Heterologous regulation of CXCR4 lysosomal trafficking

Adriana Caballero1,3, Sarah A. Mahn2, Mudassir S. Ali2, M. Rose Rogers2, and Adriano Marchese1,2,*

From the 1Department of Pharmacology, Loyola University Chicago, Maywood, IL 60153 and 2Department of Biochemistry, Medical College of Wisconsin, Milwaukee, WI 53226

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3Present address: Department of Anatomy and Cell Biology, College of Medicine, University of Illinois at Chicago, 808 S. Wood St., Chicago, IL 60612

*To whom correspondence should be addressed: Adriano Marchese: Department of Biochemistry, Medical College of Wisconsin, TBRC C3850, 8701 Watertown Plank Rd., Milwaukee, Wisconsin 53226; amarchese@mcw.edu; Tel. (414) 955-4191; Fax. (414) 955-6510.

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ABSTRACT

G-protein coupled receptor (GPCR) signaling is regulated by members of the protein kinase C (PKC) and GPCR kinase (GRK) families, although the relative contribution of each to GPCR function varies among specific GPCRs. The CXC motif receptor 4 (CXCR4) is a member of the GPCR superfamily that binds the CXC motif chemokine ligand 12 (CXCL12) initiating signaling that is subsequently terminated in part by internalization and lysosomal degradation of CXCR4. The purpose of this study is to define the relative contribution of PKC and GRK to CXCR4 signaling attenuation by studying their effects on CXCR4 lysosomal trafficking and degradation. Our results demonstrate that direct activation of PKC via the phorbol ester PMA mimics CXCL12-mediated desensitization, internalization, ubiquitination and lysosomal trafficking of CXCR4. In agreement, heterologous activation of PKC by stimulating the chemokine receptor CXCR5 with its ligand CXCL13, also mimics CXCL12-mediated desensitization, internalization, ubiquitination and lysosomal degradation of CXCR4. Similar to CXCL12, PMA promotes PKC-dependent phosphorylation of serine residues within CXCR4 C-tail that are required for binding and ubiquitination by the E3 ubiquitin ligase AIP4 (atrophin interacting protein 4). However, inhibition of PKC activity does not alter CXCL12-mediated ubiquitination and degradation of CXCR4, suggesting that other kinases are also required. Accordingly, siRNA mediated depletion of GRK6 results in decreased degradation and ubiquitination of CXCR4. Overall these results suggest that PKC and GRK6 contribute to unique aspects of CXCR4 phosphorylation and lysosomal degradation that ensue proper signal propagation and termination.

The signaling cascades elicited by the G protein-coupled receptor (GPCR) CXCR4 and its cognate ligand CXCL12 (SDF-1α) are involved in the normal development of the immune, cardiovascular, and central nervous system (1-4) as well as playing an important role in the pathophysiology of several types of cancer (5). CXCR4 levels are upregulated in at least 23 types of cancer and is correlated with a poor prognosis either by enhancing tumor growth or contributing to the tumor metastatic potential (6-10). In
addition, WHIM syndrome, a rare immunodeficiency disorder characterized by warts, hypogammaglobulinemia, bacterial infections and myelokathexis, is linked to truncations of CXCR4 C-terminus (C-tail), which ultimately confer increased signaling capabilities to the receptor (11,12). Despite its role in disease, the regulation of CXCR4 abundance or signaling remains poorly understood.

The magnitude and duration of CXCR4 signaling is under tight regulation at multiple steps of the signal transduction cascade, including at the level of the receptor itself (13). Receptor phosphorylation is regarded as a key step in mediating CXCR4 unresponsiveness to CXCL12, a process referred to as desensitization (13-16). CXCR4 phosphorylation is typically mediated by distinct serine/threonine kinases that belong to either the G protein-coupled receptor kinase (GRK) or protein kinase C (PKC) families (17). GRKs typically mediate receptor phosphorylation of agonist bound receptor leading to β-arrestin binding and subsequent G protein uncoupling, thereby terminating further receptor signaling (18). This is referred to as homologous desensitization (19). Second messenger-dependent protein kinases such as PKC also phosphorylate GPCRs, including CXCR4(17), but they are able to do it in an agonist independent manner; this is referred to as heterologous desensitization (18). Several GRKs, namely GRK6, GRK2 and GRK3, and several PKC isoforms have been implicated in phosphorylation of CXCR4 carboxyl-tail serine/threonine residues that contribute to CXCR4 desensitization (20-22).

Phosphorylation of CXCR4 is required for CXCL12-mediated receptor internalization and trafficking to lysosomes, a terminal degradative compartment (23,24). Multiple phosphorylation sites on the C-tail have been implicated in CXCL12-mediated internalization of CXCR4 (23). CXCL12-induced phosphorylation of the C-tail is also required for targeting CXCR4 for lysosomal degradation (24). Site specific phosphorylation of serine residues 324 and 325 (Ser-324/5) induced by CXCL12 promotes ubiquitination of nearby lysine residues, a prerequisite for lysosomal trafficking and receptor degradation (25). These residues are thought to be phosphorylated by PKC and GRK6, and while the contributions of PKC or GRK6 to CXCR4 internalization are known (17,23,26-30), their contribution to CXCR4 lysosomal trafficking remain unknown.

The purpose of this study is to define the relative contribution of PKC and GRK6 to CXCR4 lysosomal trafficking. Here we show that PMA, a phorbol ester that directly activates PKC (31), mimics many aspects of CXCL12 in promoting lysosomal trafficking of CXCR4. Similar to CXCL12, PMA promotes CXCR4 phosphorylation at Ser-324/5 and trafficking to a lysosomal degradative compartment. However, while PKC is dispensable for CXCL12-promoted ubiquitination and degradation, GRK6 is essential to target CXCR4 to a degradative compartment. Moreover, heterologous activation of PKC by CXCL13, the cognate ligand for the chemokine receptor CXCR5 promotes CXCR4 internalization and degradation. Accordingly, pre-treatment with PMA or CXCL13 attenuates early CXCR4 heterotrimeric G protein Gi signaling by CXCL12. Therefore, heterologous regulation of CXCR4 lysosomal trafficking by chemokines via PKC desensitizes CXCR4 signaling to its ligand CXCL12.

Results
PKC activation is sufficient to induce CXCR4 trafficking to lysosomes and degradation
Previous reports have provided evidence that direct activation of PKC by the phorbol ester PMA is sufficient to induce CXCR4 internalization in several cell types, although the fate of CXCR4 after internalization remains unclear (23,28,29). Here, we investigated whether direct PKC activation is sufficient to promote CXCR4 intracellular trafficking to terminal lysosomal compartments. In HEK293 cells stably expressing HA-tagged CXCR4, PMA induces dose-dependent (Fig. 1A) and time-dependent (Fig. 1B) degradation of HA-CXCR4. Based on these results, all subsequent experiments were performed at 3 hours after the addition of 10 nM PMA. Next, we investigated whether PMA-mediated degradation was sensitive to endosomal acidification inhibitors such as chloroquine. In the absence of
chloroquine, CXCL12 and PMA elicit 60-70% of HA-CXCR4 degradation after 3 hours of CXCL12 stimulation (Fig. 1C). Pre-treatment of HEK293 with chloroquine completely abrogates both CXCL12 and PMA-mediated HA-CXCR4 degradation (Fig. 1C), indicating that PMA, similar to CXCL12 (25), induces the delivery of CXCR4 to acidic compartments necessary for CXCR4 degradation.

