Adaptor protein interaction with specific peptide motifs found within the intracellular, carboxy terminus of chemokine receptor CXCR2 has been shown to modulate intracellular trafficking and receptor function. Efficient ligand-induced internalization of this receptor is dependent on the binding of adaptor protein 2 to the specific LLKIL motif found within the carboxy terminus (1). In this study we show that the carboxyl-terminal type 1 PDZ ligand motif (-STTL) of CXCR2 plays an essential role in both proper intracellular receptor trafficking and efficient cellular chemotaxis. First, we show that CXCR2 is sorted to and degraded in the lysosome upon long-term ligand stimulation. We also show that receptor degradation is not dependent upon receptor ubiquitination, but is instead modulated by the carboxyl-terminal type 1 PDZ ligand of CXCR2. Deletion of this ligand results in increased degradation, earlier co-localization with the lysosome, and enhanced sorting to the Rab7-positive late endosome. We also show that deletion of this ligand effects neither receptor internalization nor receptor recycling. Furthermore, we demonstrate that deletion of the PDZ ligand motif results in impaired chemotactic response. The data presented here demonstrate that the type I PDZ ligand of CXCR2 acts to both delay lysosomal sorting and facilitate proper chemotactic response.

The chemokine receptor CXCR2 is a seven transmembrane G protein-coupled receptor (GPCR) that plays an important role in many diverse physiological processes such as leukocyte chemotaxis, wound healing, angiogenesis, and inflammation (see Ref. 2 for review). Conversely, CXCR2 disregulation has been linked to several pathologies, including chronic inflammatory responses, metastatic progression, and aberrant angiogenesis during tumor progression (see Ref. 3 for review). However, the molecular mechanisms regulating the activation, signaling, desensitization, and resensitization of this receptor remain poorly characterized.

Upon ligand engagement of CXCR2, two interrelated processes occur: a conformational change that initiates several signal transduction cascades linked to cell motility and proliferation, and a phosphorylation event that facilitates the binding of adaptor proteins essential for receptor endocytosis and subsequent intracellular trafficking (see Ref. 4 for review). Like many GPCRs, CXCR2 undergoes clathrin-mediated endocytosis subsequent to agonist-induced activation by its cognate ligands that include CXCL1–3 and 5–8 (5–7). This endocytic event initiates intracellular trafficking of the receptor that results in either a re-localization of the receptor on the plasma membrane, or a down-regulation and degradation of internalized receptor (8). Intracellular trafficking plays an essential role in the regulation of signaling and reactivation, and thereby represents a mechanism that regulates proper cellular response. The interruption of this process can result in the disregulation of both signaling and functional outcomes.

Subsequent to interaction with endogenous concentrations of ligand, most of the internalized CXCR2 receptors enter the Rab11a-positive recycling endosome and are then shuttled back to the plasma membrane where they become functional for a new round of signaling (4). The small GTPase Rab11a is essential for CXCR2 recycling (8), but little else is known regarding the molecular mechanisms governing this process. Initial evidence suggested that the recycling of some membrane receptors, including the transferrin receptor, occurred by bulk flow (9). However, this “default” pathway does not appear to be the case for other receptors. The β2-adrenergic receptor was recently shown to possess a “recycling sequence” in its carboxy-terminal portion that binds to a specific protein and directs the receptor to the recycling pathway (10). Interestingly, this sequence is a type I PDZ ligand that interacts with the Na+/H+ exchanger regulatory factor/ezrin/radixin/moesin-binding phosphoprotein of 50 kDa (NHERF/EBP50), a PDZ-domain containing protein (10). Subsequently, several studies have reported the carboxy-terminal domains of GPCRs to contain “sorting sequences” that presumably interact with specific adaptor proteins and direct the receptor to its fate (11).

Upon ligand stimulation, most internalized GPCRs are either fated for recycling or for degradation (12). CXCR2 undergoes a “sorting decision” in which receptor fate is decided subsequent...
to internalization. Exposure to either high concentrations of ligand or long-term stimulation appears to shuttle CXCR2 to the lysosome and away from the recycling pathway (8). The lysosomal shuttling of GPCRs is thought to be sequence directed, requiring a monoubiquitination event followed by the binding and release of the receptor by several multiprotein complexes (13–20). These protein complexes include hepatocyte receptor substrate (Hrs), Tgs101, and vacuolar protein sortet protein 4 (Vps4). However, the molecular mechanisms that direct CXCR2 to the lysosome are largely unknown.

To identify the molecular mechanisms responsible for lysosomal shuttling of CXCR2, we first analyzed the degradation of CXCR2 mutants in which the lysine residues of the intracellular, carboxyl-terminal domain had been mutated to arginine. In addition, we examined the effect of carboxyl-terminal truncation mutants of CXCR2 on receptor degradation. Our data show that ubiquitination of CXCR2 is not essential to receptor degradation. Instead, we show that the carboxyl-terminal PDZ ligand binding motif of CXCR2 is an important regulator of this process. Our demonstration that this ligand binding motif regulates CXCR2 degradation by modulating the post-endocytic sorting of this receptor. Truncation of the PDZ ligand binding motif of CXCR2 increases ligand-mediated receptor degradation and facilitates early co-localization with the lysosome, without interrupting receptor internalization or recycling. We also show that truncation of the C-terminal PDZ ligand results in interrupted chemotaxis. Taken together, our findings define a new role for the GPCR carboxyl-terminal PDZ ligand binding motif and highlight the importance of the relationship between proper receptor trafficking and functional outcomes.

MATERIALS AND METHODS

**Eukaryotic Expression Constructs**—The pRc/CMV CXCR2, pRc/CMV 352T, and pRc/CMV 331T vectors have been previously described (21). The EGFP-Rab7 vector was a kind gift from Dr. James R. Goldenring (Department of Surgery, Vanderbilt University Medical Center).

**Cell Culture and Transfection**—HEK293 cells (American Type Culture Collection, Manassas, VA) stably expressing CXCR2, CXCR2 331T, or CXCR2 352T were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, glutamine (0.3 mg/ml), and penicillin/streptomycin (100 units/ml) at 37 °C in 5% CO2. Transient transfections were performed with FuGENE 6 (Roche Diagnostics) according to the manufacturer’s instructions, and cells were assayed 36–48 h post transfection. HEK293 cells stably expressing CXCR2 constructs were generated by FuGENE transfection of pRc/CMV CXCR2 vectors followed by 2 weeks of stable selection in 1 mg/ml G418. Stable cells were then sorted according to receptor surface expression by fluorescence-activated cell sorter analysis using a CXCR2 antibody conjugated directly to phycoerythrin (R&D Technologies, North Kingstown, RI).

