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Agonist-induced Endocytosis of CC Chemokine Receptor 5 Is Clathrin Dependent

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The signaling activity of several chemokine receptors, including CC chemokine receptor 5 (CCR5), is in part controlled by their internalization, recycling, and/or degradation. For CCR5, agonists such as the chemokine CCL5 induce internalization into early endosomes containing the transferrin receptor, a marker for clathrin-dependent endocytosis, but it has been suggested that CCR5 may also follow clathrin-independent routes of internalization. Here, we present a detailed analysis of the role of clathrin in chemokine-induced CCR5 internalization. Using CCR5-transfected cell lines, immunofluorescence, and electron microscopy, we demonstrate that CCL5 causes the rapid redistribution of scattered cell surface CCR5 into large clusters that are associated with flat clathrin lattices. Invaginated clathrin-coated pits could be seen at the edge of these lattices and, in CCL5-treated cells, these pits contain CCR5. Receptors internalized via clathrin-coated vesicles follow the clathrin-mediated endocytic pathway, and depletion of clathrin with small interfering RNAs inhibits CCL5-induced CCR5 internalization. We found no evidence for CCR5 association with caveolae during agonist-induced internalization. However, sequestration of cholesterol with filipin interferes with agonist binding to CCR5, suggesting that cholesterol and/or lipid raft domains play some role in the events required for CCR5 activation before internalization.

INTRODUCTION

Chemokine receptors are G protein-coupled receptors (GPCRs) that are activated by chemoattractant cytokines called chemokines. They play key roles in a variety of developmental and chemotactic events (Rossi and Zlotnik, 2000; Horuk, 2001). The CC chemokine receptor 5 (CCR5) is specifically expressed on subsets of leukocytes that are recruited to sites of inflammation by the CC chemokines and CCR5 ligands, CCL3 (macrophage inflammatory protein [MIP]-1α), CCL4 (MIP-1β), CCL5 (regulated on activation normal T-cell expressed and secreted [RANTES]), CCL8 (monocyte chemoattractant protein-2), and CCL3L1 (LD78β). In addition, together with CD4, CCR5 is a major cellular receptor for the human (HIV-1 and HIV-2) and simian immunodeficiency viruses (Simmons *et al*., 2000).

Chemokine receptor agonists are able to inhibit HIV infection of susceptible cells in vitro (Cocchi *et al*., 1995; Bleul *et al*., 1996; Oberlin *et al*., 1996). We and others have established that chemokines trigger endocytosis of cell surface chemokine receptors and that this internalization is a major component of the mechanism of chemokine inhibition of viral infection (Alkhatib *et al*., 1997; Amara *et al*., 1997; Signoret *et al*., 1997; Mack *et al*., 1998; Signoret *et al*., 2000). Agonist binding induces rapid CCR5 internalization and accumulation of the internalized receptor in recycling endosomes (Mack *et al*., 1998; Signoret *et al*., 1998, 2000). Although this internalization has been studied superficially, little is known of how these receptors are recruited into endocytic organelles. Initial studies of immunolabeled cryosections from agonist-treated Chinese hamster ovary (CHO)-CCR5 cells revealed the presence of recycling CCR5 in coated pits and vesicles, suggesting a role for clathrin in CCR5 endocytosis (Signoret *et al*., 2000). In addition, treatment of cells with hypertonic sucrose, a treatment that can inhibit endocytosis via clathrin-coated vesicles (CCVs), inhibits agonist-induced CCR5 uptake (Mack *et al*., 1998). CCR5 endocytosis has been shown to require nonvisual arrestins (β-arrestins) that may couple ligand-activated receptors to clathrin (Miller and Lefkowitz, 2001; Fraile-Ramos *et al*., 2003). Together, these observations suggested that CCR5 is internalized via clathrin-mediated endocytosis.

Recently, several publications have indicated that CCR5 is internalized through clathrin-independent mechanisms (Mueller *et al*., 2002; Venkatesan *et al*., 2003). For some time, it has been recognized that alternative endocytic pathways exist that do not rely on the formation of clathrin coats (Nichols and Lippincott-Schwartz, 2001; Johannes and Lamaze, 2002). However, the molecular details of these pathways are still largely lacking, and their physiological roles in cells have still to be elucidated. The best characterized of these is the caveolar pathway that depends on the

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Abbreviations used: BM, binding medium; CCP, clathrin-coated pit; CCR5, CC chemokine receptor 5; CCV, clathrin-coated vesicle; CHC, clathrin heavy chain; CHO, Chinese hamster ovary; CLC, clathrin light chain; CTxB, cholera toxin B subunit; EM, electron microscopy; GPCR, G protein-coupled receptor; HTf-R, human transferrin receptor; Mv-1-Lu, mink lung endothelial cells; PAG, protein A-gold; PBS, phosphate-buffered saline; PFA, paraformaldehyde; RBL, rat basophilic leukemia; Tf, transferrin; siRNA, small interfering RNA.

presence of integral membrane proteins called caveolins (Okamoto *et al*., 1998). Caveolin-1 and -2 (or caveolin-3 in muscle) form a protein coat on the inner face of the plasma membrane that is essential for the formation and stability of flask-shaped invaginations termed caveolae (Okamoto *et al*., 1998; Pelkmans and Helenius, 2002; Nichols, 2003). Endocytosis through these structures leads to the delivery of cargo molecules to caveolin-containing endosomes, or caveosomes, organelles that are distinct from early sorting and recycling endosomes (Pelkmans and Helenius, 2002). The caveolar pathway seems to be important not only for entry and intracellular delivery of certain bacterial toxins, viruses, and growth factors but also for endocytosis of some membrane constituents (Pelkmans and Helenius, 2002; Nichols, 2003). The findings that caveolin-1 binds cholesterol and is resistant to extraction with some nonionic detergents, together with the fact that caveolae are enriched in glycophosphatidylinositol-linked proteins, cholesterol, and glycosphingolipids, led to the suggestion that caveolae constitute a form of lipid microdomain or raft. Lately, further endocytic pathways have emerged, based on the findings that markers for raft domains can be internalized from the plasma membrane through a cholesterol-sensitive but caveolin- and clathrin-independent mechanism (Nichols, 2003; Parton and Richards, 2003; Pelkmans and Helenius, 2003). However, due to the diversity of the intracellular itineraries followed by raft markers and the lack of specific machinery associated with raft-dependent endocytosis, this pathway remains poorly understood (Parton and Richards, 2003; Helms and Zurzolo, 2004).

A number of GPCRs, including chemokine receptors, have been proposed to be enriched in lipid rafts, but the significance of these observations is unclear (Manes *et al*., 1999, 2001; Chini and Parenti, 2004; Gomez-Mouton *et al*., 2004). To determine precisely the mechanism of agonist-induced CCR5 internalization, we have conducted a detailed morphological and biochemical investigation of the events leading to CCR5 endocytosis. Using immunofluorescence and electron microscopy (EM), and CCR5 expressing cell-lines in which CCR5 has been shown to be functionally active (Mack *et al*., 1998; Zhao *et al*., 1998; Kraft *et al*., 2001), we examined the very early effects of agonist treatment on CCR5 distribution and internalization. We demonstrate that agonist binding triggers clustering of cell surface CCR5 into clathrin-coated domains of the plasma membrane containing the adaptor protein complex 2 (AP2). CCR5 molecules are then internalized through clathrin-coated pits (CCPs) and CCVs into early endosomes together with transferrin (Tf), a marker for the clathrin-mediated endocytic pathway. By suppressing the expression of clathrin heavy chain (CHC), and the formation of clathrin-coated structures, we establish that clathrin is required for ligand-induced CCR5 endocytosis. Finally, we show that cholesterol can influence agonist binding to CCR5, but we find no evidence to support a role for nonclathrin-mediated endocytosis in CCR5 internalization.

MATERIALS AND METHODS

Antibodies and Reagents

Tissue culture reagents and plastics were from Invitrogen (Paisley, United Kingdom), and chemicals were from Sigma Chemical (Poole, Dorset, United Kingdom), unless otherwise indicated. CCL5 (RANTES) was provided by A.E.I. Proudfoot (Serono Pharmaceuticals Research Institute, Geneva, Switzerland). ^{125}I-CCL4 (MIP-1β; specific activity 2000 Ci/mmol) was purchased from Amersham Biosciences UK (Little Chalfont, Buckinghamshire, United Kingdom). Fixatives and reagents for EM were from TAAB Laboratories Equipment (Aldermaston, United Kingdom) and Agar Scientific (Stanstead, United Kingdom). The murine monoclonal antibody against human CCR5, MC-5 (IgG2a), was provided by M. Mack (Medizinische Poliklinik, University of Munich, Germany). MC-5 conjugated to Alexa-Fluor 488 (Molecular Probes Europe, Leiden, The Netherlands) as described previously (Signoret *et al*., 2004) was used in some experiments. Rabbit antisera directed against the AP2 adaptor complex α-subunit (C8), early endosome antigen (EEA)-1, and clathrin light chain (CLC) were obtained from M. S. Robinson (University of Cambridge, Cambridge, United Kingdom), M. Clague and S. Urbe (University of Liverpool, Liverpool, United Kingdom), and F. Brodsky (University of California San Francisco, San Francisco, CA), respectively. Rabbit anti-caveolin-1 was purchased from BD Transduction Laboratories (Mannheim, Germany). Alexa-Fluor–conjugated goat anti-mouse IgG (^{488}GAM and ^{647}GAM), goat anti-rabbit IgG (^{488}GAR and ^{594}GAR), human transferrin (^{594}Tf and ^{647}Tf), and cholera toxin B subunit (^{488}CTxB) were purchased from Molecular Probes. Protein A-gold conjugates (10 and 15 nm, PAG_{10} or PAG_{15}) were obtained from the Department of Cell Biology, University of Utrecht, Utrecht, The Netherlands.

Cells

DHFR-deficient CHO, rat basophilic leukemia (RBL), and mink lung endothelial (Mv-1-Lu) cells stably expressing wild-type human CCR5 (CHO-CCR5, RBL-CCR5, and Mv-1-Lu-CCR5) were maintained in nucleoside-free α-minimal essential medium, 80:20 medium (80 parts of RPMI 1640 medium, 20 parts of medium 199) and DMEM, respectively (Signoret *et al*., 1998, 2000; Kraft *et al*., 2001). CHO-K1 cells were maintained in DMEM-F12. All media were supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. RBL-CCR5 and Mv-1-Lu-CCR5 were kept under selection with 0.6 and 1 mg/ml G418 (Geniticin) as described previously (Signoret *et al*., 1998; Kraft *et al*., 2001).

Transfection

CHO-CCR5 cells were transfected with a pRK5 mammalian cell expression construct for the human transferrin receptor (HTf-R, a gift from D. Cutler, Medical Research Council-Laboratory for Molecular Cell Biology, London, United Kingdom) together with pSV2-Neo at a ratio of 10:1 by using nucleofection (Amaxa, Köln, Germany). Transfected cells were selected in medium containing 1 mg/ml G418, and stable HTf-R–expressing cell lines were isolated by limiting dilution. The caveolin-1-green fluorescent protein (GFP) constructs were provided by A. Helenius (Institute of Biochemistry, Swiss Federal Institute of Technology, Zurich, Switzerland). Proteins were transiently expressed in CHO-CCR5 cells by nucleofection.

