Rescue of HIV-1 receptor function through cooperation between different forms of the CCR5 chemokine receptor.

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The abbreviation used are: MIP1-β, macrophage inflammatory protein; HIV-1, human immunodeficiency virus type 1; NT, amino-terminal extracellular domain, ECL, extracellular loop; TM, membrane-spanning domains; mAb, monoclonal antibody; WT, wild-type; GPCR, G protein coupled receptor.
SUMMARY

Interaction of the human immunodeficiency virus (HIV-1) envelope glycoproteins with the CCR5 chemokine receptor, a G-protein coupled receptor, triggers a membrane fusion process and virus entry. Cooperation for HIV-1 receptor activity was observed when two forms of CCR5 were coexpressed, either the wild-type (WT) receptor and a defective mutant with deletion of the N-terminal extracellular domain (NT), or the latter ΔNT mutant and a human-mouse CCR5 chimera bearing the NT domain from human CCR5. Cooperation was most efficient when the two forms of CCR5 were in a 1:1 ratio. It was not observed between the CCR5 ΔNT mutant and a chimeric receptor (5444) in which the NT domain of CCR5 was in the context of another GPCR, the HIV-1 receptor CXCR4. These results suggested that physical association between two forms of CCR5 was required for their cooperation. Coimmunoprecipitation experiments in transfected cell lysates indeed showed that the ΔNT CCR5 mutant formed oligomeric complexes with the WT CCR5 or the HMMM chimera, but not with the CXCR4-derived chimera 5444. These observations suggest that formation of CCR5 oligomers is a constitutive process independent from activation by chemokine ligands. The interaction of HIV-1 with independent subunits of CCR5 oligomers could favor the local recruitment of fusiogenic proteins and the formation of a fusion pore.
INTRODUCTION

Chemokines represent a family of at least 40 structurally related small proteins (8-10 K) mediating chemotactic migration of leukocytes through binding and activating receptors with seven membrane-spanning domains coupled to heterotrimeric G proteins (GPCR) (for recent reviews, see 1-4). Chemokines are classified according to the relative position of two conserved cysteine residues in their amino terminal region, the major subgroups being termed CXC (or α) and CC (or β) and the same nomenclature (CXCR, CCR) used to designate their receptors (5). In addition to their role in cell signal transduction, chemokine receptors have attracted a lot of interest since they represent cell entry portals for the human immunodeficiency viruses (HIV-1, HIV-2) and related simian or feline retroviruses. A list of chemokine receptors or related orphan GPCRs can mediate HIV-1 entry in certain experimental conditions, but only two of them, CCR5 and CXCR4, seem to be used in vivo. The predominant role of CCR5 is indicated by the resistance to HIV-1 infection of individuals genetically deficient for the expression of this receptor, the most frequent defect being a 32-nucleotide deletion (Δ32 allele) resulting in a translation frameshift after residue 187 (reviewed in 6-9). While HIV-1 strains infecting cells via CCR5 (termed R5 strains) can be isolated throughout infection, strains using CXCR4 (termed X4) or both receptors (R5X4) generally emerge at more advanced stages. Selectivity of HIV-1 for CCR5 or CXCR4 can explain differences in cell tropism, such as the ability of R5 but not X4 strains to replicate in macrophages. The inhibition of HIV-1 infection by chemokines and other CCR5 or CXCR4 ligands raises hope for novel antiviral strategies (1,7,10) and stimulates investigations on the role of these receptors in the initial steps of the virus life cycle.

The cell entry of HIV-1 and other retroviruses is mediated by their envelope glycoproteins (Env) which consist in trimeric complexes of a surface subunit (gp120 in the case of HIV-1) responsible for contacts with target cells and a transmembrane subunit (gp41) mediating membrane fusion (reviewed in 11-13). The conformation changes in the Env complex required to activate the fusiogenic properties of gp41 are considered to be triggered by the interaction of
gp120 with CCR5 or CXCR4. This interaction has been observed by different techniques, usually in presence of soluble forms of the CD4 protein, which led to consider CCR5 and CXCR4 as CD4-associated HIV-1 coreceptors (14-16). However, the possibility of CD4-independent binding of gp120 to CCR5 and CXCR4 (17-19) as well as evolutionary and mechanistic considerations also allow to view them as *stricto sensu* HIV-1 receptors. How CCR5 or CXCR4 interact with gp120 is not known at the molecular level. The current view is that the functional interaction with HIV-1 gp120, i.e. leading to infection, is mediated by the extracellular domains of chemokine receptors, in particular their amino terminal (NT) domain and second extracellular loop (ECL2), while their intracellular domains coupled to the cell signaling machinery are dispensable, at least in experimental situations (reviewed in 6,20,21). The ability of CCR5 and CXCR4 to interact with gp120 is certainly a critical parameter, but it can be wondered if other features could also contribute to their HIV-1 receptor activity. Discrepancies have indeed been reported between gp120 binding and efficiency of HIV-1 infection in certain experiments (22,23). Among parameters proposed to influence the HIV-1 receptor activity of CCR5 or CXCR4 are their association with CD4 (14,24,25) and their density at the cell surface (26,27), which both could be linked to localization in particular domains of the plasma membrane such as glycolipid-rich “rafts” (28). A high local density of HIV-1 receptors could favor the clustering of activated viral fusigenic proteins which seems important for the formation of fusion pores (29-31). In line with this view, the oligomeric status of chemokine receptors could represent an important parameter for HIV-1 infection.