**Western Blotting**—Immunoblotting to detect total cellular levels of proteins was performed as previously described (22). Briefly, cells were pre-treated for 30 min with 20 μg/ml cycloheximide (Sigma) to inhibit new protein synthesis before stimulation for various times with CXCL8 (Peprotech, Rocky Hill, NJ). Cells were then washed in ice-cold 1× phosphate-buffered saline and lysed in extraction buffer (0.05% Triton X-100, 150 mM NaCl, 1× each protease inhibitor mixture (Sigma) and phosphatase inhibitor mixture (Sigma), in 1× TBS). Extracts were then clarified by centrifugation and mixed with Laemmli sample buffer for denaturation. The proteins were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, blocked, and probed for either CXCR2 (Santa Cruz Biotechnology, Santa Cruz, CA) or actin (Santa Cruz Biotechnology) by immunoblotting using infrared-conjugated secondary antibodies goat antimouse (γg) IgG 680 or goat anti-rabbit (γr) IgG 700 (Rockland Immunochemicals, Gilbertsville, PA) and the Odyssey Detection System (LI-COR Biosciences, Lincoln, NE). Blots were processed and quantitated with Licor Odyssey Software (LI-COR Biosciences, Lincoln, NE).

**Lysosomal/Proteasomal Inhibitor Studies**—Cells were pre-treated for 30 min with either 100 μg/ml leupeptin, 50 nM NH4Cl, or 200 μM chloroquine (Sigma) before stimulation with CXCL8. Cells were lysed and analyzed for CXCR2 expression by Western blotting as described above.

**Immunofluorescence Staining and Confocal Imaging**—Visualization of CXCR2 receptors, LAMP3, and Rab11a was performed using indirect immunofluorescence staining. Briefly, cells were plated on poly-L-lysine-coated coverslips and starved prior to stimulation with CXCL8 for the indicated times. Cells were then fixed, permeabilized, blocked, and incubated with mouse anti-CXCR2 antibody (Santa Cruz Biotechnology) or rabbit anti-CXCR2 antibody (generated in our laboratory and previously characterized in Ref. 23), mouse anti-LAMP3 antibody (Santa Cruz Biotechnology), or rabbit anti-Rab11a (kind gift of Dr. James R. Goldenring, previously characterized in Ref. 24). Cells were then washed and incubated with goat anti-mouse Cy3 or goat anti-rabbit Cy5 (Jackson ImmunoResearch, West Grove, PA), washed again, and mounted with Anti-fade Gold (Invitrogen). Lysotracker Red was used to visualize the lysosomes according to the manufacturer’s instructions (Invitrogen/Molecular Probes). Briefly, cells were treated with 75 nM Lysotracker Red 2 h prior to fixation. Specimens were examined using a Zeiss LSM510 Meta Confocal Laser microscope with an oil immersion ×63/NA1.3 objective, using instrument settings verified to produce negligible bleedthrough between channels and an estimated section thickness of 1 μm. Micrographs shown are representative optical sections imaged through the center of the cell. Images were obtained and processed with LSM Imaging Software. Co-localization of CXCR2 with endosomal markers was quantified using the Metamorph Imaging System software package (Molecular Devices Corp., Sunnyvale, CA). Threshold levels for all images were kept consistent among vector and mutant expressing cells. At least 20 fields were quantified for each time point. The percent co-localization is indicative of the area of CXCR2-stained fluorescent pixels overlapping that of endocytic markers.

**Site-directed Mutagenesis**—All lysine residues in the intracellular, carboxyl-terminal domain of wild-type cxcr2 were
sequentially mutated to arginine residues by site-directed mutagenesis (QuikChange, Stratagene) according to the manufacturer’s instructions. Briefly, primers containing point mutations were designed to the COOH terminus of CXCR2 in pRC/CMV. Subsequent to PCR amplification with a high fidelity Taq polymerase, methylated parental DNA was digested with restriction enzyme DpnI. DNA was then transformed into XL1-Blue *Escherichia coli*, plated on LB-agar plates containing ampicillin, and prepared for transfection.

**Radioligand Binding Receptor Internalization Studies**—Radioligand binding internalization assays were performed as previously described (1). Briefly, cells were serum-starved overnight and then preincubated with binding buffer containing $^{125}$I-CXCL8 for 1 h at 4 °C. Unbound $^{125}$I-CXCL8 was removed and the cells were warmed to 37 °C for the indicated times. Internalized $^{125}$I-CXCL8 was quantified with a γ-counter (Beckman Coulter). Background binding was determined by acidic washing without warming to 37 °C.

**Receptor Recycling Studies and Plasma Membrane Receptor Expression**—Receptor recycling studies were performed as previously described (8), with minor modification. Briefly, cells were serum-starved for 2 h in serum-free media containing 20 μg/ml cycloheximide. Cells were then stimulated with 100 ng/ml CXCL8 for 30 min. Subsequent to stimulation, ligand was removed, and cells were allowed to recover in complete media containing 20 μg/ml cycloheximide for 0, 30, 60, or 90 min. Both unstimulated and stimulated cells were then incubated with anti-CXCR2 antibody directly conjugated to phycoerythrin for 1 h at 4 °C and surface receptor expression was analyzed by flow cytometry (Beckman Coulter). Plasma membrane receptor expression was performed similarly. Briefly, cells were serum-starved for 2 h in serum-free media. Cells were then incubated with anti-CXCR2 antibody directly conjugated to phycoerythrin for 1 h at 4 °C and surface receptor expression was analyzed by flow cytometry (Beckman Coulter).

**Chemotaxis Assays**—Chemotaxis assays were previously described in Ref. 25. Briefly, the Boyden chamber membranes (10 μm pore size, Neuroprobe, Gaithersburg, MD) were coated with 10 μg/ml collagen IV (Sigma). The Boyden chamber was then prepared with increasing concentrations of CXCL8 in the lower chambers. Cells stably expressing CXCR2 were trypsinized, recovered in complete media for 2 h, resuspended in binding buffer (0.5% bovine serum albumin in Dulbecco’s modified Eagle’s medium), and applied to the top of the Boyden chamber. Cells were allowed to migrate for 4 h, then fixed and stained with crystal violet. Those cells migrating to the underside of the membrane were counted under a ×20 objective in an upright light microscope (Nikon), and 20 random fields were counted per treatment.

**Statistical Analysis**—Statistical significance was determined by a non-parametric analysis of variance followed by a post hoc Dunn’s test, a non-parametric Mann-Whitney test, or a Student’s *t* test, as indicated.