Small Interfering RNA (siRNA) and Clathrin Knockdown

Clathrin knockdown was performed by RNA interference (RNAi) essentially as described previously (Fraile-Ramos *et al*., 2003). Cells were detached with phosphate-buffered saline (PBS)/10 mM EDTA and seeded at a density of 0.3×10^5 cells/16-mm well in tissue culture medium without antibiotics. Cells were transfected, 4–12 h later, with 60 pmol of a 21-nucleotide RNA duplex targeting the clathrin heavy chain (Motley *et al*., 2003) using Oligofectamine as recommended by the manufacturer (Invitrogen). A second transfection was performed 24 h later. Eight hours after the second transfection, the cells were detached in PBS/EDTA and plated onto coverslips. Clathrin knockdown was assessed 72 h later by immunofluorescence, and endocytosis assays were performed.

Immunofluorescence Microscopy

Cells on coverslips were washed in binding medium (BM: RPMI 1640 medium without bicarbonate containing 0.2% bovine serum albumin [BSA] and 10 mM HEPES, pH 7.0) and treated with 125 nM CCL5, 200 nM ^{594}Tf, or 10 μM ^{488}CTxB in 37°C BM for the indicated times. Cells were fixed in 3% paraformaldehyde (PFA) for 15 min, and free aldehyde groups were quenched with 50 mM NH_4Cl in PBS. To analyze the cell surface distribution of CCR5, cells were labeled intact with 3.35 nM MC-5 in PBS/0.2% gelatin for 1 h, before being permeabilized with 0.05% saponin in PBS/gelatin and labeled with rabbit anti-clathrin light chain (1/1000). After washing in PBS/0.05% saponin, cells were stained with ^{488}GAM and ^{594}GAR secondary antibodies diluted in PBS/gelatin/saponin. To localize endocytosed CCR5, live cells were prelabeled with MC-5 in BM and incubated at 37°C as indicated in the text. Cells were then fixed, quenched, and permeabilized with saponin, and the MC-5 was detected with ^{488}GAM or ^{647}GAM. In some experiments, the permeabilized samples were costained for cellular markers as indicated. Coverslips were washed extensively, mounted in Mowiol as described previously (Signoret and Marsh, 2000), and examined using a Zeiss Axioskop or a Nikon Optiphot-2 microscope equipped with an MRC Bio-Rad 1024 confocal laser scanner. Digital images were assembled using Adobe Photoshop.

^{125}I-CCL4 Binding

Confluent layers of CHO-CCR5 cells and CHO-K1 cells were incubated in BM alone or containing 5 μg/ml filipin for 45 min on ice. This medium was then made 125 pM with ^{125}I-CCL4, and the samples were incubated at 4°C for a further 90 min. The cells were washed extensively in BM and PBS, harvested,

and the cell-bound radioactivity was measured using a gamma-counter as described previously (Signoret *et al*., 2004).

Flow Cytometry

CCR5 down-modulation and transferrin uptake were measured as described previously (Signoret *et al*., 2004). Cells detached with PBS/EDTA were washed in BM, pretreated as indicated, and/or incubated for 30 min at 37°C with 125 nM CCL5 or 200 nM ^{647}Tf, to allow CCR5 or transferrin internalization, respectively. Samples were cooled on ice, washed with ice cold BM, and cell surface-bound transferrin was removed by acid washing as described previously (Fraile-Ramos *et al*., 2003). To detect CCR5, cells were labeled at 4°C with 1.75 μg/ml MC-5 in wash buffer (PBS containing 1% FCS and 0.05% azide), followed by ^{647}GAM. The cells were washed, fixed overnight in PBS containing 1% FCS and 1% PFA, and analyzed using a dual laser four-color FACSCalibur flow cytometer and the CellQuest Pro-software (BD Biosciences UK, Oxford, United Kingdom). For each sample, the mean fluorescence intensity (MFI) was determined from 10,000 accumulated events.

Electron Microscopy

Cell Surface Replicas of Whole Mount Preparations. Cells grown to 50–70% confluence on glass coverslips were rinsed in BM and incubated at 37°C in 125 nM CCL5 for various times. The cells were rinsed briefly in ice-cold PBS and fixed in 2% PFA in 0.1 M phosphate buffer, pH 7.4. CCR5 at the plasma membrane was labeled at room temperature with MC-5 (7.7 nM) in PBS containing 2% BSA (PBS/BSA), followed by PAG_{15}. After washing extensively in PBS/BSA and PBS, the cells were fixed in 4% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.6; postfixed in 1% osmium tetroxide/1.5% potassium ferricyanide; dehydrated through 70%, 90%, and absolute ethanol; and critical point dried. A thin film of platinum/carbon was evaporated onto the dried specimens by rotary shadowing at an angle of 45°, and the platinum/carbon replicas were reinforced with a layer of carbon. Cells were removed from the coverslips with 8% hydrofluoric acid and cellular material under the replicas was dissolved in 10N sodium hydroxide for 4–6 h. Replicas were placed on grids and examined with a Philips EM 420 transmission electron microscope (FEI UK, Cambridge, United Kingdom).

To measure the density of gold particles on replicas from untreated or CCL5-treated cells, random fields of cells were photographed, and 14 negatives from each condition were printed at 34,000×. Photographs were placed under a mask showing 30 randomly positioned 1-cm-diameter circles. Gold particles seen within these circles were counted and the values used to determine the number of gold particles per square micrometer of membrane. For the CCL5-treated samples, values from circles that fell over any clusters of gold particles (>11 particles per 1-cm-diameter circle, the highest number of gold particles per circle seen on untreated cells) were ignored. To determine the density of gold particles in the CCR5 clusters seen on CCL5-treated cells, gold particles within a 1-cm-diameter circle placed over the center of all obvious clusters (with >11 gold particles per circle) were counted.

Preparation of Membrane Sheets Showing the Cytoplasmic Face of the Plasma Membrane. Nearly confluent cell monolayers grown on coverslips for 3 d were treated with 125 nM CCL5 as described above and washed in ice-cold BM. CCR5 at the plasma membrane was sequentially labeled with 7.7 nM MC-5 in BM followed by PAG_{15} for 1 h at 4°C. Samples were rinsed in BM and HEPES buffer (25 mM HEPES, 25 mM KCl, and 2.5 mM Mg acetate, pH 7.0), and upper membranes were prepared using the "rip-off" technique described previously (Sanan and Anderson, 1991). Briefly, coverslips were inverted onto Formvar/carbon-coated nickel grids that had been treated with poly-L-lysine on the day of the experiment. A rubber bung was pressed onto the top of the coverslips with light finger pressure for 10 s before the coverslips were lifted away, leaving portions of the upper membrane of the cells attached to the poly-L-lysine-coated grids. The membrane preparations were washed in ice-cold HEPES buffer and fixed for 20 min in 4% glutaraldehyde in HEPES buffer. Alternatively, to immunolabel the inner face of the plasma membrane, the membrane sheets were fixed with 2% PFA/1% glutaraldehyde for 10 min at room temperature and stained with primary antibodies and PAG_{10}. Immunolabeled membranes were washed and fixed in 2% glutaraldehyde in HEPES buffer. All samples were postfixed with 1% osmium tetroxide in HEPES buffer, washed in HEPES buffer and distilled H_2O (dH_2O), and incubated 10 min in 1% tannic acid. After washing in dH_2O, samples were stained with 1% uranyl acetate, rinsed in dH_2O, and air-dried before viewing with the electron microscope.

To measure gold particle densities and the sizes of flat lattices on ripped-open untreated or CCL5-treated CHO-CCR5 cells, random fields were photographed and printed at 36,200×, and gold particle distributions were counted. The membrane areas associated with flat lattice were determined by placing photographs under a test point grid (point spacing 4 mm), counting the proportion of test points over the lattices and converting to square micrometers following standard stereological procedures (Mayhew *et al*., 2002).

Immunolabeling of Cryosections. CHO-CCR5 or Mv-1-Lu-CCR5 cells were washed in BM, treated with 125 nM CCL5 for 5 min at 37°C, and fixed immediately by adding an equal volume of prewarmed double-strength fixative (8% PFA in 0.1 M sodium phosphate buffer, pH 7.4) directly into the CCL5-containing medium. After 20 min, the medium was replaced with single-strength fixative (4% PFA) for 90 min. Fixed cells were rinsed in PBS/20 mM glycine, embedded in 12% gelatin, infiltrated with 2.3 M sucrose, and frozen in liquid nitrogen as described previously (Raposo *et al*., 1997). Cryosections (50 nm) were quenched in 50 mM glycine/50 mM NH_4Cl and labeled with primary antibodies and PAG. For double-labeling experiments, sections were first stained with MC-5 and a rabbit anti-mouse bridging antibody (DakoCytomation, Ely, Cambridgeshire, United Kingdom) and PAG_{10}. The sections were then fixed in 1% glutaraldehyde for 10 min, re-quenched, and stained with rabbit polyclonal antibodies against clathrin light chain or caveolin-1 and PAG_{15}. Sections were embedded in uranyl acetate/methyl cellulose (Raposo *et al*., 1997) and examined with the electron microscope.

Other EM Methods

Mv-1-Lu-CCR5 cells were fixed in 1.5% glutaraldehyde, 2% PFA in 0.2 M sodium cacodylate buffer, pH 7.6, for 30 min at room temperature. Cells were postfixed in 1% osmium tetroxide/1.5% potassium ferricyanide for 1 h at 4°C and stained with 1% tannic acid before dehydrating and embedding in Epon resin (TAAB 812). Ultrathin sections (70 nm) were stained with lead citrate.

RESULTS

Agonist-activated CCR5 Associates with Clathrin-positive Plasma Membrane Domains

Agonist treatment of CHO and RBL cells stably expressing CCR5 has been shown to cause receptor activation, as judged by calcium mobilization and chemotactic responses (Mack *et al*., 1998; Kraft *et al*., 2001; Chen *et al*., 2004). In RBL-CCR5 cells, this activation is also apparent as agonist-induced cell spreading (Figure 1B). We and others have shown that agonist treatment of CHO-CCR5 or RBL-CCR5 cells leads to rapid CCR5 internalization (Mack *et al*., 1998; Olbrich *et al*., 1999; Signoret *et al*., 2000; Pollok-Kopp *et al*., 2003). To investigate the mechanisms responsible for this internalization in more detail, we focused on the events occurring in the first few minutes after agonist addition.

