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## **Identification of a Post-endocytic Sorting Sequence in CCR5**

**Maurine Delhaye, Audrey Gravot, Diana Ayinde, Florence Niedergang,**

**Marc Alizon and Anne Brelot**

**Institut Cochin, Université Paris Descartes, CNRS (UMR 8104), Paris, France;**

**INSERM, U567, Paris, France (MD, AG, DA, FN, MA, AB).**

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Address correspondence to:

Anne Brelot, Institut Cochin, U567, département de biologie cellulaire,

22 rue Méchain, 75014 Paris, France,

Tel: +33-1 40 51 64 40, Fax: +33-1 40 51 64 54; E-Mail: [brelot@cochin.inserm.fr](mailto:brelot@cochin.inserm.fr)

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$\beta$ 2AR,  $\beta$ 2-adrenergic receptor; CCR2b, CC chemokine receptor 2b; D1R, D1 dopamine receptor;  $\delta$ OR, delta opioid receptor; ET<sub>A</sub>, endothelin receptor; GASP, G protein-coupled receptor-associated sorting protein; GluR2, glutamate receptor 2; GPCR, G protein-coupled receptor; GRIP, glutamate receptor-interacting protein; GRK, G protein-coupled-receptor kinase; hLHR, human lutropin receptor; mGluR7, metabotropic glutamate receptor 7;  $\mu$ OR, mu-opioid receptor; NHERF/EBP50, sodium-hydrogen exchange regulatory factor/ezrin-radixin-moesin-binding phosphoprotein of 50 kDa; NSF, N-ethylmaleimide-sensitive factor; PAR1, protease-activated receptor 1; PDZ, PSD-95/discs large/ZO-1; PDZK1, PDZ protein expressed in kidney 1; PICK-1, protein interacting with protein kinase C; PIST, PDZ protein interacting specifically with Tc10; SNX1, sorting nexin 1; SSTR5, somatostatin receptor 5.

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### Abstract

The chemokine receptor 5 (CCR5), a member of the G protein-coupled receptor family (GPCR), is used by human immunodeficiency virus type 1 (HIV-1) with a R5 tropism as an entry receptor in addition to CD4. It is a key target for an antiviral action aiming at inhibiting the HIV-1 entry process. Only few data are available today regarding the mechanism involved in the intracellular trafficking process of CCR5. Understanding how CCR5 cell surface expression is regulated is particularly important in regards to HIV-1 entry inhibition. We set out to investigate whether CCR5 molecular determinants were involved in the post-endocytic recycling and degradative pathways. We constructed progressive deletion mutants of the C-terminal domain of CCR5 that we stably expressed in HEK293 cells. All of the deletion mutants were expressed at the cell surface and were functional HIV-1 receptors. The deletion mutants were internalized after stimulation, but they lost their ability to recycle to the plasma membrane. They were re-routed towards a lysosomal degradative pathway. We identified here a sequence of 4 amino acids, present at the extreme C-terminus of CCR5, which is necessary for the recycling of the internalized receptor, independently of its phosphorylation. A detailed analysis of this sequence indicated that the 4 amino acids acted as a PDZ interacting sequence. These results show that the CCR5 cytoplasmic domain bears a sequence similar to the “recycling signals” previously identified in other GPCRs. It is likely that drugs able to disrupt the recycling pathway may constitute promising tools for therapeutic treatment.

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The chemokine receptor 5 (CCR5) belongs to the superfamily of seven transmembrane domains proteins, coupled to the heterotrimeric G proteins (GPCR) (Lefkowitz, 2004). This receptor is mainly expressed on a subset of leukocytes that are recruited to sites of inflammation in response to its ligands including Rantes (CCL5), Mip-1 $\alpha$  (CCL3), Mip-1 $\beta$  (CCL4) and MCP-2 (CCL8) (Moser et al., 2004). CCR5 stimulation triggers signal transduction cascades that promote a proper cell response. The endocytosis and post-endocytic trafficking of CCR5 represent mechanisms that regulate such a response. Detailing these intracellular trafficking mechanisms is particularly important in regards to HIV-1 entry inhibition. Together with CD4, CCR5 is used as a cell entry portal for the human immunodeficiency virus type 1 (HIV-1) with a R5 tropism (Lederman et al., 2006). It is a key target for an antiviral action aiming at inhibiting the HIV-1 entry process (Princen and Schols, 2005). Some antiviral compounds, derived from chemokines, seem especially efficient. After interacting with CCR5, these compounds induce a sequestration of the receptor from the cell surface and thus prevent HIV-1 infection (Hartley et al., 2004). However, the protective mechanism of such compounds is not fully understood yet, mainly due to our lack of knowledge of the CCR5 intracellular trafficking mechanism.

Like many GPCRs, CCR5 undergoes endocytosis after agonist-induced activation. After chemokine binding, CCR5 is phosphorylated on the C-terminal serine/threonine, leading to its desensitisation and its internalization (Oppermann, 2004). The phosphorylated amino acids are potential binding sites for  $\beta$ -arrestins, intracellular adaptors involved in the endocytosis process (Oppermann, 2004). The internalization of CCR5 depends on clathrin-mediated endocytic pathway even if some studies mention the use of the caveolae-dependent pathway in some cells (Mueller et al., 2002; Signoret et al., 2005; Venkatesan et al., 2003).

After interacting with its natural ligands, the internalized receptor is accumulated in recycling endosomes and then recycled back to the plasma membrane, where it becomes functional for a new round of stimulation (Signoret et al., 2000). Contrary to other GPCRs, like the prototype  $\beta$ 2 adrenergic receptor ( $\beta$ 2AR), CCR5 seems recycled independently of endosomes acidification (Signoret et al., 2004).

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However, only few data are available today regarding the mechanism involved in this recycling process. Initial studies on many cell surface receptors such as the transferrin receptor suggest that receptor recycling occurs « by default » following the bulk membrane flow (Gruenberg, 2001). However, this suggestion has been challenged recently for some GPCRs. The group of M. von Zastrow first showed that the C-terminal domain of  $\beta$ 2AR possesses a “sorting sequence” that is necessary for recycling. This sequence, a type I PDZ ligand, could interact with the PDZ domain-containing protein NHERF/EBP50 and promote the recycling of  $\beta$ 2AR (Cao et al., 1999). Since these findings, several studies report that the cytoplasmic domain of GPCRs could similarly own a “sorting sequence” that dictates the route followed by the internalized receptor (Tsao and von Zastrow, 2001). The interaction of this sequence with particular sorting proteins could route the receptor towards either a recycling or a degradative pathway. This leads either to a recovery of cell responsiveness or to a signal attenuation.

Depending on the GPCRs, the “sorting sequence” involved in receptor post-endocytic trafficking is divergent in both its primary structure and its location within the C-terminus (Cao et al., 1999; Paasche et al., 2005; Tanowitz and von Zastrow, 2003; Vargas and Von Zastrow, 2004). Likewise, the sorting protein is also different from one GPCR to another (Cao et al., 1999; Wentz et al., 2005; Whistler et al., 2002). Many PDZ domain-containing proteins interact with GPCR C-termini (Bockaert et al., 2003); some of them have been identified as being involved in GPCR post-endocytic trafficking (Trejo, 2005). However, any correlation between the presence of a PDZ-interacting sequence (a PDZ ligand) in the receptor and the fate of the receptor has not been shown yet. At its extreme C-terminus, CCR5 possesses such a PDZ ligand, but it is not established whether this PDZ ligand plays any role in controlling the membrane trafficking of CCR5.

Here, we sought to characterize the post-endocytic mechanism of CCR5 by investigating the molecular determinants present in its C-terminal domain. We looked at the intracellular trafficking of both progressive truncation mutants and substitution mutants of the C-terminal domain. We identified that the C-terminal tip of CCR5 possesses a sequence necessary for recycling, which acts as a PDZ ligand.