To corroborate these results we examined lysosomal trafficking of CXCR4 using multi-label fluorescence microscopy. HEK293 cells were transfected with CXCR4 tagged with YFP (CXCR4-YFP), serum starved for approximately 3 hours, and followed by treatment with vehicle, CXCL12, or PMA for another 3 hours. Following stimulation and fixation, LAMP1-positive lysosomes were immunostained for multi-label fluorescence microscopy and colocalization analysis. As shown in Fig. 2A, cells treated with PMA and CXCL12 displayed high levels of colocalization between CXCR4-YFP and LAMP1. The mean percentage of LAMP1-positive lysosomes colocalizing with CXCR4-YFP puncta was greater in cells treated with either PMA (~22%) or CXCL12 (~27%), compared to vehicle-treated cells (~13%), with no significant difference between cells treated with PMA and CXCL12 (Fig 2B). These data demonstrate PMA, similar to CXCL12, elicits an intracellular itinerary that facilitates degradation of CXCR4 in terminal endocytic compartments.

**Role of PKC in CXCR4 phosphorylation**

The similarity between CXCL12 and PMA mediated degradation raised the question of whether PKC is involved directly in CXCL12-mediated CXCR4 degradation. Previously, we have shown that CXCL12 stimulation drives CXCR4 degradation through transient phosphorylation of serine residues 324 and 325 (Ser-324/325) at the plasma membrane, using a custom antibody that specifically detects simultaneous phosphorylation of Ser324 and Ser325 (p-Ser324/325) by immunofluorescence microscopy (24). To determine whether PMA and CXCL12 elicit similar spatial patterns of Ser324 and Ser325 phosphorylation, HEK293 or HeLa cells transiently expressing CXCR4-YFP were serum starved for 3.5 hours and then stimulated with either CXCL12 or PMA for 30 minutes. The antibody against p-Ser324/325 showed robust staining at the cell periphery in both PMA and CXCL12-treated cells compared to vehicle, suggesting simultaneous phosphorylation of Ser324 and Ser325 (Fig. 3A and quantified in 3B). To determine if PKC is necessary for CXCL12-mediated phosphorylation of these residues, cells were treated with BisI, a selective PKC inhibitor (32), or vehicle before stimulation with CXCL12. Simultaneous phosphorylation of Ser324 and Ser325 was significantly inhibited by BisI compared to vehicle control (Fig. 4A and quantified in 4B). These results indicate that PKC is sufficient and necessary for simultaneous phosphorylation of Ser324 and Ser325 promoted by CXCL12.

**Role of PKC in CXCR4 degradation and ubiquitination**

To explore whether PKC is necessary for CXCR4 degradation, cells were pre-treated with BisI followed by treatment with CXCL12 or PMA for 3 hr and immunoblotting. We performed these experiments in HEK293 cells transiently transfected with HA-CXCR4, as well as in HeLa cells, which endogenously express CXCR4 (33-36). Consistent with our previous data (33,35,37), CXCL12 promoted degradation of HA-CXCR4 in HEK293 cells (Fig. 5A and quantified in 5B) as well as endogenous CXCR4 in HeLa cells (Fig. 5C and quantified in 5D). Similarly, PMA promoted CXCR4 degradation in both HEK293 and HeLa cells; however, pre-treatment with BisI did not impact CXCL12 promoted CXCR4 degradation in HEK293 or HeLa cells, whereas PMA promoted degradation was completely inhibited by BisI pre-treatment in both cell types (Figs. 5A-D). These data indicate that PMA-mediated CXCR4 degradation is entirely due to activation of PKC, whereas PKC phosphorylation of CXCR4 induced by CXCL12 is dispensable to commit CXCR4 to the degradative pathway.

Because phosphorylation of Ser-324/325 mediates AIP4 binding and ubiquitination (24), we next examined the role of PKC on the status of CXCR4 ubiquitination upon CXCL12 treatment in the presence of the PKC inhibitor BisI. Consistent with our previously published
results (25,33), CXCL12 elicits ubiquitination of CXCR4 (Fig. 5E and quantified in 5F). However, pre-treatment with BisI had no effect on CXCR4 ubiquitination upon CXCL12 treatment (Fig. 5E and quantified in 5F). Because the antibody against p-Ser324/325 only recognizes dually phosphorylated CXCR4 (24), these results suggest that PKC activity may be responsible for the phosphorylation of only one of the residues in the Ser-324/325 doublet. To explore this further, we examined CXCR4 degradation of single serine mutants S324A and S325A transiently expressed in HEK293 cells in the presence of BisI. Degradation of these mutants was not impaired compared to the double-serine mutant S324A/S325A (Fig. 6A and quantified in 6B), as we have previously reported (24). Similarly, BisI did not impact degradation of the individual mutants to the level observed with the double S324A/S325A mutant. These data suggest that phosphorylation of either Ser324 or Ser-325 is sufficient to support CXCR4 degradation by CXCL12, and although PKC may be sufficient, it also suggests that another kinase(s) is likely necessary for phosphorylation of these residues and CXCR4 degradation.

**GRK6 contributes to CXCR4 ubiquitination and degradation**

GRK6 has been previously implicated in phosphorylation of serine residues 324 and 325 induced by CXCL12 (17), suggesting it may be involved in CXCR4 degradation. Thus, we addressed whether GRK6 participated in CXCL12-mediated degradation of CXCR4 by measuring degradation of endogenous CXCR4 following CXCL12 treatment in HeLa cells transfected with siRNA against GRK6. Compared to control siRNA, CXCR4 degradation was significantly attenuated by approximately 60% in GRK6 siRNA transfected cells (Fig. 7A and quantified in 7B). Pre-treatment of BisI in the GRK6 siRNA background had no significant additional effect on CXCR4 degradation promoted by CXCL12 (data not shown). Thus, these data indicate that GRK6 has a distinctive role in targeting CXCR4 for degradation.

