ECL2) of CCR5 as the main determinant of ligand selectivity (23). The importance of the CCR5 amino-terminal domain for chemokine binding was also demonstrated, in particular structural determinants consisting of negatively charged and aromatic residues (34). A number of tyrosines in the amino-terminal domain of CCR5 are sulfated, and this post-translational modification has been proposed to provide a larger, potentially more flexible and negatively charged surface for ligand binding (35, 36). Some CCR5 mutations, including in extracellular domains, affect the pharmacological profile of the receptor, with a loss of functional response to specific chemokines but not others. This observation suggests that different chemokines interact with different residues within the extracellular domains of a given receptor (30). Such mutants are particularly interesting because they may help to test hypotheses concerning the precise way chemokines bind and activate their receptors. In this study, we have used CCR5 mutants that discriminate between MIP-1α and RANTES, to determine which chemokine domains interact with specific CCR5 sites and the role of these interactions in determining binding affinity and receptor activation.

The Globular Core of the Chemokine Interacts with the Extracellular Domain of CCR5—Using different chemokines as tracers, we found that a number of CCR5 mutants, in which charged residues in extracellular domains were substituted for alanine, continued to bind RANTES with high affinity, but not MIP-1α. These mutations were largely located in ECL2. We determined, by using a chimeric chemokine (MIP/RANTES) containing the amino-terminal domain of MIP-1α and the RANTES core, which chemokine region was responsible for this specific binding deficit. MIP/RANTES bound and activated most receptor mutants (R168A, K191A, and D276A) with affinities and potencies similar to RANTES. These results strongly suggest that important determinants involved in the differential binding of MIP-1α and RANTES are located in CCR5 ECL2 and that these determinants interact with the core domain of these chemokines. The affinity of MIP/RANTES for E172A was reduced as compared with RANTES, although much less severely than that of MIP-1α. This might be the consequence of a conformational change of this mutant receptor, as suggested by the lower fluorescence-activated cell sorting signal obtained with several conformation-sensitive mAbs (26). However, we cannot exclude the possibility that this region of ECL2 might interact both with the chemokine core and with the amino-terminal domain.

We and others have identified in different CCR5 ligands conserved or chemokine-specific residues that are important for receptor binding. A conserved aromatic residue located in

TABLE I
Binding and functional parameters for extracellular domain mutants

	RANTES			MIP-1α			MIP/RANTES			LD78β		
	Binding		Function	Binding		Function	Binding		Function	Binding		Function
	MIP-1β $pIC_{50} \pm$ S.E.	RANTES $pIC_{50} \pm$ S.E.	$pEC_{50} \pm$ S.E.	MIP-1β $pIC_{50} \pm$ S.E.	RANTES $pIC_{50} \pm$ S.E.	$pEC_{50} \pm$ S.E.	MIP-1β $pIC_{50} \pm$ S.E.	RANTES $pIC_{50} \pm$ S.E.	$pEC_{50} \pm$ S.E.	MIP-1β $pIC_{50} \pm$ S.E.	RANTES $pIC_{50} \pm$ S.E.	$pEC_{50} \pm$ S.E.
CCR5	9.64 ± 0.08	9.13 ± 0.01	8.49 ± 0.12	9.42 ± 0.05	8.77 ± 0.11	8.50 ± 0.09	9.44 ± 0.03	8.41 ± 0.11	8.17 ± 0.10	10.17 ± 0.12	9.75 ± 0.10	9.15 ± 0.15
R168A	ND[a]	9.34 ± 0.21	7.85 ± 0.08	ND	<6	<6.3	ND	8.62 ± 0.09	7.35 ± 0.05	ND	9.33 ± 0.12	7.84 ± 0.08
E172A	ND	9.81 ± 0.05	8.47 ± 0.12	ND	<6	<6.3	ND	7.60 ± 0.01	7.57 ± 0.08	ND	8.60 ± 0.01	8.39 ± 0.07
K191A	ND	ND	7.85 ± 0.05	ND	ND	<6.3	ND	ND	7.35 ± 0.07	ND	ND	NP[b]
D276A	9.57 ± 0.23	NP	8.01 ± 0.10	8.23 ± 0.17	NP	6.98 ± 0.15	9.83 ± 0.09	NP	7.14 ± 0.07	NP	NP	NP

[a] ND, not determined.
[b] NP, not performed.

