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# Extracellular Cysteines of CCR5 Are Required for Chemokine Binding, but Dispensable for HIV-1 Coreceptor Activity*

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**CCR5 is the major coreceptor for macrophage-tropic human immunodeficiency virus type I (HIV-1). For most G-protein-coupled receptors that have been tested so far, the disulfide bonds linking together the extracellular loops (ECL) are required for maintaining the structural integrity necessary for ligand binding and receptor activation. A natural mutation affecting $Cys^{20}$, which is thought to form a disulfide bond with $Cys^{269}$, has been described in various human populations, although the consequences of this mutation for CCR5 function are not known. Using site-directed mutagenesis, we mutated the four extracellular cysteines of CCR5 singly or in combination to investigate their role in maintaining the structural conformation of the receptor, its ligand binding and signal transduction properties, and its ability to function as a viral coreceptor. Alanine substitution of any single Cys residue reduced surface expression levels by 40–70%. However, mutation of $Cys^{101}$ or $Cys^{178}$, predicted to link ECL1 and ECL2 of the receptor, abolished recognition of CCR5 by a panel of conformation sensitive anti-CCR5 antibodies. The effects of the mutations on receptor expression and conformation were partially temperature-sensitive, with partial restoration of receptor expression and conformation achieved by incubating cells at 32 °C. All cysteine mutants were unable to bind detectable levels of MIP-1β, and did not respond functionally to CCR5 agonists. Surprisingly, all cysteine mutants did support infection by R5 strains of HIV, though at reduced levels. These results indicate that both disulfide bonds of CCR5 are necessary for maintaining the structural integrity of the receptor necessary for ligand binding and signaling. Env binding and the mechanisms of HIV entry appear much less sensitive to alterations of CCR5 conformation.**

The entry of human immunodeficiency virus type-1 (HIV-1)[1] into its target cells requires the interaction of the envelope glycoprotein (gp120) with CD4 and a coreceptor belonging to the rhodopsin-like G-protein-coupled receptor (GPCR) family. CCR5, a chemokine receptor for MIP-1α, MIP-1β, MCP-2, and RANTES (1) plays a key role in HIV-1 transmission and pathogenesis. It is used as the main coreceptor by macrophage-tropic HIV-1 strains and primary isolates that are responsible for viral transmission and which predominate during the asymptomatic phase of the disease (2, 3). The central role of CCR5 in HIV pathogenesis was demonstrated by the occurrence of a deletion mutant of CCR5 (CCR5Δ32 or Δ*ccr5*), frequent in populations of European origin, that confers a strong, although incomplete, protection to homozygotes (4–7). CXCR4, the receptor for the CXC chemokine SDF-1 (8, 9), acts as a coreceptor for T-cell line-adapted strains of HIV-1 and T-cell tropic strains that appear during the late stages of the disease. Other GPCRs including the chemokine receptors CCR8, CCR2, CCR3, and $CX_3CR1$, and the orphan receptors BOB/GPR15, Bonzo/STRL33, GPR1, APJ, and ChemR23 have been shown to function as co-receptors *in vitro* for subsets of HIV-1, HIV-2, or simian immunodeficiency virus strains, but their role *in vivo* remains to be clarified (10–18). Structure-function studies performed by various groups have shown the involvement of all extracellular domains of CCR5 in its HIV coreceptor function, particularly the N-terminal domain and second extracellular loop (19–23). In addition, the second extracellular loop of CCR5 is responsible for the binding selectivity of the receptor, and determines the range of chemokines to which it responds functionally (24). Detailed antigenic mapping of CCR5 also indicates that CCR5 is structurally complex, with the reactivity of many antibodies being dependent upon multiple CCR5 domains (25).

Disulfide bonds linking extracellular domains of GPCRs are thought to be important for maintaining the conformational integrity of the receptor, and in particular for allowing ligand access to the binding pocket (26). Within the rhodopsin family of GPCRs, two cysteines are almost invariably present that form a disulfide bond linking the first and second extracellular loops (26–29). Mutation of these cysteines in the $\beta_2$-adrenergic, muscarinic M1, and thyrotropin-releasing hormone receptors results in a dramatic impairment of receptor function (27, 28, 30). A few rhodopsin-like G protein-coupled receptors, such as

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[1] The abbreviations used are: HIV, human immunodeficiency virus; mAb, monoclonal antibody; GPCR, G-protein-coupled receptor; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; FACS, fluorescence-activated cell sorting; BSA, bovine serum albumin; MCF, mean channel fluorescence; wt, wild-type; DTT, dithiothreitol.


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the $CB_1$ cannabinoid receptor and the *mas* oncogene do not share this conserved disulfide bond (31, 32). In addition to this conserved disulfide bridge, the various chemokine receptors have in common two additional cysteines located in the N-terminal domain and the third extracellular loop. These two cysteines are thought to form a second disulfide bond (33), which is expected to provide an additional structural constraint that contributes to the stability and conformation of the receptor. Indeed, all GPCRs that have been demonstrated to interact with chemokines contain these additional Cys residues, suggesting that the structure imposed by the additional bond is essential for the interaction of the receptors with the common core structure of their ligands.

