

# Endocytosis and Recycling of the HIV Coreceptor CCR5<sup>☆</sup>

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**Abstract.** The chemokine receptor CCR5 is a cofactor for the entry of R5 tropic strains of human immunodeficiency viruses (HIV)-1 and -2 and simian immunodeficiency virus. Cells susceptible to infection by these viruses can be protected by treatment with the CCR5 ligands regulated on activation, normal T cell expressed and secreted (RANTES), MIP-1 $\alpha$ , and MIP-1 $\beta$ . A major component of the mechanism through which chemokines protect cells from HIV infection is by inducing endocytosis of the chemokine receptor. Aminoxypropentane (AOP)-RANTES, an NH<sub>2</sub>-terminal modified form of RANTES, is a potent inhibitor of infection by R5 HIV strains. AOP-RANTES efficiently downmodulates the cell surface expression of CCR5 and, in contrast with RANTES, appears to prevent recycling of CCR5 to the cell surface. Here, we investigate the cellular basis of this effect.

Using CHO cells expressing human CCR5, we show that both RANTES and AOP-RANTES induce rapid internalization of CCR5. In the absence of ligand, CCR5 shows constitutive turnover with a half-time of 6–9 h. Addition of RANTES or AOP-RANTES has little effect on the rate of CCR5 turnover. Immunofluorescence and

immunoelectron microscopy show that most of the CCR5 internalized after RANTES or AOP-RANTES treatment accumulates in small membrane-bound vesicles and tubules clustered in the perinuclear region of the cell. Colocalization with transferrin receptors in the same clusters of vesicles indicates that CCR5 accumulates in recycling endosomes. After the removal of RANTES, internalized CCR5 recycles to the cell surface and is sensitive to further rounds of RANTES-induced endocytosis. In contrast, after the removal of AOP-RANTES, most CCR5 remains intracellular. We show that these CCR5 molecules do recycle to the cell surface, with kinetics equivalent to those of receptors in RANTES-treated cells. However, these recycled CCR5 molecules are rapidly reinternalized. Our results indicate that AOP-RANTES-induced changes in CCR5 alter the steady-state distribution of the receptor and provide the first evidence for G protein-coupled receptor trafficking through the recycling endosome compartment.

Key words: chemokine receptor • endocytosis • CCR5 • recycling endosome • HIV

## Introduction

Chemokine receptors are members of the large family of seven transmembrane domain G protein-coupled receptors (GPCR)<sup>1</sup> that function in immune and inflammatory responses by regulating the activation and directed migration of leukocytes (Pelchen-Matthews et al., 1999; Murphy et al., 2000). These receptors have also been implicated in

hematopoiesis, development, angiogenesis, and, recently, in the entry and infection of two groups of animal viruses, the primate immunodeficiency viruses (HIV-1 and -2, and simian immunodeficiency virus) and the rabbit poxvirus responsible for myxomatosis (Berger et al., 1999; Lalani et al., 1999; Murphy et al., 2000). Although various chemokine receptors have been shown to facilitate the entry of HIV-1 and its relatives in vitro, the receptors that have been most strongly implicated in disease are the CC chemokine receptor 5 (CCR5) and CXCR4 (Berger et al., 1999; Pelchen-Matthews et al., 1999; Murphy et al., 2000). CCR5 is a receptor for the CC chemokines regulated on activation, normal T cell expressed and secreted (RANTES), MIP-1 $\alpha$ , MIP-1 $\beta$ , and MCP-3 and is expressed on peripheral blood-derived dendritic cells, subsets of Th1 lymphocytes, and CD34<sup>+</sup> haematopoietic stem

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<sup>1</sup>Abbreviations used in this paper: AOP, aminoxypropentane;  $\beta$ 2AR,  $\beta$ 2-adrenergic receptor; BM, binding medium; CCR5, CC chemokine receptor 5; CHX, cycloheximide; GAG, glycosaminoglycan; GPCR, heterotrimeric G protein-coupled receptor; GRK, GPCR kinase; HIV, human immunodeficiency virus; RANTES, regulated on activation, normal T cell expressed and secreted; TfR, transferrin receptor.

cells (Murphy et al., 2000). In contrast, CXCR4 binds uniquely the CXC chemokine stromal cell-derived factor 1, and it is expressed on most haematopoietic cells, including neutrophils, monocytes, T and B lymphocytes, dendritic cells, and macrophages, as well as vascular endothelial cells, neurons, and glia (Murphy et al., 2000). Current evidence suggests that, together with CD4, CCR5 is important for the transmission of CCR5 tropic strains of HIV (R5 viruses) and the establishment of infection. CXCR4 tropic viruses (X4 viruses) can emerge in infected individuals during the course of infection or passage of virus through T cell lines in culture (Berger et al., 1999).

The initial indication that chemokine receptors have a role in HIV infection came from the finding that the CC chemokines RANTES, MIP-1 $\alpha$ , and MIP-1 $\beta$  can inhibit HIV infection in vitro (Cocchi et al., 1995). Subsequently, stromal cell-derived factor 1 was also shown to inhibit infection by X4 viruses (Bleul et al., 1996; Oberlin et al., 1996). Two models have been proposed for the mechanism through which chemokines inhibit HIV entry (Wells et al., 1996). One proposal is that interaction of the chemokine with its receptor masks a binding site(s) on the chemokine receptor that is involved in docking of the viral envelope protein. The alternative is that the chemokine induces activation and internalization of the receptor so that it is no longer available on the cell surface for virus binding. Evidence has accumulated that certain receptor antagonists can block the viral envelope protein-binding sites on the receptor without inducing receptor endocytosis (Arenzana-Seisdedos et al., 1996; Doranz et al., 1997; Klasse et al., 1999). However, studies with native chemokines indicate that a major component of the mechanism through which these molecules inhibit HIV entry is by inducing endocytosis of the chemokine receptor (Amara et al., 1997; Signoret et al., 1997). Subsequently, a modified form of the CC chemokine RANTES, in which an aminooxypentane group (AOP) is coupled to the NH<sub>2</sub> terminus of the protein, was shown to be a particularly effective inhibitor of R5 tropic HIV strains (Simmons et al., 1997). Significantly, this activity appeared to correlate with the ability of AOP-RANTES to irreversibly downmodulate CCR5 (Mack et al., 1998). Initial studies of the fate of CCR5 in cells treated with RANTES or AOP-RANTES indicate that both ligands induce CCR5 endocytosis through clathrin-coated vesicles (Amara et al., 1997; Aramori et al., 1997; Mack et al., 1998; Signoret et al., 1998), but only in RANTES-treated cells is CCR5 recycled to the cell surface after ligand removal (Mack et al., 1998).

Here, we investigated the cellular mechanisms of ligand-induced CCR5 trafficking. We find that both RANTES and AOP-RANTES induce endocytosis of CCR5 with similar kinetics. With both ligands, internalized receptors are delivered to endosomal vesicles with properties similar to those described for recycling endosomes. After removal of RANTES, CCR5 reaccumulates on the cell surface. In contrast, on AOP-RANTES-treated cells, CCR5 appeared to remain inside the cell. However, antibody feeding experiments indicated that this CCR5 was able to recycle to the cell surface, and that the recycled receptor was rapidly reinternalized. The results suggest that AOP-RANTES is able to modify CCR5 such that the receptor continues to be recognized by the endocytosis machinery and is unable to reaccumulate on the cell surface.

## Materials and Methods

### Reagents

Tissue culture reagents and Nunc tissue culture plastic were from Life Technologies. Chemicals were from Sigma-Aldrich, unless indicated. Radioactive reagents were from Amersham Pharmacia Biotech. Recombinant RANTES was prepared as described previously (Proudfoot et al., 1996). AOP-RANTES was produced as described previously (Simmons et al., 1997) and was provided by Robin E. Offord (Centre Medical Universitaire, Geneva, Switzerland). <sup>125</sup>I-RANTES and <sup>125</sup>I-AOP-RANTES (specific activity 2,000 Ci/mmol) were prepared by enzymatic iodination with sodium [<sup>125</sup>I]iodide, hydrogen peroxide, and lactoperoxidase and then purified by high performance liquid chromatography (Nymcomed Amersham plc).

### Antibodies

The anti-CCR5 murine mAb MC-5 (IgG2a) was generated as described previously (Segerer et al., 1999), and its specificity was determined on CHO cells stably transfected with human CCR1-5 or CXCR4. Binding was only detected on cells expressing CCR5. The antibody did not react with freshly isolated peripheral blood mononuclear cells, or with cultured monocytes from donors homozygous for the CCR5  $\Delta$ 32 mutation (Segerer et al., 1999). The MC-5 epitope has been mapped to the NH<sub>2</sub>-terminal domain of the receptor, with amino acids 2–4 being crucial for binding. Purified MC-5 was radioiodinated using <sup>125</sup>I-Bolton and Hunter reagent (Amersham Pharmacia Biotech), as described previously (Signoret et al., 1997).

The anti-hamster lysosomal glycoprotein mAbs to Lgp-B (Lgp-120) UH3c and 3E9 (both IgG1), 1B3-13 (IgG2a), and hamster CD63 (ch1c9b, IgG2a) were provided by Dr. Bruce Granger (Montana State University, Bozeman, MO). The anti-lysobisphosphatidic acid (LBPA) mAb 6C4 (IgG1) (Kobayashi et al., 1998) was provided by Dr. Jean Gruenberg (Universite de Geneve, Geneva, Switzerland). The anti-transferrin receptor (TfR) mAb H68.4 (IgG1) was provided by Dr. Ian Trowbridge (Salk Institute, La Jolla, CA) or purchased from Zymed Laboratories, and anti-HRP was purchased from Jackson ImmunoResearch Laboratories.

