β-Arrestins Regulate Interleukin-8-induced CXCR1 Internalization*

Jana Barlic‡‡, Masud H. Khandaker‡‡, Elizabeth Mahon‡, Joseph Andrews‡, Mark E. DeVries‡‡, Gordon B. Mitchell‡, Rahbar Rahimpour‡, Christopher M. Tan‡‡, Stephen S. G. Ferguson¶ and David J. Kelvin‡‡

From the ‡Laboratory of Molecular Immunology and Inflammation, John P. Robarts Research Institute, London, Ontario N6G 2V4, Canada, the §Department of Microbiology and Immunology, The University of Western Ontario, London, Ontario N6A 5C1 Canada, and the ¶Department of Physiology, Pharmacology and Toxicology, The University of Western Ontario, and John P. Robarts Research Institute, London, Ontario N6A 5K8, Canada

The functional role of neutrophils during acute inflammatory responses is regulated by two high affinity interleukin-8 receptors (CXCR1 and CXCR2) that are rapidly desensitized and internalized upon binding their cognate chemokine ligands. The efficient re-expression of CXCR1 on the surface of neutrophils following agonist-induced internalization suggests that CXCR1 surface receptor turnover may involve regulatory pathways and intracellular factors similar to those regulating β2-adrenergic receptor internalization and re-expression. To examine the internalization pathway utilized by ligand-activated CXCR1, a CXCR1-GFP construct was transiently expressed in two different cell lines, HEK 293 and RBL-2H3 cells. While interleukin-8 stimulation promoted CXCR1 sequestration in RBL-2H3 cells, receptor internalization in HEK 293 cells required co-expression of G protein-coupled receptor kinase 2 and β-arrestin proteins. The importance of β-arrestins in CXCR1 internalization was confirmed by the ability of a dominant negative β-arrestin 1-V53D mutant to block internalization of CXCR1 in RBL-2H3 cells. A role for dynamin was also demonstrated by the lack of CXCR1 internalization in dynamin-1-K44A dominant negative mutant-transfected RBL-2H3 cells. Agonist-promoted co-localization of transferrin and CXCR1-GFP in endosomes of RBL-2H3 cells confirmed that receptor internalization occurs via clathrin-coated vesicles. Our data provides a direct link between agonist-induced internalization of CXCR1 and a requirement for G protein-coupled receptor kinase 2, β-arrestins, and dynamin during this process.

A general characteristic of inflammatory responses is the migration of leukocytes from the blood to sites of injury or infection. A number of chemoattractants have been shown to cause the directed migration of leukocytes in vitro and in vivo. These include complement fragment C5a, formylated bacterial peptides (fMLP), arachidonic acid metabolites (LTB4), and a group of low-molecular weight pro-inflammatory cytokines known as chemokines (1–3). The superfamily of chemokines is divided into CC, CXC, and CX3C subfamilies. All known neutrophil-targeted human chemokines belonging to the CXC subfamily (IL-8,1 GROα, GROβ, NAP-2, ENA78, and GCP-2) are defined by the presence of a glutamic acid-leucine-arginine motif (ELR motif) in the portion of the molecule that lies N-terminal to the first highly conserved cysteine, thus representing the ELR-CXC chemokine subclass of CXC chemokines (3–5).

IL-8 and other neutrophil-directed chemokines stimulate neutrophils via specific seven-transmembrane guanine nucleotide-binding protein-coupled receptors (GPCRs) (5, 7). The two human IL-8 receptors, CXCR1 and CXCR2, have 77% overall sequence homology. The two receptor subtypes differ notably in their N-terminal extracellular domains, as well as in their C-terminal intracellular domains, and possess differences in their ligand specificities. CXCR1 displays greater ligand specificity by binding to IL-8 and GCP-2 with high affinity, whereas CXCR2 binds with high affinity multiple CXC chemokines in addition to IL-8, including ENA 78, NAP-2, GROα, and GROβ (6–9). Binding of the ligand to high affinity IL-8 receptors initiates a variety of cellular responses, including calcium translocation, chemotaxis, alterations in cytoskeletal architecture as well as cellular morphology, degranulation, and respiratory burst activation (3, 10–12). ELR-CXC chemokines are produced by a variety of cell types including monocytes, T lymphocytes, fibroblasts, and endothelial cells (3, 5, 6).

It has been well documented that IL-8 receptors become rapidly desensitized and internalized upon agonist stimulation (13, 14). The molecular mechanism(s) and cellular factors required for translocation of these agonist-occupied receptors from the membrane to cytosolic compartments are not well characterized. However, the rapid sequestration and re-expression of CXCR1 (14, 16) is similar to the well described model of β2-adrenergic receptor (β2-AR) regulation.