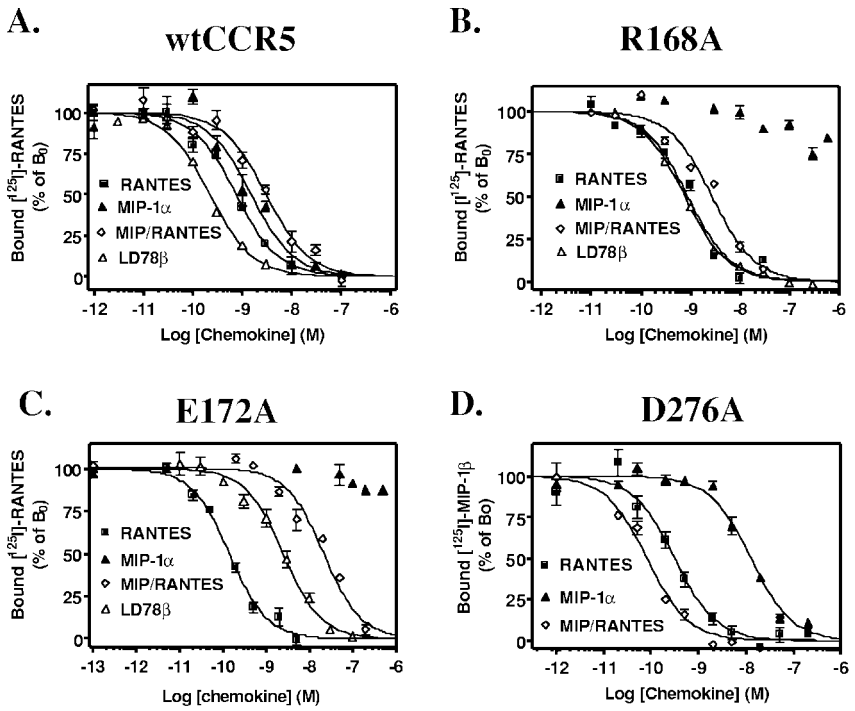

FIG. 4. **Chemokine binding to CCR5 extracellular loop mutants.** Competition binding curves were performed on CHO-K1 cell lines expressing wtCCR5 (*A*), R168A (*B*), E172A (*C*), or D276A (*D*) mutants using 0.05 nM [^{125}I]RANTES or 0.1 nM [^{125}I]MIP-1β as tracer. Results were analyzed by the Graphpad Prism software, using a single site model, and the data were normalized for nonspecific (0%) and specific binding in the absence of competitor (100%). All points were run in triplicate (*error bars* represent S.E.). Data are representative of three independent experiments.

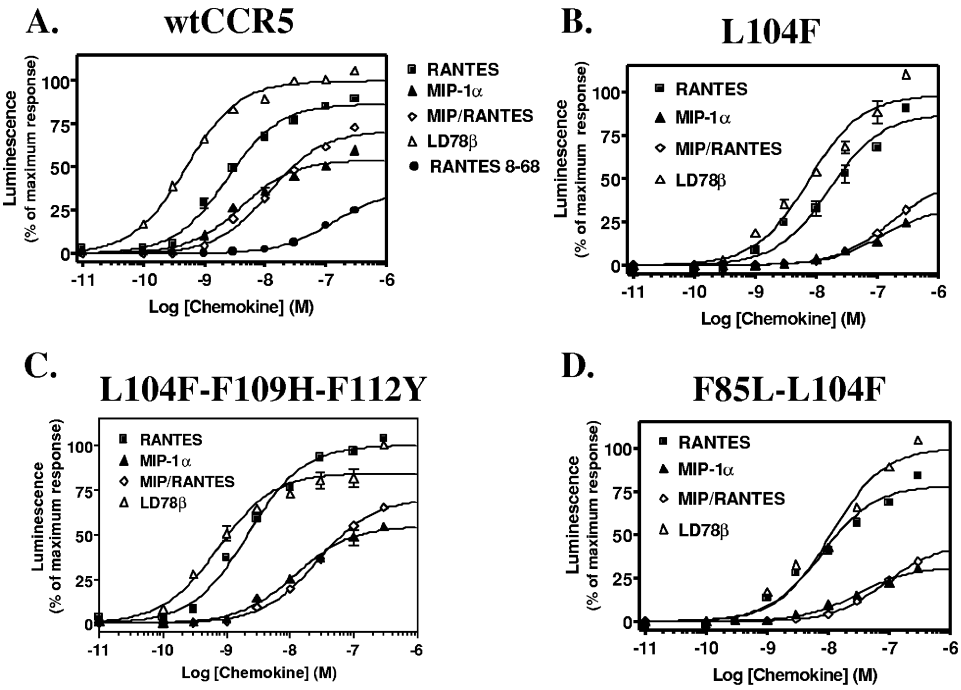

FIG. 5. **Functional response of CCR5 transmembrane helix mutants.** The functional response of cell lines co-expressing apoaequorin and CCR5 transmembrane mutants was tested following the addition of MIP-1α, RANTES, MIP/RANTES, and LD78β. *A*, wtCCR5; *B*, L104F; *C*, L104F/F109H/F112Y; *D*, F85L/L104F. The results were analyzed and normalized as described for Fig. 4. All points were run in triplicate (*error bars* represent S.E.). The displayed curves represent a typical experiment out of three performed independently.