### Cells

DHFR-deficient CHO cells stably expressing human CCR5 (CHO-CCR5) were maintained in nucleoside-free  $\alpha$ MEM with 10% FCS, as described previously (Mack et al., 1998). CHO cells defective in glycosaminoglycan (GAG) synthesis (CHO-pgsA-745) and CHO-pgsA-745 expressing human CCR5 were obtained from Dr. A. Trkola (University Hospital, Zurich, Switzerland; Trkola et al., 1999). CHO-pgsA-745 cell lines and wild-type CHO-K1 cells were maintained in DMEM-F12 containing 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin.

### Binding and Elution of <sup>125</sup>I-RANTES and <sup>125</sup>I-AOP-RANTES

CHO-CCR5 and CHO-K1 cells were plated in 24  $\times$  16-mm well plates and grown to confluence over 2 d. <sup>125</sup>I-labeled chemokines (250  $\mu$ l at 125 pM), diluted in binding medium (BM: RPMI-1640 without bicarbonate, containing 0.2% BSA, 10 mM Hepes and adjusted to pH 7.0 or 7.4, as indicated in the text), were added to each well and bound for 90 min at 4°C on a reciprocal shaker. Subsequently, free ligand was removed by washing twice with 4°C BM. Half of the wells were then washed twice (3 min each) with 500  $\mu$ l pH 2.0 medium (RPMI-1640 without bicarbonate, containing 0.2% BSA, 10 mM MES, and adjusted to pH 2.0) or 500  $\mu$ l pH 11.6 buffer (0.25 M NaH<sub>2</sub>PO<sub>4</sub> adjusted to pH 11.6) at 4°C. The cells were washed again in cold BM, harvested in 400  $\mu$ l of 0.2 M NaOH, and transferred to tubes for  $\gamma$ -counting.

### Internalization of Radiolabeled Chemokines

Cells plated in 16-mm wells and grown to 85% confluence over 2 d were incubated in BM, or BM containing 125 pM <sup>125</sup>I-RANTES or <sup>125</sup>I-AOP-RANTES, for 90 min at 4°C. Four wells were used for each time point. Unbound chemokines were removed by washing with 4°C BM, and the cells were incubated in 37°C BM for the indicated times. The incubations were stopped by cooling the plates on ice and washing the cells twice with 4°C BM. To determine the proportion of iodinated ligand internalized during the 37°C incubation, half the wells for each time point were rinsed and washed twice (3 min/wash) with pH 11.6 buffer. All wells were then rinsed with BM and the cells were harvested, as described above.

## Ligand-induced CCR5 Downmodulation and Recycling

Cells were plated, as described above, and incubated in 37°C BM or BM containing either RANTES (500 nM) or AOP-RANTES (100 nM). After treatment, the cells were placed on ice and washed four times with 4°C BM to remove the free chemokine. Selected plates were then incubated in 37°C BM for 1 h and then cooled on ice. The cells were incubated in 4°C BM containing 6.1 nM <sup>125</sup>I-MC-5 for 2 h at 4°C. Finally, all cells were washed in 4°C BM to remove free antibody, and the cell-associated activity was measured, as described above.

## <sup>125</sup>I-MC-5 Antibody Feeding

Cells were treated with RANTES or AOP-RANTES, as described above, to internalize cell surface CCR5. After endocytosis, all cells were labeled with 4°C BM, which contained 6.1 nM <sup>125</sup>I-MC-5, for 2 h to saturate any CCR5 receptors remaining at the cell surface. Selected cultures were then incubated in 37°C BM containing 6.1 nM <sup>125</sup>I-MC-5. At the indicated times, the cells were cooled to 4°C, free antibody was washed away, and the cell-associated activity was determined, as described above. The cell protein concentration was determined using bicinchoninic acid (Pierce and Warriner [UK] Ltd.). Where indicated, 100 µg/ml cycloheximide (CHX) was included in the medium. At this concentration, CHX blocked virtually all [<sup>35</sup>S]methionine/cysteine incorporation in a 5-min pulse label.

## Western Blotting

CHO-CCR5 (6.5 × 10<sup>4</sup> cells/well) were grown in 16-mm wells for 2 d. The cells were washed twice in BM at room temperature and incubated in 250 µl of prewarmed BM containing 100 µg/ml CHX without chemokine or with 500 nM RANTES or 100 nM AOP-RANTES. At the indicated times, the plates were placed on ice, and the cells were washed twice in 4°C BM and then in 4°C PBS. Cells were lysed in 100 µl of lysis buffer (1% NP-40, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8) containing the protease inhibitors PMSF (1 mM), 5 µg/ml each of chymostatin, pepstatin A, antipain hydrochloride, and 10 µg/ml leupeptin hemisulphate, as well as the phosphatase inhibitors sodium orthovanadate (0.1 mM) and sodium fluoride (50 mM). The wells were scraped and the cell lysates were collected into 1-ml screw cap tubes and left on ice for 30 min. Samples were centrifuged at 13,000 rpm for 10 min at 4°C, to remove the nuclei and cell debris, and the supernatants were transferred to new 1-ml tubes. The samples were either used fresh or frozen at -70°C. Samples (10 µl of cell lysates) were mixed with 3.3 µl of 4× reducing SDS sample buffer and 10-µl aliquots were loaded onto 10% SDS-polyacrylamide gels without boiling. After electrophoresis, proteins were transferred to nitrocellulose membranes (Schleicher & Schuell). The blots were incubated in blocking buffer (5% skimmed milk, 5% FCS, 0.1% Tween 20 in PBS) for 1 h and then in blocking buffer containing 1.1 nM <sup>125</sup>I-MC-5 for at least 2 h at room temperature. Blots were washed four times 10 min each in PBS containing 0.2% Tween 20 and two times 10 min each in PBS containing 0.1% Tween 20. The blots were left to dry at room temperature and exposed for a minimum of 6 h to Kodak X-Omat AR film (Eastman Kodak Co.). The blots were quantitated using a Molecular Imager (Bio-Rad Laboratories). In addition, bands were cut from the nitrocellulose and γ-counted directly. Results acquired by both methods were comparable.

## Immunofluorescence Microscopy

CHO-CCR5 cells were grown on coverslips for 2 d. The cells were washed with BM at room temperature and incubated in 37°C BM with or without chemokines. Then, the coverslips were placed on ice and washed extensively with cold BM. To study CCR5 receptor recycling, some coverslips were reincubated at 37°C in BM containing 5 µg/ml of MC-5 for 1 h. The cells were washed and fixed in PBS containing 3% paraformaldehyde for 10 min. Subsequently, the cells were washed with PBS, the free aldehyde groups were quenched with 50 mM NH<sub>4</sub>Cl in PBS, and nonspecific-binding sites were saturated with PBS containing 0.2% gelatin (PBS/gelatin). For internal staining, cells were permeabilized during the saturation step by adding 0.05% saponin into the PBS/gelatin solution. Intact or permeabilized cells were labeled for 1 h with MC-5 (5 µg/ml) in PBS/gelatin with or without 0.05% saponin. The cells were washed to remove free antibody and then stained with a FITC-conjugated anti-mouse antibody (1:500 in PBS/gelatin with or without 0.05% saponin; Pierce and Warriner).

For colocalization experiments, cells were labeled with MC-5 (5 µg/ml) and either 6C4 or 3E9 (1:50 and 1:2 dilutions of hybridoma supernatant, respectively) in PBS/gelatin. Cells were then stained with a mix of FITC-conjugated anti-mouse IgG2a (1:100; Nordic Immunological Laborato-

ries) and biotin-conjugated anti-mouse IgG1 (1:100; Amersham Pharmacia Biotech). Finally, the cells were stained with streptavidin-Texas red (1:500; Pierce and Warriner). The coverslips were mounted in Mowiol and examined using a Nikon Optiphot-2 microscope equipped with an MRC BioRad 1024 confocal laser scanner. The images were assembled using Adobe Photoshop® software.

## Flow Cytometry and FACS® Analysis

CHO-pgsA-745-CCR5 cells were detached in PBS containing 10 mM EDTA, washed, and resuspended at 10<sup>7</sup> cells/ml in BM or BM containing RANTES or AOP-RANTES at 100 nM. Cells were incubated at 37°C for 1 h, cooled on ice, centrifuged (1,500 rpm, 5 min), and washed three times in cold BM. For each condition, an aliquot of 100 µl (10<sup>6</sup> cells) was washed in cold PBS and fixed in PBS containing 1% FCS and 1% formaldehyde. The rest of the cells were centrifuged once more, resuspended in prewarmed BM, and incubated for another 60 min at 37°C. Cells were pelleted by centrifugation, washed in cold PBS, and fixed as above. After two washes in PBS containing 1% FCS and 0.05% azide (wash buffer), all cells were labeled with MC-5 (5 µg/ml in wash buffer) and with a FITC-conjugated goat anti-mouse antibody (1:100 in wash buffer) for 1 h, and, lastly, washed three times in wash buffer. Cells were analyzed using a FACS® Calibur flow cytometer (Becton Dickinson).

