Multiple nonfunctional alleles of CCR5 are frequent in various human populations

Cédric Blanpain, Benhur Lee, Marie Tackoen, Bridget Puffer, Alain Boom, Frédérick Libert, Mathew Sharron, Valérie Wittamer, Gilbert Vassart, Robert W. Doms, and Marc Parmentier

CCR5 is the major coreceptor for macrophage-tropic strains of the human immunodeficiency virus type I (HIV-1). Homozygotes for a 32-base pair (bp) deletion in the coding sequence of the receptor (CCR5Δ32) were found to be highly resistant to viral infection, and CCR5 became, therefore, one of the paradigms illustrating the influence of genetic variability onto individual susceptibility to infectious and other diseases. We investigated the functional consequences of 16 other natural CCR5 mutations described in various human populations. We found that 10 of these variants are efficiently expressed at the cell surface, bind [^{125}I]-MIP-1β with affinities similar to wtCCR5, respond functionally to chemokines, and act as HIV-1 coreceptors. In addition to Δ32, six mutations were characterized by major alterations in their functional response to chemokines, as a consequence of intracellular trapping and poor expression at the cell surface (C101X, FS299), general or specific alteration of ligand binding affinities (C20S, C178R, A29S), or relative inability to mediate receptor activation (L55Q). A29S displayed an unusual pharmacological profile, binding and responding to MCP-2 similarly to wtCCR5, but exhibiting severely impaired binding and functional responses to MIP-1α, MIP-1β, and RANTES. In addition to Δ32, only C101X was totally unable to mediate entry of HIV-1. The fact that nonfunctional CCR5 alleles are relatively frequent in various human populations reinforces the hypothesis of a selective pressure favoring these alleles. (Blood. 2000;96:1638-1645)

Introduction

Human immunodeficiency virus type I (HIV-1) infection is initiated by the interaction of the virion envelope glycoprotein (gp120) with CD4 and coreceptors belonging to the G-protein coupled receptor (GPCR) family.[1]

CCR5, a functional receptor for the CC-chemokines MIP-1α, MIP-1β, RANTES, MCP-2, and MCP-4,[2-5] is the principal coreceptor used by macrophage-tropic HIV-1 strains and primary isolates that are responsible for HIV-1 transmission and that predominate during the asymptomatic phase of the disease.[1] It is expressed on memory T cells, macrophages, dendritic cells, hematopoietic stem cells, and microglial cells.[6-9] The key role of CCR5 in HIV-1 pathogenesis has been demonstrated by the almost complete resistance to HIV-1 infection of individuals homozygous for a 32-base pair (bp) deletion in the coding region of the receptor. This deletion results in the absence of functional receptors at the cell surface as well as in a slower progression to acquired immunodeficiency syndrome (AIDS) for heterozygous individuals.[10-15] CCR5 has also been involved in other human diseases, such as rheumatoid arthritis and multiple sclerosis.[16-19] The absence of pathological phenotype in individuals homozygous for the Δ32 allele and the potent in vitro and ex vivo HIV-1 suppressive activity of CCR5 ligands or derivatives, monoclonal antibodies (MAbs), and small molecular weight antagonists makes CCR5 an ideal candidate for pharmacological interventions.[6,20-23] The Δ32 allele has a unique and recent origin (about 2000 years ago), and the high frequency of the mutant allele in the European population (10% on average) cannot be explained by random genetic drift, suggesting that a selection advantage has been conferred to carriers of this nonfunctional allele.[24]

Sixteen other mutations in the CCR5 coding region have been described in various human populations,[25,26] but the consequences of these mutations on chemokine-receptor and HIV-1 coreceptor functions are largely unknown. It is, therefore, of interest to determine whether CCR5 mutations other than Δ32 may also confer resistance to HIV-1 infection and whether nonfunctional CCR5 variants are more prevalent than functional variants, as suggested by the higher frequency of nonsynonymous mutations as compared with neutral mutations. To address those questions, we recreated all CCR5 natural mutants described to date by site-directed mutagenesis and expressed them in recombinant cell lines. We investigated their expression at the cell surface by flow cytometry using MAbs of various specificities, their subcellular distribution by confocal microscopy, their ability to bind and functionally respond to various chemokines, as well as their ability to bind the Env protein of R5 HIV-1 strains and mediate viral entry.

From the IRIBHN, Laboratoire d'Histologie, de Neuroanatomie et de Neuropathologie and Service de Génétique Médicale, Université Libre de Bruxelles, Bruxelles, Belgium; and Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA.

Submitted January 7, 2000; accepted May 5, 2000.

Supported by the Actions de Recherche Concertées of the Communauté Française de Belgique, the French Agence Nationale de Recherche sur le SIDA, 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 BIOMED and BIOTECH programmes of the European Community (grants BIO4-CT98-0543 and BMH4-CT98-2343), the Fonds de la Recherche Scientifique Médicale of Belgium, Télévie and the Fondation Médicale Reine Elisabeth to M.P.; by grant R01 40880 from the National Institutes of Health and a grant from the Burroughs Wellcome Fund to R.W.D.

R.W.D. is a recipient of an Elizabeth Glaser Scientist Award. C.B. and F.L. are respectively Aspirant and Chercheur Qualifié of the Belgian Fonds National de la Recherche Scientifique. V.W. is recipient of a First fellowship of the Région Wallonne.

Reprints: Marc Parmentier, IRIBHN, ULB Campus Erasme, 808 route de Lennik, B-1070 Bruxelles, Belgium; e-mail: mparment@ ulb.ac.be.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked ''advertisement'' in accordance with 18 U.S.C. section 1734.

Materials and methods

CCR5 mutants

Plasmids encoding the natural CCR5 mutants described in the literature were constructed by site-directed mutagenesis by using the Quickchange method (Stratagene). Following sequencing of the constructs, the mutated coding sequences were subcloned into the bicistronic expression vector pEFIN3 as previously described[27] for generation of stable cell lines and in pcDNA3 (Invitrogen) for HIV-1 envelope binding and infection assays. All constructs were verified by sequencing before transfection.

Expression of mutant receptors in CHO-K1 cells

CHO-K1 cells were cultured in HAM F12 medium supplemented with 10% fetal calf serum (Life Technologies), 100 U/mL penicillin, and 100 μg/mL streptomycin (Life Technologies). A plasmid encoding an apoaequorin variant targeted to mitochondria,[28] under control of the SRα promoter[29] was transfected into CHO-K1 cells, using Fugene 6 (Boerhinger Mannheim). Selection with zeocin (250 μg/mL; Invitrogen) was initiated 2 days later. Individual clones were isolated 3 weeks after transfection, and the most responding clone was selected on the basis of its luminescent response to ionomycin A (100 nmol/L) and ATP (10 μmol/L). Constructs encoding wild-type or mutant CCR5 in the pEFIN3 bicistronic vector were further transfected by using Fugene 6 in this apoaequorin-expressing cell line. Selection of transfected cells was made for 14 days with 400 μg/mL G418 (Life Technologies), 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 by using MAbs recognizing different CCR5 epitopes: 2D7 (phycoerythrin-conjugated; Pharmingen), MC-5 (kindly provided by M. Mack, Munich, Germany), CTC5 (Protein Design Lab), and 5523 (R&D Systems).[22]

