Determinants of the \textit{trans}-Dominant Negative Effect of Truncated Forms of the CCR5 Chemokine Receptor*

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The human immunodeficiency virus, type 1 (HIV-1) entry process is triggered by interaction between the viral envelope and a seven membrane-spanning domain receptor at the cell surface, usually the CCR5 chemokine receptor. Different naturally occurring mutations in the \textit{CCR5} gene abolish receptor function, the most frequent being a 32-nucleotide deletion resulting in a truncated protein (Δ32) lacking the last three transmembrane domains (TM5–7). This mutant is retained in the endoplasmic reticulum and exerts a \textit{trans}-dominant negative (TDN) effect on the wild type, preventing its exit from this compartment. This TDN effect is often considered as evidence for the oligomerization of CCR5 during transport to the cell surface. Here we use a genetic approach to define the structural determinants of the TDN effect of the Δ32 mutant. It was abolished by certain deletions and by mutations of cysteine residues preventing formation of a disulfide link between the first and second extracellular loops, suggesting that conformation of Δ32 is important for its interaction with CCR5. To circumvent this problem, we used chimeric forms of the Δ32 and wild type CCR5, consisting in substitutions with homologous domains from the mouse CCR5. All chimeric full-length receptors were expressed at the cell surface and were functional for interaction with HIV-1 or with a chemokine ligand, when assayed. The TDN effect was only observed if both the TM3 domain in CCR5 and the TM4 domain in Δ32 were from human origin, whereas the rest of the proteins could be from either origin. This suggests that the TDN effect involves some form of interaction between these transmembrane domains. Alternatively, but less likely to us, substitutions in TM4 could affect the conformation of CCR5 in the endoplasmic reticulum but not at the cell surface. However, that may be, it seems that the TDN effect of the Δ32 mutant has no bearing to the issue of CCR5 dimerization and to its possible role in the processing of the receptor to the cell surface.

Chemokines are a family of structurally related cytokines mediating cell activation and chemotaxis upon binding to receptors with a seven membrane-spanning domain (7TM)\textsuperscript{1}

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\textsuperscript{1} The abbreviations used are: \textit{7TM}, seven membrane-spanning domains; HIV-1, human immunodeficiency virus type 1; NT, amineterminal; ECL, extracellular loop; R5, CCR5-dependent; TDN, \textit{trans}-dominant negative; TM, transmembrane domain; mAb, monoclonal coupled to heterotrimeric G-proteins (reviewed in Refs. 1–3). Most chemokines can be classified in the CC or CXC subgroups depending upon the relative position of two conserved cysteines in their amino-terminal region, and the same terminology (CCR or CXCR) is used for their receptors. The presence of chemokine receptors at the surface of cells allows their infection by the human immunodeficiency virus, type 1 (HIV-1) or by related retroviruses. At least 10 chemokine receptors can confer permissivity to HIV-1 under experimental conditions (4), but only two, CCR5 and CXCR4, seem to be used by HIV-1 \textit{in vivo} (reviewed in Refs. 5–7). The interaction of CCR5 or CXCR4 with the HIV-1 surface envelope glycoprotein gp120 usually occurs after a prior contact of gp120 with another cell surface protein, CD4, leading to a view of chemokine receptors as CD4-associated HIV-1 co-receptors. The selectivity of HIV-1 strains for CCR5 or for CXCR4 determines biological properties, in particular cell tropism, and could also influence the evolution of infection \textit{in vivo}. Indeed, viral strains able to use CXCR4 (termed X4) or both CCR5 and CXCR4 (R5X4 strains) usually emerge at later stages of infection, whereas strictly CCR5-dependent strains (R5) are present throughout infection (7).