## Immunolabeling of Cryosections for Electron Microscopy

Monolayers of CHO-CCR5 cells in 60- or 90-mm tissue culture dishes were incubated in BM (pH 7.4) or BM containing 500 nM RANTES or 125 nM AOP-RANTES for 2 or 4 h at 37°C. In one experiment, cells were pre-treated for 20 min with medium containing BSA conjugated to 5-nm gold particles (BSA-G<sub>5</sub>, OD<sub>520</sub> = 5) (provided by Dr. Clare Futter, MRC-LMCB, London, England), then by a 4 h chase in normal medium; RANTES or AOP-RANTES was added during the last 2 h of the chase. Early endosomes were labeled with a pulse of HRP (type II at 10 mg/ml), which was added during the final 5–10 min of the incubation. Cells were cooled on ice, washed extensively with cold medium, and fixed in 4% paraformaldehyde alone or together with 0.05 or 0.2% glutaraldehyde. Fixed cells were washed, embedded in 10% gelatin (ICN Biomedicals), infiltrated with 2.3 M sucrose, and frozen in liquid nitrogen, as described previously (Raposo et al., 1997). Cryosections (~60-nm thick) were labeled with MC-5 at 1.15 µg/ml (~7.7 nM). In addition, some sections were costained with antibodies against hamster Lgp-B (UH3c), LBPA (6C4), hamster CD63 (ch1c9b), TfR (H68.4), or HRP. To visualize the mouse IgG1 antibodies, a bridging rabbit anti-mouse antibody was used (Dako). Bound antibodies were detected with protein A-gold (EM Lab., Utrecht University, The Netherlands) and sections were examined with a transmission electron microscope (EM420; Phillips, Eindhoven, The Netherlands).

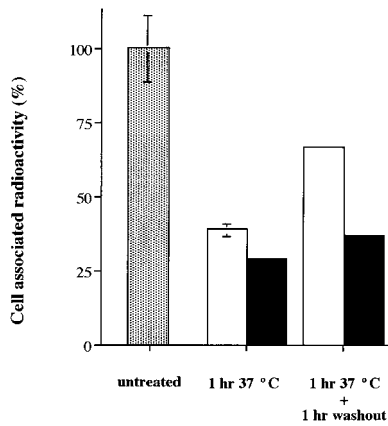
## Online Supplemental Materials

<sup>125</sup>I-RANTES feeding. CHO-CCR5 cells plated in 16-mm wells were grown to confluence over 3 d. Cells were incubated in pH 7-binding medium (BM; see Materials and Methods) containing 50 nM of RANTES or AOP-RANTES for 1 h at 37°C (to internalize cell surface CCR5). Subsequently, the cells were extensively washed with ice-cold BM and incubated in BM containing 125 pM <sup>125</sup>I-RANTES for 2 h at 4°C to equilibrate CCR5 receptors remaining at the cell surface. One sample for each condition was left on ice to give the initial cell-associated <sup>125</sup>I-RANTES binding. The other samples were incubated for 10, 30, or 60 min at 37°C in the continued presence of <sup>125</sup>I-RANTES. All cells were then washed four times with 4°C BM and twice with 4°C PBS. Finally, the cells were harvested and the cell-associated radioactivity was determined, as described in Materials and Methods. Online supplemental material available at <http://www.jcb.org/cgi/content/full/151/6/1281/DC1>.

## Results

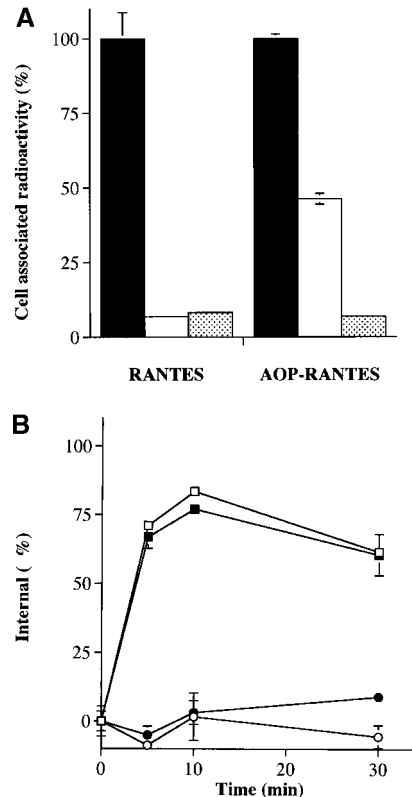
### Endocytosis and Recycling of CCR5

We reported previously that RANTES and AOP-RANTES downmodulate CCR5 from the surface of peripheral blood mononuclear cells and transfected CHO cells (Mack et al., 1998). On cells treated with RANTES,



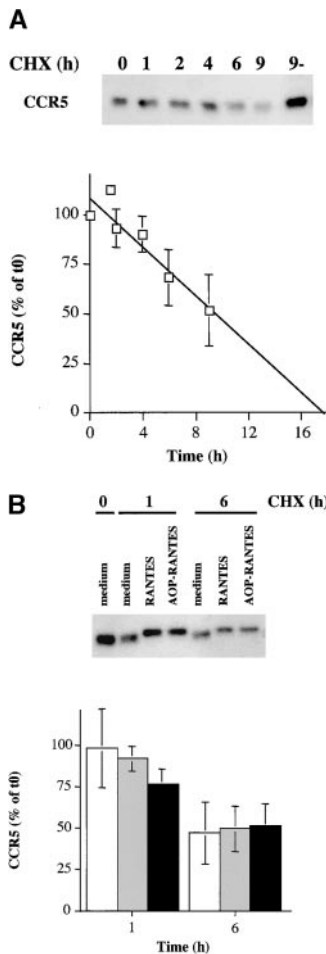
**Figure 1.** CCR5 downmodulation and recycling. Duplicate sets of CHO-CCR5 cells were treated with RANTES (500 nM, white bars) or AOP-RANTES (100 nM, black bars) for 1 h at 37°C or were left untreated (stippled bar). One set of treated cells was cooled to 4°C, whereas the other was washed and incubated in the absence of ligand for a further 1 h at 37°C. Subsequently, all cells were placed on ice and incubated for 2 h at 4°C with <sup>125</sup>I-MC-5. Cell-associated radioactivity was determined as described in Materials and Methods. The cell-associated counts are expressed as a percent of the untreated controls. The means and standard deviations for quadruplicate samples are shown for a representative experiment.

CCR5 recycles to the cell surface after ligand removal. However, after removal of AOP-RANTES, apparently, CCR5 is not recycled to the cell surface (Mack et al., 1998). To understand the cellular basis to these different effects, we followed CCR5 through the endocytic pathway after treatment of cells with RANTES or AOP-RANTES. Both ligands bind to multiple CC chemokine receptors, including CCR1, CCR3, and CCR5 (Murphy et al., 2000). In previous binding studies, radiolabeled MIP-1 $\alpha$  displacement has been used to analyze the binding properties of RANTES and AOP-RANTES for CCR5 (Simmons et al., 1997). However, binding studies with these ligands are complicated, since both can oligomerize and bind GAGs (Hoogewerf et al., 1997; Oravec et al., 1997; Ali et al., 2000). These activities are most pronounced at concentrations of ligand in excess of 1 nM and at pH values >7.0 (Proudfoot, A.E.I., unpublished data). To facilitate our studies, we used a CHO cell line stably transfected to express human CCR5 (Mack et al., 1998). Thus, the only RANTES/AOP-RANTES binding receptor on these cells is the CCR5 molecule. Nontransfected CHO-K1 cells were used throughout as a CCR5-negative control. For some experiments, we also used CHO-psgA-745 cells that are defective in GAG synthesis (Trkola et al., 1999). For experiments with <sup>125</sup>I-labeled RANTES and AOP-RANTES, ligand concentrations of 125 pM were routinely used in media adjusted to pH 7.0. At this concentration, ligand oligomerization and GAG binding is minimal, but receptor occupancy is subsaturating (data not shown). To saturate cell surface receptors, we routinely used ligands at 100–500 nM in media adjusted to pH 7.0. At this concentration, a significant fraction of the ligand is bound to cell surface proteoglycans on CHO-CCR5 and -K1 cells, but receptor downmodulation is maximal (data not shown).



**Figure 2.** Binding and internalization of iodinated RANTES and AOP-RANTES. (A) CHO-CCR5 cells were incubated with 125 pM <sup>125</sup>I-RANTES or <sup>125</sup>I-AOP-RANTES for 90 min at 4°C. Bound-radiolabeled ligands (black bars) were eluted from the cell surface by washes at pH 2 (white bars) or pH 11.6 (stippled bars). The means and standard deviations for triplicate samples are shown for a representative experiment. (B) <sup>125</sup>I-RANTES (closed symbols) and <sup>125</sup>I-AOP-RANTES (open symbols) were bound to CHO-CCR5 (■ and □) or CHO-K1 (●, ○) cells as described in A. Cells were then warmed to 37°C for the indicated times, and the ligand remaining on the cell surface was removed by treatment with pH 11.6 buffer at 4°C, as described in A. Each time point indicates the alkaline resistant (internal) radioactivity as a proportion of the total cell-associated activity, after subtraction of the background activity at time zero.