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Our study strengthened the key role played by PDZ ligands in the intracellular sorting. It also reinforced the emerging concept that GPCR recycling is a regulated process.

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### Materials and methods

*Cell lines and chemokines*—The human cell lines U373MG-CD4 and HeLa-P4 stably transfected with *Escherichia coli lacZ* under the transcriptional control of the HIV-1 long terminal repeat (LTR) have been described (Clavel and Charneau, 1994; Harrington and Geballe, 1993) as well as the HeLa-Env/ADA cell lines stably expressing the envelope glycoproteins (Env) from an R5 HIV-1 strains (Pleskoff et al., 1997). Stably transfected human embryonic kidney HEK293 cells expressing FLAG-tagged CCR5 were generated by calcium phosphate coprecipitation of the appropriate constructs followed by culture of cells for several weeks in 1 mg/ml G418 (Geneticin; Invitrogen). Cell clones were screened by flow cytometric assay to specifically detect FLAG-tagged receptors using the M2 anti-FLAG monoclonal antibody (Sigma). Cell clones exhibiting comparable amounts of cell surface receptors were used in the following experiments. These cells were grown in Dulbecco modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and antibiotics. Human peripheral blood T lymphocytes (PBTs) were isolated by Ficoll density gradient centrifugation from healthy blood donors obtained from Etablissement Français du Sang (EFS) (Rungis, France) in accordance with a convention signed between the Institut Cochin and EFS. Peripheral blood mononuclear cells (PBMCs) were depleted of erythrocytes, platelets and peripheral leukocytes that are not T lymphocytes with a 'cocktail' of monoclonal biotin-conjugated anti-CD11b/Mac1, CD16, CD19, CD36, CD41, CD56 and CD235a antibodies (Human T lymphocyte enrichment set- DM; BD Biosciences). Human PBTs and T cell lines (MOLT-4 and Jurkat) were transfected with expression vectors for CCR5 and green fluorescent protein (GFP) (pEGFP-N1 vector; Clontech) (ratio 6:1) using Amaxa nucleofector technology (Amaxa) according to the manufacturer's instructions and were used 18 hours after transfection. These cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics. The chemokines Mip-1 $\beta$  (CCL4) and Rantes (CCL5) were purchased from Peptotech (Tebu-bio).

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*cDNA constructs*—The human CCR5 cDNA and derived mutants were subcloned downstream to the cytomegalovirus immediate-early promoter in the expression vectors pcDNA3-FLAG (Clontech). The epitope-tagged receptor has a 23-amino acid sequence corresponding to the sequence **MKTIIASYIFCLVFADYKDDDA**, containing the epitope of the M1 and M2 monoclonal antibodies (MAbs) at its amino-terminus. The deleted CCR5 C-terminal mutants were obtained by introducing a stop codon at positions 336, 341 and 349, relative to the initial ATG codon, by site-directed mutagenesis on a single-stranded human CCR5 template. Site-directed mutagenesis was also performed to obtain CCR5-ala (addition of a single alanine to CCR5 C-terminus), S349A and L352A (substitution of S349 and L352 by an alanine), S349E (substitution of S349 by a glutamic acid) as well as CCR5-4A (substitution of the last 4 amino acids of CCR5 by alanine residues). Mutants were screened for the creation of restriction enzyme sites and checked by sequencing. Deletions are depicted in Fig. 1A.

*Detection of receptor expression at the cell surface*—HEK293 (or HeLa-P4) cells were transfected with expression vectors for CCR5 and green fluorescent protein (GFP) (pEGFP-N1 vector; Clontech) (ratio 6:1) by calcium phosphate precipitation. Cells were detached with phosphate-buffered saline (PBS) containing 1 mM EDTA and pelleted 36 hours after transfection. About  $10^6$  cells were incubated for 1 hour at 4°C with the anti-FLAG M2 monoclonal antibody (Sigma) at a concentration of 10 µg/ml (or with 1 µg/ml anti-CCR5 2D7-Becton-dickinson) in phosphate-buffered saline containing 2% bovine serum. Cells were washed three times and incubated for 1 hour with phycoerythrin-conjugated rabbit anti-mouse immunoglobulins (Dako). Stained cells were washed, fixed in 1.5% paraformaldehyde (PFA), and analyzed on a Cytomics™ FC 500 flow cytometer (Beckman Coulter).

To study CCR5 turnover, HEK293 stably expressing FLAG-CCR5 were grown on 12-well tissue dishes and incubated in pre-warmed medium containing 100 µM of cycloheximide. At the indicated times, culture dishes were placed on ice, and cells were washed in cold PBS. Cells were then detached, fixed in 4% PFA and stained as described above.



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*Coreceptor functional assays*—The U373MG-CD4 (or HeLa-P4) cells were transfected with wild type or mutant CCR5 expression vectors in 6-well culture dishes by calcium phosphate precipitation. Twenty-four hours after transfection, HeLa-Env/ADA cells were added to the culture dishes (1:1 ratio). After 24 hours of co-cultures, cells were fixed in 0.5% glutaraldehyde and stained for  $\beta$ -galactosidase activity with the X-Gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) substrate, as described previously (Dragic et al., 1992). Blue-stained foci were scored under a magnification of x 20.

*Fluorescence flow cytometric assay of receptor internalization and recycling*—Cells were grown on 12-well tissue culture dishes and incubated with a saturable amount of M1 antibody (5  $\mu$ g/ml) for 30 minutes to label receptors present in the plasma membrane, then incubated in the absence ('untreated' condition) or presence of 100 nM agonist for 90 minutes at 37°C. Untreated cells and agonist treated cells ('ago' condition) were then chilled to 4°C and washed with PBS containing 1 mM CaCl<sub>2</sub>. For the recycling experiments, additional dishes of agonist treated cells were washed with calcium-free PBS supplemented with 1 mM EDTA to dissociate antibodies from the receptors remaining in the plasma membrane. These latter agonist-treated dishes were either left at 4°C ('edta' condition), or washed and incubated in media for 30, 60 or 90 minutes at 37°C ('rec' condition). Cells were collected from dishes by gentle mechanical dissociation and approximately 2 x 10<sup>5</sup> cells were incubated for 1 hour at 4°C in 100  $\mu$ l of phosphate-buffered saline, 2% fetal bovine serum containing a phycoerythrin (PE)-conjugated goat anti-mouse IgG secondary antibody (Dako) and fixed in 1.5% paraformaldehyde (Sigma). Cells were analyzed for PE fluorescence using a Cytomics™ FC 500 flow cytometer (Beckman Coulter). Mean fluorescence intensities were determined from fluorescence histograms (representing analysis of 5,000 cells per data point) using FC 500 software. Mean values were used to compute the proportion of internalized receptors as indicated by a decrease of immunoreactive surface receptor with agonist compared to untreated cells:

$$\% \text{ of internalization} = 1 - \frac{\text{mean PE 'ago'}}{\text{mean PE 'untreated'}}$$

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Percentage of receptor recycling was the proportion of receptors that was recovered at the cell surface out

$$\text{of the internalized receptors: \% of recycling} = \frac{\text{mean PE 'rec'} - \text{mean PE 'edta'}}{\text{mean PE 'untreated'} - \text{mean PE 'ago'}} \times 100$$

The same protocol was used to study the recycling of CCR5 in human T cells (MOLT-4, Jurkat and PBT).  $10^6$  Amaxa transfected cells were used for each condition.