A natural variant of CCR5 has been described in which the N-terminal domain $Cys^{20}$ is replaced by Ser (34). Although the consequences of this mutation on receptor function was not described, this allele was found in two seropositive non-progressors, suggesting that the mutant receptor could play a role similar to that of the prevalent CCR5 mutant allele CCR5Δ32. In order to address the role of the disulfide bonds of CCR5 for its receptor and coreceptor functions and to investigate the consequences of the $Cys^{20}$ polymorphism, we substituted alanine residues for the four extracellular cysteines, individually or in combination. The various mutants were tested for their ability to bind chemokines, to respond functionally to agonists and to allow infection by macrophage-tropic HIV-1 strains. The mutants were also tested by flow cytometry, using a panel of well defined monoclonal antibodies to determine surface expression and/or modifications of receptor conformation. We show that alanine substitution of any of the four conserved cysteines resulted in a moderate decrease of CCR5 expression level, but abolished completely chemokine binding and functional response to chemokines. The disulfide bond bridging extracellular loops 1 and 2 was found to be necessary for maintaining the complex conformational structure of the extracellular domains. Finally, none of the disulfide bonds was an absolute requirement for the coreceptor function of CCR5.

## EXPERIMENTAL PROCEDURES

*Chemokines*—Recombinant MIP-1β was obtained from R&D Systems. It was shown locally to be active at adequate concentrations on wild-type CCR5 expressed in CHO-K1 cells. The lyophilized chemokine was dissolved as a 10 $\mu$M solution in sterile phosphate-buffered saline (PBS) and stored at −20 °C in aliquots. It was diluted to the working concentration immediately before use. $^{125}$I-MIP-1β (specific activity, 2200 Ci/mmol) was obtained from NEN Life Science Products.

*Generation of Mutant Constructs*—A plasmid containing the coding region of the CCR5 gene (1) in the pcDNA3 vector (Stratagene) was used as a template for site directed mutagenesis using the Quick-Change kit (Stratagene) as described by the manufacturer. After sequencing, the mutated cassettes were transferred into pcDNA3-CCR5. Double and quadruple mutants were generated by further transfer of mutated cassettes. The mutant CCR5 genes were also transferred into a bicistronic vector (pEFIN3) as described previously (24). All final constructs were verified by sequencing.

*Cell Culture*—293T cells were maintained in DMEM (Life Technologies) supplemented with 10% fetal calf serum (HyClone), 100 units/ml penicillin, 100 $\mu$g/ml streptomycin, and 2 mM glutamine. CHO-K1 cells were cultured using Ham's F-12 medium supplemented with 10% fetal calf serum (Life Technologies, Inc.), 100 units/ml penicillin, and 100 $\mu$g/ml streptomycin (Life Technologies, Inc.).

*FACS Analysis*—The 2D7 and the 3A9 monoclonal antibodies (35, 36) were kindly provided by Charles Mackay (Leukocyte). The monoclonal antibodies CTC5 and CTC8 were received from Protein Design Labs (25). The monoclonal antibodies 501, 531, and 549 were donated by R&D Systems (25). 293T cells were transfected with wild-type CCR5 or the various cysteine mutants in the pcDNA3 vector using the calcium phosphate method (37). 18 h following transfection, the cells were suspended in PBS containing 2 mM EDTA and washed with PBS. The cells were incubated in FACS staining buffer (PBS supplemented with 2.5% calf serum, 0.5% BSA, and 0.02% sodium azide), containing 10 $\mu$g/ml monoclonal antibody, followed by a phycoerythrin-conjugated horse anti-mouse secondary antibody (Vector Laboratories) at a 1/100 dilution. The concentration used for the monoclonal antibodies was at least 2-fold the saturating concentration. FACS analysis was performed on a FACScan flow cytometer using the CellQuest software (Becton Dickinson). The mean channel fluorescence (MCF) was used to compare the levels of receptor expression at the cell surface. Results were normalized for the MCF obtained for a particular antibody against wild-type (wt) CCR5 (normalized as 100%) after subtraction of the background MCF obtained against pcDNA3 transfected cells (normalized as 0%).

*Expression of Mutant Receptors in CHO-K1 Cells*—A plasmid encoding apoaequorin and $G\alpha_{16}$ under control of the SRα promoter (38) was transfected into CHO-K1 cells, using Fusgene 6 (Roche Molecular Biochemicals). Zeocin (250 $\mu$g/ml, Invitrogen) selection of transfectants was initiated 2 days after transfection, and 3 weeks later, stably transfected cell lines were cloned by limit dilution. The best responding clone was selected on the basis of the functional responses to ionomycin A (100 nM) and ATP (10 $\mu$M). Constructs encoding wild-type CCR5 and alanine substitution mutants were transfected using Fusgene 6 in this apoaequorin expressing cell line. Selection of stably transfected cells was made for 14 days with 400 $\mu$g/ml G418 (Life Technologies), and the population of mixed cell clones expressing each of the constructs was used for binding and functional studies. The level of receptor expression was measured by flow cytometry using antibodies directed against the N terminus (3A9) and second extracellular loop (2D7) of CCR5.