In the case of β2-AR, agonist binding induces a change in the receptor conformation, which is necessary for the interaction of the receptor with G protein-coupled receptor kinases (GRKs) (17, 19). GRK-mediated phosphorylation of the β2-AR C terminus promotes binding of arrestin proteins (β-arrestins) which when bound, elicit uncoupling of the receptor from its G protein (18–20). Recent data suggests that the synergistic action of cellular GRKs and β-arrestins determines the kinetics of β2-AR internalization (21). Moreover, it was demonstrated that β-arrestins serve as adaptor proteins, specifically targeting agonist-occupied receptors to clathrin-coated vesicles (CCVs) (19, 20).

1 The abbreviations used are: IL-8, interleukin-8; GPCR, guanine nucleotide-binding protein-coupled receptor; β2-AR, β2-adrenergic receptor; GRK, G protein-coupled receptor kinase; GFP, green fluorescent protein; HEK, human embryonic kidney; RBL, rat basophilic leukemia; CCV, clathrin-coated vesicle; EMEM, Eagle’s minimal essential medium; PBS, phosphate-buffered saline.

* This work was supported by grants from the Medical Research Council of Canada, the Medical Research Council-Juvenile Diabetes Foundation International, and the Heart and Stroke Foundation of Canada. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

† To whom correspondence should be addressed. Tel.: 519-663-3877; Fax: 519-663-3847; E-mail: kelvin@rri.on.ca.

This paper is available on line at http://www.jbc.org
22. A critical step in receptor-mediated endocytosis of β2-AR is the translocation of CCVs saturated with agonist-occupied receptor to the cytosol, which is a dynamin-regulated event (23). Desensitized β2-ARs, internalized via CCVs, are thought to be re-sensitized in the acidified endosomal environment and recycled back to the cell surface to re-establish normal receptor signaling (24).

In the present work we examined the role of GRK2, β-arrestins, and dynamin in regulating CXCR1 internalization. For this purpose a CXCR1-green fluorescent protein (GFP) construct (CXCR1-GFP) was transiently expressed in human embryonic kidney 293 (HEK 293) and rat basophilic leukemia 2H3 (RBL-2H3) cell lines. We determined that GRK2, β-arrestins, and dynamin are required for rapid agonist-induced internalization of CXCR1.

**EXPERIMENTAL PROCEDURES**

**Materials**—HEK 293 and RBL-2H3 cell lines were obtained from American Type Culture Collection (Manassas, VA). Dulbecco’s modified essential medium and Eagle’s modified essential medium (EMEM) were purchased from Biowhittaker (Walkersville, MD). Chemiluminescent substrates and horseradish peroxidase-coupled donkey anti-rabbit antibody were purchased from Amersham (Amersham International). The plasmid containing a variant of a green fluorescence protein (pEGFP-N1) and a green fluorescence protein directed polyclonal antibody were purchased from CLONTECH ( Palo Alto, CA). Human recombinant interleukin-8 (IL-8) was obtained from R&D Systems (Minneapolis, MN) and 111-IL-8 was obtained from Amersham. Texas Red-transferrin conjugates were purchased from Molecular Probes (Eugene, OR).

**Construction of CXCR1-GFP**—RNA from human neutrophils was isolated using TriPure™ Isolation Reagent (Roche Molecular Biochemicals) and CXCR1 cdNA synthesized using Superscript™ II reverse transcriptase (Life Technologies, Inc.) according to the manufacturer’s instructions. The coding sequence of CXCR1 was amplified using forward primer 5’-GTCTCATGATGAGGTCTCCCGAG-3’ fixed in 5’ (351 bp) and reverse primer 5’-GGTGGTCTCCCGAGG-3’ primers carrying EcoRI restriction enzyme recognition sequence. Polymerase chain reaction products were 5’ and 3’ terminal digested with EcoRI and cloned into pEGFP-N1 cloning vector. The final construct was sequenced through the region that was generated by polymerase chain reaction to confirm sequence fidelity.

**Cell Cultures and Transfections**—HEK 293 cells were grown in Dulbecco’s modified essential medium whereas RBL-2H3 cells were grown in EMEM, both containing 10% fetal bovine serum and 1:100 dilution of penicillin/streptomycin (BioWhittaker) at 37 °C in a humidified atmosphere in EMEM, both containing 10% fetal bovine serum and 1:100 dilution of penicillin/streptomycin (BioWhittaker) at 37 °C in a humidified atmosphere.

