On the Mechanism and Significance of Ligand-induced Internalization of Human Neutrophil Chemokine Receptors CXCR1 and CXCR2*

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It is well established that leukocyte chemotactic receptors, a subset of G protein-coupled receptors, undergo endocytosis after stimulation by ligand. However, the significance of this phenomenon to cell motility and other important leukocyte functions induced by chemotactants has not been clearly defined. Here we show that in primary human neutrophils, the threshold levels of agonist required for endocytosis of the chemotactic receptors CXCR1 and CXCR2 were 10-fold or higher than those needed for maximal chemotactic and calcium flux responses. Moreover, when stimulated by agonists at concentrations that are high enough for chemotaxis but too low for receptor endocytosis, neutrophil CXCR1 and CXCR2 could be reactivated in response to repeated application of the same agonist. Both receptors were excluded from Triton X-100-insoluble lipid rafts, and at high agonist concentrations were rapidly endocytosed by a clathrin/rab5/dynamin-dependent pathway. These data support the conclusion that neutrophil migration in response to CXCR1 or CXCR2 agonists is not dependent on endocytosis of CXCR1 or CXCR2. Rather than being integral to the process of cell migration, receptor endocytosis may be a terminal stop signal when cells reach the focus of inflammation where the chemotactrant concentrations are the highest.

A diverse family of chemotactants and their cognate seven-transmembrane domain G-protein-coupled receptors (GPCRs) regulate leukocyte trafficking in the immune system. Included among these GPCRs are 18 chemokine receptor subtypes (CKRs), which are divided among four subgroups named CC, CXC, CX3C, and C according to the structure of their chemokine ligands (1). Most chemokine receptors signal predominantly via members of the G, class of pertussis toxin (PTX)-sensitive G proteins, and activate multiple phospholipases (A2, beta and rho, and D) and lipid and protein kinases (phosphoinositidol 3-kinase, protein kinase C, and tyrosine kinases). This results in accumulation of lipid mediators such as phosphatidylinositol 3,4,5-triphosphate, calcium flux, and activation of small monomeric GTPases, which coordinate cell adhesion processes, F-actin polymerization and contractile events, polarize the cell, and allow cell movement (2, 3).

Like other GPCRs, CXCR1 and CXCR2 are rapidly desensitized following a burst of agonist-mediated signaling. The mechanism appears to be similar to that used by many other GPCRs (10–12): G protein-coupled receptor kinase (GRK)-mediated phosphorylation of the agonist-occupied receptor followed by beta-arrestin binding, which un copples receptors from G protein activation. Interaction of beta-arrestin with clathrin and the beta subunit of the AP-2 adapter complex facilitates endocytosis of agonist-bound CKRs. Following agonist removal, internalized CXCR1 and CXCR2 may be recycled to the cell surface, thereby enabling a subsequent round of signaling (13–15). The importance of CXCR1 and CXCR2 internalization to chemotactic migration is controversial. Internalization of agonist-occupied CXCR2 was shown to be critical for chemotaxis (16, 17) and neutrophil function in the course of inflammation (18, 19). In contrast, a recent study (20) using CXCR1 and CXCR2 receptor mutants and chimera that were refractory to agonist-mediated endocytosis concluded that receptor internalization was not required for chemotaxis. However, the above studies were done in expression systems at maximal agonist levels that induced quantitative receptor internalization, far removed from the physiological conditions experienced by migrating leukocytes sensing an agonist gradient.

Recycling rates of agonist-occupied receptors may have a bearing on the processive nature of chemotactic signaling in an agonist gradient. Endocytic rates of agonist-occupied receptors depend on their respective trafficking itineraries. The clathrin-dependent pathway is efficient and is commonly used by many receptors (21, 22). In recent years, other slower clathrin-independent pathways have been described. Viruses, toxins, and...
certain receptors lacking conventional endocytic signals (23), may also localize to and endocytose from lipid rafts (also known as detergent-resistant membrane domains or detergent-insoluble glycolipid-rich membrane (DIG)), discrete domains of lipid-ordered lateral heterogeneities in the plasma membrane that are enriched in cholesterol and sphingomyelin (24–27). This process may be slower than clathrin-dependent endocytosis. Recycling proteins with GPI anchors or those covalently modified by addition of long unsaturated fatty acyl chains, and some membrane spanning proteins are preferentially associated with rafts (24–26, 28).

In the present study, we have investigated the lipid microdomain association and endocytic pathways of CXCR1 and CXCR2 in primary neutrophils and placed this phenomenon in the context of conditions required for neutrophil migration. Our results suggest that continuous chemotactic signaling does not require parallel receptor trafficking.

**EXPERIMENTAL PROCEDURES**

**Expression Plasmids, Cell Lines, and Cell Transfection—**Expression plasmids for CXCR1 and CXCR2 have been described (29). The Eps15 deletion mutant fused amino-terminal to GFP (GFP-Eps15Δ/295) was obtained from Alexandre Benmerah (INSERM, Paris, France) (30). GFP-tagged WT dynamin and the K44A dominant negative dynamin mutant (31) were from Julie Donaldson (NICHD, National Institutes of Health, Bethesda, MD). Granulocyte-enriched leukopaks were provided by the Department of Transfusion Medicine at the Clinical Center, NIH. Residual red blood cells and platelets in the leukopaks were disrupted by suspending the packed cells in 10× volumes of ACK lysis buffer at room temperature for 5–6 min. The granulocytes were collected by centrifugation, rinsed twice with PBS, and suspended in RPMI (with 10% fetal bovine serum) at 106/ml. HEK293 cells stably expressing CXCR1 or CXCR2 have been described (32). Epithelial cells were transfected using FuGENE (Roche Diagnostics) or poly-fect (Qiagen Inc.) protocols according to the manufacturer’s instructions. For FACS analysis of transient transfectants, CD4 or CD8 was co-expressed to control for variation. For microscopy of epithelial cells, DNA transfection was carried out on cells plated on glass coverslips in 24-well plates or on Nunc Titer-Tek coverglass chambers.