Confocal microscopy

Stable CHO-K1 cell lines coexpressing apoaequorin and wild-type or mutant CCR5 were grown on uncoated glass coverslips. Coverslips were rinsed with phosphate-buffered saline (PBS, pH 7.4), fixed for 10 minutes in 3% paraformaldehyde in PBS, and washed 3 times for 10 minutes with tris-buffered saline (TBS). For intracellular staining, the cells were permeabilized with 0.15% Triton X-100 in TBS for 5 minutes and washed three time in TBS. Fixed or permeabilized cells were incubated for 1 hour at room temperature with 5% normal sheep serum (NSS) in TBS. Incubation with the MC-5 MAb at 1:4 dilution was performed overnight in the presence of 5% NSS. Cells were rinsed three times in TBS, incubated for 60 minutes in the dark with an FITC-labeled sheep antimouse immunoglobulin G (IgG) antibody (1:30 dilution, Amersham), washed three times in TBS and once in water, and mounted with a drop of gelvatol solution containing 100 mg/mL Dabco reagent (Sigma). Cells were observed under a Zeiss Axiovert fluorescence microscope and images were collected with the use of a Laser-Scanning Confocal Microscope (MRC 1000, BioRad) equipped with an argon-krypton laser (excitation wavelength of 488 nm) and the Laser-Sharp software (BioRad). Images where further analyzed by using NIH-Image 1.62 and Image Space software programs (Molecular Dynamics). No labeling was observed on untransfected CHO cells using MC-5 or on transfected cells using a control IgG MAb (data not shown).

[^{125}I]-MIP-1β binding assays

CHO-K1 cells expressing wild-type or mutant CCR5 were collected from plates with Ca^{++} and Mg^{2+}-free PBS supplemented with 5 mmol/L EDTA, gently pelleted for 2 minutes at 1000*g* and resuspended in binding buffer (50 mmol/L Hepes pH 7.4, 1 mmol/L $CaCl_2$, 5 mmol/L $MgCl_2$, 0.5% BSA). Competition binding assays were performed in Minisorb tubes (Nunc), using 0.08 nmol/L of [^{125}I]-MIP-1β or [^{125}I]-MCP-2 (2200 Ci/mmol, NEN) as tracer, variable concentrations of competitors (R&D Systems) 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 minutes at 27°C, then bound tracer was separated by filtration through GF/B filters presoaked in 1% BSA for [^{125}I]-MIP-1β or 0.3% polyethylenimine (Sigma) for [^{125}I]-MCP-2. Filters were counted in a β-scintillation counter. Binding parameters were determined with the Prism software (GraphPad Softwares) by using nonlinear regression applied to a one-site competition model.

Functional assays

Functional response to chemokines was analyzed by measuring the luminescence of aequorin as described.[30,31] Cells were collected from plates with Ca^{++} and Mg^{2+}-free DMEM supplemented with 5 mmol/L EDTA, pelleted for 2 minutes at 1000*g*, resuspended in DMEM at a density of 5×10^6 cells/mL, and incubated for 2 hours in the dark in the presence of 5 μmol/L coelenterazine H (Molecular Probes). Cells were diluted 7.5-fold before use. Agonists in a volume of 50 μL DMEM were added to 50 μL of cell suspension (33 000 cells) and luminescence was measured for 30 seconds in a Berthold Luminometer.

HIV-1 infection assay

Plasmids encoding the HIV-1 ADA and JRFL Envs were provided by J. Moore (Aaron Diamond AIDS Research Center, New York, NY). The NL4-3 luciferase virus backbone (pNL-Luc-E$^-$ R$^-$) was provided by N. Landau (Aaron Diamond AIDS Research Center). Luciferase reporter viruses were prepared as previously described by cotransfecting 293T cells with the indicated Env and the NL4-3 luciferase virus backbone.[32] Target cells were prepared by cotransfecting 293T cells with CD4 and a constant amount of coreceptor-encoding plasmid. Incubation was done at 37°C. Three days after infection, cells were lysed with 0.5% Triton X-100 in PBS and analyzed for luciferase activity.

[^{125}I]-gp120 binding assays

Soluble JRFL gp120 was iodinated as described elsewhere.[22] Env binding assays were performed by resuspending 2×10^5 transfected 293T cells in a total volume of 100 μL of 50 mmol/L Hepes pH 7.4, 5 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, and 5% BSA. Iodinated JRFL gp120 and 100 nmol/L sCD4 were added to cells, and incubation was carried out at room temperature for 1 hour. Cells were filtered through Whatman GF/C filters presoaked in 0.2% polyethylenimine (Sigma) and washed twice with 4 mL of 50 mmol/L Hepes pH 7.4, 500 mmol/L NaCl, 5 mmol/L $MgCl_2$, and 1 mmol/L $CaCl_2$. Filters were counted in a Wallac 1470 Wizard gamma counter.

Results

Expression of CCR5 mutants and exporting to cell surface

A schematic representation of the putative transmembrane organization of CCR5, highlighting the natural mutations analyzed in this work, is presented in Figure 1. Plasmids encoding wtCCR5 or the mutated receptors were stably expressed in CHO-K1 cells coexpressing apoaequorin. Cell surface expression of the receptor was assayed by flow cytometry by using 2D7, an anti-CCR5 MAb recognizing a conformational epitope located in the second extracellular loop (ECL2) of the receptor (Figure 1).[33] As shown in Figure 2A, wtCCR5 was highly expressed at the cell surface, with a rightward shift of mean channel fluorescence (MCF) by over 3 logs as compared with untransfected CHO-K1 cells. Many mutants were expressed at levels similar (MCFs ranging from 60% to 160% of wild type) to that of wtCCR5 (Figure 2A and B). However, a few mutants appeared to be poorly expressed at the cell surface, including C20S and C178R, the amino acid substitution that is involved in disulfide bonds linking the N-terminal domain and ECL3, or ECL1 and ECL2, respectively (Figure 1). We have shown

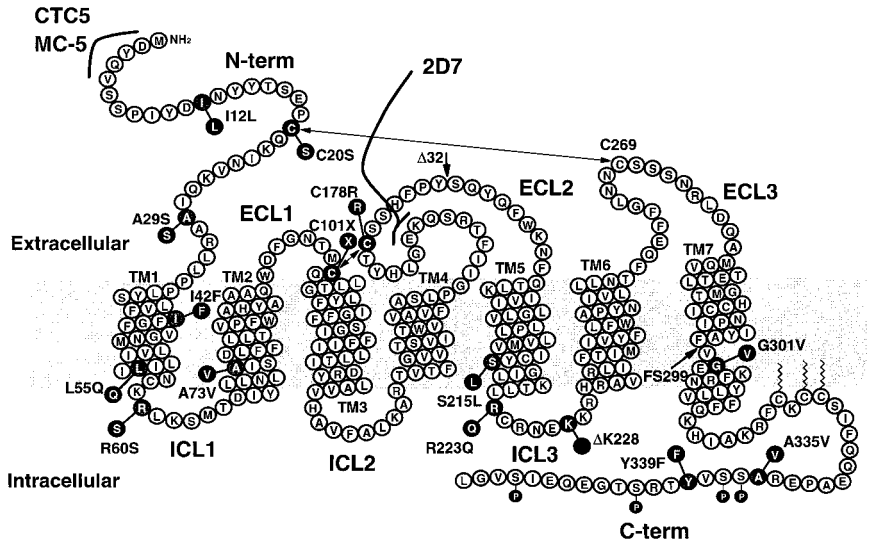

Figure 1. Natural CCR5 mutants. The putative transmembrane organization of CCR5 is represented, as well as the location and the nature of the CCR5 natural mutations (in black) analyzed in this study. Extracellular loops (ECL), intracellular loops (ICL), and transmembrane domains (TM) are numbered. Disulfide bonds linking together CCR5 extracellular domains (C20-C269 and C101-C178) as well as the epitopes recognized by the MAbs 2D7, MC-5, and CTC5 are indicated. The 55 523 MAb recognizes a conformational epitope that includes the N-terminal domain and second ECL.