**RESULTS**

**Chemokine Receptor CXCR2 Is Degraded in the Lysosome**—Previous studies have shown that upon ligand stimulation, CXCR2 is endocytosed via clathrin-mediated endocytosis and intracellularly trafficked through endosomal compartments (8, 26). This intracellular trafficking can result in either entry into the recycling endosome and subsequent re-appearance on the plasma membrane where it can continue to receive and transmit signals, or it can result in shuttling to the lysosome where the receptor is degraded and permanently silenced. Previously, we observed that upon ligand stimulation CXCR2 co-localized with recycling endosome marker Rab11a at 30 min post-stimulation, and co-localized with late endosomal marker Rab7-GFP and the lysosomal marker LAMP-1 at 4 h post-stimulation (8). These results suggested that upon prolonged ligand stimulation with a high concentration of ligand, CXCR2 is shuttled to the lysosome via the endosomal pathway where it is subsequently degraded. To confirm these findings, we first used a series of lysosomal inhibitors to block total CXCR2 degradation. HEK293 cells stably expressing wild-type CXCR2 receptor, treated first with vehicle alone and then stimulated with CXCL8, exhibited a 35–45% degradation of receptor (Fig. 1, A and B). Upon stimulation, CXCR2 undergoes a mass shift due to differential phosphorylation of the serine residues found within the carboxyl terminus of the receptor (21). Those cells treated first with specific lysosomal inhibitor and then stimulated with CXCL8 exhibited 0–10% degradation of receptor, significantly less degradation than was observed with vehicle alone (Fig. 1, A and B). Consistent with these results, confocal analysis of CXCL8-stimulated cells revealed co-localization between CXCR2 and the lysosomal marker lysosomal-associated membrane protein 3 (LAMP-3) (Fig. 1C).

**Neither the Lysosomal Shuttling nor Degradation of CXCR2 Is Dependent Upon Ubiquitination**—Previous studies have shown that many transmembrane receptors, including both single transmembrane growth factor receptors as well as heptathelial G protein-coupled receptors, must be monoubiquitinated on one or more lysine residues located within the intracellular, carboxyl terminus to be shuttled to the lysosome (27). This monoubiquitination event results in molecular recognition by the lysosomal shuttling machinery, and targets specific receptors for degradation (28). To determine whether ubiquitination was indeed a necessary signal for the lysosomal shuttling of CXCR2, we first identified the lysine residues found within the intracellular, carboxyl terminus (Fig. 2A). We then progressively mutated all 4 lysine residues found within the carboxyl terminus to arginine residues with successive rounds of site-directed mutagenesis (Fig. 2A). Subsequent to expression of mutant CXCR2 receptors in HEK293 cells, we found that those receptors mutant for either 3 or 4 lysine residues were degraded upon prolonged ligand stimulation (Fig. 2, B and C). Furthermore, it was noted that the degradation of either CXCR2 3KR or CXCR2 4KR did not differ significantly from that of wild-type CXCR2 (Fig. 2C). We also confirm that the mutant receptors CXCR2 3KR and CXCR2 4KR were both expressed on the surface of HEK293 cells subsequent to transfection, and internalized upon stimulation with CXCL8 (Fig. 2D). In addition, we expressed hemagglutinin-ubiquitin in HEK293 cells stably expressing wild-type CXCR2 and were unable to detect any hemagglutinin-ubiquitin in CXCR2 immunoprecipitates (data not shown). Taken together, these data demonstrate that ubiquitination regulates the degradation of CXCR2 through the lysosomal pathway.
PDZ Ligand of CXCR2 Regulates Trafficking and Chemotaxis

The only PDZ domain-containing protein shown to bind to the carboxyl terminus of CXCR2 is RGS12 (35). However, the functional consequences of this putative PDZ interaction with CXCR2 are unknown. The portion of CXCR2 that is located distal to amino acid residue 331 contains several serine residues that are phosphorylated upon ligand stimulation (1). Phosphorylated CXCR2 is desensitized to subsequent ligand challenge, and phosphorylated receptor stably associates with adapter protein β-arrestin, resulting in enhanced endocytosis (1).

We demonstrated here that upon ligand stimulation, both the CXCR2 331T and CXCR2 352T truncated receptors exhibited significantly enhanced degradation compared with that of the wild-type control (Fig. 3, B and C). We also found that degradation of the PDZ ligand deletion mutant increased over that of CXCR2 331T (Fig. 3C). Furthermore, we found that upon CXCL8 stimulation, the absence of the COOH-terminal PDZ ligand motif resulted in earlier co-localization with the lysosome (Fig. 3D). Using Lysotracker red as a lysosomal marker, we showed that at 6 h post-CXCL8 stimulation, both wild-type receptor and the CXCR2 352T mutant co-localized with the lysosome (Fig. 3D, lower panels). At 2 h post-CXCL8 stimulation, CXCR2 352T co-localized extensively with the lysosome while the wild-type receptor did not (Fig. 3D, middle panels). Not surprisingly, there was little co-localization of either receptor with the lysosome prior to ligand stimulation (Fig. 3D, upper panels). Taken together, these data demonstrate that the presence of the COOH-terminal PDZ ligand motif slows both receptor degradation as well as co-localization with the lysosome.

COOH-terminal PDZ Ligand Deletion Has No Effect on Either Receptor Internalization or Receptor Recycling—PDZ domain containing proteins have been shown to act as scaffolds that anchor receptors to the plasma membrane and affect receptor expression (36–39). Studies have demonstrated that knockdown of specific PDZ domain containing proteins results in increased internalization of receptors subsequent to ligand stimulation (40, 41). Because an increased rate of internalization of the CXCR2 352T mutant could explain the increased degradation and earlier lysosome co-localization as compared

FIGURE 1. CXCR2 is degraded in the lysosome. A, lysosomal inhibitors attenuate CXCL8-mediated CXCR2 degradation. HEK293 CXCR2 WT cells were pretreated with cycloheximide, then treated with vehicle alone, chloroquine (CHLQ), ammonium chloride (NH₄Cl), or leupeptin 30 min prior to stimulation with 50 nm CXCL8 for 4 h. Immunoblots were simultaneously probed for both CXCR2 and actin using the Licor Odyssey Infrared Imaging System, and is representative of four independent experiments. B, graphical representation of CXCR2 degradation. Immunoblots in A were subjected to densitometric analysis (Licor Odyssey 2.0 software) to quantify receptor levels. The bar graphs represent the average percentages of CXCL8-mediated receptor degradation in cells treated with lysosomal inhibitors as compared with vehicle-treated cells after normalization with actin levels. The error bars represent the mean ± S.E. from four independent experiments. The data were subjected to statistical analysis by a non-parametric analysis of variance followed by a post-hoc Dunn’s test (* indicates p < 0.05). C, CXCR2 co-localizes with the lysosomal marker LAMP-3 subsequent to stimulation with CXCL8. HEK293 CXCR2 WT cells were stimulated with either vehicle alone or 50 nm CXCL8 for 4 h. LAMP-3 is shown white (left panels) and green (right panels). CXCR2 is shown white (middle panels) and red (right panels). Puncta that appear yellow in the merged images (right panels) indicate co-localization of CXCR2 with the late endosomal/lysosomal marker. The insets represent enlarged views of the boxed regions. The scale bars represent 10 μm. Confocal images were taken on an LSM META 150 inverted confocal fluorescence scope and processed with LSM imaging software. Similar results were observed from three independent experiments with 30–40 cells analyzed per experiment.