In our previous study, we used the mAb MC-1 to monitor ligand-induced CCR5 downmodulation and cycling (Mack et al., 1998). MC-1 binds to an epitope associated with the NH<sub>2</sub>-terminal half of the second extracellular loop of CCR5, which overlaps the ligand-binding site and can itself induce CCR5 internalization (Mack, M., unpublished results). Here, we used a second mAb, MC-5, that binds an epitope associated with the NH<sub>2</sub>-terminal domain of human CCR5. This mAb does not induce CCR5 internalization or affect RANTES binding (Mack, M., unpublished results). MC-5 was radioiodinated and used for quantitative analysis of CCR5–cell surface expression. Significantly, MC-5 could not be eluted from its binding site at either acidic or basic pH (within the pH range of 2–12) and, consequently, could not be used to measure endocytosis rates directly. To determine whether CCR5 endocytosis and cycling exhibited similar properties when assessed using MC-5, CHO-CCR5 were treated with

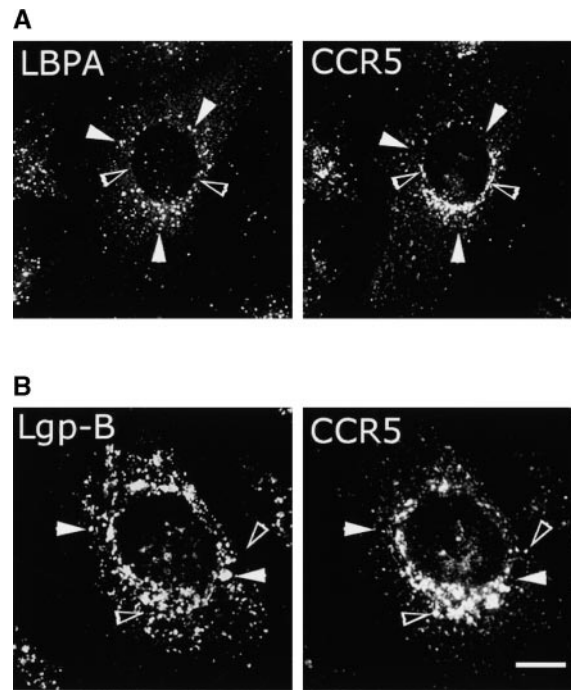


**Figure 3.** CCR5 turnover in ligand-treated CHO cells. (A) CHO-CCR5 cells were incubated in BM or BM-containing CHX for up to 9 h at 37°C. One tenth of each cell lysate was loaded per lane and Western blot analysis was performed using <sup>125</sup>I-MC-5. Antibody binding was determined for each band and is illustrated as the mean ± SD for three independent experiments, and one representative blot is depicted. 9- indicates cells incubated in BM for 9 h without CHX. (B) Cells were incubated in BM plus CHX (white bars) or BM/CHX containing 500 nM RANTES (gray bars) or 100 nM AOP-RANTES (black bars) for 1 or 6 h and the amount of cellular CCR5 was analyzed as described in A.

RANTES or AOP-RANTES for 1 h at 37°C or left untreated. The cells were then cooled to 4°C or washed and reincubated in the absence of ligand for 1 h at 37°C. Subsequently, all cells were incubated at 4°C with <sup>125</sup>I-MC-5, and the amount of cell surface bound mAb was determined, as described in Materials and Methods. Fig. 1 indicates that, as seen previously, both ligands induced downmodulation of cell surface CCR5, giving a decrease of 65–70% in the level of cell surface receptor. After RANTES washout, cell surface CCR5 levels recovered by ~50%. In contrast, little recovery (~7%) was detected after AOP-RANTES removal.

#### Endocytosis of <sup>125</sup>I-labeled RANTES and AOP-RANTES

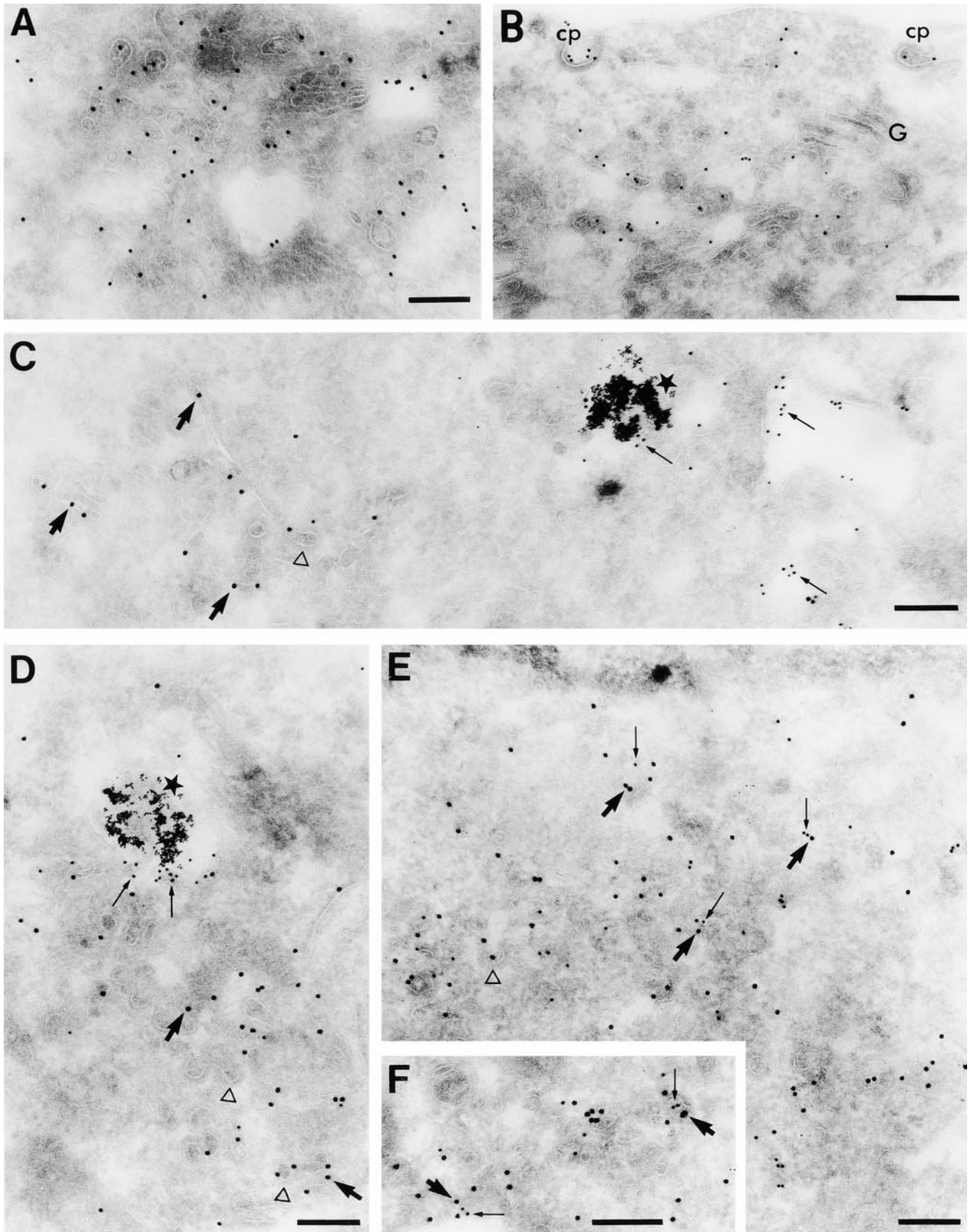
To determine the ability of RANTES and AOP-RANTES to induce endocytosis of CCR5, without the possible complications of recycling, we developed a direct receptor endocytosis assay using <sup>125</sup>I-labeled ligands. For this assay we needed to distinguish between ligand located on the cell surface and ligand in intracellular organelles. In previous endocytosis assays we used low pH to elute cell surface chemokines (Signoret et al., 1997). To test the effect of low pH media on RANTES and AOP-RANTES binding, <sup>125</sup>I-labeled ligands were bound to cells at 4°C for 90 min. The cells were then washed and treated with 4°C media adjusted to pH 7.0–2.0. We found



**Figure 4.** Localization of downmodulated CCR5. CHO-CCR5 cells were treated with RANTES (500 nM) for 4 h at 37°C. After fixation and permeabilization, the cells were stained for CCR5 and the lysosomal markers LBPA (A) and Lgp-B (B), respectively. Open arrowheads indicate CCR5-positive, but LBPA- or Lgp-B-negative, vesicles; white arrowheads indicate LBPA- (A) or Lgp-B-positive (B), but CCR5-negative, late endocytic structures. Bar, 10 μm.

that, though RANTES could be eluted from the cells at pH values <4.0, at least 50% of the cell surface AOP-RANTES remained bound at pH 2.0 (Fig. 2 A). We also investigated the effect of increasing the pH of the elution solution. When we used pH 11.6 buffer, >95% of both ligands were eluted without lysing the cells (Fig. 2 A). We used these alkaline conditions for the subsequent endocytosis assays.

To measure the ability of RANTES and AOP-RANTES to induce CCR5 endocytosis, radioiodinated ligands were bound to CHO-CCR5 and CHO-K1 cells on ice. The cells were then washed and warmed to 37°C. At the indicated times (Fig. 2 B), duplicate samples were returned to 4°C. The total cell-associated radioactivity was determined on one of the samples and the intracellular activity was determined in the other after pH 11.6 elution of the cell surface radioactivity. After warming cells to 37°C, both RANTES and AOP-RANTES underwent endocytosis with equivalent rates (Fig. 2 B). Approximately 70% of the surface-bound ligand was internalized in the first 5 min after warmup and the amount of internal ligand reached a maximum at 75–80% by 10 min. The intracellular activity dropped slightly with longer periods of incubation at 37°C (Fig. 2 B). No internalization was seen for ligands bound to CHO-K1 cells, indicating that ligand internalization was dependent on binding to CCR5. Taken together, the data indicate that both RANTES and AOP-RANTES induce CCR5 endocytosis with equivalent kinetics.



