CC Chemokine Receptor-3 Undergoes Prolonged Ligand-induced Internalization*

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Nives Zimmermann‡, Juliana J. Conkright§, and Marc E. Rothenberg¶

From the ‡Division of Pulmonary Medicine, Allergy, and Clinical Immunology and the §Division of Pulmonary Biology and Neonatology, Department of Pediatrics, Children’s Hospital Medical Center, Cincinnati, Ohio 45229

CC chemokine receptor-3 (CCR-3) is a major receptor involved in regulating eosinophil trafficking; therefore, elucidation of ligand-induced CCR-3 events has important implications in understanding the biological and pathological properties of eosinophils. Previous studies have demonstrated that unique receptor events occur in different cell types supporting investigation of CCR-3-mediated events in eosinophilic cells. We now report biochemical characterization of CCR-3 internalization following exposure of eosinophils to CCR-3 ligands. Treatment of freshly isolated human eosinophils with CCR-3 ligands resulted in marked and differential internalization of CCR-3 in a dose-dependent manner. Exposure to 100 ng/ml eotaxin reduced surface expression to 43, 43, and 76% at 15 min, 1 h, and 3 h, respectively. RANTES (reduced on activation T cell expressed and secreted) treatment induced more significant and prolonged internalization of CCR-3 than eotaxin; following 100 ng/ml of RANTES, 29, 24, and 47% of the receptor was expressed at 15 min, 3 h, and 18 h, respectively. Confocal microscopy demonstrated that receptor modulation involved receptor internalization by an endocytic pathway shared with the transferrin receptor. Receptor internalization was accompanied by partial degradation of CCR-3, and reexpression of CCR-3 was dependent in part upon de novo protein synthesis. Internalization was not blocked by pretreatment of eosinophils with pertussis toxin. Furthermore, staurosporine did not inhibit internalization although it blocked phorbol 12-myristate 13-acetate-induced CCR-3 down-modulation. These results demonstrate that CCR-3 ligands induce differential receptor internalization that is not dependent upon G-protein coupling, calcium transients, or protein kinase C.

Eosinophils are bone marrow-derived granulocytes associated with numerous diseases including allergic disorders, parasitic infections, and malignancies (1–3). Elucidating the processes that regulate eosinophil tissue accumulation is fundamental, since these cells markedly increase in tissue locations and cause potent proinflammatory effects in numerous diseases.

Recent studies have focused on the orchestration of eosinophil tissue accumulation by chemokines, especially eotaxin, the most selective eosinophil chemoattractant identified to date (4, 5). Chemokines are grouped into the CXC, CC, C, and CXC subfamilies on the basis of the arrangement of the conserved cysteine residues (6–8). The CXC and CC groups, in contrast to the C and CX3C groups, contain many members and have been studied in detail. The specific effects of chemokines are mediated by a family of seven-transmembrane spanning G-protein-coupled receptors (GPCR)1 (9, 10). To date, 17 chemokine receptors have been described: receptors for the CXC chemokines CXCR-1 through -5; the CC chemokines CCR-1 through -10; the receptor for C5a, C chemokine CXCR-1; and the C chemokine XCR-1. Most CXC chemokines are active on neutrophils, while CC chemokines have variable potencies for monocytes, lymphocytes, eosinophils, and basophils. In addition to mediating leukocyte chemoattraction and activation, selected chemokine receptors (e.g. CXCR-4, CCR-2, CCR-3, and CCR-5) serve as co-receptors for the entry of human immunodeficiency virus type 1 into cells (11).

The major chemokine receptor operational in eosinophils is CCR-3. This receptor appears to play a central role in allergic responses, since it is not only expressed on eosinophils but also on basophils and Th2 lymphocytes, other cells central in allergic responses (12, 14–17). CCR-3 binds multiple ligands including eotaxin, RANTES, MCP-2, MCP-3, and MCP-4. Of these chemokines, only eotaxin signals exclusively through CCR-3. Eotaxin has been shown to be responsible for eosinophil trafficking during baseline and inflammatory processes (18–23).