Given the role of CXCR4 ubiquitination in CXCR4 degradation (24,25), we investigated whether GRK6 contributes to receptor ubiquitination. CXCL12-induced ubiquitination of CXCR4 occurs normally in cells transfected with control siRNA, whereas GRK6 siRNA consistently decreased the fraction of ubiquitinated CXCR4 (Fig. 7D and quantified in 7E). Because impairment of internalization could also explain the impact on CXCR4 degradation and ubiquitination we also assessed whether GRK6 siRNA impaired early steps of receptor endocytosis from the plasma membrane. For this purpose, HeLa cells were pre-treated with increasing amounts of CXCL12 for 30 min at 37°C and the level of remaining endogenous CXCR4 was measured by staining with a PE-labeled anti-CXCR4 (12G5) and analyzed by flow cytometry. In control siRNA transfected cells, CXCR4 shows a dose-dependent decrease in surface staining, consistent with receptor sequestration (Fig. 7E). Cells transfected with GRK6 siRNA also elicited a dose-dependent decrease in receptor levels upon CXCL12 treatment, but to a significantly lower extent (Fig. 7E). These data indicate that GRK6 contributes to CXCR4 endocytosis, but since CXCR4 internalization is not a rate-limiting step in its lysosomal trafficking (25), it is unlikely this accounted for the defect observed at the level of degradation (Fig. 7B). In addition, because CXCR4 ubiquitination occurs at the level of the plasma membrane (25), the defect on internalization is unlikely to be responsible for decreased ubiquitination (Fig. 7D), further suggesting that GRK6 activity is required for proper ubiquitination of CXCR4. Taken together, our data suggest that GRK6 contributes to CXCR4 degradation by facilitating ubiquitination of CXCR4.

**Heterologous regulation of CXCR4 lysosomal degradation by CXCR5**

Next, we examined whether PKC activation elicited by signaling from other members of the chemokine receptor family could promote CXCR4 downregulation. We focused on the chemokine receptor CXCR5 because it is endogenously expressed in HeLa cells and because its cognate ligand CXCL13 promotes signaling via a pertussis toxin sensitive G protein and is likely to activate PKC in these cells (36). We first examined whether CXCL13 mediates
CXCR4 internalization because it is a prerequisite for CXCR4 degradation (25). Stimulation with 100 nM CXCL13 promoted CXCR4 internalization, similar to levels that are observed with CXCL12 or PMA stimulation (Fig. 8A). Treatment with BisI almost completely blocked internalization by CXCL13 or PMA, but not CXCL12 (Fig. 8A) Additionally, CXCL13, similar to PMA, promoted rapid CXCR4 degradation (Fig. 8B and quantified in 8C), which was also almost completely blocked by BisI (Fig. 8D and quantified in 8E). Accordingly, PMA- or CXCL13-induced ubiquitination of CXCR4 was inhibited by BisI (Fig. 8E and quantified in 8G). Taken together, these data provide evidence that CXCR5 induces PKC-dependent heterologous internalization, ubiquitination and degradation of CXCR4.

**Discussion**

This study extends our knowledge of the role that kinases play in CXCR4 regulation. Here, we show that CXCR4 downregulation can be heterologously regulated by PKC or via PKC-dependent CXCR5 signaling. The phorbol ester PMA, a potent activator of PKC, mimics several aspects of CXCL12-promoted endocytic trafficking of CXCR4, including internalization and lysosomal targeting, leading to CXCR4 degradation (Figs. 1 & 2). Similar to CXCL12 (24), PMA induces dual phosphorylation of C-tail Ser residues (Ser-324/5) in a PKC-dependent manner (Fig. 3). Although PKC is necessary and sufficient for Ser-324/5 phosphorylation, it is not necessary for promoting CXCL12-mediated CXCR4 ubiquitination and degradation (Fig. 5). This suggested another kinase and/or phosphorylation of other C-tail sites were also able to support CXCL12-mediated degradation of CXCR4. Consistent with this hypothesis, depletion of GRK6 reduced CXCL12-mediated ubiquitination and degradation of CXCR4 (Figs. 7A-E). Accordingly, GRK6 has been previously linked to CXCL12-mediated dual phosphorylation of Ser-324/5 within the C-tail of CXCR4 (17). We also provide evidence that heterologous regulation of CXCR4 can occur in a PKC-dependent mechanism through activation of CXCR5 by its cognate chemokine CXCL13, resulting in CXCR4 internalization, ubiquitination and degradation (Fig. 8). Moreover, pretreatment of HeLa cells with either PMA or CXCL13 desensitizes early CXCR4 signaling as evidenced by reduced cAMP inhibition when challenged acutely with CXCL12 (Figs. 9B-C). Our data support the hypothesis that CXCR4 lysosomal trafficking is under tight heterologous regulation by PKC via activation of GPCRs, such as CXCR5, providing an additional layer of regulation that is likely required to fine-tune CXCR4 signaling.

PKC is responsible for phosphorylating multiple serine or threonine residues within the CXCR4 C-tail (27), however phosphorylation of Ser-324/325 is particularly important because it is necessary for mediating CXCR4 lysosomal trafficking (24,25). Dual phosphorylation of Ser-324/5 is required for recruitment of the E3
ubiquitin ligase AIP4 at or near the plasma membrane and subsequent ubiquitination of CXCR4, a prerequisite for its lysosomal trafficking and degradation (24). The precise PKC isoform responsible for phosphorylating Ser-324/5 cannot be discerned from our data because PMA activates (31) and BisI inhibits both conventional and novel PKC isoforms (39). However, a recent study suggests that a conventional PKC isoform may phosphorylate Ser-324/325 (40). Albeit sufficient, PKC is not necessary for CXCL12-stimulated ubiquitination and degradation of CXCR4 (Fig. 5). Inhibition of PKC with BisI completely blocked PMA promoted degradation of CXCR4, but it did not impact its ubiquitination or degradation by CXCL12 even though BisI blocks phosphorylation of Ser-324/325 (Fig. 4). Because the antibody used to detect phosphorylation at Ser-324/325 specifically recognizes the dual phosphorylated sites, it is possible that phosphorylation of only one of these sites still occurs following stimulation with CXCL12 in the presence of BisI, albeit by a kinase other than PKC. We have previously shown that phospho-mimetic mutants of CXCR4 at either Ser-324 or Ser-325 fully supports AIP4 binding (24). Thus, phosphorylation of either Ser-324 or Ser-325 may be sufficient to support ubiquitination and degradation of CXCR4. Whether Ser-324 or Ser-325 are individually phosphorylated by CXCL12 in the presence of BisI remains unknown, but a mass spectrometry based approach has suggested that Ser-325 can be individually phosphorylated (17). Alternatively, CXCL12 may promote phosphorylation of other serine or threonine residues in the C-tail of CXCR4 in the presence of BisI, a number of which have been shown to be phosphorylated by CXCL12 stimulation (17,40,41). The CXCR4 C-tail has 18 Ser/Thr residues, however the role of other residues on CXCR4 degradation in the context of PKC inhibition remains to be examined (17,41).