**Materials—** HEK 293 and RBL-2H3 cells were subcultured in 6-well plates (1 × 105 cells/well) in complete EMEM. Cells were washed twice with serum-free EMEM containing 1% bovine serum albumin, and 25 mM HEPES (pH 7.2) and preincubated in the same media 1 h prior to 125I-IL-8 treatment. Nontransfected RBL-2H3 and RBL-2H3 cells stably expressing CXCR1-GFP were stimulated with 50 nM 125I-IL-8 (3000 Ci/mmol) at 4 °C and 37 °C for 45 min. The reaction was stopped with 1 ml of ice-cold PBS (pH 7.4) supplemented with 1% bovine serum albumin, the cells were washed with the same buffer three times and lysed in the lysis buffer (0.5% Nonidet P-40 and 0.5% Triton X-100 in PBS) on ice for 30 min. After incubation, the total volume of the well was transferred onto 10% sucrose, PBS cushion and pelleted at 10,000 rpm for 20 min. Equal volumes of the supernatant (100 μl) were aliquoted and the amount of 125I-IL-8 in supernatants was measured using a γ-counter (LKB/Wallac, Turku, Finland). Incorporation of non-specific radioactivity was determined in supernatants of nontransfected RBL-2H3 cells.

**Sectretion of β-Hexosaminidase**—Cells were seeded as described for the “Radioligand Sequestration Assay,” washed, and preincubated in serum-free EMEM for 30 min. Nontransfected and RBL-2H3 cells stably expressing CXCR1-GFP were then stimulated with 50 nM IL-8 for 60 min at 37 °C. The reaction was terminated by placing 6-well plates on ice for 15 min. The amount of the secreted β-hexosaminidase was determined by incubating the well overlay of a 1:1 mx p-(nitrophenyl)-N-acetyl-β-D-glucosamide in 0.1 mM sodium citrate buffer (pH 4.5) at 37 °C for 1 h. At the end of the incubation 500 μl of a 0.1 M Na2CO3, NaHCO3 buffer (pH 4.5) was added and the absorbance was measured at 400 nm.

**Confocal Microscopy of Single Cell Time Courses and Colocalization Studies**—Confocal microscopy was performed on a Bio-Rad MRC-600 confocal microscope under x 60 oil immersion objective, using a fluorescein isothiocyanate filter with the emission wavelength of 488 nm. Transiently transfected HEK 293 and RBL-2H3 cells were maintained in fresh complete media. For time course studies, the cells were treated with increasing concentrations of IL-8 (10, 25, 40, 50, and 75 nM) and events following agonist stimulation were observed in 5 min time intervals up to 90 min post-agonist stimulation. To determine the effect of de novo protein synthesis that occurs during the time of observation, RBL-2H3 cells transiently expressing CXCR1-GFP were first pretreated with cyclohexamide (10 ng/ml) for 45 min at 37 °C and then stimulated with 50 nM IL-8. Events following agonist treatment were observed under confocal microscope.

For colocalization studies, RBL-2H3 cells transiently expressing CXCR1-GFP were stimulated with 50 nM IL-8 and labeled with Texas Red-transferrin conjugates (15 ng/ml) for 45 min at 37 °C. The reaction was terminated by washing the cells twice with ice-cold PBS (pH 7.4). Cells were then permeabilized with 0.1% saponin and washed with acidic methanol solution and confocal microscopy was performed as described above.

**Subcellular Cell Fractionation**—HEK 293 cells transiently expressing CXCR1-GFP, CXCR1-GFP, and GRK2, and CXCR1-GFP, GRK2, and β-arrestin 1 were stimulated with 50 nM IL-8 for 45 min at 37 °C.

The cells were washed twice with ice-cold PBS (pH 7.4), removed from plates by gentle washing, and pelleted at 100 rpm for 10 min. The cell pellet was resuspended in 3 ml of buffer A (10 mM Tris-HCl, pH 7.4, 2 mM NaCl, 1 mM EDTA) and incubated on ice for 30 min allowing a Dounce homogenizer. Nuclei were removed by centrifugation at 200 rpm for 10 min. The supernatant was loaded on a stepwise sucrose cushion (35 and 5% sucrose in PBS) and centrifuged at 35,000 rpm for 90 min at 4 °C. The supernatant was removed and the 35% sucrose interface fraction containing endosomes (the light vesicular fraction) was collected, diluted in buffer A, and re-centrifuged at 35,000 rpm for 45 min at 4 °C. The pellets were resuspended in 100 μl of buffer A containing 2 × SDS sample buffer and 100 μg of each protein sample was loaded onto SDS-polyacrylamide gel electrophoresis.

