Role of Clathrin-mediated Endocytosis in CXCR2 Sequestration,
Resensitization, and Signal Transduction*

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CXCR2 is a seven-transmembrane receptor that transduces intracellular signals in response to the chemokines interleukin-8, melanoma growth-stimulatory activity/growth-regulatory protein, and other ELR motif-containing CXC chemokines by coupling to heterotrimeric GTP-binding proteins. In this study, we explored the mechanism responsible for ligand-induced CXCR2 endocytosis. Here, we demonstrate that dynamin, a component of clathrin-mediated endocytosis, is essential for CXCR2 endocytosis and resensitization. In HEK293 cells, dynamin I K44A, a dominant-negative mutant of dynamin that inhibits the clathrin-mediated endocytosis, blocks the ligand-stimulated CXCR2 sequestration. Furthermore, co-expression of dynamin I K44A significantly delays dephosphorylation of CXCR2 after ligand stimulation, suggesting that clathrin-mediated endocytosis plays an important role in receptor dephosphorylation and resensitization. In addition, ligand-mediated receptor down-regulation is attenuated when receptor internalization is inhibited by dynamin I K44A. Interestingly, inhibition of receptor endocytosis by dynamin I K44A does not affect the CXCR2-mediated stimulation of mitogen-activated protein kinase. Most significantly, our data indicate that the ligand-stimulated receptor endocytosis is required for CXCR2-mediated chemotaxis in HEK293 cells. Taken together, our findings suggest that clathrin-mediated CXCR2 internalization is crucial for receptor endocytosis, resensitization, and chemotaxis.

Melanoma growth-stimulatory activity (MGSA)1/growth-regulatory protein (GRO), interleukin-8 (IL-8), neutrophil-activating peptide-2, epithelial derived neutrophil-activating peptide 78, and granulocyte chemotactic protein-2 are members of a family of structurally related cytokines that induce chemotaxis and respiratory burst in neutrophils (1–3). These chemokines belong to the CXC chemokine subfamily in which the first two conserved cysteine residues are separated by an intervening amino acid (4). Several chemokine receptors have been cloned that bind MGSA: CXCR2 (formerly called IL-8 RB) (5), Duffy antigen receptor for chemokine, and two herpesvirus-encoded receptors: the herpesvirus saimiri ECRF-3 and the human herpesvirus-8 G protein-coupled receptor (6). IL-8 binds to these four receptors as well as CXCR1 (formerly called IL-8 RA) (7), which is a receptor for IL-8 and granulocyte chemotactic protein-2. CXCR2 is a shared receptor that binds to IL-8, MGSA, neutrophil-activating peptide-2, epithelial derived neutrophil-activating peptide 78, and granulocyte chemotactic protein-2 (8).

Like all of the chemokine receptors cloned to date, CXCR2 is a member of a superfamily of G protein-coupled receptors (GPCRs) that transduce signals to the interior of the cell through heterotrimeric guanine nucleotide-binding proteins (G proteins). These receptors share a common putative structural topology composed of seven-transmembrane domains separated by three extracellular and three intracellular loops. Upon agonist binding, CXCR2 activates G protein-mediated phosphoinositide hydrolysis to generate diacylglycerol and inositol 1,4,5-trisphosphate, thereby activating protein kinase C and mobilizing Ca2+ to initiate a variety of cellular responses (9). Receptor activation is followed by receptor phosphorylation on multiple serine residues and subsequent desensitization of the receptor to further stimulations. These events are usually accompanied by receptor endocytosis and/or recycling of the receptor (10).

A large body of evidence suggests that the major route of sequestration of GPCRs is via clathrin-coated pits and into early endosomes (11). The β2-adrenergic receptor (β2-AR), a prototypical GPCR, becomes phosphorylated upon ligand binding and then exhibits an increased binding affinity for the adaptor molecules (either β-arrestin or arrestin 3), which prevent further coupling between the receptor and G proteins (12, 13). The arrestins are subsequently involved in β2-AR sequestration by specifically binding to clathrin, a major component of clathrin-coated pits (14, 15). It has been suggested that sequestration of GPCRs to early endosomes and lysosomes might be responsible for receptor down-regulation and resensitization (16, 17). Within the acidic environment of the endosomes and lysosomes, the receptors either undergo dephosphorylation and recycle back to the cell surface and thus are resensitized or are degraded and down-regulated (11).