Following ligand binding to GPCRs, cellular responses are rapidly attenuated. This may be particularly important in eosinophils, since treatment of eosinophils with eotaxin, a chemokine specific for CCR-3, for only 10 min in vitro results in chemokine unresponsiveness for at least 8 h when the cells are adoptively transferred in vivo (21). A variety of mechanisms may be responsible for signal attenuation including receptor desensitization, endocytosis, and down-regulation (24). Each receptor and each cell type utilize unique mechanisms indicating the importance of elucidating individual receptor events in the appropriate cell type (25). It is therefore important to analyze CCR-3-mediated events in eosinophilic cells rather than CCR-3-transfected heterologous cells. We now report biochemical analysis of CCR-3 internalization following exposure of human eosinophils to CCR-3 ligands.

EXPERIMENTAL PROCEDURES

Cell Culture—The AML14.3D10 cell line (kindly provided by C. Paul and M. Baumann, Dayton Veterans Affairs Medical Center, Dayton, OH) was grown in RPMI 1640 (Life Technologies, Inc.) containing 10% FCS, 0.1 mg/ml Penicillin, 100 U/ml Streptomycin, and 2 mM L-glutamine. The cells were maintained at 37°C in a humidified atmosphere of 5% CO2/95% air.

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† To whom correspondence should be addressed: Division of Pulmonary Medicine, Allergy and Clinical Immunology, Department of Pediatrics, Children’s Hospital Medical Center, Cincinnati, Ohio 45229. Tel.: 513-636-7177; Fax: 513-636-3310; E-mail: rothm@chmc.org.

1 The abbreviations used are: GPCR, G-protein-coupled receptor; CCR, CC chemokine receptor; CXCR, CXC chemokine receptor; FACS, fluorescence-activated cell sorter; IL, interleukin; MCP, monocyte chemotactic protein; PBS, phosphate-buffered saline; PKC, protein kinase C; RANTES, reduced on activation T cell expressed and secreted; PMA, phorbol 12-myristate 13-acetate.
fetal calf serum (Life Technologies), 50 μg 2-mercaptoethanol (Sigma), 0.1 mM nonessential amino acids (Life Technologies), 1 mM sodium pyruvate (Sigma), and penicillin-streptomycin (Life Technologies). Initially, the pH of the media was titrated to 7.8, but after it was established that CCR-3 expression does not depend upon the pH of the media (data not shown) this practice was discontinued. A stock of 50 mM butyric acid (Sigma) in phosphate-buffered saline (PBS) was prepared and stored at 4 °C prior to use. IL-5 (R&D, Minneapolis, MN) was prepared as a stock of 100 ng/ml and stored at −80 °C prior to use.

Induction experiments were performed by growing cells in six-well plates on glass coverslips, starting with a concentration of ~10^6 cells in 3 ml media. Cells were incubated with 0.5 mM butyric acid. 2 days later, 10 ng/ml IL-5 was added, and experiments were performed on day 7. Medium was not replenished during the induction period. These cells will be referred to as DAML.

Eosinophil Purification—Eosinophils were purified from healthy or mildly atopic volunteers by negative immunomagnetic selection based on the method of Hansel et al. (26). Briefly, granulocytes were isolated from heparin-anticoagulated whole blood by dextran sedimentation, Percoll centrifugation, and hypotonic lysis of red blood cells. Cells were resuspended in Hanks’ buffered salt solution (Life Technologies, Inc.) with 2% fetal calf serum and incubated with 0.75 μM10^6 cells of anti-CD16-conjugated microbeads (MACS; Miltenyi Biotech Inc., Sunnyvale, CA) for 30 min at 4 °C. The cell suspension was then applied onto a CS MACS column, and negative populations were collected through a magnetic field. The isolates routinely contained >95% eosinophils. Viability of >95% as assessed by trypan blue exclusion. For internalization experiments, freshly isolated eosinophils were plated at 0.5 × 10^6/ml in the same medium used for AML cells but supplemented with 50 μM IL-5. Cells were cultured at 37 °C, 5% CO2 for a maximum of 22 h, and viability was >85%.