Genetic defects resulting in the absence of CCR5 expression are relatively frequent and apparently without pathological consequences (8, 9). Infection by HIV-1 is usually not seen in CCR5-negative individuals, although exceptions have been reported (10–12). The most frequent mutation in the \textit{CCR5} gene associated with a loss of function is a 32-nucleotide deletion (Δ32) in the open reading frame yielding a truncated protein with only four transmembrane domains (8, 9, 13). The level of CCR5 expression at the cell surface was found to be highly reduced in the blood lymphocytes of individuals heterozygous for the \textit{CCR5} Δ32 allele (Δ32/) (14, 15) and in cells co-transfected with expression vectors for the wild type (WT) CCR5 and the Δ32 mutant (9, 16). The Δ32 mutant is not processed to the cell surface and accumulates with the WT receptor in a compartment likely to be the endoplasmic reticulum (16). The \textit{trans}-inhibitory effect of the Δ32 mutant on the expression of WT CCR5 was proposed to result from the formation of heterodimers unable to undergo normal processing to the cell surface. This could also represent an indirect argument for the ability of CCR5 to form homo-oligomers and for the role of this process in the routing of the receptor (16, 17), because it is the case for a number of other cell surface proteins (18). There is growing evidence that \textit{7TM} receptors can undergo oligomerization, but the relevance of this process to their natural function, \textit{i.e.} ligand binding and signal transduction, is unclear (19–21). Oligomers of \textit{7TM} receptor, usually dimers, have been directly detected by co-precipitation usually after antibody; WT, wild type; X4, CXCR4-dependent; GFP, green fluorescent protein; PBS, phosphate-buffered saline; Ab, antibody.
stimulation of cells with the cognate ligand or indirectly for example by energy transfer techniques (22). The dimerization of 7TM receptors is also suggested by the trans-complementation of two defective mutant (23–26) and by the trans-inhibitory effect of certain mutants (27–29). In the case of the chemokine receptors CCR2, CXCR4, and CCR5, dimerization has been directly observed in cells stimulated by specific ligands, either chemokines or antibodies (30–35). Spontaneously formed CXCR4 dimers have also been observed in macrophages and proposed to play a role in the resistance of these cells to infection by X4 HIV-1 strains (36).

Here we have used a genetic approach to study the trans-inhibitory effect of the Δ32 CCR5 mutant on the expression of the WT receptor. Our aim was to define the structural requirements for this effect and hence for the interaction of these forms of CCR5. Determinants for this interaction were located in different TM domains of the mutant and the WT receptor. A variant of CCR5 fully resistant to the trans-inhibitory effect was apparently processed normally to the cell surface and was functional for interaction with a chemokine ligand. This suggests that the interaction between the Δ32 mutant and the WT CCR5 does not mimic the formation of CCR5 homo-oligomers or that CCR5 oligomerization is not required for processing to the cell surface.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents—All cell lines were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin). The HeLa-P4 (37) and U373MG-CD4 cell lines (38) are stably transfected HeLa-P4 (37) and U373MG-CD4 cell lines (38) are stably transfected with a HIV-1 integrase reporter gene (LTR-lacZ) induced upon fusion with the transactivator Tat, such as the HeLa-Env/LAI (39) and HeLa-Env/ADA (40) cell lines stably express the envelope glycoproteins (Env) from an X4 strain and an R5 strain, respectively. The anti-CCR5 monoclonal antibodies (mAb) 2D7 (41) and 3A9 (14) and peroxidase-conjugated anti-mouse IgG were obtained from Pharmingen (San Diego, CA), the anti-FLAG mAb M2 and its agarose-coupled derivative were from Sigma, and the phycoerythrin- and fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulins were from Dako (Glostrup, Denmark). The MIP1β chemokine was purchased from Peprotech, Inc.