**Antibody Binding and Flow Cytometric Analysis—**Dye-conjugated or unconjugated mAbs or rabbit antisera against various CKRs, CD4, CD8, CD16, CD32, CD45, and CD71 were obtained either from commercial sources (BD Biosciences, Caltag Corp., R and D Systems, or Zymed Labs) or gifted by the National Institutes of Health AIDS Reference and Reagent Program. For secondary staining, dye-conjugated purified Fab fragments with the relevant species-specific reactivity were obtained from commercial sources (Molecular Probes and Jackson ImmunoResearch Lab, West Grove, PA). Dye-conjugated Tfn, low density lipoprotein, and Ctx-B were from Molecular Probes. Cell surface receptor density was quantified by FACS analysis (29). Typically, 106 cells were incubated for 15 min at 25 °C with the appropriate antibodies in 0.1 ml of PBS containing 1% bovine serum albumin or 1% FCS and 0.02% sodium azide. Flow cytometric data acquisition was carried out using a dual laser four-color BD Biosciences FACSort™ flow cytometer. Data analysis was done using CELLQUEST™ version 3.3 (BD Pharmingen) and FlowJo version 3.3.4 (Tree Star Inc., San Carlos, CA) software.

**Steady State and Kinetic Evaluation of Receptor Internalization and Recycling—**Experimental conditions were as described (33). For receptor internalization assay by FACS, cells were starved in serum-free medium for 20 min. 106 cells were then incubated in RPMI containing 1% bovine serum albumin and challenged with the appropriate agonists at different concentrations for the indicated times. For kinetic analysis, chemokine concentrations were optimized to induce 50% receptor loss on the cell surface after 30 min incubation. For receptor recycling experiments, cell lines expressing the indicated receptor(s) were treated for 30 min with cycloheximide and anisomycin (50 and 25 μg/ml, respectively) before and during the course of the experiment.

**Microscopic Visualization of Receptor Internalization—**Endocytosis of agonist-stimulated CKRs and ligand-bound Tfn-R was visualized in antibody feeding experiments using HEK293 cells stably expressing the indicated receptors, or HeLa cell transfectants as described before (33). In some experiments (indicated in the relevant figure legends), cell surface bound antibody was stripped by treatment for 2 min with 0.5% acetic acid in 500 mM NaCl.

**RESULTS**

**Differential Trafficking Kinetics of Neutrophil CXCR1 and CXCR2 in Response to IL-8—**Multiple studies (35, 36) have shown that the binding affinities of CXCR1 and CXCR2 for IL-8 are similar, and that IL-8 was an agonist at both receptors in several but not all functional assays. We found that in vitro IL-8 treatment of fresh neutrophils in suspension reduced the cell surface immunoreactivity for both CXCR1 and CXCR2, confirming a previous report (13) (Fig. 1A, upper panels). However, IL-8 potency was not identical for the two receptors in this assay: EC50 = 50 nM for CXCR1 and 20 nM for CXCR2 after 20 min incubation with IL-8. CXCL1 (GROα), a selective agonist of CXCR2, did not show similar effects. This suggests that CXCR2 may be more sensitive to chemokine treatment than CXCR1. These experiments, cell lines expressing the indicated receptor(s) were treated for 30 min with cycloheximide and anisomycin (50 and 25 μg/ml, respectively) before and during the course of the experiment.
At CXCR2, was slightly less potent than IL-8 in down-regulating neutrophil CXCR2 (EC_{50} = 50 nM, 20 min of incubation). Moreover, at 100 nM, the highest concentration tested, IL-8 reduced the cell surface densities of CXCR1 and CXCR2 on neutrophils to a different extent, 3–10-fold, respectively. GROα at 100 nM also reduced the surface density of CXCR2 by 10-fold. In summary, in human neutrophils, IL-8-induced down-regulation of CXCR1 mirrored that of GROα occupied CXCR2, but IL-8 was more potent than GROα in down-regulating CXCR2.

A similar rank order was observed in time course experiments using human neutrophils. Thus, treatment with 25 nM IL-8 reduced CXCR1 surface expression 5-fold after 20 min with a \( t_{1/2} \) of \(-8 \) min (Fig. 1A, lower left panel), whereas the same concentration of GROα or IL-8 induced more rapid and complete clearance of CXCR2 (\( t_{1/2} \) = 2 min; 10-fold reduction at 10 min). CXCR2 down-regulation was also slightly faster and more extensive than CXCR1 in agonist-treated HEK293 cells stably expressing individual receptors (Fig. 1A, right panel).
Confocal microscopy of neutrophils revealed a pattern of ligand-induced trafficking for CXCR1 and CXCR2 similar to that of the transferrin receptor (Tfn-R); this pattern could also be induced by PMA treatment (Fig. 1B). Thus, down-regulation of these chemokine receptors in response to ligand appears to be because of receptor internalization or endocytosis. After maximal internalization occurred (100 nM chemokine agonist treatment for 15 or 30 min in neutrophils and HEK293 cells, respectively), both CXCR1 and CXCR2 were able to recycle to the plasma membrane in the absence of new protein synthesis when the cells were washed free of chemokine, albeit to different extents and with different kinetics. Thus in neutrophils CXCR1 reached about 60% of pre-treatment levels after 3 h (Fig. 1C, top left panel), whereas CXCR2, whether down-regulated by IL-8 or GROα, recycled to a much lower extent (Fig. 1C). In HEK293 cells the pattern differed: both CXCR1 and CXCR2 began to recycle to the cell surface shortly after agonist removal, and within 3 h reached 80% of pre-treatment levels (Fig. 1C, right panel). The patterns of CXCR1 and CXCR2 trafficking in neutrophils that we have described are similar to those reported (13). In epithelial cells, both receptors retained substantial recycling potential.