**Protein Determination**—Protein levels in the whole cell lysates of HEK 293 and RBL-2H3 cells were determined using Bio-Rad protein assay (Richmond, CA) with bovine serum albumin as a standard.

**Western Blotting**—Expression levels of β-arrestin 1, β-arrestin 2, and GRK 2 in HEK 293 and RBL-2H3 cells were examined using specific polyclonal antibodies as described previously (20). Equivalent amounts (100 μg) of total cell protein were separated on a 10% polyacrylamide gel and transferred onto nitrocellulose membrane (Bio-Rad). The endogenous amounts of β-arrestin 1, β-arrestin 2, and GRK 2 were determined using anti-β-arrestin 2 and anti-GRK2 rabbit polyclonal sera at a dilution of 1:2500 and horseradish peroxidase-conjugated anti-rabbit secondary antibodies using the ECL system (Amersham) according to manufacturer’s instructions. The amount of total β-arrestin 1, β-arrestin 2, and GRK 2 in RBL-2H3 cells were determined relative to their respective endogenous expression in HEK 293 cells. Amounts of CXCR1-GFP in the light vesicular subcellular fraction were determined using GFP-directed polyclonal antibody at dilution 1:1000 (CLONTECH).

**Statistical Analysis of the Sequestration Data**—The relative membrane and cytosol luminosity was measured using SigmaScan Pro software. Data was statistically analyzed and plotted using Microsoft Excel software. Results are the average ± S.D. from three separate identical experiments.
RESULTS

Agonist-promoted Internalization of CXCR1-GFP in HEK 293 and RBL-2H3 Cells—Cells transfected with CXCR1-GFP were positive for fusion protein expression within 24–36 h post-transfection as evidenced by robust membrane fluorescence in 15% of the RBL-2H3 and 70% of the HEK 293 cells visualized by confocal microscopy. A representative single cell time courses are shown. B, the relative membrane and cytosolic luminosity of RBL-2H3 cells overexpressing CXCR1-GFP was plotted versus time prior to and post-agonist stimulation for \( n = 3 \) (± S.D.) experiments. A, membrane; B, cytoplasm. C, detection of \(^{125}\)I-IL-8 binding was performed as described under “Experimental Procedures.” \(^{125}\)I-IL-8 accumulation in control RBL-2H3 cells was compared with \(^{125}\)I-IL-8 accumulation in CXCR1-GFP expressing RBL-2H3 cells at 4 and 37 °C. * represents statistical significance (\( p < 0.05 \)) using one-way ANOVA as compared with control nontransfected RBL-2H3 cells. □, RBL-2H3 cells nontransfected; ▣, RBL-2H3 cells CXCR1-GFP transfected (4 °C); □, RBL-2H3 cells CXCR1-GFP transfected (37 °C). D, the release of \( \beta \)-hexosaminidase was performed as described under “Experimental Procedures.” Data are represented as percentage of total \( \beta \)-hexosaminidase in the cell. * represents statistical significance (\( p < 0.05 \)) using one-way ANOVA as compared with group 1 (RBL-2H3 cells nonstimulated). Lanes indicate RBL cells: 1, nonstimulated; 2, IL-8 stimulated; 3, MCP-1 stimulated; 4, CXCR1-GFP transfected, nonstimulated; 5, CXCR1-GFP transfected, IL-8 stimulated; 6, CXCR1-GFP transfected, MCP-1 stimulated.
GFP over the same time frame (Fig. 1A, ii). Stably transfected CXCR1-GFP cells internalized $^{125}$I-IL-8 at 37 °C whereas at 4 °C, CXCR1-GFP transfected cells failed to internalize $^{125}$I-IL-8 (Fig. 1C), indicating that internalization of CXCR1-GFP is an agonist and temperature-dependent process, which is similar to IL-8 receptor internalization observed in neutrophils (14). To assess whether CXCR1-GFP transduced functional responses, we performed $\beta$-hexosaminidase assays on IL-8 stimulated and unstimulated CXCR1-GFP stably transfected cells. IL-8 stimulation of CXCR1-GFP transfected cells resulted in a 13.4% release of hexosaminidase compared with a 4.8% release from untransfected RBL-2H3 cells stimulated with IL-8 (Fig. 1D). Stimulation with MCP-1, a CC chemokine that does not bind CXCR1, did not induce hexosaminidase release. These results show that CXCR1-GFP expressed in RBL-2H3 cells retains several features of the wild type receptor expressed in neutrophils; the receptor can transduce signals that result in granule release, undergo agonist-induced internalization, and sequester IL-8.