Plasmid Vectors—All WT and mutant chemokine receptors cDNAs were subcloned downstream to the cytomegalovirus immediate-early promoter. Site-directed mutagenesis (42) was performed on a single-stranded template corresponding to the human or the mouse CCR5 promoter. Site-directed mutagenesis (42) was performed on a single-stranded template corresponding to the human or the mouse CCR5 open reading frame. The Stranded template corresponding to the human or the mouse CCR5 promoter. Site-directed mutagenesis (42) was performed on a single-stranded template corresponding to the human or the mouse CCR5 open reading frame. The Plasmid Vectors—All WT and mutant chemokine receptors cDNAs were subcloned downstream to the cytomegalovirus immediate-early promoter. Site-directed mutagenesis (42) was performed on a single-stranded template corresponding to the human or the mouse CCR5 promoter. Site-directed mutagenesis (42) was performed on a single-stranded template corresponding to the human or the mouse CCR5 open reading frame. The Stranded template corresponding to the human or the mouse CCR5 open reading frame. The Plasmid Vectors—All WT and mutant chemokine receptors cDNAs were subcloned downstream to the cytomegalovirus immediate-early promoter. Site-directed mutagenesis (42) was performed on a single-stranded template corresponding to the human or the mouse CCR5 open reading frame. The Stranded template corresponding to the human or the mouse CCR5 open reading frame. The Plasmid Vectors—All WT and mutant chemokine receptors cDNAs were subcloned downstream to the cytomegalovirus immediate-early promoter. Site-directed mutagenesis (42) was performed on a single-stranded template corresponding to the human or the mouse CCR5 open reading frame. The Stranded template corresponding to the human or the mouse CCR5 open reading frame. The Plasmid Vectors—All WT and mutant chemokine receptors cDNAs were subcloned downstream to the cytomegalovirus immediate-early promoter. Site-directed mutagenesis (42) was performed on a single-stranded template corresponding to the human or the mouse CCR5 open reading frame. The Stranded template corresponding to the human or the mouse CCR5 open reading frame. The Plasmid Vectors—All WT and mutant chemokine receptors cDNAs were subcloned downstream to the cytomegalovirus immediate-early promoter. Site-directed mutagenesis (42) was performed on a single-stranded template corresponding to the human or the mouse CCR5 open reading frame. The Stranded template corresponding to the human or the mouse CCR5 open reading frame. The Plasmid Vectors—All WT and mutant chemokine receptors cDNAs were subcloned downstream to the cytomegalovirus immediate-early promoter. Site-directed mutagenesis (42) was performed on a single-stranded template corresponding to the human or the mouse CCR5 open reading frame. The Stranded template corresponding to the human or the mouse CCR5 open reading frame. The Plasmid Vectors—All WT and mutant chemokine receptors cDNAs were subcloned downstream to the cytomegalovirus immediate-early promoter. Site-directed mutagenesis (42) was performed on a single-stranded template corresponding to the human or the mouse CCR5 open reading frame. The Stranded template corresponding to the human or the mouse CCR5 open reading frame.

RESULTS AND DISCUSSION

trans-Inhibition of CCR5 Expression—the expression of CCR5 at the surface of transfected HeLa-P4 cells was analyzed by flow cytometry after staining with the 2D7 mAb directed against a conformational epitope formed at least in part by the second extracellular loop (ECL2) of the receptor (41). As expected, the fraction of stained cells and mean fluorescence intensities were markedly reduced when cells were co-transfected with expression vectors for the WT CCR5 and the Δ32 CCR5 mutants in equal amounts, relative to cells transfected with the WT CCR5 vector and a control plasmid (Fig. 1A). Similar results were obtained using other cell types (COS, HEK, and U373MG-CD4 cells) or when cells were stained with the 3A9 mAb, which detects a conformation-independent epitope in the amino-terminal (NT) extracellular domain of CCR5 (data not shown). When cells co-transfected with the WT CCR5 and Δ32 vectors were stained with the 2D7 mAb, confocal microscopy revealed accumulation of CCR5 in a perinuclear compartment probably corresponding to the endoplasmic reticulum (Fig. 1B), in agreement with results obtained by Benkirane et al. (16).
The level of expression of CCR5 at the surface of cells can be indirectly assessed from their relative permissivity to HIV-1 infection or to formation of syncytia with cells expressing the HIV-1 envelope proteins (Env). Fusion of U373MG-CD4 human astrogloma cells transfected with CCR5 vectors and HeLa-Env/ADA cells (R5 strain) can be readily detected and quantitated by means of a simple β-galactosidase assay (40). The efficiency of fusion was markedly lower for U373MG-CD4 cells co-transfected with WT and ΔH900432 CCR5 vectors (1:1) relative to cells transfected with the same amount of the WT vector (35%; Fig. 2A). There was no detectable fusion when cells were transfected only with the ΔH900432 vector. Also, co-transfection with this vector had no apparent effect on the expression of the CXCR4 chemokine receptor measured by its HIV-1 co-receptor activity (Fig. 2B) or on the surface expression of a number of other markers, including CD4 (data not shown). These results seem to rule out an effect of the ΔH900432 mutant on the machinery responsible for the routing of proteins to the cell surface.