Intercellular [Ca2+] Measurement—Cells (2 × 10^6/ml) were loaded with 5 μM Fura-2/AM (Molecular Probes, Inc., Eugene, OR) in Hanks’ buffered salt solution with 1% fetal calf serum for 60 min at 37 °C in the dark. After two washes in flux buffer (145 mM NaCl, 4 mM KCl, 1 mM NaHPO4, 0.8 mM MgCl2, 1.8 mM CaCl2, 25 mM Hepes, and 22 mM glucose), cells were resuspended at 2 × 10^6 cells/ml and maintained on ice. Cells (2 ml) were prewarmed to 37 °C and stimulated in a cuvette with a continuously stirring magnetic bar using a RatioMaster fluorimeter (Photon Technology, Inc., South Brunswick, NJ). Data were recorded as the relative ratio of fluorescence emitted at 510 nm after excitation at 340 and 380 nm (y axis) over time (x axis).

Flow Cytometry—Cells (5 × 10^6) were washed with FACS buffer (2% bovine serum albumin, 0.1% sodium azide in PBS) and incubated with 0.5 μg CCR-3 antibody (clone 7B11, kindly provided by Dr. Paul Panath, Leukosite, Cambridge, MA), 0.5 μg anti-CD18 antibody (clone TS1/18, ATCC), or the mouse isotype-matched control IgG2a or IgG1, respectively (Pharmingen, San Diego, CA) for 30 min at 4 °C. After two washes in FACS buffer, cells were incubated with 0.5 μg of fluorescein isothiocyanate-conjugated isotype-specific secondary antibody (Pharmingen) for 30 min at 4 °C in the dark. After two washes, labeled cells were subjected to flow cytometry using a FACScan flow cytometer (Becton Dickinson) and analyzed using the CELLQuest software (Becton Dickinson). Internalization of surface CCR-3 was assayed by incubating cells at 37 °C for the indicated lengths of time with 0–100 ng/ml human eosinophil or human RANTES (Peprotech, Rocky Hill, NJ). In other experiments, eosinophils were exposed to pertussis toxin (List Laboratories, Campbell, CA) at a dose of 20–1000 ng/ml for 3 h, and chemokine was added for the last hour of the incubation period. In other experiments, eosinophils were exposed to 1–100 ng/ml staurosporine (Sigma) for 3 h, and chemokine or phorbol 12-myristate 13-acetate (PMA; Sigma) was added for the last hour of the 3-h incubation period. Following chemokine exposure, cells were immediately placed on ice and washed with at least twice the volume of cold FACS buffer. Receptor density (percentage) was calculated as 100 × (mean channel fluorescence of chemokine − mean channel fluorescence of isotype-matched control)/mean channel fluorescence of medium − mean channel fluorescence of isotype-matched control. Results are expressed as mean ± S.E.

Western Blotting—Whole cell lysates were prepared from eosinophils by washing twice in cold PBS and lysing in radioimmune precipitation assay buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS in PBS) with 10 μg/ml aprotinin, 10 μg/ml antipain, 10 μg/ml chymostatin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A (all from Boehringer Mannheim) and 2 mM phenylmethylsulfonyl fluoride (Sigma). Detergent-insoluble material was removed by centrifugation at 12,000 × g for 15 min at 4 °C. Supernatants were stored in siliconized tubes and either used immediately or stored at −80 °C. The protein concentration was determined using the bicinchoninic acid assay (Pierce), and 50 μg were separated by electrophoresis on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Equal loading was verified by staining with Ponceau S (Sigma). After blocking the membrane for 1 h at room temperature in Tris-buffered saline with 0.2% Tween 20 (TBST) with 5% dry milk, the anti-CCR-3 polyclonal rabbit antiserum (kindly provided by Dr. Bruce Daugherty, Merck) was added for 1 h at room temperature (1:5000 in TBST), followed by goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1: 10,000 in TBST, Calbiochem). The signal was developed using enhanced chemiluminescence (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. Antibody specificity was determined on lysates obtained from HOS.CD4 cells transfected with CCR-1 or CCR-3 (AIDS Research and Reference Reagent Program, Rockville, MD) (27, 28). Cycloheximide (Sigma) was used at 10 μg/ml for 3 h. Inhibition of protein synthesis (>80%) was verified by [35S]methionine incorporation for 3 h in the presence or absence of cycloheximide. Protein was precipitated with trichloroacetic acid, and radioactivity was measured in a β-counter.