*Binding Assays*—Transfected CHO-K1 cells stably expressing wild-type or mutant CCR5 were collected from plates with $Ca^{2+}/Mg^{2+}$-free PBS 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% BSA). Competition binding assays were performed in Minisorb tubes (Nunc), using 0.24 mM $^{125}$I-MIP-1β (2200 Ci/mmol, NEN Life Science Products) 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, then bound tracer was separated by filtration through GF/B filters presoaked in 1% BSA. Filters were counted in a β-scintillation counter. Binding parameters were determined with PRISM software (Graph-Pad software) using nonlinear regression applied to a one-site competition model. Dithiothreitol (DTT) treatment on wtCCR5 was carried out with 100 nM at 37° for 1 h. Cells were than washed three times, and binding experiments were performed as described above.

*Functional Assay*—Functional response to chemokines was analyzed by measuring the luminescence of aequorin as described (39). The stably transfected cell lines were collected from plates with $Ca^{2+}/Mg^{2+}$-free DMEM supplemented with 5 mM EDTA, pelleted for 2 min at $1000 \times g$, resuspended in DMEM at a density of $5 \times 10^6$ cells/ml and incubated for 2 h in the dark in the presence of coelenterazine H (Molecular Probes) at a final concentration of 5 $\mu$M. Cells were diluted 7.5-fold before use. Agonists in a volume of 50 $\mu$l were added to 50 $\mu$l of cell suspension (33,000 cells), and luminescence was measured for 30 s in a Packard luminometer.

*Infection Assays*—Plasmids encoding the HIV-1 ADA and BaL Env. were provided by John Moore (Aaron Diamond AIDS Research Center, New York, NY). The NL4–3 luciferase virus backbone (pNL-Luc-$E^-$ $R^-$) was provided by Ned Landau (Aaron Diamond AIDS Research Center). Luciferase reporter viruses were prepared as described previously by cotransfecting 293T cells with the indicated Env and the NL4–3 luciferase virus backbone (40). Target cells were prepared by co-transfecting 293T cells with CD4 and a constant amount of appropriate coreceptor cloned in pcDNA3 vector. Incubation was done at 37 or 32 °C as indicated. Four days after infection, cells were lysed with 0.5% Triton X-100 in PBS, and an appropriate aliquot was analyzed for luciferase activity.

## RESULTS

*Disulfide Bonds Linking CCR5 Extracellular Domains Are Not Necessary for Cell Surface Expression of the Receptor but Contribute to Efficient Transport*—The four extracellular cysteines of CCR5 believed to be involved in the formation of disulfide bonds were mutated to alanine, individually or in combination. A schematic representation of the CCR5 extracellular domains highlighting the position of the four cysteines and the location of the two putative bonds is presented in Fig. 1. The disulfide bond linking $Cys^{101}$ and $Cys^{178}$ is common to most G protein-coupled receptors, while the bond linking $Cys^{20}$

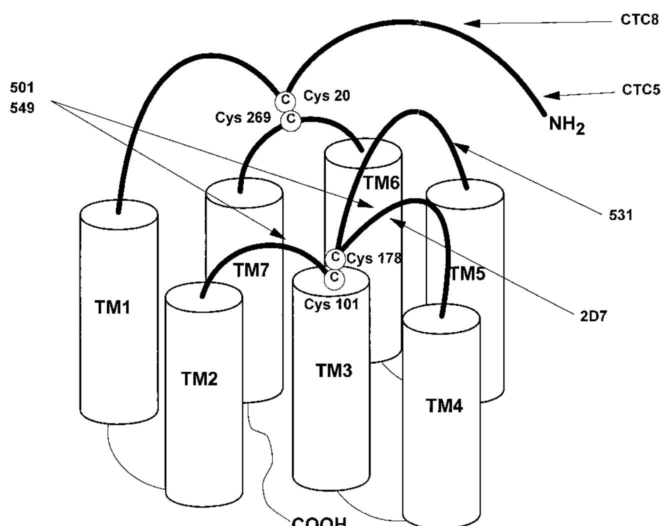


FIG. 1. **Schematic representation of CCR5.** The extracellular domains of CCR5 are indicated, and the position of the four extracellular cysteines and the two putative disulfide bonds are highlighted, as well as the location of the epitopes recognized by the various mAbs.