In addition to PKC, CXCR4 is phosphorylated by several GRKs, including GRK6, GRK2 and GRK3 (17,40). We focused on GRK6 in this study mainly because it has been linked to phosphorylation of Ser-324/325 (21,22,42). Accordingly, depletion of GRK6 by siRNA attenuated CXCR4 ubiquitination (Fig. 7D) and degradation (Fig. 7A), indicating that GRK6 is necessary for CXCR4 degradation. Similar to inhibiting PKC, it is possible that GRK siRNA impacts phosphorylation of either Ser-324 or Ser-325 individually, yet in contrast to inhibiting PKC, GRK6 siRNA reduces CXCR4 degradation by CXCL12 (Fig. 7A). One reason for this may be because in addition to Ser-324/325, GRK6 mediates phosphorylation of serine residues 330 and 339 following CXCL12 stimulation (17). Along with Ser-324/325, Ser-330 is part of a previously described degradation motif (25). Although we have determined that Ser-324/325 serve as a phospho-based interaction site for the E3 ubiquitin ligase AIP4 (24), the precise function of Ser-330 in CXCR4 lysosomal trafficking has yet to be defined. It has been previously shown that mutation of Ser-330 to alanine reduces CXCR4 degradation by CXCL12, but not its internalization (25), suggesting that phosphorylation of this residue is important for sorting CXCR4 into the degradative pathway at the level of the endosome. GRK6 also phosphorylates Ser-339 and adjacent Ser-338 (17), which are dually phosphorylated following stimulation with CXCL12 and also PMA (41). Mutation of these residues individually or simultaneously to alanine, did not have an impact on CXCR4 degradation induced by PMA or CXCL12 (data not shown). Taken together our data suggest that GRK6 may regulate CXCR4 lysosomal trafficking at the level of the plasma membrane and endosome via site-specific phosphorylation of CXCR4.

Although GRK6 may play an essential role in CXCR4 lysosomal degradation, it is possible that GRK2 and GRK3 may also have a role. GRK2 and GRK3 phosphorylate the C-tail of CXCR4 at sites that are not redundant to GRK6 (17,40). There is evidence to suggest phosphorylation of Ser-324/325 is under hierarchical regulation (41); that is, CXCL12 promoted phosphorylation of distal C-tail serine residues Ser-346/347 may be a prerequisite for efficient phosphorylation of Ser-324/325 or Ser-338/339 (41). Ser-346/347 may be phosphorylated by GRK2 or GRK3 (40,41), suggesting these kinases may also be involved in CXCR4 ubiquitination and degradation. However, in a previous study examining C-tail truncation mutants without Ser-
CXCR4 degradation by CXCL12 was not impacted (25). A reason for this may be because C-tail truncation mutants or Ser-346/347 point mutants only show reduced phosphorylation at Ser-324/325 (41), suggesting that sub-stoichiometric phosphorylation of these residues is not rate limiting and will only modestly impact receptor turnover by ubiquitination and lysosomal degradation. This is important because mutations in the gene encoding CXCR4 lead to C-tail truncation mutants which have been linked to the rare immunodeficiency disorder known as WHIM syndrome characterized by warts, hypogammaglobulinemia, infections and myelokathexis (43). Most of these truncation receptor mutants have an intact degradation motif, which contains Ser-324/325 and Ser-330 (43). Although these mutant receptors are typically unable to efficiently terminate acute signaling (12), however because the degradation motif is intact they are likely able to undergo efficient lysosomal degradation. The implication is that heterologous degradation of CXCR4 may represent a bona fide strategy to control excessive CXCL12 signaling associated with WHIM syndrome.

We provide evidence that CXCR4 lysosomal trafficking is cross-regulated by the chemokine receptor CXCR5 (Figs. 8 & 9). The cognate ligand for CXCR5, CXCL13, robustly mimics PMA- and CXCL12-mediated internalization, ubiquitination and degradation of CXCR4 (Fig. 8). BisI significantly attenuated CXCL13- and PMA-mediated regulation of CXCR4 internalization, suggesting that canonical Gαi-mediated signaling and PKC activation are responsible for these effects on CXCR4 trafficking. CXCL13 activation of PKC likely results in phosphorylation of Ser-324/325, the key serine residues that drive receptor ubiquitination and degradation (24,25). A recent study has suggested that CXCR4 and CXCR5 cross-talk through receptor heterodimerization to possibly regulate their respective signaling (44). In contrast, our data are consistent with the classical heterologous second-messenger dependent mechanism of regulation, which may not require an heteromeric receptor complex. Heterologous regulation of CXCR4 trafficking by other chemokine receptors has been previously reported (45), which may represent a common manner of regulation in the chemokine receptor family (46). Agonist activation of CXCR1 mediates phosphorylation and internalization of CXCR4 in a PKC-dependent mechanism (45). In addition to GPCRs, CXCR4 is heterologously regulated by non-GPCR signaling receptors. Activation of the antigen-specific B cell receptor in (BCR) triggers CXCR4 internalization through a PKC-mediated mechanism (26). Whether CXCR1 or BCR activation also promotes CXCR4 lysosomal degradation remains to be examined.

Heterologous degradation of CXCR4 regulates its acute signaling. We provide evidence that PMA or CXCL13 leads to desensitization of acute CXCL12-mediated Gαi responses, namely inhibition of adenylyl cyclase (AC) activity (Fig. 9). Because PMA or CXCL13 mediate CXCR4 internalization and sorting into the degradative pathway, it is likely that a loss in the full complement of receptor leads to a reduction in CXCR4 signaling. We propose that this is mediated in part by PKC phosphorylation of Ser-324/325, which promotes CXCR4 ubiquitination and degradation (24), suggesting a role for these residues in long-term downregulation of signaling. However, desensitization of CXCR4 signaling is also mediated by β-arrestins (18). CXCL12 stimulated phosphorylation of the C-tail promotes β-arrestin recruitment to the receptor (17,40) and rapid desensitization of CXCR4 signaling through G protein uncoupling (13). β-arrestin-2 recruitment to CXCR4 upon CXCL12 stimulation may be mediated by GRK3, but also PKC (40), therefore β-arrestins could account in part for the loss of CXCR4 signaling observed by PMA or CXCL13 pretreatment (Fig. 9). However, Ser-324/325 are not required for β-arrestin1/2 recruitment to CXCR4 by CXCL12 (17). Previously, we have shown that Ser-324/325 are required for recruiting the E3 ubiquitin ligase AIP4 to CXCR4 (24), further suggesting these residues have a role in long-term attenuation of signaling by controlling ubiquitination and lysosomal degradation of CXCR4. It is important to note that we have previously shown that β-arrestins do in fact
mediate CXCR4 lysosomal degradation, but not CXCR4 ubiquitination, suggesting they act at the level of the endosome and not the plasma membrane (47).

In summary, our study provides evidence that CXCR4 lysosomal degradation is subject to heterologous regulation via activation of CXCR5 and PKC. CXCR4 lysosomal degradation is subject to homologous regulation, which is at least in part mediated by GRK6. GRK6 or PKC mediates phosphorylation of Ser-324/325, which previously we have shown is essential for AIP recruitment, ubiquitination and ultimately CXCR4 lysosomal degradation (24). Targeting CXCR4 heterologously via other GPCRs might represent a useful strategy to antagonize aberrant CXCR4 signaling found in some chronic diseases.