recently that the two disulfide bonds linking together CCR5 extracellular domains (C20-C269 and C101-C178) contribute to the maintenance of a conformation compatible with the efficient trafficking of the receptor to the cell surface.[31] As previously described, C101X, a mutant characterized by the presence of a stop codon before the third transmembrane domain, and the Δ32 mutant, with a 32-bp deletion in ECL2 resulting in a premature stop codon,[11,34] were not detected at the cell surface. FS299, a mutant with a single base pair deletion causing a frame shift at the end of the seventh transmembrane domain and leading to premature termination and the absence of intracellular C-terminal domain, was also poorly expressed (Figure 2A and B). Relative surface expression levels were found to be similar, following transient expression of the mutant receptors in 293 T cells (data not shown).

To test whether some mutations affected specifically the recognition of the receptor by 2D7, transfected cells were assayed with MAbs directed against other CCR5 regions. MAbs MC-5 and CTC5 recognize linear epitopes in the CCR5 N-terminal domain (Figure 1), whereas MAb 55 523 recognizes a conformational epitope involving several extracellular domains of the receptor.[22] For most mutants, a fairly good correlation was found for the fluorescence obtained with the four MAbs. In general, mutations resulting in low 2D7 staining were also poorly labeled by MC-5, CTC5, and 55 523 (data not shown), suggesting reduced surface expression of the mutants rather than direct alteration or impaired accessibility of the 2D7 epitope. A single exception was the C178R mutant, which was not detected by 2D7, but for which low but significant fluorescence levels were obtained with MC-5 and CTC5 (18% of wtCCR5 MCF for MC-5).

To investigate whether the low surface expression of some mutants (eg, C178R, Δ32, or FS299) was due to a defect in receptor trafficking to the plasma membrane or to other causes, such as impaired synthesis, we analyzed the subcellular distribution of receptor immunoreactivity by confocal microscopy, using the MC-5 MAb. In the absence of cell permeabilization, staining was intense for wtCCR5 and markedly reduced for FS299 and C178R (Figure 2C). In contrast, when cells were permeabilized, strong intracellular staining could be seen in all cases, demonstrating that the mutated receptors were synthesized efficiently, but sequestered intracellularly. Similarly, and as previously described,[35] no extracellular staining was seen for Δ32, although intracellular staining was readily detected (data not shown).

Binding assays and functional response to chemokines

We first measured the specific [^{125}I]-MIP-1β binding for all mutants, using a single concentration of the tracer (0.08 nmol/L), and estimated grossly the affinity by competing with 50 nmol/L MIP-1β (nonspecific binding) or with a concentration of unlabeled MIP-1β (0.5 nmol/L) corresponding to the IC_{50} for wtCCR5. No specific [^{125}I]-MIP-1β binding could be detected for C20S, A29S, C101X, Δ32, C178R, and FS299 (data not shown), and these receptors could not be studied further in MIP-1β binding assays. Specific [^{125}I]-MIP-1β binding was obtained for all other mutants (75% to 90% of the binding to wtCCR5), and 0.5 nmol/L MIP-1β competed for about half of the specific binding, suggesting that the binding affinity of these mutants was similar to that of the wild-type receptor. Competition binding experiments were then conducted, whenever possible, for mutations affecting extracellular domains of the receptor (I12L) and for a set of representative mutations affecting intracellular domains (L55Q, S215L, ΔK228, G301V). All these receptors exhibited similar binding affinities for MIP-1β, with K_i values ranging from 0.3 to 0.7 nmol/L (Figure 3A and Table 1), although total binding capacity varied in accordance with surface expression levels.

Because ligand binding does not necessarily correlate with the ability to activate intracellular cascades, we tested the functional response of all CCR5 mutants by using a reporter system based on the coexpression of the receptor with apoaequorin.[30,31] As shown in Figure 3B, wtCCR5 responded to MIP-1β with an EC_{50} of 2.8 nmol/L, which is similar to that obtained with other functional assays. C20S, C101X, C178R, Δ32, and FS299 did not signal even in response to the highest concentration of MIP-1β tested (500 nmol/L), whereas the same cells generated a strong signal in response to 10 μmol/L ATP (data not shown). A29S, and to a lower extent L55Q, exhibited a significant impairment in their functional response to MIP-1β with EC_{50} values of 200 and 30 nmol/L, respectively. The other mutants (I12L, I42F, R60S, A73V, S215L, R223Q, ΔK228, G301V, A335V, and Y339F) promoted functional responses similar to that of wtCCR5 (Figure 3B and Table 1).

The various agonists of CCR5 likely interact with different extracellular residues of the receptor. Therefore, receptors with mutations in the N-terminal domain (I12L, C20S, A29S), ECLs (C178R), as well as the I42F mutant that affects the outer part of the