The TDN effect of the Δ32 mutant on the HIV-1 co-receptor activity of CCR5 could be observed using different ratios between transfected expression vectors (Fig. 2C), even when the WT CCR5 plasmid was in excess (5:1). Such an assay can be used to predict the stoichiometry of the interaction between a functional protein and a nonfunctional mutant, assuming a similar efficiency of expression for both plasmids, and assuming that the heterodimers are not functional (47, 48). With these assumptions, the co-receptor activity curve was very close to the profile predicted in the case of a 1:1 interaction between the WT CCR5 and Δ32 (Fig. 2C). Overall, these initial experiments confirmed the view that the Δ32 mutant and the WT CCR5 form complexes unable to reach the plasma membrane.

**Requirements in ΔH900432 CCR5 for the TDN Effect**—We next engineered a series of deletions in the ΔH900432 mutant and assessed their effects on the surface expression of CCR5, judged from the ability of cells to engage fusion with EnvΔH11001 cells (Fig. 3). There was a similar decrease in the efficiency of cell fusion when U373MG-CD4 cells were co-transfected with WT and Δ32 CCR5 vectors (1:1) relative to cells transfected with the same amount of the WT vector (−35%; Fig. 2A). There was no detectable fusion when cells were transfected only with the Δ32 vector. Also, co-transfection with this vector had no apparent effect on the expression of the CXCR4 chemokine receptor measured by its HIV-1 co-receptor activity (Fig. 2B) or on the surface expression of a number of other markers, including CD4 (data not shown). These results seem to rule out an effect of the Δ32 mutant on the machinery responsible for the routing of proteins to the cell surface.

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**Fig. 1. Inhibition of CCR5 surface expression.** A, flow cytometry analysis of HeLa-P4 cells co-transfected with GFP, CCR5, and either Δ32 or pCDNA3 vectors (0.25:1.5:1.5) after staining with the 2D7 anti-CCR5 mAb and phycoerythrin-coupled secondary Ab or with secondary Ab only (shaded curve). The red fluorescence analysis was performed on the GFP-positive fraction. B, confocal microscopy analysis of HeLa-P4 cells transfected with the Δ32 expression vector alone (panel a), CCR5 and Δ32 vectors (1:1, panel c), or CCR5 vector alone (panel b) after staining with 2D7 and fluorescein isothiocyanate-coupled secondary Ab.
The in-frame deletion of the TM1 domain (deletion of residues 27–56) reduced but did not abolish the TDN effect of the Δ32 mutant, which was the case upon truncation at the level of ECL1 (mutant 1–100), TM3 (mutant 1–127), or ECL2 (mutant 1–176). The result seen with the last mutant was unexpected, because it only differed from the 1–184 mutant by a few residues in an extracellular loop. To rule out the possibility that the lack of TDN effect was due to insufficient expression, HEK 293T cells were transfected with epitope-tagged forms of the Δ32 mutant and derivatives, corresponding to in-frame insertion of a FLAG sequence at their N terminus. Immunoprecipitations with anti-FLAG antibodies showed a similar level of expression for the Δ32, 1–178, and 1–100 CCR5 mutants in cell lysates (Fig. 4). We were therefore led to consider that the integrity of the ECL2, or in fact its initial nine residues, was required for the TDN effect of the Δ32 mutant.