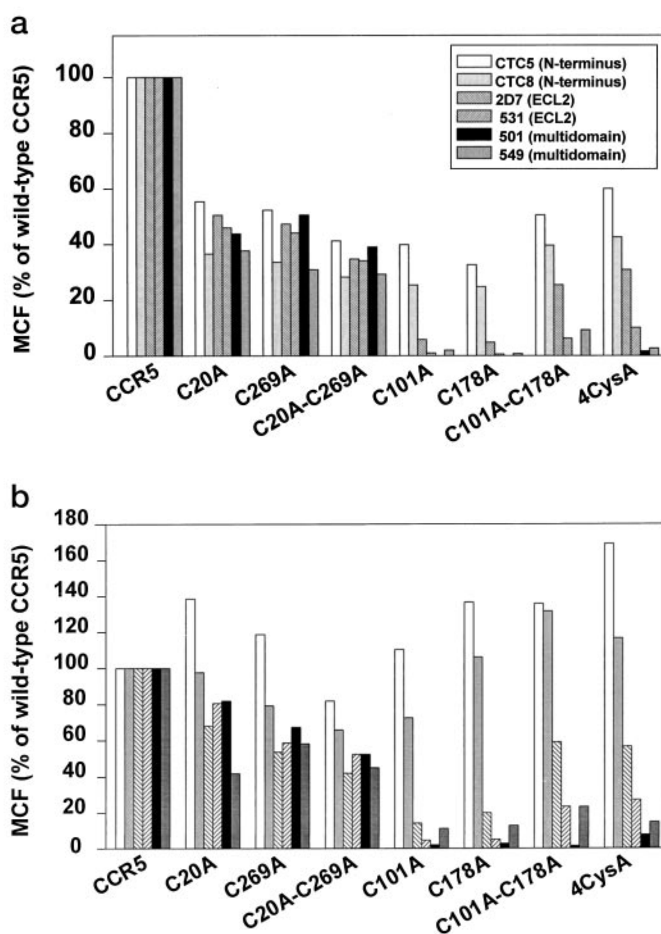


FIG. 2. *a*, FACS analysis of CCR5 mutants using different anti-CCR5 mAbs. 293T cells were transfected with wild-type CCR5 or mutant receptors, kept for 18 h at 37 °C, and stained with six mAbs directed against different domains of CCR5. CTC5 and CTC8 recognize well defined linear epitopes at the N terminus of the receptor. 2D7 and 531 recognize epitopes of the second extracellular loop, located, respectively, before and after Cys[178]. mAbs 501 and 549 recognize multidomain epitopes involving at least ECL1 and ECL2. Results have been normalized against the MCF values obtained with each antibody for wild-type CCR5 (set at 100%) after subtraction of background fluorescence (MCF value obtained for pcDNA3 transfected cells). The results shown are representative of two to three independent experiments (depending on the antibodies) that provided similar results (less than 10% variation between experiments). *b*, influence of temperature on CCR5 mutant conformation. Following transfection, the cells were incubated for 18 h at 32 °C instead of 37 °C. FACS analysis was performed with the same panel of mAbs as that used in Fig. 1. Results were normalized as for Fig. 1. Mutation of Cys[101] and Cys[178], or their combination, strongly impaired the recognition by multi-domain mAbs 501 and 549 without altering the surface expression of the mutants.

and Cys[269] is specific to chemokine receptors and is not found outside this subfamily. Plasmids encoding the mutant receptors were transfected into 293T cells and cell surface expression evaluated by FACS using two monoclonal antibodies (mAbs) directed against linear epitopes located within the N-terminal domain of CCR5 (CTC5 and CTC8, Figs. 1 and 2*a*). CTC5 recognizes the very distal part of CCR5 N terminus, including Asp[2]; CTC8 reacts with a more proximal region involving Tyr[10] to Asn[13] (25). The location of these epitopes coupled with the fact that these mAbs recognize fully denatured and reduced CCR5 by Western blot argues that binding of these mAbs to CCR5 is unlikely to be affected by the Cys mutations, either directly or indirectly through altered receptor conformation. Using these two mAbs, we found that surface expression of the CCR5 mutants was consistently reduced by 40–70%, demonstrating that the disulfide bonds are dispensable for surface expression but nevertheless contribute to the generation of a receptor conformation compatible with efficient trafficking to the cell surface. Simultaneous substitution of the two cysteines involved in a putative disulfide bond (*e.g.* Cys[101] and Cys[178] or Cys[20] and Cys[269]) did not result in further impairment of surface expression, which is compatible with the hypothesis that disulfide bond disruption is more important than the actual amino acid replacements. Disruption of both disulfide bonds by concomitant replacement of the four cysteines by alanine did not affect expression more dramatically than single bond disruption.

*The Disulfide Bond Linking the First and Second Extracellular Loops Is Essential for Maintaining the Conformational Integrity of CCR5*—The reduced levels of surface expression observed after mutation of any single Cys residue suggested that disulfide bond disruption could affect receptor conformation, resulting in partial impairment of receptor trafficking through the endoplasmic reticulum and Golgi complex. To test this hypothesis further, mAbs recognizing other regions of the receptors were used. mAbs 2D7 and 531 recognize conformation-dependent determinants in the second extracellular loop of CCR5 (Fig. 1). The epitope of 2D7 maps to the first part of ECL2 (35) and involves Lys[172] and Asp[173], while that of mAb 531 maps to the second half of ECL2, from Tyr[184] to Phe[189]. In addition, two mAbs (mAb 501 and mAb 549) which interact with multiple CCR5 extracellular domains were also used (Fig. 1). mAb 501 requires both the first and second loops of CCR5, while mAb 549 requires the combination of the N terminus and the first two extracellular loops for efficient recognition of this receptor (25). None of the four mAbs recognizes CCR5 by Western blot.