The possibility that GPCRs form oligomeric complexes has been the matter of a long debate but now seems an accepted fact supported by a list of coimmunoprecipitation and transcomplementation experiments and more recently evidenced by biophysical techniques based on light resonance energy transfer (for reviews see 32-34). There is still no consensus on the possible function(s) of such dimers or higher order oligomers of GPCRs. In most instances, the formation of such structures appears to be a constitutive process, independent from activation of these receptors by their ligands (32). Conflicting results have been obtained in the case of
chemokine receptors. In a series of studies, formation of CCR5, CCR2 or CXCR4 homodimers as well as CCR5/CCR2 heterodimers was found to occur upon treatment of cells with the cognate chemokine ligands (35-38). On the contrary, Benkirane et al. found that wild-type CCR5 could be trapped in the endoplasmic reticulum of cells expressing the truncated form of CCR5 (residues 1-187) encoded by the Δ32 allele and proposed that formation of dimers was a constitutive process necessary for efficient processing of the receptor to the cell surface (39). Although such an effect of the Δ32 mutant on the cell surface expression of CCR5 could not be evidenced in a recent study (40), we have reported experiments confirming the finding of Benkirane et al. and suggesting that the trans-dominant negative effect involved interactions between membrane-spanning domains of the two proteins, in particular TM3 of CCR5 and TM4 of Δ32 (41). In similar experiments, we found that the HIV-1 receptor activity of CCR5 was enhanced when cells coexpressed a functionally defective CCR5 mutant, obtained by complete deletion of the N-terminal extracellular domain. Another example of cooperation between two forms of CCR5 was obtained by rescuing HIV-1 receptor activity upon expressing two defective CCR5 mutants in transfected cells. This functional transcomplementation apparently required physical association of the different forms of CCR5, detected in coimmunoprecipitation experiments. These results provide strong and direct evidence for the formation of constitutive CCR5 oligomers and shed new light on the molecular interplay between the HIV-1 envelope proteins and the target cell membrane.
EXPERIMENTAL PROCEDURES

Cell Lines, HIV-1 strains, and other biological materials.

The HEK293T and HeLa-derived cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS) and antibiotics (100 units/ml penicillin, 100 µg/ml streptomycin). The CEM.NKR cell line expressing CCR5 (42) was grown in RPMI with 10% FCS and antibiotics. The HeLa-P4 cell line (43) and CCR5+ derivative (44) are CD4+ and stably transfected with a HIV-inducible β-galactosidase reporter gene (LTR-lacZ). HeLa-Env/ADA cells are Tat+ and stably express the envelope glycoproteins of the R5 HIV-1 strain ADA (44). Viral stocks corresponding to the R5 HIV-1 strains YU-2 (45), JRCSF (46), and ADA (44,47) were produced by transfection of cloned proviruses in HEK293T cells. Titers were measured in CCR5+ HeLa-P4 cells were ~10^5 infectious units (I.U.) per ml for YU2 and JRCSF and 10^6 I.U. per ml for ADA as described (44). The anti-CCR5 mAbs 2D7 (48) and 3A9 (49) and peroxidase-conjugated anti-mouse IgGs were obtained from Pharmingen (San Diego, CA), anti-CXCR4 mAb 12G5 (50) from Pharmingen, phycoerythrin-conjugated rabbit anti-mouse IgGs from Dako (Glostrub, Denmark), M2 anti-FLAG and 9E10 anti-c-MYC mAbs from Sigma and Boehringer Mannheim, respectively. Recombinant MIP-1β was purchased from Peprotech, Inc.

Plasmid Vectors.

All WT and mutant chemokine receptors cDNAs were expressed from the cytomegalovirus immediate-early promoter using a standard calcium phosphate technique. The expression vectors for WT and MYC-tagged CCR5 human CCR5 (44) the HMMM human-mouse chimeric CCR5 (51), truncated CCR5 1-100 (41), WT CXCR4 (52) have been described. The CCR5 ΔNT mutant corresponding to an in-frame deletion between Asp_2 and Lys_26 was obtained by site-directed mutagenesis on a single-stranded template. PCR amplification strategies were used to obtain the FLAG-tagged forms of CCR5 (in-frame insertion of the amino acid sequence DYKDDDDDK after the initiation codon) and the 5444 chimera, which has the NT domain of human CCR5 (M_1
to $K_{26}$) and the other domains of human CXCR4 ($C_{28}$ to $S_{352}$). The green fluorescent protein (GFP) was expressed from the EGFP-N1 vector (Clontech).