CXCR1 and CXCR2 Endocytosis Involves Clathrin-coated Vesicles, Rab5 and Dynamin—We next investigated the factors that regulate agonist-induced internalization of CXCR1 and CXCR2. We found that pre-treatment with hyperosmotic sucrose, high salt, or 50 mM NH₄Cl partially inhibited receptor internalization in both neutrophils and HEK293 cells, suggesting a pathway involving clathrin-dependent endocytosis (not shown). This conclusion was supported more directly in an antibody feeding experiment using transiently transfected HeLa cells or HEK293 cells (Fig. 2A) in which CXCR1 and...
CXCR1 and CXCR2 could be reactivated by repeated agonist stimulation below the critical threshold required for endocytosis. A, CXCR1 and CXCR2 were saturable for chemokine signaling at 10-fold lower levels than the respective EC_{50} values for receptor clearance. Dose-response curves of granulocytes to stimulation with SDF-1α, GROα, and IL-8 are shown. 1 × 10^6 cells preloaded with Fura-2 AM in 2 ml of HBSS with 1% fetal bovine serum were sequentially stimulated with the indicated amounts of chemokines at the denoted times with continuous data recording. Relative fluorescence ratios are plotted as a function of time. The plots represent results obtained with polymorpho-
CXCR2 colocalized with clathrin-enriched vesicles within 2 min of agonist treatment. The kinetics of GROα driven CXCR2 endocytosis was consistent with the \( t_{1/2} \) value obtained by FACS analysis (Fig. 1, A and C), whereas CXCR1 appeared to traffic faster in the microscopic assay than the FACS based assay. Furthermore, the endocytic kinetics of CXCR1 and CXCR2 in the microscopic assay was virtually indistinguishable from that of TfR uptake (data not shown).

Ligand-dependent endosomal traffic of CXCR1 or CXCR2 was also critically dependent on the functional expression of Rab5 (data not shown), consistent with the findings in a recent report (15). Dynamin (Dyn), another GTPase known to regulate endocytosis (37), also appeared to regulate CXCR1 and CXCR2 internalization. Thus, in an antibody feeding experiment we found that endocytosis of agonist-occupied CXCR1 or CXCR2 as well as TfR, which served as a positive control, was completely blocked in cells expressing the K44A dominant negative dynamin mutant (yellow arrows) (Fig. 2B). All three receptors were internalized as efficiently in WT Dyn-GFP expressing HeLa cells (green arrows) as in control cells.

To more precisely establish that agonist-occupied CXCR1 and CXCR2 traffic via a clathrin-dependent pathway, we examined receptor trafficking in cells expressing a dominant negative mutant of Eps15, a critical regulator of clathrin-coated vesicle (CCV) assembly. GFP-tagged E295/295, a dominant negative inhibitor of Eps15, a key regulator of CCV assembly, was able to block internalization of agonist-occupied CXCR1 and CXCR2 in cotransfected HeLa cells. Inhibition was greater for CXCR1 than CXCR2 (Fig. 2C, white arrows). In a quantitative FACS assay of endocytosis, the Eps15-GFP mutant reversed the 3- or 10-fold reduction in the cell surface distribution of CXCR2 or CXCR1 induced by agonist treatment (Fig. 2D). As shown previously (30, 34, 38), the Eps15 mutant was also able to block TfR uptake in these cells. Endocytic trafficking of agonist-occupied CXCR1 and CXCR2 was also severely impaired in cells expressing another inhibitor of CCV assembly, namely the COOH-terminal fragment of the clathrin adapter, AP180 (39) (data not shown).

**CXCR1 and CXCR2 Oscillate between Active and Inactive States with Increasing Agonist Levels below the Threshold for Endocytosis**—We investigated the relationship between chemotactic signaling and endocytic trafficking of agonist-occupied CXCR1 and CXCR2. As indicated by the intracellular Ca\(^{2+}\) flux profiles in Fig. 3A, neutrophil chemokine receptors were stimulated maximally at about 2 nM agonist input. At these agonist levels, which are ~10-fold higher than the \( K_d \) values for the individual receptor:ligand combinations (13, 35), receptor sites are saturated, yet, as previously shown in Fig. 1A, there was no evidence of receptor clearance within the time frame of the signaling assay. This suggested that \( G_i \) activation and chemotactic signaling might not be required for endocytosis of these receptors and vice versa.

Because maximal calcium flux signaling from CXCR1 and CXCR2 occurred at levels that were 10-fold less than the respective EC\(_{50}\) for endocytosis for the respective ligand:receptor combinations, we investigated the extent to which the chemotactic pathway was desensitized by agonist. Following initiation of Ca\(^{2+}\) flux in response to 5 nM agonist, the cells were refractory to re-stimulation (S2) with the same concentration of the same chemokine, confirming multiple previous reports. However, when the dose of S2 was increased, we observed a progressive increase in the cellular response, reaching 100% of the response to S1 at an S2 concentration of 50 nM (Fig. 3B). The failure to respond to low levels of S2 was not because of rapid inactivation of the added chemokine because, when fresh cells were added to the pre-stimulated cells within seconds after the initial response, we were able to elicit significant signaling (40% of the response to S1, data not shown). Therefore, the need for higher agonist input to re-stimulate pre-treated cells might reflect underlying kinetic parameters (i.e. \( k_{off} \) rates).