The first and second extracellular loops of 7TM receptors contain conserved cysteine residues (Cys\(^{101}\) and Cys\(^{178}\)) in the case of CCR5 capable of engaging a disulfide bridge (49, 50). We envisioned that the absence of this bridge could be detrimental to the TDN effect of the 1–178 mutant and other truncated forms of CCR5, for example by impairing their spatial structure. The mutation of either the ECL1 cysteine (C101A) or the ECL2 cysteine (C178A) into alanine was indeed sufficient to abolish the TDN activity of the 1–176 mutant (Fig. 3), indicating that the ECL2 residues upstream of the C101A mutation were sufficient to restore the TDN activity of the Δ32 mutant.

Interestingly, a TDN effect similar to that of the Δ32 mutant was observed when both cysteine residues or the putative disulfide link were simultaneously mutated (C101A/C178A). We therefore considered that the 1–176 mutant might be capable of engaging a disulfide bridge (Fig. 3).

The C101A/C178A mutation was sufficient to restore the TDN activity of the 1–176 mutant (Fig. 3), indicating that the ECL2 residues present in the Δ32 mutant (177–184) were not directly required for the TDN effect. These results suggest that the presence of a single cysteine, either in ECL1 or in ECL2, in the context of
Δ32 and other CCR5 mutants indirectly affect the TDN activity, most likely by modifying the conformation of the protein. A possible mechanism could be the engagement of Cys101 (or Cys176) into inadequate disulfide links, either intrachain with Cys20 in the NT domain or with other protein chains. The C101A mutation did not restore TDN activity for the 1–126 mutant (Fig. 3). It shows that residues 127–176, comprising in particular the TM3 and TM4 domains, directly contribute to the TDN activity of the Δ32 mutant and therefore to its interaction with CCR5.

We sought to further define the interface of interaction by assaying the TDN activity of chimeric proteins corresponding to reciprocal substitutions between the Δ32 mutant and the mouse CCR5. We indeed observed that co-expression with a mouse CCR5 mutant equivalent to Δ32 (frameshift in ECL2 downstream to the conserved cysteine) had no inhibitory effect on the HIV-1 co-receptor activity of human CCR5 (Fig. 5) or its cell surface expression measured by flow cytometry (data not shown). This mouse CCR5 mutant (MΔ32) seems therefore unable to interact with the WT human CCR5, despite a relatively high level of sequence identity (83%). An epitope-tagged form of MΔ32 was readily detected by immunoprecipitation (Fig. 4). Chimeric human/mouse forms of Δ32 were co-expressed with WT human CCR5 in HeLa-P4 cells and fusion assays performed with Env+ cells (Fig. 5). There was no inhibitory effect for chimeras Δ32.A, Δ32.B, and Δ32.C, and there was the same high efficiency of fusion with the latter two chimeras. In contrast, there was a reduction in fusion efficiency similar to that induced by the human Δ32 for chimeras Δ32.D, Δ32.E, and Δ32.F, indicating that sequence differences between human and mouse Δ32 in the region corresponding to residues 1–127 (TM1–3) have no apparent role. Consequently, residues 128–176 of human Δ32, containing TM4 and the adjacent intracellular (i2) and extracellular loops (ECL2) seem to be involved in the interaction with CCR5. The analysis of this region was refined, and the minimal region of human CCR5 sufficient to confer TDN activity in the MΔ32 context was contained in 25 residues from TM4 and 4 residues from ECL2 (chimera Δ32.H). The reciprocal substitution (chimera Δ32.G) was sufficient to suppress the inhibitory activity of human Δ32. Epitope-tagged forms of the Δ32.G and Δ32.H constructs were expressed at similar levels (Fig. 4). There are only four differences in TM4 and ECL2 supporting the phenotype difference between MΔ32 and the Δ32.H chimera (Fig. 5). Replacing any of these residues in the MΔ32 context by the corresponding human CCR5 residue was not sufficient to restore a TDN effect (data not shown). Overall, these experiments suggest that the TM4 domain of human Δ32 has a central role in the interaction with CCR5, explaining the lack of TDN activity of mutants 1–126 and 1–100.