**Syncytia formation assays and HIV-1 infections.**

Infection of HeLa-P4 cells expressing different forms of CCR5 or cocultures with Env$^+$ cells were performed essentially as described (44). Briefly, cells were transfected in 6-well trays ($5 \times 10^5$ cells/well) with a total amount of 3 µg of DNA per well. Cocultures or infections were initiated 24 h later by adding an equivalent number of freshly trypsinized HeLa-Env/ADA cells or $10^5$ I.U of each R5 HIV-1 strains. After 24 h (or 36 h for infections), the cells were washed, fixed in 0.5% glutaraldehyde, and stained for β-galactosidase activity with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal). Blue-stained foci were scored under 20X magnification. Cell counts $>200$ were obtained by extrapolation from randomly selected fields.

**Flow cytometry analysis**

The surface expression of chemokine receptor was monitored as described (52). Briefly, HEK293T cells were detached with phosphate-buffered saline (PBS), 1 mM EDTA 36 h after cotransfection with GPCR and GFP expression vectors (6:1 ratio). Cells were incubated 1 h at 4°C in PBS 2% FCS with 1 µg/ml of anti-CCR5 (2D7 or 3A9) or anti-CXCR4 (12G5) mAb, then washed and stained 1 h at 4°C with phycoerythrin (PE)-conjugated secondary Ab before fixation in 4% paraformaldehyde. The green (GFP) and red (CCR5 or CXCR4) fluorescence was analyzed on an Epics Elite flow cytometer (Coultronics). Results are shown as percentage of PE-positive cells or mean PE intensity in the GFP-positive fraction, as indicated.

**Immunoprecipitations and western blots.**

Approximately $10^7$ transfected HEK293 T cells or CEM cells were lysed by incubation 1 h at 4°C in 1 ml of 0.5% n-dodecyl-D-maltoside (Sigma), 25 mM Tris-HCl (pH 7.4), 140 mM NaCl, 2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (Sigma), protease and phosphatase inhibitors.
Cell lysates were clarified by centrifugation (12,000g, 15 min) and immunoprecipitations performed at 4 °C on 1 ml samples (1 mg of proteins) by contact with 1µg of 2D7 or anti-MYC mAb (2 h) and protein G-agarose (Roche). Proteins were resolved by SDS-PAGE and transferred to nylon membranes before contact with the 3A9, M2 (anti-FLAG) or 9E10 (anti-c-MYC) mAb (all at 1 µg/ml) and then with peroxidase-coupled anti-mouse IgGs (0.5 µg/ml). The reaction was revealed with ECL™ reagents (Amersham).
RESULTS

Cooperation of wild-type CCR5 and a defective mutant.

The HIV-1 receptor activity of CCR5 was assayed by transient transfection of CD4\(^+\) HeLa-P4 cells followed by infection by R5 HIV-1 strains (ADA, JRCSF or YU2) or coculture with HeLa-Env/ADA cells (44). Infection by HIV-1 or fusion with Env\(^+\) cells (also expressing the HIV-1 transactivating protein, Tat) activates a β-galactosidase (\(lacZ\)) transgene in HeLa-P4 cells, allowing to quantify these events by staining with X-gal. Syncytia formation and HIV-1 infection were not detected when HeLa-P4 cells expressed the ΔNT CCR5 mutant, corresponding to a 25-residue deletion in the N-terminal extracellular domain (Figure 1A, B). Unexpectedly, cotransfection of this defective mutant and the wild-type (WT) CCR5 resulted in markedly higher numbers of syncytia with Env\(^+\) cells and HIV-1 infection events, by comparison with cells transfected with the WT CCR5 only (Figure 1A, B). The efficiency of cell fusion or infection was similar for cells transfected with equal amounts (1.5 µg) of WT and ΔNT CCR5 vectors or with 3 µg of the WT CCR5 vector. The cooperation observed in this experiment could either be due to functional complementation of the defective mutant or to enhanced expression of the WT receptor.