**Figure 5.** Ultrastructural localization of CCR5. CHO-CCR5 cells were pulsed with BSA-gold (5 nM, BSA-G<sub>5</sub>) for 20 min and chased with medium for 4 h to mark late endocytic organelles. Cells were treated with 500 nM RANTES (A and D) or 125 nM AOP-RANTES (B, C, E, and F) during the last 2 h of this chase, washed, and processed for cryosection immunolabeling EM. CCR5 was detected by staining with MC-5 and 10 nm (B) or 15 nm (A and C-F) protein A-gold particles. cp, coated pit; G, Golgi complex. C-F show double label staining of sections for CCR5 (15 nm gold particles, large arrows) with the markers of the endocytic pathway Lgp-B (C), CD63

### CCR5 Turnover

In many cases, ligand-induced receptor internalization can lead to the delivery of receptors to hydrolytic compartments of the endocytic pathway, where they are degraded. To determine whether either RANTES or AOP-RANTES caused enhanced degradation of CCR5, we examined the turnover of this receptor on ligand-treated and -untreated cells. The MC-5 antibody effectively detected CCR5 in Western blot analysis (Fig. 3). Therefore, we treated cells with CHX for up to 9 h to block protein synthesis, and then analyzed cell lysates by SDS-PAGE and Western blotting using <sup>125</sup>I-MC-5. Initial experiments indicated that the cellular CCR5 content decreased with a half-time of 6–9 h in these transfected-CHO cells (Fig. 3 A), similar to previous reports (Mirzabekov et al., 1999). When cells were treated with RANTES or AOP-RANTES for either 1 or 6 h, the electrophoretic mobility of CCR5 decreased (Fig. 3 B). This apparent increase in molecular weight correlates with ligand-induced serine phosphorylation in the cytoplasmic COOH-terminal domain of CCR5 (Oppermann et al., 1999). However, the amount of CCR5 in samples from ligand-treated cells was similar to that recovered from untreated cells (Fig. 3 B). The data indicate that neither RANTES nor AOP-RANTES significantly affect the turnover of CCR5 and that ligand-induced endocytosis does not lead to increased CCR5 degradation.

### Location of Internalized CCR5

The finding that RANTES and AOP-RANTES induced rapid endocytosis of CCR5, but did not affect the rate of turnover of the receptor, prompted us to look closely at the intracellular distribution of CCR5 after ligand-induced internalization. Using immunofluorescence, we found previously that after 1-h treatment with saturating concentrations of ligand, most CCR5 was redistributed from the cell surface and was seen in endosomal elements that overlapped with vesicles containing TfR. There was little detectable delivery of internalized CCR5 to vesicles that labeled for the lysosomal membrane glycoprotein Lgp-B (Lgp-120) (Mack et al., 1998). To determine whether longer incubations with ligand changed the distribution of CCR5, we treated CHO-CCR5 cells for 4 h or overnight at 37°C with RANTES or AOP-RANTES. Cells were then fixed, permeabilized, and stained for CCR5 and markers of late endosomes and lysosomes, Lgp-B, and LBPA (Kobayashi et al., 1998). Fig. 4 shows cells labeled after 4 h of RANTES treatment. CCR5 was detected primarily in intracellular structures located in the perinuclear region. These were mostly distinct from LBPA-containing vesicles that were also located around the nucleus. Similarly, staining with anti-Lgp-B indicated that after longer periods of ligand treatment little CCR5 was located in late endosomes and lysosomes. The distribution was indistinguishable for cells treated with AOP-RANTES and for cells treated overnight with either ligand (data not shown). Together with the turnover data (Fig. 3), these re-

sults indicate that neither RANTES nor AOP-RANTES induce the accumulation of internalized CCR5 into late endosomes or lysosomes.

### Ultrastructural Localization of Internalized CCR5

To identify the subcellular compartment(s) in which internalized CCR5 accumulated, we used immunolabeling of ultrathin cryosections and EM. MC-5 can label CCR5 on cryosections, even after fixation in solutions containing up to 0.2% glutaraldehyde, and can interact directly with protein A–gold. Labeling of untreated cells demonstrated that CCR5 is localized almost exclusively at the plasma membrane, including microvilli (not shown). Nonspecific binding, indicated by gold particles over the nucleus, or on CHO-K1 cells, was very low (not shown). Within minutes of treatment with RANTES or AOP-RANTES, CCR5 could be seen in coated vesicles and tubular vesicular profiles similar to those described for sorting endosomes (data not shown). When cells were treated with RANTES or AOP-RANTES for 2 or 4 h, CCR5 was relocated from the plasma membrane into intracellular structures. Most of the internalized CCR5 was observed in small membrane-bound vesicles and tubules of ~50-nm diameter (Fig. 5, A and B). These tubules and vesicles were often clustered in the perinuclear area of the cell and frequently close to stacked cisternae of the Golgi apparatus. At least 60% of all internalized CCR5 was found over such small tubules and vesicles. A smaller proportion of the internalized CCR5 (<20%) could also be observed in larger vacuoles, which were usually devoid of content or contained one or a few internal vesicles.

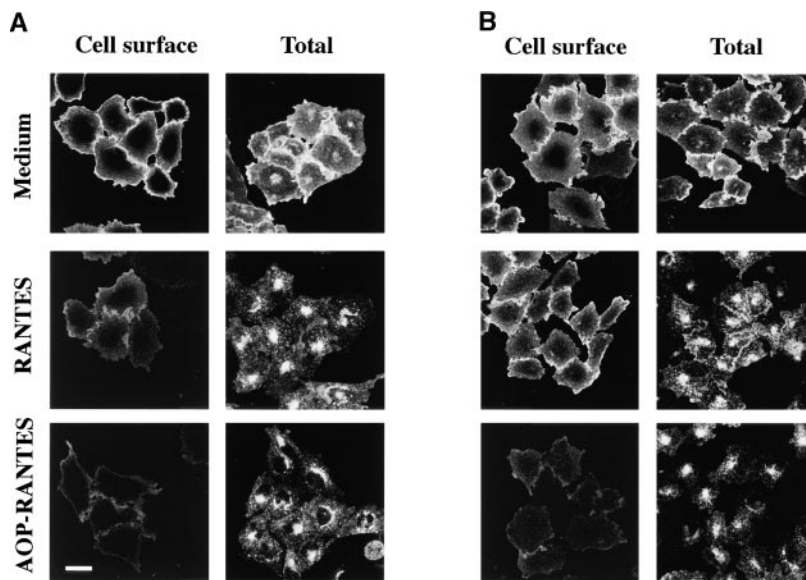
We used antibody markers to identify specific endocytic compartments in double-staining studies together with MC-5. Late endocytic structures and lysosomes were marked by feeding cells BSA coupled to 5-nm gold (BSA-G<sub>5</sub>) for 20 min followed by a 4 h chase in normal medium; RANTES or AOP-RANTES was added during the last 2 h of the chase. Here, <2% of intracellular-labeled CCR5 molecules were seen in BSA–gold-containing structures (Fig. 5, C and D). Furthermore, double-labeling studies showed that there was little colocalization of CCR5 with Lgp-B (Fig. 5 C), CD63 (Fig. 5 D), or LBPA (not shown). These EM studies support the notion that internalized CCR5 is not delivered to late endosomes or lysosomes after treatment of cells with RANTES or AOP-RANTES.

To determine whether CCR5 accumulates in early endosomes in ligand-treated cells, cells were pulsed with the fluid-phase marker HRP for 5 or 10 min at the end of the incubations with RANTES or AOP-RANTES. Internalized HRP was detected with a rabbit polyclonal anti-HRP antibody and protein A–gold. Internalized HRP was also found in small vesicles or tubules that were usually located in the periphery of the cell close to the plasma membrane, and were distinct from the more perinuclear clusters of vesicles containing CCR5 (not shown). Occasional MC-5

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(D), or the TfR (E and F) labeled with 10 nM gold particles (small arrows). BSA–G<sub>5</sub>–containing late endocytic vacuoles are marked with an asterisk. The CCR5-containing tubules and vesicles are distinct from the organelles labeled with Lgp-B (C) or CD63 (D), even though these markers can occasionally be observed in the same part of the cell. In contrast, the TfR-containing tubules are intermingled with MC-5–labeled tubules and vesicles (E and F), with the large and small gold particles are occasionally found in the same vesicles. Note the prominent coats on some of the CCR5-containing vesicles (open arrowheads in C–E). Bars, 200 nm.





**Figure 6.** Recycling of CCR5. (A) CHO-CCR5 cells were incubated in BM or BM with 100 nM RANTES or 100 nM AOP-RANTES for 1 h at 37°C. Cells were then fixed and stained with MC-5 and a FITC-conjugated goat anti-mouse antibody. Cells were stained intact to show cell surface CCR5 or after permeabilization to show intracellular plus cell surface CCR5 (Total). (B) CHO-CCR5 cells were initially treated with RANTES or AOP-RANTES as in A, but after downmodulation, the cells were washed extensively at 4°C in BM, and then incubated in BM containing MC-5 for 1 h at 37°C. The cells were then fixed and stained intact, or after permeabilization with Saponin, with the FITC-conjugated anti-mouse antibody. Bar, 20  $\mu$ m.

labeling was seen in larger (0.2–0.5- $\mu$ m diameter) empty vacuoles that contained HRP. However, the majority of the two labels was distinct.

The distribution of CCR5 in tubules and vesicles clustered in the vicinity of the Golgi apparatus suggested that CCR5 may accumulate in recycling endosomes (Yamashiro et al., 1984; Hopkins et al., 1994). The TfR also traffics through this juxtannuclear compartment and is found primarily within recycling endosomes in CHO cells (Yamashiro et al., 1984). To determine whether CCR5 is associated with recycling endosomes in RANTES or AOP-RANTES-treated cells, cryosections were costained with the anti-TfR antibody H68.4 and with MC-5. The TfR was also seen to be located in clusters of small vesicles and tubules frequently close to stacks of Golgi cisternae. Significant overlap between the TfR and CCR5 was seen (Fig. 5, E and F). Due to the low density of gold particles, the two markers were only occasionally seen together in the same vesicles. Nevertheless, the accumulation of both CCR5 and TfR in the same clusters of vesicles indicates that CCR5 does indeed enter the recycling endosome. Moreover, as the distribution of internalized CCR5 was indistinguishable in RANTES- or AOP-RANTES-treated cells, both ligands appear to induce redistribution of CCR5 from the cell surface to recycling endosomes.

