The Chemokine Receptor D6 Constitutively Traffics to and from the Cell Surface to Internalize and Degrade Chemokines[□]

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The D6 heptahelical membrane protein, expressed by lymphatic endothelial cells, is able to bind with high affinity to multiple proinflammatory CC chemokines. However, this binding does not allow D6 to couple to the signaling pathways activated by typical chemokine receptors such as CC-chemokine receptor-5 (CCR5). Here, we show that D6, like CCR5, can rapidly internalize chemokines. However, D6-internalized chemokines are more effectively retained intracellularly because they more readily dissociate from the receptor during vesicle acidification. These chemokines are then degraded while the receptor recycles to the cell surface. Interestingly, D6-mediated chemokine internalization occurs without bringing about a reduction in cell surface D6 levels. This is possible because unlike CCR5, D6 is predominantly localized in recycling endosomes capable of trafficking to and from the cell surface in the absence of ligand. When chemokine is present, it can enter the cells associated with D6 already destined for internalization. By this mechanism, D6 can target chemokines for degradation without the necessity for cell signaling, and without desensitizing the cell to subsequent chemokine exposure.

INTRODUCTION

The correct positioning of leukocytes is of fundamental importance for a fully functional immune system. Chemokines represent part of a tissue "address" system allowing the selective movement of specific leukocyte subsets into, and between, microanatomical domains within tissues (Rossi and Zlotnik, 2000; Mackay, 2001; Kunkel and Butcher, 2002). This is achieved by the tightly regulated expression of members of a family of seven-transmembrane (7-TM) chemokine receptors on the surface of leukocytes. Functional differentiation is coupled to chemokine receptor switching to alter intratissue localization and change tissue tropism. Because leukocytes are intimately involved in many diseases, it is of little surprise that chemokines are implicated in pathologies

such as chronic inflammatory disease, autoimmunity, atherosclerosis, allergy, allograft rejection, and cancer (Gerard and Rollins, 2001). In addition, chemokine receptors are pirated for cellular entry by HIV, with the ligands blocking viral entry (Simmons *et al.*, 2000). Their importance is emphasized by the association of allelic variants of genes within the chemokine system with variable disease susceptibility, severity, or progression (O'Brien and Moore, 2000; Gonzalez *et al.*, 2002; Cybulsky and Hegele, 2003) and by the analysis of animal models of disease in mice carrying genetic modifications of the chemokine network (Gerard and Rollins, 2001; Power, 2003).

Chemokines show obvious sequence homology, in particular a conserved cysteine motif, which has led to their subdivision (Zlotnik and Yoshie, 2000; Mackay, 2001; Bacon *et al.*, 2002). In the 15 human CXC chemokines, a single nonconserved amino acid separates the first two cysteines, whereas in the 25 human CC chemokines, they are directly juxtaposed. The C chemokines lack one of these first two cysteine residues, whereas three amino acids separate these cysteines in the single CX₃C chemokine. In general, this motif limits a chemokine to a receptor subgroup, with six receptors identified for CXC chemokines, 10 for the CC subfamily, and one each for the C and CX₃C proteins. These receptors often bind multiple ligands, and many ligands can use multiple receptors, allowing for enhanced flexibility in the regulation of responses. Each of these 18 receptors is found on subsets of leukocytes where they act to define migratory responses to chemokines. However, it is clear that

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Abbreviations used: 7-TM, seven-transmembrane; CCR, CC-chemokine receptor; CCL, CC-chemokine ligand; CMV, cytomegalovirus; DARC, Duffy antigen receptor for chemokines; GFP, green fluorescent protein; h, human; HEK, human embryonic kidney; HA, hemagglutinin; LEC, lymphatic endothelial cell; m, murine; PBS, phosphate buffered saline; PE, phycoerythrin; PFA, paraformaldehyde; S-PE, Streptavidin-PE; TCA, trichloroacetic acid; TRITC, tetramethylrhodamine isothiocyanate.

expression is often not restricted to leukocytes (allowing chemokines to exert biological effects on other cell types) and that migration is not the only biological response mediated by chemokines.

Because chemokines play a major role in immune regulation, mechanisms must exist to tightly control their production, distribution, and destruction. Decoy receptors are well characterized in mammalian cytokine biology, providing additional ways to regulate responses (Mantovani *et al.*, 2001). Some viruses encode soluble chemokine-neutralizing proteins among their immunomodulatory armory (Alcami, 2003), but mammalian counterparts have not been described. Instead, specialized 7-TM chemokine decoy receptors may have evolved to fulfill this function, a strategy also apparently used by certain viruses (Bodaghi *et al.*, 1998; Alcami 2003). In mammals, three 7-TM proteins exist that differ in a number of important aspects from the 18 known leukocyte migratory receptors. These are Duffy antigen receptor for chemokines (DARC), CCX-CKR, and D6 (Chaudhuri *et al.*, 1993; Nibbs *et al.*, 1997, 2003; Gosling *et al.*, 2000; Townson and Nibbs, 2002; Lee *et al.*, 2003). Despite structural similarities and high-affinity ligand binding, they cannot couple to the major signaling pathways used by typical chemokine receptors, and no alternative signals have been described. Also, they are predominantly expressed on nonleukocytic cell types and seem unlikely to be directly involved in leukocyte migration. Consequently, transport and/or decoy receptor-like functions have been proposed for these molecules, although little firm evidence exists. For example, DARC expression on red blood cells has been seen as an indication of a sink or reservoir function (Horuk, 1994; Fukuma *et al.*, 2003). Alternatively, DARC expressed by blood vessel endothelial cells (Hadley *et al.*, 1994) may act as a chemokine transporter, presenting tissue-derived chemokines on the luminal surface of blood vessels (Middleton *et al.*, 1997; Lee *et al.*, 2003). We, and others, have proposed decoy and/or DARC-like transport functions for D6 on lymphatic endothelial cells (LECs), a major site of D6 expression (Mantovani *et al.*, 2001; Nibbs *et al.*, 2001, 2003). Indeed, recent work has indicated that despite being "silent," D6 internalizes chemokines and can degrade them over time (Fra *et al.*, 2003). Although this lends support to the decoy receptor hypothesis, it is unclear as to whether ligand degradation is simply a consequence of internalization or a specific property of D6. Also, no mechanistic details behind ligand internalization have been revealed.

Here, we have examined D6-mediated chemokine uptake compared with CCR5, a receptor that also interacts with many of the D6 ligands. We demonstrate that D6-expressing cells internalize CC-chemokine ligand 3 (CCL3) at a rate indistinguishable from CCR5, but unlike CCR5, this is done without reducing the surface levels of receptor, or desensitizing subsequent ligand uptake. This is possible because D6 is constitutively cycling to and from the cell surface in the absence of ligand. In fact, D6 is predominantly an intracellular protein, associated with the early/recycling endosomal compartment with <5% on the surface at any one time. In addition, D6-internalized ligands are more readily retained in the cell than those internalized through CCR5. This is because passage through the acidic endosomal compartment displaces chemokines from D6 but slows their dissociation from CCR5. Thus, by associating with the constitutive recycling endosome pool, and readily disengaging from its ligands at lower pH, D6 can internalize ligand without the need for ligand-induced signaling, and seems specifically designed for targeting CC chemokines for destruction. We also show, using a novel flow cytometric protocol, that

interfering individually with the activity of the early endosomal GTPase (rab5), a regulator of clathrin-coated vesicle formation (Eps15), or the endocytosis mediator dynamin I, can all reduce ligand uptake via D6.

MATERIALS AND METHODS

Generation and Maintenance of Cell Lines

Human embryonic kidney (HEK)293 cells were maintained at 37°C in 5% CO₂ in 293 medium (1× Dulbecco's minimal essential medium [Sigma, Gillingham, United Kingdom], 10% fetal calf serum [Perbio, Tattenhall, United Kingdom], 4 mM glutamine [Invitrogen, Inchinnan, United Kingdom], and streptomycin and penicillin [Invitrogen]). Expression constructs encoding human D6 or CCR5 carrying an N-terminal hemagglutinin (HA) tag (MYPY-DVPDYAGPG) (in pcDNA3; Invitrogen) or C-terminal green fluorescent protein (GFP) (in pEGFP-N2; Clontech, Basingstoke, United Kingdom) were generated by polymerase chain reaction (PCR) and verified by sequencing. These vectors, and controls lacking receptor inserts, were transfected into HEK293 cells by using FuGENE (Roche, Lewes, United Kingdom) and pools of stable transfectants selected in 0.8 mg/ml Geneticin (Invitrogen). Single cell clones were selected by plating at low density in 293 medium (with 0.8 mg/ml Geneticin) and picking at least 10 individual colonies. Each colony was assessed using confocal microscopy and flow cytometry and representative clones used along with pools.

Plasmids

Constructs encoding GFP-tagged canine wild-type, S34N, or Q79L rab5, or rat dynamin I (K44A) or rat β -arrestin-1 (V53D) were generous gifts from S. Ferguson (Robarts Research Institute, London, Canada) (Barlic *et al.*, 1999; Seachrist *et al.*, 2000). Those encoding GFP-tagged mutants of human eps15 (i.e., E495/295, DIII, and DIIIΔ2) were kindly provided by A. Benmerah (Cochin Institute, Paris, France) (Benmerah *et al.*, 1998, 1999).