The cell surface expression of the two forms of CCR5 was assayed by flow cytometry after staining with the 3A9 or 2D7 monoclonal antibodies (mAbs), which react with the N-terminal domain (NT) and the second extracellular loop (ECL2) of CCR5, respectively. Similar flow cytometry profiles were obtained for cells transfected with the WT CCR5 or the ΔNT mutant upon staining with the 2D7 mAb, indicating that the deletion did not affect cell surface expression, while the 3A9 mAb only stained cells expressing WT CCR5, as expected (Figure 2A, Table 1). Analysis of HeLa-P4 cells cotransfected with equal amounts of the WT CCR5 vector and either a control plasmid or the ΔNT CCR5 vector revealed similar staining efficiency with the 3A9 mAb (Figure 2B). The enhanced HIV-1 receptor activity of cells coexpressing WT and ΔNT CCR5 seemed therefore due to a complementation effect.
Coexpression of CCR5 mutants restores HIV-1 receptor activity

To obtain further evidence of cooperation between two forms of CCR5, we sought to rescue HIV-1 receptor function by coexpressing the ΔNT mutant and another defective forms of CCR5. In our hands, point mutations in the extracellular loops of CCR5 had a very limited functional effect, while deletions were not compatible with transport of CCR5 to the cell surface, likely because they caused improper folding and intracellular retention of the polypeptide chain. Our option was therefore to use a CCR5 chimera (HMMM) in which the NT and TM1 domains derived from human CCR5 (H) and other domains from its mouse homologue (M). Although such a chimeric receptor has been reported to be a functional HIV-1 receptor (51,53), its activity was very low in our assays. It was less than 10 % relatively to WT CCR5 in fusion assays between transfected HeLa-P4 cells and HeLa-Env/ADA cells (Figure 3A) and in infections with the YU-2 and JRCSF HIV-1 strains (Figure 3B), while infection by HIV-1 ADA was somehow more efficient (~30 % relatively to WT CCR5). Flow cytometry analysis with the 3A9 mAb indicates that the lower HIV-1 receptor activity of the HMMM chimera does not result from reduced surface expression (Table 1, Figure 2A).

Cotransfection of CCR5 ΔNT and HMMM expression vectors (1.5 µg each) in HeLa-P4 cells resulted in efficient fusion with HeLa-Env/ADA cells and infection by the HIV-1 JRCSF and YU-2 strains, while infection by HIV-1 ADA strain was markedly increased (Figure 3A,B). The number of syncytia or infection events were comparable to those seen when HeLa-P4 cells were transfected with 1.5 µg of WT CCR5, again indicating that the functional complementation of these two CCR5 mutants is a relatively efficient process. Flow cytometry analysis confirmed that the ΔNT mutant did not upregulate the surface expression of the HMMM receptor (Figure 2B). In similar experiments, the CCR5 ΔNT mutant was not rescued by coexpressing the mouse CCR5 or other chimeric receptors not containing the N-terminal domain of human CCR5 (unpublished results) or by the CCR51-100 mutant corresponding to the NT and TM1 domains (Figure 3A) and previously shown not to be processed to the cell surface (41).
To find out if complementation was possible when the NT domain of CCR5 was in the context of a more distant GPCR, it was substituted to the homologous domain of CXCR4. Flow cytometry analysis showed that the resulting 5444 chimera and WT CXCR4 were expressed at a similar level in transfected cells, while reactivity with the 3A9 mAb was ~ 50 % relatively to WT CCR5 (Table 1), indicating that the N-terminal domain of CCR5 was accessible. Expression of the 5444 chimera in HeLa-P4 cells allowed to detect fusion with Hela-Env/ADA cells at a relatively low level (~ 20 % relatively to WT CCR5) (Figure 3A), which is in agreement with observations made with similar chimeric receptors (54). The efficiency of fusion was not enhanced when HeLa-P4 cells coexpressed the 5444 chimeric receptor and the ΔNT mutant (Figure 3A). These different results suggested that cooperation of different forms of CCR5 for HIV-1 receptor activity required some form of interplay that cannot take place with a more distant GPCR, such as CXCR4. It obviously led to envision that functional complementation involved formation of dimers or higher order oligomers.

In cells expressing two receptors capable to efficiently associate, the relative proportions of homodimers and heterodimers can be deduced from simple equations and follow theoretical curves shown in Figure 4A. The efficiency of fusion with Env⁺ cells and the level of CCR5 at the cell surface were in approximately linear relationship with the amount of CCR5 vector transfected, at least for DNA amounts ranging between 0.5 and 3 µg (Figure 4B and other unpublished data). It allowed to use this type of assay to estimate the stoichiometry of the different forms of CCR5 engaged in a functional complex. For cells cotransfected with the WT and ΔNT CCR5 vectors in different ratios, the fusion efficiency closely followed the theoretical curve corresponding to addition of the two curves corresponding to relative concentrations of the putative WT/WT and WT/ΔNT dimers (Figure 4B). When cells coexpressed the ΔNT CCR5 and HMMM chimera, the shape of the fusion efficiency curve was similar to that of the heterodimer concentration, although it was more compact (Figure 4C), which could indicate that only a fraction of the two forms of CCR5 could associate or that the putative complex they form is a less efficient HIV-1 receptor than WT CCR5. These experiments nevertheless indicated that cooperation is most efficient when
the different forms of CCR5 are in a 1:1 stoichiometry. Also, an important fraction of CCR5 or derived mutant seemed capable to form functional complexes.