the N-loop of MIP-1β (Phe^{13}) plays a critical role in CCR5 binding (17). The same residue is also important for the binding of other CC-chemokines, including RANTES, to their respective receptors (15, 20, 21, 37, 38). Conserved basic residues located in the N-loop (Arg^{18}, Lys^{19}), 3^{10}-helix (Arg^{22}), and 40s loop (Arg^{46}–Lys^{48}) of MIP-1β also contribute to CCR5 binding (18, 22), and homologous residues of MCP-1 and eotaxin play a similar role for CCR2b and CCR3 binding, respectively (20, 21). This suggests that a common binding surface of CC-chemokines to their receptors involves patches of basic residues separated by an hydrophobic groove. With the exception of Lys^{33}, single substitutions of basic residues in RANTES do not result in a significant decrease of affinity for CCR5, although combinations of these mutations result in a progressive reduction of binding affinity (19).[2] The functional response of CCR5 to these chemokine mutants correlates well with their binding affinity, suggesting that these residues are involved in binding but not directly in the activation process (18, 19, 22).

[2] C. Blanpain, B. J. Doranz, A. Bondue, C. Govaerts, A. De Leener, G. Vassart, R. W. Doms, A. Proudfoot, and M. Parmentier, unpublished results.

Motifs of acidic and hydrophobic residues located in the amino-terminal domain of chemokine receptors represent a common binding motif for chemokines (20, 34). It is therefore tempting to speculate that the conserved binding sites identified in chemokines and in chemokine receptors interact with one another. This common interaction surface might account for the promiscuous character of CC-chemokine binding to their receptors. In addition to this conserved binding site in the N-terminal domain, CCR5-specific residues, in particular those in ECL2, are involved in the selective binding of a chemokine subset. As discussed above, the MIP-1α residues involved in ECL2 binding are expected to be charged amino acids located in the core domain of the protein. Although these residues are presently unknown, the alignment of MIP-1α and RANTES sequences (Fig. 1*B*) shows that MIP-1α is much more acidic than RANTES. It exhibits several clusters of acidic residues, especially in the 30s and 50s loops and in the carboxyl-terminal α-helix, but also a cluster of basic residues in the 40s loop. Identifying which of these residues interacts with charged amino acids of CCR5 extracellular domain will require further investigation.

The Amino Terminus of the Chemokine Interacts with the TM Domain of CCR5—Activation of G protein-coupled receptors is thought to involve conformational changes within transmembrane helices, which are either induced or stabilized upon agonist binding and which allow the receptor to trigger signaling through G proteins (39). We have recently identified a key motif (T*X*P) in CCR5 transmembrane helix 2 that plays a major role in chemokine-induced receptor activation (32). We found that mutations of this T*X*P motif induce a profound alteration of CCR5 activation, although this functional defect was strongly chemokine-selective. RANTES responses were the least affected, whereas MCP-2 effects were highly dependent on the integrity of this motif. Molecular dynamics simulations predicted that the Pro residue of this structural determinant would orient the extracellular part of TM2 toward TM3, and not toward TM1 as seen in the structure of rhodopsin, suggesting a direct interaction between TM2 and TM3 at this level. To analyze further the residues involved in the activation switch of CCR5, we studied the role of a cluster of aromatic residues located at close proximity of the T*X*P motif in TM helices 2 and 3. The substitution of these aromatic residues in CCR5 by their CCR2b counterparts resulted, for some of the mutants, in a profound alteration of their ability to functionally respond to chemokines, while retaining their ability to bind them with high affinity. As for T*X*P mutants, the functional alteration induced by mutations of aromatic residues was chemokine-selective, some mutants presenting a clear difference in their respective response to MIP-1α or RANTES (33). Because the amino-terminal domain of chemokines is known to be important for receptor activation, it is tempting to hypothesize that this part of the ligand interacts with a CCR5 domain involved in receptor activation. This hypothesis was tested by measuring the biological activity of chemokines, differing in their amino-terminal domain, onto these CCR5 mutants. The binding and functional properties of amino-terminally truncated chemokines were not affected by mutations of the aromatic cluster of CCR5. In contrast to what was found for extracellular mutants, the chimeric chemokine MIP/RANTES displayed a biological activity similar to that of MIP-1α on CCR5 aromatic cluster mutants, while retaining a normal binding affinity. LD78β activated these mutants as well as RANTES, which can probably be attributed to the presence of a Pro residue in position 2 (5), as found in RANTES. These results strongly argue for an interaction between the chemokine N terminus and a site in the helix bundle of the receptor, necessary for triggering the activation process.