At present, much of the information concerning the molecular mechanisms for GPCR sequestration and down-regulation is derived from the studies of β2-AR. Due to the enormous number of GPCRs and the diverse cellular function evoked by each individual receptor, it is conceivable that multiple mechanisms may be employed for receptor sequestration, down-regulation, and resensitization. This is exemplified by the finding that while clathrin-coated pits are essential for agonist-promoted β2-AR sequestration, sequestration of the angiotensin II type 1A receptor does not require intact function of clathrin-coated pits (18).

In contrast to the β2-AR, little is known regarding the se-
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Fig. 1. Colocalization of CXCR2 with the transferrin receptor. HEK293 cells expressing CXCR2 were incubated with fluorescein isothiocyanate-conjugated transferrin for 2 h, washed, and chased for 30 min. The cells were treated with 100 ng/ml MGSAGRO for 0 min (A, B) or 3 h (C, D). The cells were permeabilized, and stained for CXCR2 with a rabbit anti-CXCR2 antibody followed by rhodamine-conjugated goat anti-rabbit antibody (A, C). The emission of fluorescein isothiocyanate-transferrin is shown in B and D. The arrows indicate the examples of co-localization of CXCR2 and transferrin receptor. Images were processed using Photoshop software.

**Experimental Procedures**

Cell Culture and Transfections—293 human embryonic kidney cells (HEK293) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. HEK293 cells cultured to 80% confluence were transfected with the CXCR2 cDNA in the pRe/cytovector virus vector using the LipofectAMINE PLUS reagent (Life Technologies, Inc.) according to the manufacturer's recommendation. Before each experiment, an indirect immunofluorescence assay was used to assess the transfection efficiency. The transfection efficiency was approximately 80% in each experiment.

Receptor Internalization Assay—The acid/buffer wash technique was used to determine the kinetics of MGSAGRO-induced internalization of CXCR2.

In Vivo Phosphorylation and Western Blot Analysis—Receptor phosphorylation assays were performed as described previously (20). In brief, the transfected cells were plated on six-well plates 1 day after the transfection. On the following day, after incubating in serum- and phosphate-free medium for 1 h, cells were then labeled by incubating in [32P]orthophosphate (100 Ci/mmol) in the same medium at 37 °C for 2 h. Cells were then stimulated with or without 100 ng/ml MGSAGRO for 10 min. Diphosphorylation was performed by allowing cells to recover in fresh serum-free media without ligand at 37 °C for 1 h. The cells were then lysed in RIPA buffer containing 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 10 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. CXCR2 was immunoprecipitated with a polyclonal rabbit anti-CXCR2 antibody (20) from cleared supernatants containing approximately 300 μg of protein (estimated by the BCA method; Pierce). The immunoprecipitates were electrophoresed through a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Bio-Rad). The phosphorylated receptors were then detected by autoradiography, and the total amount of receptor was determined by Western blotting using a monoclonal antibody against CXCR2 to ensure equal loading.

Indirect Immunofluorescence—HEK293 cells stably expressing CXCR2 (10) were grown on 10-mm coverslips (Fisher). To compare the distribution of CXCR2 and the transferrin receptor, cells were washed once with serum-free medium and then incubated at 37 °C with the same medium containing 100 μg/ml of fluorescein isothiocyanate-conjugated transferrin (Molecular Probes, Inc., Eugene, OR) for 2 h. The cells were washed and then chased for 30 min. Following MGSAGRO or IL-8 (100 ng/ml) treatment for the indicated time periods, cells were processed for indirect immunofluorescence to detect the subcellular localization of CXCR2 (19).