Carboxyl-terminal Truncation of CXCR2 Results in Increased Receptor Degradation and Earlier Co-localization with the Lysosome—The intracellular, carboxyl-terminal domains of GPCRs have been shown to bind to a myriad of adapter proteins, and regulate such diverse processes as signaling and receptor trafficking (29–32). Adaptor protein binding of the carboxyl-terminal domain of CXCR2 in particular has been shown to regulate ligand-mediated endocytosis, receptor recycling, and cell-surface expression (1, 33, 34). Therefore, we decided to use progressive carboxyl-terminal truncation mutants to decipher the contribution of specific protein motifs to receptor degradation (Fig. 3A). The four amino acid residues distal to residue 352 (-STTL) encode a putative PDZ ligand (20).

utilization is not necessary for the lysosomal shuttling of CXCR2.

Phosphorylation of the 331T and 352T mutants of CXCR2 arrests receptor internalization (1). CXCR2 WT cells pretreated with PKA or PKC inhibitors were stimulated with CXCL8, then subjected to immunoblots (A). Immunoblots in A were simultaneously probed for both CXCR2 and actin using the Licor Odyssey Infrared Imaging System, and is representative of four independent experiments. B, graphical representation of CXCR2 degradation. Immunoblots in A were subjected to densitometric analysis (Licor Odyssey 2.0 software) to quantify receptor levels. The bar graphs represent the average percentages of CXCL8-mediated receptor degradation in cells treated with lysosomal inhibitors as compared with vehicle-treated cells after normalization with actin levels. The error bars represent the mean ± S.E. from four independent experiments. The data were subjected to statistical analysis by a non-parametric analysis of variance followed by a post-hoc Dunn’s test (* indicates p < 0.05). C, CXCR2 co-localizes with the lysosomal marker LAMP-3 subsequent to stimulation with CXCL8. HEK293 CXCR2 WT cells were stimulated with either vehicle alone or 50 nm CXCL8 for 4 h. LAMP-3 is shown white (left panels) and green (right panels). CXCR2 is shown white (middle panels) and red (right panels). Puncta that appear yellow in the merged images (right panels) indicate co-localization of CXCR2 with the late endosomal/lysosomal marker. The insets represent enlarged views of the boxed regions. The scale bars represent 10 μm. Confocal images were taken on an LSM META 150 inverted confocal fluorescence scope and processed with LSM imaging software. Similar results were observed from three independent experiments with 30–40 cells analyzed per experiment.

utilization is not necessary for the lysosomal shuttling of CXCR2.

Phosphorylation of the 331T and 352T mutants of CXCR2 arrests receptor internalization (1). CXCR2 WT cells pretreated with PKA or PKC inhibitors were stimulated with CXCL8, then subjected to immunoblots (A). Immunoblots in A were simultaneously probed for both CXCR2 and actin using the Licor Odyssey Infrared Imaging System, and is representative of four independent experiments. B, graphical representation of CXCR2 degradation. Immunoblots in A were subjected to densitometric analysis (Licor Odyssey 2.0 software) to quantify receptor levels. The bar graphs represent the average percentages of CXCL8-mediated receptor degradation in cells treated with lysosomal inhibitors as compared with vehicle-treated cells after normalization with actin levels. The error bars represent the mean ± S.E. from four independent experiments. The data were subjected to statistical analysis by a non-parametric analysis of variance followed by a post-hoc Dunn’s test (* indicates p < 0.05). C, CXCR2 co-localizes with the lysosomal marker LAMP-3 subsequent to stimulation with CXCL8. HEK293 CXCR2 WT cells were stimulated with either vehicle alone or 50 nm CXCL8 for 4 h. LAMP-3 is shown white (left panels) and green (right panels). CXCR2 is shown white (middle panels) and red (right panels). Puncta that appear yellow in the merged images (right panels) indicate co-localization of CXCR2 with the late endosomal/lysosomal marker. The insets represent enlarged views of the boxed regions. The scale bars represent 10 μm. Confocal images were taken on an LSM META 150 inverted confocal fluorescence scope and processed with LSM imaging software. Similar results were observed from three independent experiments with 30–40 cells analyzed per experiment.
PDZ Ligand of CXCR2 Regulates Trafficking and Chemotaxis

Neither the lysosomal shuttling nor degradation of CXCR2 is dependent upon ubiquitination. A, amino acid sequence of the carboxyl terminus of CXCR2. Shown is the amino acid sequence (single-letter code) of the carboxyl terminus of wild-type CXCR2 and the various lysine mutants used in this study. Amino acid sequence represents the entire cytoplasmic sequence of the receptor, distal to the seventh transmembrane domain. B, CXCR2 lysine mutants undergo CXCL8-mediated degradation. HEK293 cells were transfected with CXCR2 WT, CXCR2 3KR, or CXCR2 4KR. Cells were stimulated with 10 nM CXCL8 for the indicated times. Immunoblots are representative of three independent experiments. The molecular weights are indicated.

PDZ Ligand Deletion Facilitates Early Entry into the Rab7-positive Late Endosome—Because deletion of the C-terminal PDZ ligand increased receptor degradation and caused earlier co-localization with the lysosome over that of wild-type CXCR2 without affecting either receptor internalization or recycling, we hypothesized that the presence of the PDZ ligand would increase the probability of receptor recycling and decrease the probability of localization within the late endosome. To test this hypothesis, we stimulated HEK293 cells stably expressing either wild-type CXCR2 or CXCR2 352T for 30 min or 1 h with CXCL8. We then examined the localization of CXCR2 WT and CXCR2 352T within the late endosome using an expressed GFP-tagged Rab7. Before ligand stimulation, we found little co-localization of either CXCR2 WT or CXCR2 352T with the Rab7-positive endosome (Fig. 5, A and C). Subsequent to the 1-h CXCL8 stimulation, we found an apparent increased co-localization of CXCR2 352T with Rab7 as compared with CXCR2 WT (Fig. 5A). Upon quantitation, CXCR2 352T co-localized at significantly higher levels with GFP-Rab7 at both 30 min and 1 h post-ligand stimulation than did CXCR2 WT (Fig. 5C). We also found that both CXCR2 WT and CXCR2 352T co-localized with Rab11a subsequent to 30 min and 1 h of ligand stimulation (Fig. 5, A and B). Upon quantitation, CXCR2 WT was found to co-localize significantly more extensively with Rab11a at 30 min and 1 h post-ligand stimulation than did CXCR2 352T (Fig. 5B). These data demonstrate that even...
PDZ Ligand of CXCR2 Regulates Trafficking and Chemotaxis

though a percentage of the mutant 352T receptor will enter the recycling endosome from the early endosome and be re-expressed on the surface, there is a higher probability that it will be sorted to the late endosome from the early endosome, and eventually make its way to the lysosome for degradation.