Experimental procedures

Cell lines, antibodies and reagents - HEK 293 (Microbix, Toronto, Canada) and HeLa cells (ATCC) were cultured in DMEM or EMEM supplemented with 10% FBS (Atlanta Biologicals). Plasmid DNA transfections were done using the Trans-IT transfection reagent from Mirrus (Madison, WI) according to manufacturer’s instructions as we have previously described (47) or polyethylenimine (PEI, Polysciences Inc., catalog # 23966) (48). All siRNA transfections were done using Lipofectamine2000 (Invitrogen; Carlsbad, CA) according to manufacturer’s instructions, as we have previously described (36). Bisindolylmaleimide I (BisI; catalog no. 0741) and V (BisV; catalog no. 20303) were purchased from Tocris and Calbiochem, respectively. Forskolin (FSK) was purchased from Biomol (Plymouth, PA). Isobutylmethylxanthine (IBMX), N-ethylmaleimide (NEM), poly-L-lysine, dimethyl sulfoxide (DMSO) and the phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma. The p-nitrophenyl phosphate and diethanolamine buffer (catalog no. 9701861) was from Bio-Rad Laboratories. For RNA interference studies, the control and siGENOME SMARTpool targeting GRK6 was obtained from Dharmacon RNA Technologies (Lafayette, CO). Non-enzymatic cell dissociation solution, CellStripper was from Mediatech (Manassas, VA). The monoclonal antibody (2B11) and the phycoerythrin-labeled (12G5-PE) monoclonal antibody against CXCR4 were obtained from BD. The monoclonal antibody (5E11) against dual phosphorylated serine residues 324 and 325 was custom made and previously described (24). The mouse monoclonal antibodies against actin and GAPDH were purchased from MP Biomedicals (Solon, OH) and Abcam, respectively. The rabbit anti-GRK6 antibody (C-20) was purchased from Santa Cruz Biotechnology (Sta. Cruz, CA). DNA constructs (HA-CXCR4, FLAG-ubiquitin) and antibodies (monoclonal and polyclonal anti-HA, anti-LAMP1, anti-FLAG and anti-mouse IgG alkaline phosphatase conjugated) have been previously described in detail (25,37,49).

Analysis of CXCR4 degradation - Degradation of CXCR4 was measured essentially as previously described (25). For studies requiring siRNA transfection, HEK 293 or HeLa cells were transfected with 600 pmol/10-cm dish of control or GRK6 siRNA for a total of 72 h before performing degradation experiment. The day before experiments, cells were passaged into 6-well plates at 3.0×10^5 - 4.5×10^5 viable cells/well. Whenever using inhibitors, these were added in DMEM or EMEM for 30 min before agonist treatment unless otherwise noted. Subsequently, cells were incubated for 3 h with 10 nM CXCL12, 100 nM CXCL13 or 10 nM PMA using corresponding vehicle as control (water for CXCL12 or CXCL13 and ethanol for PMA) in the presence of 50 µg/mL of cycloheximide and the inhibitors. Cells were lysed in 2× sample buffer (8% SDS, 10% glycerol, 0.7 M mercaptoethanol, 37.5 mM Tris-HCl, pH 6.5, and 0.003% bromphenol blue) and equal amounts were resolved by 10% SDS-PAGE, followed by immunoblotting. HA-CXCR4 was detected using a monoclonal anti-HA antibody, while endogenous CXCR4 was detected with an anti-CXCR4 (clone 2B11) antibody. Immunoblots were subsequently stripped and re-probed with anti-actin or samples were run in parallel and immunoblotted for GAPDH. Bands were analyzed by densitometry and adjusted for loading using actin or GAPDH density as a correction factor.
Analysis of CXCR4 internalization by flow cytometry - HeLa cells transfected with control or GRK6 siRNA were dissociated using CellStripper according to manufacturer's instructions. For all subsequent steps, cells were washed and collected in PBS containing 0.1% BSA unless otherwise noted. For each sample, 5×10^5 cells were incubated at 37°C for 30 min with indicated concentrations of CXCL12. After centrifugation in cold PBS, cells were fixed with a solution of 3.7% paraformaldehyde in PBS, followed by two washes with PBS. Cells were then stained with the monoclonal anti-CXCR4 (12G5-PE) for 1h, followed by two PBS/BSA washes to remove unbound antibody. Fluorescent intensity was acquired using a FACSCanto II and analyzed using the FlowJo Software package (TreeStar, Ashland, OR).

Analysis of CXCR4 internalization by whole cell ELISA – Whole cell ELISA was used to measure CXCR4 internalization, essentially as we have previously described (36,48). HeLa cells grown on 10-cm dishes were transiently transfected with HA-CXCR4 (6 µg) using polyethylenimine (PEI). The next day, ~2×10^5 cells were seeded onto poly-L-lysine–coated 24-well plates. The next day, cells were serum starved for 1 h; pretreated with DMSO, 10 µM Bis I or BisV for 30 min, followed by stimulation with 10 nM CXCL12, 100 nM CXCL13, or 10 nM PMA for 5 min. Cells were fixed with 3.7% paraformaldehyde in Tris-buffered saline (TBS) for 5-10 min at room temperature. Cells were washed three times with TBS and incubated for 45 min in TBS supplemented with 1% bovine serum albumin (BSA) to block non-specific binding sites. Cells were incubated with an anti-HA monoclonal antibody diluted to 1:1000 for 1 h at room temperature. Cells were washed and incubated with alkaline phosphatase conjugated goat anti-mouse IgG diluted to 1:1000 in 1% BSA/TBS for 1 h at room temperature. After three rapid rinses with TBS, antibody binding was detected by adding 0.25 mL the alkaline phosphatase substrate p-nitrophenyl phosphate diluted in diethanolamine buffer. Reactions were stopped by removing 0.1 mL and adding it to a well of a 96-well microtiter plate containing 0.1 mL 0.4 M NaOH. The absorbance at 405 nm was read using a microtiter plate reader (FlexStation 3, Molecular Devices). Percent receptor internalization was calculated by subtracting the fraction of absorbance after agonist treatment and vehicle treatment (following background subtraction) from 1 and multiplied by 100.

Analysis of CXCR4 ubiquitination - Ubiquitination of CXCR4 was determined as we have previously described (49). Briefly, HEK293 cells stably expressing HA-CXCR4 were transfected with 3 µg of FLAG-tagged ubiquitin and 7 µg of empty vector (pCMV) per 10 cm-dish using Transit-LT1 transfection reagent following the manufacturer's instructions. Additionally, HEK293 cells were transiently transfected with HA-CXCR4 (3 µg) and FLAG-ubiquitin (3 µg) using PEI transfection reagent. Twenty-four hours after transfection, cells were passed into four 6-cm-dishes and grown for an additional 24 hours. The day of the experiment, cells were washed two times with DMEM containing 20mM HEPES and pre-treated with vehicle/10 µM BisI for 30 min before treating with 30 nM CXCL12 for 20 min or 10 nM PMA or 100 nM CXCL13 for 30 min. Medium was aspirated and cells were washed once with cold PBS followed by the addition of 1 mL of cold lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% Na-Deoxycholate, 1% NP40, 0.1% SDS) freshly supplemented with protease inhibitors (10 µg/mL each of aprotinin, pepstatin A, leupeptin) and 20 nM NEM. Subsequent HA-CXCR4 immunoprecipitation using 120-250 µg of lysate per samples and detection of ubiquitin-FLAG were performed as previously described (49).