Next, we investigated the extent of functional desensitization of CXCR1 and CXCR2 in neutrophils stimulated with both GROα and IL-8 at 100 nM over a 5-min time span, a protocol able to induce substantial clearance of the receptors from the cell surface (Fig. 1A). Within 10 min of agonist removal, the cells were unable to respond to 100 nM GROα, but were almost fully responsive to 100 nM IL-8, suggesting a quantitative (>70%) recovery of functionally active CXCR1 and no recovery of functional CXCR2. Prolonging the incubation after agonist removal up to 40 min did not significantly improve recovery of CXCR1 (Fig. 3C). Although functionally active CXCR2 did recover with time, even prolonged incubation allowed recovery of only 10–20% of CXCR2-specific signaling. This suggested that sequential pre-stimulation with 100 nM GROα and IL-8 over a long time period (>5 min) probably induced endocytic degradation of CXCR2 as was also evident in the cells pretreated with 100 nM of either chemokine for 20 min (Fig. 1C). However, when the neutrophils were pre-stimulated with GROα alone for <2 min, substantial (>70%) functionally active CXCR2 was recovered within 20 min after agonist removal (Fig. 3C, right panel).

The above results, which suggested that CXCR1 or CXCR2 signaling did not require receptor internalization, prompted us to inquire whether the converse situation was true by examining the endocytic kinetics of CXCR1 and CXCR2 in neutrophils arrested for CKR signaling. For this purpose, we used a 4-h pretreatment with PTX to inhibit the Gαi subunits before carrying out agonist-mediated receptor clearance determinations. As shown in Fig. 4A, neutrophils that were pretreated with PTX to inhibit Gαi subunits were devoid of chemotactic potential toward IL-8 or GROα. Whereas untreated cells displayed a typical bell-shaped profile of chemotactic response to agonist inputs of 1–100 nM, PTX-treated cells were inactive (not shown). The dose-response and rate curves of agonist-mediated receptor clearance from PTX-treated or untreated cells are shown in Fig. 4B. EC\(_{50}\) and \( t_{1/2} \) values particularly for GROα versus CXCR2 (and less so for IL-8 versus CXCR1) in neutrophils incubated in HBSS for 3 h were consistently lower than the corresponding values with fresh granulocytes (Fig. 1A).
Endocytosis of agonist-occupied CXCR1 and CXCR2 does not require G protein signaling.

A, histogram of chemotaxis of neutrophils that were pretreated with PTX or left untreated before stimulation with IL-8 or GROα. Chemotaxis assay was done as described under “Experimental Procedures.” The number of migrated cells was expressed as a percentage of the input cells. Duplicate wells were used for each condition, with error bars representing S.E. for the duplicate values. Data shown are representative of three experiments.

B, dose-response and rate curves of endocytosis. Fresh neutrophils (10^7 cells/ml) were incubated at 37 °C for 3 h in HBSS buffer with Ca^{2+} and Mg^{2+} and bovine serum albumin (100 μg/ml) in the presence or absence of B. pertussis toxin (PTX, 4 μg/ml) after which they were stimulated for 20 min with varying amounts of IL-8 or GROα (top plots). Receptor densities were evaluated by FACS analysis as described in the legend to Fig. 1. Rate curves of receptor clearing after treatment with 100 nM of either chemokine are shown at the bottom. Data are expressed as percent of MFV values for the untreated sample(s) and data from four different donors were used to fit polynomial regression curves.
However, PTX treatment induced little if any changes in the endocytic potential of agonist-occupied CXCR1 or CXCR2 (Fig. 4B). These findings strongly endorse the conclusion that receptor internalization, which requires GRK-mediated receptor phosphorylation and β-arrestin recruitment, does not require ongoing chemotactic signaling.
CXCR1 and CXCR2 Endocytosis and Early Signaling Events Are Not Dependent on Lipid Raft Integrity—Next we inquired whether the functional response of desensitized CXCR1 and CXCR2 to repeated agonist stimulation reflected slow endocytic itineraries resulting from the receptor association with plasma membrane rafts. Several lines of evidence indicated that the endocytic pathway for CXCR1 and CXCR2 in neutrophils did not involve lipid rafts. First, up to 4 mM cyclodextrin treatment, which disrupts rafts by depleting plasma membrane cholesterol (40), induced little loss in the cell surface density of CXCR1 or CXCR2 in transfected HeLa cells or in neutrophils (Fig. 5B). CXCR1 expression was reduced when higher concentrations of cyclodextrin were used, however, CXCR2 expression remained relatively stable (Fig. 5B). It should be noted that levels of cyclodextrin >5 mM were toxic and resulted in severe morphological changes to neutrophils (Fig. 5A). When analyzed by confocal microscopy, both CXCR1 and CXCR2 and Tf-R appeared to undergo robust endocytosis after agonist binding or PMA treatment in cells treated with even 10 mM cyclodextrin (Fig. 5D). The reason for this discrepancy with the FACS results is unclear. CXCR1 or CXCR2 function, as measured by calcium flux signals induced by 100 nM IL-8 or GROα in neutrophils, was also insensitive to cholesterol depletion by cyclodextrin (Fig. 5E).