*Visualization of receptor trafficking by fluorescence microscopy*—Cells were grown on poly-D-lysine (Sigma) coated coverslips and incubated 30 minutes with 10  $\mu\text{g/ml}$  of anti-FLAG (M1) monoclonal antibodies (Sigma) and then incubated with 100 nM agonist (Mip-1 $\beta$  or Rantes) at 37°C for 90 minutes to drive the internalization of CCR5. A first set of coverslips was then washed with ice-cold PBS to remove residual agonist and immediately fixed, to determine total internalized receptors. A second set of coverslips was washed with PBS/EDTA to remove surface-bound antibodies and then fixed. A third set of coverslips, washed with PBS/EDTA, was incubated for the indicated times at 37°C with media to allow receptor recycling. Cells were fixed in 4% paraformaldehyde in PBS, pH 7.4 for 15 minutes and quenched with 3 washes of Tris-buffered saline (TBS) + 1 mM CaCl<sub>2</sub>. Specimens were permeabilized with 3% bovine serum albumin (Sigma), 0.15% TX-100 in TBS, and incubated with a Cy3-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch) to specifically detect labelled FLAG-CCR5. Epifluorescence microscopy was carried out using a 63X objective and standard filter sets on a Zeiss Axiovert200.

*Colocalization assay using confocal imaging*—Cells were grown on poly-D-lysine (Sigma) coated coverslips. After 30 minutes incubation with rabbit anti-FLAG antibody (5  $\mu\text{g/ml}$ , Sigma) at 37°C, treatments with Mip-1 $\beta$  were carried out during 90 minutes. These incubations were performed in the presence of leupeptin (400  $\mu\text{g/ml}$ ) to avoid any proteolysis of the FLAG epitope (Moore et al., 1999). Cells were fixed in 4% paraformaldehyde in PBS, pH 7.4, permeabilized with 3% bovine serum albumin

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(Sigma), 0.05% saponin (Sigma) in TBS, and incubated with a mouse anti-human lysosome-associated membrane protein 1 (LAMP1) monoclonal antibody (Beckton Dickinson). Then, the cells were washed and incubated with a Cy3-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch) and a Cy2-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch). They were visualized using a Leica SP2 confocal microscope (model DMIRE2) equipped with Ar and HeNe lasers. Optical sections were recorded with a 63X, 1.4 NA oil immersion lens.

*Cell surface biotinylation and assay of agonist-induced proteolysis*—Cells were grown on poly-D-lysine (Sigma) coated 6-well tissue culture dishes. Cell surface biotinylation was conducted by incubating monolayers with 300 µg/ml sulfo-NHS-biotin (Pierce) in phosphate-buffered saline, pH 7.4 (PBS) at 4°C for 30 minutes. Unreacted sulfo-NHS-biotin was quenched by 3 washes with ice-cold Tris-buffered saline pH 7.5 (TBS). Cells were incubated with media at 37°C for 4 hours in the absence or presence of 100 nM agonist (Mip-1β or Rantes) and in the absence or presence of 400 µg/ml leupeptin to block lysosomal degradation. They were then chilled on ice, washed 3 times with PBS and extracted with TX-100 extraction buffer (0.5% (v/v) TX-100, 10 mM Tris-HCl, pH 7.5, 120 mM NaCl, 25 mM KCl, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 2 µg/ml aprotinin, 2 µg/ml PMSF). Extracts were clarified by centrifugation in a microcentrifuge (14,000 x rpm for 15 minutes) prior to a Bradford analysis. Equal amounts of proteins were incubated with 25 µl of streptavidin beads (Pierce) overnight (O/N). Washed beads were extracted with SDS sample buffer and eluted proteins were resolved by SDS-PAGE. Resolved proteins were transferred to a PVDF membrane and blocked O/N at 4°C in 5% dry milk, 0.1% Tween-20 in TBS + 1 mM CaCl<sub>2</sub> (MTBST). Detection of receptors containing FLAG epitope was carried out by incubation of blots with M1 anti-FLAG antibody (15 µg/ml) in MTBST for 60 minutes. Blots were washed and incubated for another 60 minutes in MTBST containing 400 ng/ml HRP-coupled goat anti-mouse IgG (Jackson ImmunoResearch). After washing, proteins were detected by enzyme-linked chemiluminescence (ECL system, Amersham Life Sciences). A control of the quantity of proteins per lane was performed

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after an acid strip followed by incubation of blots with an avidin-HRP complex (Sigma). Band intensities were quantified by densitometry of films.

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### Results

Previous studies on GPCRs indicate that the sequences controlling the trafficking of internalized receptors are located at the C-terminal domain (Tsao and von Zastrow, 2001). We sought out to determine whether the C-terminal domain of CCR5 could similarly contain a sequence involved in its intracellular trafficking after agonist-induced endocytosis. For this purpose, we progressively truncated the C-terminal domain of CCR5 (Fig. 1A). The truncations were performed after a motif of palmitoylation that is required for the correct expression of the receptor at the cell surface (Percherancier et al., 2001). We constructed a series of deletion mutants in a N-terminal FLAG-tagged version of CCR5 by introducing stop codons at the following positions relative to the initial codon: 336 (CCR5-336), 341 (CCR5-341) and 349 (CCR5-349). The three resulting mutants lacked the 17, 12 and 4 last amino acids of CCR5 respectively (Fig. 1A). The tag introduced in the N-terminal part of CCR5 has no influence on the correct expression or function of the receptor (Chelli and Alizon, 2002).

#### *The deleted mutants are correctly express at the cell surface and are functional HIV-1 receptors*

These mutants, transiently transfected into human cell lines, were first examined for their correct expression at the cell surface and for their ability to mediate fusion with HIV-1 envelope expressing cells (see materials and methods). The cell surface expression of wild type (WT) and mutant CCR5 was monitored in HEK293 cells by flow cytometry after immunostaining. The transfected cells were stained with the anti-FLAG M2 monoclonal antibody as well as with the anti-CCR5 2D7 monoclonal antibody that recognizes a conformational epitope in the second extracellular loop (Lee et al., 1999). As shown in figure 1B, the extent of staining of the three mutants was at least 70% of the WT receptor. This suggested that the overall conformation of each receptor was not significantly altered. We observed similar results with the two monoclonal antibodies (Fig. 1B) and in different cell lines (data not shown).

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We tested the ability of CCR5 mutants to behave as HIV-1 receptors by expressing them in the CD4<sup>+</sup> human glioma cell line U373MG-CD4 (or the HeLa-P4 cell line). These cell lines are naturally resistant to fusion with cells that stably express the envelope glycoproteins Env/ADA (HeLa-Env/ADA cells) (Pleskoff et al., 1997). In addition, these cell lines were stably transfected with a Tat-inducible *lacZ* reporter gene (LTR*lacZ*). This allowed us to detect complementation for fusion with Tat<sup>+</sup> Env<sup>+</sup> cells by a simple in situ  $\beta$ -galactosidase assay, as described previously (Dragic et al., 1992). The expression of CCR5 WT or the three deleted mutants enabled CD4 cells to fuse with HeLa-Env/ADA cells (Fig. 1C). The deletions introduced in the C-terminal domain of CCR5 were thus fully compatible with its HIV-1 receptor activity, which confirms published results (Alkhatib et al., 1997).

### *The distal portion of the carboxy-terminal domain of CCR5 is not required for agonist-induced endocytosis of the receptor*

The C-terminal domain of GPCRs was shown to be involved in receptor endocytosis (Moore et al., 2007). We first tested whether the truncation of CCR5 prevented the ability of the receptors to undergo initial agonist-induced endocytosis. We examined the internalization of FLAG-tagged CCR5 WT and mutants stably expressed in HEK293 cells. These cells were incubated with a saturating amount of M1 antibody during 30 minutes at 37°C, then stimulated with 100 nM chemokine during the indicated times and finally analyzed by flow cytometry after staining with PE-coupled anti-mouse IgG antibodies (Fig. 2, A and B). We used the M1 antibody in this experiment since it neither induces the internalization of CCR5 nor prevents agonist binding (Fig. 3). After stimulation, the surface expression of FLAG-CCR5 WT showed a 30% decrease and reached a plateau (Fig. 2B). CCR5 was therefore internalized in response to stimulation by chemokines as previously shown in HEK293 cells (Venkatesan et al., 2003). Kinetic studies showed that loss of receptors was detectable after 5 minutes and reached a maximum after

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90 minutes (Fig. 2B). The plateau observed could correspond to a steady state reached between endocytosis and recycling (Koenig and Edwardson, 1997).