TABLE II
Binding and functional parameters for transmembrane domain mutants

	RANTES			MIP-1α			MIP/RANTES			LD78β		
	Binding		Function	Binding		Function	Binding		Function	Binding		Function
	MIP-1β $pIC_{50} \pm$ S.E.	RANTES $pIC_{50} \pm$ S.E.	$pEC_{50} \pm$ S.E.	MIP-1β $pIC_{50} \pm$ S.E.	RANTES $pIC_{50} \pm$ S.E.	$pEC_{50} \pm$ S.E.	MIP-1β $pIC_{50} \pm$ S.E.	RANTES $pIC_{50} \pm$ S.E.	$pEC_{50} \pm$ S.E.	MIP-1β $pIC_{50} \pm$ S.E.	RANTES $pIC_{50} \pm$ S.E.	$pEC_{50} \pm$ S.E.
CCR5	9.64 ± 0.08	9.13 ± 0.01	8.49 ± 0.12	9.42 ± 0.05	8.77 ± 0.11	8.50 ± 0.09	9.44 ± 0.03	8.41 ± 0.11	8.17 ± 0.10	10.17 ± 0.12	9.75 ± 0.10	9.15 ± 0.15
L104F	9.78 ± 0.17	NP[a]	8.07 ± 0.11	9.00 ± 0.06	NP	7.25 ± 0.11	9.62 ± 0.02	NP	7.02 ± 0.13	10.20 ± 0.08	NP	8.40 ± 0.28
F85L/L104F	ND	9.20 ± 0.19	8.01 ± 0.06	ND[b]	8.36 ± 0.05	7.34 ± 0.17	ND	8.46 ± 0.09	6.75 ± 0.30	NP	NP	8.19 ± 0.24
L104F/F109H/F112Y	9.81 ± 0.07	NP	8.62 ± 0.13	9.74 ± 0.13	NP	7.88 ± 0.02	9.96 ± 0.15	NP	7.55 ± 0.01	10.42 ± 0.10	NP	8.96 ± 0.17

[a] NP, not performed.
[b] ND, not determined.

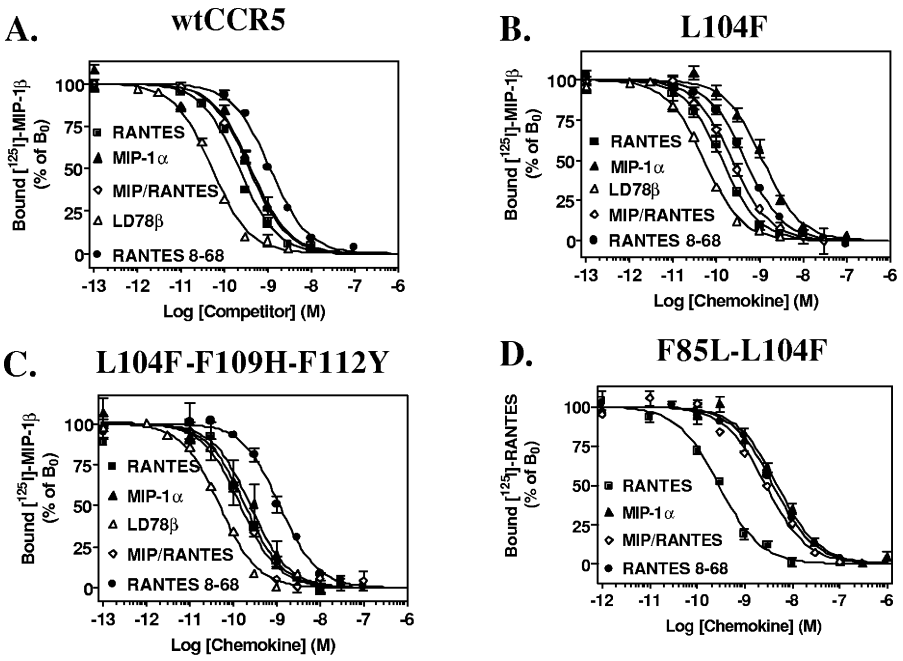

FIG. 6. **Chemokines binding to CCR5 transmembrane helix mutants.** Competition binding curves were performed on CHO-K1 cell lines expressing wtCCR5 (*A*) or the L104F (*B*), L104F/F109H/F112Y (*C*), or F85L/L104F (*D*) mutants using 0.05 nM [^{125}I]RANTES or 0.1 nM [^{125}I]MIP-1β as tracer. The results were analyzed and normalized as described for Fig. 3. All points were run in triplicate (*error bars* represent S.E.). Data are representative of three independent experiments.