Measurement of cAMP levels - HEK293 or HeLa cells grown in 10-cm dishes were transfected with plasmid DNA and control or GRK6 siRNA and split at 24-48 hrs later in 6-well plates (HeLa cells) or 24-well plates (HEK293 cells). At 48 h after transfection, cells were incubated with serum-free medium for 2-4 h, followed by a 30 min-incubation at 37°C with DMEM containing 1 mM IBMX and 20 mM HEPES (DMEM/IBMX). All subsequent incubations were performed at 37°C in IBMX/DMEM. HEK293 cells were first treated with 1 µM forskolin (FSK) for 5 min and subsequently
incubated with vehicle or increasing doses of CXCL12 for an additional 5 min. For HeLa cells, FSK and CXCL12 were added at the same time and incubated at 37°C for 10 min. After treatment, agonists were removed by aspiration and one wash with cold PBS containing 1mM IBMX. Thereafter, cells were lysed at approximately 1×10^6 cells/mL using 0.1 N HCl/0.5% TX-100 and incubated for 15 min at room temperature while rocking to allow for complete lysis. The concentration of cAMP in each sample was determined using a colorimetric cAMP EIA kit (Assay Designs) following the manufacturer’s instructions. Samples and standards were run in duplicate and averaged. Concentration of cAMP was determined by extrapolation of sample average into the standard curve subjected to a four-parameter fit as recommended by the manufacturer. To calculate the effect of CXCL12 treatment on cAMP levels, FSK+CXCL12 values were divided by the cAMP levels of samples treated with FSK+vehicle multiplied by 100.

pGlo-based cAMP reporter assay - HeLa cells were transfected in a 10-cm dish with 2 µg pGlo-22 sensor plasmid obtained from Mark Von Zastrow and Nikoleta Tsvetanova (UCSF) using PEI. Twenty-four hours later, cells were passed in triplicate into a white-walled 96-well plate (CELLSTAR) at a seeding density of 30,000 cells/well. The following day, cells were incubated for 1 hr at 37°C with phenol-free MEM (Gibco) containing 10% FBS (Atlanta Biologicals), 3.2 mM luciferin (GoldBio), and 50 µg/mL cycloheximide (Sigma). Cells were pre-treated for 30 minutes with increasing concentrations of CXCL13 (0 nM, 10 nM, 100 nM, 1 µM) at 37°C. Luminescent readings were taken at 25°C, the optimal temperature for the pGlo sensor (50), at an integration of 1 second every minute for 20 minutes to obtain a baseline measurement using a Flex Station 3 plate reader (Molecular Devices). Then, vehicle or 10nM CXCL12 was added in the presence of 10 µM forskolin (Millipore-Sigma) and 500µM IBMX (Biomol), and luminescence was measured for 10 minutes at 25°C. Values were plotted for each condition, and the area under each curve was calculated to determine cAMP levels. Vehicle and CXCL12 values within the same pre-treatment group were compared to determine % cAMP inhibition, with the resulting value being normalized to the control pre-treatment group not receiving CXCL13.

Confocal immunofluorescence microscopy – For detection of phosphorylated CXCR4 serine residues 324 and 325, HEK293 or HeLa cells grown on 10-cm dishes were transfected with 5 µg of HA-CXCR4-YFP and passed 24 h later onto coverslips pre-treated with poly-lysine, essentially as we have previously described (51). The next day, cells were washed once with DMEM/20 mM HEPES and serum-starved for 3-4 h in the same medium, followed by treatment with vehicle, 10 nM CXCL12 or 10 nM PMA for 15-30 min at 37°C. For the PKC inhibitor experiment, cells were pre-treated with 10 µM BisI or DMSO for 30 min at 37°C. Following treatment, cells were fixed, permeabilized and immunostained using a custom mouse monoclonal antibody specific for dually phosphorylated serine residues 324 and 325 (pSer324/325) (Clone 5E11), as previously described (24,52). Images were acquired using a Zeiss LSM 510 laser scanning confocal microscope with a 63x W Apochromat oil-immersion objective. Image acquisition settings across parallel samples were identical. Image analysis was done with Image J software (NIH). Individual cells were traced in each field of view to define regions of interest (ROI) and the mean fluorescence intensity for CXCR4-YFP and pSer324/325 was measured. For each ROI, pSer324/325 intensity was divided by HA-CXCR4-YFP intensity to normalize CXCR4 phosphorylation to receptor expression.

For detection of LAMP1-positive lysosomes (LAMP1+) and colocalization analysis, HEK293 cells were grown in 6-well plates and transfected with 250ng HA-CXCR4-YFP. Cells were passaged onto PLL-coated coverslips and serum-starved as above, followed by treatment with vehicle, 10 nM CXCL12 or 10 nM PMA for 3 hours. Following treatment, cells were fixed in ice-cold 4% PFA, 0.1 M sodium phosphate buffer (pH 7.0) for 15 minutes and immunostained with an antibody against LAMP1, as we have previously described (37). Images were acquired
as z-stacks at 60× (N.A. 1.4) using an Olympus Disk Spinning confocal microscope controlled by CellSens Dimensions software (Olympus). Acquisition settings for each channel were identical across parallel samples. Z-slices were spaced at 0.3 µm and acquired using the online deblur tool to remove out of focus light. The total number of LAMP1+ lysosomes and colocalization with CXCR4-YFP+ puncta were determined using 3D object-based analysis (52-54). Briefly, background was subtracted and auto-thresholding was used to create binary images. HA-CXCR4-YFP+ and LAMP1+ puncta were segmented and quantified using the 3D Objects Counter Plugin (ImageJ)(52). Centroids from HA-CXCR4-YFP+ puncta and LAMP1+ objects from resultant labeled images were converted to binary masks and then multiplied to quantify the number of overlapping HA-CXCR4-YFP+ and LAMP1+ puncta.

Statistical Analysis - Data are represented as the mean ± standard deviation of at least three experiments or determinations. All statistical tests were done using GraphPad Prism version 7.0d for Mac OS X (GraphPad Software, San Diego, CA). Student’s t-test was used to compare the difference between two groups, one-way analysis of variance (ANOVA) was used to compare the difference between three or more groups, and two-way ANOVA was used to compare the difference between different groups under different treatment conditions. ANOVA was followed by Tukey’s or Bonferroni’s post-hoc test, as indicated in figure legends. Colocalization studies were analyzed using Welch’s ANOVA followed by Tamhane’s T2 multiple comparisons test. A probability (p) value < 0.05 was considered significant. Specific values are provided in the figure panels or in the figure legends.
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FOOTNOTES
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The abbreviations used are: CXCR, CXC motif chemokine receptor; CXCL, CXC motif chemokine ligand; PKC, protein kinase C; GPCR, G protein-coupled receptor; GRK, GPCR kinase; AIP4, atrophin interacting protein 4; PMA, phorbol myristate acetate; EtOH, ethanol; BisI, Bisindolylmaleimide I; cAMP, cyclic AMP;