In contrast to RBL-2H3 cells, HEK 293 cells transiently expressing the fusion protein construct did not internalize CXCR1-GFP when stimulated with IL-8 (Fig. 2A, i). Since previous studies (30) have demonstrated that HEK 293 cells require increased expression of $\beta$-arrestins and GRKs for internalization of some GPCRs we explored whether co-expression of these two classes of molecules with CXCR1-GFP could restore agonist-induced receptor internalization in HEK 293 cells.

Co-expression of CXCR1-GFP with either GRK2 (Fig. 2A, ii) or $\beta$-arrestin 1 alone (Fig. 2A, iii) in HEK 293 cells failed to facilitate IL-8 induced CXCR1-GFP internalization. However, expression of CXCR1-GFP with GRK2 and $\beta$-arrestins together resulted in IL-8-induced receptor internalization (Fig. 2A, iv) showing a 3.5-fold increase of cytosolic fluorescence and a 60% decrease in membrane fluorescence (Fig. 2B). The increased cytosolic receptor fluorescence was associated with increased receptor labeling of the intracellular vesicles. Similar results were obtained with $\beta$-arrestin 2, another closely related member of the $\beta$-arrestin family, again co-expressed with GRK2 and CXCR1-GFP (Fig. 2A, v). These results suggest that both GRKs and $\beta$-arrestins are required for CXCR1 internalization. Western analysis of GRK2, $\beta$-arrestin 1, and $\beta$-arrestin 2 from HEK 293 cells and RBL-2H3 cells (Fig. 3, A and B), shows a substantial difference in $\beta$-arrestin 1, $\beta$-arrestin 2, and GRK2 expression between HEK 293 cells and RBL-2H3 cells. The
higher levels of GRK2 and β-arrestin 2 expression in RBL-2H3 cells likely explain why CXCR1-GFP undergoes agonist-induced internalization in RBL-2H3 cells without the requirement for co-expression with GRKs or β-arrestins.

Inhibition of CXCR1-GFP Sequestration in RBL-2H3 Cells by Overexpression of β-Arrestin 1-V53D and Dynamin I-K44A Mutants—To explore the role of β-arrestins in CXCR1 internalization in RBL-2H3 cells we co-expressed CXCR1-GFP along with the β-arrestin 1-V53D dominant negative mutant in RBL-2H3 cells and stimulated with IL-8. In the presence of β-arrestin 1-V53D there was no redistribution of membrane fluorescence that followed IL-8 stimulation (Fig. 4, A, ii, and B). This was in sharp contrast to cells expressing CXCR1-GFP alone (Fig. 4A, i) or cells expressing CXCR1-GFP and wild type β-arrestin 1 (data not shown), which showed marked receptor internalization following IL-8 stimulation. These observations complement the results obtained with HEK 293 cells and clearly demonstrate a role of β-arrestins in agonist-induced CXCR1 internalization.

β-Arrestins are thought to act as scaffolding proteins in coupling GPCRs to CCVs (22, 24–26). Agonist stimulation promotes the formation of CXCR1-GFP containing vesicles, which are pinched off from the plasma membrane and translocated into post-endocytic compartments (19, 24, 26). The pinching or sealing off of the vesicles from the plasma membrane is dependent upon dynamin, a GTPase containing molecule (27–29). We explored whether CXCR1 required dynamin for agonist-induced receptor internalization by co-expressing CXCR1-GFP with the dynamin I-K44A dominant negative mutant. The expression of dynamin I-K44A successfully blocked redistribution of CXCR1-GFP from the membrane to the cytosol. Vesicles formed in cells expressing the dynamin I-K44A mutant simply did not pinch off from the inner surface of the plasma membrane (Fig. 4A, iii, 45 min). These results indicate that agonist-induced internalization of CXCR1 occurs via CCVs and requires functional β-arrestins and dynamin molecules.

Agonist-induced Colocalization of Transferrin and CXCR1-GFP in Endosomes and the Presence of CXCR1-GFP Conjugates in the Light Vesicular Subcellular Fraction—To further investigate and confirm the identity of membrane-derived vesicles that translocate agonist-occupied CXCR1-GFP from the membrane to post-endocytic compartments, we labeled RBL-2H3 cells transiently expressing the receptor-GFP construct with a Texas Red-transferrin conjugate. Transferrin has been shown to undergo receptor-mediated endocytosis through CCVs upon binding to its cognate transferrin receptor and it has been described as a significant endosomal marker (37, 38). Agonist stimulation promoted colocalization of CXCR1-GFP and dye-labeled transferrin conjugate within CCVs (Fig. 5, ii) whereas unstimulated RBL-2H3 cells transiently expressing CXCR1-GFP did not display any colocalization (Fig. 5, i). These results were further supported by isolation of the light vesicular (endosomal) subcellular fraction from transiently transfected HEK 293 cells expressing CXCR1-GFP and CXCR1-GFP, GRK2, and β-arrestin 1 that were stimulated or unstimulated with IL-8. A 9.5-fold increase in CXCR1-GFP was found in the light vesicular subcellular fraction isolated from IL-8 stimulated HEK 293 cells expressing CXCR1-GFP, GRK2, and β-arrestin 1 (Fig. 6, lane 4 versus lane 2). However, only a modest increase (3-fold) in CXCR1-GFP was found in the light vesicular subcellular fraction isolated from HEK 293 cells in the absence of transfected GRK2 and β-arrestin 1 (Fig. 6, lane 3 versus lane 1) indicating that GRK2 and β-arrestin 1 substantially enhance the efficiency of CXCR1 internalization. Together these results support a model for CXCR1 sequestration via clathrin-coated pits that are regulated by GRKs, β-arrestins, and dynamin.