Antibodies

α -Human D6 antibodies are described previously (Nibbs *et al.*, 2001). Other primary antibodies were uncoupled and fluorescein isothiocyanate-coupled α -HA (Cambridge Bioscience, Cambridge, United Kingdom), phycoerythrin (PE)-coupled and -uncoupled α -CCR5 (R&D Systems, Abingdon, United Kingdom). α -CCR5 clone CTC-5 was selected because 1) it bound better to CCR5 than other α -CCR5 clones in preliminary studies, and 2) it did not interfere with ligand binding (Lee *et al.*, 1999; our unpublished data). Other antibodies were α - β -arrestin-1, α -dynamin I, α -rab5, and α -rab11 (BD Biosciences, Oxford, United Kingdom), α -CD63 (Chemicon, Harrow, United Kingdom), α -CD71 (transferrin receptor), and α -golgin-97 (Molecular Probes, Leiden, The Netherlands) and α -clathrin (Affinity Bioreagents via Cambridge Bioscience). Tetramethylrhodamine isothiocyanate (TRITC)-, Cy3-, and PE-coupled α -mouse IgG were all from Sigma (Poole, Dorset, United Kingdom). Antibodies were optimized by preliminary investigations suggested by the suppliers.

Chemokines

The murine (m) CCL3 used was a nonaggregating mutant version, with identical *in vitro* bioactivity to wild-type murine CCL3 (Graham *et al.*, 1994). Human (h) CCL3-L1 was produced as reported previously (Nibbs *et al.*, 1999) and has identical bioactivity to hCCL3-L1 from R&D Systems. Biotinylated mCCL3 (Bio-CCL3) was synthesized by Albachem (Gladsmiter, East Lothian, United Kingdom) and is identical in sequence to the nonaggregating mutant of mCCL3 described above except it carried an additional biotinylated lysine residue on the C terminus. Other chemokines were from either R&D Systems or Peprotech (London, United Kingdom), were full length according to accepted sites of signal peptidase cleavage, and showed bioactivity on expected receptors. Chemokines were labeled with radioiodine as described previously (Graham *et al.*, 1993).

Radioligand Internalization Assays

mCCL3 Internalization Rate Determination. Cells were harvested by mechanical disruption or brief trypsinization, washed in phosphate-buffered saline (PBS), and 0.5–1 × 10⁶ cells were resuspended in 50 μ l of 293 medium containing 12 nM radioligand and incubated at 4°C for ~1 h with regular gentle agitation. Preliminary experiments showed that radioligand binding reaches equilibrium within 30 min. Cells were then spun (2600 rpm, 5 min, 4°C), washed in ice-cold PBS, and resuspended in 200 μ l of 293 medium. After shift to 37°C, tubes were spun as described above, the medium removed (which contained minimal radioligand), and the cells washed with either PBS or acid wash (0.2 M acetic acid, 0.5 M NaCl), both ice-cold, for 5 min. Finally, tubes were spun as described above, and the cell pellet counted in a Beckman Gamma 5500B counter (Beckman, High Wycombe, United Kingdom). Ligand

internalization was determined by the ratio of radioactivity in acid stripped versus PBS-washed cell pellets.

Ligand Uptake after hCCL3-L1 Pretreatment. Harvested cells were incubated at 5×10^6 cells/ml in binding buffer (293 medium plus 10 mM HEPES, pH 7.4) with or without 200 nM hCCL3-L1 for 1 h on ice and then shifted to 37°C for 45 min. After two washes in 50 ml of ice-cold PBS, cells were resuspended in ice-cold binding buffer to $\sim 1 \times 10^7$ cells/ml, and 40- μ l aliquots were taken. Then, 10 μ l of 30 nM 125 I-hCCL3-L1, prediluted in binding buffer, was added, and samples were incubated at 37°C for up to 30 min, after which cells were spun (2600 rpm, 5 min, 4°C) and washed with ice-cold PBS. Cell-associated radioactivity was determined using a Beckman Gamma 5500B counter.

Surface Receptor Assessments by Flow Cytometry

For simple flow cytometry, cells were washed with fluorescence-activated cell sorting (FACS) buffer (PBS/2% fetal calf serum) and resuspended in 100 μ l of ice-cold FACS buffer. Antibodies were added and the cells left on ice for 30–45 min with occasional gentle agitation. Where α -IgG secondary antibodies were required, 1–1.4 ml of ice-cold FACS buffer was added, the samples spun (2600 rpm, 5 min, 4°C), and the cell pellet was resuspended in 50–100 μ l of ice cold FACS buffer containing the α -IgG antibody and left on ice for 30–45 min with occasional gentle agitation. After the antibody incubation steps, and another cold FACS buffer wash, cells were resuspended in 400 μ l of FACS buffer and passed through a FACScan flow cytometer (Becton Dickinson, Cowley, United Kingdom). As a negative control, samples of untransfected cells were used alongside test samples.

SDS-PAGE and Western Blotting

Samples from the radioligand degradation experiments (below) were boiled for 10 min, spun (13,000 rpm, 3 min), and 25 μ l electrophoresed on a 4–12% Bis-Tris gradient acrylamide gel (Invitrogen) in $1 \times$ MES buffer (Invitrogen). An aliquot of 125 I-hCCL3-L1 stock, containing an equivalent number of cpm to that found in 25 μ l of the most radioactive test sample, was similarly prepared in $1 \times$ LB and loaded on the gel. Samples were run adjacent to MultiMark protein markers (Invitrogen). After electrophoresis, gels were dried onto filter paper and exposed to x-ray film. For α -D6 probed Western blots, D6 was purified from L1.2 cells expressing HA, His₁₀-tagged D6 and quantified by bicinchoninic acid assay (Blackburn *et al.*, 2004). Cell lysates from cells treated with or without mCCL3, and with or without cycloheximide (CHX; Sigma Chemical) (20 μ g/ml), were prepared in CellLytic-M mammalian cell lysis buffer (Sigma Chemical). An equal volume of HU buffer (8 M urea, 5% SDS, 200 mM Tris-HCl, pH 8, 0.1 mM EDTA, 100 mM dithiothreitol, 0.5% bromophenol blue) was added. Samples were not boiled (this causes D6 aggregation; Blackburn *et al.*, 2004), but they were left at room temperature for 10 min, subjected to SDS-PAGE, electrophoretically transferred onto polyvinylidene difluoride membrane, and blocked overnight in 10% milk/PBS. Blots were incubated with α -D6 antibody, washed in PBS/0.1% Tween, visualized with α -mouse horseradish peroxidase-coupled secondary antibodies (Amersham Biosciences UK, Little Chalfont, Buckinghamshire, United Kingdom), developed using a WestPico kit (Pierce Chemical, Rockford, IL), and exposed to x-ray film.

Immunostaining Protocols

Cells were grown on fibronectin-treated eight-well chamber slides overnight, washed with PBS, fixed for 10 min in 3.5% paraformaldehyde (PFA), washed twice with PBS, and then incubated for 20 min in 50 mM NH₄Cl. After a PBS wash, cells were incubated in PGS (PBS, 0.2% gelatin, 0.05% saponin) for 30 min and then in PGS containing primary antibody for 1 h. After two PGS washes, PGS containing fluorophore-coupled α -IgG antibodies was added for 1 h, and cells washed twice with PGS and fixed in 3.5% PFA. Slides were mounted in Vectashield (with or without 4,6-diamidino-2-phenylindole; DAPI) (Vector Laboratories, Peterborough, United Kingdom), a coverslip applied, and sealed with nail varnish.

Immunohistochemistry on Human Tissue Samples

This was performed as published previously (Nibbs *et al.*, 2001) on paraffin-embedded sections obtained from the Glasgow University Pathology Department.

Antibody Feeding and Recycling

Antibody Feeding. Cells on fibronectin-treated eight-well chamber slides were washed twice with PBS and incubated in BM (serum-free 293 medium, 10 mM HEPES pH 7.4, 0.2% bovine serum albumin) at 4°C for at least 30 min. Antibody was then added and cells left at 4°C for a further 30–45 min. Cells were then washed with ice-cold BM (or ice cold BM adjusted to pH 3 where appropriate) and then either directly fixed (10 min in 3.5% PFA) or BM added, and the slides transferred to 37°C in the presence or absence of mCCL3 (200 nM). Cells washed with BM, pH 3, were washed twice with BM to return pH to 7.4 before 37°C incubation. After incubation, cells were washed with PBS, fixed for 10 min in 3.5% PFA, and washed twice in PBS. All slides were then

treated as described above for the immunostaining protocols with incubation with 50 mM NH₄Cl and then PGS and finally PGS containing fluorophore-coupled antibodies. After two PGS washes, cells were fixed for 10 min in 3.5% PFA and mounted as described above. Control experiments were performed in the absence of saponin.

Antibody Recycling Experiments for Confocal Microscopy Visualization.

Cells plated as described above were loaded for up to 1 h at 37°C with antibody, washed with cold BM, pH 3, and then twice with cold BM and incubated in BM containing fluorophore-coupled α -IgG antibodies for up to 1 h at 37°C. Cells were washed in PBS, fixed in 3.5% PFA, washed again with PBS, and mounted as described above.

Flow Cytometric Analysis of Recycling. Cells were harvested by mechanical disruption or brief trypsinization, and 5×10^5 cells were loaded with anti-receptor antibodies at 37°C for 45–60 min in BM. Cells were then washed twice with cold BM, pH 3, and twice with cold BM, and finally incubated at 37 or 4°C in BM containing PE-coupled α -mouse IgG antibodies for up to 90 min. Cells were then washed in 1 ml of PBS and analyzed by flow cytometry using a FACScan flow cytometer.