Receptor Down-regulation Assay—Cells were collected by trypsinization 24 h after transfection and plated onto 12-well plates. The next day, the cells were treated with or without 100 ng/ml MGSAGRO for 0.5–8 h. Cells were lysed in 0.5 ml/well RIPA buffer with proteinase and phosphatase inhibitors described as above. Aliquots of lysates containing 30 μg of protein were subjected to electrophoresis (10% SDS-polyacrylamide gel electrophoresis) and then transferred to nitrocellulose membrane. The amount of CXCR2 was detected by Western blot using the previously described rabbit anti-CXCR2 antibody.

MAP Kinase Assay—Agonist-treated cells were lysed by RIPA buffer. Lysates containing equal amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis. Phosphorylated MAP kinase (ERK1/ERK2) was detected by a phosphospecific MAP kinase antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Chemotaxis Assay—Chemotaxis assays were performed on transiently transfected HEK293 cells as described previously (10) using a 96-well chemotaxis chamber (Neuroprobe Inc.).

Results

Redistribution of CXCR2 to Endosomes following MGSAGRO Treatment—Immunofluorescence microscopy was used to define the subcellular distribution of CXCR2 in stably transfected HEK293 cells using rabbit anti-CXCR2 polyclonal antibody. The localization of endosomes was detected using the transferrin-fluorescein isothiocyanate complex as described previously. The majority of CXCR2 was targeted to the plasma membrane in untreated cells (Fig. 1A). A small portion of CXCR2 was located intracellularly as punctate structures that colocalized with the transferrin receptor (Fig. 1, A and B). Following MGSAGRO (100 ng/ml) treatment for 3 h, agonist...
stimulation resulted in sequestration of CXCR2, observable as a loss of cell surface immunofluorescence with a concomitant increase in the number of intracellular punctate structures. The distribution of the punctate structures overlapped extensively with those of the transferrin receptor (Fig. 1, C and D), suggesting that agonist stimulation enhances CXCR2 redistribution into endosomes. This phenomenon was observable as early as 10 min with MGSAGRO treatment (data not shown). IL-8 treatment induced a similar redistribution of CXCR2 to the endosomes (data not shown).

Effect of Dynamin I K44A on CXCR2 Endocytosis—It has been shown previously that CXCR2 undergoes a rapid endocytosis within minutes in response to either MGSAGRO or IL-8 stimulation. To determine whether the agonist-promoted CXCR2 endocytosis is a clathrin-mediated process, HEK293 cells were transiently co-transfected with cDNAs encoding CXCR2 and dynamin I K44A. Agonist-promoted endocytosis was determined and compared with that observed in cells transfected only with CXCR2 or cells co-transfected with CXCR2 and wild-type dynamin I. Cells expressing only CXCR2 showed rapid receptor internalization after MGSAGRO treatment (Fig. 2). The MGSAGRO-stimulated sequestration of CXCR2 occurred in a time-dependent manner. The percentage of endocytosed receptor increased progressively from 20% at 1 min to approximately 60% at 20 min. Co-expression of wild-type dynamin I with CXCR2 seemed neither to promote nor inhibit the rate of CXCR2 endocytosis. In contrast, in cells co-expressing CXCR2 and dynamin I K44A, there was a profound inhibition of the MGSAGRO-promoted CXCR2 endocytosis, with less than 15% of receptor endocytosed at 20 min. These data clearly indicate that agonist-promoted endocytosis of CXCR2 depends on functional expression of dynamin I and thus is a clathrin-mediated process. This is further supported by the fact that internalized CXCR2 colocalized with the transferrin receptor. Based on ligand binding data, we observed similar CXCR2 cell surface expression level in cells transfected with CXCR2 and dynamin I K44A as compared with those transfected with only CXCR2. We also observed a similar effect on receptor endocytosis when the cells were treated with IL-8 (data not shown).