Cholesterol depletion experiments were also carried out in HEK293 and HeLa cell transfectants expressing CXCR1 or CXCR2. Treatment with 10 mM cyclodextrin or 10 μg/ml filipin, a second cholesterol depleting agent, both led to a dramatic loss of cholesterol from these cells (Fig. 6A and data not shown), but did not affect cell surface expression of CXCR1 or CXCR2 when evaluated either by FACS (Fig. 6B) or by confocal microscopy (Fig. 6C). Endocytosis of CXCR1 and CXCR2 in HeLa cells treated with 10 mM cyclodextrin was also unaffected. As expected, pretreating cells with PTX, which inhibits CXCR1 and CXCR2 signaling by inactivating Goq subunits, did not inhibit endocytosis of these receptors (Fig. 6D). Finally, cyclodextrin induced only a slight reduction in the calcium flux response when cell lines expressing CXCR1 or CXCR2 were treated with agonist, whereas PTX pretreatment abolished signaling. Goq-dependent signaling from ATP receptors was not affected by PTX in these cells, as expected.

Independence of CXCR1 and CXCR2 from lipid rafts was also suggested by tests of receptor solubility in panels of non-ionic detergents of variable lipophilicity. Raft proteins resist extraction by non-ionic detergents at 0–4 °C (24, 26, 41). In one protocol, cells stained with fluorescent mAbs were fixed and then extracted with detergent. This protocol is abbreviated “SFT,” an acronym for “Stain, Fix, Triton extract,” the order of the procedures. In a second protocol the cells were stained with mAbs, and then extracted with Triton X-100 prior to fixation. This protocol was abbreviated “STF” for “Stain, Triton extraction, Fix.”

Both by immunocytochemical and FACS analysis, CXCR1 and CXCR2 appeared to be almost completely extracted when neutrophils were treated with ice-cold detergent prior to fixation (Figs. 7A, STF, and 8A, top plot SFT versus STF). When neutrophils were treated with detergent after fixation the results were not consistent: by immunocytochemistry the receptors appeared to resist detergent extraction (Fig. 7A, SFT versus STF), whereas by FACS, cell surface receptor density was decreased when detergent extraction followed fixation (Fig. 8A, top plot SFT versus STF). Triton X-100 at 0–4 °C also solubilized CXCR1 and CXCR2 from the corresponding HEK293 and HeLa cell transfectants (Fig. 7B and data not shown). Detergent solubility of CD45, a known non-raft marker (42) mirrored that of the chemokine receptors (Fig. 8A, SFT in top plot). As expected, GPI anchored CD14 and the neutrophil membrane protein FcγRIIB (CD16), both known raft markers (43, 44), were stable to detergent extraction both before and after fixation (Figs. 7A, and 8A, top plot). Another Fc receptor, FcγRIIA (CD32), which is not GPI anchored (44), also resisted detergent extraction (Fig. 8A, top plot). Lipid raft disruption resulting from cholesterol depletion by cyclodextrin treatment would be expected to render some raft proteins susceptible to detergent solubilization. Consistent with this, both CD16 and CD32 were solubilized by detergent extraction of cyclodextrin-treated cells, although CD32 was more sensitive in this regard, being susceptible even to detergent treatment of fixed cells (Fig. 8A, top panel cyclodextrin 6, SPT, and STP bars).

Whereas insolvency of a membrane protein in Triton X-100 is sufficient to classify it as a raft protein, Triton X-100 solubility does not rule out the possibility that a protein resides in rafts. For instance, prominin, an apically sorted plasma membrane pentaspan protein, was shown to be associated with cholesterol-based rafts that were soluble in Triton X-100 but insoluble in other non-ionic detergents (33). The differential solubility of prominin in various detergents reflected their hydrophilic-lipophilic balance (HLB) (33). Therefore we compared the solubility of CXCR1 and CXCR2 in four detergents, which differed from one another in the size of the polar polyethyleneglycol head group (HLB of 13.5 for Triton X-100 versus 14.6 for Triton X-102; HLB of 16.9 for Brij 35 versus 15.8 for Brij 58) (33). The chemokine receptors were least soluble in Triton X-102, in the order of CXCR1 > CXCR2 > CXCR4 (Fig. 8A, bottom panel). They were more soluble in Brij 35, which has a nominally higher HLB value. Brij 58 was the most potent detergent and partially solubilized even the predominantly raft-associated CD16 and CD32 proteins, which resisted extraction by other detergents (Fig. 8A, bottom panel). The relative insolubility of CXCR1 in detergents with larger polar head groups together with its solubility at high cyclodextrin levels (Fig. 5B) suggested that a fraction of CXCR1 might be associated with alternative forms of cholesterol-based rafts.

Consistent with the above FACS results, most if not all CXCR1 and CXCR2 from detergent-extracted neutrophils was identified by immunoblotting in the soluble fraction of detergent-treated cells after centrifugation at 17,000 × g for 10 min (Fig. 8B). Cyclodextrin treatment at 5 mM did not change this behavior of CXCR2 (Fig. 8B) or CXCR1 (not shown). With 10 mM cyclodextrin treatment, which caused considerable cell damage (Fig. 5A), there was a modest decrease in the recovery of CXCR2 (Fig. 8B) and CXCR1 (not shown). Neutrophil CXCR2 was not redistributed from the detergent-soluble fraction after agonist stimulation (100 nM GROα for 15 min) or protein kinase C activation by PMA treatment (10−7 M PMA for 20 min). However, there was a considerable loss of CXCR2 after these treatments (Fig. 8B, right panel), which induced degradative endocytosis as shown in Fig. 1C and in an earlier report (13).