The endocytosis process of CCR5-336 was significantly altered compared to the WT receptor after agonist exposure (Fig. 2B). This might be due to the loss of 4 phosphorylable serines needed for  $\beta$ -arrestin binding (Oppermann, 2004). In contrast, the two mutants, CCR5-349 and CCR5-341, were correctly internalized but they never reached a plateau even after 60 minutes with Mip-1 $\beta$  (Fig. 2B). After 120 minutes, between 50% and 70% of the mutants disappeared from the cell surface compared to 30% for the WT receptor (Fig. 2B). Similar results were obtained after Rantes stimulation (data not shown). This effect could result either from an increase in receptor endocytosis or from an inhibition of rapid recycling of receptors even before agonist washout. We compared the internalization of FLAG-CCR5 WT and the two mutants (CCR5-349 and CCR5-341) at early time points after agonist addition when initial endocytosis is expected to be the major determinant of net internalization (Koenig and Edwardson, 1997). We observed the same internalization rate for the WT receptor and the two mutants (CCR5-349 and CCR5-341) in the first 10 minutes following the addition of ligands (Fig. 2C). This observation indicated that the deletion performed did not detectably increase the initial agonist-induced endocytosis of receptors but should inhibit the recycling of internalized receptors. Since the two mutants (CCR5-349 and CCR5-341) were efficiently internalized after stimulation, we could study their trafficking after internalization.

### *The last 4 amino acids of CCR5 C-terminus are essential for recycling*

We investigated whether the C-terminal deletions affected the trafficking of CCR5 after internalization. We used fluorescence microscopy in order to visualize the distribution of internalized receptors. Surface receptors were labelled with the calcium-dependent M1 anti-FLAG monoclonal antibody. Then we added 100 nM chemokine to the cell culture to drive the endocytosis of receptors. We

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next washed the cells in PBS/EDTA so as to remove surface-bound antibody. Finally, we incubated them in fresh medium at 37°C to allow CCR5 recycling. The reappearance of previously internalized receptor-bound antibody was detected 30 minutes after agonist removal.

Prior to chemokine addition, the FLAG-CCR5 WT and the aforementioned mutants were primarily localized at the plasma membrane (Fig. 3 a,e,i). After 90 minutes of stimulation by Mip-1 $\beta$ , the receptors moved into intracellular vesicles (Fig. 3 b,f,j). The FLAG-CCR5 WT was still noticeable at the cell surface, whereas the mutant receptors were almost completely redistributed from the plasma membrane to intracellular vesicles. This observation confirmed our previous result obtained in the internalization assay (Fig. 2). Fig. 3 c,g,k showed that M1 antibody was actually removed from the cell surface after a wash with PBS/EDTA. After agonist washout, FLAG-CCR5 WT returned to the cell surface almost completely within 30 minutes (Fig. 3d). In contrast, the mutant receptors remained in intracellular vesicles (Fig. 3 h,l). The same results were obtained for three different cell clones as well as by using transient expressing cells (data not shown). These results indicated that the truncation performed in the C-terminal part of CCR5 affected receptor trafficking by reducing its recycling. Based on the low recycling of the CCR5-349 mutant, we assumed that the last 4 amino acids of CCR5 (SVGL) were needed for efficient CCR5 recycling.

To quantify the effect of the C-terminal deletions on recycling, we next used a flow cytometric assay and followed previously internalized receptor-bound antibody. The recovery of receptor immunoreactivity to the cell surface was calculated from mean fluorescence intensities at different times after agonist washout (see materials and methods). The recycling proportion of FLAG-CCR5 WT reached 90%, 90 minutes after Mip-1 $\beta$  removal (Fig. 4B). As expected, FLAG-CCR5 mutant receptors recycled to a much smaller extent: 90 minutes after Mip-1 $\beta$  washout, we detected that only 5% to 10% of the internalized CCR5-341 and CCR5-349 recycled (Fig. 4, A and B). We obtained similar results after



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Rantes stimulation (data not shown). These results showed that the truncations of CCR5 profoundly impaired the recycling of the receptors and confirmed the observations obtained by microscopy. Therefore, our results suggested that the C-terminal SVGL motif of CCR5 played a key role in the recycling process.

To further confirm the role played by the last 4 amino acids of CCR5, we substituted them by 4 alanine residues (CCR5-4A). This mutant behaved like CCR5-349 when stably expressed in HEK293 cells. CCR5-4A was correctly expressed at the cell surface, functioned as a HIV-1 receptor and was highly internalized after chemokine stimulation (data not shown). In addition, this mutant lost its ability to recycle back to the cell surface (data not shown). These results reinforced the importance of the last 4 amino acids (SVGL) of CCR5 in its recycling.

Finally, CCR5 trafficking might be different according to the cell type considered (Koenig and Edwardson, 1996). To ensure that the results obtained with the CCR5 mutants are not dependent on the cell type, we compared the recycling of both FLAG-CCR5 WT and CCR5-349 when transiently expressed in T cell lines (MOLT-4 and Jurkat) and in human peripheral blood T cells (PBT). The data presented in Figure 4C showed that the recycling of the receptors internalized in these cells was similar to the one observed in HEK293 cells. The recycling process of CCR5 should use identical cellular machinery whatever the cell type.

### *Removal of the last 4 amino acids of CCR5 directs the receptor toward a degradative pathway*

We next set out to analyze the fate of the mutant receptors that exhibited a reduced recycling. Did they stay inside the cell for a longer time, or did they undergo a proteolytic degradation, as described for other receptors (Cao et al., 1999)? To answer this question, we first analyzed the turnover of FLAG-

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CCR5 WT and FLAG-CCR5 mutants at the cell surface of the HEK293 stably expressing cells in the absence of agonist. We treated the cells with cycloheximide during 9 hours and followed FLAG-CCR5 surface expression by FACS after immunostaining. The half-life times for CCR5-349 and for CCR5-341 were shorter compared to the half-life time for CCR5 WT (Fig.5 A). These results showed that the deletion of at least the last 4 amino acids significantly altered the turnover of CCR5.

To further investigate the fate of mutant receptors, we followed the degradation of biotinylated receptors (see materials and methods). After surface biotinylation, HEK293 cells were treated in the absence or continuous presence of Mip-1 $\beta$  for various times (1, 2, 4 hours) and lysed. Biotinylated proteins were pulled-down with streptavidin beads, and immunoblotted with anti-FLAG antibodies to detect the FLAG-CCR5 constructs. We observed an immunoreactive protein species of 40 kDa, corresponding to the mature CCR5 protein (Fig. 5B). In accordance with its efficient recycling, negligible proteolysis of wild type CCR5 was observed even after 4 hours of agonist exposure. In contrast, CCR5-349 was extensively proteolyzed 4 hours after agonist addition to the culture medium (Fig. 5, B and C). More than 50% of mutant receptors were proteolysed (Fig. 5C). Similar results were obtained after Rantes stimulation of CCR5-349 and CCR5-341 (data not shown). These results indicated that mutants, once they lost the ability to recycle, were re-routed towards a degradative pathway. To find out whether the observed proteolysis occurred through a lysosomal degradation mechanism, we performed the same experiment in the presence of the lysosomal protease inhibitor leupeptin (Moore et al., 1999). Following such a treatment, we did not observe any proteolysis of CCR5-349 after agonist stimulation (Fig. 5B, 349+leup). About 90% of biotinylated receptors were still detected after 4 hours (Fig. 5C). The same result was obtained for CCR5-341 (Fig. 5C) and after Rantes exposure (data not shown). This result suggested that the internalized mutant receptor was directed towards a lysosomal degradative pathway.