FIGURE LEGENDS

Figure 1. Characterization of PMA-mediated CXCR4 degradation.
(A-B) HEK293 cells stably expressing HA-CXCR4 were treated with increasing doses of PMA for 3 hr (n=6) (A) or for the indicated times with 10 nM of PMA (n=3) (B). Graphs represent the average amount of HA-CXCR4 degraded compared to its vehicle control at each dose (A) or time (B) ± S.D. Data were analyzed by a one-way ANOVA, followed by Tukey’s post hoc test. In panel A, the adjusted p values between: *, 1 and 10 nM = 0.0185; **, 1 and 100 nM = 0.0088; ***, 1 and 1000 = 0.0078. In panel B, the adjusted p values between: *, 0.5 and 3 hr = 0.0032; **, 0.5 and 6 hr = 0.0042. (C) HEK293 cells stably expressing HA-CXCR4 were pre-treated with 100 µM of chloroquine (+CQ) or not (No CQ) for 30 min before treatment with 10 nM CXCL12, 10 nM PMA or corresponding vehicle (n=3) for 3 hr. Representative immunoblots are shown. The positions of the molecular weight markers are indicated. Average percent degradation of HA-CXCR4 was calculated as described in Experimental Procedures. Error bars represent S.D. In panel C, data were analyzed by a two-way ANOVA, followed by Tukey’s post hoc test. Adjusted p values are indicated.

Figure 2. PMA and CXCL12 promote trafficking of CXCR4 to lysosomes.
(A-C) HEK293 cells were transiently expressing HA-CXCR4-YFP were treated with either vehicle, 100 nM PMA or 10 nM CXCL12 for 3 hours and then coimmunostained with antibodies against LAMP1 for multilabel immunofluorescence microscopy and colocalization analysis. (A) Representative micrographs of cells treated with vehicle (top panel), PMA (middle panel), or CXCL12 (bottom panel) showing CXCR4-YFP+ puncta (outer left panel) and LAMP1+ lysosomes (inner left panel) in single-channel and merged images (outer right panel). Insets show boxed areas enlarged 5×. Scale bar equals 10 µm. (B) Quantification of the mean percentage of LAMP1+ lysosomes colocalized with CXCR4-YFP+ puncta in cells treated with vehicle, PMA, or CXCL12. The total number of LAMP1+ lysosomes and colocalization with CXCR4-YFP+ puncta were determined using 3D object-based analysis, as described in Experimental Procedures. Bars represent the average from 3 independent experiments. Error bars represent 95.00% CI. The following number of cells were used in the analysis: vehicle, n = 32 cells; for PMA, n = 21 cells; and for CXCL12, n = 31 cells. Data were analyzed using Welch’s ANOVA followed by Tamhane’s T2 multiple comparisons test. Adjusted p values are indicated.

Figure 3. PKC is sufficient to mediate site-specific C-tail phosphorylation of CXCR4.
(A) Representative micrographs of HEK293 cells transiently expressing HA-CXCR4-YFP and immunostained to detect simultaneous phosphorylation of serine residues 324 and 325 (pSer324/325). Cells were serum-starved for 3.5 hours then stimulated with either vehicle, 10 nM PMA or 10 nM CXCL12 for 30 minutes. Panels show HA-CXCR4-YFP (left panel) and
pSer324/325 (center panel) as single-channel grayscale and pseudocolored, merged images (right panel) for the indicated treatment conditions. Images were processed in ImageJ, as described in Experimental Procedures. For optimal viewing, HA-CXCR4-YFP and pSer324/325 signal intensities were adjusted with opacity screens in Photoshop. Screens for HA-CXCR-YFP were adjusted to offset between group variation in expression; screens for pSer324/325 were adjusted within each group to match adjustments in HA-CXCR4-YFP. Scale bar equals 10 µm. (B) Quantification of pSer324/325:HA-CXCR4-YFP fluorescence intensities. Bars represent the average from 3 independent experiments. Error bars represent 95.00% CI. For Vehicle, n = 13 cells; for PMA, n = 15 cells; for CXCL12, n = 17 cells. Data are presented as mean pSer324/325:HA-CXCR4-YFP fluorescence intensity ratios. Data were analyzed using Welch’s ANOVA followed by Tamhane’s T2 multiple comparisons test. Adjusted p values are indicated.

Figure 4. PKC is necessary for site-specific C-tail phosphorylation of CXCR4.
(A) Representative micrographs of HEK293 cells transiently expressing HA-CXCR4-YFP and immunostained to detect simultaneous phosphorylation of serine residues 324 and 325 (pSer324/325). Cells were serum-starved for 3.5 hours and then pre-treated with either vehicle (DMSO) or BisI for 30 minutes and then stimulated with either vehicle or 10 nM CXCL12 for 30 minutes. Panels show HA-CXCR4-YFP (left panel) and pSer324/325 (center panel) as single-channel grayscale and pseudocolored, merged images (right panel) for the indicated treatment conditions. Images were processed in ImageJ, as described in Experimental Procedures. For optimal viewing, HA-CXCR4-YFP and pSer324/325 signal intensities were adjusted with opacity screens in Photoshop. Screens for HA-CXCR-YFP were adjusted to offset between group variation in expression; screens for pSer324/325 were adjusted within each group to match adjustments in HA-CXCR4-YFP. Scale bar equals 10 µm. (B) Quantification of pSer324/325:HA-CXCR4-YFP fluorescence intensities. Bars represent the average from 3 independent experiments. Error bars represent 95.00% CI. For DMSO: vehicle, n = 8 cells; for DMSO: CXCL12, n = 19 cells; for BisI: vehicle, n = 5 cells, for BisI: CXCL12, n = 14 cells. Data were analyzed using a two-way ANOVA followed by Tukey’s test for multiple comparisons. Adjusted p values are indicated.

Figure 5. PKC is sufficient, but not necessary, for CXCL12-mediated CXCR4 degradation.
(A-D) HEK293 cells transiently transfected with HA-CXCR4 (A) or HeLa cells (C) were pre-treated with 10 µM BisI or DMSO equivalent for 30 min, before treatment with 10 nM each of CXCL12 or PMA for 3 hours. (A,C) Representative immunoblots are shown. The positions of the molecular weight markers are indicated. (B,D) Degradation of HA-CXCR4 (n = 5-6) (B) and endogenous CXCR4 (4-5) (D) was measured and calculated as described in Experimental Procedures. Bars represent the average receptor degraded ± S.D. Data were analyzed by a two-way ANOVA followed by Tukey’s post hoc test. Adjusted p values are indicated. (E) HEK293 cells stably expressing HA-CXCR4 and transiently transfected with FLAG-ubiquitin were pre-treated with 10 µM BisI or DMSO equivalent and stimulated for 20 min with 30 nM CXCL12 in the presence or absence of BisI. Detection of ubiquitinated CXCR4 was performed as described in Experimental Procedures. Representative immunoblots are shown. (F) CXCR4 ubiquitination was quantified by densitometric analysis. Ubiquitin levels were normalized to CXCR4 levels in immunoprecipitates and CXCR4 ubiquitination is expressed as a fraction of control, or the values from DMSO and CXCL12 treated cells. Bars represent the average from 3 independent experiments ± S.D. Data were analyzed by a two-way ANOVA and there was a marginally significant effect between vehicle and CXCL12 (p 0.0519).
Figure 6. Analysis of the role of S324 and S325 in PKC-mediated CXCR4 degradation.