We next confirmed these results by microscopy. In co-patching experiments, we co-stained neutrophils with antibodies against individual receptors and CxT-B, which binds penta-valently to GM1 ganglioside in lipid rafts, or antibody against Tfn-R, a non-raft marker. As shown by the confocal images in Fig. 8C, the GPI-anchored CD16 exhibited extensive co-localization with CxT-B-stained patches. In contrast, CXCR1 and CXCR2 positive zones were separate from CxT-B positive raft patches. There was an almost complete reversal of the co-staining pattern with Tfn-R. CD16 was completely excluded from Tfn-R positive zones, whereas CXCR1 and CXCR2 partially colocalized with Tfn-R (Fig. 8C).
DISCUSSION

In this study, we have demonstrated through multiple lines of evidence that the neutrophil chemokine receptors CXCR1 and CXCR2 were excluded from plasma membrane rafts in neutrophils and were rapidly endocytosed via clathrin-dependent pathways upon agonist binding. We have also shown that cholesterol depletion and raft disruption did not alter the cell surface densities, trafficking, and signaling potential of CXCR1 and CXCR2 in expression systems. A, cholesterol staining of HEK293 cell lines expressing CXCR1 or CXCR2 as in Fig. 5. B, FACS histograms of CKR expression in HEK293 cell lines treated with cyclodextrin, filipin, or left untreated. Cyclodextrin (CyDx) was at 10 mM and filipin at 10 µg/ml. Conditions are as described for Fig. 5. C, cell surface distribution of CXCR1 or CXCR2 in cyclodextrin-treated or untreated HEK293 cell lines visualized by confocal microscopy. Cells were fixed before staining with fluorescein isothiocyanate-labeled mAbs. D, effects of cyclodextrin and PTX on agonist (IL-8 or GROα at 50 nM for 30 min) induced endocytosis of CXCR1 and CXCR2 in HeLa cell transfectants. Cyclodextrin treatment was at 10 mM for 30 min and PTX treatment was at 250 ng/ml at 37 °C for incomplete medium. Internalization of cell surface-bound fluorescein isothiocyanate mAbs monitored CKR trafficking. Results represent three experiments. E, signaling response of CXCR1 and CXCR2 to their respective agonists (100 nM) in HEK293 cell lines that had been treated with cyclodextrin (10 mM), PTX (250 ng/ml), or untreated. Intracellular Ca²⁺ flux profiles represent two experiments.

Mechanism and Significance of CXCR1 and CXCR2 Endocytosis
Unlike authentic raft-associated proteins such as CD14 and CD16, cell surface-associated CXCR1 and CXCR2 were extracted by ice-cold detergent treatment. Cells were stained for the respective receptors, and fixed before (SFT) or after (STF) detergent extraction (0.25% Triton X-100 at 0–4 °C). Granulocytes were stained with a mixture of allophycocyanin-conjugated CD14 or CD16 mAb with fluorescein isothiocyanate-labeled CXCR1 or CXCR2 mAb. HeLa cell transfectants were stained with CXCR1- or CXCR2-fluorescein isothiocyanate. Single channel images of cells representing three experiments are shown.
the agonist input level for maximal signaling was considerably lower than the critical threshold required for receptor endocytosis. Beneath this threshold, both receptors oscillated between sensitive and desensitized states on exposure to increasing agonist inputs.

CXCR1 and CXCR2 differ with respect to agonist selectivity, ligand binding affinity, receptor trafficking, and downstream signaling pathways such as phospholipase D activation (6, 8, 9, 13). Our results showing that CXCR2 was internalized at lower agonist inputs with faster kinetics than CXCR1 in neutrophils and expression systems were in essential agreement with the earlier reports (13, 20). CXCR1 and CXCR2 were also endocytosed in PMA-treated neutrophils suggesting that protein kinase C-mediated phosphorylation probably rendered the receptors susceptible to endocytosis by recruiting AP2 complexes (17).

Endocytosis of agonist-occupied CXCR1 and CXCR2 receptors was rapid and followed a clathrin-dependent itinerary paralleling closely the intracellular uptake of Tfn. Receptor transport to CCVs in the microscopic assay was substantial by 5 min in agreement with the \( t_{1/2} \) for receptor clearance in the FACS assay. Consistent with a recent report (15), and similar to the behavior of CXCR4 (29), the endosomal delivery of CXCR1 and CXCR2 was dependent on Rab5 function (45). Endocytic trafficking of agonist-bound CKRs and Tfn were substantially inhibited in cells expressing the dominant negative mutant inhibitor of dynamin GTPase function essential for both clathrin and non-clathrin-dependent vesicular traffic (37, 46, 47). More direct evidence for receptor trafficking via the clathrin pathway was provided by the genetic inhibition of CCV assembly by the dominant negative Eps15 mutant (30, 38). Quantitative FACS analysis showed 60–80% inhibition of receptor clearance by agonist binding.

Internalized CXCR1 and CXCR2 exhibited divergent recycling patterns after agonist removal in neutrophils versus epithelial cells. As shown before (13), internalized CXCR1 was recovered more rapidly and efficiently than CXCR2 in neutrophils. A substantial fraction of internalized CXCR2 was sequestered in both early and late endosomal vesicles of neutrophils (data not shown) and was probably degraded (13, 48). In contrast, both CXCR1 and CXCR2 recycled efficiently to the cell surface in HEK293 cell lines after removal of the agonist and a similar behavior of CXCR2 in HEK293 cells was reported (13, 15). Whether or not these differences reflect relative efficien-