All four conformation-dependent mAbs recognized the C20A and C269A single and combination mutants as well as the N-terminal mAbs CTC5 and CTC8. Thus, disruption of the disulfide bond involving the N-terminal domain of CCR5 and the third extracellular loop did not affect conformationally sensitive antigenic determinants involving extracellular loops 1 and 2 (Fig. 2*a*). By sharp contrast, 2D7 barely detected the C101A and C178A mutants, while mAbs 531, 501, and 549 were totally unable to label these mutants. Interestingly, the mutant combining C101A and C178A was recognized more efficiently by 2D7 and mAb 531 than the individual mutants (Fig. 2*a*). These results are consistent with these two cysteine

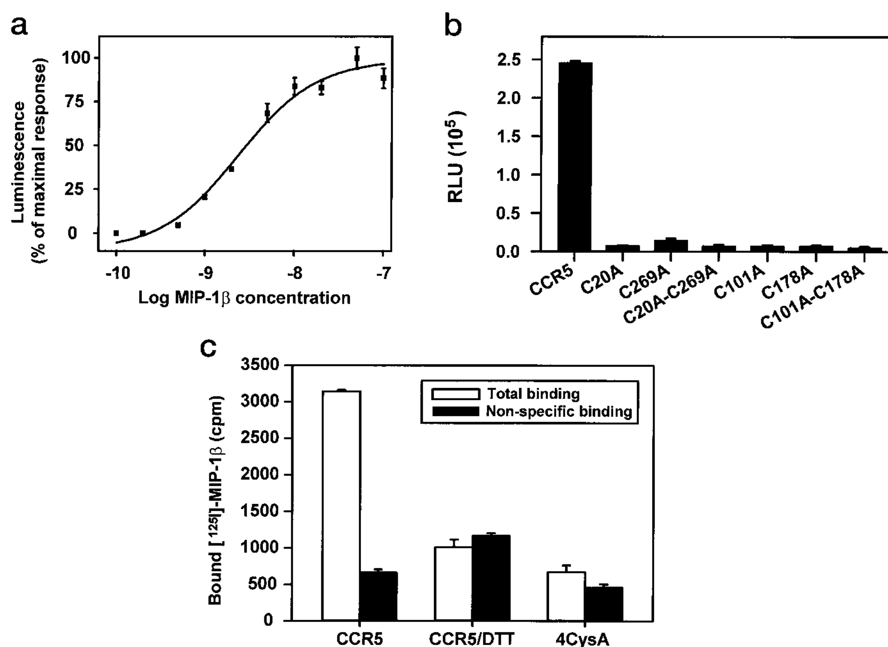


FIG. 3. **Binding and functional response of CCR5 mutants to MIP-1β.** Stable CHO-K1 lines coexpressing wild-type or mutant CCR5 together with apoaequorin, were established. Surface expression of receptors was verified by FACS analysis using the 2D7 and 3A9 mAbs, previously mapped to the second extracellular loop and N-terminal domain of CCR5, respectively (data not shown). Cells were preincubated with coelenterazine for 2 h, and tested for their functional response to 100 nM MIP-1β using a luminometer. Background activity was subtracted from all values. The data represent the average of two independent experiments, each performed in duplicate. The dose-response curve for wild-type CCR5 was performed in triplicate, and the $EC_{50}$ was calculated by non-linear regression using Graph-Pad software. The stably transfected cell lines expressing wtCCR5 preincubated or not with 100 mM DTT, or the four-cysteine mutant (*4CysA*) were tested for their ability to bind MIP-1β. Bound $^{125}$I-MIP-1β in the absence (total binding) or presence of 100 nM (nonspecific binding) of unlabeled MIP-1β is represented. *Error bars*, S.E.

residues forming a disulfide bond, since elimination of one Cys residue involved in a disulfide bond leaves behind an unpaired, reactive Cys residue that has the potential to form aberrant intra- or intermolecular disulfide bonds. By contrast, simultaneous replacement of both cysteines involved in a disulfide bond will not leave an unpaired Cys, and so might result in more efficient protein folding. These data demonstrate that the disulfide bond linking ECL1 and ECL2, but not the bond linking the N terminus to ECL3, is essential for rendering the extracellular domains of CCR5 compatible with their recognition by conformation-sensitive antibodies.

Mutations that affect protein folding are sometimes temperature-sensitive. Thus, a mutation that results in misfolding at 37 °C may not do so at 32 °C. To determine if the Cys mutants exhibited a temperature-sensitive phenotype, cells were incubated for 18 h at 32 °C following transfection and CCR5 expression monitored by FACS using the panel of mAbs described above (Fig. 2*b*). We found that incubation at 32 °C dramatically increased the relative expression level of all the CCR5 mutants as detected by the N terminus mAbs CTC5 and CTC8, resulting in FACS signals in the same range as for the wild-type receptor (81–138% of control). Incubation at 32 °C also increased the labeling of C20A, C269A, and the double mutant C20A,C269A by the ECL2 (2D7 and mAb 531) and multidomain (mAb 501 and mAb 549) mAbs, though not to wild type levels. However, while incubation at 32° increased the expression of the C101A and C178A mutants as judged by staining with the N-terminal mAbs, these mutants were still poorly recognized by the conformationdependent mAbs. Interestingly, the double mutant C101A,C178A was recognized reasonably well by the ECL2 mAbs 2D7 and 531 (Fig. 2*b*), again suggesting that these two residues are involved in a disulfide bond that is important for receptor conformation.