Confocal Microscopy

All fixed cells were processed on glass slides mounted under a glass coverslip as described above. For live imaging, cells were grown overnight on fibronectin-treated glass-bottomed culture dishes (MatTek, Ashland, MA), the medium was replaced with complete 293 medium containing 10 mM HEPES, pH 7.4, and the cells were examined directly on the confocal microscope fitted with a stage heated to 37°C. All images were captured using a Leica SP-2 confocal (Leica, Milton Keynes, United Kingdom) configured with Leica confocal software, with a 40 $\times$ or 63 $\times$ oil immersion objective and digital zoom. Fluorochromes were excited sequentially with lasers at 488 nm (GFP) or 543 nm (Cy3 and TRITC), and with a UV laser to excite DAPI. Images were superimposed using Leica Confocal software and assembled using Thumbnail software (Cerious Software, Charlotte, NC). In all experiments, at least eight fields of cells were examined and representative images collected. Serial z-sectioning was used to confirm the intracellular localization, and colocalization, of fluorescence emission where appropriate.

Ligand Degradation Assays

Harvested cells (5×10^5) were incubated at 4°C with radioligand for at least 1 h, washed with ice-cold PBS, resuspended in 100 μ l of 293 medium, and incubated at 37°C for up to 2.5 h. In some instances, cells were omitted from the tubes. Where appropriate, 50 mM NH₄Cl was included in the 293 medium: cells treated in this way bind radioligand and internalize it upon temperature shift (as revealed by acid stripping) in a manner indistinguishable from non-NH₄Cl-treated cells (our unpublished data). In other experiments, 200 nM unlabeled hCCL3-L1 was included during the 37°C incubation. For each time point, triplicate samples were spun (2600 rpm, 5 min, 4°C), the supernatant taken, and the cell pellet washed in 200 μ l of 293 medium, which was subsequently combined with the first 100 μ l of supernatant. Cell pellets were resuspended in 100 μ l of PBS, and 50 μ l was counted in a Beckman Gamma 5500B counter. To the remainder, 50 μ l of 2 $\times$ LB (100 mM Tris-HCl, pH 6.5, 4% SDS, 20 mM dithiothreitol, 20% glycerol, 0.2% bromophenol blue) was added in readiness for SDS-PAGE (see above). With the 300 μ l of supernatant, 150 μ l was taken and an equal volume of 25% trichloroacetic acid (TCA) added. These were incubated at 4°C for 15 min, centrifuged (13,000 rpm, 4°C, 15 min), supernatant taken, and the TCA precipitate washed in 300 μ l of ice-cold acetone: the acetone wash was combined with the non-TCA precipitable supernatant. The TCA pellet and non-TCA precipitable material were counted in a Beckman Gamma 5500B counter. The percentage of retrieved counts of associated radioiodine in cells, TCA pellet, and non-TCA precipitable was then calculated. To the remaining 150 μ l of supernatant taken from the incubated cells, an equivalent volume of 2 $\times$ LB was added for SDS-PAGE analysis.

Radioligand Dissociation Assays

Harvested cells (5×10^5) were incubated at 4°C with 12 nM 125 I-hCCL3-L1 for at least 1 h, spun (2600 rpm, 5 min, 4°C), and washed with regular agitation for 5–60 min in 1.5 ml of ice-cold 293 medium adjusted to a given pH (2–7) with HCl. Medium was buffered with 10 mM HEPES for pH 6 and above, but below this 10 mM MES was used. Control cells were washed briefly with ice-cold PBS. Cells were retrieved by centrifugation (2600 rpm, 5 min, 4°C) and counted in a Beckman Gamma 5500B counter. Further washing (0.2 M acetic acid, 0.5 M NaCl) was used on some samples to confirm surface localization of radioligand. Each treatment was done in triplicate.

Transient Transfection

HA-D6 cells (2×10^5) were plated into the wells of a six-well dish and incubated for 48 h (37°C, 5% CO₂). Effectene (QIAGEN, Valencia, CA) was used for transfection with suppliers' buffers. Except where indicated 1 μ g of plasmid DNA was mixed with 100 μ l of EC buffer, 3.2 μ l of Enhancer added,

Figure 1. ^{125}I -mCCL3 is internalized by D6 without reducing surface receptor levels. (A) Cells were preloaded with ^{125}I -mCCL3 at 4°C, washed, and shifted to 37°C, and internalization was determined by the ratio of acid resistant- to acid-sensitive radioactivity associated with the cell pellet. Data are representative of three repeats, with each point done in triplicate. (B) Cells were incubated with 200 nM mCCL3 at 37°C, and receptor levels were assessed by flow cytometry by using α -D6 or α -CCR5 antibodies. Each time point was done in triplicate, and mean fluorescence intensity readings were compared with cells that did not receive mCCL3, but otherwise underwent the same protocol. Untransfected HEK293 cells were used as a negative control, and their mean fluorescence readings subtracted from all transfected cell data. (C) Synchronized ligand internalization. Cells were preloaded with or without 200 nM mCCL3 at 4°C, shifted to 37°C, and receptor levels assessed by flow cytometry. (D) Cells were incubated with or without 200 nM hCCL3-L1 for 45 min at 37°C, washed, and then incubated in the presence of 6 nM ^{125}I -hCCL3-L1 at 37°C for the given time. Cell-associated radioactivity was determined. Data are presented as a percentage of radioligand internalized by hCCL3-L1 pretreated cells compared with those receiving no hCCL3-L1 pretreatment. Each point was done in triplicate and the experiment repeated three times, with a representative example shown.

left for 5 min at room temperature, 10 μl of Effectene added, and the sample mixed and incubated for a further 5–10 min at room temperature. Then, 600 μl of 293 medium was added, and the whole mix was added dropwise to one well of cells bathed in 2 ml of warm 293 medium. Mock transfectants, omitting DNA, were done as a control for each test construct, and pEGFP-N2 was used as an untagged GFP control. Twenty-four hours after transfection, cells were harvested and analyzed for ligand uptake or surface D6 expression.

Flow Cytometric Fluorescent Ligand Uptake Assay

Bio-CCL3 (250 ng) was mixed in PBS with 3 μg of Streptavidin-PE (S-PE) (Molecular Probes) and incubated at room temperature for 45–60 min. Control samples lacked Bio-CCL3 (i.e., S-PE alone). Cells ($2\text{--}4 \times 10^5$) were resuspended in 40 μl of binding buffer, the premixed Bio-CCL3/S-PE or S-PE alone was added, and the cells were incubated at 37°C for 30–150 min with regular gentle agitation. Inclusion of 50 mM NH_4Cl only slightly enhances Bio-CCL3/S-PE uptake over this time frame (our unpublished data). Ice-cold FACS buffer (1.4 ml) was added, and the cells were retrieved by centrifugation (2600 rpm, 5 min, 4°C), resuspended in 400 μl of FACS buffer, and passed through a FACScan flow cytometer. For each transient transfection assessed, controls used were untransfected parental HEK293 or mock transfected HA-D6 cells treated with Bio-CCL3/S-PE or S-PE alone. For GFP-tagged constructs, after setting detection parameters to avoid fluorescence bleed-through, GFP- and PE-negative regions were gated using controls (mock-transfected HA-D6 plus Bio-CCL3/S-PE [red only] and GFP-transfected HA-D6 cells plus S-PE [green only]) such that >98% of the cells in these controls fell in the negative gate. More than 98% of all transiently transfected cells, irrespective of the construct used, consistently fell into this negative gate when S-PE alone was used in the incubation. Then data from test samples

(transiently transfected plus Bio-CCL3/S-PE) were collected. On analysis, gates of high and low GFP expressers were set, and the number of PE-positive cells was determined and compared with identical gates set on pEGFP-N2 transfectants. For β -arrestin-1 and dynamin I constructs, mock-transfected S-PE-treated HA-D6 cells were used to define the PE-negative gate, and transfectants were compared with mock-transfected HA-D6 cells after Bio-CCL3/S-PE uptake for the presence of PE-positive cells. Cell lysates were prepared from transfections as described above. Each sample was done in triplicate, and experiments repeated at least once.

RESULTS

Internalization of mCCL3 by D6 and CCR5

HEK293 cells expressing N-terminally HA-tagged human D6 or CCR5 accumulate radioligands for these receptors to a far greater extent than untransfected parental cells. When done at 4°C, ligands can be removed by washing in a high salt acid solution, indicating that the chemokine remains on the cell surface: at 37°C, very little (<10%) can be removed, indicative of ligand internalization. To examine the rapidity of internalization, cells were loaded at 4°C with ^{125}I -mCCL3, washed, and then incubated at 37°C. Internalization was measured by the extent of cell-associated radioactivity that became resistant to acid washing. As shown in Figure 1A,

Figure 2. D6 is found predominantly inside the cell. (A) Autoradiograph of Western blot, revealed with α -D6 antibodies, of known quantities of purified D6 and lysate from 10⁵ HA-D6-expressing HEK293 cells. Size of bands (in kilodaltons) is indicated to the left, as determined by the position of molecular weight markers ran adjacent to the samples shown. (B) Immunofluorescence analysis of paraformaldehyde-fixed HA-D6-expressing HEK293 cells by using α -D6 revealed with a TRITC-coupled α -mouse IgG secondary (red). The nucleus (blue) is revealed using DAPI. (C and D) Representative images of live HEK293 cells stably expressing either CCR5-GFP (C) or D6-GFP (D). Bar (B–D), 20 μ m. (E) Immunohistochemical staining of LECs in a paraffin-embedded section of human tonsil, with α -D6 revealed enzymatically using peroxidase-coupled secondary reagents to give a brown-red stain. Nuclei are stained with hematoxylin. The lumen of this flattened lymphatic vessel is indicated by the dotted line. Bar, 10 μ m.