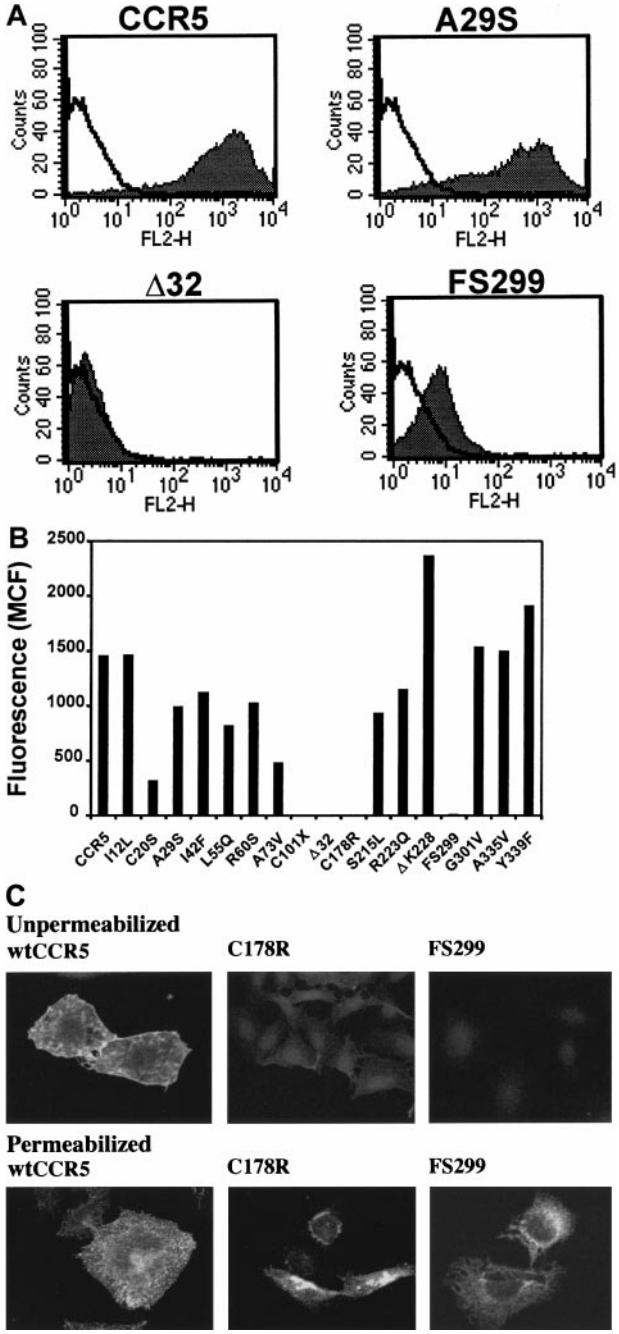

Figure 2. Surface expression and cellular trafficking of CCR5 natural mutants. (A) Cell surface expression of wtCCR5 and the A29S, Δ32, and FS299 mutants as analyzed by FACS using 2D7-PE, a MAb mapping to the second extracellular loop of the receptor. The displayed patterns are representative of the surface expression observed for the various mutants. Staining of untransfected cells with 2D7 was used as a negative control. (B) Mean channel fluorescence (MCF) obtained for all mutants using the 2D7 MAb. A typical experiment out of the two performed independently is represented. (C) Subcellular distribution of wt CCR5, C178R, and FS299 as analyzed by confocal microscopy by using MC-5, a MAb recognizing a linear epitope on the receptor N-terminus. Paraformaldehyde-fixed cells were permeabilized or not with 0.15% Triton X-100 before staining. The figure presented is representative of two experiments with similar results.

first transmembrane segment, were tested for their functional response to other high-affinity ligands of CCR5 (MIP-1α, RANTES, and MCP-2). Wild-type CCR5 was characterized by an EC_{50} of 15 nmol/L for MIP-1α, 2.5 nmol/L for RANTES, and 11.8 nmol/L for MCP-2 (Figure 3C). This order of potency was similar for I12L and I42F (data not shown). Mutants that did not respond to 500 nmol/L MIP-1β (C20S, C101X, C178R, Δ32, and FS299) did not respond to the same concentration of MIP-1α, RANTES, or MCP-2 (data not shown). Interestingly, A29S displayed an atypical order of potency: MCP-2 appeared as the best agonist, with an EC_{50} similar to that on wtCCR5, whereas RANTES and MIP-1β had a potency decreased by about an order of magnitude, and no functional response was detected following addition of 500 nmol/L MIP-1α (Figure 3C). To investigate whether the poor response to MIP-1β, MIP-1α, and RANTES was due exclusively to decreased binding affinities, we performed competition-binding experiments on wtCCR5 and A29S, using [^{125}I]-MCP-2 as tracer. MCP-2, RANTES, MIP-1β, and MIP-1α were characterized by IC_{50} values of 0.24 nmol/L, 0.72 nmol/L, 0.67nmol/L, and 0.93 nmol/L for wtCCR5, in agreement with previously reported data.[5] It should be noted that no significant difference in affinities was observed for any of the competitors depending on the tracer used, in contrast to what has been reported for other receptors.[36] As shown in Figure 3D, the affinities of A29S for the various chemokines were severely affected: The IC_{50} for MCP-2 was unchanged (0.06 nmol/L) as compared with wtCCR5, that for MIP-1β was increased by 2 orders of magnitude (4.3 nmol/L), whereas MIP-1α hardly competed for [^{125}I]-MCP-2 binding at a concentration of 200 nmol/L ($IC_{50} > 200$ nmol/L).

Env binding and HIV-1 infection assays

To determine whether natural CCR5 mutations, other than Δ32, could provide resistance toward HIV-1 infection, we first investigated the ability of 293T cells transfected with wtCCR5 or the natural mutants to bind the Env protein of the R5 HIV-1 strain JRFL, in the presence of an excess of soluble CD4. As shown in Figure 4A, C20S, C101X, C178R, Δ32, and FS299 did not bind [^{125}I]-JRFL gp120. R60S, A73V, and R223Q bound lower amounts of gp120, presumably as a result of their reduced surface expression. The other mutants behaved similarly to wtCCR5. Because moderate reductions in affinity may prevent the detection of gp120 binding capability,[37] coreceptor function was also investigated by an infection assay. GFP reporter viruses pseudotyped with two R5 Envs (ADA and JRFL) were used to infect human 293T cells expressing CD4 and the various CCR5 mutants. As shown in Figure 4B, only C101X and Δ32 were totally resistant to HIV-1 infection, whereas C178R, R223Q, and FS299 showed an important (more than 70%) and significant decrease in coreceptor function. There were no significant differences in coreceptor function for the various mutants when viruses were pseudotyped with ADA Env instead of JRFL (data not shown).

Discussion

The strong resistance to HIV-1 infection that characterizes individuals homozygous for the Δ32 gene variant has highlighted the key role played by CCR5 in HIV-1 pathogenesis and has stimulated the search for other such alleles. The genetic analysis of various populations around the world has, to our knowledge, resulted in the identification of 16 additional natural CCR5 variants. Each of these alleles is usually restricted to a specific population and is relatively rare (1%-5% allele frequency within the affected population; Table 1). We and others have previously studied the relative contribution of the different domains of CCR5 to its receptor and HIV coreceptor functions. These studies have shown that the N-terminal domain of CCR5 is the most important region involved in coreceptor activity, but that the three extracellular loops also