Confocal Microscopy—Induced AML cells were cultured in six-well plates on glass coverslips. For experiments, the chemokine was added to the growth medium for 3 h at 37 °C. To stop the reaction, cells were placed on ice and fixed with 3% paraformaldehyde in PBS. The fixed cells were washed with PBS, quenched with 15 mM glycine in PBS, and permeabilized with 0.2% saponin in permeabilization buffer (1% cold fish gelatin and 1% bovine serum albumin in PBS). Staining was achieved with the 7B11 antibody (0.75 μg/coverslip) in 1% cold fish gelatin and 3% bovine serum albumin in PBS for 1 h at room temperature. Following three washes in 1% cold fish gelatin in PBS, Texas Red-conjugated anti-mouse IgG (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) was added for 1 h at room temperature. Finally, cells were stained with fluorescein isothiocyanate-conjugated anti-CD71 (transferrin receptor) antibody (Research Diagnostics, Inc., Flanders, NJ). Cells were washed three times with 1% cold fish gelatin, twice with PBS, and once with water. Coverslips were mounted onto slides, sealed, and stored at −20 °C until analysis on a Leica DMRBE inverted microscope equipped with a confocal laser scanner. Images were analyzed with Metamorph (Universal Imaging Corporation, West Chester, PA) and printed in Adobe Photoshop (Adobe Systems Inc., Mountain View, CA). In some experiments, fresh human eosinophils were treated with chemokine for 15 min, cytotoxicnferred, and stained for CCR-3 as above.

**RESULTS**

**Ligand-induced Internalization of CCR-3**—In order to investigate whether CCR-3 attenuation involves modulation of receptor expression, we examined receptor internalization following ligand binding. First, the surface expression of CCR-3 over
18 h following exposure of eosinophils to eotaxin was investigated by FACS analysis. Eotaxin (100 ng/ml) caused receptor loss, which was detectable after 15 min, remained reduced at 3 h, and returned to baseline at 18 h. The combined results are shown in Fig. 1, which demonstrate only 43±6, 43±2, and 76±4% of the original receptor level present on the surface after 15 min, 1 h, and 3 h, respectively. A representative experiment is shown in Fig. 2. Exposure of eosinophils to RANTES (100 ng/ml), another CCR-3 ligand, also internalized CCR-3. Only 29±6, 24±2, 24±6, and 47±7% of the receptor remain on the cell surface after 15 min, 1 h, 3 h, and 18 h, respectively (Fig. 1). A representative experiment for RANTES is shown in Fig. 2.

In all instances, RANTES induced a greater magnitude of CCR-3 internalization and had a longer duration compared with eotaxin (p < 0.05). We were interested in determining if this difference could be related to different potencies of these two chemokines. Therefore, several doses of both chemokines were analyzed. As shown in Fig. 3, RANTES and eotaxin induced a dose-dependent internalization of CCR-3. At all doses, eotaxin was less potent than RANTES. At the highest dose of eotaxin (1000 ng/ml), there was still reduction in CCR-3 surface expression at 18 h. At 100–250 ng/ml RANTES, there was also reduction in CCR-3 surface expression at 18 h.

We were interested in determining the effect of chemokine removal on the surface expression of CCR-3 following receptor internalization. Eosinophils were exposed to eotaxin or RAN-

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**Fig. 2.** CCR-3 cell surface expression after stimulation with eotaxin or RANTES. Eosinophils were incubated with eotaxin (A–D) or RANTES (E–H) for 15 min (A, E), 1 h (B, F), 3 h (C, G), and 18 h (D, H) and the cell surface expression was assessed by FACS analysis. Results are of a representative experiment (n = 3). The isotype-matched control is depicted as the filled histogram, CCR-3 expression without chemokine as a solid line, and expression with chemokine as a dashed line.