**Detection of CCR5 oligomers**

Immunoprecipitation of CCR5 in apparently correct conformation, i.e. capable to bind gp120, seems critically dependent upon experimental conditions, in particular anti-CCR5 antibodies and reagents used for membrane solubilization (55). Based on these indications, cells were lysed with n-dodecyl-D-maltoside and a conformation-dependent mAb (2D7) used for immunoprecipitations. Using these conditions, SDS-PAGE allowed to detect as ~ 40 K species, which corresponds to the expected size of a CCR5 monomer (38 K), in transfected HeLa-P4 or HEK293T cells (data not shown). Monomers were also the predominant CCR5 species in the stably transfected CEM-CCR5 cell line (42), although a ~ 80 K band, consistent with the size of homodimers was detected upon longer exposure and was when the sample was treated with 4 M urea (Figure 5A). To address the effect of agonist binding on oligomerization, CEM-CCR5 cells were treated with the macrophage inflammatory protein (MIP-1β) chemokine at a relatively high concentration (100 nM). This treatment resulted in almost complete loss of CCR5 expression at the cell surface after 30 min, due to internalization (unpublished results). It indicates efficient binding of MIP-1β to most receptor sites in these conditions. Treatment of cells with MIP-1β did not seem to modify the relative abundance of the ~ 80 K CCR5 species but resulted in the apparition of heavier species that could correspond to oligomers or aggregates of CCR5 (Figure 5A). It also resulted in a slower migration of the different CCR5 species, particularly evident after 15 and 30 min (Figure 5A). Such an effect was previously reported for CCR5 monomers upon treatment with another ligand (AOP-Rantes) and seems due to phosphorylation of serine residues in the C-terminal domain of the receptor (56).

The results obtained in CEM-CCR5 cells indicate that CCR5 can form dimers in the absence of chemokine activation. However, such dimers represent a minor fraction of CCR5 in these cells and were not even detected in transiently transfected cells, which was in apparent
contradiction with the efficiency of functional complementation. This could indicate that cooperation between different forms of CCR5 mutants does not require their physical association. However, before accepting this conclusion, we had to envision the possibility that our experimental setting was not adapted to the detection of CCR5 oligomers, for example because of their relative instability in SDS-PAGE conditions. To circumvent this problem, we sought to address the oligomerization of CCR5 in assays based on coimmunoprecipitation.

In a first series of experiments, HEK293T cells were cotransfected with two epitope-tagged forms of CCR5 obtained by insertion of a FLAG or a c-MYC sequence at their N-terminus. Immunoprecipitation of cell lysates with anti-MYC mAb followed by SDS-PAGE and western blot analysis with anti-FLAG mAb allowed to detect a ~ 40 K band, consistent with the size of a CCR5 monomer (Figure 5B). It indicates that the FLAG- and MYC-CCR5 formed a complex that was disrupted during SDS-PAGE. By the same technique, it was possible to immunoprecipitate a FLAG-tagged ΔNT mutant with the MYC-CCR5, which indicates that the N-terminal region of CCR5 is dispensable for this association (Figure 5B). Bands with similar intensities were detected when the same western blot was analyzed with the anti-MYC mAb (Figure 5B). An important fraction of the MYC-tagged and FLAG-tagged CCR5 seemed therefore to form stable complexes.

Physical association of the ΔNT CCR5 and HMMM chimera was also evidenced in transfected cells by immunoprecipitation with the 2D7 mAb (only reacting with the ΔNT CCR5) followed by western blot with the 3A9 mAb selectively detecting the HMMM chimera (Figure 5C). The same approach yielded negative results when cells coexpressed the ΔNT CCR5 and the 5444 chimera (data not shown), or when immunoprecipitation was performed after mixing lysates of cells independently transfected to express the ΔNT CCR5 and the HMMM chimera (Figure 5C), ruling out artifactual formation of the CCR5 complexes.
DISCUSSION

Our finding that different forms of the CCR5 chemokine receptor can cooperate to fulfil the HIV-1 receptor function and form complexes detected by coimmunoprecipitation experiments represents further evidence for the oligomerization of this family of G-protein coupled receptors. It also suggests that formation of CCR5 oligomers is independent from chemokine binding and from the resulting cell activation process. Before discussing the implications of these findings for the different functions of CCR5, we shall briefly come back on the oligomerization of CCR5 and other chemokine receptors.