The Effect of Dynamin K44A on CXCR2 Phosphorylation and Dephosphorylation—We have previously shown that CXCR2 undergoes a rapid phosphorylation and desensitization after MGSAGRO treatment (10). To further investigate the effects of the inhibition of receptor endocytosis on CXCR2 dephosphorylation and resensitization, transiently transfected 293 cells were metabolically labeled with [32P]orthophosphate, treated with MGSAGRO, and allowed to recover from ligand. In the absence of dynamin I K44A expression, phosphorylation of CXCR2 was detected after 10-min treatment of 100 ng/ml MGSAGRO. After a 1-h recovery period following removal of ligand, CXCR2 underwent dephosphorylation to 44% of the stimulated level. However, the dephosphorylation of CXCR2 in dynamin I K44A co-transfected cells was greatly attenuated. After the same recovery period, about 80% of receptor still remained in the phosphorylated form as compared with ligand-stimulated cells without recovery (Fig. 3, A and D). The equal loading of CXCR2 and expression of dynamin I K44A were confirmed by Western blots using antibodies against CXCR2 (Fig. 3B) and dynamin I (Fig. 3C). The rate of CXCR2 dephosphorylation with co-expression of wild-type dynamin is comparable with that which occurred in cells transfected with CXCR2 alone.

Effect of Dynamin K44A on CXCR2 Down-regulation—Previously, we have observed that MGSAGRO treatment resulted in a concentration-dependent decrease in the level of CXCR2 in stably transfected 3A5SubE P-3 and HEK293 T2 cells (10, 20). Based on information from a β2-AR down-regulation experiment (16), we hypothesized that a portion of the internalized CXCR2 may be subject to proteolytic degradation in the endosome or lysosome following receptor endocytosis. Moreover, reducing this portion of receptors by blocking endocytosis should have a concomitant effect on receptor degradation and down-regulation. To test this hypothesis, we determined the effect of the dynamin I K44A mutant on CXCR2 degradation and down-regulation in HEK293 cells. Transiently transfected cells were stimulated with MGSAGRO for various time periods and lysed. The relative amount of CXCR2 was assessed by Western blot using whole cell lysates. As demonstrated in Fig. 4, MGSAGRO treatment promoted progressive CXCR2 degradation in a time-dependent fashion. After 2 h of treatment, cells lost 14% of receptor. The percentage of CXCR2 loss was increased to 49% following 8 h MGSAGRO incubation. In contrast, the dynamin I K44A mutant effectively reduced the agonist-promoted CXCR2 degradation, with only 15% of CXCR2 being degraded after 8 h of MGSAGRO treatment (Fig. 4). These data suggest that a portion of endocytosed CXCR2 undergoes degradation and down-regulation via a clathrin-mediated event.

Effect of Dynamin K44A on CXCR2-mediated MAP Kinase Activation—Previous studies demonstrated that IL-8 stimulation on its receptors in neutrophils resulted in activation of MAP kinase pathway (21). We also confirmed the activation of MAP kinase following MGSAGRO stimulation in HEK293 T2 cells. To study the downstream CXCR2 signal transduction pathway, we next examined the effect of dynamin I K44A expression on MAP kinase activation. The MGSAGRO-dependent activation of MAP kinase in cells expressing both CXCR2 and dynamin I K44A was compared with that of cells expressing only CXCR2 by using a phoshospecific ERK1/ERK2 antibody. As demonstrated in Fig. 5, A and C, dynamin I K44A cells exhibited an identical MAP kinase activation response following 5 min of MGSAGRO or IL-8 (100 ng/ml) stimulation. Equal loading was confirmed by immunoblotting with an antibody against ERK-2.
CXCR2 phosphorylation. Dotted bars indicate CXCR2; striped bars, CXCR2 plus dynamin I K44A.

**Fig. 3.** The effect of the dynamin I K44A mutant on CXCR2 phosphorylation and dephosphorylation. HEK293 cells were transiently transfected with plasmids expressing CXCR2 with or without rat dynamin I K44A. A, a representative autoradiograph from two independent experiments showing the phosphorylation and dephosphorylation of CXCR2 in the whole cell lysates. Cells were treated with 100 ng/ml MGSA/GRO for 0 min (lanes 1 and 4) or 10 min (lanes 2, 3, 5, and 6). Cells were then washed, incubated in fresh medium without ligand for 1 h (lanes 3 and 6), or kept on ice (lanes 2 and 5). B, the equal amount of receptor loading was confirmed by Western blot using a monoclonal anti-CXCR2 antibody. C, the expression of dynamin I K44A on the transfected cell lysates was detected by Western blot using anti-dynamin antibody. D, quantification of the mean ± S.E. of two different experiments performed in duplicate. The data were normalized to MGSA-stimulated CXCR2 phosphorylation. Dotted bars, phosphorylated; striped bars, dephosphorylated.