CCR5 and D6 behaved in a similar manner. Specifically, within 2 min >70% of the radiolabeled protein bound to the cells was internalized. Similar profiles were obtained for both receptors with ¹²⁵I-hCCL3-L1 (our unpublished data).

Surface Levels of D6 Are Not Decreased during Ligand Internalization

For most chemokine receptors, ligand binding increases receptor internalization rate, measured by a reduction in cell surface receptor. hCCL3-L1 or mCCL3 addition, at 37°C, to cultures of cells expressing HA-CCR5 causes a rapid reduction in cell surface receptor detectable with either α -HA or α -CCR5 antibodies (Figure 1B; our unpublished data). Surprisingly, when these chemokines were individually added to cultures of HEK293 cells expressing HA-D6, this reduction did not occur. Instead, there was a slight, but reproducible, increase in surface D6 levels (Figure 1B) as detected by either α -D6 or α -HA antibody immunoreactivity. To examine this more closely, we synchronized ligand internalization by loading D6 or CCR5 expressing HEK293 cells at 4°C with a high concentration of hCCL3-L1 (200 nM), a ligand that binds with similar affinity to CCR5 and D6 (Nibbs *et al.*, 1999). Preliminary radioligand binding assays indicated that at this concentration, most available cell surface D6 and CCR5 should be occupied with ligand. On shift to 37°C, the majority of surface ligand is internalized within 10 min (Figure 1A). Despite this, there is no reduction in surface D6 levels compared with unloaded cells over this time frame (or up to an hour at 37°C), as detectable with α -D6 or α -HA antibodies (Figure 1C). Concentrations of ligand as high as 1 μ M were unable to reduce detectable D6 surface protein. On the other hand, surface HA-CCR5 levels are rapidly and extensively down-regulated in a manner coincident with ligand internalization (Figure 1C). Control experiments have demonstrated that there is minimal displacement of any of the antibodies used by hCCL3-L1. In case α -D6 or α -HA were limiting, we performed studies with a range of higher, supersaturating, concentrations of antibody, or lower numbers of cells. No decreases in surface HA-D6 were detected after hCCL3-L1 or mCCL3 treatment, and a small increase was consistently observed at later time points (our unpublished data). The D6 ligands CCL2, 4, 5, 7, 8, 11, 13, or 14 (all

used at 200 nM) did not reduce surface HA-D6 levels, whereas CCL4 or 5 caused a reduction in surface CCR5 detectability (our unpublished data).

To test the functional activity of the receptors after hCCL3-L1 exposure, we examined the ability of hCCL3-L1-treated cells to subsequently take up ¹²⁵I-hCCL3-L1. There was no reduction in ¹²⁵I-hCCL3-L1 uptake by HA-D6 cells after preincubation with 200 nM hCCL3-L1, whereas similarly pretreated HA-CCR5 cells only internalized ~50–60% of the amount seen with the untreated cells (Figure 1D). Estimates of receptor number using saturation radioligand binding assays consistently showed no reduction in surface D6 after ligand pretreatment (our unpublished data), although these experiments and others indicated a change in receptor affinity, revealed most clearly when full homologous displacement curves were performed (Supplemental Figure 1). This phenomenon, along with the apparent increase in receptor surface levels described above, does not seem to dramatically alter the rate of ligand internalization at 37°C (Figure 1D), but is intriguing because it represents the first demonstration of a downstream consequence of ligand occupation of D6, suggestive of signaling.

Thus, in contrast to CCR5, D6 can internalize chemokines without undergoing either a reduction in surface receptor levels or desensitization to subsequent chemokine uptake. One possible explanation for this is that D6 constitutively cycles to and from the surface, allowing ligand to enter cells on D6 molecules already destined for internalization. Internalized D6 is replaced by other intracellular D6 molecules predestined for the cell surface. A similar mechanism has been proposed for the cytomegalovirus (CMV) US28 protein (Fraile-Ramos *et al.*, 2001). The following series of experiments lend support to this model for D6.

Most Cellular D6 Is Found in Early and Recycling Endosomes

US28 and other constitutively recycling proteins are found predominantly in endosomes (Fraile-Ramos *et al.*, 2001; Royle and Murrell-Lagnado, 2003), so we investigated the subcellular localization of D6. First, we used biochemical approaches to estimate the percentage of D6 molecules, as a fraction of the full cellular complement, which are on the

Figure 3. D6 localizes to the early/recycling endosomal compartment. Paraformaldehyde-fixed HEK293 expressing D6-GFP (green) were permeabilized with 0.05% saponin and incubated with antibodies to CD63 (A), rab5 (B), or transferrin receptor (TfR) (C), which were then visualized using Cy3-coupled α -mouse IgG antibodies (red). Confocal microscopy was used to generate consecutive images to localize D6-GFP and Cy3, which were then superimposed, giving yellow upon colocalization. In B and C, the white boxes on the overlaid image correspond to the position of the close-up image shown to the right. Nuclei were revealed with DAPI. Bar on the overlaid images, 20 μ m.

surface at any one time. Lysates from HA-D6 HEK293 cells were compared, by Western blot analysis, with known quantities of purified D6 (Figure 2A), taking advantage of our recent isolation of pure preparations of D6 (Blackburn *et al.*, 2004). Two bands 46–49 kDa are seen: the upper band is a glycosylated version of the lower with carbohydrate decoration on asparagine 17, a modification not required for function (Blackburn *et al.*, 2004). By comparison of band intensity, we estimate that 10^5 cells contain ~ 70 ng of HA-D6 protein. Parallel radioligand binding assays revealed, on average, 3×10^5 functional surface receptors per cell. Thus, assuming all surface D6 can bind ligand, 10^5 cells have ~ 2.5 ng of HA-D6 protein on the surface at any one time, representing only $\sim 3.5\%$ of the total.

Next, immunofluorescent staining of HA-D6 cells with the α -D6 antibody revealed that the majority of the HA-D6 protein is in intracellular vesicles often clustered around the nucleus (Figure 2B). HA-CCR5 was found predominantly on the cell surface as expected (our unpublished observation). These distributions are recapitulated in live stable HEK293

cell transfectants (pools or single cell clones) expressing CCR5 or D6 carrying a C-terminal GFP tag (Figure 2, C and D): GFP was found throughout the cell, as expected, after transfection of expression vectors lacking receptor inserts. It is noteworthy that the fusion proteins had similar mCCL3 or hCCL3-L1 binding affinity to wild-type or HA-tagged proteins and behaved indistinguishably from their HA-tagged counterparts with respect to all the parameters described herein. This differential subcellular distribution is not due to differences in levels of expression, because even when D6-GFP is present at a low level, green fluorescence is predominantly vesicular. In addition, flow cytometric analysis of pools of D6-GFP transfected HEK293 cells stained with α -D6 antibodies (detected with PE-coupled α -mouse IgG secondary antibodies) showed that as total D6-GFP increased in the cell, the amount detectable on the surface also increased, i.e., there was not a point at which surface expression became saturated (our unpublished data). A similar distribution of endogenous or transfected D6 was observed in other cell lines, and often very little surface D6 was detectable (by flow

Figure 4. D6-GFP internalizes α -D6 antibodies in the absence of chemokine. HEK293 cells expressing D6-GFP (green) were loaded at 4°C with α -D6 antibodies, washed, and then either immediately fixed (top row) or shifted to 37°C for 5 (middle row) or 15 min (bottom row) before fixation. Cells were then permeabilized in 0.05% saponin, and the location of the α -D6 was visualized with Cy3-coupled α -mouse IgG antibodies (red). Confocal microscopy was used to generate consecutive images to localize D6-GFP and Cy3, which were then superimposed (overlay, yellow indicating colocalization). The white boxes on the overlaid image correspond to the position of the close-up image shown to the right. Nuclei were revealed with DAPI. Bar on the overlaid images, 20 μ m.

cytometry and radioligand binding) despite abundant total cellular expression (our unpublished data). This distribution of D6 is reminiscent of granular α -D6 immunoreactivity seen in human tissue sections. This can be seen in LECs (Figure 2E; Nibbs *et al.*, 2001) and is particularly manifest in angiosarcoma or Kaposi's sarcoma samples, transformed derivatives of LECs (our unpublished data). These observations provide significant circumstantial evidence that our *in vitro* systems are recapitulating genuine intracellular distribution *in vivo*.

To identify the nature of these vesicles, we have stained cells expressing D6-GFP with markers of various vesicular compartments. Unlike US28 (Fraile-Ramos *et al.*, 2001), little D6-GFP is found to colocalize with CD63, a marker of late endosomes and lysosomes (Figure 3A), although occasionally single D6-GFP-containing vesicles seem to stain with α -CD63. Instead, markers of early endosomes and the recycling endosomal compartment, namely, rab5 (Figure 3B), the transferrin receptor (Figure 3C), and rab11 (our unpublished data), were consistently found in D6-GFP+ vesicles. A small proportion of D6-GFP+ vesicles also stained with antibodies to clathrin, but the Golgi (visualized with α -Golgin 97 antibodies) contained little, if any, D6-GFP (our unpublished data).

We next examined chemokine production by the transfectants, screening for all known D6 ligands by reverse transcription-PCR. CCL22 was also included: recent unpublished work has revealed it to be a high-affinity ligand for D6 (previous work used a -2 variant that does not bind D6; Nibbs *et al.*, 1997). CCL2, 7, and 8 mRNA only were detectable at similar very low levels (requiring sensitive reverse transcription-PCR techniques and >30 rounds of amplification) in both transfected and untransfected HEK293 cells. Thus, autocrine ligand production is not responsible for the differences in intracellular distribution observed between D6 and CCR5. Also, D6 distribution is unchanged in the absence of serum, excluding serum proteins as responsible for D6 localization.