It has been reported for some other GPCRs, such as the β_2-adrenergic receptor (β_2-AR), that agonist-activated receptors concentrate in areas of the plasma membrane where they colocalize with clathrin before being endocytosed by a β-arrestin- and clathrin-dependent mechanism (Goodman *et al*., 1996; Scott *et al*., 2002). To examine whether CCR5 can associate with clathrin, we carried out immunofluorescence experiments on CHO-CCR5 and RBL-CCR5 cells. Cells were exposed to CCL5 for up to 5 min at 37°C, before fixation and staining intact with an anti-CCR5 antibody (MC-5) to detect only the plasma membrane CCR5. CCL5 treatment caused a change in the cell surface staining pattern of CCR5 in both cell lines (Figure 1, A and B). On untreated cells CCR5 seemed uniformly distributed. After incubation with CCL5, CCR5 staining became more punctate, a pattern clearly visible on single confocal sections, especially at the edges of the cells (Figure 1, A and B). When cells were permeabilized and costained for clathrin, there was no detectable overlap of CCR5 with clathrin on untreated cells, but some of the punctate CCR5 cell surface staining seen after CCL5 treatment coincided with clathrin-labeled structures (Figure 1, A and B). These initial observations suggested that agonist-stimulation triggers the translocation of CCR5 molecules into clathrin-positive domains of the plasma membrane.

Agonist Treatment Causes CCR5 Clustering at the Plasma Membrane

To study the distribution of CCR5 at higher resolution, we examined immunolabeled cell surface replicas of CHO-CCR5 and RBL-CCR5 cells by EM (Miller *et al*., 1991). Cells

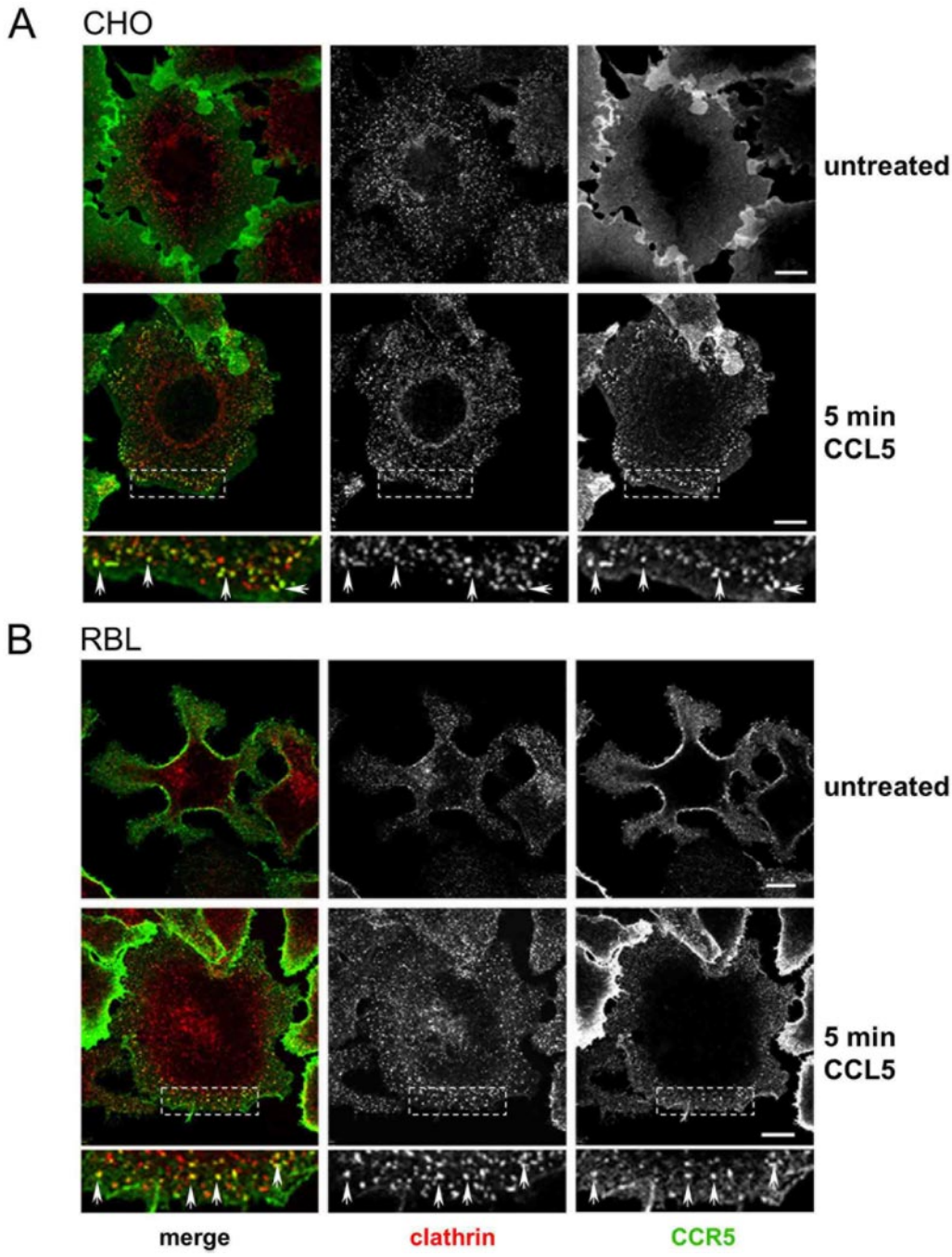

Figure 1. Agonist-induced accumulation of CCR5 in clathrin-positive plasma membrane domains. CHO-CCR5 cells (A) or RBL-CCR5 cells (B) were left untreated or treated with CCL5 for 5 min, fixed, and labeled intact for cell surface CCR5 with MC-5. Cells were permeabilized and stained with a rabbit anti-clathrin light chain and then with ^{488}GAM and ^{594}GAR secondary antibodies to detect CCR5 and clathrin, respectively. The figure shows single confocal sections from a representative experiment. Boxed areas are shown at higher magnification under each panel. Bars, 10 μm.

cultured on coverslips were kept in medium or treated with CCL5 for up to 5 min at 37°C and fixed. CCR5 at the cell surface was detected with the MC-5 antibody and 15-nm protein A-gold particles (PAG_{15}).

Images of replicas of whole mount preparations from untreated CHO-CCR5 and RBL-CCR5 cells are shown in Figure 2, a and d, respectively. These replicas revealed a dispersed distribution for CCR5 on untreated cells. In contrast, on samples treated for as little as 30 s with CCL5, CCR5 molecules seemed clustered into patches (Figure 2, b, c, and e). These clusters were visible within 30 s of ligand treatment (Figure 2b). Note that features of the cell surface such as microvilli and invaginations that may represent the openings of pits also were seen, and in some cases these structures were labeled for CCR5 (Figure 2b). The size of CCR5 clusters varied somewhat. Measurements on CHO-CCR5 cells treated with CCL5 for 2 min indicated an average diameter of 0.28 $\pm$ 0.09 μm, but patches as large as 0.45 μm

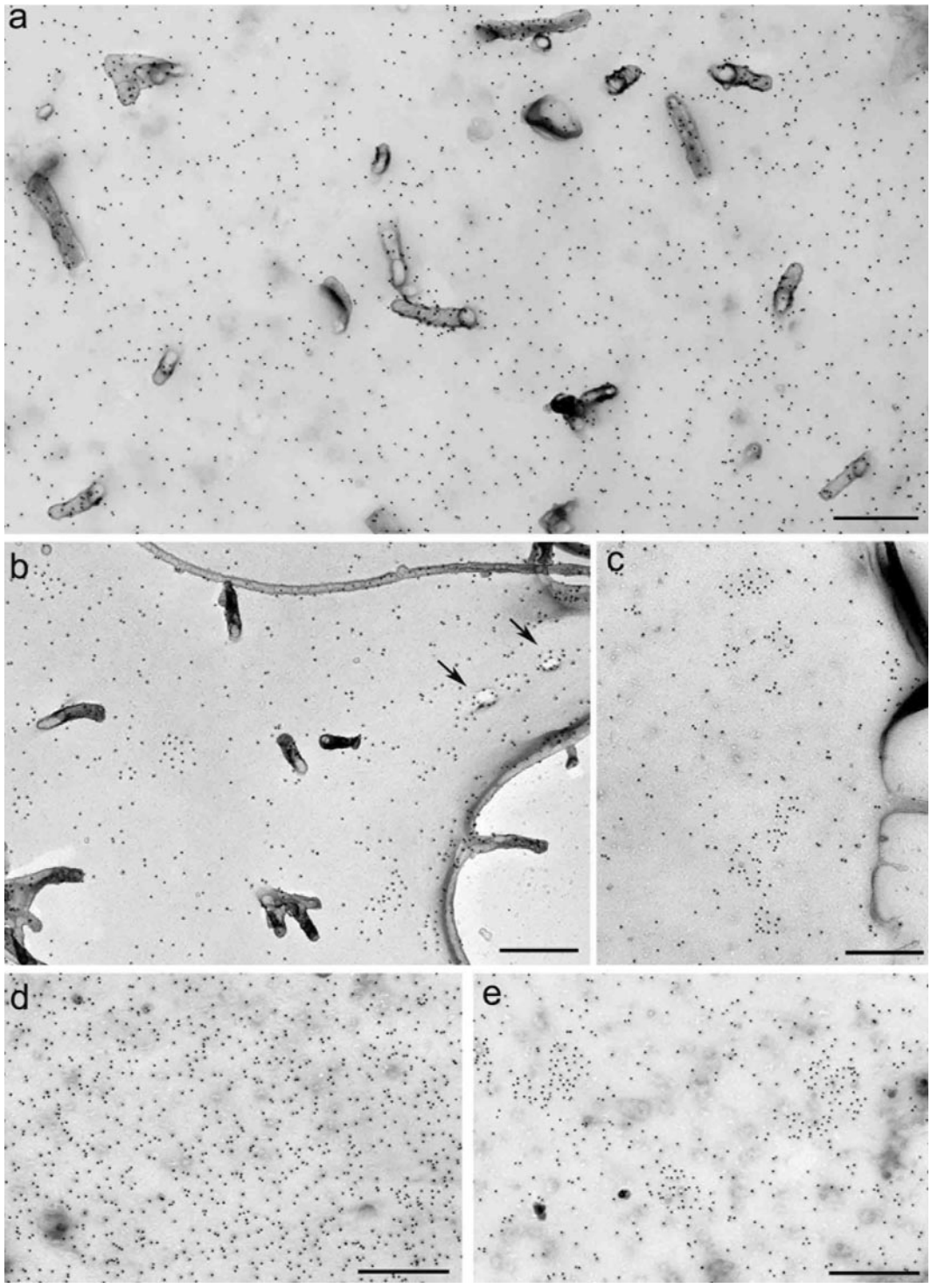

Figure 2. CCR5 cell surface distribution. CHO-CCR5 (a–c) and RBL-CCR5 (d and e) cells were treated in BM alone (a and d) or with CCL5 for 30 s (b) or 5 min (c and e) at 37°C before fixation. Surface CCR5 was detected by labeling with MC-5 and PAG_{15}, and cell surface replicas were prepared as described in *Materials and Methods*. Agonist-treated CCR5 was occasionally seen associated with shadowed invaginations of the membrane (arrows). Bars, 500 nm.

were sometimes observed. On RBL-CCR5 cells, the clustering was more dramatic, and the clusters were larger with an average diameter of 0.44 ± 0.09 μm. In general, the CCR5-containing patches were much larger than the estimated diameter of a CCP (0.05–0.1 μm).