The most widely accepted model for G protein-coupled receptor activation is the ternary complex model (39). According to this model, the receptor exists in a equilibrium between an inactive conformation (R) and an active conformation (R*). Agonists are predicted to bind with high affinity to the R* conformation, and due to the mass action law, to increase the proportion of R*. It is somewhat surprising that the mutation of residues expected to interact with the N-terminal domain of chemokines does not result in a significant decrease in the measured binding affinity. It is therefore likely that the interaction responsible for receptor activation does not contribute much to the overall stability of the chemokine-receptor complex, in which the contacts between the core of the chemokine and the extracellular domains of the receptor play the major role. Mutations strongly impairing activation without significantly affecting ligand binding have been described for other G protein-coupled receptors, including the C5a receptor (40) and the thyrotropin receptor (41).

Several structural differences between the amino-terminal domain of the various chemokines might account for the differences in their ability to activate receptors mutated in the aromatic cluster. The N terminus of RANTES is shorter (9 residues) than that of other CCR5 ligands (10 residues). RANTES, like LD78β and MIP-1β, have a Pro in position 2, whereas MIP-1α has a Ser. This Pro residue certainly plays an important role in chemokine function, since the P2A substitution significantly decreases the biological activity of RANTES (38). This role is, however, highly context-dependent, since cleavage of RANTES by the CD26 peptidase, removing the Pro and generating RANTES-(3–68), does not affect its activity (42). Mutational analyses of different chemokines has shown that high sequence variability in the N-terminal domain is well tolerated for normal function, suggesting that interactions between this domain and the receptor might involve primarily backbone atoms (13, 15, 31, 43).

In summary, we can now propose a more detailed scheme for the interaction between CCR5 and its ligands (Fig. 7). The core domain of chemokines mediates high affinity receptor binding through interactions with various residues located in CCR5 extracellular domains, whereas their amino-terminal domain interacts with TM residues and mediates receptor activation. Further studies will be needed to understand more precisely the molecular details of this process.

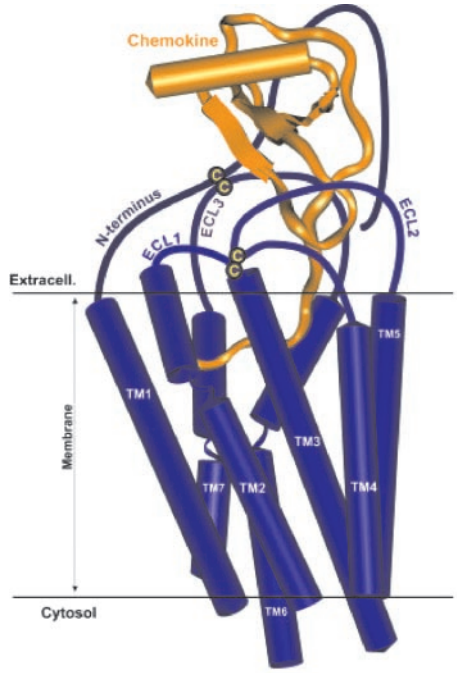

FIG. 7. **Schematic representation of the proposed receptor-chemokine interaction.** The receptor is depicted in *blue* with the transmembrane helices shown as *solid tubes*. The chemokine is in *orange*, with the C-terminal helix shown as a *solid tube* and the three β-strands as *flat ribbons*. In the proposed mode of binding, the core of the chemokine would interact mainly with the extracellular loops (*ECL*) and in particular with the N terminus and the second part of ECL2. The N-terminal portion of the ligand would interact specifically with residues inside the transmembrane bundle. The displayed conformations of the extracellular loops are not relevant *per se* and are shown for illustration purposes of the binding mode. In particular, no attempt was made to predict secondary structure elements. The exact relative orientations of the receptor and its ligand have also been chosen arbitrarily for this purpose. The intracellular loops are not displayed.

Acknowledgment—Expert technical assistance was provided by M. J. Simons.

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