*Both Disulfide Bonds Are Critical for MIP-1β Binding and Receptor Activation*—The cysteine mutants were tested for their ability to bind chemokines, and/or to respond functionally to them. For monitoring the activation of intracellular cascades, we used a very sensitive calcium reporter system based on light emission by the apoaequorin-coelenterazine complex following intracellular calcium release. Stable cell lines expressing high levels of the mutant receptors together with apoaequorin were used for this purpose. In each of these cell lines, receptor expression levels were determined by FACS analysis using two different mAbs (2D7 and 3A9) recognizing the ECL-2 or the N-terminal region, respectively. All mutants were found to be expressed at levels similar to those achieved in transient expression experiments (data not shown). Apoaequorin expression was functionally tested through the activation of endogenous P2 receptors by 100 μM ATP (data not shown).

Using $^{125}$I-MIP-1β as a ligand, we were able to detect a high affinity binding site on cells expressing the wild-type receptor, as previously reported (24). However, no binding activity was detected for any of the single Cys mutations or combinations thereof (Fig. 3*c* and data not shown). Since a reduction of the affinity of the mutant receptors for chemokines by 1 order of magnitude would result in a barely detectable binding activity, we tested the activation of the mutant receptors by MIP-1β concentrations ranging from 0.05 to 100 nM. Chemokine receptors are coupled to intracellular calcium release through pertussis-toxin sensitive G proteins. As shown in Fig. 3*a*, stimulation of wild-type CCR5 by MIP-1β resulted in the generation of a strong luminescent signal in the apoaequorin-expressing cell line, with an $EC_{50}$ (2.4 nM) comparable with that obtained previously with other functional assays. None of the Cys mutants was able to generate a signal in response to MIP-1β up to 100 nM, and the combination of mutations did not rescue the phenotype (Fig. 3*b*). The absence of signaling was confirmed by using a microphysiometer-based functional assay as described previously (1). Once again, wild-type CCR5 responded to MIP-1β with an $EC_{50}$ of 2.6 nM, and none of the mutants responded to up to 100 nM MIP-1β or RANTES. Incubation of the cells at 32 °C for 12 h prior to the assay did not rescue the

binding or functional properties of the mutant receptors (data not shown).

*DTT Treatment Abolishes Chemokine Binding*—From the binding and functional results using the mutant receptors, it appeared that the presence of the four extracellular cysteines is required for the binding activity of CCR5. In order to verify that the disulfide bonds were necessary, rather than the cysteines themselves, we evaluated the effect of disulfide bond reduction on the binding properties of wild-type CCR5. CHO-K1 cells stably expressing wtCCR5 were tested for their ability to bind MIP-1$\beta$ in the presence or absence of 100 mM DTT. In the absence of DTT, MIP-1$\beta$ binding was obtained with an $IC_{50}$ of 0.4 nM. No specific binding for MIP-1$\beta$ could be detected on cells pretreated with DTT (Fig. 3*c*). This experiment suggests the importance of solvent-accessible disulfide bond(s) for high affinity chemokine binding.

*CCR5 Cysteine Mutants Retain Significant Coreceptor Activity*—In order to assess the importance of disulfide bonds for HIV infection, we tested the coreceptor function of CCR5 cysteine mutants using a luciferase-based reporter virus assay. Luciferase reporter viruses pseudotyped with two R5 Envs (ADA and BaL) were used to infect human U87 cells expressing CD4 and the various CCR5 mutants. As shown in Fig. 4*a*, mutation of $Cys^{20}$ and/or $Cys^{269}$ resulted in a moderate decrease in coreceptor function (40–62% of wtCCR5 activity for BaL), while mutants involving $Cys^{101}$ and/or $Cys^{178}$ affected infection efficiency more dramatically (14–20% of wtCCR5 activity for BaL). The concurrent mutation of all four extracellular cysteines gave similar results (10–17% of wtCCR5 activity for BaL and ADA). Since CCR5 expression levels are known to be correlated with infection efficiency (36), we tested whether decreased coreceptor activity of the mutants could be explained partially by lower expression level, as compared with wtCCR5. Following transfection, the cells were therefore incubated at 32 °C, in order to increase the expression level of the four CysA mutants to wild-type levels before infection with pseudotyped viruses. In these conditions, the surface expression level, as measured by FACS analysis with the CTC5 mAb was similar to that of wtCCR5 (60–169%), and we observed also an increase in coreceptor function from 10% to 39% of wtCCR5. These experiments demonstrate that the coreceptor activity of CCR5, although impaired by the disruption of extracellular disulfide bridges, was much less affected by these mutations than was the binding of chemokines.