To further analyze whether the disruption of the last 4 amino acids leads the receptors to traffic to lysosomes, we followed CCR5 subcellular localization by confocal microscopy (Fig. 6). We treated

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FLAG-CCR5 WT and CCR5-349 expressing cells with Mip-1 $\beta$  in the presence of leupeptin to avoid any proteolysis of the FLAG epitope (Moore et al., 1999). Then, we visualized the localization of internalized CCR5 relative to the late endosomal/lysosomal membrane marker LAMP1. CCR5-349 colocalized with LAMP1 within 90 minutes after agonist addition (Fig. 6). In contrast, the wild type receptor examined under similar conditions was sparsely observed in LAMP1 positive structures (Fig. 6). These results were consistent with the CCR5-349 proteolysis observed after receptor biotinylation and confirmed that the internalized mutant receptor were directed towards a lysosomal pathway.

Above, we described mutations in the C-terminus of CCR5 that affected receptor recycling by routing mutated proteins toward a lysosomal degradative pathway. The deletion of the last 4 amino acids (SVGL) prevented receptor recycling, suggesting that these residues were the key determinants of CCR5 trafficking after endocytosis.

### *The SVGL motif of CCR5 C-terminus acts as a PDZ interacting sequence*

PDZ domains are modular protein interaction domains that bind in a sequence-specific manner to short carboxy-terminal peptides (Hung and Sheng, 2002). Any addition of amino acid at the end of the carboxy-terminal peptide prevents it from interacting with the PDZ domains and inhibits its function (Cao et al., 1999; Gage et al., 2005). For the  $\beta$ 2AR, such an addition at the end of its PDZ ligand inhibits the receptor recycling to the cell surface.

The SVGL motif of CCR5 conforms to a putative PDZ ligand. To find out whether this motif actually acts as a PDZ interacting sequence, we inserted an additional alanine at the end of the CCR5 C-terminus. The resulting mutant (CCR5-ala) was expressed at the cell surface and was functional as a HIV-1 receptor (data not shown). Like previously, we stably expressed this mutant in HEK293 cells and

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analyzed its intracellular trafficking process. This mutant was efficiently internalized after Mip-1 $\beta$  or Rantes stimulation: we observed a 40% decrease of receptor cell surface expression after 90 minutes of chemokine exposure (Fig 7A). However, the addition of the single alanine to the SVGL motif significantly impaired CCR5 recycling: less than 30% of the internalized CCR5-ala recycled back to the cell surface after agonist removal (Fig. 7B). This result suggested that the additional alanine could inhibit the interaction between the SVGL motif and a cytoplasmic protein required for recycling. The SVGL motif of CCR5 could act as a PDZ ligand.

The presence of a phosphate acceptor and a C-terminal hydrophobic amino acid are structural characteristics of PDZ ligands (Hung and Sheng, 2002). In order to reinforce our findings showing that the SVGL motif of CCR5 acted as a PDZ ligand, we substituted the amino acid S349 and L352 of this motif by an alanine (mutants S349A and L352A). As previously performed, we first controlled the expression of these mutants at the cell surface (data not shown), their HIV-1 receptor function (data not shown) and their correct internalization after chemokine stimulation (Fig. 7A). We finally analyzed their ability to recycle after agonist removal in stably expressing HEK293 cells. We observed that the ability of these two mutants to recycle back to the cell surface was diminished (Fig. 7B). This result showed that only one substitution in the SVGL motif of CCR5 disrupted its recycling, the amino acid L being of particular importance. The structural wholeness of this motif seemed clearly needed for CCR5 recycling. All together these results led us to suggest that the SVGL motif of CCR5 acts as a PDZ ligand.

Contrary to other GPCR receptors (Cao et al., 1999), previous studies suggested that CCR5 recycling is independent of receptor dephosphorylation and ligand dissociation (Signoret et al., 2004). The serine in the PDZ ligand motif of CCR5 is shown to be GRK phosphorylated (Oppermann, 2004). To further investigate the role played by this phosphorylation state during recycling, we substituted the amino acid S349 of the PDZ ligand motif by a glutamic acid (mutants S349E). Such a substitution should mimic the negative charge of phosphorylated S349. We analyzed the trafficking of this mutant after a

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transient expression in Jurkat cells (Fig. 7C). We observed that this mutant was recycled with the same efficiency as the wild type receptor, suggesting that the phosphorylation state of this residue did not alter its trafficking. In addition, as already shown in Figure 7B, the recycling of CCR5 S349A was reduced compared to the wild type receptor. In accordance with previous studies, these results suggested that CCR5 recycling was independent of receptor dephosphorylation. The phosphorylation state of CCR5 PDZ ligand seemed to favour the recycling.

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### Discussion

In this study, we sought to elucidate whether the C-terminal domain of CCR5 was involved in its recycling. We performed different mutations (progressive truncations and punctual substitutions) of this domain and expressed the resulting mutants in HEK293 cells. We studied their functionality as HIV-1 receptors and their intracellular trafficking.

The present results identified a sequence in the C-terminal tip of CCR5, dispensable for receptor internalization, but necessary for CCR5 to recycle back to the cell surface. In the absence of this sequence, CCR5 mutants were redirected towards a lysosomal degradative pathway. Finally, this sequence seemed to act through its association with PDZ domain-containing proteins. To our knowledge these results were the first evidence of the presence of a sequence similar to a “recycling signal” in CCR5.

Our results indicated that the CCR5 C-terminal deletion did not prevent the correct cell surface expression and the HIV-1 receptor function of CCR5 (Fig. 1), as previously reported (Alkhatib et al., 1997). We also found that all of the deleted CCR5 mutants except CCR5-336 were correctly internalized after stimulation by two of its ligands (Rantes or Mip-1 $\beta$ ). However, the truncated CCR5 mutants lost serines likely phosphorylated and needed for  $\beta$  arrestin association and for GPCRs internalization (Oppermann, 2004). These findings suggested that only two phosphorylated serines are sufficient to trigger receptor internalization, which confirms previous studies (Oppermann, 2004). However, other intracellular domains or mechanisms might intervene in receptor internalization (Moore et al., 2007).

As already described (Signoret et al., 2004; Venkatesan et al., 2003), we also found that once internalized, CCR5 is rapidly recycled back to the cell surface (Fig. 3 and 4). Five minutes after ligand removal, 20% of the receptors recycled. This recycling process was complete after 60 minutes. The present results also indicated that the C-terminal deleted mutants lost their recycling ability. We

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determined that the extreme C-terminus of CCR5, and especially the last 4 amino acids, was necessary for CCR5 recycling. The loss of the 4 amino acids was closely connected to the proteolysis of the receptor and to its routing towards the lysosomal pathway. On one hand, this lysosomal trafficking could merely be a default way that came as a consequence of the 4 amino acids removal. Even if we could not exclude that CCR5 might lose its integrity, we favoured the hypothesis that CCR5 lost a signal that guides its recovery to the cell surface (see below). On the other hand, the proteolysis could result from the functional activity of a degradative signal: the removal of the last 4 amino acids might unmask a signal that targets CCR5 towards degradation. Such a mechanism was described for  $\beta$ 2AR: the recycling signal of  $\beta$ 2AR protects the receptor from ubiquitylation-dependent degradation (Shenoy et al., 2001) and/or from binding to the sorting protein GASP (Thompson et al., 2007). The recycling signal of  $\beta$ 2AR appears to function as a dominant trafficking signal (Tanowitz and von Zastrow, 2003). No degradative sorting mechanism has been described for CCR5 yet.