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**Fig. 8.** Whereas CXCR1 and CXCR2 were soluble in Triton X-100 and co-patched with non-raft rather than raft markers, a fraction of CXCR1 and CXCR2 was associated with alternative cholesterol-based rafts. **A:** Top, differential solubility of various granulocyte receptors in ice-cold Triton X-100 in cells treated with 6 mM cycloheximide (CyDs) or untreated before fixation and extraction at 0–4°C with 0.25% Triton X-100. SF denotes no detergent extraction, whereas STF and SFT denote detergent extraction before or after fixation, respectively. The histograms reflect mean fluorescent values (MFV) of individual receptors (by FACS analysis) after various treatments expressed relative to those in cells that were stained and fixed. Bottom, relative stabilities of granulocyte receptors in detergent with different hydrophilic-lipophilic balance. Granulocytes were stained for the indicated receptors prior to detergent (0.25%) extraction at 0–4°C. Fluorescence intensities were determined immediately by FACS analysis and MFVs are expressed relative to values obtained without detergent treatment. Circles denote results from individual experiments. B, CXCR1 and CXCR2 are concentrated in detergent-soluble membranes of fresh granulocytes. Granulocytes were extracted with 0.2% Triton X-100 at 0–4°C and centrifuged at 17,000 × g for 15 min. Supernatant (S) and pellet (P) fractions were analyzed by SDS-PAGE followed by immunoblotting for CXCR1 (left) or CXCR2 (right) using the respective mAbs. CXCR2 distribution was also analyzed in granulocytes that had been pretreated with 5 or 10 mM CyDs or untreated before fixation and extraction at 0–4°C with 0.25% Triton X-100. CyD6–STF denotes no detergent extraction, whereas CyD6–SFT and CyD6–ST denote detergent extraction before or after fixation, respectively. The Immunoblot profiles are representative of results from two donors for CXCR2 and three donors for CXCR1. C, copatching of CD16, CXCR1, and CXCR2 in granulocytes with raft (CTX-B) and non-raft (Tfn-R) markers. For copatching with Tfn-R, cells were stained at 20°C for 5 min with a mixture of CD71-allophycocyanin and fluorescein isothiocyanate-labeled mAbs against CD16, CXCR1, or CXCR2 followed by cross-linking with anti-mouse IgG. For copatching with CTX-B, cells were co-stained with Alexa 594-CTX-B. RGB images were separated into pseudo-colored two channel images to show CKRs in red and CTX-B or Tfn-R in green. Images represent three experiments.
cies of Rab7 versus Rab11 assisted lysosomal sorting versus recycling pathways (49–52) in these cell types, the balance between these pathways may determine the agonist threshold for receptor sequestration and also fine tune the chemotactic response of neutrophils in an agonist gradient (13, 15).

The role(s) of endocytic trafficking of agonist-occupied receptor during chemotactic signaling remain unresolved. Several studies using immunocytochemical techniques have shown that polarized cells undergoing directional movement in a shallow agonist gradient or unpolaredized cells exposed to uniform agonist concentration redistributed and concentrated the receptors at the leading edge (53–57). Moreover, neutrophil chemokine receptors mutated to abolish GRK-mediated phosphorylation, arrestin interaction, and endocytosis were inefficient in mediating chemotaxis (16, 17). In contrast, more recent studies have shown that neither the neutrophil C5a receptor (58), nor the cAR1 receptor of Dictyostelium (59), was concentrated at the leading edge or internalized during chemotaxis in an agonist gradient.

By monitoring the dose-response kinetics of phospholipase β activation (by dissociated Gβγ subunits), we have shown that CXCR1 and CXCR2 on fresh neutrophils were saturated by agonist levels that were 10-fold higher than the Kᵦᵦ values for the respective receptor-agonist pairs (13, 35).2 The minimal agonist levels that elicited intracellular Ca²⁺ flux were 10-fold lower than the EC₅₀ values for receptor internalization (Fig. 1A). Although the receptors were refractory to restimulation with the same agonist concentration, a 4–10-fold higher agonist input elicited maximal signaling response (S2) instantaneously. The S2 response did not reflect recruitment or priming of a dormant class of low-affinity receptors because the magnitude of signaling remained the same over a 50-fold range of the respective agonist input (Fig. 3A). Intraacellular pools of G-protein subunits were probably not limiting for the signaling magnitude; sequential stimulation of unrelated Go₁-‐coupled chemokine receptors (i.e. CXCR4, Fig. 3A, and MLF-R, not shown) generated a comparable Ca²⁺ flux response. This was consistent with previous work (60) that showed that in Dictyostelium, the G-protein cycle is preserved during the adaptation response of cAR1 receptor thereby allowing re-stimulation.

Although there was no evidence of receptor clearance from the cell surface at agonist levels that permitted re-stimulation (Fig. 1A), it is possible that the receptors might have been desensitized by GRK-mediated phosphorylation and β-arrestin recruitment (10, 11) and resensitized by PP2A-mediated dephosphorylation (61, 62). Cyclical GPCR phosphorylation and dephosphorylation might have accounted for the oscillatory pattern of signaling response that we observed. Alternatively, receptor phosphorylation at low agonist input might have lowered the ligand binding affinity as was shown for the cAR1 receptor in Dictyostelium (63). However, GRK-mediated receptor phosphorylation requires high agonist treatment (>50 nM) for 5 min or more (64–66) and as such might not have targeted more than a small fraction of agonist-bound receptor under our conditions. Even if a significant fraction of receptors were phosphorylated by GRKs, dephosphorylation would only occur during endosomal transit of recycling receptor (61, 62, 64). Although physical interaction between PP2A and CXCR2 does not require receptor phosphorylation, such an interaction was shown to require receptor trafficking to the endosomes (64). When the cells were stimulated with >EC₅₀ agonist levels that facilitated receptor endocytosis, there was a significant delay (~20 min) in the recovery of receptor response (Fig. 3C). This lag time in response recovery reflects the temporal constraints of receptor recycling.