CCR5 oligomerization

The first evidence that chemokine receptors can physically associate was the detection of CCR5 homodimers in cells transfected with an epitope-tagged receptor (39). This process was apparently constitutive and proposed to play a role in the routing of CCR5 to the cell surface. But in other studies, dimers of the CCR2, CCR5 or CXCR4 were detected only if cells were treated with the cognate chemokine ligands (35-37), or with a monoclonal antibody in the case of CCR5 (36). In these different studies, dimers of chemokine receptors detected by SDS-PAGE and western blot techniques seemed to represent a small fraction relatively to monomers. The formation of CCR5 / CCR2 heterodimers was observed by coimmunoprecipitation and also required activation of cells by chemokine ligands (38).

We have used these two types of techniques to investigate the physical association of CCR5 monomers and of different mutant forms of CCR5. Immunoprecipitation of cell lysates followed by SDS-PAGE and western blot only allowed to detect CCR5 monomers in transiently transfected cells. A CCR5 species with apparent size of a dimer could be detected in a stably transfected T-cell line (CEM-CCR5) but represented a very minor fraction of total CCR5 which was mainly monomeric. Treatment of CEM-CCR5 cells with the MIP-1β chemokine did not
enhance the relative abundance of the putative CCR5 dimers although it resulted in the apparition of higher molecular weight species possibly representing tetramers or aggregates. According to these results, formation of dimers seemed to be a marginally important process in the case of CCR5 and therefore unlikely to account for the apparent efficiency of functional complementation.

A different case could be made from results obtained in coimmunoprecipitation experiments. Indeed, the association of CCR5 monomers bearing different epitope tags, and of the ΔNT mutant with the HMMM chimera was readily detected in transiently transfected cells, in the absence of chemokine activation or contact with HIV-1 envelope proteins. Coimmunoprecipitation was not observed between CCR5 monomers that were independently expressed in different cells, or between CCR5 and CXCR4 although these GPCRs were found to be clustered at the membrane in different cell types (57). It indicates that complexes detected in these experiments were not due to artifactual aggregation of chemokine receptors during cell lysis. Although these experiments did not allow precise quantification, they were consistent with the view that a high fraction of CCR5 was engaged in complexes, at least when expressed by transient transfection. The coimmunoprecipitation and functional complementation assays were therefore in complete agreement, since physical association was only detected between receptors cooperating for HIV-1 receptor activity and seemed an efficient process. This view is also in agreement with the recent detection of constitutive CCR5 oligomers by bioluminescence resonance energy transfer (BRET). By this approach, the BRET signal was not enhanced when cells were treated with CCR5 chemokine ligands and CCR5 appeared unable to form heterodimers with CXCR4, even in cells expressing relatively high levels of both receptors (58,59).

The relative instability of CCR5 complexes in SDS-PAGE and other technical issues, such as reagent used for membrane solubilization (55) likely account for the discrepancies between reports with regard to abundance of dimers as well as their constitutive or ligand-activated nature. If assays relying upon SDS-PAGE detect only the most stable fraction of dimers or oligomers, the apparent upregulation of these structures may not allow to infer that agonist binding induces
dimerization. As recently discussed, chemokines or other GPCR ligands could indeed bind to preexisting dimers or oligomers and thereby modify their conformation, rendering them more stable in detergent or more accessible to antibodies, hence more easily detected in certain experimental conditions (32). In the case of the glutamate receptor, there is direct crystallographic evidence that the ligand binds to preexisting dimers and induces conformation changes in its receptor (60).

**Interaction of CCR5 and HIV-1**

Binding of the HIV-1 envelope glycoprotein gp120 to CCR5 or CXCR4 at the surface of target cells, usually after prior contact with CD4, is considered to trigger the virus entry process. How gp120 interacts with chemokine receptors is not known in molecular details, although indirect elements suggest that the N-terminal domain and second loop of CCR5 and CXCR4 are involved, as well as the third hypervariable domain (V3) and a pocket formed by more inner and conserved domains of gp120 (12,61). Our finding that a defective form of CCR5 due to deletion of the N-terminal domain (ΔNT) was rescued by a chimeric receptor bearing this domain suggests that gp120 must engage distinct contacts with two sites in CCR5, one being entirely located in the N-terminal domain and the other formed by the extracellular loops. A similar model is usually envisioned for the interaction of chemokines and chemoattractants with their receptors (62).