D6 Internalizes in the Absence of Chemokine

If D6 undergoes constitutive endocytosis, it should be possible to internalize antibodies against the receptor in the absence of ligand. To test this, we have used antibody feeding techniques, by using either α -D6 or α -HA antibodies, and cells expressing D6-GFP or HA-D6. Similar results were obtained with all these reagents, and data are shown from D6-GFP cells and α -D6 antibodies. Cells were loaded at 4°C

Figure 5. CCR5-GFP requires ligand to undergo significant receptor and antibody internalization. HEK293 cells expressing CCR5-GFP (green) were loaded at 4°C with α -CCR5 antibodies, washed, and then either immediately fixed (images in A) or shifted to 37°C for 5 (B, top row) or 10 min (B, bottom row) in the presence or absence of 200 nM mCCL3, before fixation. Cells were then permeabilized in 0.05% saponin, and the location of the α -CCR5 was visualized with Cy3-coupled α -mouse IgG antibodies (red). Confocal microscopy was used to generate consecutive images to localize CCR5-GFP and Cy3, which were then superimposed (overlay, yellow indicating colocalization). Nuclei were revealed with DAPI. Bar on the overlaid images, 20 μ m.

with antibody, washed (with buffer at pH 7.4, or pH 3 to strip off antibody), and then either fixed or shifted to 37°C to encourage internalization and fixed. α -D6 location was revealed with Cy3-coupled α -mouse IgG antibodies, with or without cell permeabilization with saponin. Without shifting to 37°C, the antibodies remained on the cell surface, with D6-GFP+ vesicles just under the cell surface not colocalized with Cy3 (Figure 4A). Washing with acidic (pH 3) medium before visualization removed all antibody. However, α -D6 antibodies are taken into the cell after 5 min at 37°C, were protected from acid washing, and remained colocalized with D6-GFP+ vesicles around the cell periphery (Figure 4B). After 15 min, the majority of the D6-GFP-containing vesicles are labeled with antibodies (Figure 4C), despite only a small proportion of the total cellular D6-GFP (i.e., those molecules on the surface) being loaded with antibodies at the start of the experiment. This implies that vesicles of internalized D6-GFP, carrying antibodies, rapidly fuse with preexisting intracellular D6-GFP+ vesicles. Antibody uptake was further confirmed by the lack of vesicular staining in nonpermeabilized α -D6-fed D6-expressing cells and was dependent on D6 expression because untransfected cells showed no detectable internalization (our unpublished data). The inclusion of 200 nM hCCL3-L1 during these treatments had no discernible effect on the rate of internalization, or the gross distribution of the internalized antibody. Flow

cytometric data, detecting surface versus intracellular antibodies associated with antibody-fed cells, support these observations (our unpublished data). Significantly, we have also used flow cytometry to show that α -D6 antibody uptake does not alter the size of the surface D6 pool, consistent with constitutive D6 internalization driving α -D6 uptake (Supplemental Figure 2).

Similar experiments were performed on HA-CCR5 or CCR5-GFP cells by using either α -HA or α -CCR5 antibodies. As with D6, no internalization of antibody was seen after 4°C loading without shifting to 37°C (Figure 5A). In the absence of exogenous chemokine, very little antibody was internalized after shift to 37°C (Figure 5B, far right). In fact, the distribution of antibody and CCR5-GFP, even after 1 h at 37°C, did not look dissimilar to cells that had not been shifted, although less antibody remained associated with the cells. However, when hCCL3-L1 or mCCL3 was added just before the 37°C incubation, CCR5 and associated antibodies were rapidly internalized and remained colocalized inside the cell (Figure 5B). These data are consistent with the requirement for ligand-induced signaling for significant internalization of CCR5.

Internalized D6 Recycles to the Cell Surface

Next, we asked whether internalized D6 recycles to the cell surface. D6-GFP expressing cells were loaded with α -D6

A **B**

antibodies at 37°C for 30–60 min, and surface antibodies were removed by acid stripping. Flow cytometry confirmed that the acid wash removed the majority of the antibodies from the cell surface (see below). Stripped cells were then reincubated at 37°C in the presence of TRITC- or Cy3-coupled α -mouse IgG antibodies. If α -D6 antibodies return to the surface, these secondary antibodies should become internalized. As shown in Figure 6A, there is significant internalization of the labeled secondary antibody that colocalizes completely with D6-GFP. Large vesicles are formed during these experiments, likely as a result of cross-linking within the D6-GFP/ α -D6/ α -mouse IgG complexes disrupting vesicle trafficking. Control experiments showed that secondary antibody uptake is dependent on both D6-GFP and the preincubation step with α -D6 antibodies (our unpublished data).

To assess recycling rate, similar protocols were used, but the cells were subsequently assessed by flow cytometry. Cells were loaded at 37°C with α -D6 antibodies, acid washed, and incubated for varying times at 37 or 4°C, with PE-coupled α -mouse IgG. As shown in Figure 6B, acid washing effectively removes surface α -D6 antibodies from the cells. However, if the cells are incubated at 37°C they internalize the secondary antibody. Within 90 min, cells exhibit fluorescent levels equivalent to those seen on cells that had simply been incubated with primary and secondary antibodies at 4°C (right-hand column).

These combined data clearly demonstrate that internalized α -D6 antibodies return to the surface of the cells and then reinternalize after picking up secondary antibody, results indicative of receptor recycling.

CCL3 Internalized by D6 Is Trapped Inside the Cell and Degraded

Ligands internalized by D6 are reportedly degraded by lysosomes, indicated by the emergence of TCA nonprecipitable radioactivity from D6-expressing cells loaded with radioligand, which can be inhibited by neutralization of vesicle acidification by NH_4Cl (Fra *et al.*, 2003). To examine

Figure 6. Internalized α -D6 antibodies recycle back to the cell surface. (A) Confocal microscopy. HEK293 cells expressing D6-GFP were loaded at 37°C for 60 min with α -D6 antibodies, surface antibody removed by acid washing, and the cells then incubated at 37°C with Cy3-coupled α -mouse IgG antibodies (red) for 60 min. Cells were then fixed and confocal microscopy used to generate consecutive images to localize D6-GFP and Cy3, which were then superimposed. Nuclei were revealed with DAPI. Bar on the overlaid images, 20 μm . (B) Flow cytometry. HEK293 cells expressing HA-D6 were loaded at 37°C for 60 min with α -D6 antibodies, surface antibody removed by acid washing where indicated, and the cells then incubated at 37°C (black columns) or 4°C (hatched columns) with PE-coupled α -mouse IgG antibodies for the time specified beneath the graph. Alternatively (white column, labeled 4°C control), cells were loaded at 4°C for 60 min with α -D6 antibodies, washed with PBS, and then incubated at 4°C with PE-coupled α -mouse IgG antibodies. For all samples, after a final wash, cells were subjected to flow cytometric analysis and mean fluorescence intensity values were determined. Each point was done in triplicate, and data are representative of repeated data sets.

the fate of the internalized ligand in HEK293 cells, HA-D6 cells, loaded with ^{125}I -mCCL3, were shifted to 37°C and the cells and supernatant were analyzed over time. At early time points, most radioactivity (>90%) was associated with the cells, but there was the accumulation of degraded, non-TCA-precipitable products in the supernatant of HA-D6-expressing cells over time (Figure 7A). By 30 min, ~30% of the retrieved radioactivity is in the non-TCA precipitable fraction, increasing to ~80% after 150 min. In the presence of 50 mM NH_4Cl , nearly all the radioactivity remained associated with the cell pellet, consistent with previous observations (Fra *et al.*, 2003). Untransfected cells took up little radiolabel, and there was minimal radioactivity in the non-TCA-precipitable fraction (Figure 7A).

SDS-PAGE analysis of the supernatants revealed the appearance over time of diffuse bands with a slower electrophoretic mobility than intact ^{125}I -mCCL3 (Figure 7B). These most likely represent iodinated amino acids or short peptides that do not become shrouded in SDS to allow them to electrophorese effectively on SDS-PAGE. They were coincident with the accumulation of non-TCA precipitable radioactive material and were not seen in supernatants from NH_4Cl -treated cells, which kept all their internalized ^{125}I -mCCL3 intact over the 150 min of the experiment. Intracellular ^{125}I -mCCL3 migrated with indistinguishable motility to intact ^{125}I -mCCL3 incubated in the absence of cells (Figure 7B). The smeared high-mobility bands seen in the supernatants were occasionally just detectable in cell lysates, but acid washing before lysate preparation removed these degradation products, suggesting they are attached to the cell surface (our unpublished observations). Next, we examined D6 protein levels during ligand degradation. Cell lysates were made from 5×10^6 HEK293 cells expressing HA-D6 or D6-GFP, cultured continuously in 20 ml of 200 nM mCCL3 for 1, 2, 4, 8, or 24 h in the presence or absence of CHX (Figure 7C; our unpublished data). Over this time frame, there was little, if any, detectable change in D6 protein levels, even in the presence of CHX, and mCCL3 was unable to enhance D6 destruction or bring about a change in

Figure 7. CCL3 internalized by D6 is degraded by an NH_4Cl -sensitive pathway. (A and B) HA-D6 transfectants, or untransfected HEK293 cell controls, were loaded at 4°C with ^{125}I -mCCL3, washed, shifted to 37°C for the specified time, and the cell pellet and supernatant were harvested. Where indicated, NH_4Cl (50 mM) was present throughout the experiment. (A) Supernatant was subjected to TCA precipitation. Radioactivity associated with the TCA pellet, the non-TCA precipitable fraction, and the cells themselves was counted and is presented as a percentage of the total retrieved radioactivity for HA-D6 and untransfected cells. Samples at each time point were done in triplicate. Repeat experiments gave similar data sets. (B) Ligand fate in HA-D6 cells. Matched samples of cell lysate ($\sim 12.5\%$ of total) (labeled C) or supernatant ($\sim 4\%$ of total) (labeled S) were run on SDS-polyacrylamide gels adjacent to samples in which no cells were included (labeled 0). Gels were dried onto filter paper and exposed to x-ray film. Intact ^{125}I -mCCL3 is marked with an arrowhead, and high-mobility smeared bands are marked with arrows. (C) α -D6-probed Western blot of aliquots of cell lysates made from 5×10^6 HA-D6-expressing HEK293 cells treated for the given times in 20 ml of medium containing mCCL3 (200 nM) and CHX (20 $\mu\text{g}/\text{ml}$) where indicated. Arrow to the right of the panel indicates the apparent molecular weight calculated from the positions of protein markers electrophoresed adjacent to the samples.

the electrophoretic mobility of the receptor. D6 is therefore a stable protein that does not seem to undergo degradation concomitant with D6-mediated ligand destruction. Control blots probed with α -actin showed the CHX to be active on these cells (our unpublished data).