To establish whether the observed clustering of CCR5 was significant, we analyzed quantitatively the distribution of gold particles on untreated and on CCL5-treated samples of CHO-CCR5 cells (Table 1). For each sample, we examined a series of random images and calculated the density of gold

Table 1. Density of gold particles on membranes from CHO-CCR5 cells

	Average density/μm^2 ± SD	
	Random areas	in clusters
Untreated	30.05 ± 10.87	
CCL5 5 min	26.89 ± 14.28	203.54 ± 23.39

Arbitrarily selected fields of membrane were photographed and the average density of gold particles was determined by counting particles over randomly selected areas of these fields or in apparent clusters, as described in *Materials and Methods*.

particles per square micrometer of membrane. The overall density of gold particles at the plasma membrane was only slightly reduced after 5 min of agonist treatment, suggesting that the majority of CCR5 molecules were still at the cell surface at this time (Table 1, random areas). The gold particle density in the clusters on CCL5-treated samples averaged >200 particles/μm^2, indicating an enrichment of 6.7-fold compared with untreated cells (Table 1).

Agonist-induced CCR5 Clusters Are Associated with Clathrin Lattices

To identify architectural features of the inner face of the plasma membrane that might be associated with the CCR5 clusters, we generated membrane sheets from the upper surface of CHO-CCR5 or RBL-CCR5 cells by using a so-called rip-off protocol (Sanan and Anderson, 1991; Wilson *et al.*, 2000; Lamaze *et al.*, 2001). Cells on coverslips were treated with CCL5 as described above and labeled on ice for surface CCR5 using MC-5 and PAG_{15}. Subsequently, the coverslips were pressed down onto EM grids and then lifted away, ripping the cell open, and leaving pieces of the plasma membrane from the tops of the cells on the grids. After fixation, the sheets were viewed by EM.

CCR5-labeled membrane sheets from CHO-CCR5 or RBL-CCR5 cells are shown in Figure 3. In these membrane sheets, we observed filamentous material (arrowheads) that labeled with phalloidin (our unpublished data) and corresponded to elements of the cortical actin cytoskeleton. In addition, large areas of hexagonal lattice (L), characteristic of clathrin lattices (see below), as well as small electron-dense deeply invaginated coated structures (arrows) were seen. On untreated CHO-CCR5 or RBL-CCR5 cells, gold particles were distributed throughout the area of the membranes. These particles were often seen associated with the filamentous material but were mainly excluded from the flat lattices (Figure 3A, a and c). After a short stimulation with CCL5, gold particles were found clustered over the areas of flat lattice (Figure 3A, b, d, and e). Occasionally, particles were seen associated with coated invaginations at the edge of these lattices, as presented here for RBL-CCR5 cells (Figure 3A, e). Quantitative analysis of the distribution of gold particles on the membrane preparations from CHO-CCR5 cells showed a 14-fold increase in the density of gold particles per area of lattice after 2 min of treatment with CCL5 (Table 2). Significantly, the area and frequency of lattice in CHO cells did not change after agonist treatment, suggesting that clathrin recruitment to the membrane was not increased by CCR5 activation.

The polygonal appearance of the flat lattices and of the coat seen on the invaginations strongly suggested the presence of clathrin. We confirmed the nature of the coat by immunolabeling the cytoplasmic face of the ripped-off membrane sheets with an antibody against clathrin and PAG_{10} (Figure 3B, a and b). Lattices on membranes from both untreated and agonist-treated cells were labeled strongly for clathrin. These lattices were also labeled with an antibody against the α-subunit of the AP2 clathrin adaptor complex (Figure 3B, c and d). The colocalization between the 15-nm gold particles associated with CCR5 on the external face of the membrane and the 10-nm particles associated with clathrin or AP2 on the inside face of the membrane demonstrate that CCR5 relocated into AP2-positive clathrin-coated domains after agonist treatment.

Endocytosis of Agonist-treated CCR5 by CCVs

Because these studies indicated that CCR5 could associate with clathrin-coated structures, we used immunolabeling of ultrathin cryosections to identify the early vesicular structures containing agonist-activated CCR5 on CHO-CCR5 cells (Figure 4). Sections were doubly stained for CCR5 (PAG_{10}) and clathrin (PAG_{15}). After 5 min of treatment with agonist, CCR5 was visible in vesicles just beneath the plasma membrane with electron-dense coats on the cytoplasmic sides of their membranes (Figure 4, b–d, arrows). These vesicles also labeled for clathrin (PAG_{15}), indicating that they are CCVs In addition, CCR5 could be observed associated with flat areas of the plasma membrane that also labeled for clathrin (Figure 4a) and seemed to correspond to the regions of flat lattice observed in the rip-offs. A similar study on another CCR5 expressing cell line, Mv-1-Lu-CCR5, also showed that CCR5 internalized via CCPs and CCVs (Figure 6B).

Internalized CCR5 Follows the Clathrin-mediated Endocytic Pathway

Plasma membrane components internalized through CCVs are usually delivered to early sorting endosomes where they are sorted for direct or indirect recycling, or degradation. To establish whether CCR5 is transported along a similar route, we used CHO-CCR5 cells overexpressing the HTf-R, a characteristic marker for the clathrin-mediated endocytic pathway. This allowed us to trace the uptake of fluorescent transferrin (^{594}Tf) and simultaneously follow the internalization of ligand-activated CCR5. Cell surface receptors were prelabeled with fluorescent MC-5 for 1 h at room temperature, conditions that do not induce receptor internalization nor affect CCR5 distribution (Signoret *et al.*, 2000). Cells were then washed and incubated for 10 min in medium containing ^{594}Tf before adding CCL5 to the culture for a further 5 or 10 min. We observed a considerable overlap of internalized CCR5 with ^{594}Tf (Figure 5A). By 5 min of CCL5 treatment, a significant portion of CCR5 positive structures at the edges of the cells were costained with ^{594}Tf. Because this experiment does not distinguish internalized receptors from those still at the cell surface, the Tf-negative CCR5 puncta may correspond to clusters of CCR5 molecules at the plasma membrane. After 10 min nearly all the CCR5 was found in ^{594}Tf-containing structures, indicating that CCR5 and transferrin are internalized via the same pathway.

We also double-stained agonist-treated CHO-CCR5 cells for CCR5 and the early sorting endosome marker EEA-1. As shown in Figure 5B, CCR5 colocalized with EEA-1, but only after 10 min of stimulation. These results are consistent with the movement of activated CCR5 through the clathrin-mediated endocytic pathway from CCPs, to transferrin and EEA-1–positive early endosomes, and subsequently to perinuclear recycling endosomes (Signoret *et al.*, 2000).

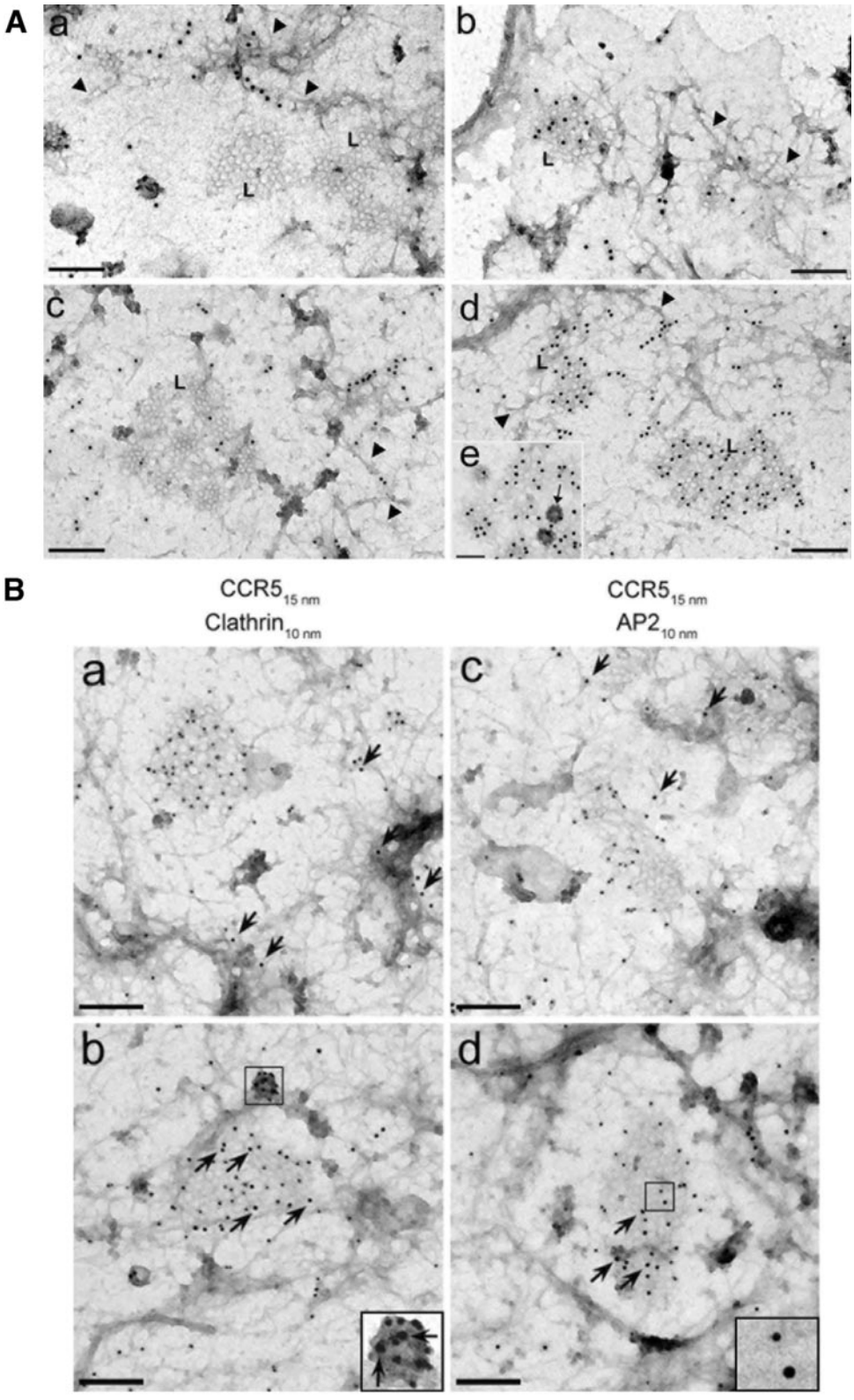

Figure 3. Agonist-induced redistribution of CCR5 into clathrin lattices. (A) CHO-CCR5 (a and b) and RBL-CCR5 (c–e) cells were incubated in BM alone (a and c) or with CCL5 (b, d, and e) for 2 min at 37°C, rinsed at 4°C, and labeled with MC-5 and PAG_{15} on ice before being ripped open as described in *Materials and Methods*. The cortical filamentous network (arrowheads), large areas of flat lattice characterized by their polygonal coat (L), and small electron-dense coated invaginations (arrow) are visible on the inner face of the plasma membrane. (B) Membrane sheets from CHO-CCR5 cells treated for 2 min in BM alone (a and c) or with CCL5 (b and d) were prepared as described above and labeled on the cytoplasmic surface with antibodies against clathrin (a and b) or AP2 (c and d) followed by PAG_{10}. Arrows indicate some of the large gold particles (PAG_{15}) marking CCR5. A clathrin-labeled coated invagination containing CCR5 is enlarged in b. Bars, 200 nm except A, e, which is 100 nm.