**Fig. 3.** Dose-dependent ligand-induced CCR-3 internalization on eosinophils. Peripheral blood eosinophils were cultured with eotaxin (A) or RANTES (B) for 3 h (solid line) or 18 h (dashed line). Cell surface expression of CCR-3 was measured by FACS analysis and compared with CCR-3 expression of eosinophils not treated with the chemokine.

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calcium flux and chemotaxis assays. These cells, unlike fresh eosinophils, express the transferrin receptor, which was useful for co-localization studies. Induced eosinophilic cells were grown on glass coverslips and treated with eotaxin or medium alone for 3 h at 37 °C. This treatment resulted in 36 ± 6% (n = 12) CCR-3 expression. Subsequently, cells were fixed, permeabilized, and stained with the anti-CCR-3 antibody (A and B). Alternatively, cells were co-stained with the anti-transferrin receptor antibody anti-CCR-3 (C and D). Staining was analyzed using confocal microscopy. CCR-3 staining is depicted in red, and transferrin receptor staining is shown in green. Co-localization is shown as yellow.

**Fig. 4. Confocal microscopy.** Cells (dAML) were grown on glass coverslips and incubated in medium alone (A and C) or with eotaxin (B and D) for 3 h at 37 °C. Subsequently, cells were fixed, permeabilized, and stained with anti-CCR-3 antibody (A and B). Alternatively, cells were co-stained with the anti-transferrin receptor antibody anti-CCR-3 (C and D). Staining was analyzed using confocal microscopy. CCR-3 staining is depicted in red, and transferrin receptor staining is shown in green. Co-localization is shown as yellow.

Ligand-induced Modulation of CCR-3 Protein Level—Internalization of CCR-3 may induce receptor degradation. Therefore, it was of interest to examine total cell protein for the level of CCR-3 protein by Western blot analysis. The conditions for Western blot analysis were first established from control cells: human eosinophils, untransfected HOS.CD4 cells, or HOS.CD4 cells transfected with CCR-1 or CCR-3. A polyclonal rabbit anti-human CCR-3 serum detected multiple background bands but showed a strong, large band between 50 and 60 kDa in HOS.CD4 cells transfected with CCR-1 or CCR-3. A polyclonal rabbit anti-human CCR-3 serum detected multiple background bands but showed a strong, large band between 50 and 60 kDa, and this band was not present in granulocytes or mononuclear cells (Fig. 5A). Human eosinophils had a strong band between 50–60 kDa, and this band was not present in granulocytes or mononuclear cells (Fig. 5B). In all cases, detection of immunoreactive CCR-3 protein was lost if the protein extract was boiled prior to electrophoresis. Eosinophils were then treated with medium alone or medium supplemented with eotaxin (250 ng/ml) or RANTES (100 ng/ml) for 3 h, conditions that promote optimal chemokine-induced internalization. Western blot analysis of whole cell lysates revealed that the level of CCR-3 protein decreased by ~30% (Fig. 5C). The decreased level of CCR-3 was further reduced (by ~60%) when eosinophils were treated with cycloheximide for the 3 h during the exposure to the chemokine. Cells treated with cycloheximide alone had no change in the level of immunoreactive CCR-3 protein compared with cells incubated in medium alone (Fig. 5C). Since the antibody used in the Western blot recognizes the carboxy-terminal region of CCR-3, the absence of degradation products may be due to the loss of this epitope in the degradation products. These experiments demonstrate that internalization of CCR-3 is accompanied by receptor degradation and that de novo synthesis of CCR-3 protein significantly contributes to the total level of CCR-3 protein following chemokine binding. In order to investigate if reexpression of CCR-3 on the cell surface
is dependent upon protein synthesis, we monitored CCR-3 expression by FACS analysis on cells treated with eotaxin in the presence or absence of cycloheximide. Following treatment with 100 ng/ml eotaxin for 3 h, the receptor was beginning to reappear on the cell surface. In contrast, on cells treated with eotaxin in the presence of cycloheximide, the receptor expression on the cell surface remained low (Fig. 5D). These data suggest that reexpression of CCR-3 on the surface depends in part upon de novo protein synthesis.