This series of experiments confirm and extend previous observations (Fra *et al.*, 2003) and show that ligand internalized by D6 1) is degraded, 2) is released from the cells as soon as it is degraded, 3) is not released from the cells unless it is degraded, 4) involves acidic vesicles in the degradation process, and 5) does not bring about detectable D6 degradation.

Next, we wanted to examine whether these phenomena occurred after ligand internalization by a typical chemokine receptor, CCR5, so experiments were performed side by side on either HA-D6 or HA-CCR5 cells. ^{125}I -hCCL3-L1 was used because its equivalent affinity for D6 and CCR5 (Nibbs *et al.*, 1999) ensures that similar amounts of ^{125}I -hCCL3-L1 are taken up by the two cell lines. Intriguingly, these experiments showed that although internalized ligand is also degraded after CCR5-mediated internalization (and is NH_4Cl sensitive), it occurs much more slowly (Figure 8A). This is apparent using either media TCA precipitation (top) or SDS-

PAGE of cell lysates (bottom). However, internalized ^{125}I -hCCL3-L1 can be retrieved from CCR5 cells by incubation with unlabeled hCCL3-L1 after the radioligand has been internalized (Figure 8B). Radioligand degradation proceeds at the same rate whether unlabeled hCCL3-L1 is present or not, but the unlabeled hCCL3-L1 successfully chases out internalized ^{125}I -hCCL3-L1 from CCR5 cells only. This ^{125}I -hCCL3-L1 is intact according to its ability to be TCA precipitated (Figure 8B, bottom) and comigrates with intact ^{125}I -hCCL3-L1 by SDS-PAGE (our unpublished data). There is a small amount of intact ^{125}I -hCCL3-L1 displaced from D6 cells by the unlabeled hCCL3-L1 (Figure 8B, top), but this is equivalent to the amount of protein that is still on the cell surface after ^{125}I -hCCL3-L1 loading (as indicated by acid washing). Interestingly, when the competitive elution experiments were repeated in the presence of 50 mM NH_4Cl , internalized radioligand was released from D6-expressing cells (Figure 8C). In fact, NH_4Cl treatment alone was sufficient to double the amount of intact ^{125}I -hCCL3-L1 release, and this was further enhanced by the presence of excess unlabeled hCCL3-L1. On the other hand, ^{125}I -hCCL3-L1 release from HA-CCR5 cells was reduced by combined unlabeled hCCL3-L1/ NH_4Cl treatment. In all cases, intracellular

Figure 8. ^{125}I -hCCL3-L1 internalized by D6, in contrast to CCR5, is rapidly degraded and cannot be “washed” from the cells with unlabeled hCCL3-L1. (A) Top, cells expressing HA-D6 or HA-CCR5 were surface loaded at 4°C for 1 h in the presence of 12 nM ^{125}I -hCCL3-L1, washed, and shifted to 37°C. At the given time points after temperature shift, cells were spun out, and the supernatant was subjected to TCA precipitation. Radioactivity associated with the TCA pellet, the non-TCA precipitable fraction, and the cells themselves was counted and is presented as a percentage of the total retrieved radioactivity. Samples at each time point were done in triplicate. Bottom, cell lysates from the experiment depicted in the top panel were subjected to SDS-PAGE. The gel was dried and exposed to x-ray film, and an autoradiograph is shown. The position of intact ^{125}I -hCCL3-L1 is indicated. (B) HEK293 cells expressing HA-D6 (top) or HA-CCR5 (bottom), preloaded with ^{125}I -hCCL3-L1, were washed and shifted to 37°C in the presence or absence of 200 nM unlabeled hCCL3-L1 (as indicated under each graph). At the given time points, cells were spun out and the supernatant was subjected to TCA precipitation. Radioactivity associated with the TCA pellet, the non-TCA precipitable fraction, and the cells themselves was counted and is presented as a percentage of the total retrieved radioactivity. Samples at each time point were done in triplicate. (C) HEK293 cells expressing HA-D6 (top) or HA-CCR5 (bottom) were subjected to the same analysis described in B, except that 50 mM NH_4Cl was added to some samples where indicated throughout the incubations. The presence of 200 nM hCCL3-L1 is indicated along with the time of incubation at 37°C. Samples at each time point were done in triplicate.

and TCA-precipitable radioligand from NH_4Cl -treated samples exhibited identical motility in SDS-PAGE to intact ^{125}I -hCCL3-L1 (our unpublished data). Notably, neutralizing vesicle acidity made D6 and CCR5 behave in a similar manner in terms of radioligand release in this assay, a phenomenon investigated further below. As for D6 (Figure 7C), Western blot analysis was done on lysates from mCCL3-treated or untreated CCR5-expressing HEK293 cells, in the presence of CHX. These gave results consistent with previously published data (Signoret *et al.*, 2000), confirming that CCR5 has a half-life of ~8 h, which is not noticeably enhanced by ligand, although ligand does reduce the electro-

phoretic mobility of CCR5 (most likely due to increased receptor phosphorylation) (our unpublished data).

These data, taken as a whole, demonstrate that when hCCL3-L1 is internalized into HEK293 cells by D6, rather than by CCR5, its fate is sealed, and it will be targeted for eventual degradation. They also suggest that in an environment high in CCR5/D6 chemokines, D6 would be considerably more effective than CCR5 at neutralizing these molecules. Indeed, head-to-head comparisons of cell lines expressing similar surface levels of D6 or CCR5 show that in continuous culture D6 proves to be a more effective chemokine destroyer (Supplemental Figure 3). Finally, experiments

Figure 9. ^{125}I -hCCL3-L1 interactions with CCR5 and D6 show opposing sensitivities to reduced pH. HEK293 cells expressing HA-D6 or HA-CCR5 were loaded at 4°C with ^{125}I -hCCL3-L1 and then washed for the indicated time at 4°C with buffered 293 medium set to pH 5, 6, or 7 with HCl. Remaining radioactivity was compared with radioligand-loaded cells washed briefly in ice-cold PBS. Each point shows the mean $\pm$ SD of results from three identically treated samples.

individually using two other ligands for D6 and CCR5, ^{125}I -hCCL4 and 5, showed that as with hCCL3-L1 these ligands are internalized by both receptors, but they are more effectively retained and targeted for degradation when D6 is used (our unpublished data).

Opposing Effects of Lowered pH on hCCL3-L1 Dissociation from D6 and CCR5

The observation that NH_4Cl -treated D6- or CCR5-expressing cells behaved in a similar manner in the competitive elution experiments (Figure 8C) led us to hypothesize that the differences between these two receptors may lie in their sensitivity to reduced pH during endosomal passage. We therefore examined the effect of reduced pH on ligand/receptor interactions. D6HA-, D6GFP-, or CCR5GFP-expressing HEK293 cells, loaded with ^{125}I -hCCL3-L1 on ice, were washed at 4°C with PBS or with DMEM adjusted to pH values ranging from 2 to 7. A brief 5-min wash dissociated most ligand from both receptors at pH <4. Under less acidic conditions, there was an indication that D6 held onto ^{125}I -hCCL3-L1 less well than CCR5. These differences were dramatically enhanced when the incubation period was extended (Figure 9). At pH 7, CCR5 and D6 behaved alike, losing 40–60% of surface bound radioligand after 1 h, with D6 consistently releasing slightly more. However, as the pH dropped to levels similar to those encountered in endosomes, progressively less radioligand could be eluted from CCR5, whereas there was a more rapid dissociation from D6. Thus, at pH5, CCR5 holds onto nearly all the loaded ligand for at least an hour, whereas it is essentially all lost from D6 in 30 min. These data suggest that the pH sensitivity of ligand/receptor interaction is responsible for ensuring chemokines internalized by D6 are retained inside cells.