Significantly, some CCR5 was seen at the plasma membrane even after 2 or 4 h treatment with RANTES or AOP-RANTES, and CCR5 could be found in coated-pit and coated-vesicle profiles at these times (Fig. 5 B). The significance of these observations is discussed in more detail below.

### CCR5 Recycling

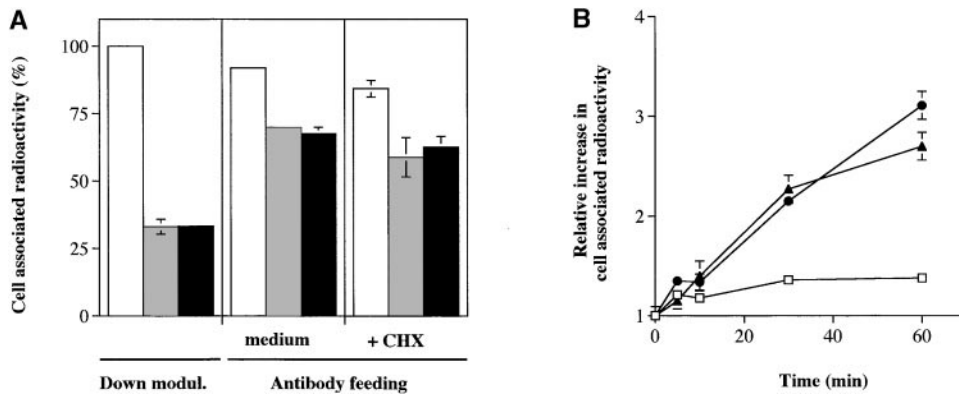
The observations that (a) both RANTES and AOP-RANTES induce redistribution of CCR5 to recycling endosomes, (b) CCR5 appears to recycle to the cell surface only after RANTES washout, and (c) CCR5 can be found in coated vesicles even after 2 or 4 h of AOP-RANTES treatment prompted us to reinvestigate the recycling of CCR5. Our previous recycling assays would detect receptors that recycle to the cell surface and remain there. However, it would not detect receptors that recycle and then re-internalize. Alternatively, AOP-RANTES may selectively

induce the sequestration of CCR5 in recycling endosomes. To investigate these alternatives we performed antibody feeding experiments in which cells that had been treated with chemokine were washed to remove free chemokine and then reincubated at 37°C in the presence of MC-5. For cells to be labeled with MC-5 during this incubation, CCR5 must return to the plasma membrane and be exposed to the extracellular medium containing the antibody.

We first monitored CCR5 cycling by immunofluorescence. CHO-CCR5 cells were incubated for 1 h at 37°C in normal medium or medium containing RANTES or AOP-RANTES. The cells were then placed on ice and washed extensively with cold medium. At this stage, some cells were fixed, labeled with MC-5, and stained with a fluorescent anti-mouse antibody to assess the efficiency of chemokine-induced CCR5 internalization. The rest of the cells were incubated for a further 1 h at 37°C in ligand-free medium containing MC-5. Subsequently, these cells were washed in cold medium, fixed, and labeled with only the fluorescent anti-mouse reagent with or without permeabilization with saponin. With untreated cells, MC-5 staining indicated that the majority of CCR5 was located at the cell surface and there was little detectable intracellular staining (Fig. 6 A). Treatment with RANTES or AOP-RANTES induced redistribution of the bulk of CCR5 from the cell surface to intracellular organelles (Fig. 6 A). When RANTES was washed away and the cells fed with MC-5, we observed a recovery of the cell surface staining visible on intact cells, indicating that some CCR5 receptors had recycled to the plasma membrane (Fig. 6 B). In addition, permeabilized RANTES-treated cells showed some internal punctate staining, suggesting that some CCR5 labeled with MC-5 antibodies had reinternalized into intracellular vesicles (Fig. 6 B).

In keeping with previous results (Mack et al., 1998), little cell surface staining was seen on AOP-RANTES-treated cells fed with MC-5. However, prominent intracellular perinuclear staining was observed on permeabilized cells (Fig. 6 B). This pattern was similar to that seen with cells fixed and stained at the end of the initial AOP-RANTES treatment (Fig. 6 A). If the receptors internalized during the AOP-RANTES treatment were sequestered in an en-





**Figure 7.** Quantitative analysis of  $^{125}\text{I}$ -MC-5 feeding. CHO-CCR5 cells were incubated in BM (white bars, and □) or BM containing RANTES (500 nM, gray bars and ●) or AOP-RANTES (100 nM, black bars and ▲) for 1 h at 37°C. CCR5 molecules remaining at the plasma membrane were then labeled on ice with  $^{125}\text{I}$ -MC-5 to determine the extent of downmodulation. After this saturation step, some cells were reincubated in prewarmed medium

containing  $^{125}\text{I}$ -MC-5 to allow iodinated antibody to label recycling CCR5. (A) Cells were fed with the  $^{125}\text{I}$ -MC-5 for 1 h in the presence or absence of CHX. The graph indicates the cell-associated radioactivity for each point as a percent of the radioactivity bound on untreated cells and is the mean  $\pm$  SD for triplicate samples. (B) To determine the kinetics of  $^{125}\text{I}$ -MC-5 uptake, cells were fed with  $^{125}\text{I}$ -MC-5 for various times after washing out RANTES or AOP-RANTES. The graph shows the relative increase in cell-associated radioactivity at each time point, calculated by dividing the total cell-associated radioactivity at each time point by the cell-associated activity at time zero. Each point represents the mean  $\pm$  SD of triplicate samples from a representative experiment.

docytic compartment, they should not have access to the extracellular MC-5. The finding that AOP-RANTES-treated cells can be labeled with MC-5 indicates that, even on these cells, CCR5 has recycled to the cell surface where it encountered MC-5 and was then reinternalized. The fact that fluid-phase markers are not delivered efficiently to recycling endosomes (Sheff et al., 1999), as indicated by the HRP uptake (see above), and that untreated CHO-CCR5 cells fed with MC-5 show only cell surface staining (Fig. 6 B) makes it unlikely that this labeling could have occurred by fluid-phase endocytosis of MC-5.

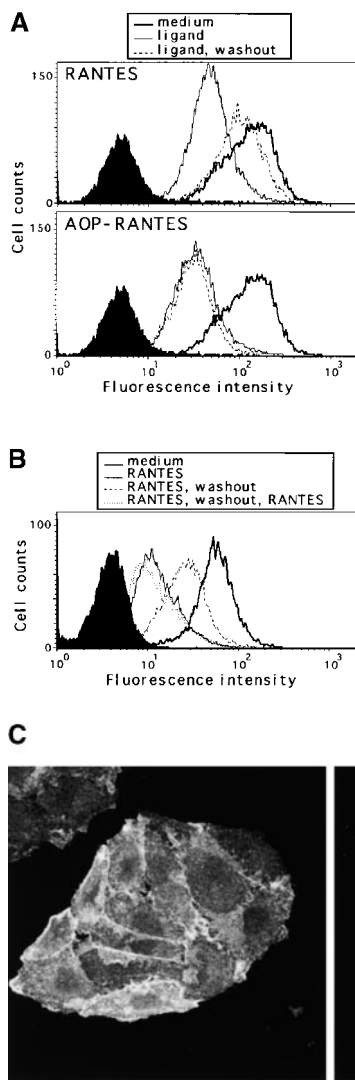
### Biochemical Analysis of CCR5 Recycling

To examine CCR5 cycling quantitatively, we performed similar antibody feeding experiments using  $^{125}\text{I}$ -MC-5. CHO-CCR5 cells were treated with medium or medium containing either RANTES or AOP-RANTES for 1 h at 37°C. The cells were then labeled with saturating concentrations of  $^{125}\text{I}$ -MC-5 for 2 h at 4°C to block all cell surface MC-5-binding sites. Subsequently, the cells were washed with cold medium to remove the excess antibody. One sample for each condition was left on ice to give the amount of antibody bound to the cell surface after CCR5 downmodulation. The other samples were incubated for 1 h at 37°C in the continued presence of  $^{125}\text{I}$ -MC-5. Subsequently, all the cells were washed and the cell-associated radioactivity was determined. RANTES and AOP-RANTES treatment induced 65–70% CCR5 downmodulation, respectively (Fig. 7 A). Since 30–35% of the initial cell surface pool of CCR5 remained at the plasma membrane, the initial incubation with  $^{125}\text{I}$ -MC-5 at 4°C saturated these sites and enabled us to quantitate any internal receptor that recycled to the cell surface. This experiment does not discriminate between recycling receptors that remain at the plasma membrane and recycled receptors that are rapidly reinternalized, rather it assesses the total number of receptors recycling from endosomes. With both RANTES and AOP-RANTES-treated cells, the total counts detected after 1 h of  $^{125}\text{I}$ -MC-5 feeding at 37°C were  $\sim$ 70% of the counts initially bound to the surface of untreated cells (Fig. 7 A). This indicates that at least 35–

40% of the receptors that were internalized during the initial exposure to the chemokines were able to recycle when the chemokine was removed. Moreover, no significant difference was seen for cells treated with RANTES versus cells treated with AOP-RANTES. Similar results were obtained when the experiment was done using cells that had been treated with CHX before the start of the assay and throughout the course of the experiment, ruling out the possibility that uptake of MC-5 occurred on newly synthesized CCR5 molecules (Fig. 7 A).