Complementation for HIV-1 receptor activity only occurred between chemokine receptors capable to form complexes detected in coimmunoprecipitation experiments. It suggests that functional interaction of HIV-1 with the different domains of CCR5 has spatial constraints and requires the N-terminal domain and loops to be in close vicinity. Whether these domains of CCR5 reconstitute a single gp120 binding site or engage contact with two gp120 subunits of a trimeric Env complex cannot be decided from our results. Assaying the binding of recombinant monomeric gp120 to cells coexpressing the ΔNT CCR5 mutant and HMMM chimera could help clarify this issue. Of note, complementation experiments have shown the possibility of cooperative subunit interactions within HIV-1 Env (63).
The finding that complexes of defective CCR5 mutants mediate HIV-1 infection can seem in contradiction with conclusions of a study linking the antiviral activity of an anti-CCR5 mAb (designated CCR5-02) to the dimerization of CCR5 that it apparently induced (36). Since the CCR5-02 mAb did not interfere with the surface expression and chemokine receptor activity of CCR5, the authors inferred that its conformation in the context of dimers does not permit a functional interaction with HIV-1. However, this mechanism seems difficult to reconcile with apparently normal gp120 binding in presence of CCR5-02 mAb. Also, the antiviral activity was monitored at day 7 after infection, after several replicative cycles, which allows to envision indirect effects of the mAb on other steps of the virus cycle.

On the contrary, our results seem fully consistent with the observation that several CCR5 receptors, probably 4 to 6, must cooperate in order to mediate HIV-1 infection (27). This conclusion was based on the shape of curves depicting efficiency of infection relatively to amount of CCR5 at the cell surface. Analysis of membrane fusion mediated by the hemagglutinin (HA) of influenza virus has shown that several HA trimers must cooperate to form a fusion pore (29,30,64). In line with this view, the ability of HIV-1 to engage a functional interaction with different domains of a receptor prone to oligomerization can represent an advantage in terms of efficiency of infection. It can indeed favor the activation of a sufficient number of gp41 in the area of virus-cell contact to enable membrane fusion.

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FIGURES LEGENDS

Fig. 1. Cooperation of wild-type and defective CCR5 mutants for HIV-1 receptor activity.
(A) Syncytium-formation assays between HeLa-P4 cells (CD4+, LTR-lacZ) transfected with indicated amounts (µg) of different expression vectors and Hela-Env/ADA cells stably expressing R5 Env glycoproteins R5. Results represent numbers of syncytia detected by X-gal staining after 24 h coculture. (B) Infections of transfected HeLa-P4 cells with three different R5 HIV-1 strains (10^3 units), scored at 36 h by X-gal staining. Results are shown relatively to cells transfected with 3 µg of WT CCR5. Data shown are the average from three independent transfections.

Fig. 2. Relative cell surface expression of wild-type and mutant CCR5. (A) Flow cytometry analysis of HEK293T cells transfected with 3 µg of indicated expression vector (WT CCR5, ΔNT mutant, HMMM chimera) or control DNA (pCDNA3) (grey shading) and 0.5 µg of pEGFP-N1, after staining with the 3A9 or 2D7 anti-CCR5 mAbs, and phycoerythrin (PE)-coupled secondary antibody. PE fluorescence was analyzed for GFP-positive cells. (B) Same experiment in HeLa-P4 cells transfected with equal amounts (1.5 µg) of WT CCR5 vector or HMMM vector and either ΔNT CCR5 vector or pCDNA3.

Fig. 3. Trans-complementation of CCR5 mutants for HIV-1 receptor activity. (A) Syncytium-formation assays between transfected HeLa-P4 cells and Hela-Env/ADA cells and (B) infections of with R5 HIV-1 strains were performed and monitored as in Fig. 1. CCR51-100 is a truncated form corresponding to residues 1-100. HMMM and 5444 are chimeric receptors with N-terminal domain from human CCR5 and other domains from mouse CCR5 and human CXCR4, respectively.
Fig. 4. Stoichiometry of the cooperation between different forms of CCR5. (A) Relative concentrations of homodimers and heterodimers as function of the relative concentration of two proteins, assuming complete dimerization. (B) HIV-1 receptor activity measured by number of syncytia formed with Hela-Env/ADA cells for cells transfected with variable amounts of CCR5 expression vector (0 to 3µg) and either pCDNA3 or ΔNT CCR5 vector, so that a total amount of 3 µg of DNA was transfected. Results (means of three independent assays with standard error) are shown as percentage, relative to HeLa-P4 cells transfected with 3µg of WT CCR5. Dotted line is the addition of curves corresponding to relative concentrations of one type of homodimers (p²) and heterodimers (2p(1-p)) in panel A. (C) Same experiment except that the WT CCR5 vector was replaced by HMMM vector. Dotted line is the curve corresponding to relative heterodimer concentration in panel A.