Effect of Pertussis Toxin on CCR-3 Internalization—We were interested in determining if G-protein coupling was required for CCR-3 internalization. CCR-3-induced calcium transients are known to be inhibited by pertussis toxin, suggesting that CCR-3 couples to G-proteins (32). We first determined a dose of pertussis toxin that was able to completely inhibit eotaxin-induced calcium transients in eosinophils. Treatment of eosinophils with 100–1000 ng/ml of pertussis toxin for 3 h inhibited calcium flux, but we were surprised to see that these doses also reduced CCR-3 expression down to 30% (data not shown). At a lower dose of 20 ng/ml pertussis toxin, inhibition of eotaxin-induced calcium transients was maintained (Fig. 6, A and B), but the level of CCR-3 was not significantly reduced. Exposure of eosinophils to this dose of pertussis toxin (20 ng/ml) did not block eotaxin (100 ng/ml)-induced receptor internalization (Fig. 6, C and D). There was also no evidence of an effect of pertussis toxin using eotaxin at 10 or 500 ng/ml (data not shown). These data indicate that CCR-3 internalization is not dependent upon G-protein coupling or calcium transients in human eosinophils.

Involvement of Protein Kinase C in CCR-3 Internalization—We were next interested in determining if protein kinase C (PKC) was involved in ligand-induced CCR-3 internalization, since the internalization of some GPCRs is dependent upon PKC. We first treated human eosinophils with PMA in order to determine if pharmacological activation of PKC was able to cause CCR-3 down-regulation. As shown in Fig. 7A, treatment of eosinophils with PMA for 1 h resulted in a dose-dependent down-modulation of CCR-3 surface expression. We next determined the nontoxic dose of staurosporine that would effectively inhibit PMA-induced CCR-3 down-regulation. As shown in Fig. 7B, pretreatment of eosinophils with 10 ng/ml of staurosporine for 2 h prior to PMA completely inhibited PMA induced CCR-3 down-modulation. We next determined the effect of staurosporine treatment on chemokine-induced CCR-3 internalization. As shown in Fig. 7C, staurosporine had no effect on the level of eotaxin (100 ng/ml)-induced CCR-3 internalization. There was also no evidence of an effect of staurosporine using eotaxin at 10 or 500 ng/ml (data not shown). These results indicate that although PMA induces CCR-3 down-modulation, PKC is not involved in ligand-induced CCR-3 internalization.