Table 2. Density of gold particles in flat lattices on CHO-CCR5 cells

	Average ± SD	
	Untreated	CCL5 (2 min)
No. of lattices analyzed	163	168
Lattice (% of total membrane)	1.20 ± 0.28	1.08 ± 0.51
Area/lattice (μm^2)	0.05 ± 0.02	0.04 ± 0.01
Gold particles/μm^2 of lattice	0.58 ± 0.47	8.30 ± 2.89

Arbitrarily selected fields of membranes prepared from ripped-open cells were photographed. Equivalent surface areas of membranes from untreated or 2-min CCL5-treated samples were analyzed. The total number of flat lattices found on these membranes was similar. The area of membrane covered by lattice is expressed as % of total membrane area analyzed. The average size of a lattice and the average density of gold particles per square micrometer of lattice were determined as described in *Materials and Methods*.

Caveolae Are Not Involved in CCR5 Internalization

To determine whether CCR5 may also be internalized via a clathrin-independent pathway, we used CCR5-expressing mink lung endothelial cells (Mv-1-Lu-CCR5) that have a prominent caveolar pathway (Di Guglielmo *et al*., 2003). Caveolae and caveosomes can be easily seen by EM on Epon-embedded cell preparations (Figure 6A, a) or when cryosections from these cells are stained with an anti-caveolin antibody (Figure 6A, b). We have previously shown that CCR5 undergoes rapid agonist-induced endocytosis in these cells (Signoret *et al*., 1998). EM analysis of cryosections from CCL5-treated Mv-1-Lu-CCR5 cells showed gold-labeled CCR5 molecules in coated structures invaginating from the plasma membrane and in coated vesicles that colabeled for clathrin (Figure 6B). Thus, CCR5 also undergoes clathrin-mediated endocytosis in Mv-1-Lu-CCR5 cells.

To investigate a role for caveolae, we first used immunofluorescence to localize CCR5 and endogenous caveolin-1 in Mv-1-Lu-CCR5 cells. We followed prelabeled cell surface CCR5 on cells kept in medium or exposed to CCL5 for 5 or 10 min and costained for caveolin-1 (Figure 6C). Caveolin-1 was found at the plasma membrane as well as in internal vesicular structures. There was no detectable overlap of the CCR5 and caveolin-1 labeling before or after agonist treatment (Figure 6C). Note that, unlike CCR5, caveolin-1 was not uniformly expressed on the surface of these cells, as observed previously (Di Guglielmo *et al*., 2003). The detection of caveolin-1 by immunofluorescence required cell permeabilization, which could potentially disturb caveolin-1 distribution. We therefore investigated the subcellular distribution of CCR5 and caveolin-1 on ultrathin cryosections (Figure 6A, c and d). In agreement with the immunofluorescence experiment, caveolin and caveosomes showed a restricted distribution. Cryosections from 5 min CCL5-treated samples costained for CCR5 failed to show the receptor in caveolin-positive invaginations or vesicles (Figure 6A, c and d).

To further examine whether caveolin could influence the internalization of CCR5, we transfected CHO-CCR5 cells with GFP-tagged constructs for caveolin-1. The C-terminal caveolin-1-GFP construct (Cav-1-GFP) has been shown to behave like the wild-type protein, whereas the N-terminal GFP-tagged caveolin-1 molecule (GFP-Cav-1) acts as dominant negative (DN) inhibitor of caveolae-mediated endocytosis (Pelkmans *et al*., 2001). CHO-CCR5 were transiently transfected with either of these GFP-tagged caveolin-1 constructs or with GFP alone. At 48 h posttransfection, ~50% of the transfected cells were expressing the GFP proteins (our unpublished data). To assess agonist-induced CCR5 down-modulation, transfected cells were treated in suspension with CCL5 for 30 min at 37°C and then labeled on ice with MC-5 and a fluorescent goat anti-mouse antibody. The cell surface fluorescence on GFP-positive cells was then measured by fluorescence-activated cell sorting (FACS). Figure 7A shows a similar level of CCR5 down-modulation in cells expressing Cav-1-GFP, GFP-Cav-1 (DN), or GFP alone, the latter being identical to nontransfected cells (our unpub-

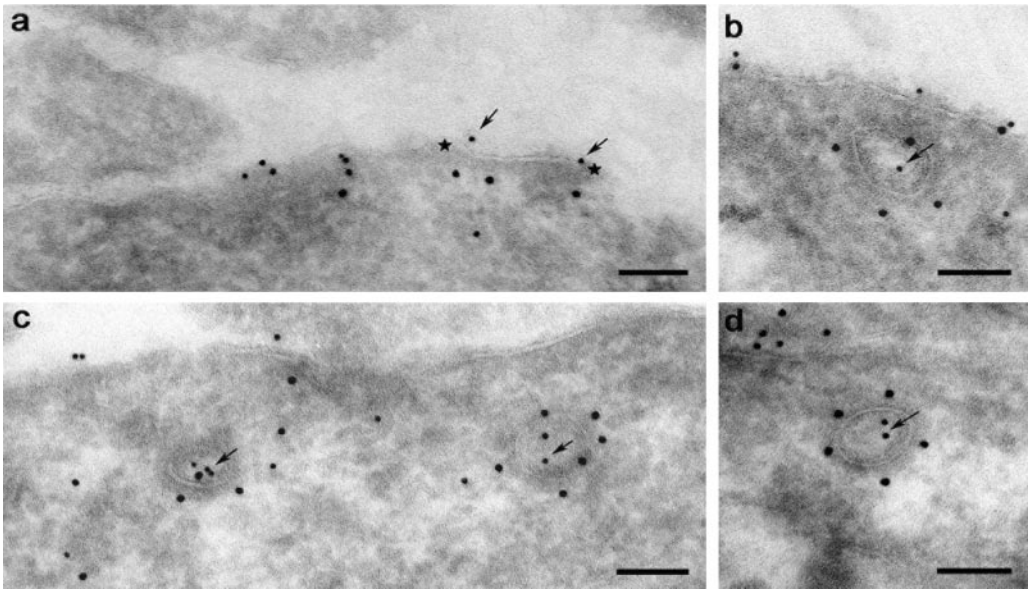

Figure 4. CCR5 internalized in CCVs. Cryosections of CHO-CCR5 cells treated with CCL5 for 5 min were double labeled with MC-5 plus PAG_{10} and anti-clathrin plus PAG_{15}, respectively. CCR5 (arrows) is seen in coated areas of the plasma membrane (a, between stars) and in coated vesicles (b–d) that are labeled for clathrin. Bars, 100 nm.

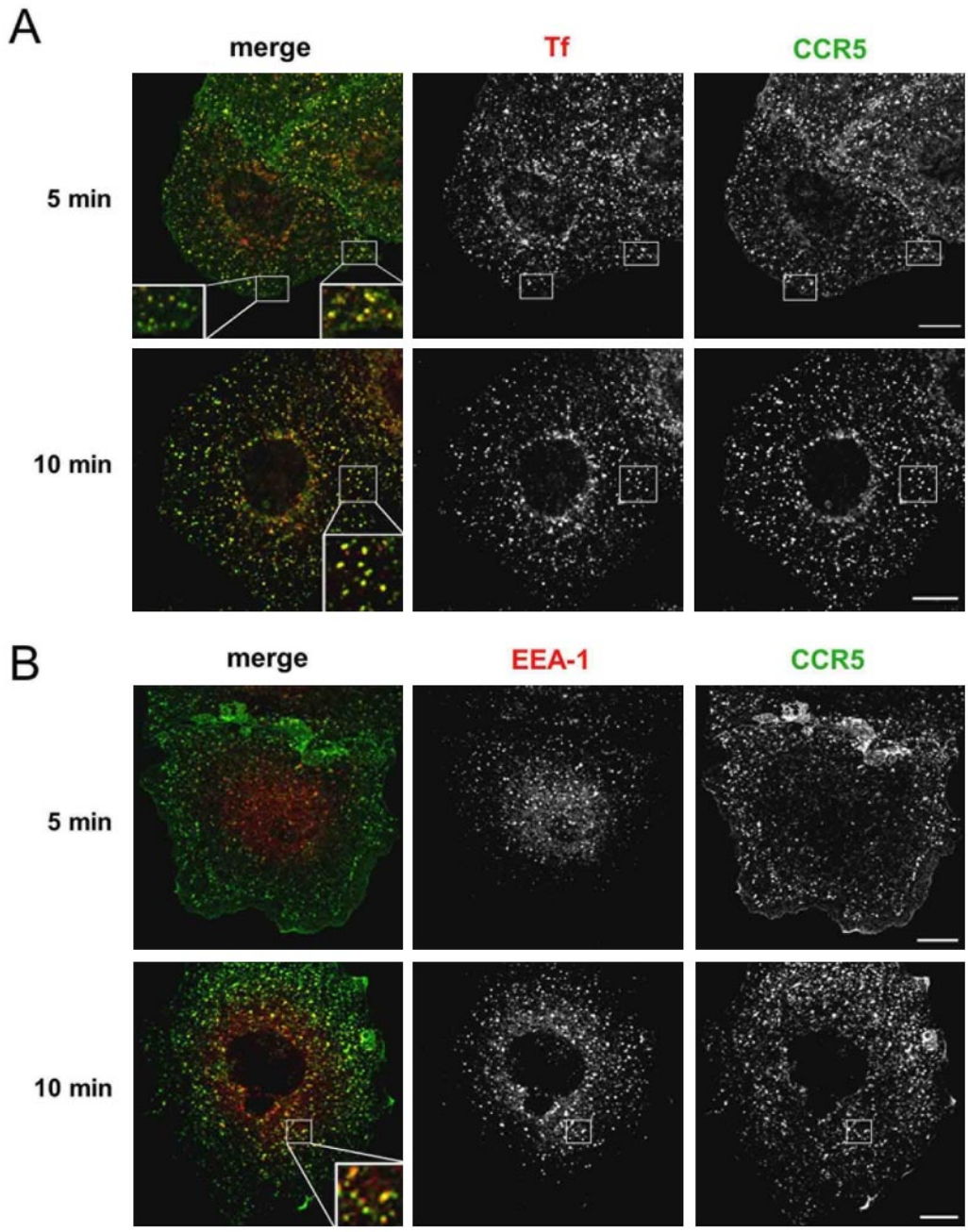

Figure 5. Internalized CCR5 follows the clathrin-mediated endocytic pathway. Internalization of prelabeled cell surface CCR5 on CHO-CCR5 cells overexpressing HTf-R. (A) Cells prelabeled with fluorescent ^{488}MC-5 were allowed to take up ^{594}Tf for 10 min, and CCL5 was then added for a further 5 or 10 min. Cells were fixed, and the distribution of the fluorescent signals was analyzed using a confocal microscope. (B) Cells prelabeled with MC-5 were treated with CCL5 for 5 or 10 min, fixed, permeabilized with saponin, and stained with a rabbit anti-EEA-1 antibody. CCR5 and EEA-1 were then detected using Alexa-Fluor–conjugated secondary antibodies. The figure shows single confocal sections from a representative experiment. Boxed areas are shown at higher magnification in the merged panels. Bars, 10 μm.

lished data). Immunofluorescence analysis of CCL5-treated cells expressing GFP-Cav-1 (DN) confirmed that CCR5 was internalized and sorted to perinuclear recycling endosomes, as described previously (Signoret *et al*., 2000), in the absence of a functional caveolin-dependent pathway (Figure 7B).