The key role of GPCRs last 4 amino acids were described for other receptors like  $\beta$ 2AR. In this case, the amino acids DSLL act as a type I PDZ ligand (consensus sequence as X-S/T-X- $\Phi$ , where X is any amino acid,  $\Phi$  is a hydrophobic amino acid) that enables the receptor to bind NHERF/EBP50. This binding is described as needed for  $\beta$ 2AR recycling (Cao et al., 1999). This recycling is disrupted by a mutation of the last leucine of the DSLL motif and inhibited by the phosphorylation of the serine after GRK5 overexpression. This phosphorylation could prevent  $\beta$ 2AR from binding NHERF/EBP50. In our case, the last 4 amino acids involved (SVGL) belong to a type II PDZ ligand (X- $\Phi$ -X- $\Phi$ ). This SVGL motif possesses structural characteristics close to the DSLL motif: the presence of a phosphate acceptor as well as a leucine at the extreme C-terminus. We identified that both serine and leucine were required for proper recycling of CCR5. The serine S349 is a substrate for GRK phosphorylation (Oppermann, 2004). Although GRK-mediated phosphorylation regulates  $\beta$ 2AR recycling, we showed that CCR5 recycling seems independent of the receptor's phosphorylation (Fig. 7) (see discussion below). However, the

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similar characteristics between  $\beta$ 2AR and CCR5 last 4 amino acids led us to suggest that the SVGL motif of CCR5 could act as a “recycling signal”. Such a “recycling signal” is also found in other GPCRs like hLHR (Galet et al., 2004),  $\mu$ OR (Tanowitz and von Zastrow, 2003), D1R (Vargas and Von Zastrow, 2004), ET<sub>A</sub> receptor (Paasche et al., 2005), and SSTR5 (Wente et al., 2005). Whatever the recycling signal, they all share a leucine and/or a phosphate acceptor. This structural characteristic might represent the signature of a “recycling signal”.

How do these sorting sequences intervene in the GPCR sorting process? Several hypotheses have been developed: (i) the sorting sequence might be associated with enzymes involved in receptor phosphorylation or ubiquitination; (ii) the sorting sequence might be associated with proteins involved in the post-endocytosis. The identification of the proteins that mediate post-endocytic sorting has just started. NHERF/EBP50 and NSF seem involved in the recycling process of  $\beta$ 2AR (Cao et al., 1999; Cong et al., 2001), PIST and PDZK1 in the recycling process of SSTR5 (Wente et al., 2005), while SNX1 and GASP seem involved in the degradation process of PAR1 and  $\delta$ OR respectively (Wang et al., 2002; Whistler et al., 2002). These proteins may promote receptor sorting by linking them with the cytoskeleton. It has been shown, for instance, that NHERF/EBP50 interacts with the C-terminal tail of  $\beta$ 2AR through a PDZ domain, and with the cortical actin cytoskeleton through an ezrin-radixin-moesin (ERM) binding domain. A role of the actin cytoskeleton in receptor recycling is also proposed for CCR5 (Neel et al., 2005).

How do the last 4 amino acids (SVGL) of CCR5 intervene in its sorting process? The SVGL motif of CCR5 should act by linking particular proteins involved in the recycling process. We could assume this for the following reasons: (i) the SVGL motif of CCR5 constitutes a putative PDZ ligand; (ii) the sorting of some GPCRs requires an interaction between a C-terminal sequence and cytoplasmic proteins; (iii) we identified that mutations in the CCR5 SVGL motif, which make the motif very unlikely



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to be a PDZ domain target, inhibited CCR5 recycling. We suggested that the SVGL motif of CCR5 could act as a “recycling signal” linking PDZ domains.

To ascertain this sorting mechanism, the next step will be to identify the protein involved in CCR5 recycling. A type II PDZ ligand has been found in other receptors like CCR2b, GluR2 or mGluR7 (Hung and Sheng, 2002). For the two latter, the type II PDZ ligand is described as being able to bind proteins like PICK-1, syntenin or GRIP (Bockaert et al., 2003). The role played by these proteins in the recycling process of GPCRs has been poorly studied. It would be interesting to investigate whether these proteins, or other PDZ domain-containing proteins, could bind the SVGL motif of CCR5 and could intervene in the recycling process. We detected *in vitro* a weak binding of CCR5 to NHERF/EBP50 (data not shown). This result was in accordance with previous studies showing that NHERF/EBP50 interacts mainly with  $\beta$ 2AR (Heydorn et al., 2004).

All together, our results suggested that, like other GPCRs, CCR5 contained a post-endocytic sorting signal. However, contrary to other GPCRs, CCR5 recycling seems independent of this sorting signal dephosphorylation. This means that, if a sorting protein is involved in CCR5 recycling, then this protein might interact with the receptor whatever its phosphorylation status. This protein might be less “selective” than the protein involved in  $\beta$ 2AR recycling, whose interaction depends on dephosphorylation. Such a “selective” protein has already been described for the regulation of GluR2 trafficking. The serine phosphorylation of the PDZ ligand “SVKI” prevents GluR2 from binding to GRIP-1 but not to PICK-1 (Chung et al., 2000). We could suggest that if GPCRs apparently share similar sorting mechanisms, their membrane trafficking seems differently regulated. Further studies could investigate how CCR5 recycling is regulated.  $\beta$ -arrestins are described as controlling the intracellular trafficking of some GPCRs (Moore et al., 2007). Since CCR5 is internalized in the presence of these regulatory proteins, the role played by  $\beta$ -arrestins in CCR5 recycling may represent a way to explore.

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Other mechanisms might also control the recycling process: for instance the ability of CCR5 to oligomerize, as suggested for  $\beta$ 2AR (Cao et al., 2005).

We provide here new insight on CCR5 post-endocytic mechanism. Understanding the processes involved in the sorting of internalized CCR5 is particularly important in regards to HIV entry inhibition. The HIV entry directly depends on the receptor quantity at the cell surface. The disappearance of receptors from the cell surface contributes to the antiviral effect of chemokines. Some antiviral compounds internalize and keep the receptor inside the cell (Hartley et al., 2004). It would be interesting to determine the exact fate of the receptor after such a treatment. This study opens new perspective towards our understanding of the antiviral compounds mechanisms.

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### **Footnotes**

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### Legends for figures

#### Figure 1: Cell surface expression and HIV-1 receptor activity of CCR5 C-terminal mutants.

A, **Amino acids sequences of the CCR5 C-terminal WT and mutants.** The numbered sequence of CCR5 WT from amino acid 320 to 352 is underlined. The C-terminal deletions are represented ending at the designated sites. Cysteines in the palmitoylation motif are shaded. CCR5 WT and the three deletion mutants were tagged with a FLAG epitope at the N-termini.

B, **Relative cell surface expression of CCR5 mutants.** HEK293 cells transfected with WT or mutant CCR5 were stained with the anti-FLAG M2 mAb or with the anti-CCR5 2D7 mAb. Fractions of M2 (or 2D7) positive cells were determined by flow cytometry. Bars represent staining efficiency for cells expressing CCR5 mutants relatively to cells expressing CCR5 WT. Data shown represent the mean +/- SEM from 3 independent transfections.

C, **HIV-1 receptor activity of CCR5 mutants.** Target cells (U373MG-CD4) transfected with WT or mutant CCR5 were cocultured with HeLa cell line stably expressing Env/ADA. Fusion with HeLa-Env/ADA cells results in transactivation of the LTR*lacZ* reporter gene and high  $\beta$ -galactosidase activity. Cells were stained with the X-Gal substrate 24 hours after coculture and blue-stained foci were counted. Results are shown as percentage of fusion efficiency relative to the WT receptor (mean +/- SEM from 3 independent transfections). The same results were obtained in HeLa-P4 cells (data not shown).



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### Figure 2: Agonist-induced internalization of CCR5 WT and mutants.