The instantaneous Ca²⁺ flux response during repeated agonist stimulation that we observed is a snapshot of leukocyte behavior in an agonist gradient. Polarized leukocytes migrating in a chemotactrant gradient continuously ratchet their response to ever increasing agonist levels, by adjusting the intracellular levels of asymmetrically distributed 2° messengers (3). When the extracellular cue is removed, the asymmetric accumulation of phosphatidylinositol 3,4,5-triphosphate and F-actin polymerization at the leading edge decay rapidly (67, 68). Increased duration of receptor occupancy on cells navigating in an agonist gradient or re-exposed to an agonist pulse would shift the intracellular equilibrium among the feedback controlled positive and negative signals to a different set point. A potential mechanism for attenuating the receptor response was provided by a recent study of cAR1-mediated chemotaxis in Dictyostelium where agonist stimulation led to a rapid increase of inositol pyrophosphates, InaP7 and InaP8 (69). Because InaP7 was a strong competitor of phosphatidylinositol 3,4,5-triphosphate for binding to pleckstrin homology domain proteins, InaP7 was presumed to inhibit membrane binding and activation of several chemotactic signal transducers. Similar regulation of intracellular signals may be operative in leukocytes.

Just as repeated chemokine receptor signaling was observed at agonist levels too low to induce receptor internalization, inhibition of G-protein signaling had no effect on agonist-driven endocytosis. Previous studies with CXCR2 suggested that chemotaxis required internalization of agonist occupied receptors (61, 62, 64). However, a recent report (20) showed that CXCR1 and CXCR2 mutants and chimeras that were refractory to agonist-mediated endocytosis, nevertheless, supported phosphatidylinositol 4,5-diphosphate hydrolysis, granulocyte chemotaxis, and intracellular Ca²⁺ flux albeit with reduced chemotaxis. And, the non-phosphorylatable CCR2B receptor mutant, which failed to be internalized supported chemotaxis in B cells (70). Our results with fresh human neutrophils support the latter two conclusions.

Slow endocytic trafficking rates of agonist-occupied receptor may allow ligand dissociation and receptor resensitization. Alternative trafficking itineraries may result in different signaling schemes as was shown for the epidermal growth factor receptor (71, 72). We have shown that both CXCR1 and CXCR2 followed almost exclusively clathrin-dependent endocytic pathways. However, clathrin independent trafficking has been demonstrated for other GPCRs such as CCR2B in the neurovascular lature (73), fMLF and C5a receptors in leukocytes (74), endothelin receptor-type A in Chinese hamster ovary cells (75), and CCR5 in epithelial cells (34). Some GPCRs and G protein subunits are raft-associated by virtue of fatty acylation (34, 76) and raft association of CCR5 coupled it to a non-clathrin trafficking itinerary (34). By these criteria, neither CXCR1 nor CXCR2 would be expected to associate with lipid rafts. In agreement, extraction with Triton X-100 at 0–4 °C completely solubilized CXCR1 and CXCR2 from neutrophils and epithelial cell lines. Chemokine binding or PMA treatment did not alter the detergent solubility of either receptor, thus excluding the possibility of stimulated receptors being recruited into raft-associated signaling complexes. Copatching of CXCR1 with the non-raft marker Tfn-R but not with the raft-associated GM1 ganglioside further supported these conclusions.

Global extraction of cholesterol by cyclodextrin and the resultant raft disruption did not alter the cell surface density or endocytic trafficking of CXCR1 or CXCR2 in epithelial cell lines. In contrast, there was a progressive loss of receptor density at the cell surface of neutrophils with increasing cyclodextrin extraction. Whereas the phospholipase β activation potential (as measured by the initial peak of Ca²⁺ flux) from
agonist-bound CXCR1 or CXCR2 was intact in cholesterol-depleted cells, there was a measurable decrease in the magnitude of agonist-mediated receptor clearance in the FACs assay. This decrease in receptor clearance probably reflected the reduced receptor-staining in the cyclodextrin-treated cells because a similar reduction in the endocytic trafficking was not obvious in the microscopic assay and was not associated with a corresponding loss in the Ca\(^{2+}\) flux response.

Recent studies have indicated that rafts are heterogeneous with respect to lipid composition, biochemical properties, detergent sensitivities, and protein cosegregation (33, 77–81). Furthermore, in chemotaxing cells, the leading edge and the uropod are composed of different raft subtypes containing distinct gangliosides and membrane proteins (77, 82–85). Variable detergent solubilities of membrane proteins probably reflect their association with different raft subtypes (33, 78, 86). By evaluating the solubility of neutrophil receptors in detergents with different hydrophilic-lipophilic balances, the raft-associated, GPI anchored CD16 isoform was judged to be the most insoluble. Of the three chemokine receptors tested, CXCR1 displayed the greatest resistant to detergent extraction. The graded resistance of CXCR1 to various detergents (Fig. 8A) correlated with the progressive loss of cell surface CXCR1 in cyclodextrin-treated cells (Fig. 5B) and implied that a fraction of CXCR1 may be associated with a different raft subtype. This behavior of CXCR1 was somewhat reminiscent of prominin, the penta-span plasma membrane protein associated with microvilli and plasmalemmal protrusions (33).

Although we have shown that cholesterol depletion had no significant effect on the initial Ca\(^{2+}\) flux response from stimulated CXCR1 or CXCR2, recent studies have shown that raft integrity is essential for the gamut of the chemotactic response in neutrophils including sustained intracellular Ca\(^{2+}\) flux, membrane polarization, F-actin polymerization at the leading edge, and myosin phosphorylation at the uropod (82, 87). Moreover, non-chemokine-initiated leukocyte activation complexes originating at receptors mediating acquired (88) or innate (89) inflammation may be mobilized to the lipid rafts on the leading edge of neutrophils. However, it is possible that CXCR1 and CXCR2 (89), we have shown that neither CXCR1 nor CXCR2 was significant effect on the initial Ca\(^{2+}\) flux response from stimu-

Mechanism and Significance of CXCR1 and CXCR2 Endocytosis

In conclusion, CXCR1 and CXCR2 can be thought of as...
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