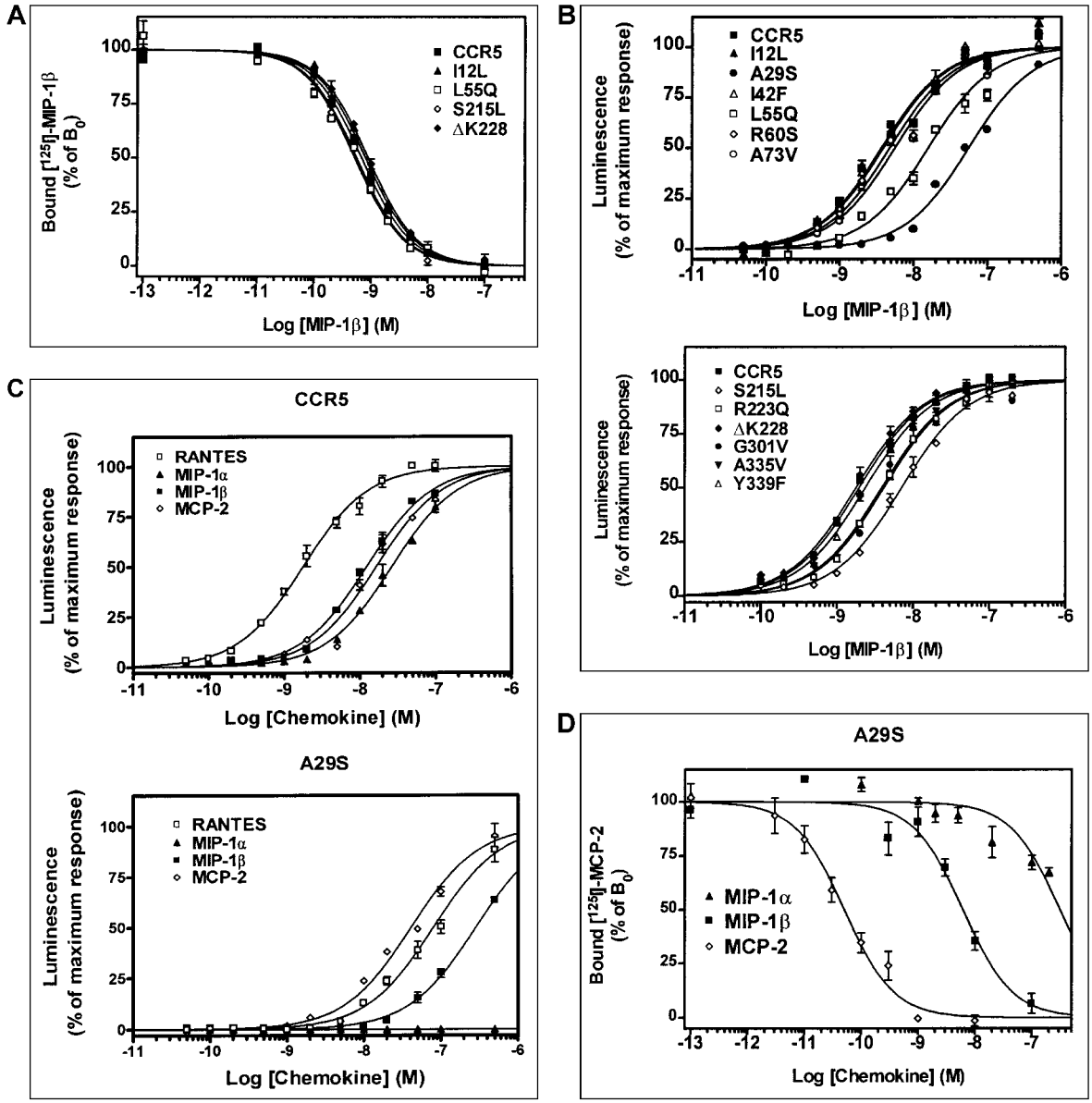

Figure 3. Chemokine binding and functional assays. (A) Competition binding curves were performed on CHO-K1 cell lines expressing wtCCR5 or 12L, L55Q, S215L, and ΔK228 mutants using 0.08 nmol/L [125I]-MIP-1β as tracer. Results were analyzed by the Graphpad Prism software, using a single-site model, and the data were normalized for nonspecific (0%) and specific binding in the absence of competitor (100%). All points were run in triplicate (error bars: SEM). Data are representative of two independent experiments. (B) The functional response of the cell lines coexpressing apoaequorin and CCR5 natural mutants was tested following addition of MIP-1β. The luminescent signal resulting from the activation of the apoaequorin-coelenterazine complex was recorded for 30 seconds in a luminometer. Results were analyzed by nonlinear regression by using the Graphpad Prism software. The data were normalized for basal (0%) and maximal luminescence (100%). All points were run in triplicate (error bars: SEM). The displayed curves represent a typical experiment out of three performed independently. (C) The functional response to MIP-1β, MIP-1α, RANTES, and MCP-2 was tested on CHO-K1 cells expressing wtCCR5 or the A29S mutant using the aequorin assay. All points were run in triplicate (error bars: SEM). The displayed curves represent a typical experiment out of three performed independently. (D) Competition binding assay, using [125I]-MCP-2 as tracer, and MIP-1α, MIP-1β, and MCP-2 as competitors, was performed on CHO-K1 cell lines expressing A29S. The data were analyzed and normalized as in Figure 3A. All points were run in triplicate (error bars: SEM). The displayed curves represent a typical experiment out of two performed independently.

contribute to the interaction with gp120.[38-40] It was also shown that the second extracellular loop is the principal determinant of ligand selectivity,[27] that the disulfide bonds structuring the extracellular domains of CCR5 are required for chemokine binding,[31] and that motifs of charged and aromatic residues in the amino-terminal domain of the receptor are involved in high-affinity binding of both chemokines and gp120.[41-44] We have now analyzed the function of the natural CCR5 mutants described so far in terms of surface expression, chemokine receptor, and HIV coreceptor functions.

Among the mutations affecting extracellular residues, I12L had no effect on receptor and coreceptor activity, in agreement with the results of the substitution by alanine at the same site.[44] C20S, a mutation disrupting the disulfide bond linking the N-terminus to ECL3, resulted in a strong reduction of surface expression of the receptor, and the mutant did not bind chemokines nor functionally responded to them but still functioned as a coreceptor for HIV-1. C178R, another mutation affecting a highly conserved extracellular cysteine involved in the formation of a disulfide bond linking ECL1 and ECL2, resulted in a more severe drop in surface expression, as a consequence of the intracellular trapping of the misfolded receptor. In contrast to its inability to bind and functionally respond to chemokines, this mutant kept part of its coreceptor function,

Table 1. Overview of the expression, binding and functional parameters of the CCR5 mutants*

	Allelic frequency (estimation from available data)	Surface expression (2D7 MCF)	MIP-1β binding PIC_{50} −Log(M) ± SEM	MIP-1β functional response pEC_{50} −Log(M) ± SEM	HIV-1 infection
CCR5		1463	9.12 ± 0.15	8.57 ± 0.15	++
I12L	Afro-Americans: 0.000 Caucasians: 0.003	1469	9.14 ± 0.07	8.18 ± 0.19	++
C20S	Afro-Americans: 0.000 Caucasians: 0.003	318.5	nm	>6	++
A29S	Afro-Americans: 0.015	997	nm	6.70 ± 0.44	++
I42F	Afro-Americans: 0.007 Caucasians: 0.001	1130	nd	8.17 ± 0.33	++
L55Q	Afro-Americans: 0.007 Caucasians: 0.041	823	9.28 ± 0.05	7.53 ± 0.31	++
R60S	Afro-Americans: 0.013	1030	nd	8.07 ± 0.25	++
A73V	Afro-Americans: 0.000 Caucasians: 0.002	481	nd	7.73 ± 0.40	++
C101X	Afro-Americans: 0.014	5	nm	>6	−
C178R	Asians	8	nm	>6	+
Δ32	Caucasians: 0.100 Africans: 0.000	4	nm	>6	−
S215L	Afro-Americans: 0.010	941	9.16 ± 0.06	8.3 ± 0.28	++
R223Q	Caucasians: 0.013	1153	N.D.	8.35 ± 0.07	+
ΔK228	Afro-Americans: 0.000 Caucasians: 0.002	2373	8.99 ± 0.07	8.9 ± 0.28	++
FS299	Asians: 0.035	14.7	nm	>6	+
G301V	Afro-Americans: 0.000 Caucasians: 0.011	1542	8.96 ± 0.05	8.45 ± 0.07	++
A335V	Afro-Americans: 0.025 Caucasians: 0.006	1504	nd	8.55 ± 0.07	++
Y339F	Afro-Americans: 0.026 Caucasians: 0.000	1916	nd	8.6 ± 0.05	++