To investigate the kinetics of CCR5 recycling after treatment with RANTES or AOP-RANTES, we analyzed  $^{125}\text{I}$ -MC-5 feeding on CHO-CCR5 cells for up to 60 min after ligand-induced downmodulation. In these experiments, MC-5 labeling of ligand treated or untreated cells were compared. For the latter cells, recycling should not occur, but the initial labeling with  $^{125}\text{I}$ -MC-5 would be predicted to be high. Therefore, we plotted the data for each time point relative to the cell-associated activity after the initial  $^{125}\text{I}$ -MC-5 labeling at 4°C. Fig. 7 B shows a rapid increase in  $^{125}\text{I}$ -MC-5 labeling for cells exposed to ligand, with virtually identical rates of labeling for RANTES and AOP-RANTES-treated cells.

Since removal of RANTES allowed CCR5 to recycle to the cell surface after the initial endocytosis, we asked if this CCR5 is sensitive to a second exposure to RANTES, as already observed on primary cells (Mack et al., 1998). CHO cells expressing CCR5 were treated in suspension with RANTES for 1 h at 37°C, or left untreated. The cells were then cooled to 4°C or washed and reincubated in the absence of ligand for 1 h at 37°C. Some cells were then treated with RANTES for a second time. Cell surface expression of CCR5 was monitored on fixed cells using MC-5 and analyzed by flow cytometry. This type of experiment was performed on different CHO-CCR5 lines with similar results, and Fig. 8 B shows one of these experiments done with CHO-pgsA-745 CCR5 cells (see below). The increase in fluorescence intensity seen after removal of the ligand was lost after the second treatment with RANTES, indicating that recycled CCR5 molecules are sensitive to a second round of RANTES-induced internalization.



**Figure 8.** Downmodulation and recycling of CCR5 on CHO cells deficient in GAG synthesis. (A) CHO-pgsA-745-expressing CCR5 (medium) were treated in suspension with 100 nM RANTES or 100 nM AOP-RANTES for 1 h (ligand). After washout of the chemokines, some cells were incubated in fresh medium for a further 1 h (ligand, washout). Cells were then fixed and stained with MC-5 and FITC-conjugated anti-mouse antibody and the level of cell-surface fluorescence was measured by flow cytometry. Cells labeled with the secondary antibody only were used as a negative control (shaded peak). (B) CHO-pgsA-745-CCR5 cells were incubated in medium alone (medium) or medium containing 100 nM RANTES for 1 h (RANTES). Excess chemokine was removed by washing and the cells were incubated in fresh medium for one more hour (RANTES, washout). Cells were then treated for a second time with medium containing 100 nM RANTES for 1 h (RANTES, washout, RANTES). Finally, cells were fixed, stained, and analyzed, as described in A. (C) CHO-pgsA-745-CCR5 cells were incubated in medium alone (left) or medium containing 100 nM AOP-RANTES (right) for 1 h. Excess chemokine was removed by washing and then the cells were fed with MC-5 for 1 h at 37°C. Cells were fixed, permeabilized, and stained with the FITC-conjugated anti-mouse antibody. Bar, 20 μm.

Taken together, the antibody feeding assays indicate that CCR5 can recycle to the cell surface after washout of both RANTES and AOP-RANTES. Moreover, the extent and kinetics of recycling appear to be similar after treatment with either ligand. However, on AOP-RANTES-treated

cells, the recycled CCR5 continues to undergo endocytosis. As a consequence, the major steady-state pool of CCR5 is relocated from the cell surface to recycling endosomes.

### Cell Surface Proteoglycans Do Not Influence CCR5 Cycling

In addition to their high affinity for CCR5, RANTES and AOP-RANTES can bind to GAG, such as heparan sulfate (Kuschert et al., 1999; Trkola et al., 1999). It has been suggested that GAG binding may serve to increase the local concentration of chemokines on the cell surface by inducing chemokine oligomerization (Hoogewerf et al., 1997). As many of the experiments discussed above used high concentrations of ligand in which significant cell surface GAG binding could have occurred, we sought to determine whether cell surface GAGs might influence the internalization and recycling of CCR5 seen on CHO cells. CHO-pgsA-745, a CHO line defective in GAG synthesis (Esko et al., 1985; Trkola et al., 1999), and CHO-pgsA-745-expressing human CCR5 were treated in suspension with RANTES or AOP-RANTES for 1 h to induce CCR5 downmodulation. The cells were then washed and some cells were reincubated at 37°C for 1 h in ligand-free medium. Cell surface expression of CCR5 was detected by immunofluorescence using MC-5 and was analyzed by flow cytometry. As shown in Fig. 8 A, both RANTES and AOP-RANTES induced CCR5 downmodulation on the transfected CHO-pgsA-745 cells, indicating that the absence of GAG did not interfere with the activity of the chemokines. Furthermore, by 1 h after the removal of RANTES, a large part of the CCR5 molecules had recycled to the cell surface (Fig. 8, A and B). In contrast, and in keeping with the CHO-CCR5 cells, no significant recovery of cell surface-CCR5 expression was seen on AOP-RANTES-treated cells (Fig. 8 A). Nevertheless, feeding of MC-5 after washout of AOP-RANTES showed uptake of the antibody and an intracellular fluorescent pattern (Fig. 8 C) similar to that described for CHO-CCR5 cells (Fig. 6 B). These results indicate that the binding of RANTES and AOP-RANTES to GAG is not required for either CCR5 internalization or recycling.

### Discussion

Interest in chemokines and chemokine receptors has been prompted by the recent discovery that these proteins play important roles not only in inflammation and regulation of immune responses, but also in the entry and pathogenesis of the primate immunodeficiency viruses (Murphy et al., 2000). Considerable effort has been directed towards understanding the binding properties and specificities of these molecules and to the development of antagonists for specific chemokine receptors (Baggiolini et al., 1997; Murphy et al., 2000). However, relatively little attention has been paid to understanding the trafficking properties of chemokine receptors and the mechanisms through which trafficking pathways are coupled to receptor downmodulation, signal transduction, and other functions of these proteins. Recently, we and others demonstrated that ligand-induced endocytosis of CXCR4 and CCR5 plays a major role in the mechanisms through which chemokines protect cells from HIV infection (Amara et al., 1997; Signoret et al., 1997). This conclusion was subsequently supported by the obser-

vation that the effective anti-HIV activity of a chemically modified form of the CC chemokine RANTES, AOP-RANTES, correlated with the ability of this ligand to induce efficient and apparently irreversible downmodulation of CCR5 (Simmons et al., 1997; Mack et al., 1998). Moreover, recent data have suggested that the ability of chemokines produced in vivo to downmodulate chemokine-receptor expression on target cells for HIV infection may have a significant influence on viral transmission and the course of pathogenesis (Agace et al., 2000). Here, we have investigated the cell biological mechanism(s) through which RANTES and AOP-RANTES differentially downmodulate cell surface-CCR5 expression. In particular, we sought to understand why CCR5 internalized in the presence of RANTES can be reexpressed on the cell surface when the ligand is removed, but CCR5 internalized in the presence of AOP-RANTES is not. Our data indicate that this effect is due to an irreversible, or only slowly reversible, activation of CCR5 endocytosis by AOP-RANTES.

AOP-RANTES was initially characterized as a RANTES receptor antagonist due to its ability to inhibit chemotaxis induced by RANTES and MIP-1 $\beta$  (Simmons et al., 1997). Subsequent studies indicated that though it is a weak partial agonist for CCR1 and CCR3, AOP-RANTES is in fact a full agonist for CCR5 and can induce calcium flux, GPCR kinase (GRK)-mediated receptor phosphorylation, and receptor endocytosis (Mack et al., 1998; Oppermann et al., 1999; Proudfoot et al., 1999; Elsner et al., 2000). In these activities, AOP-RANTES is similar to RANTES. Nevertheless, AOP-RANTES is clearly more effective in blocking CCR5-dependent HIV entry than native RANTES (Simmons et al., 1997; Mack et al., 1998). However, the activities of AOP-RANTES on other chemokine receptors, including CCR1 and CCR3, are distinctly different and the properties described here for CCR5 do not extend to other receptors. Moreover, though more effective than RANTES on CCR5, AOP-RANTES does not block entry of CCR3 tropic HIV-1 strains (Elsner et al., 2000).

Ligand-induced endocytosis of CCR5 is believed to involve mechanisms similar to those described for the  $\beta$ 2-adrenergic receptor ( $\beta$ 2AR) (Lefkowitz, 1998). Ligand binding induces receptor phosphorylation, calcium influx, G protein activation, GRK-dependent recruitment of a nonvisual arrestin, and internalization (Aramori et al., 1997; Oppermann et al., 1999; Rodriguez-Frade et al., 1999; Vila-Coro et al., 1999).  $\beta$ -Arrestin 1 and 2 have been implicated in coupling activated  $\beta$ 2AR and other GPCRs to clathrin and the endocytosis of these receptors through clathrin-coated pits (Ferguson et al., 1996; Goodman et al., 1997; Krupnick et al., 1997). Inhibitor studies (Mack et al., 1998), together with the transient expression of exogenous or dominant-negative GRKs and/or arrestins (Aramori et al., 1997) and the morphological experiments reported here, have indicated that CCR5 is also endocytosed through a mechanism that involves nonvisual arrestins and clathrin-coated vesicles. The subsequent fate of CCR5 is less well established. We showed previously that the distribution of internalized CCR5 overlaps closely with that of endosomal markers, in particular the TfR, and that, at least after RANTES washout, CCR5 can recycle to the cell surface.