DISCUSSION

We report that CCR-3 is rapidly removed from the cell surface into an intracellular endocytic compartment following ligand binding. The two CCR-3 ligands studied, eotaxin and RANTES, induced a different magnitude and duration of receptor internalization. In all cases, RANTES was more potent and induced a longer duration of internalization than eotaxin although it was not a stronger chemotactant in previous studies (15) and in eosinophil transmigration assays (data not shown). This suggests that internalization of CCR-3 involves molecular events that are dissociated, at least in part, from the signals involved in triggering chemotaxis. Consistent with this, chemokine-induced internalization was not dependent upon G-protein coupling or calcium transients although both of these processes are involved in chemotraction. Interestingly, a derivative of RANTES that functions as an antagonist, aminoxy-pentane-RANTES, induces a stronger and more prolonged internalization of CCR-5 in transfected Chinese hamster ovary cells compared with RANTES itself (33). Additionally, IL-8 derivatives that have equal chemotactic activity can have differential ability to induce elastase release, suggesting that they trigger independent events involving the same receptor (34). We also demonstrate that the half-life of CCR-3 is prolonged (>3 h), since treatment of eosinophils with cycloheximide did not change the level of CCR-3 protein in the whole cell lysate.
However, following 3 h of chemokine treatment, the level of cell-associated CCR-3 protein was decreased in a manner augmented by cycloheximide treatment. This indicates that chemokine treatment induces protein degradation and that new protein synthesis is involved in maintaining the level of the CCR-3 protein following chemokine treatment. Concomitantly, the surface expression of CCR-3 was further decreased with cycloheximide treatment, indicating that reexpression of the receptor on the cell surface is dependent upon de novo protein synthesis. Ligand-induced modulation of chemokine receptor expression has only been examined for a limited number of chemokine receptors, and variable pathways have been reported. IL-8 induces rapid internalization of its receptor in neutrophils (29). Stromal cell-derived factor-1α induces a decrease of cell surface CXCR-4 in the CEM T-cell line, HeLa cells, and peripheral blood mononuclear cells (35, 36). Additionally, CCR-5 ligands induce receptor internalization in lymphocytes, monocytes/macrophages, and CCR-5-transfected Chinese hamster ovary cells (33). In these cases, the receptors enter an endocytic pathway but recycle by 3 h after ligand binding. In contrast, CXCR-2 undergoes internalization and does not recycle, since it enters a degradative lysosomal pathway (37). Rapid ligand-induced internalization of CCR-1 in transfected Chinese hamster ovary cells (38) and CCR-2b in transfected HEK-293 cells (39) have been described, but their intracellular processing was not studied. The diverse mechanisms of ligand-induced modulation of chemokine receptors indicate the importance of dissecting these processes for each chemokine receptor.

Because most experiments were done by FACS analysis utilizing an antibody against CCR-3, it remained possible that the apparent decrease in the level of CCR-3 expression was dependent upon blockade of the CCR-3 immunoreactive epitope by the chemokine. Therefore, experiments were performed at 4°C in order to block internalization. These experiments showed no modulation of CCR-3 expression, indicating that ligand binding alone was not sufficient to decrease CCR-3 detection. Furthermore, confocal microscopy was performed in order to directly demonstrate ligand-induced CCR-3 internalization. These experiments were performed on an eosinophilic cell line that expressed high levels of functionally active CCR-3. This cell line was useful especially because it maintained expression of the transferrin receptor, which allowed co-localization studies to be performed. On resting cells, most CCR-3 was demonstrated to be on the cell membrane; however, following ligand treatment, the majority of the CCR-3 protein was detected in an intracellular location. Co-staining studies with the transferrin receptor revealed that intracellular CCR-3 was predominantly co-localized with the transferrin receptor, which is known to recycle via the early endosome compartment. This

**Fig. 6. Effect of pertussis toxin on CCR-3 internalization.** Eosinophils were cultured for 3 h in medium alone (A, C) or medium containing pertussis toxin (20 ng/ml) (B, D), and calcium transients (A, B) induced by eotaxin treatment (500 ng/ml) are shown. Data are presented as the relative ratio of fluorescence emitted at 510 nm after excitation at 340 and 380 nm (y axis) over time (x axis). Replicate cells were also exposed to eotaxin for the last 1 h of the culture, and the level of CCR-3 expression was determined (C, D). Cell surface expression of CCR-3 was measured by FACS analysis and compared between eotaxin-treated cells (dashed histogram) and non-chemokine-treated cells (boldface histogram). The level of isotype matched control antibody expression is indicated with the filled histogram. The insets in C and D represent data expressed as percentage of CCR-3 expression.
Eosinophils were treated with increasing doses of 0.1 ng/ml PMA and staurosporine (ST) inhibited the PMA-induced down-modulation of CCR-3 expression (solid line) (B). C, the eotaxin-induced reduction of CCR-3 surface expression (solid line) was not inhibited by staurosporine treatment (10 ng/ml, dashed line). The level of CCR-3 expression in untreated cells is indicated by the filled histogram. The insets in B and C represent data expressed as percentage of CCR-3 expression.