*The allelic frequencies of each mutant were estimated from the data available in the populations studied so far.[10,25,26] Surface expression of the mutant receptors in transfected CHO-K1 cells is presented as the mean channel fluorescence (MCF) using the 2D7 monoclonal antibody (Mab). The binding (pIC_{50}) and functional (pEC_{50}) parameters are given, as well as the ability of the mutants to support human immunodeficiency virus (HIV) infection. Means and errors (SEM) were calculated from at least two independent experiments. nm, not measurable given the low binding affinity; nd, not determined.

demonstrating that HIV infection may occur even through improperly folded receptors. Alternatively, a small fraction of C178R may fold correctly, giving rise to residual coreceptor activity. The two mutants with a dramatically affected structure (Δ32 and C101X that contain only four and two transmembrane domains, respectively) were not detectable at the cell surface as a result of intracellular trapping of the unfolded receptors. They did not function as chemokine receptors or HIV coreceptors in accordance with previous studies.[10,11,34] A29S was expressed at the cell surface as well as the wtCCR5 but did not bind detectable levels of [^{125}I]-MIP-1β, and its functional response to MIP-1β was strongly impaired. Interestingly, the mutation did not alter MCP-2 binding or the functional response to this chemokine, whereas the response to MIP-1α was totally abolished. This is the first example of a natural mutation affecting a chemokine receptor that results in a modified pharmacological profile of the receptor, with a loss of functional response to specific chemokines, and not to others. These results also suggest that different chemokines interact with different residues on the extracellular domains of the same receptor.

The activation of GPCRs, following the binding of an agonist, is believed to involve relative movements within the transmembrane

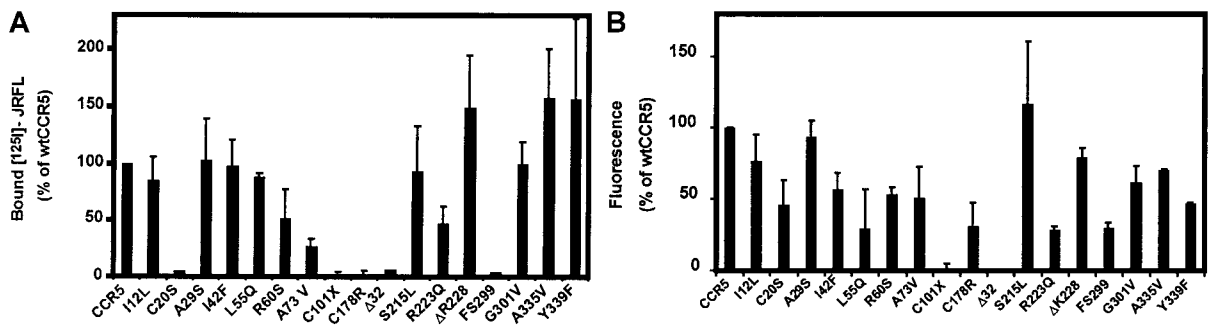

Figure 4. gp120 binding and HIV-1 coreceptor function of CCR5 natural mutants. (A) 293T cells transfected with wtCCR5 or mutants were tested for their ability to bind [^{125}I]-gp120 from the M-tropic HIV-1 strain JRFL in the presence of soluble CD4 (100 nmol/L). The data were normalized for the specific binding on wtCCR5 (100%) after deduction of nonspecific binding (bound [^{125}I]-gp120 on cells transfected with the pcDNA3 vector alone). Results represent the mean and SEM of four experiments performed separately. (B) The ability of GFP reporter viruses pseudotyped with R5 HIV-1 Envs (JRFL) to infect 293T cells expressing CD4 and CCR5 natural mutants was assayed. The data were normalized to the luminescence obtained with wtCCR5 (100%) and represent the mean and SEM of two independent experiments.

helix bundle, unmasking intracellular sites that mediate heterotrimeric G protein activation. Among the natural mutations studied, five affected transmembrane domains (I42F, L55Q, A73V, S215L, and FS299), three concerned the C-terminal tail (G301V, A335V, and Y339F). L55Q, a mutant in which an hydrophobic residue highly conserved among chemokine receptors is substituted by a polar residue in the first transmembrane domain, was characterized by a moderate decrease in its functional response to MIP-1β, even though its binding affinity for MIP-1β was not affected. This finding suggested a partial defect in the activation of this receptor mutant. R223Q, affecting another residue conserved across chemokine receptors, exhibited a significant decrease in its coreceptor function even though it could bind [^{125}I]-gp120, suggesting that changes in intracellular loops may influence HIV-1 coreceptor function. FS299 contained a frame shift, resulting in a modified end of the seventh transmembrane domain and the absence of intracellular C-terminal tail. It was poorly expressed at the cell surface, as a result of intracellular trapping, did not bind detectable levels of chemokines nor functionally responded to them, but could still function as an HIV-1 coreceptor, although with a lower efficiency than the wild-type receptor. The other natural mutations affecting transmembrane and intracellular domains did not alter notably CCR5 receptor or coreceptor functions.

The functional analysis of some of the mutants studied here was reported recently by others,[45] and our observations differ from the results of that study. No reduction of the expression level was observed in our hands for I12L, A29S, I42F, or L55Q. No functional impairment could be found for I12L, none of the mutants exhibited increased affinities for chemokines, and the functional deficit of A29S was restricted, in our study, to some of the CCR5 ligands only.

Among the natural mutants that have been analyzed in this study, it appears that nonfunctional CCR5 alleles are among the most frequent variants in the populations in which they were described. Δ32 is by far the most frequent natural mutant in Europe, with an average allele frequency of 10%. FS299 is the most frequent mutant allele in Asia (3%-4%), whereas the nonfunctional C101X mutant and the partially impaired A29S variant are among the most frequent mutant CCR5 alleles found in central Africa. The high frequencies observed for nonfunctional CCR5 alleles in diverse human populations, as compared with functional CCR5 variants, might suggest that a selective advantage has been associated with the partial loss of CCR5 function. In the case of the Δ32 mutant, we and others have collected strong genetic elements suggesting a selective advantage for carriers of this allele, although the nature of the selective factor is presently unknown.[24,46] Because of its recent origin, the HIV-1 epidemic was certainly not the driving force of this selection. CCR5 has been involved in the development of a range of human inflammatory diseases, including multiple sclerosis and rheumatoid arthritis,[16-18] and an impaired CCR5 function might provide some protection to otherwise genetically or environmentally predisposed individuals. Indeed, it has been shown recently that the CCR5Δ32 allele was associated with a lower risk of recurrent disease activity in patients with multiple sclerosis and with a reduced risk of developing asthma.[47,48] It remains to be determined whether nonfunctional CCR5 variants, other than Δ32, also have a relatively recent origin in their respective populations and whether a selective advantage has to be taken into account as well. It should be stressed that variants of the CCR5 gene promoter,[49,50] presumably resulting in lower CCR5 expression, have been described and that these alleles might have been selected as well.