Chemokine Internalization by D6 Is Reduced by Genetic Modulation of the rab5 Activity and Eps15 Function

To examine the mechanism of ligand internalization through D6, we used transient transfection methodology to intro-

duce mutant versions of proteins known to interfere with endocytotic pathways and then assessed their impact on ligand uptake through D6. The constructs selected have been widely used and successfully used in the analysis of other chemokine receptors (Barlic *et al.*, 1999; Fan *et al.*, 2003; Venkatesan *et al.*, 2003). To facilitate unbiased, accurate quantitation of ligand uptake relative to expression from the transfected construct, we developed a novel fluorescence-based assay by using GFP-tagged versions of the transfected proteins, and a custom-made biotinylated version of mCCL3, termed Bio-CCL3. This molecule has bioactivity and receptor binding parameters indistinguishable from unmodified mCCL3 and behaves like mCCL3 when radiolabeled and incorporated into the assays described above (our unpublished data). Moreover, we can premix this molecule with fluorescently coupled Streptavidin (such as S-PE) and quantify uptake. An example is shown in Figure 10A, where mock, or untagged GFP-transfected HA-D6 cells show a Bio-CCL3-dependent uptake of S-PE that can be assessed relative to the expression levels of GFP ("low" and "high" gates). HEK293 cells lacking D6 or GFP constructs produce plots like the "Mock (S-PE only)" plot in Figure 10A, irrespective of the presence of Bio-CCL3. Confocal microscopy, in which internalized Bio-CCL3 is detected either by premixing or postinternalization detection methods, shows that premixing does not significantly impact on the rate or extent of ligand uptake (our unpublished data).

Using this assay, we first investigated the early endosomal GTPase, rab5. HA-D6-expressing cells were transiently transfected with plasmids expressing GFP-tagged versions of wild-type (wt) rab5, a constitutively active rab5 (Q79L), or a dominant-negative version (S34N), and 24 h later ligand uptake examined by flow cytometry. As shown in Figure 10B, both mutated forms of rab5, particularly when expressed at high levels, caused a reduction in the number of cells able to uptake Bio-CCL3/S-PE during a 1-h incubation relative to control HA-D6 cells expressing an equivalent amount of untagged GFP. Wt rab5 only marginally reduced ligand uptake. Similar experimental analyses were then performed on cells transiently transfected with plasmids encoding forms of Eps15, fused to GFP, known to inhibit clathrin-mediated endocytosis and pit assembly (E495/295 and DIII) (Benmerah *et al.*, 1998, 1999). A noninhibitory form of DIII, termed DIIIΔ2, was used as a control (Benmerah *et al.*, 1998). Fewer cells expressing E495/295 or DIII were able to internalize ligand, whereas in those expressing DIIIΔ2 this was much less pronounced (Figure 10C). In all transient transfectants, shorter incubations with Bio-CCL3/S-PE reduced the amount of ligand taken into the cells, but the impact of the constructs was consistent with the data presented above (our unpublished data). Finally, confocal examination of HA-D6 cells transiently transfected with the above-mentioned constructs revealed the GFP fusion proteins to be subcellularly distributed in a manner consistent with previous reports. The use of confocal microscopy to assess Bio-CCL3 uptake (by premixing with, or postinternalization detection by Streptavidin-Cy3) showed effects of the constructs consistent with the flow cytometric data, but presented considerable difficulties of quantitation (our unpublished data). As a whole, these results demonstrate that modulating rab5 activity or disrupting clathrin-coated vesicle formation can impact on the ability of D6 to internalize ligand.

Ligand Internalization Is Reduced by Dominant-Negative Dynamin I but Not by Dominant-Negative β -Arrestin-1

Next, plasmids encoding well-characterized dominant-negative forms of dynamin I (K44A) and β -arrestin-1 (V53D)

Figure 10. CCL3 uptake via D6 is reduced by genetic modulation of the activity of rab5, Eps15, or dynamin I, but not β -arrestin-1. (A–C) Controls and rab5 and Eps15 data. Representative flow cytometric profiles of HA-D6-expressing HEK293 cells transiently transfected with constructs expressing the proteins given underneath each plot. Mock transfected cells are labeled Mock. Twenty-four hours after transfection, cells were harvested and incubated with Bio-CCL3/S-PE (or S-PE only where indicated), for 1 h at 37°C, washed, and data were collected

were introduced transiently into HA-D6 cells, and ligand uptake was assessed (Figure 10, D–F). These molecules were not GFP tagged, so increasing amounts of plasmid (1–3 μ g) were individually transfected. The amounts used were chosen because of data from pEGFP-N2 transfections, which showed that between 1 and 3 μ g of plasmid there is an increase in the number of HA-D6 cells expressing low or high levels of GFP (Figure 10D). In the dynamin I (K44A) and β -arrestin-1 (V53D) transfectants, the amount of plasmid-encoded protein was assessed by Western blot analysis (Figure 10E). This reveals robust expression well above levels in untransfected cells (seen only upon prolonged exposure of the blot) and increasing stepwise with the amount of DNA used. Bio-CCL3/S-PE uptake was sensitive to the progressive increase in expression of dynamin I K44A (with up to a ~50% reduction in ligand uptake) but was relatively unaffected by the introduction of β -arrestin-1 (V53D) plasmid, with only moderate inhibition when very high levels of the protein were expressed (Figure 10F). On the other hand, hCCL3-L1-driven CCR5 internalization was reduced by up to 50% when β -arrestin-1 (V53D) was introduced into CCR5-expressing cells, with even the 1 μ g transfection showing some inhibition (Supplemental Figure 4). These data suggest that perturbing dynamin I function in HEK293 cells can reduce Bio-CCL3/S-PE uptake through D6 but that this internalization is able to occur in the absence of functional β -arrestin-1.

DISCUSSION

D6 has proven to be an enigmatic member of the chemokine receptor family. It binds 11 proinflammatory CC chemokines with high affinity, yet is not coupled to the signaling pathways used by typical chemokine receptors such as CCR5 and is often

referred to as a silent chemokine receptor. In addition, it is undetectable on peripheral blood cells and instead is expressed on LECs lining a subset of lymphatic channels (Nibbs *et al.*, 2001). These properties make it unlikely to be directly involved in mediating leukocyte chemotaxis, and indeed, heterologous expression of D6 is unable to make cells chemotactic to D6 ligands. A decoy function has been proposed for this molecule (Mantovani *et al.*, 2001; Nibbs *et al.*, 2001, 2003) and has received some support recently (Fra *et al.*, 2003). Our data add further weight to this idea and provide mechanistic insight into how D6 can internalize ligand without the need for ligand-induced mechanisms of receptor internalization used by other more typical chemokine receptors.

The antibody feeding experiments we have performed show that D6 is able to cycle to and from the cell surface in the absence of ligand. Surface D6 is not reduced after CCL3 exposure, despite rapid ligand uptake. Although we cannot exclude the possibility that ligands enhance the rates of endocytosis and exocytosis of D6 by a similar extent to bring about this phenomenon, the fact that antibodies to the receptor are internalized at a similar speed whether CCL3 is present or not suggests that receptor internalization is not enhanced by this ligand. These data are in contrast to those shown here for CCR5, which fit in with the accepted mechanism that ligand-induced signals mediate the internalization of CCR5 and other 7-TM receptors (Thelen, 2001; Tsao *et al.*, 2001; Pierce *et al.*, 2002). Instead, D6 seems to be acting rather like a patenoster lift, constitutively trafficking to and from the cell surface, with the ligand simply hitching a ride into the cell.

Once internalized, D6 is particularly adept at targeting chemokines for destruction, doing this much more effectively than CCR5. Although the rate of internalization would seem to be the most significant requirement to remove chemokines from the extracellular milieu (which CCR5 and D6 both do well), holding internalized ligand inside cells is also necessary. D6-expressing cells can do this, but interestingly, CCR5-expressing cells release their internalized cargo back into the medium upon addition of further ligand. CCR5 has been suggested not to require ligand dissociation for receptor recycling (Signoret *et al.*, 2000), and it seems likely that the ability to displace internalized 125 I-hCCL3-L1 reflects competition for the recycled cell surface CCR5, between labeled and unlabeled ligand. These fundamental differences between CCR5 and D6 seem to be a consequence of their intrinsic differential abilities to release ligand in the acidic environment of the endosomal compartment. Thus, in an environment high in CCR5/D6 ligands, such as inflamed tissue, degradation of these molecules by CCR5 may be minimal. Moreover, CCR5 surface down-regulation should also contrive to reduce CCR5-mediated ligand internalization and degradation. On the other hand, D6-expressing cells will internalize, retain, and target chemokines for degradation, and our data suggest it will continue to do this, because the receptor is not subject to ligand-induced desensitization. Therefore, the biochemical properties of D6 make it ideally suited to act as a decoy receptor.