DISCUSSION

Agonist-dependent regulation of chemokine receptor desensitization, internalization, and sequestration is an important mechanism for regulating leukocyte responsiveness to chemokine stimulation. Several studies have demonstrated that exposure of neutrophils to high concentrations of ELR-CXC chemokines renders the exposed neutrophils unresponsive to additional homologous chemokine stimulation. The refractory state of neutrophils following stimulation with high chemokine concentrations appears to be dependent upon desensitization, internalization, and sequestration of CXCR1, CXCR2, or both IL-8 receptor subtypes. Initial studies using radiolabeled IL-8 showed that IL-8-binding sites were rapidly lost from the neutrophil surface following stimulation of high concentrations of IL-8 (13). These initial studies were later confirmed by additional work utilizing monoclonal antibodies directed at the external domains of CXCR1 and CXCR2, that demonstrated a rapid loss of CXCR1 and CXCR2 following stimulation of neutrophils with high concentrations of ELR-CXC chemokines (14, 33).

Recent studies by Richardson et al. (32) have demonstrated that phosphorylation of critical serine residues in the C-terminal region of CXCR1 is important for internalization of the receptor following agonist stimulation. Our work here compliments these observations by demonstrating that GRK2 is necessary for internalization of CXCR1 in HEK 293 cells (Fig. 2A, iv and v). GRK2 is a serine-threonine kinase and a member of...
a multigene family whose members regulate GPCR function and internalization by phosphorylating serine/threonine residues located within the cytoplasmic regions of various receptors (17). GRK2 is abundantly expressed in human peripheral leukocytes (Ref. 31 and data not shown) and may represent the endogenous kinase responsible for CXCR1 phosphorylation in neutrophils. Alternatively one of the other members of the GRK family (GRK1 and 3–5) may serve the same function in regulating phosphorylation of CXCR1 in neutrophils. CCR5, a member of the CC chemokine family of receptors (4), is preferentially phosphorylated by GRK2 and GRK3 indicating that GRK phosphorylation likely represents a common feature of both CC and CXC chemokine receptor regulation (30).

While GRK phosphorylation represents a critical step in regulating the desensitization and internalization of a subset of GPCRs, it is the β-arrestin proteins which facilitate the translocation of GPCRs from the plasma membrane to CCVs. Our data in the present study places β-arrestins as central regulators of CXCR1 internalization in response to agonist stimulation. This has been substantiated using two separate cell lines displaying two different phenotypes. HEK 293 cells which have low expression of β-arrestins require expression of β-arrestin 1 or β-arrestin 2 for agonist induced internalization. In sharp contrast to HEK 293 cells, RBL-2H3 cells express higher levels of β-arrestins and do not require additional expression of β-arrestins for CXCR1 internalization (Fig. 3A). However, agonist-induced internalization of CXCR1 could be blocked by co-expressing the dominant negative β-arrestin 1-V53D mutant in RBL-2H3 cells (Fig. 4, A, ii, and B). These experiments provide strong evidence for β-arrestin regulation of agonist-induced CXCR1 internalization.

**Fig. 4.** Effects of β-arrestin 1-V53D and dynamin 1-K44A mutants overexpression on the agonist-promoted internalization of CXCR1-GFP conjugate in RBL-2H3 cells. A, RBL-2H3 cells transiently expressing CXCR1-GFP alone (i), CXCR1-GFP and β-arrestin 1-V53D (ii), or dynamin 1-K44A mutant (iii). Cells were stimulated with IL-8 (50 nM) and observed using a confocal microscope. B, the relative membrane and cytosolic luminosity of RBL-2H3 cells overexpressing CXCR1-GFP and β-arrestin 1-V53D mutant and (C) RBL-2H3 cells overexpressing CXCR1-GFP and dynamin 1-K44A mutant was plotted versus time prior to and post-agonist treatment for n = 3 (± S.D.) experiments.