Cholesterol Is Required for CCR5 Endocytosis

Recent reports implicated lipid rafts in agonist-induced CCR5 internalization, based on experiments using drugs that interfere with membrane cholesterol to disorganize so-called raft microdomains (Mueller *et al*., 2002; Venkatesan *et al*., 2003). However, other studies have suggested that membrane cholesterol is crucial for maintaining CCR5 in a structural conformation capable of binding agonist (Nguyen and Taub, 2002; Nguyen and Taub, 2003a). To identify which of the events leading to CCR5 internalization was affected when disturbing membrane cholesterol, we studied the effect of filipin on agonist binding, CCR5 clustering and

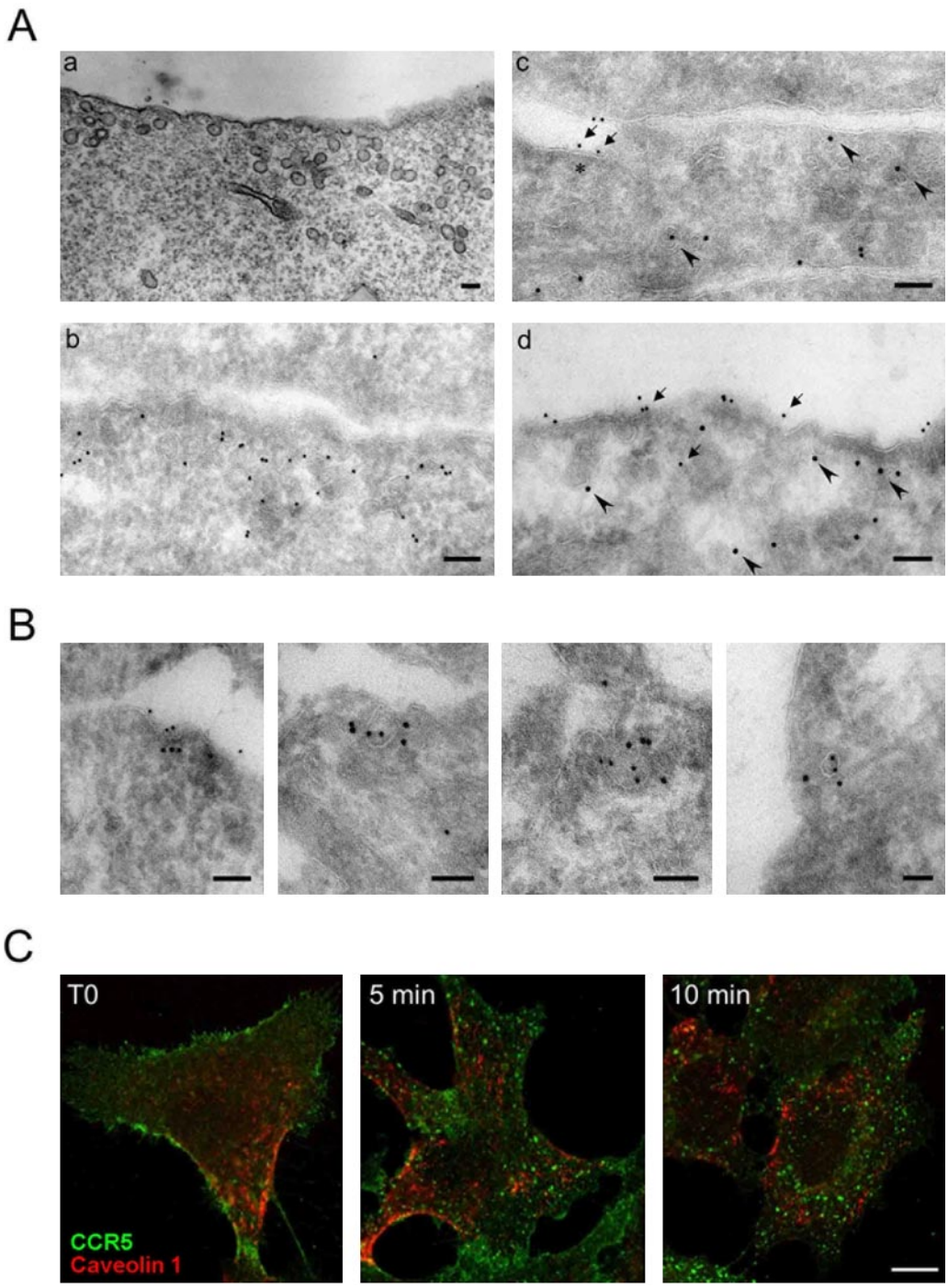

Figure 6. Distribution of agonist-treated CCR5 in Mv-1-Lu cells. (A) Caveolae in Mv-1-Lu-CCR5 cells. Uniform small membrane invaginations resembling caveolae were observed at the cell surface and beneath the plasma membrane on Epon-embedded Mv-1-Lu-CCR5 cells (a) and can be labeled for caveolin-1/PAG_{10} on cryosections (b). In c and d, CCL5-treated Mv-1-Lu-CCR5 cells (125 nM CCL5, 5 min) were double labeled for CCR5 (MC-5 and PAG_{10}, arrows) and caveolin-1 (PAG_{15}, arrowheads). Although some CCR5 can be seen in regions containing PAG_{15}-labeled caveolae, it is not found associated with the invaginations. Some CCR5 is located near an electron-dense flat, presumably clathrin-coated, region of the plasma membrane (c, *). Bars, 100 nm. (B) Cryosections of Mv-1-Lu-CCR5 cells treated with CCL5 for 5 min were double labeled for CCR5 (MC-5 and PAG_{10}) and clathrin (PAG_{15}). CCR5 was seen in clathrin-coated pits and vesicles. Bars, 100 nm. (C) Mv-1-Lu-CCR5 cells prelabeled with MC-5 for cell surface CCR5 were treated with CCL5 for 5 or 10 min, fixed, permeabilized with saponin, and costained for caveolin-1. Samples were analyzed by confocal microscopy. Bars, 10 μm.

down-modulation on CHO-CCR5 cells. We first compared the capacity of agonist to bind CCR5 in the presence or absence of filipin by using ^{125}I-CCL4 (Figure 8A). The amount of ^{125}I-CCL4 bound was significantly reduced on filipin-treated cells. Thus, cholesterol sequestration by filipin seems to interfere with CCL4 binding to CCR5.

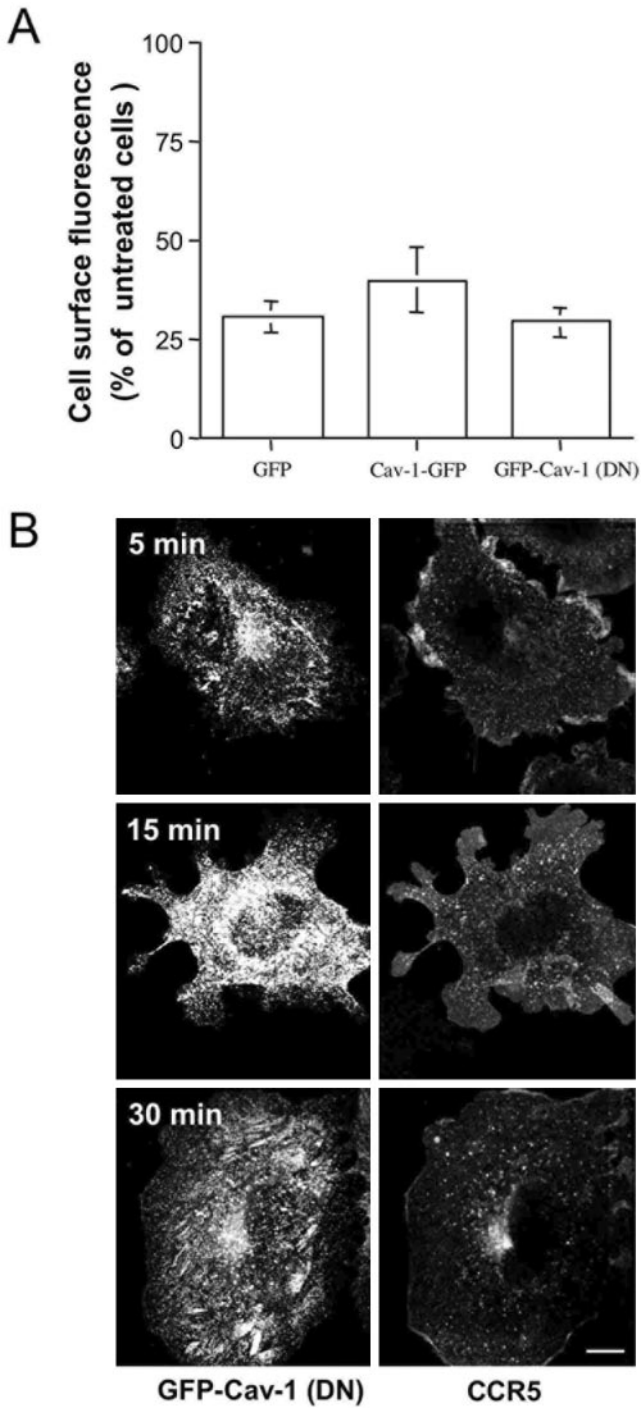

Figure 7. Effect of GFP-tagged caveolin-1 proteins on CCR5 endocytosis. (A) CHO-CCR5 cells transiently transfected with plasmids for GFP alone, Cav-1-GFP or GFP-Cav-1 (DN) were treated with CCL5 for 30 min at 37°C. Cell surface CCR5 down-modulation was monitored by staining with MC-5 and ^{647}GAM and quantified by FACS analysis of samples gated on GFP-positive cells. The results show the percentage of the ^{647}GAM fluorescence ± SD compared with untreated cells for a representative experiment performed in triplicate. (B) CCR5 internalization in cells expressing GFP-Cav-1 (DN) prelabeled with MC-5 and incubated with CCL5 for up to 30 min. CCR5 was detected on fixed and saponin-permeabilized cells by staining with ^{647}GAM. The figure shows single confocal sections. Bars, 10 μm.

Immunofluorescence staining and confocal analysis demonstrated that CCL5 was able to induce changes in the plasma membrane distribution of CCR5 in the presence of filipin at 37°C, indicating that CCR5 clustering could still take place (Figure 8B). However, CCL5-induced CCR5 down-modulation was completely inhibited by filipin treatment (Figure 8C). When CHO-CCR5 cells were treated with CCL5 in suspension in the presence of filipin and the level of CCR5 cell surface expression was determined by FACS analysis as described above, there was no significant reduction in cell surface-associated fluorescence of CCL5-treated samples, indicating that filipin prevented the internalization of CCR5. Significantly, the same treatment also inhibited the uptake of fluorescent transferrin in these cells (Figure 8D). However, fluorescent transferrin could still bind to the surface of filipin-treated cells (our unpublished data). This experiment suggested a general interference of filipin with the clathrin-mediated pathway, similar to that described for methyl-β-cyclodextrin-treated cells (Rodal *et al*., 1999; Subtil *et al*., 1999). Thus, cholesterol seems to be required both for CCR5 agonist binding and for efficient clathrin-mediated endocytosis.