Fig. 7. Effect of PMA and staurosporine on CCR-3 internalization. Eosinophils were treated with increasing doses of 0.1 ng/ml PMA (solid line) and 1 ng/ml PMA (dashed line), and the level of CCR-3 was analyzed by FACS analysis (A). Staurosporine (ST, 10 ng/ml, depicted as a dashed line) inhibited the PMA-induced down-modulation of CCR-3 expression (solid line) (B). C, the eotaxin-induced reduction of CCR-3 surface expression (solid line) was not inhibited by staurosporine treatment (10 ng/ml, dashed line). The level of CCR-3 expression in untreated cells is indicated by the filled histogram. The insets in B and C represent data expressed as percentage of CCR-3 expression.

pathway exists in numerous cells and is commonly involved in receptor endocytosis. It is interesting that the cytoplasmic motif (YXXF) required for internalization of other receptors (e.g. transferrin receptor) is conserved in CCR-3 (40).

We have begun to elucidate mechanisms of CCR-3 internalization. Experiments conducted with pertussis toxin demonstrated that G-protein coupling is not necessary for internalization. Interestingly, high doses of pertussis toxin alone down-regulated the level of surface CCR-3 expression. The dissociation of G-protein coupling and GPCR internalization has been reported for other selected GPCRs such as CXCR-4 (35, 36). We also tested the involvement of PKC in mediating ligand-induced CCR-3 internalization. Using staurosporine to inhibit PKC, we demonstrated that ligand-induced CCR-3 internalization was not dependent upon PKC. However, in these experiments, CCR-3 down-modulation was induced by activation of PKC with PMA. Similarly, although PMA and ligands induce rapid phosphorylation of CXCR-2, only PMA-induced phosphorylation is inhibited by staurosporine (37). These data indicate that at least two pathways exist for down-modulating CCR-3 expression: one mediated by PKC and another mediated by chemokines and independent of PKC. The latter pathway may be dependent upon G-protein-related kinases, such as GRK-2, which has been shown to be involved in CCR-5 internalization (41).

The present results have several biological implications, especially since CCR-3 events have been investigated specifically in eosinophils. Conclusions concerning eosinophil chemokine receptor events have often been drawn from investigation of heterologous cell lines transfected with CCR-3. Since individual cell types often utilize distinct signaling events, it is important to examine chemokine-triggered biochemical events in eosinophils. For example, fMLP inhibits adenylyl cyclase in fMLP receptor-transfected cells but not in human neutrophils (25). We have therefore performed all experiments in eosinophilic cells. The finding that chemokines induce CCR-3 to undergo significant and prolonged receptor internalization has mechanistic implications for understanding eosinophil trafficking in vivo. It is widely accepted that chemokines induce cellular activation and chemotaxis. However, it is unclear if chemokines are also involved in stopping leukocyte movement. For example, eotaxin has been demonstrated to be required for the maintenance of gastrointestinal eosinophils at base line (23). If eotaxin also induces receptor internalization with subsequent cellular hyporesponsiveness, then eosinophils would likely home into the intestine and become localized there, since they would no longer be responsive to other chemokine gradients operating through CCR-3. In contrast, eosinophils in hematopoietic organs, tissues that do not express eotaxin, would remain responsive to the induction of subsequent chemokine gradients. Additionally, the observed inefficiency of receptor recycling following ligand binding, especially for RANTES, may have mechanistic implications concerning the difficulty in expressing CCR-3 on the surface of transfected cell lines. In one study, only 2–5% of CCR-3-transfected cells expressed CCR-3 on their surface although substantial intracellular protein could be detected in most transfected cells (15), suggesting a problem with CCR-3 protein trafficking. Last, the current study sheds light on possible mechanisms by which eotaxin may block cellular entry of CCR-3 trophic human immunodeficiency virus type 1 strains (13). Besides directly blocking human immunodeficiency virus binding to CCR-3, eotaxin may inhibit human immunodeficiency virus uptake by causing internalization of the CCR-3 co-receptor. Further studies are under way analyzing the long term biological consequences of exposing eosinophils to chemokines and a further elucidation of the biochemical events associated with CCR-3 signaling.

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