Internalization of receptors following exposure to agonist was measured by flow cytometry. HEK293 stably expressing WT or mutant receptors were incubated with the M1 anti-FLAG antibody for 30 minutes at 37°C. Unbound antibody were washed and cells were incubated in the presence or the absence of 100 nM of agonist for various time at 37°C. Cells were cooled on ice and the receptors remaining at the cell surface were stained with a secondary anti-mouse PE antibody. Results are expressed as the percentage of total antibody bound, calculated from mean fluorescence intensity, relative to untreated cells. (A) cell surface expression in the absence of agonist; (B) cell surface expression after Mip-1 $\beta$  exposure; (C) cell surface expression during the first ten minutes after Mip-1 $\beta$  exposure. The data represent the mean  $\pm$  SEM from three separate experiments.

### Figure 3: Endocytic trafficking of CCR5 WT and mutants visualized by fluorescence microscopy.

Cells expressing FLAG-tagged CCR5 were labelled at the cell surface with the M1 anti-FLAG monoclonal antibody, and receptor localization was visualized under the indicated conditions by epifluorescence microscopy. (a-d) localization of WT receptor; (e-h) localization of CCR5-349 mutant; (i-l) localization of CCR5-341 mutant. The results shown are representative of three experiments using two clones of each transfected cell population.

### Figure 4: Quantitative analysis of recycling by flow cytometry.

A, Fluorescence histograms representing surface-accessible FLAG-tagged receptor immunoreactivity in cells expressing WT receptor (left), in cells expressing CCR5-349 (middle) and CCR5-341 (right) 90

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minutes after Mip-1 $\beta$  exposure. Results are representative of three different clones and of at least 3 independent experiments.

B, Recovery of internalized antibody-labelled FLAG-CCR5 to the plasma membrane after Mip-1 $\beta$  washout obtained by a flow cytometric analysis. Points represent the percentage of receptor recycling (calculated from mean fluorescence histograms) at the indicated time points after agonist washout (3 independent experiments).

C, Recycling of internalized antibody-labelled FLAG-CCR5 in T cell lines (MOLT-4 and Jurkat) and in human peripheral blood T cells (PBT). Cells were transiently transfected with FLAG-CCR5 WT or CCR5-349 and the recycling was measured in a flow cytometric assay after Mip-1 $\beta$  washout. Bars represent the percentage of CCR5 WT or CCR5-349 recycling 90 minutes after agonist washout (mean +/- SEM from 2 independent experiments).

### Figure 5: Fate of CCR5 deletion mutants.

A, **Half-life times of CCR5 WT and mutants.** Cell surface expression of FLAG-CCR5 WT and mutants on cells treated for different times with 100  $\mu$ M cycloheximide was assessed by flow cytometry. Times indicated are after a 1-hour pre-incubation in cycloheximide. Mean +/- SEM from 3 independent experiments is shown.

B, **Effect of CCR5 C-terminal deletion on agonist-induced proteolysis.** HEK293 cells stably expressing the indicated FLAG-tagged receptors were surface-biotinylated and exposed or not to 100 nM of Mip-1 $\beta$  for the indicated times. Equal amounts of cell extracts were pull-downed by streptavidine, separated on SDS-PAGE, transferred to a PVDF membrane, and blotted with the M1 anti-FLAG mAb.

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The same experiment was performed in the presence of leupeptine (349+leup). Representative blots out of 3 are shown.

**C, Quantification of anti-FLAG Western blots from experiments performed as in B.** The amount of surface receptor proteins was quantified by scanned densitometry and plotted relative to the amount of receptor detected in untreated cells (mean +/- SEM from two independent experiments).

### **Figure 6: Colocalization of mutant receptors with lysosomal markers using confocal imaging.**

HEK293 stably expressing FLAG-CCR5 WT and CCR5-349 cells were fed with anti-FLAG antibody to label surface receptor prior to treatment with Mip-1 $\beta$  (100 nM) during 90 minutes at 37°C in the presence of leupeptin. Cells were fixed, permeabilized, stained for LAMP1 and visualized by confocal microscopy as described in materials and methods. A medial optical section is shown. Receptor is in green and LAMP1 in red in the combined images presented (Merge). Bar = 15  $\mu$ m.

### **Figure 7: Effect of CCR5 C-terminal point mutations on recycling.**

A, Cell surface expression after Mip-1 $\beta$  exposure. Experiments were performed as defined in the legend of the Figure 2.

B, Recycling of internalized antibody-labelled FLAG-CCR5 to the plasma membrane of stably expressing HEK293 cells after Mip-1 $\beta$  washout obtained by a flow cytometric analysis. Bars represent the percentage of receptor recycling 90 minutes after agonist washout (mean +/- SEM from 3 independent experiments).

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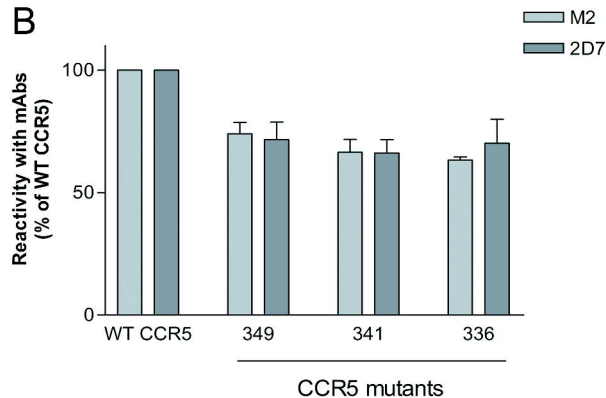
C, Recycling of internalized antibody-labelled FLAG-CCR5 in Jurkat cells. Cells were transfected with FLAG-CCR5 WT, CCR5-349, CCR5 S349A and CCR5 S349E expressing vectors. The recycling was measured in a flow cytometric assay after Mip-1 $\beta$  washout. Bars represent the percentage of receptors recycling 90 minutes after agonist washout (mean  $\pm$  SEM from 3 independent transfections).

Figure 1

A

320 336 352  
WT CCR5 : CKCCSIFQQEAPERASSVYTRSTGEQEISVGL-COOH  
CCR5-349 : CKCCSIFQQEAPERASSVYTRSTGEQEI-  
CCR5-341 : CKCCSIFQQEAPERASSVYT-  
CCR5-336 : CKCCSIFQQEAPER-

B



C

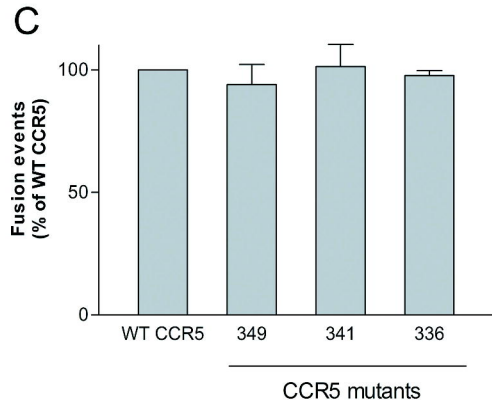
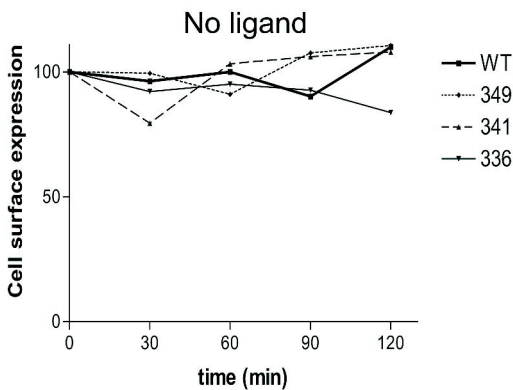
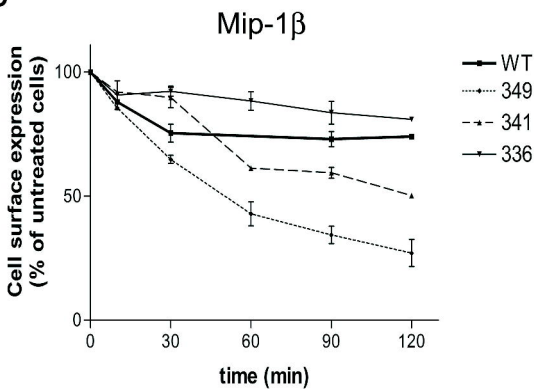