Fig. 5. Detection of CCR5 oligomers. (A) Immunoprecipitation (IP) and western blot of lysates from CEM cells (lane 1) or CEM-CCR5 cells (lanes 2-7). The 2D7 anti-CCR5 mAb (ECL2 epitope) was used for IP and the 3A9 mAb (NT epitope) to reveal western blot. Lanes 3-6 correspond to cells treated with 100 nM MIP-1β for indicated time. Sample in lane 7 was loaded in the presence 4M urea. Arrows indicate expected size for CCR5 monomers and dimers. The band at ~55 K corresponds to the immunoglobulin heavy chain detected by the secondary antibody. (B) Coimmunoprecipitation of MYC- and FLAG-tagged forms of CCR5. The 9E10 anti-MYC mAb was used for IP of lysates from HEK293T cells cotransfected with equal amounts of indicated vectors. Western blot was revealed with the M2 anti-FLAG mAb (left panel) or with the 9E10 mAb after stripping (right panel). (C) Coimmunoprecipitation of HMMM and ΔNT CCR5. Lysates from HEK293T cells transfected with 3 µg of WT CCR5, ΔNT CCR5 or HMMM vector (lanes 1-3), or cotransfected with equal amounts of ΔNT CCR5 or HMMM vectors (lane 4) or from equal numbers of independently transfected cells (lane 5) were subjected to IP with the 2D7 mAb and western blot revealed with the 3A9 mAb. Arrow indicates...
CCR5 monomers. The ~55 K band corresponds to the immunoglobulin heavy chains detected by the secondary antibody.

**Table I.** Relative cell surface expression of CCR5, CXCR4 and derivatives in transfected HEK293T cells. Flow cytometry analysis was performed as in Fig. 2.
Table I : M. Chelli and M. Alizon

|               | 2D7 | 3A9 | 12G5 |
|---------------|-----|-----|------|
| CCR5          | 100 | 100 | 0    |
| ΔNT           | 112 | 0   | 0    |
| HMIMM         | 3.2 | 89  | 0    |
| CXCR4         | 0   | 0   | 100  |
| 5444          | 0   | 52  | 89   |
Figure 1: M. Chelli and M. Alizon

A

![Graph A](https://via.placeholder.com/150)

Vector: (µg)

|                | CCR5 (3) | CCR5 (1.5) | ΔNT (1.5) | CCR5+ΔNT (1.5/1.5) |
|----------------|----------|------------|-----------|-------------------|
| Fusion events  |          |            |           |                   |

B

![Graph B](https://via.placeholder.com/150)

Vector: (µg)

|                | CCR5 (3) | CCR5 (1.5) | ΔNT (1.5) | CCR5+ΔNT (1.5/1.5) |
|----------------|----------|------------|-----------|-------------------|
| Infected cells (% of WT CCR5) |          |            |           |                   |

HIV-1 R5 strains:
- ADA
- YU-2
- JRCSF
**Figure 2:** M. Chelli and M. Alizon

**A**

|   | 2D7  | 3A9 |
|---|------|------|
| CCR5 | ![CCR5](image1) | ![CCR5](image2) |
| ΔNT | ![ΔNT](image3) | ![ΔNT](image4) |
| HMMM | ![HMMM](image5) | ![HMMM](image6) |

**B**

|   | 3A9 |
|---|-----|
| CCR5 | ![CCR5](image7) |
| CCR5+ΔNT | ![CCR5+ΔNT](image8) |
| HMMM | ![HMMM](image9) |
| HMMM+ΔNT | ![HMMM+ΔNT](image10) |
Figure 3: M. Chelli and M. Alizon

A

![Graph A showing fusion events](graph)

B

![Graph B showing infected cells](graph)
Figure 4: M. Chelli and M. Alizon

**A**

- Graph showing the proportion of different combinations.
- The equation $p^2 + 2p(1-p)$ is plotted.
- The graph includes data points for $(1-p)^2$, $p^2$, and $2p(1-p)$.

**B**

- Graph showing fusion efficiency (%) vs. fraction of transfected CCR5.
- Curves for CCR5+pcDNA3 and CCR5+ΔNT are plotted.

**C**

- Graph showing fusion efficiency (%) vs. fraction of transfected ΔNT.
- Curves for HMM+ΔNT and ΔNT are plotted.
Figure 5: M. Chelli and M. Alizon

A

CEM-CCR5

+MIP-1β

1' 5' 15' 30' Urea

B

MYC-CCR5

+ FLAG ΔNT CCR5 pcDNA3

MYC-CCR5

+ FLAG ΔNT CCR5 pcDNA3

IP anti-MYC
WB anti-FLAG

IP anti-MYC
WB anti-MYC

C

CCR5 ΔNT HMMM ΔNT+ HMMM ΔNT / HMMM

Mr(K)

1 2 3 4 5 6 7

36 49 60 79 171

Mr(K)

1 2 3 4 5

36 49 60 79 171
Rescue of HIV-1 receptor function through cooperation between different forms of the CCR5 chemokine receptor
Maurice Chelli and Marc Alizon

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