**Fig. 5.** IL-8 stimulates CXCR1-GFP and transferrin colocalization in endosomes of RBL-2H3 cells. RBL-2H3 cells transiently expressing CXCR1-GFP fusion protein were stained with Texas Red-transferrin conjugate and samples processed as described under "Experimental Procedures." Unstimulated conditions (i) and agonist-induced CXCR1-GFP-transferrin endosomal colocalization (ii) are shown. Areas of colocalization are indicated yellow.
Additionally, it is also clear from our studies that cellular factors other than GRK2 and β-arrestin are involved in the CXCR1 internalization machinery. Dynamas have been previously described as key proteins involved in the pinching off or sealing of CCVs from the membrane by stimulating GTP/GDP exchange which facilitates endocytic vesicle release (27, 28). In contrast to the angiotensin II type 1A receptor, which is able to undergo dynamin-independent endocytosis (23), our studies indicate that in the presence of dynamin I-K44A mutant, CCVs saturated with agonist-occupied receptor are not released from the membrane into the cytosolic compartment (Fig. 4A, iii). Thus CXCR1 appears to undergo internalization and sequestration through a dynamin-driven and clathrin-dependent internalization pathway similar to several other GPCRs (20, 26, 30). This is supported by two additional pieces of data, agonist-promoted colocalization of transferrin and CXCR1-GFP in endosomal vesicles of RBL-2H3 cells (Fig. 5) and redistribution of CXCR1-GFP to the light vesicular fraction following IL-8 stimulation in HEK 293 cells transiently expressing CXCR1-GFP, GRK2, and β-arrestin 1 (lanes 1 and 3) and CXCR1-GFP, GRK2, and β-arrestin 1 (lanes 2 and 4) pre- and post-stimulation were detected using GFP-directed polyclonal antibody.

β-arrestins in the activation of tyrosine kinases (36). Thus, it is conceivable that some of the functional responses elicited by CXCR1 are due to signals transduced by β-arrestins coupling to the CXCR1 receptor.

While there are at least five independent mechanisms for endocytotic internalization including the clathrin- and non-clathrin-coated pits, micropinocytosis, caveolae, and phagocytosis, GPCRs appear to utilize only two: clathrin-dependent and dynamin-independent endocytotic pathways (23, 34, 35). We present here a previously undescribed model for CXCR1 chemokine receptor internalization whereby we have demonstrated that GRK2, β-arrestin, and dynamin are necessary molecules for the entry of CXCR1 into the cell. Upon IL-8 binding, GRK2 phosphorylates C-terminal serine-threonine residues on CXCR1 allowing β-arrestins to couple phosphorylated receptor to cytoplasmic complexes containing clathrin. Furthermore, our data demonstrates that dynamin is required to pinch off CCVs containing CXCR1 and allow vesicular entry of the activated receptor into the cell. The importance of chemokine receptor internalization may be to serve as a mechanism of reducing the chemotactic activity of leukocytes under conditions of high exposure to inflammatory stimuli thereby preventing their continued migration and departure from the site of inflammation. These studies provide insight into the biochemical factors involved in chemokine receptor entry into the cell and thus may further our understanding of the inflammatory process.

Acknowledgments—We thank Dr. Bruce M. Gill for critical review of this paper and Anne Leaist for excellent technical assistance.