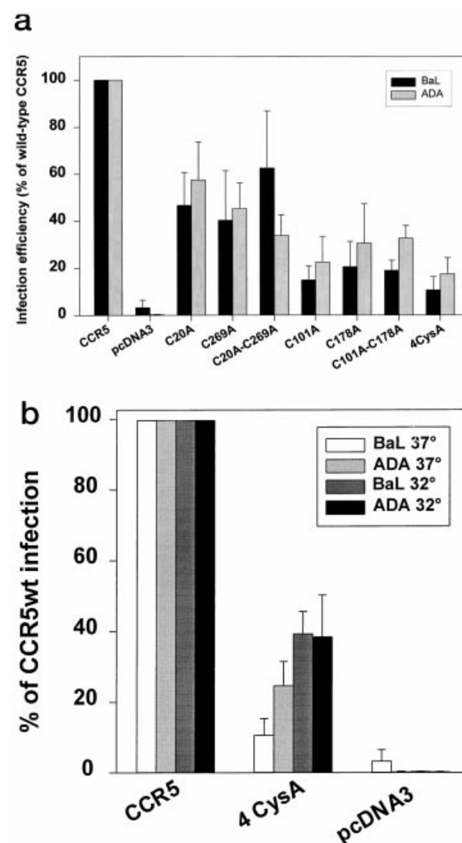


FIG. 4. **Coreceptor function of cysteine mutants.** Wild-type CCR5 and extracellular cysteine mutants were tested for their ability to act as HIV coreceptors in an infection assay, using pseudotyped viruses. Plasmids encoding wild-type CCR5 or mutants were transfected in 293T cells and subsequently infected by a luciferase reporter virus pseudotyped with BaL or ADA envelopes as indicated. The luciferase activity was measured 4 days after infection, and the results were normalized with regard to the light signal obtained with wild-type CCR5. Data are presented as mean ± S.E. of three independent experiments. All mutants retained substantial coreceptor activity. wtCCR5 or the quadruple-cysteine mutant were infected by the same viruses. The cells were incubated at 37 °C (*a*) or 32 °C (*b*). The reduction of coreceptor function was partially due to a reduction of expression levels.

## DISCUSSION

HIV infection is initiated by the interaction of virion envelope glycoprotein (gp120/41) with CD4 and a coreceptor belonging to the G protein-coupled receptor family (41). Macrophage-tropic strains of HIV-1 that are responsible for virus transmission and predominate in the early stages following infection use CCR5 as the main coreceptor (2, 3) and have therefore been renamed R5 strains (42). The CD4-gp120 interaction induces conformational changes in gp120, unmasking a binding domain for the coreceptor (43). The direct interaction of gp120 with CCR5 involves both conserved and variable regions of the viral glycoprotein (44–46). It is believed that, following this interaction, additional conformational changes of the envelope allow the insertion of the fusion peptide of gp41 into the target cell membrane, triggering the ill defined fusion process itself (47). The central role of CCR5 in HIV pathogenesis and the absence of overt phenotype in individuals homozygotes for non-functional alleles of the CCR5 gene (5) has suggested that CCR5 might be an ideal target for pharmacological agents preventing viral entry. Chemokines or analogs (48–51), mAbs (35), or small molecule antagonists directed against co-receptors (52–54) have already been shown to efficiently inhibit HIV infection *in vitro*. Nevertheless, the presently available molecules will probably be of limited use *in vivo*, and a better understanding of the molecular interaction between CCR5 and gp120 on one hand, and between CCR5 and its natural chemokine ligands on the other hand, would be helpful in designing new anti-HIV therapeutics on a rational basis.

We and others have previously studied the respective role of the different domains of CCR5 for its receptor and HIV coreceptor functions. It was found that the N-terminal domain of CCR5 is the most important region involved in coreceptor activity, but that all extracellular domains contribute to this function (19–23). Interestingly, different strains of HIV were shown to exhibit a variable sensitivity to specific alterations of the receptor (19, 21, 55). For the chemokine receptor function of CCR5, we have shown previously that its second extracellular loop represents the principal determinant of ligand specificity (24).

In the present work, we have extended our analysis of the structural determinants of CCR5 that are required for its receptor and coreceptor functions by investigating the role of the disulfide bonds believed to link the extracellular domains of the receptors. With rare exceptions, members of the main family of G protein-coupled receptors (rhodopsin-like) contain two conserved cysteines located, respectively, in the first and second

extracellular loops. For a few receptors (rhodopsin, $\beta_2$-adrenergic, and TRH receptor), these cysteines have been demonstrated to be involved in a disulfide bond essential for the binding and functional properties of the receptors (26–29). This bond is therefore believed to be present in most receptors belonging to that group. Chemokine receptors, including CCR5, share two additional conserved cysteines, located in the N-terminal domain and the third extracellular loop. These cysteines are believed to form an additional disulfide bond but this hypothesis has not been demonstrated experimentally.