**Fig. 4.** The effect of dynamin I K44A mutant on MGSA-stimulated CXCR2 degradation and down-regulation. A, HEK293 cells were transiently transfected with plasmids expressing CXCR2 with (lanes 5–8) or without (lanes 1–4) rat dynamin I K44A. The cells were harvested and plated equally onto 12-well plates. The cells were then treated with 100 ng/ml MGSA/GRO for 0 min (lanes 1 and 5), 0.5 h (lanes 2 and 6), 2 h (lanes 3 and 7), and 8 h (lanes 4 and 8). 30 μg of protein from clarified RIPA lysates were loaded per lane, and Western blot analysis was performed with rabbit anti-CXCR2 polyclonal antibody and visualized by horseradish peroxidase-conjugated secondary antibody. The arrow indicates CXCR2 epitope. B, the density of the bands (mean ± S.E.) representing CXCR2 was determined by densitometric scanning. The results represent one of two experiments performed in duplicate. Dotted bars, CXCR2; striped bars, CXCR2 plus dynamin I K44A.

**Effect of Dynamin K44A on CXCR2-mediated Chemotaxis**—Ligand-stimulated CXCR2-mediated chemotaxis is a direct and effective functional test to access chemokine receptor signal transduction. We used chemotaxis assays to determine if blocking of receptor endocytosis by co-expression of dynamin I K44A could abolish the CXCR2-mediated chemotaxis toward a gradient of IL-8 (Fig. 6) or MGSA/GRO (data not shown). We observed an IL-8 concentration-dependent chemotactic response in cells expressing wild-type receptor, with a peak migration occurring at a concentration around 25–50 ng/ml IL-8. Also, the cellular migration response followed a typical bell-shaped curve in which the chemotaxis was inhibited at higher concentrations of IL-8 (Fig. 6). Strikingly, overexpression of dynamin I K44A resulted in marked attenuation of CXCR2-mediated chemotaxis. These data suggest that agonist-mediated endocytosis and resensitization are crucial for CXCR2-mediated chemotaxis.

**DISCUSSION**

CXCR2 is internalized into vesicular compartments following agonist stimulation and phosphorylation at multiple serine residues in the carboxyl-terminal domain in a manner similar to that observed for several other GPCRs. However, the mechanisms responsible for receptor endocytosis and the functional significance of this event regarding receptor signal transduction are largely unknown. Our studies provide direct biochemical evidence for the requirement of a dynamin-mediated formation of clathrin-coated pits in CXCR2 sequestration, resensitization, and down-regulation. More importantly, our data suggest that endocytosis of receptor is prerequisite to its ability to continuously sense the agonist stimulation that is unique for chemokine receptor-mediated chemotaxis.

Previous studies have identified dynamin as a major component of clathrin-mediated endocytosis (22). Dynamin associates with the adapter protein AP2 complex (23). Once localized to the clathrin-coated pits, dynamin promotes endocytosis by catalyzing the pinching off process of coated vesicles (24, 25). Dynamin GTPase activity is essential for the formation of clathrin-coated pits. This is confirmed by the observation that expression of a GTPase-defective dynamin mutant prevents formation of the endocytic clathrin-coated vesicles (25, 26).

Using a dominant-negative mutant of dynamin I (K44A), we have demonstrated inhibition of ligand-induced CXCR2 endocytosis. This observation suggests that CXCR2 internalization is mediated by a highly conserved mechanism that is dynamin and clathrin-dependent. Our immunofluorescence experiments revealed that CXCR2 internalization in HEK293 cells reached to endosomal compartments following MGSA/GRO treatment. Colocalization of CXCR2 with the transferrin receptor in transfected HEK293 cells is consistent with the concept that both receptors utilize the same clathrin-dependent pathway.
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and 5 (were transiently transfected with plasmids expressing CXCR2 with treated with buffer (lanes 1–3) or without (lanes 4–6) dynamin I K44A. The cells were harvested and plated equally onto 60-mm plates. The cells were then evaluated CXCR2-mediated chemotaxis of HEK293 cells.