According to our patenoster lift-like model of D6-mediated ligand internalization, it is possible to envisage how this could proceed without evoking intracellular signals. However, it should be noted that this predicts that there should be no change in the properties of D6 after ligand treatment, yet D6-expressing HEK293 cells consistently exhibit some alterations after treatment with D6 ligands. This is manifest by a slight, but consistent, increase in surface α -D6 immunoreactivity, and a reduction in subsequent affinity of D6 for ligand, data suggesting ligand effects on 1) the rate of endocytosis or recycling, 2) D6 conformation, or 3) the behavior of factors required for optimal ligand binding. We do not yet understand the

Figure 10 (cont.) for GFP expression and PE uptake. (A) Controls, i.e., mock-transfected and untagged GFP-transfected cells. Gates of low and high expressers were selected and further separated into those +ve or -ve for PE uptake based on controls like these. (B and C) Data from HA-D6-expressing HEK293 cells transfected with rab5 or Eps15 constructs, respectively. The graphs on the right-hand side of B and C show the mean percentage of PE+ cells ($\pm$ SD) from four identically treated samples. Repeat experiments produced similar data sets. (D) GFP control transfection. Expression of GFP, determined by flow cytometry, in HA-D6 cells transiently transfected with 1–3 μ g of pEGFP-N2. The nonexpresser gate was set using mock-transfected HA-D6 cells. GFP+ cells were split into high expressers (white bars) and low expressers (black bars) arbitrarily, by dividing the remaining region of the plot into two regions of equal size (according to the FL1 logarithmic axis). (E) Western blot analysis of lysates from β -arrestin-1 (V53D) or dynamin I (K44A) transfectants. Cell lysates were prepared from aliquots of the cells used in F, subjected to SDS-PAGE, and Western blots prepared. β -Arrestin-1 or dynamin I proteins were visualized by chemiluminescence, by using antibodies against these proteins and appropriate secondary reagents. The antibody used, and the estimated molecular weight of the protein detected, is given under each autoradiograph. (F) Impact of expression of β -arrestin-1 (V53D) or dynamin I (K44A) on BioCCL3/S-PE uptake. HA-D6 cells were transiently transfected with the quantity and type of plasmid indicated beneath the graph. Twenty-four hours later, cells were assessed for Bio-CCL3/S-PE uptake (1-h incubation). Mean fluorescence intensity (MFI) of cell-associated PE was determined by flow cytometry in test samples and is presented as a percentage (mean $\pm$ SD from four replicates) of the MFI seen in HA-D6 cells transiently transfected with a similar quantity of pEGFP-N2 plasmid, and that had been similarly treated for Bio-CCL3/S-PE uptake. On repeat, a similar data set was observed.

significance of these observations and further experiments are required to dissect these apparently ligand-driven responses.

How is the endocytosis of D6 regulated? Our experiments targeting known components of endocytotic machinery are beginning to shed some light on this, and implicate rab5, Eps15, and dynamin I. Antagonizing the activity of any of these proteins is able to reduce Bio-CCl3 uptake via D6. Along with our colocalization data (Figure 3), a simple model for D6 internalization emerges in which the receptor localizes to clathrin-coated pits, buds with the help of dynamin I, and enters the rab5+ early endosomal compartment. The interpretation of the data from the rab5 mutants is complex because dominant-negative (S34N) or constitutively active (Q79L) forms bring about a comparable reduction in ligand uptake. Rab5 is involved in the transport and fusion of vesicles of recently endocytosed membrane with early endosomes, and the S34N mutant would be anticipated to retard this process, thereby reducing ligand uptake. The Q79L mutant may be predicted to have the reverse effect, but in a ligand-independent constitutively cycling system such as seen here with D6, this mutant may encourage internalization of the receptor before it has had a chance to bind ligand, reducing cell surface residence time. Alternatively, the previously published observation that expression of this mutant causes the formation of enlarged endosomes in HEK293 cells (Seachrist *et al.*, 2000), and that we also see in our transfectants, may be responsible for interfering with D6 recycling. In support of these models, HA-D6 cells expressing abundant rab5(Q79L) have on average ~40% less D6 on the cell surface than mock-transfected cells (our unpublished data). On rab5(S34N) transfectants, on the other hand, slowed endocytotic uptake does not alter surface D6 levels (our unpublished data), indicating that compensatory mechanisms may be at work to limit surface accumulation of the receptor.

Many aspects of the behavior of D6 show more similarity to the CMV chemokine receptor homologue US28 than they do to CCR5. US28 undergoes constitutive ligand-independent internalization and recycling, and it has been proposed that one function of US28 is to remove chemokines from the extracellular milieu in an effort to prevent immune detection of CMV-infected cells (Bodaghi *et al.*, 1998). Moreover, both receptors are able to use clathrin-coated vesicles, and more significantly, our results with the V53D mutant of β -arrestin-1 suggest the internalization of D6, like US28 (Fraile-Ramos *et al.*, 2003), does not require arrestin function. For many 7-TM G protein-coupled receptors, ligand-driven interaction between arrestins and the intracellular domains of the receptor aids internalization (by linking them to clathrin and AP2) and coordinates some aspects of intracellular signaling (Pierce *et al.*, 2002). Our observations require further validation, by using β -arrestin null cells, for example, but it is tempting to speculate that constitutive internalization of D6 and US28 uses a common pathway for the same outcome, i.e., chemokine degradation. Intriguingly, D6, like US28 (Mokros *et al.*, 2002; Miller *et al.*, 2003), undergoes constitutive phosphorylation in mouse pre-B L1.2 and HEK293 cells (Blackburn *et al.*, 2004; our unpublished observations). Unlike CCR5, but again in common with US28 (Mokros *et al.*, 2002; Miller *et al.*, 2003), the extent of this phosphorylation is unaltered by the addition of ligand (Blackburn *et al.*, 2004; our unpublished observations). However, although US28 cycling is more rapid than typical human chemokine receptors, it seems to be significantly slower than D6 (Fraile-Ramos *et al.*, 2001), although a direct comparison in the same cell background would be useful. Also, US28 is localized predominantly in the CD63+ late endosome/lysosome compartment (Fraile-Ramos *et al.*, 2001), exhibits ligand-independent signaling, and will respond to ligands with typical chemokine receptor signals and chemotaxis (Streblow *et al.*, 1999; Casarosa

et al., 2001; Minisini *et al.*, 2003). These properties, to our knowledge, are not apparent in D6.

Currently, we have not identified the molecular determinants in D6 that encourage constitutive recycling. With US28, this property is controlled by the C-terminal cytoplasmic domain of the protein, the probable target of ligand-independent phosphorylation, which, when transferred to other 7-TM receptors, induces their constitutive cycling (Mokros *et al.*, 2002; Waldhoer *et al.*, 2003). Moreover, this domain modulates the signaling capacity of US28, with variants truncated at the C terminus showing altered constitutive and ligand-induced coupling to downstream signal molecules (Miller *et al.*, 2003; Waldhoer *et al.*, 2003). In addition to the C terminus, the DKYLEIV motif in the second intracellular loop of D6 may play a role. Mutation of the glutamic acid residue to alanine (seen in CCR5) unmasks weak pertussis toxin-sensitive ligand-induced coupling to calcium ion fluxes and allows some down-regulation of surface receptor molecules in response to ligand (deMendonca and Nibbs, unpublished data). This mutant still undergoes ligand-independent endocytosis and recycling, but the data would indicate that some D6 mutant molecules leave this cycle and are available for typical chemokine receptor signaling.

Constitutive endocytosis and recycling have been described for a number of receptors and transporters, and in many cases they allow for rapid changes in cell surface expression (Royle and Murrell-Lagnado, 2003). By changing the rates of internalization or recycling, environmental stimuli can quickly modulate surface receptor availability without altering gene transcription, or mRNA translation or stability. This is well demonstrated for insulin regulation of GLUT4, a glucose transporter, and the GABA-regulated ion channel GABA_A (Wan *et al.*, 1997; Pessin *et al.*, 1999). Whether such stimuli exist to control D6 surface levels *in vivo* remains to be determined, but modulation of D6 cycling could provide a mechanism to easily and rapidly modulate chemokine uptake by these cells, depending on the immediate needs of the tissue.

What is the biological impact of D6-mediated chemokine neutralization by LECs? One possibility is that it may act simply as a method of intratissue chemokine neutralization to help resolve or modulate inflammation. Alternatively, D6 may affect the extent of chemokine drainage into lymphatics to influence the phenomenon of "remote control," whereby distal chemokine production alters leukocyte infiltration into lymph nodes (Palfreman *et al.*, 2001). A third possibility is that it acts to control the passage of leukocytes into lymphatics by modifying the concentrations of proinflammatory CC chemokines around these conduits. When CC chemokines are kept low by D6, leukocytes expressing typical signaling receptors for these molecules may be discouraged from leaving the tissue, whereas those without them may be able to exit. This latter group could include mature dendritic cells that, as part of the program of maturation, switch off CCR1, 2, and 5, and turn on CCR7, a receptor essential for dendritic cell migration into draining lymph nodes activated by CCL21 and the LEC-expressed chemokine CCL19 (Cyster, 1999; Robbiani *et al.*, 2000). However, it is worth noting that despite compelling data implicating D6 as a decoy receptor, we cannot formally exclude the possibility that it may, under some circumstances, be able bring about a different fate for internalized chemokine. Although US28 expression in late endosomes/lysosomes may indicate a predilection for chemokine degradation (Fraile-Ramos *et al.*, 2001), the localization of D6 within the recycling endosomal pool could allow ligand to be alternatively targeted for, for example, transcytosis or storage, depending on cellular background or environmental stimulus. Fra *et al.* (2003) used a lymphangioma cell line to attempt to exclude transcytosis of

chemokines across LECs by D6, and indeed, these cells degraded rather than transported ligand. However, it is possible that this cell line may not truly recapitulate the *in vivo* biology of LECs, and subtle environmental changes could potentially alter intracellular trafficking of D6-internalized chemokines. Dissecting these possible functions of D6 will require more elaborate *in vitro* experimentation and the implementation of suitable *in vivo* models employing genetically modified mice.

In conclusion, this work has shed light onto novel mechanisms and functions apparent in the human chemokine receptor family. Interestingly, the other atypical human chemokine receptors, DARC and CCX CKR, can internalize their ligands (Comerford, Nibbs, and Rot, unpublished data), yet typical chemokine receptor coupling to the signal transduction machinery is absent. Further studies are required on these molecules to see whether they use similar mechanisms to D6 to regulate the distribution and/or destruction of their ligands, and indeed, to determine whether, and how, signals are elicited on ligand binding. Ultimately, a further understanding of the biology of these proteins will enhance our knowledge of the chemokine system, and hopefully lead to new therapeutic approaches to modulating its activity in disease.

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