A number of sometimes conflicting studies has demonstrated the dual role of chemokines and chemokine-receptor stimulation in the frame of HIV-1 pathogenesis.[20,51] In most instances, the chemokines acting on established coreceptors were found to be protective in vitro and ex vivo.[20,52,53] Nevertheless, it was also conclusively demonstrated that signaling through chemokine receptors (acting or not as coreceptors for the infecting virus) may enhance dramatically viral replication in other conditions.[52,54,55] Signaling through HIV coreceptors following gp120 binding was also demonstrated,[56-58] although the functional relevance of this signaling for the virus life cycle is presently unknown. It is, therefore, not clear whether mutations affecting the functional response of CCR5 to chemokines, while maintaining its HIV-1 coreceptor activity, might also affect HIV-1 disease progression.

A number of mutants analyzed in this study were not found to display significant alterations of their receptor or coreceptor properties. It remains possible that some of these mutations might promote subtle modifications of receptor properties that were not detected in our assays. Minor alterations of receptor function would, however, not provide a significant selective advantage to heterozygotes, as it is believed to happen for the Δ32 allele. If the frequency of nonsynonymous mutations is indeed higher than expected as compared with synonymous mutations,[25,26] it remains to be determined what advantages may be associated with variants that have kept normal (or near normal) receptor and coreceptor functions.

Acknowledgments

Expert technical assistance was provided by M. J. Simons. We thank Mathias Mack for kindly providing monoclonal antibodies.

References

1. Littman DR. Chemokine receptors: keys to AIDS pathogenesis? Cell. 1998;93:677-680.
2. Samson M, Labbe O, Mollereau C, Vassart G, Parmentier M. Molecular cloning and functional expression of a new human CC-chemokine receptor gene. Biochemistry. 1996;35:3362-3367.
3. Gong W, Howard OM, Turpin JA, et al. Monocyte chemotactic protein-2 activates CCR5 and blocks CD4/CCR5-mediated HIV-1 entry/replication. J Biol Chem. 1998;273:4289-4292.
4. Ruffing N, Sullivan N, Sharmeen L, Sodroski J, Wu L. CCR5 has an expanded ligand-binding repertoire and is the primary receptor used by MCP-2 on activated T cells. Cell Immunol. 1998; 189:160-168.
5. Blanpain C, Migeotte I, Lee B, et al. CCR5 binds multiple CC-chemokines: MCP-3 acts as a natural antagonist. Blood. 1999;94:1899-1905.
6. Wu L, Paxton WA, Kassam N, et al. CCR5 levels and expression pattern correlate with infectability by macrophage-tropic HIV-1, in vitro. J Exp Med. 1997;185:1681-1691.
7. Lee B, Sharron M, Montaner LJ, Weissman D, Doms RW. Quantification of CD4, CCR5, and CXCR4 levels on lymphocyte subsets, dendritic cells, and differentially conditioned monocyte-derived macrophages. Proc Natl Acad Sci U S A. 1999;96:5215-5220.
8. Lee B, Ratajczak J, Doms RW, Gewirtz AM, Ratajczak MZ. Coreceptor/chemokine receptor expression on human hematopoietic cells: biological implications for human immunodeficiency virus-type 1 infection. Blood. 1999;93:1145-1156.
9. He J, Chen Y, Farzan M, et al. CCR3 and CCR5 are co-receptors for HIV-1 infection of microglia. Nature. 1997;385:645-649.
10. Samson M, Libert F, Doranz BJ, et al. Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. Nature. 1996;382:722-725.
11. Liu R, Paxton WA, Choe S, et al. Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. Cell. 1996;86:367-377.
12. Dean M, Carrington M, Winkler C, et al. Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural

gene. Hemophilia Growth and Development Study, Multicenter AIDS Cohort Study, Multicenter Hemophilia Cohort Study, San Francisco City Cohort, ALIVE Study. Science. 1996;273:1856-1862.