A

B

C

Phospho-MAPK
p42MAPK

ERK2

Relative Density

control MGS/GRO IL-8

CXCR2 CXCR2+dynamin 1 K44A

chemotaxis index

0 1 2 3 4 5 6

IL-8 Concentration (ng/ml)

Fig. 5. The effect of dynamin I K44A mutant on MGS/GRO or IL-8-stimulated ERK activation in HEK293 cells. A, HEK293 cells were transiently transfected with plasmids expressing CXCR2 with (lanes 1–3) or without (lanes 4–6) dynamin I K44A. The cells were harvested and plated equally onto 60-mm plates. The cells were then treated with buffer (lanes 1 and 4) or 100 ng/ml MGS/GRO (lanes 2 and 5) or IL-8 (lanes 3 and 6) for 5 min. 30 μg of protein from clarified RIPA lysates were loaded per lane, and Western blot analysis was performed with a phosphospecific MAP kinase antibody and visualized by an horseradish peroxidase-conjugated secondary antibody. B, the blot from A was stripped and reprobed with an antibody against ERK-2 to ensure the equal loading. C, the density of the bands (mean ± S.E.) representing phosphorylated ERK1 and ERK2 was determined by densitometric scanning. The results were obtained from three independent experiments. Dotted bars, CXCR2; black bars, CXCR2 plus dynamin I K44A.

Fig. 6. The effect of the dynamin I K44A mutant on IL-8-stimulated CXCR2-mediated chemotaxis of HEK293 cells. HEK293 cells were transiently transfected with plasmids expressing CXCR2 with (striped bars) or without (dotted bars) rat dynamin I K44A. Two days after transfection, cells were compared for chemotaxis in response to IL-8 stimulation as described under “Experimental Procedures.” Values represent the means ± S.E. of three independent experiments performed on different days. The data were analyzed using Student’s paired t test (*, p < 0.05).

The chemotaxis assay is believed to be the most useful assay to evaluate the signal transduction capability of chemokine receptors, since it measures the ultimate result of a cascade of intracellular events that are activated by ligand-receptor interaction. More importantly, this assay also provides a functional read out for a process composed of sequential desensitization and resensitization events with respect to receptor activation. Our results clearly demonstrate that overexpression of dynamin I K44A abolished the CXCR2-mediated HEK293 cell chemotaxis toward both IL-8 and MGS/GRO. The equal or similar cell surface expression of receptors has been confirmed in each experiment by immunofluorescence assay to exclude the possibility that the reduced chemotaxis in cells expressing dynamin I K44A is due to reduced receptor expression or cell surface targeting. The specific signal pathway responsible for chemotaxis is poorly understood. In human neutrophils, phosphatidylinositol 3-kinase activity is required for both induction of chemotaxis and MAP kinase activation (32). When phosphatidylinositol 3-kinase is blocked with wortmannin, IL-8-induced MAP kinase is also blocked (21). The fact that MAP kinase activation in cells expressing dynamin I K44A is normal absence of agonist, a small but detectable portion of CXCR2 seems to be localized in the endosomes (Fig. 1A). This may be due to constitutive receptor internalization and recycling. This idea is supported by the finding that overexpression of dynamin I K44A reduces basal intracellular localization of CXCR2 (data not shown).

After internalization to the endosome, the receptor will be dephosphorylated and either recycled back to cell surface or degraded by proteolytic enzymes (11, 27). Using the dynamin I K44A mutant, we have shown that clathrin-mediated receptor internalization is a critical initial step for CXCR2 resensitization and down-regulation. Co-expression of dynamin I K44A resulted in significant diminution of both CXCR2 dephosphorylation and down-regulation. Although the exact mechanism responsible for dephosphorylation of GPCRs remains unclear, it is postulated that the conformational change of the receptor in the acidic environment facilitates dephosphorylation by a G protein-coupled receptor phosphatase (28). Recycling and resensitization of dephosphorylated receptor is believed to allow the chemokine receptors to sense and respond to a continuous gradient of chemokine stimulation.