COOH-terminal PDZ Deletion Enhances Receptor Degradation and Interrupts Chemotaxis—Because COOH-terminal PDZ deleted CXCR2 is degraded faster that wild-type receptor, together, these results demonstrate that the COOH-terminal PDZ ligand is necessary for efficient migration and chemotaxis in response to chemokine CXCL8.

DISCUSSION

The molecular basis of the intracellular sorting decision of GPCRs that takes place subsequent to endocytosis is largely variable. Work with the \( \beta_2 \)-adrenergic, V2 vasopressin, and...
CXCR4 chemokine receptors has revealed a complex and elegant sorting mechanism that involves post-translational modification of the receptor at the plasma membrane subsequent to ligand engagement (19, 45, 46). Other work involving the protease-activated receptor 1, the β2-adrenergic receptor, as well as CXCR2, has examined short peptide sorting sequences found within the carboxy-terminal domain that dictate sorting decisions (47–49). These amino acid sequences essentially act as binding motifs that allow the association of specific adaptor proteins subsequent to the conformational change facilitated by ligand binding (29). Bound adaptor proteins then allow the receptor to associate with either the lysosomal sorting machinery or the recycling sorting machinery, depending on specific associations found within the early endosome (12, 20). Still other studies have found evidence for sorting that takes place in specific subsets of clathrin-coated vesicles (50). In truth, molecular sorting machinery is likely GPCR specific, and depends on both the specific arrangement of amino acids within the carboxy terminus of the receptor and the cellular context within which that receptor is found.

Consistent with previous studies, we show that CXCR2 is degraded within the lysosome (8) (Fig. 1). We also show that the lysosomal shuttling of CXCR2 is not dependent upon ubiquitination. The most extensively characterized ligand-mediated lysosomal sorting mechanism for GPCRs involves monoubiquitination of specific lysine residues in the carboxy-terminal

FIGURE 4. Carboxy-terminal PDZ ligand motif deletion does not impair either agonist-induced receptor internalization rates or receptor recycling efficiency. A, CXCR2 WT and CXCR2 352T exhibit similar agonist-induced internalization rates. Internalization rates were analyzed as described under “Materials and Methods.” Percent receptor internalization was calculated as internalized counts/min divided by the total specific counts/min bound prior to incubation at 37 °C. Error bars represent the S.E. from four independent experiments performed in triplicate. The data were subjected to statistical analysis by a non-parametric Mann-Whitney test (* indicates \( p < 0.05 \)). B, CXCR2 WT and CXCR2 352T exhibit similar receptor recycling efficiencies. Receptor recycling was analyzed as described under “Materials and Methods.” Percent receptor surface expression was calculated as mean fluorescence intensity detected on vehicle-stimulated cells (base). Error bars represent S.E. for four independent experiments. The data were subjected to statistical analysis by a non-parametric Mann-Whitney test (* indicates \( p < 0.05 \)).

FIGURE 5. Carboxy-terminal PDZ ligand motif deletion facilitates early entry into the late endosome upon agonist stimulation. A, HEK293 cells stably expressing either CXCR2 wild-type or CXCR2 352T were transiently transfected with late-endosomal marker Rab7-GFP. Cells were either treated with vehicle alone or stimulated with 10 nM CXCL8 for 1 h. Cells were then fixed, permeabilized, and immunostained for both CXCR2 and recycling endosome marker Rab11a. Rab7 is shown in white (1st panel column) and green (4th panel column). CXCR2 is shown in white (2nd column panel) and red (4th column panel). Rab11a is shown in white (3rd column panel) and blue (4th column panel). Areas that appear magenta in the merged images indicate co-localization of CXCR2 and recycling marker Rab11a. Areas that appear yellow in the merged images indicate co-localization of CXCR2 and late endosomal marker Rab7. The insets represent enlarged views of the boxed regions. The scale bars represent 10 μm. Confocal images were taken on a LSM META 150 inverted confocal fluorescence microscope and processed with LSM imaging software. Similar results were observed from three independent experiments. B, quantification of either CXCR2 WT or CXCR2 352T and Rab11a co-localization as seen in A. 30–40 cells were analyzed per experiment and the graph represents an average of three independent experiments. Data were subjected to a non-parametric Mann-Whitney test (** indicates \( p < 0.0005 \)). C, quantification of either CXCR2 wild-type or CXCR2 352T with Rab7-GFP as seen in A. 30–40 cells were analyzed per experiment and the graph represents an average of three independent experiments. Data were subjected to a non-parametric Mann-Whitney test (** indicates \( p < 0.0005 \)).
domain (51). This ubiquitination event is then followed by recognition of the modified GPCR by a series of protein complexes (ESCRTs) that associate with the receptor via UIM domains at the early endosome (52–55). These protein complexes bind and then dissociate in sequence, allowing both the formation of the multivesicular body and the sorting of the targeted GPCR into that sorting compartment (56). Here, we investigated the possibility that CXCR2 was sorted to the lysosome via this specific machinery.

Using a series of lysine mutants, we show that ubiquitination is not necessary for the lysosomal sorting of CXCR2. Consistent with this finding, there are reports suggesting that ubiquitination is not necessary for the association of HRS with certain GPCRs, and the subsequent sorting via the ESCRT machinery (57). We found extensive co-localization of HRS with CXCR2 at the early endosome, both with endogenous HRS and exogenous myc-tagged protein (data not shown). However, blocking Hrs activation by overexpression did not appear to block CXCR2 degradation (data not shown). Blocking Vps4 activation also did not appear to block CXCR2 degradation. Therefore, it appears that CXCR2 is shuttled to the lysosome via a different mechanism than the highly related chemokine receptor

**FIGURE 6.** Carboxyl-terminal PDZ ligand motif deletion enhances receptor degradation and impairs chemotaxis. A, degradation of both the wild-type receptor and CXCR2 352T is reduced upon stimulation with physiologically relevant concentrations of CXCL8. HEK293 cells stably expressing either wild-type CXCR2 or CXCR2 352T were stimulated with 10 ng/ml CXCL8 for the indicated times. Immunoblots are representative of three independent experiments. The molecular weights are indicated. B, graphical representation of receptor degradation. Immunoblots (IB) in A were subjected to densitometric analysis (LICOR Odyssey 2.0 software) to quantify receptor levels. The line graph represents the average percentages of CXCL8-mediated receptor degradation in cells subsequent to agonist stimulation after normalization with actin levels. Error bars represent the S.E. from three independent experiments. The data were subjected to statistical analysis by a non-parametric Mann-Whitney test (* indicates p < 0.05). C, HEK293 cells expressing CXCR2 352T exhibit decreased chemotaxis as compared with those cells expressing CXCR2 WT. Cells stably expressing either CXCR2 WT or CXCR2 352T were subjected to a Boyden chamber assay assessing cellular chemotaxis, and cells/field counts were calculated from 20 random fields per ligand concentration, using the ×20 objective of an upright light microscope (Zeiss). Error bars represent S.E. Data were subjected to statistical analysis by a Student’s t test (* indicates p < 0.05; ** indicates p < 0.005). Data are representative of four independent experiments. D, data in C are represented as chemotactic index. Data were subjected to statistical analysis by a Student’s t test (* indicates p < 0.05) and graph represents an average of four independent experiments. E, surface receptor expression was analyzed as described under “Materials and Methods.”
PDZ Ligand of CXCR2 Regulates Trafficking and Chemotaxis

CXCR4. Because post-translational ubiquitination does not appear to play a role in CXCR2 sorting, we examined specific motifs found within the intracellular portion of the receptor to determine their role in post-endocytic sorting.