Here, we present evidence that the extracellular cysteines of CCR5 effectively form two disulfide bonds, one ($Cys^{20}$-$Cys^{269}$) linking the N-terminal domain and ECL3, the second ($Cys^{101}$-$Cys^{178}$) linking ECL1 and ECL2. The existence of these two disulfide bonds was demonstrated by the alteration of CCR5 properties following the replacement of one or several of the four cysteines into alanine. Identical impairment of CCR5 functions resulted from the substitution of any of the two cysteines belonging to a putative bond, demonstrating the importance of the bond itself, rather than the nature of the cysteine side chain. Mutations of $Cys^{101}$ and $Cys^{178}$ had much stronger effects on CCR5 functions that mutations of $Cys^{20}$ and $Cys^{269}$ (Figs. 2 and 4), confirming the identity of the cysteines involved in each bond. Moreover, the simultaneous substitution of the two cysteines involved in a bond did not result in further alteration of receptor functions, and in some cases improved the function (Figs. 2 and 4). Finally, the partial restoration of receptor expression and conformation by incubation of cells at 32 °C further supports the idea that the effects of the mutations are primarily conformational in nature.

FACS analysis was performed using mAbs directed at various domains of CCR5, in order to evaluate the role of disulfide bonds on receptor expression and conformation. Staining with mAbs recognizing linear epitopes within the N-terminal domain of the receptor demonstrated that disulfide bond disruption resulted in a moderate reduction of CCR5 surface expression, and that this phenotype, probably resulting from improper folding and trafficking of the receptor, could be rescued by maintaining the cells at lower temperature (32 °C). The absolute or relative inability of mAbs recognizing conformation-sensitive epitopes to label CCR5 mutants suggested major alterations of the receptor conformation following the disruption of the $Cys^{101}$-$Cys^{178}$ bond. This altered conformation was only mildly restored by culturing the cells at 32 °C. Disruption of the bond linking $Cys^{20}$ and $Cys^{269}$ did not seem to affect the CCR5 conformation in a similar way. It should be noted, however, that the conformation-sensitive mAbs used in this study recognize epitopes either restricted to the second loop of CCR5 or spread over different loops, including ECL2. Their binding is therefore expected to be affected more dramatically by disruption of the bond involving this loop.

Mutation of any of the extracellular cysteines resulted in the loss of detectable chemokine binding activity. Similarly, activation of intracellular cascades by the mutant receptors was totally abolished, as demonstrated by the use of two sensitive functional assays. One of these assays (microphysiometer) is able to detect the activation of any intracellular cascade (56), the other (apoaequorin co-expression) is specific for intracellular calcium release, the main cascade stimulated by CCR5. The reduction in surface expression of the receptor could not account for the lack of binding and functional response of the CCR5 mutants. Indeed, FACS analysis demonstrated that surface expression of the mutant receptors is similar to that obtained in transient expression experiments (Fig. 2), and binding and functional activation could be demonstrated for other mutants expressed at much lower levels (data not shown). Since ECL2 was shown to constitute the major determinant responsible for the ligand specificity of CCR5 (24), it is not surprising that disruption of the bond linking $Cys^{101}$ and $Cys^{178}$, which affects the conformation of ECL2, resulted in the loss of chemokine binding. The impairment of chemokine binding by the C20A or C269A substitutions suggested a similar alteration of the conformation of other receptor regions, such as the N terminus and ECL3. These alterations, although undetected by our panel of antibodies, are likely to prevent the efficient interaction of CCR5 with its ligands.

Interestingly, disulfide bonds did not appear to be absolutely necessary for the coreceptor function of CCR5. Infection efficiency was significantly reduced as compared with wtCCR5, although this reduction could be attributed in part to the mild reduction in cell surface expression. Other authors have reported during the course of this study that some of the cysteine substitutions did not affect the coreceptor function of CCR5 for the JR-FL strain (57), or instead totally impaired coreceptor function for JR-FL and Gun-1 HIV-1 strains (58, 59). These conflicting observations may reflect variations in the experimental procedures, and/or the use of different HIV-1 strains. Nevertheless, Env binding appears to have much lower requirements for a precise structural conformation of CCR5, as compared with chemokine binding. In line with this observation, all functional chemokine receptors do share the two disulfide bonds, while some HIV co-receptors, such as the orphan receptors Bob/GPR15, Bonzo/STRL33, and ChemR23 (11, 14–17) lack the bond linking the N terminus to ECL3. A natural human variant of CCR5 (C20S) has been reported in two long term nonprogressors (34). Although it remains to be determined whether the substitution into serine provides exactly the same phenotype as the alanine replacement we described here, it is likely that the C20S mutant is nonfunctional in terms of chemokine binding and signaling, reinforcing the hypothesis (34) that mutations affecting the CCR5 function have been selected over neutral substitutions. In addition, this mutant receptor is likely to exhibit impaired, though not completely ablated, coreceptor activity by analogy with our C20A mutant.

As a conclusion, we have presented studies that argue strongly for the presence of two disulfide bonds in CCR5, and show that these bonds play important roles in maintaining the complex structural organization of the receptor outer domains. This structural organization is an absolute requirement for chemokine binding and signaling, while alteration of this structure is better tolerated by HIV-1. The relative tolerance of the virus for modifications of CCR5 conformation may be correlated to the ability of HIV to modify the way it interacts with the coreceptor, and to expand its range of coreceptor usage. It may also imply that classical receptor antagonists that are believed to restrict the range of receptor conformations, without affecting dramatically the conformation of extracellular domains, could exhibit moderate efficacy on coreceptor function.

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