The functional role of agonist-promoted CXCR2 endocytosis has long been elusive in the field of CXCR2 signal transduction. The result of MGS/GRO and IL-8-mediated MAP kinase experiments suggests that CXCR2 endocytosis is not required for its immediate downstream signaling. These data are supported by the previous observation that a mutant CXCR2 deleting 31 carboxyl-terminal amino acids exhibited significantly impaired agonist-induced receptor endocytosis but not inhibition of adenyl cyclase or MAP kinase activation (29). Further evidence suggesting the separation between CXCR2 endocytosis and immediate downstream signal transduction events came from the studies of another CXCR2 deletion mutant (30). Richardson et al. (30) demonstrated that a CXCR2 mutant (331T), exhibiting a deletion of the last 25 amino acid residues of the carboxyl-terminal domain, lost agonist-stimulated phosphorylation and sequestration when expressed in the placental cell RBL-2H3 cells. However, the immediate downstream signal transduction events such as GTPase activity, phosphatidylinositol hydrolysis, β-hexosaminidase release, and Ca²⁺ mobilization were not impaired. Studies of the β₂-AR indicate that the capacity of the receptor to signal through adenyl cyclase remains intact following total inhibition of receptor endocytosis by dynamin K44A (17). However, others demonstrate that in HEK293 cells, the β₂-AR-mediated activation of MAP kinase is inhibited by blocking receptor internalization although tyrosine phosphorylation-mediated activation of Shc and Raf kinase are unaffected (31). The reasons for the discrepancy in ligand-mediated receptor endocytosis and downstream MAP kinase activation between CXCR2 and β₂-AR are not clear.
makes it unlikely that phosphatidylinositol 3-kinase activity is affected in these cells. Considering the fact that endocytosis permits CXCR2 dephosphorylation and resensitization, we therefore postulate that endocytosis, dephosphorylation, resensitization, and recycling of CXCR2 is required for graded response to ligand stimulation.

Blocking receptor internalization does not have major effects on the generation of immediate downstream signals but does alter the ability of cells to respond to a continuous signal generated over a concentration gradient, based upon the loss of receptor dephosphorylation and the subsequent receptor recycling. Another possible explanation for the blocking effects of dynamin I K44A on cell chemotaxis may be due to the inhibition of the redistribution of cell surface chemokine receptor and receptors for matrix attachment. One model of cell migration suggests that there is an increased exocytosis at the leading edge of moving cells (33, 34). This polarized exocytosis may be involved in delivery of fresh integrins and chemokine receptors to the cell front. This process enables the migrating cells to attach to the extracellular matrix and to sense the chemokine stimulation.

Following ligand-stimulated receptor phosphorylation, GPCRs bind to adaptor molecules that directly inhibit the interaction between receptor and G-protein (12, 13). In the case of the β2-AR, the binding of β-arrestin and arrestin-3 is also an initial event required for receptor internalization (14, 15). Both β-arrestin and arrestin-3 can function as adaptors. The association of these molecules with phosphorylated receptor and clathrin is a key event in the formation of clathrin-coated pits. Recently, Aragay et al. (35) reported that β-arrestin associates with CCR2 shortly after agonist stimulation, suggesting that arrestins might play a universal role in mediating GPCR desensitization and internalization. Studies are currently in progress to test the role of arrestins and other adaptor molecules in CXCR2 desensitization and internalization.

In summary, we have established that agonist-promoted CXCR2 proceeds through the formation of clathrin-coated pits to endosomes. Furthermore, we demonstrated that the CXCR2 internalization is required for receptor dephosphorylation, resensitization, and down-regulation but not for immediate downstream signaling. More importantly, our chemotaxis experiments indicate that agonist-stimulated receptor endocytosis and dephosphorylation is essential for normal chemokine receptor-mediated chemotaxis in HEK293 cells.

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