We show that truncation of specific motifs at the carboxy-terminal domain of CXCR2 increase receptor degradation and facilitate earlier co-localization with the lysosome. Residues 331–355 of the carboxy-terminal domain of CXCR2 contain several serine residues that have been shown to facilitate β-arrestin binding (21). β-Arrestin binds clathrin and facilitates clathrin-mediated endocytosis, and it has also been shown to be involved in the degradative sorting decision of several receptors, including CXCR4 (46, 58–60). Truncation of the β-arrestin binding motif of CXCR2 does not block or retard receptor degradation (Fig. 3). Surprisingly, truncation of this motif appears to increase receptor degradation (Fig. 3). To further pinpoint the motifs responsible for this result, we examined the different motifs within amino acid residues 331–355. The last four residues (-STTL) encode a PDZ ligand binding motif. Many GPCRs contain PDZ ligand binding motifs at the extreme COOH terminus, and these motifs have been shown in some cases to affect receptor trafficking (20). Therefore, we evaluated the effect of deletion of the PDZ ligand motif on lysosomal sorting and receptor degradation. This deletion increased ligand-dependent receptor degradation significantly above the wild-type receptor, and above that of the 331T truncation deletion. Furthermore, deletion of the motif appears to facilitate early co-localization with the lysosome, suggesting early association of the 352T mutant with the lysosomal shuttling machinery. This result suggests that the CXCR2 PDZ ligand associates with a PDZ domain containing protein, or complex of proteins, that prevents association with the lysosomal sorting machinery and retards sorting to the lysosome.

One potential explanation for degradation and lysosomal sorting of the CXCR2 352T is that there is an increase in the rate of ligand-induced receptor trafficking as a result of an increase in receptor internalization. Increased receptor internalization could simply increase the speed of receptor trafficking, resulting in earlier degradation and localization in the lysosome. Furthermore, many published reports demonstrate that PDZ ligand-PDZ domain containing protein interactions facilitate plasma membrane localization (40, 41, 61, 62). Interruption of this interaction has been shown to allow faster internalization of ligand-stimulated receptor (40, 61). However, the PDZ ligand deletion mutant of CXCR2 does not appear to internalize significantly faster that the wild-type receptor. Therefore, it appears that the affect of the PDZ ligand deletion occurs at a post-endocytic time point.

It has been shown that blocking receptor degradation can shunt receptor trafficking toward the recycling arm of the sorting decision (63). Conversely, blocking receptor recycling has been shown to increase receptor degradation (10). Therefore, we decided to investigate the effect of the PDZ ligand deletion on CXCR2 receptor recycling. We found that deleting the PDZ ligand motif has no effect on receptor recycling (Fig. 4). This result suggests that the PDZ deletion mutant is indeed capable of recycling, and does so for at least one round of internalization and re-expression on the cell surface. Furthermore, this result suggests that truncation of CXCR2 at serine 352 simply increases the probability that the receptor will be sorted from the early endosome to the late endosome, and decreases the probability that the mutant receptor will be sorted from the early endosome to the Rab11a-positive recycling endosome.

In validation of this hypothesis, we show that truncation of the PDZ ligand motif of CXCR2 facilitates early co-localization with the Rab7-positive late endosome, compared with wild-type CXCR2. This result suggests that the mutant receptor is more likely to be sorted to the late endosome upon ligand stimulation than the wild-type receptor. We also demonstrate in this study that truncation of the PDZ ligand motif reduces co-localization with the Rab11a-positive recycling endosome, as compared with wild-type receptor (Fig. 5). However, our data show that CXCR2 352T recycles back to the plasma membrane with similar efficiency to that of wild-type receptor (Fig. 4). It is therefore possible that deletion of the PDZ ligand of CXCR2 facilitates alternative receptor trafficking pathways. The mannose 6-phosphate receptor has been reported to recycle from the Rab7-containing late endosome (64, 65).

Our data demonstrate that deletion of the PDZ ligand of CXCR2 does not completely block co-localization with the Rab11a-positive recycling endosome, but merely reduces it (Fig. 5). Therefore, the apparent efficient recycling of this mutant receptor could be explained by complete re-expression of the CXCR2 352T receptor found within the Rab11a-positive compartment, as compared with only partial re-expression of the CXCR2 wild-type receptor found within the Rab11a-containing endosome. This hypothesis would suggest that the presence of the PDZ ligand retains CXCR2 in the recycling endosome, whereas the absence of the PDZ ligand allows efficient sorting of the receptor back to the plasma membrane. There are a number of reports that suggest PDZ domain containing proteins affect intracellular trafficking and receptor recycling (31, 43, 66–70). Additionally, Lin and Huganir (71) report that association of glutamate receptor 2 with PDZ domain-containing protein PICK1 results in delayed recycling with no reported affects on receptor internalization.

We also found that deletion of the PDZ ligand impaired cellular chemotaxis (Fig. 6). It has been observed previously that progressive carboxyl terminus deletions of CXCR2 result in the interruption of chemotaxis to CXCL8 in both HEK293 cells as well as RBL cells (1, 34, 72, 73). However, these experiments were performed using CXCR2 truncation mutants that left 6, 14, 15, 25, or 38 amino acid residues remaining distal to the transmembrane domain out of the 45 found at the carboxyl terminus of the wild-type receptor. The CXCR2 352T mutant receptor used in this study leaves 42 amino acid residues remaining distal to the transmembrane domain of the receptor. Therefore, our data suggest that in HEK293 cells, the PDZ ligand motif is a critical carboxyl-terminal domain for the regulation of chemotaxis in response to CXCL8.