REFERENCES

1. Fernandez, H. N., Henson, P. M., Otani, A., and Hugli, T. E. (1978) J. Immunol. 120, 109–115
2. Schifflmann, E., Corcoran, B. A., and Wahl, S. M. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 1055–1062
3. Baggioni, M., Dewald B., and Moser M. (1994) Adv. Immunol. 55, 97–179
4. Rolls, B. J. (1997) Blood 90, 909–928
5. Murphy, P. M. (1996) Cytochrome Lysogen. Fact. Rev. 7, 47–64
6. Murphy, P. M. (1997) Semin. Hematol. 34, 311–318
7. Kalin, D. J., Michiel, D. F., Johnston, J. A., Lloyd, R. A., Sprenger, H., Oppenheim, J. J., and Wang, M. J. (1993) J. Leukocyte Biol. 54, 604–612
8. Holmes, W. E., Lee, J., Kuang, W. J., Rice, G. C., and Wood, W. I. (1992) Science 253, 1278–1280
9. Lee, J., Horak, R., Rice, G. C., Bennett, G. L., Camerato, T., and Wood, W. I. (1992) J. Biol. Chem. 267, 16283–16287
10. Hammond, M., Lapointe, G., Feucht, P. S., Hilt, S., Gallegos, C., and Gardon, J. C. (1995) J. Immunol. 155, 1428–1433
11. Shams, R. L., Phatak, P. D., Inne, T. P., Abnous, J. C., and Packham, C. H. (1993) Blood 82, 2546–2551
12. Camps, M., Carrozi, A., Schnabel, P., Scheer, A., Parker, P. J., and Gierschik, P. (1992) Nature 360, 684–686
13. Sama, A. K., Oppenheim, J. J., and Matsuhashi, K. (1990) J. Biol. Chem. 265, 183–189
14. Chuntharapai, A., and Kim, K. J. (1995) J. Immunol. 155, 2557–2564
15. Prado, N. P., Suzuki, H., Wilkinson, N., Cousins, B., and Navarro, J. (1996) J. Biol. Chem. 271, 19186–19190
16. Sabroe, I., Williams, T. J., Hebert, C. A., and Collins, P. D. (1997) J. Immunol. 158, 1361–1369
17. Premont, T. P., Inglese, J., and Lefkowitz, R. J. (1995) FASEB J. 9, 175–182
18. Lohse, M. J., Benovic, J. L., Codina, J., Caron, M. G., and Lefkowitz, R. J. (1990) Science 248, 1547–1559
19. Ferguson, S. S. G., Barak, L. S., Zhang, J., and Caron, M. G. (1996) Can. J. Physiol. Pharmacol. 74, 1905–1195
20. Ferguson, S. S. G., Downey, W. E., III, Cipolletto, A. M., Barak, L. S., Menard, L., and Caron, M. G. (1996) Science 271, 363–366
21. Menard, L., Ferguson, S. S. G., Zhang, J., Lin, P. T., Lefkowitz, R. J., Caron, M. G., and Barak, L. S. (1997) Mol. Pharmacol. 51, 800–808
22. Goodman, O. B., Krupnick, J. G., Santini F., Gurevich, V. V., Penn, R. B., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1996) Nature 383, 447–450
23. Zhang, J., Ferguson, S. S. G., Barak, L. S., Menard, L., and Caron, M. G. (1997) J. Biol. Chem. 272, 18302–18305
24. Zhang, J., Barak, L. S., Winkler, K. E., Caron, M. G., and Ferguson, S. S. G. (1997) J. Biol. Chem. 272, 27065–27074
25. Barak, L. S., Ferguson, S. S. G., Zhang, J., and Caron, M. G. (1997) J. Biol. Chem. 272, 27497–27500
26. Kallal, L., Gagnon, A. W., Penn, R. B., and Benovic, J. L. (1997) J. Biol. Chem.
27. Damke, H. (1996) FEBS Lett. 389, 48–51
28. Muhlberg, A. B., Warnock, D. E., and Schmid, S. L. (1997) EMBO J. 16, 6676–6683
29. Damke, H., Baba, T., Warnock, D. E., and Schmid, S. L. (1994) J. Cell Biol. 127, 915–934
30. Aramori, I., Zhang, J., Ferguson, S. G., Bieniasz, P. D., Cullen, B. R., and Caron, M. G. (1997) EMBO J. 16, 4606–4616
31. Chuang, T. T., Sallese, M., Ambrosini, G., Paruti, G., and De Blasi, A. (1991) J. Biol. Chem. 267, 6886–6892
32. Richardson, R. M., Ali, H., Priddgen, B. C., Haribabu, B., and Snyderman, R. (1998) J. Biol. Chem. 273, 10690–10695
33. Khandaker, M. H., Xu, L., Rahimpour, R., Mitchell, G., DeVries, M., Pickering, J. G., Singhal, S. K., Feldmann, R. D., and Kelvin, D. J. (1998) J. Immunol. 161, 1930–1938
34. Oh, P., McIntosh, D. P., and Schnitzer, J. E. (1998) J. Cell Biol. 141, 101–114
35. Henley, J. R., Krueger, E. W. A., Oswald, B. J., and McNiven, M. A. (1998) J. Cell Biol. 141, 85–99
36. Luttrell, L. M., Ferguson, S. G., Daaka, Y., Miller, W. E., Maudsley, S. R., Della Rocca, G. J., Lin, F-T., Kawakatsu, Owada, K., Luttrell, D. K., Caron, M. G., and Lefkowitz, R. J. (1999) Science 283, 655–661
37. Hirst, J., and Robinson, M. S. (1998) Biochim. Biophys. Acta 1404, 173–183
38. Ponka, P., Beaumont, C., and Richardson, D. R. (1998) Semin. Hematol. 35, 35–54