Figure 2

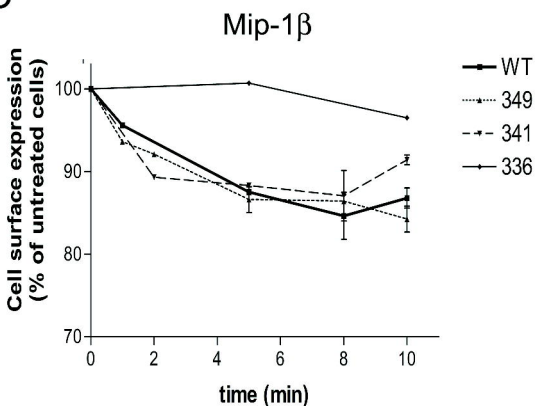
A



B



C



**Figure 3-**

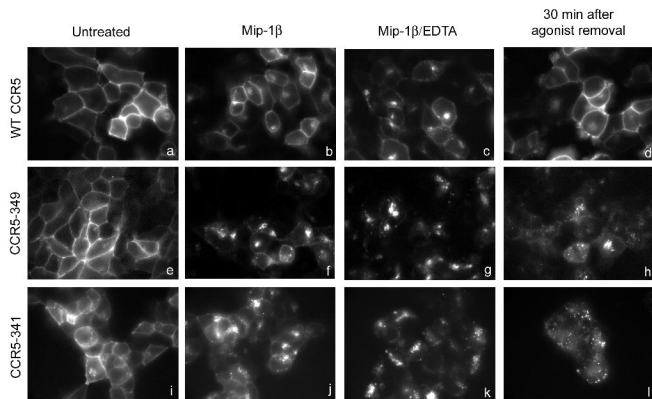
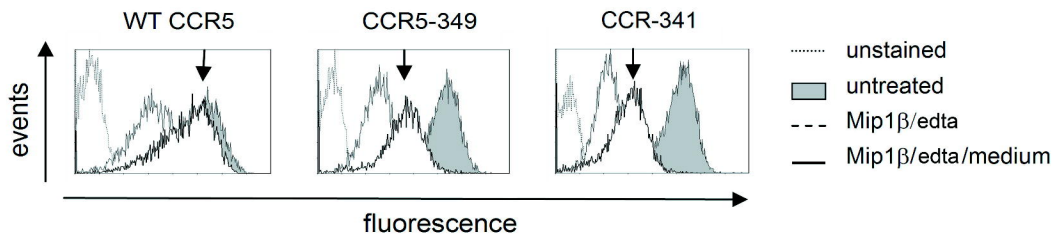
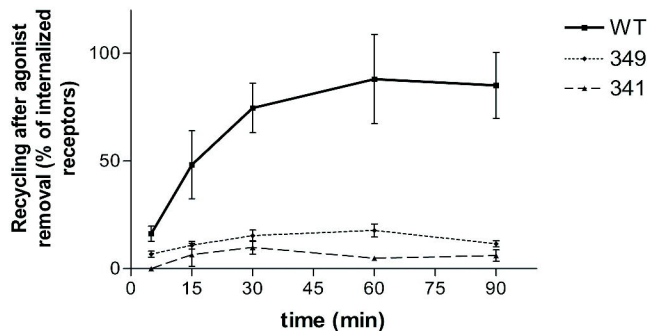


Figure 4

A



B



C

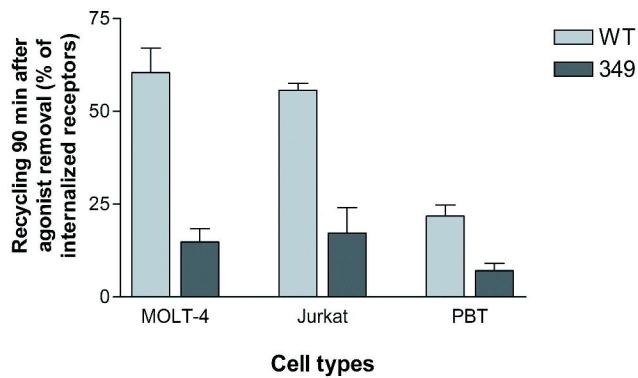
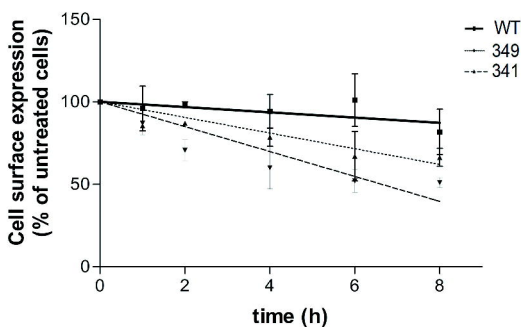


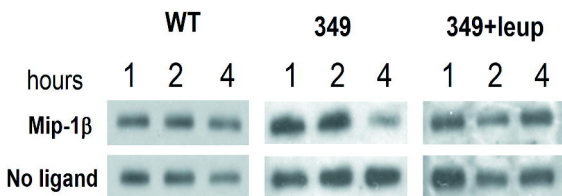


Figure 5

A



B



C

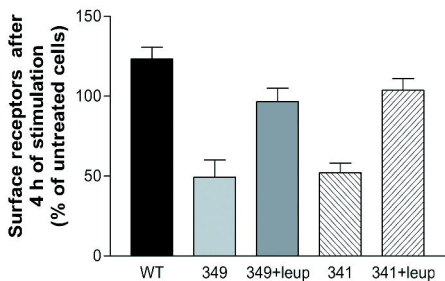
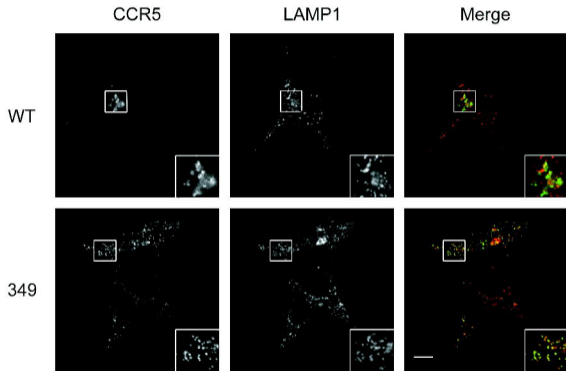
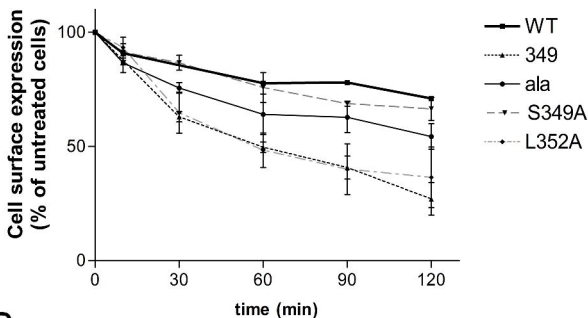


Figure 6

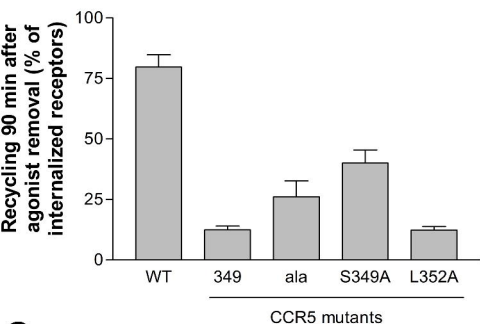


# Figure 7

## A



## B



## C