Our data also suggest that the absence of adaptor protein binding to the PDZ-ligand of CXCR2 results in early sorting to the late endosome, early localization in the lysosome, and increased receptor degradation. Unfortunately, the adaptor proteins that bind to the PDZ ligand of CXCR2 are largely unknown. One report states that the PDZ domain containing
protein RGS12 binds to the carboxyl-terminal domain of CXCR2 (10, 74). This binding was shown by a gel overlay assay as well as GST pull-down assays, but not by co-immunoprecipitation (74). We were unable to co-immunoprecipitate RGS12 with either full-length CXCR2 WT or CXCR2 352T expressed in HEK293 cells (data not shown). Because RGS12 is a protein important for the desensitization of G-protein signaling (75) and we observed that deletion of the PDZ ligand of CXCR2 had no effect on short-term extracellular signal-regulated kinase (ERK) signaling compared with the wild-type receptor (data not shown), our data do not support the idea that the lack of RGS12 binding is responsible for the noted effect on receptor-mediated chemotaxis or trafficking. Moreover, recent reports show that PDZ domain containing scaffolding proteins PSD95 and NHERF do not bind to CXCR2, further adding to the mystery of the protein that binds the PDZ ligand of CXCR2 (76).

Based upon prior studies, it has been shown that certain 14-3-3 proteins can bind to carboxyl-terminal PDZ ligands of GPCRs and effect trafficking decisions (77). It is possible that specific 14-3-3 scaffolding proteins bind to CXCR2 and regulate trafficking. Immunoprecipitation of CXCR2 in HL-60 cells stably expressing CXCR2 followed by LC/MS/MS proteomics analysis of associating proteins identified both 14-3-3 scaffolding proteins bind to CXCR2 and regulated chemotaxis or trafficking. Moreover, recent reports show that PDZ domain containing scaffold proteins PSD95 and RGS12 bind to the carboxyl-terminal domain of CXCR2 (76).

Acknowledgments—We acknowledge Sam Wells, Dawn Kilkenny, and Lynn Lapierre as well as Jiqing Sai, Nicole F. Neel, and Dayanidhi Raman.

REFERENCES

1. Fan, G. H., Yang, W., Wang, X. J., Qian, Q., and Richmond, A. (2001) Biochemistry 40, 791–800
2. Raman, D., Baugher, P. J., Thu, Y. M., and Richmond, A. (2007) Cancer Lett. 256, 137–165
3. Striet, R. M., Burdick, M. D., Mestas, J., Comperts, B., Keane, M. P., and Belzerio, J. A. (2006) Eur. J. Cancer 42, 768–778
4. Neel, N. F., Schuttyser, E., Sai, J., Fan, G. H., and Richmond, A. (2005) Cytokine Growth Factor Rev. 16, 637–658
5. Lee, J., Horuk, R., Rice, G. C., Bennett, G. L., Camerato, T., and Wood, W. I. (1992) J. Biol. Chem. 267, 16283–16287
6. Loetscher, P., Seitz, M., Clark-Lewis, I., Baggioni, M., and Moser, B. (1994) FEBS Lett. 341, 187–192
7. Ahuja, S. K., and Murphy, P. M. (1996) J. Biol. Chem. 271, 20545–20550
8. Fan, G. H., Lapierre, L. A., Goldenring, J. R., and Richmond, A. (2003) Blood 101, 2115–2124
9. Gruenberg, J. (2001) Nat. Rev. Mol. Cell. Biol. 2, 721–730
10. Cao, T. T., Deacon, H. W., Reczek, D., Bretscher, A., and von Zastrow, M. (1999) Nature 401, 286–290
11. Tsao, P. I., and von Zastrow, M. (2001) Pharmacol. Ther. 89, 139–147
12. Hanyaloglu, A. C., and von Zastrow, M. (2007) Annu. Rev. Pharmacol. Toxicol. 48, 537–568
13. Hicke, L., and Riezman, H. (1996) Cell 84, 277–287
14. Babst, M., Sato, T. K., Banta, L. M., and Emr, S. D. (1997) EMBO J. 16, 1820–1831
15. Katzmann, D. J., Babst, M., and Emr, S. D. (2001) Cell 106, 145–155
16. Babst, M., Katzmann, D. J., Estepa-Sabal, E. J., Meerloo, T., and Emr, S. D. (2002) Dev. Cell 3, 271–282
17. Babst, M., Katzmann, D. J., Snyder, W. B., Wendland, B., and Emr, S. D. (2002) Dev. Cell 3, 283–289
18. Shih, S. C., Katzmann, D. J., Schnell, D. J., Sutanto, M., Emr, S. D., and Hicke, L. (2002) Nat. Cell Biol. 4, 389–393
19. Shenoy, S. K., McDonald, P. H., Kohout, T. A., and Lefkowitz, R. J. (2001) Science 294, 1307–1313
20. Marchese, A., Paing, M. M., Temple, B. R., and Trejo, J. (2008) Annu. Rev. Pharmacol. Toxicol. 48, 601–629
21. Mueller, S. G., White, J. R., Schwag, W. P., Lam, V., and Richmond, A. (1997) J. Biol. Chem. 272, 8207–8214
22. Neel, N. F., Lapierre, L. A., Goldenring, J. R., and Richmond, A. (2007) J. Cell Sci. 120, 1559–1571
23. Mueller, S. G., Schrag, W. P., and Richmond, A. (1994) J. Biol. Chem. 269, 1973–1980
24. Lapierre, L. A., Avant, K. M., Caldwell, C. M., Ham, A. J., Hill, S., Williams, J. A., Smolka, A. I., and Goldenring, J. R. (2007) Am. J. Physiol. 292, G1249–G1262
25. Fan, G. H., Lapierre, L. A., Goldenring, J. R., Sai, J., and Richmond, A. (2004) Mol. Biol. Cell 15, 2456–2469
26. Yang, W., Wang, D., and Richmond, A. (1999) J. Biol. Chem. 274, 11328–11333
27. Urbe, S. (2005) Essays Biochem. 41, 81–98
28. Mukhopadhyay, D., and Riezman, H. (2007) Science 315, 201–205
29. Wolfe, B. L., and Trejo, J. (2007) Traffic 8, 462–470
30. Moore, C. A., Milano, S. K., and Benovic, J. L. (2007) Annu. Rev. Physiol. 69, 451–482
31. Trejo, J. (2005) Mol. Pharmacol. 67, 1388–1390
32. Hooen, R. J., and Berk, B. C. (2006) J. Cell Sci. 119, 1469–1475
33. Baric, J., Khandaker, M. H., Mahon, E., Andrews, J., DeVries, M. E., Mitchell, G. B., Rahimpour, R., Tan, C. M., Ferguson, S. S., and Kelvin, D. J. (1999) J. Biol. Chem. 274, 16287–16294
34. Richardson, R. M., Marjoram, R. J., Barak, L. S., and Snyderman, R. (2003) J. Immunol. 170, 2904–2911
35. Snow, B. E., Betts, L., Mangion, J., Sondek, J., and Siderovski, D. P. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 6489–6494
36. Brune, B., and Eggemont, J. (2005) Am. J. Physiol. 288, C20–C29
37. Kim, E., and Sheng, M. (2004) Nat. Rev. Neurosci. 5, 771–781
38. Jelen, F., Oleksy, A., Smietana, K., and Otelwski, J. (2003) Acta Biochem. Pol. 50, 985–1017
PDZ Ligand of CXCR2 Regulates Trafficking and Chemotaxis