Structural Requirements in CCR5—A similar strategy was used to define the interface of interaction in human CCR5. The mouse CCR5 cannot mediate fusion with Env+ cells, but chimeric human/mouse CCR5s bearing at least one extracellular domain from human CCR5 are endowed with such activity (43, 52). The ability of CCR5 chimeras to interact with the Δ32 mutant could therefore be functionally tested (Fig. 6). Chimera C in which ECL1 was the only extracellular domain derived from human CCR5 was less efficient at mediating cell fusion, but the number of syncytia was sufficient for a valid assay. Comparison of the results obtained with the B and F chimeras showed that the amino-terminal part of human CCR5, comprising in particular the TM1–3 domains, was required for a functional interaction with Δ32, whereas the rest of the receptor could be either from human or from mouse CCR5. The analysis of results obtained with chimeras C, E, and K allowed us to rule out a role for differences between human and mouse CCR5 in the TM1, TM2, and adjacent domains (NT, ECL1, and i1). The sensitivity to the TDN effect observed for the I but not the F chimera CCR5 indicated the importance of the TM3 domain. Conversely, the substitution of the mouse TM3 in the human CCR5 rendered chimera K resistant to the inhibitory effect of Δ32 (fusion efficiency was 89%). The TM3 domain of CCR5 could therefore be part of the interface between this receptor and the Δ32 mutant. This domain is relatively divergent, with
eight differences between the human and murine CCR5 sequences. In particular, we note the absence in the mouse CCR5 of the LXXXGXXXG motif proposed to play a role in the oligomerization of other receptors (53). We note that the divergence between human and mouse CCR5 in TM3 had no apparent role in the lack of interaction of M/H900432 with human CCR5, which confers further validity to our experiments with M/H900432 chimeras. Overall, these results are consistent with the view that the M/H900432/CCR5 interaction occurs through contact of non-homologous transmembrane domains (TM3 in CCR5 and TM4 in M/H900432). Similar observations have been made for the oligomerization of other 7TM receptors (20).

An alternative possibility is that the conformational forms of chimeric CCR5 differ from that of the wild type receptor, thereby preventing interaction with the Δ32 mutant in the endoplasmic reticulum but allowing exit from the compartment and transport to the cell surface. This possibility would be difficult to confirm, and we are not aware of comparable findings for other 7TM receptors. On the contrary, mutations that impair protein folding are well known to prevent their exit from the endoplasmic reticulum (54–56), even if examples of pharmacological rescue have been (57, 58). Thus, the conformational effects should selectively occur for chimeric CCR5 that contain the mouse TM3 domain, which as previously noted, has no apparent effect in the chimeric forms of the M/H900432 mutant.

Surface Expression and Function of a Δ32-resistant CCR5 Mutant—The minimal mutant form of CCR5 fully resistant to the inhibitory activity of Δ32 (chimera K) was studied in more detail for its processing and function. Flow cytometric analysis of transfected cells showed that it was expressed at the same level as the WT CCR5 at the cell surface (Fig. 7), which was consistent with the results obtained in cell fusion assays (Fig. 6).
6). Treatment of cells expressing either the K chimera or the WT CCR5 with the MIP-1β chemokine resulted in similar down-regulation of cell surface expression (Fig. 7), indicating that both forms of CCR5 were functional in terms of ligand binding and interplay with the receptor endocytosis machinery (59). It seems, therefore, that the ability of CCR5 to interact with Δ32 is independent of its ability to be transported to the cell surface in a functional form. Either CCR5 does not require oligomerization for normal processing or the formation of CCR5 oligomers involves a type of interaction different from the CCR5/Δ32 interaction. Our experiments do not allow us to distinguish between these possibilities. However, our results suggest that the interaction of the Δ32 mutant with CCR5 does not relate to “natural” oligomerization of 7TMR receptors but rather to the inhibition of their processing and/or function by peptides corresponding to their membrane-spanning domains (53, 60, 61). These observations strengthen the view that the tridimensional barrel-like structure of 7TM receptors, which must involve interactions between membrane-spanning domains, is relatively open and flexible (41). Disruption of this structure by agents mimicking the activity of peptides or truncated receptors could be a means of pharmacological intervention.

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