The binding of AOP-RANTES to CCR5, measured by displacement of radiolabeled MIP-1 $\alpha$ , indicates a single

high affinity-binding site with an inhibitory concentration of 50% (IC<sub>50</sub>) of 0.072 nM, whereas RANTES displayed a two component displacement curve, suggesting both a high and a low affinity site with affinities of 0.022 and 18 nM, respectively (Simmons et al., 1997). AOP-RANTES also appears to induce higher levels and more sustained phosphorylation of CCR5 compared with RANTES (Oppermann et al., 1999; Vila-Coro et al., 1999). However, the roles of these events in the irreversible loss of CCR5 cell surface expression by AOP-RANTES remain to be established. Moreover, the ability of both RANTES and AOP-RANTES to oligomerize and bind proteoglycans (Hoogewerf et al., 1997; Kuschert et al., 1998) makes the interpretation of some of these observations problematic. Here, we show that radiolabeled RANTES and AOP-RANTES, bound at subsaturating concentrations, are endocytosed with very similar kinetics on CCR5-transfected CHO cells. More than 70% of the ligand bound at 4°C is internalized in 5 min after warming cells to 37°C. Approximately 70–80% of the bound material was endocytosed by 10 min, but internal radioactivity levels then dropped to ~60% by 30 min. Whether this drop represents recycling of internalized ligand-receptor complexes has yet to be established, but is consistent with the observation that CCR5 can recycle on both RANTES and AOP-RANTES-treated cells. After internalization, CCR5 molecules are delivered to early endosomes. We show that neither RANTES nor AOP-RANTES affects the constitutive turnover of the CCR5 protein. Even 6 h after treating cells with saturating concentrations of ligand, similar levels of protein are seen in treated and untreated cells.

Using both immunofluorescence and immunolabeling of cryosections and EM, we found that most CCR5 molecules were initially located on the surfaces of untreated cells. Early after treatment with RANTES or AOP-RANTES, CCR5 was seen in coated vesicles and tubular vesicular profiles similar to those described for sorting endosomes (Marsh et al., 1986; Griffiths et al., 1989). After 2 h, some labeling was still seen at the cell surface by EM, consistent with biochemical experiments that indicated that only 70–80% of cell surface CCR5 was downmodulated by either RANTES or AOP-RANTES. At 2 h, most of the intracellular CCR5 labeling in both RANTES and AOP-RANTES-treated cells was seen to be associated with clusters of small tubules and vesicles, often located close to stacks of Golgi cisternae. At these times, little CCR5 was seen in sorting endosomes, marked with internalized HRP, and only occasional labeling was seen in late endocytic compartments, marked with either internalized BSA-gold or antibodies directed against three antigens found in late endosomes and lysosomes (Lgp-B/Lgp-120, CD63, and LBPA). Recycling endosomes were initially described in CHO cells, and they were observed as a perinuclear compartment where the bulk of the TfR is located and where internalized transferrin can be seen after endocytosis (Yamashiro et al., 1984). The compartment was subsequently seen by EM to consist of clusters of small 60-nm diameter vesicles and tubules (Hopkins et al., 1994). The tubular vesicular clusters in which we observe internalized CCR5 were not labeled with fluid-phase markers, but were labeled with antibodies directed against the cytoplasmic domain of the TfR. By the criteria currently available, these structures have the characteristics of recycling endosomes.

Thus, we propose that RANTES or AOP-RANTES-bound CCR5 molecules are internalized via clathrin-coated vesicles, delivered initially to sorting endosomes, and then directed to recycling endosomes. Although many GPCRs, including  $\beta$ 2AR, have been shown to undergo endocytosis and recycling, this is the first demonstration of which we are aware that indicates a role for the recycling endosome compartment in the trafficking of a GPCR.

The finding that both RANTES and AOP-RANTES induced relocation of CCR5 from the cell surface to recycling endosomes caused us to reexamine the finding that AOP-RANTES induces irreversible downmodulation of CCR5 (Mack et al., 1998). If the AOP-RANTES-induced CCR5 downmodulation cannot be reversed after ligand washout, this ligand must cause the receptor to be sequestered within recycling endosomes. To address this issue, we used antibody feeding experiments to investigate whether CCR5 might be recycled and then reinternalized in AOP-RANTES-treated cells. After washing out the ligand from RANTES-treated cells and incubating the cells in ligand-free medium containing MC-5 for 1 h at 37°C, we found that the antibody was located both at the cell surface and within punctate cytoplasmic organelles. This is consistent with previous observations that CCR5 can recycle on RANTES-treated cells. We also found MC-5 labeling in cells that had been treated with AOP-RANTES. However, this labeling was largely confined to intracellular compartments with a distribution indistinguishable from that seen after AOP-RANTES-induced CCR5 downmodulation. The most likely way for this CCR5 to have become labeled is for CCR5 to have recycled to the cell surface, become labeled with mAb, and then rapidly reinternalized. Quantitative analysis using iodinated MC-5 indicated that the extent of MC-5 labeling and the kinetics of labeling were very similar for both RANTES and AOP-RANTES-treated cells. Thus, CCR5 recycling is similar on cells treated with RANTES and AOP-RANTES, except that on AOP-RANTES-treated cells receptors that reappear on the cell surface are rapidly reinternalized. The fact that the rate of  $^{125}\text{I}$ -MC-5 labeling is the same on both RANTES and AOP-RANTES-treated cells argues against labeling of intracellular CCR5 by fluid-phase endocytosis of radiolabeled antibody. Moreover, previous studies have indicated that fluid-phase markers do not access recycling endosomes efficiently (Sheff et al., 1999). Finally, we observed CCR5 in clathrin-coated pits even after 4 h of AOP-RANTES treatment, which is consistent with the notion that this receptor undergoes rounds of endocytosis and recycling. Taken together, the data indicate that CCR5 recycles to the cell surface after removal of ligand from RANTES-treated cells and that recycling is unimpaired on AOP-RANTES-treated cells. The principal difference in the two ligands is that the ligand-induced conversion of CCR5 to a form that undergoes efficient endocytosis can be reversed when RANTES is washed out, but not when AOP-RANTES is removed. Thus, a stable, or only slowly reversible, AOP-RANTES-induced activation of CCR5 leads to the relocation of the cell surface pool of CCR5 to the recycling endosome compartment.

The mechanism through which AOP-RANTES irreversibly activates CCR5 endocytosis remains unclear. For other GPCRs, such as the  $\beta$ 2AR, trafficking through endosomes has been linked to acid-induced receptor–ligand dissociation (Krueger et al., 1997; Lefkowitz, 1998). Although we found some differences in the pH dependence of RANTES or

AOP-RANTES dissociation from CCR5, neither ligand exhibited significant release at pH values above 4, i.e., within the pH range found in endocytic organelles. However, we have shown that the CCR5 that recycles to the cell surface after RANTES removal is resensitized and can be reinternalized when the cells are exposed to a second round of RANTES. Thus, trafficking of RANTES-occupied CCR5 to recycling endosomes presumably leads to removal of at least some of the ligand from CCR5 and allows resensitized receptors to recycle to, and reaccumulate on, the cell surface. Preliminary  $^{125}\text{I}$ -RANTES feeding experiments, similar to those illustrated here for  $^{125}\text{I}$ -MC-5, indicate that only CCR5 molecules that recycle after RANTES washout are able to bind the iodinated ligand. CCR5 recycling after AOP-RANTES removal does not bind  $^{125}\text{I}$ -RANTES (see Online Supplemental Materials), suggesting that in this case the receptor may still be occupied, preventing further ligand binding. Sustained binding of AOP-RANTES, or an AOP-RANTES fragment, may maintain the receptor in a form that is recognized by the endocytic machinery, relocating the major steady-state pool of CCR5 to recycling endosomes.

Although these mechanisms remain to be clarified in detail, we show that CCR5 is recycled efficiently in both RANTES and AOP-RANTES-treated cells. Moreover, the recycling of CCR5 in cells treated with AOP-RANTES may not be coupled to receptor resensitization. In this respect, the recycling of CCR5 appears to differ from that described for GPCRs, such as  $\beta$ 2AR. In the latter case, endocytosis and trafficking through endosomes is required for ligand dissociation, receptor dephosphorylation, and recycling of resensitized receptors to the cell surface (Krueger et al., 1997; Lefkowitz, 1998). Whether these receptors also undergo recycling and reinternalization if ligand is not removed, as appears to be the case for CCR5, is unclear. However, one implication of our data is that CCR5 recycling may not be regulated through the ability of endosomes to distinguish receptors that have been resensitized by ligand removal from receptors that remain at least partially activated. Rather, receptors may recycle at similar rates regardless of their activation state. Thus, transit through an endosome compartment may be necessary to remove ligand and resensitize receptors, but in the event that this does not occur, activated receptors that recycle to the cell surface are rapidly reinternalized and reexposed to the endosome environment. Therefore, resensitization for CCR5 may be an iterative process requiring multiple cycles through the endosome compartment.

Finally, with regard to the ability of AOP-RANTES to protect cells from HIV infection, both RANTES and AOP-RANTES can downmodulate the cell surface expression of CCR5. At present, we know little of the requirements, in terms of receptor density, for HIV entry. The fact that the effects of AOP-RANTES appear at best to be only slowly reversible may be an important component of its increased efficacy. Our data suggest that a small molecular therapeutic agent aimed at preventing HIV entry would be most effective if it could mimic the properties displayed by AOP-RANTES in modulating the endocytic trafficking of coreceptor molecules.

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