CCR5 Internalization Is Inhibited by Clathrin Knockdown

Together, our results suggest that agonist-induced CCR5 internalization occurs through a clathrin-mediated pathway. To further examine the role of clathrin, we used RNAi to knock down expression of the CHC. We used the chc-2 siRNA sequence described previously (Motley *et al*., 2003) that efficiently depletes human HeLa cells of CHC and disrupts the assembly of clathrin coats. Although DNA sequences for hamster and mink CHC are not available, the region targeted with the chc-2 siRNA is conserved in mammalian CHC genes sequenced to date. The experiments were performed in CHO-CCR5 cells overexpressing the HTf-R or Mv-1-Lu-CCR5 cells. Chc-2, but not a scrambled siRNA, efficiently knocked down clathrin in both cell lines, although the proportion of depleted cells was lower than that seen with HeLa cells (our unpublished data). To assess for clathrin depletion, fixed and permeabilized cells were stained with an anti-CLC antibody. Knocked down cells lost the characteristic peripheral punctate pattern associated with clathrin-containing structures, as well as the perinuclear labeling associated with the *trans*-Golgi network (Figure 9A). To verify that the knockdown inhibited clathrin-mediated endocytosis, we followed the uptake of Tf in chc2 siRNA-treated CHO-CCR5 cells. As shown in Figure 9B, ^{594}Tf was not detected in clathrin-negative cells. We evaluated the internalization of prelabeled surface CCR5 in these cells after a 15-min incubation period with CCL5. CHO-CCR5 cells still expressing clathrin show a punctate staining of CCR5 throughout the cell with some perinuclear accumulation indicative of agonist-induced internalization. By contrast, knocked down cells retained a uniform plasma membrane staining as seen on untreated cells, with little indication of punctate intracellular accumulation in endosomes (Figure 9A). The same results were obtained when chc2 siRNA-treated Mv-1-Lu-CCR5 cells were exposed to agonist (Figure 9A). To ensure that the lack of CCR5 internalization was due to a specific inhibition of the clathrin pathway, we also examined the uptake of the CTxB in Mv-1-Lu-CCR5 cells. CTxB binds to the glycosphingolipid GM1 at the cell surface and is endocytosed, at least in part, through a clathrin-independent mechanism (Nichols *et al*., 2001; Torgersen *et al*., 2001). Mv-1-Lu-CCR5 cells depleted of clathrin were still able to internalize ^{488}CTxB, indicating that clathrin-independent endocytic pathways remained intact

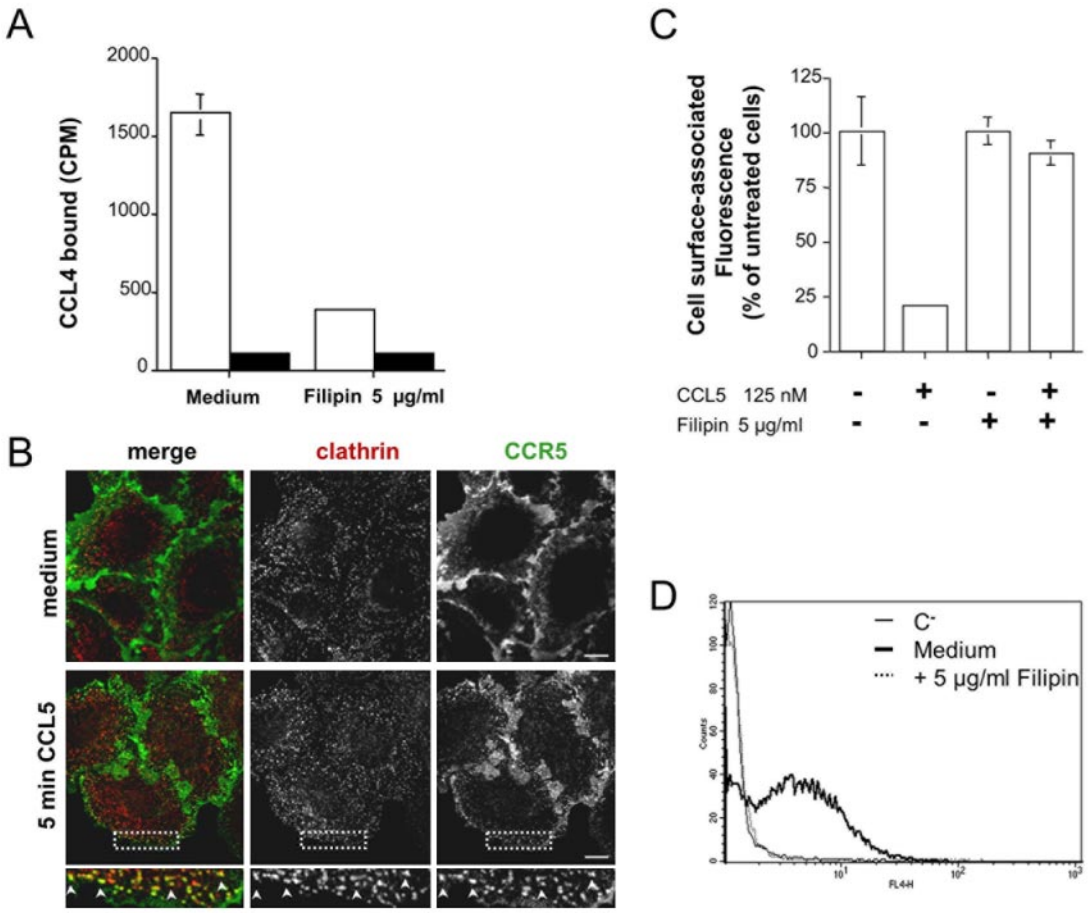

Figure 8. Filipin inhibits CCR5 agonist activation. (A) ^{125}I-CCL4 was bound, at 4°C, in medium alone or with 5 μg/ml filipin, to CHO-CCR5 cells (□) and CHO-K1 cells (■) pretreated or not with filipin. Samples were washed extensively and the amount of cell-associated radioactivity was determined by gamma counting. The means and SD of quadruplicate samples from a representative experiment are shown. (B) CHO-CCR5 cells on coverslips pretreated with filipin were incubated for 5 min with or without CCL5 at 37°C. The cells were fixed and labeled intact for cell surface CCR5 with MC-5 and then permeabilized, stained for clathrin, and analyzed as in Figure 1. Bars, 10 μm. (C) Effect of filipin on CCL5-induced CCR5 down-modulation was monitored by FACS analysis. The graph shows the mean ± SD of a representative experiment performed in triplicate. (D) Filipin treatment also inhibits the uptake of fluorescent transferrin in CHO-CCR5 cells overexpressing HTf-R. The histogram overlay represents the cell-associated fluorescence intensity before (C^-) or after a 30-min incubation with ^{647}Tf of filipin-treated or untreated (medium) cells.

(Figure 9B). This set of experiments clearly demonstrates that agonist-induced CCR5 internalization is clathrin dependent.

DISCUSSION

One of the mechanisms modulating GPCR responsiveness after agonist occupancy is the removal of receptors from the cell surface by endocytosis. This so-called agonist-induced down-modulation is the result of a combination of events, including receptor internalization, sequestration, recycling, and degradation. Its efficiency varies between GPCRs and is thought to depend mainly on intrinsic properties of the receptor (Tan *et al*., 2004). Over the last decade, β-arrestins have emerged as key regulatory proteins involved in agonist-induced endocytosis of many GPCRs. β-Arrestins are known to participate in internalization by linking phosphorylated forms of many GPCR to clathrin and AP2 complexes, thus mediating their recruitment to CCPs (Goodman *et al*., 1996; Scott *et al*., 2002). However, β-arrestin–independent pathways also have been reported (Claing *et al*., 2002). For example, the protease-activated receptor-1 and the viral GPCR-like protein US28 have been shown to be internalized via a clathrin-dependent but β-arrestin–independent mechanism (Zhang *et al*., 1996; Pals-Rylaarsdam *et al*., 1997; Fraile-Ramos *et al*., 2003), whereas the m2 muscarinic acetylcholine receptor and the *N*-formyl peptide receptor are endocytosed independently of both β-arrestin and clathrin (Gilbert *et al*., 2001; Vines *et al*., 2003). For GPCRs behaving like the m2 muscarinic receptor, it has been proposed that lipid rafts and/or caveolae are involved in their internalization (Chini and Parenti, 2004).

We and others have established that the chemokine receptor CCR5 is down-modulated in response to agonist stimulation. This was shown with primary cells expressing endogenous CCR5, such as peripheral blood mononuclear cells, purified human $CD4^+$ T-cells (Mack *et al*., 1998; Sabbe *et al*., 2001) and monocyte-derived macrophages (Signoret, unpublished observations), as well as with various CCR5-transfected cell lines (Amara *et al*., 1997; Mack *et al*., 1998; Signoret *et al*., 1998, 2000, 2004; Vila-Coro *et al*., 1999; Kraft *et al*., 2001; Brandt *et al*., 2002). Down-modulation of cell sur-