39. Nourry, C., Grant, S. G., and Borg, I. P. (2003) Sci. STKE 2003, RE7
40. Wang, B., Bisello, A., Yang, Y., Romero, G. G., and Friedman, P. A. (2007) J. Biol. Chem. 282, 36214–36222
41. Hryciw, D. H., Ekberg, J., Ferguson, C., Lee, A., Wang, D., Parton, R. G., Pollock, C. A., Yun, C. C., and Pronnink, P. (2006) J. Biol. Chem. 281, 16068–16077
42. Babst, M., Odorizzi, G., Estepa, E. J., and Emr, S. D. (2000) Traffic 1, 248–258
43. Gage, R. M., Kim, K. A., Cao, T. T., and von Zastrow, M. (2001) J. Biol. Chem. 276, 44712–44720
44. Martin, N. P., Lefkowitz, R. J., and Shenoy, S. K. (2003) J. Biol. Chem. 278, 24412–24419
45. Marchese, A., and Benovic, J. L. (2001) J. Biol. Chem. 276, 22522–22531
46. Nickerson, D. P., Russell, M. R., and Odorizzi, G. (2007) Mol. Pharmacol. 72, 32419–32425
47. Paing, M. M., Johnston, C. A., Siderovski, D. P., and Trejo, J. (2006) Mol. Cell. Biol. 26, 3231–3242
48. Gabilondo, A. M., Meana, J. J., Barturen, F., Sastre, M., and Garcia-Sevilla, J. A. (1994) Psychopharmacology 115, 135–140
49. Orsini, M. J., Parent, J. L., Mundell, S. I., Marchese, A., and Benovic, J. L. (1999) J. Biol. Chem. 274, 31076–31086; Correction (2000) J. Biol. Chem. 275, 25876
50. Lakadamyali, M., Rust, M. J., and Zhuang, X. (2006) Cell 124, 997–1009
51. Shenoy, S. K. (2007) Circ. Res. 100, 1142–1154
52. Williams, R. L., and Urbe, S. (2007) Nat. Rev. Mol. Cell. Biol. 8, 355–368
53. Hurley, J. H., and Emr, S. D. (2006) Annu. Rev. Biophys. Biomol. Struct. 35, 277–298
54. Winter, V., and Hauser, M. T. (2006) Trends Plant Sci. 11, 115–123
55. Babst, M. (2005) Traffic 6, 2–9
56. Nickerson, D. P., Russell, M. R., and Odorizzi, G. (2007) EMBO Rep. 8, 644–650
57. Hislop, J. N., Marley, A., and Von Zastrow, M. (2004) J. Biol. Chem. 279, 22522–22531
58. Bhandari, D., Trejo, J., Benovic, J. L., and Marchese, A. (2007) J. Biol. Chem. 282, 36971–36979
59. Girnita, L., Shenoy, S. K., Sehat, B., Vasilcanu, R., Girnita, A., Lefkowitz, R. J., and Larsson, O. (2005) J. Biol. Chem. 280, 24412–24419
60. Tulipano, G., Stamm, R., Pfeiffer, M., Kreienkamp, H. J., Hollt, V., and Schulz, S. (2004) J. Biol. Chem. 279, 21374–21382
61. Wheeler, D., Sneddon, W. B., Wang, B., Friedman, P. A., and Romero, G. (2007) J. Biol. Chem. 282, 25076–25087
62. James, M. F., Beauchamp, R. L., Manchanda, N., Kazlauskas, A., and Ramesh, V. (2004) J. Cell Sci. 117, 2951–2961
63. Grovdal, L. M., Stang, E., Sorkin, A., and Madshus, I. H. (2004) Exp. Cell Res. 300, 388–395
64. Ghosh, P., Dahms, N. M., and Kornfeld, S. (2003) Nat. Rev. Mol. Cell. Biol. 4, 202–212
65. Barbero, P., Bittova, L., and Pfeffer, S. R. (2002) J. Cell Biol. 155, 511–518
66. Paasche, J. D., Attramadal, T., Kristiansen, K., Oksvold, M. P., Johansen, H. K., Huitfeldt, H. S., Dahl, S. G., and Attramadal, H. (2005) Mol. Pharmacol. 67, 1581–1590
67. Zimmermann, P., Zhang, Z., Degeest, G., Mortier, E., Leenaerts, I., Coomans, C., Schulz, J., N’Kuli, F., Courtroy, P. J., and David, G. (2005) Dev. Cell 9, 377–388
68. Wente, W., Stroh, T., Beaudet, A., Richter, D., and Kreienkamp, H. J. (2005) J. Biol. Chem. 280, 32419–32425
69. Delhaye, M., Gravot, A., Ayinde, D., Niedergang, F., Alizon, M., and Brelot, A. (2007) Mol. Pharmacol. 72, 1497–1507
70. Rincon, E., Santos, T., Avila-Flores, A., Albar, J. P., Lalioti, V., Lei, C., Hong, W., and Merida, I. (2007) Mol. Cell Proteomics 6, 1073–1087
71. Lin, D. T., and Huganir, R. L. (2007) J. Neurosci. 27, 13903–13908
72. Ben-Baruch, A., Bengali, K. M., Biragyn, A., Johnston, J. I., Wang, J. M., Kim, J., Chuntharapai, A., Michel, D. F., Oppenheim, J. J., and Kelvin, D. J. (1995) J. Biol. Chem. 270, 9121–9128
73. Neptune, E. R., and Bourne, H. R. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 14489–14494
74. Snow, B. E., Hall, R. A., Krumins, A. M., Brothers, G. M., Bouchard, D., Brothers, C. A., Chung, S., Mangion, J., Gilman, A. G., Lefkowitz, R. J., and Siderovski, D. P. (1998) J. Biol. Chem. 273, 17749–17755
75. Ishii, M., and Kurachi, Y. (2003) Life Sci. 74, 163–171
76. Heydorn, A., Sondergaard, B. P., Ersboll, B., Holst, B., Nielsen, F. C., Attramadal, H. S., Dahl, S. G., and Attramadal, H. (2005) Mol. Pharmacol. 67, 1581–1590
77. Heydorn, A., Sondergaard, B. P., Ersboll, B., Holst, B., Nielsen, F. C., Haft, C. R., Whistler, J., and Schwartz, T. W. (2004) Mol. Pharmacol. 67, 1581–1590
78. Chabrier, B., Wu, M., Shikano, S., and Li, M. (2006) FEBS Lett. 580, 1531–1535