13. Smith MW, Dean M, Carrington M, et al. Contrasting genetic influence of CCR2 and CCR5 variants on HIV-1 infection and disease progression. Hemophilia Growth and Development Study (HGDS), Multicenter AIDS Cohort Study (MACS), Multicenter Hemophilia Cohort Study (MHCS), San Francisco City Cohort (SFCC), ALIVE Study. Science. 1997;277:959-965.
14. Michael NL, Louie LG, Rohrbaugh AL, et al. The role of CCR5 and CCR2 polymorphisms in HIV-1 transmission and disease progression. Nat Med. 1997;3:1160-1162.
15. Zimmerman PA, Buckler-White A, Alkhatib G, et al. Inherited resistance to HIV-1 conferred by an inactivating mutation in CC chemokine receptor 5: studies in populations with contrasting clinical phenotypes, defined racial background, and quantified risk. Mol Med. 1997;3:23-36.
16. Garred P, Madsen HO, Petersen J, et al. CC chemokine receptor 5 polymorphism in rheumatoid arthritis. J Rheumatol. 1998;25:1462-1465.
17. Qin S, Rottman JB, Myers P, et al. The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions. J Clin Invest. 1998;101:746-754.
18. Balashov KE, Rottman JB, Weiner HL, Hancock WW. CCR5(+) and CXCR3(+) T cells are increased in multiple sclerosis and their ligands MIP-1alpha and IP-10 are expressed in demyelinating brain lesions. Proc Natl Acad Sci U S A. 1999;96:6873-6878.
19. Mack M, Bruhl H, Gruber R, et al. Predominance of mononuclear cells expressing the chemokine receptor CCR5 in synovial effusions of patients with different forms of arthritis. Arthritis Rheum. 1999;42:981-988.
20. Cocchi F, DeVico AL, Garzino-Demo A, et al. Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells. Science. 1995;270:1811-1815.
21. Simmons G, Clapham PR, Picard L, et al. Potent inhibition of HIV-1 infectivity in macrophages and lymphocytes by a novel CCR5 antagonist. Science. 1997;276:276-279.
22. Lee B, Sharron M, Blanpain C, et al. Epitope mapping of CCR5 reveals multiple conformational states and distinct but overlapping structures involved in chemokine and coreceptor function. J Biol Chem. 1999;274:9617-9626.
23. Baba M, Nishimura O, Kanzaki N, et al. A small-molecule, nonpeptide CCR5 antagonist with highly potent and selective anti-HIV-1 activity. Proc Natl Acad Sci U S A. 1999;96:5698-5703.
24. Libert F, Cochaux P, Beckman G, et al. The deltaccr5 mutation conferring protection against HIV-1 in Caucasian populations has a single and recent origin in Northeastern Europe. Hum Mol Genet. 1998;7:399-406.
25. Ansari-Lari MA, Liu XM, Metzker ML, Rut AR, Gibbs RA. The extent of genetic variation in the CCR5 gene. Nat Genet. 1997;16:221-222.
26. Carrington M, Kissner T, Gerrard B, et al. Novel alleles of the chemokine-receptor gene CCR5. Am J Hum Genet. 1997;61:1261-1267.
27. Samson M, LaRosa G, Libert F, et al. The second extracellular loop of CCR5 is the major determinant of ligand specificity. J Biol Chem. 1997;272:24934-24941.
28. Rizzuto R, Pinton P, Carrington W, et al. Close contacts with the endoplasmic reticulum as determinants of mitochondrial Ca2+ responses. Science. 1998;280:1763-1766.
29. Takebe Y, Seiki M, Fujisawa J, et al. SR alpha promoter: an efficient and versatile mammalian cDNA expression system composed of the simian virus 40 early promoter and the R-U5 segment of human T-cell leukemia virus type 1 long terminal repeat. Mol Cell Biol. 1988;8:466-472.
30. Stables J, Green A, Marshall F, et al. A bioluminescent assay for agonist activity at potentially any G-protein-coupled receptor. Anal Biochem. 1997;252:115-126.
31. Blanpain C, Lee B, Vakili J, et al. Extracellular cysteines of CCR5 are required for chemokine binding, but dispensable for HIV-1 coreceptor activity. J Biol Chem. 1999;274:18902-18908.
32. Connor RI, Chen BK, Choe S, Landau NR. Vpr is required for efficient replication of human immunodeficiency virus type-1 in mononuclear phagocytes. Virology. 1995;206:935-944.
33. Wu L, LaRosa G, Kassam N, et al. Interaction of chemokine receptor CCR5 with its ligands: multiple domains for HIV-1 gp120 binding and a single domain for chemokine binding. J Exp Med. 1997;186:1373-1381.
34. Quillent C, Oberlin E, Braun J, et al. HIV-1-resistance phenotype conferred by combination of two separate inherited mutations of CCR5 gene. Lancet. 1998;351:14-18.
35. Rana S, Besson G, Cook DG, et al. Role of CCR5 in infection of primary macrophages and lymphocytes by macrophage-tropic strains of human immunodeficiency virus: resistance to patient-derived and prototype isolates resulting from the delta ccr5 mutation. J Virol. 1997;71:3219-3227.
36. Maggi CA, Schwartz TW. The dual nature of the tachykinin NK1 receptor. Trends Pharmacol Sci. 1997;18:351-355.
37. Baik SS, Doms RW, Doranz BJ. HIV and SIV gp120 binding does not predict coreceptor function. Virology. 1999;259:267-273.
38. Rucker J, Samson M, Doranz BJ, et al. Regions in beta-chemokine receptors CCR5 and CCR2b that determine HIV-1 cofactor specificity. Cell. 1996;87:437-446.
39. Atchison RE, Gosling J, Monteclaro FS, et al. Multiple extracellular elements of CCR5 and HIV-1 entry: dissociation from response to chemokines. Science. 1996;274:1924-1926.
40. Bieniasz PD, Fridell RA, Aramori I, et al. HIV-1-induced cell fusion is mediated by multiple regions within both the viral envelope and the CCR-5 co-receptor. EMBO J. 1997;16:2599-2609.
41. Farzan M, Choe H, Vaca L, et al. A tyrosine-rich region in the N terminus of CCR5 is important for human immunodeficiency virus type 1 entry and mediates an association between gp120 and CCR5. J Virol. 1998;72:1160-1164.
42. Dragic T, Trkola A, Lin SW, et al. Amino-terminal substitutions in the CCR5 coreceptor impair gp120 binding and human immunodeficiency virus type 1 entry. J Virol. 1998;72:279-285.
43. Rabut GE, Konner JA, Kajumo F, Moore JP, Dragic T. Alanine substitutions of polar and nonpolar residues in the amino-terminal domain of CCR5 differently impair entry of macrophage- and dualtropic isolates of human immunodeficiency virus type 1. J Virol. 1998;72:3464-3468.
44. Blanpain C, Doranz BJ, Vakili J, et al. Multiple charged and aromatic residues in CCR5 amino-terminal domain are involved in high affinity binding of both chemokines and HIV-1 env protein. J Biol Chem. 1999;274:34719-34727.
45. Howard OM, Shirakawa AK, Turpin JA, et al. Naturally occurring CCR5 extracellular and transmembrane domain variants affect HIV-1 Co-receptor and ligand binding function. J Biol Chem. 1999;274:16228-16234.
46. Stephens JC, Reich DE, Goldstein DB, et al. Dating the origin of the CCR5-Delta32 AIDS-resistance allele by the coalescence of haplotypes. Am J Hum Genet. 1998;62:1507-1515.
47. Sellebjerg F, Madsen HO, Jensen CV, Jensen J, Garred P. CCR5 delta32, matrix metalloproteinase-9 and disease activity in multiple sclerosis. J Neuroimmunol. 2000;102:98-106.
48. Hall IP, Wheatley A, Christie G, et al. Association of CCR5 delta32 with reduced risk of asthma. Lancet. 1999;354:1264-1265.
49. Martin MP, Dean M, Smith MW, et al. Genetic acceleration of AIDS progression by a promoter variant of CCR5. Science. 1998;282:1907-1911.
50. McDermott DH, Zimmerman PA, Guignard F, et al. CCR5 promoter polymorphism and HIV-1 disease progression. Multicenter AIDS Cohort Study (MACS). Lancet. 1998;352:866-870.
51. Schmidtmayerova H, Sherry B, Bukrinsky M. Chemokines and HIV replication. Nature. 1996;382:767.
52. Trkola A, Paxton WA, Monard SP, et al. Genetic subtype-independent inhibition of human immunodeficiency virus type 1 replication by CC and CXC chemokines. J Virol. 1998;72:396-404.
53. Margolis LB, Glushakova S, Grivel JC, Murphy PM. Blockade of CC chemokine receptor 5 (CCR5)-tropic human immunodeficiency virus-1 replication in human lymphoid tissue by CC chemokines. J Clin Invest. 1998;101:1876-1880.
54. Kinter A, Catanzaro A, Monaco J, et al. CC-chemokines enhance the replication of T-tropic strains of HIV-1 in CD4(+) T cells: role of signal transduction. Proc Natl Acad Sci U S A. 1998;95:11880-11885.
55. Trkola A, Gordon C, Matthews J, et al. The CC-chemokine RANTES increases the attachment of human immunodeficiency virus type 1 to target cells via glycosaminoglycans and also activates a signal transduction pathway that enhances viral infectivity. J Virol. 1999;73:6370-6379.
56. Davis CB, Dikic I, Unutmaz D, et al. Signal transduction due to HIV-1 envelope interactions with chemokine receptors CXCR4 or CCR5. J Exp Med. 1997;186:1793-1798.
57. Weissman D, Rabin RL, Arthos J, et al. Macrophage-tropic HIV and SIV envelope proteins induce a signal through the CCR5 chemokine receptor. Nature. 1997;389:981-985.
58. Herbein G, Mahlknecht U, Batliwalla F, et al. Apoptosis of CD8+ T cells is mediated by macrophages through interaction of HIV gp120 with chemokine receptor CXCR4. Nature. 1998;395:189-194.