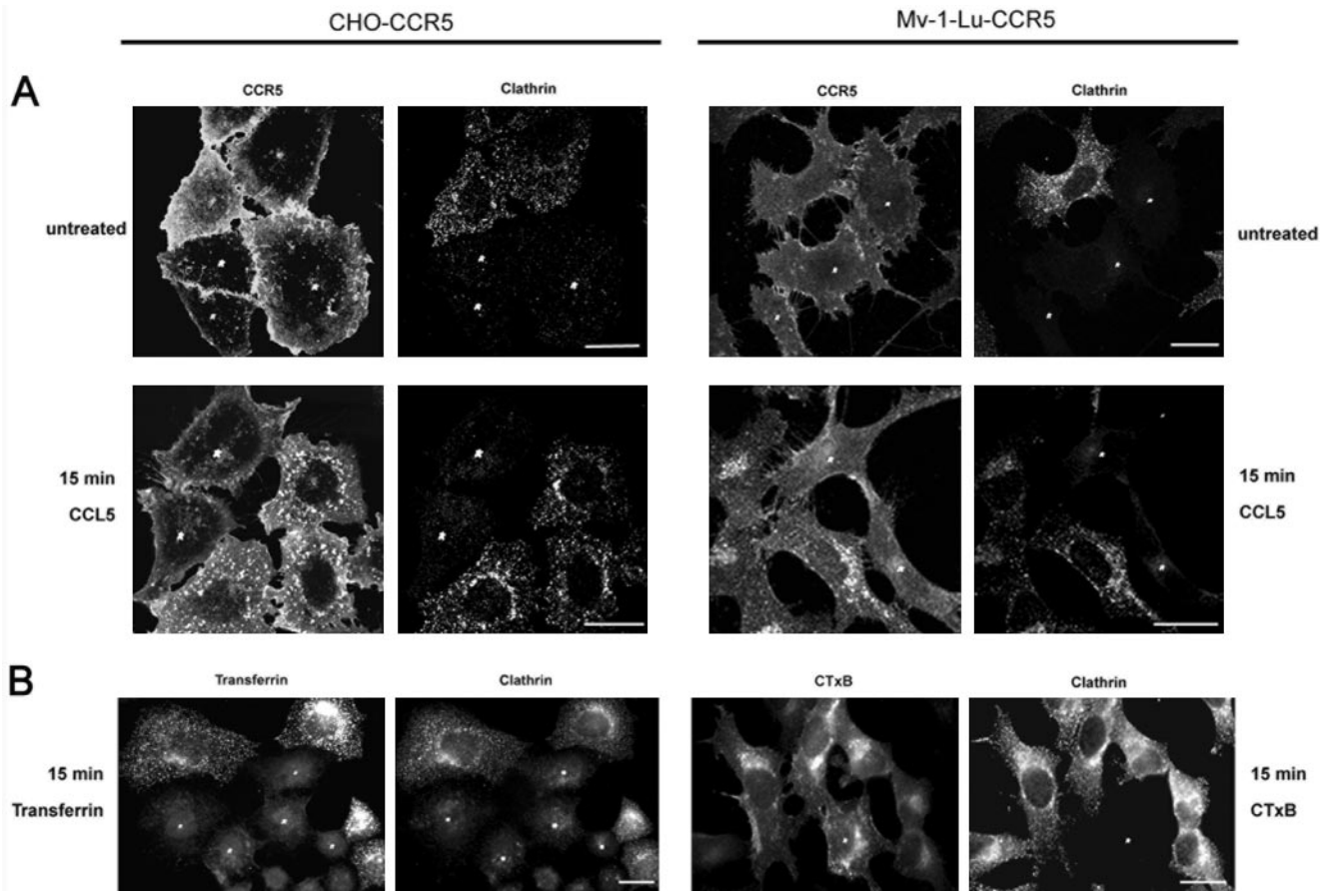

Figure 9. Clathrin-dependent CCR5 endocytosis in CHO and Mv-1-Lu cells. CHO-CCR5 and Mv-1-Lu-CCR5 cells were twice transfected with chc-2 siRNAs. Cells prelabeled with MC-5 were incubated in medium alone or treated with CCL5 (A). Some cells were incubated for 15 min with ^{594}Tf or ^{488}CTxB (B). All samples were then fixed, permeabilized with saponin, and costained for clathrin using a rabbit anti-clathrin light chain antibody. CCR5 and clathrin were detected with Alexa-Fluor–conjugated secondary antibodies. The figure shows single confocal sections (A) and digital images recorded with a Zeiss Axioscope (B). Clathrin knocked down cells are marked with asterisks. Bars, 20 μm.

face CCR5 is due to rapid agonist-induced endocytosis and accumulation of the receptors inside the cell. CCR5 was found to be internalized into the perinuclear recycling compartment and presumed to use a clathrin-dependent pathway (Amara *et al*., 1997; Mack *et al*., 1998; Signoret *et al*., 2000; Pollok-Kopp *et al*., 2003). β-Arrestins have been shown not only to influence agonist-mediated endocytosis of CCR5 but also to be required for this process (Aramori *et al*., 1997; Fraile-Ramos *et al*., 2003). On agonist treatment, β-arrestins are recruited to the plasma membrane where they interact with phosphorylated CCR5. Moreover, β-arrestins can be immunoprecipitated with CCR5 and clathrin from CCR5-transfected cells (Vila-Coro *et al*., 1999; Kraft *et al*., 2001; Huttenrauch *et al*., 2002; Mueller *et al*., 2002). Together, these observations strongly suggest that CCR5 is internalized through a β-arrestin–dependent interaction with the clathrin-mediated endocytic pathway. However, recent studies have challenged this model and evoked a clathrin-independent mechanism for agonist-mediated CCR5 internalization (Mueller *et al*., 2002; Venkatesan *et al*., 2003).

Here we demonstrate the direct involvement of clathrin in agonist-induced CCR5 internalization. Our morphological and ultrastructural analyses provide evidence of CCR5 association with clathrin-coated structures after agonist treatment. This includes structures that can be labeled for clathrin by immunofluorescence and by EM and correspond to flat lattices and invaginations of the plasma membrane, as well as coated vesicles characteristic of clathrin-dependent endocytosis. Besides, our finding that depletion of CHC by siRNA interfered with CCR5 internalization further indicates that clathrin is needed for agonist-induced CCR5 uptake. We confirmed our previous findings suggesting that once inside the cell CCR5 follows a pathway, namely, sorting and recycling endosomes, common to a number of receptors that internalize via clathrin-dependent mechanisms. In all situations examined, we found no evidence for clathrin-independent pathways that might mediate CCR5 internalization. In contrast to a recently published work (Venkatesan *et al*., 2003), we saw no evidence for CCR5 association with caveolae or caveolin-positive structures either before or after agonist treatment. This clearly differs from the transforming growth factor receptor that can be endocytosed through both clathrin-dependent and the caveolar pathways and is easily detected in caveolin-containing structures of Mv-1-Lu cells (Di Guglielmo *et al*., 2003). In addition, expression of a dominant negative form of GFP-tagged caveolin-1 did not interfere with the endocytosis of agonist-treated receptors. Moreover, although the internalization of caveolae has been shown to depend on an intact actin cytoskeleton, and at least in CHO cells, on mi-

crotubules (Mundy *et al*., 2002; Van Deurs *et al*., 2003), depolymerization of actin filaments or microtubules had no effect on ligand-induced CCR5 down-modulation (our unpublished data). Although we cannot exclude small amounts of CCR5 internalizing via a nonclathrin pathway, our results indicate that the major route for agonist-induced CCR5 endocytosis is via the clathrin-mediated pathway and in the absence of clathrin there is little, if any, CCR5 internalization detected.

The evidence in favor of raft-dependent internalization was based on the finding that CCR5 endocytosis seemed to be inhibited by compounds that destabilize lipid rafts by interfering with or removing membrane cholesterol (Mueller *et al*., 2002; Venkatesan *et al*., 2003). It has been proposed that in chemotactic cells, rafts impose a lateral organization to plasma membrane constituents, including chemokine receptors, that is important for many aspects of cell function, including cell polarization (Manes *et al*., 2001). CCR5 has been seen to accumulate at the leading edges of migrating cells where it colocalizes with markers for raft domains (Gomez-Mouton *et al*., 2004). In addition, CCR5 has been shown to be palmitoylated, a modification that is often associated with targeting of membrane proteins into raft domains (Blanpain *et al*., 2001; Kraft *et al*., 2001; Percherancier *et al*., 2001). Nevertheless, the association of CCR5 with lipid rafts remains controversial (Manes *et al*., 1999; Nguyen and Taub, 2002; Percherancier *et al*., 2003; Venkatesan *et al*., 2003). Our present finding that filipin impairs CCL4 binding to CCR5 agree with results obtained with other compounds that interfere with membrane cholesterol (Nguyen and Taub, 2002, 2003a,b) and confirm the idea that membrane cholesterol is required to maintain CCR5 and other GPCRs in a conformation competent to bind agonistic ligands (Gimpl *et al*., 1997; Nguyen and Taub, 2002).

We also found that although clustering of cell surface CCR5 could occur in the presence of filipin, suggesting that raft domains may not be essential for this event, CCR5 internalization was inhibited. Interestingly, filipin also inhibited transferrin uptake. Thus, these data, along with the observation that cholesterol extraction can interfere with the formation of CCVs (Rodal *et al*., 1999; Subtil *et al*., 1999), demonstrate an indirect effect of cholesterol perturbation on agonist-induced CCR5 internalization.

One of the first visible consequences of agonist stimulation of CCR5 is the clustering of cell surface receptors into domains associated with flat clathrin lattices. The presence of flat clathrin lattices has long been recognized, although their function has been unclear (Aggeler *et al*., 1983; Heuser, 1989; Miller *et al*., 1991; Sanan and Anderson, 1991; Sachse *et al*., 2001). Molecules such as the HTf-R, the growth hormone receptor and now CCR5, which are internalized via the clathrin-dependent pathway, can be found in these lattices. By contrast, the interleukin-2 receptor, which is internalized in a clathrin-independent manner, is excluded from these domains (Miller *et al*., 1991; Lamaze *et al*., 2001; Sachse *et al*., 2001). Note that for our cells, clathrin lattices were detected in the absence of CCR5 agonist. Moreover, morphometric analysis indicated that agonist activation of CCR5 did not increase the size or frequency of these domains, at least in CHO cells (Table 2). Although a large number of GPCRs are endocytosed in a clathrin-dependent manner, it is unclear whether clustering into flat clathrin lattice domains is a process common to all these receptors. Changes in cell surface distribution of agonist-treated receptors have been seen for the β_2-AR, and it was concluded from immunofluorescence and colocalization experiments that the receptors translocated to preexisting CCPs (Goodman *et al*., 1996; Cao *et al*., 1998; Santini *et al*., 2002; Scott *et al*., 2002). However, ultrastructural analyses would be needed to determine whether these domains correspond to the region of flat lattice we observed here.

The most obvious reason for accumulating receptors in clathrin lattices would be to concentrate the receptors in areas containing the components necessary for active endocytosis. The fact that we found the AP2 complex present in preexisting lattices and that CCR5 can be found in CCPs budding from the edges of these lattices would support this hypothesis. It has been shown, by using GFP-tagged CLC and live microscopy on various cell lines, including CHO and RBL, that budding of CCPs can occur from "hot spots" where vesicles are repeatedly observed to form and leave the plasma membrane (Gaidarov *et al*., 1999; Rappoport and Simon, 2003), although recently published work has questioned these observations (Ehrlich *et al*., 2004). Nevertheless, flat lattices may correspond to these hot spots. As such they may represent precursors to endocytic CCPs and CCVs. Studies of the HTf-R and the GHR have suggested that the recruitment of these receptors into the lattices and their incorporation into CCPs are two differently regulated events (Miller *et al*., 1991; Sachse *et al*., 2001). Future work will be needed to identify the determinants within CCR5 that regulate its recruitment to clathrin-coated structures.

The flat lattices may have additional functions. Another example of receptor clustering upon stimulation is the high-affinity IgE receptor FcεRI for which membrane segregation was suggested to regulate its signaling activity (Wilson *et al*., 2000). This also could be the case for CCR5, where clustering may sequester the activated receptor away from the G protein and other signaling molecules (Wong and Fish, 2003). Given the pivotal role of β-arrestin in uncoupling G proteins and recruiting receptors for internalization, β-arrestin binding may also influence the clustering of CCR5. The question of how receptor clustering relates to signal transduction and β-arrestin recruitment will be a topic for further study.

In conclusion, our results show that agonist-induced internalization of CCR5 is a clathrin-dependent mechanism involving the recruitment of receptors into clathrin-coated domains of the plasma membrane.

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