(A) HEK293 cells transfected with wild-type and serine receptor mutants were pre-treated with 10 µM BisI or vehicle (Veh; DMSO) for 30 min, before treatment with 10 nM CXCL12 for 3 hours. Representative immunoblots are shown. The positions of the molecular weight markers are indicated. (B) Densitometric analysis of CXCR4 degradation. Bars represent the average receptor degraded from 4 independent experiments ± S.D. Data were analyzed by two-way ANOVA, followed by Tukey’s multiple comparisons test. The adjusted p values between the following comparisons are: #, WT:Veh vs. S324/5A:Veh = 0.0003; *, WT:BisI vs. S324/5A:BisI = 0.0004; ##, WT:BisI vs. S325A:BisI = 0.0175; **, S325A:Veh vs. S325A:BisI = 0.0523.

Figure 7. Role of GRK6 in CXCR4 ubiquitination and degradation.

(A) HeLa cells transfected with control (Ctrl) or GRK6 siRNA were stimulated with vehicle or 10 nM CXCR4 for 3 hours. Representative immunoblots are shown. The positions of the molecular weight markers are indicated. (B) Samples prepared in parallel from cells transfected with siCTRL or siGRK were analyzed by immunoblotting for GRK6 abundance. Representative immunoblots are shown. (C) CXCR4 degradation was measured and calculated as described in Experimental Procedures. Bars represent the average receptor degradation from 6 independent experiments ± S.D. Data were analyzed by a Student’s t-test. Adjusted p value is indicated. (D) HEK293 cells stably expressing HA-CXCR4 were transfected with control (CTRL) or GRK6 siRNA and co-transfected one day later with FLAG-ubiquitin. The day of the experiment cells were treated with 30 nM CXCL12 or vehicle for 20 min. Detection of ubiquitinated CXCR4 was performed as described in Experimental Procedures. Representative immunoblots are shown. (E) CXCR4 ubiquitination was quantified by densitometric analysis. Ubiquitin levels were normalized to CXCR4 levels in immunoprecipitates and CXCR ubiquitination is expressed as a fraction of the siCTRL and CXCL12 treated cells. Bars represent the average from 3 independent experiments ± S.D. Data were analyzed by two-way ANOVA, followed by Sidak’s post hoc test. Adjusted p values are indicated. (F) HeLa cells transfected as described in A were dissociated and aliquoted at 5´10^5 viable cells per tube. Each tube received the indicated concentrations of CXCL12 and was immediately incubated at 37°C for 30 min. Thereafter, cells were stained with the mouse anti-CXCR4 12G5-PE antibody, as described in Experimental Procedures. Data are presented as percent of the geometric mean fluorescent intensity of each sample relative to that of vehicle treated cells. Data represent the average of 3-5 independent experiments ± S.D. The dashed line indicates the location of X-axis segmentation. Data were analyzed by two-way ANOVA followed by Tukey’s post hoc test. Asterisk (*) represents adjusted p value 0.0355.

Figure 8. Role of CXCR5 on cross-regulation of CXCR4.

(A) CXCR4 internalization was examined in HeLa cells stimulated with 10 nM CXCL12, 100 nM CXCL13, or 10 nM PMA for 5 min. Bars represent the average receptor internalized from 5 independent experiments ± S.D. (B) CXCR4 degradation was examined in HeLa cells stimulated with 10 nM CXCL12 or 100 nM CXCL13 for the indicated times. Representative CXCR4 immunoblot is shown. Lower panel, section of stain-free loading control used for densitometric analysis. The positions of the molecular weight markers are indicated. (C) Densitometric analysis of CXCR4 levels. Bars represent the average receptor degraded normalized to a stain-free loading control at each time point relative to vehicle. Error bars represent the S.D. (D) HeLa cells were pretreated with DMSO 10 µM BisI or 10 µM BisV for 30 min, before treatment with 10 nM CXCL12, 100 nM CXCL13, or 10 nM PMA for 3 h. Whole cell lysates were analyzed by immunoblotting for CXCR4. Lower panel, GAPDH immunoblot shown as loading control. Positions of molecular weight markers are indicated. (E) CXCR4 degradation was measured by densitometric analysis. Bars represent the average receptor degraded normalized to GAPDH with each ligand treatment compared to vehicle. Error bars represent the S.D. The data in A and E were analyzed by a two-way ANOVA, followed by Tukey’s multiple comparisons test. Adjusted p
values are indicated. (F) PKC is necessary for CXCL13-promoted ubiquitination of CXCR4. HEK293 cells transfected with FLAG-ubiquitin and HA-CXCR4 were treated with 10 nM PMA or 100 nM CXCL13 for 30 minutes in the presence or absence of 10 μM BisI. Detection of ubiquitinated CXCR4 was performed as described in Experimental Procedures. Representative immunoblots are shown. (G) CXCR4 ubiquitination was quantified by densitometric analysis. Ubiquitin levels were normalized to CXCR4 levels in immunoprecipitates and CXCR4 ubiquitination is expressed as a fraction of control, or the values from the DMSO and vehicle treated cells. Bars represent the average from 3 independent experiments ± S.D. Data were analyzed by two-way ANOVA, followed by Tukey’s post hoc test. Adjusted p value between vehicle:DMSO vs. CXCL13:DMSO is indicated. The asterisk (*) represents the adjusted p value 0.0467 between CXCL13:DMSO vs. CXCL13:BisI.

Figure 9. PMA and CXCL13 promote desensitization of CXCR4.

(A) HeLa cells were pulsed for 10 min with either vehicle or 10 nM CXCL12 in the presence of 1 μM forskolin (FSK), after which cells were lysed and processed to determine the concentration of cAMP by an EIA assay, as described in Experimental Procedures. Bars represent the average cAMP levels of 8 samples from 4 independent experiments ± SD normalized to cAMP levels of forskolin (FSK) in the absence of CXCL12. Data were analyzed by an unpaired t-test. The adjusted p value is indicated. (B) HeLa cells were pre-treated with PMA (1 nM or 10 nM) or vehicle for 1 hr at 37°C. Subsequently, cells were pulsed for 10 min with 10 nM CXCL12 in the presence of 1 μM FSK. Cells were lysed and cAMP levels were determined by EIA as in panel A. Bars represent the average desensitization of CXCL12-induced inhibition of cAMP levels from 4 independent experiments ± SD. CXCL12 promoted inhibition of cAMP compared to vehicle in cells pretreated with 1nM or 10 nM PMA was 72% ± 10% and 20% ± 7%, respectively. Data were analyzed by a one-way ANOVA, followed by Tukey’s post hoc test. The adjusted p values are as follows: vehicle vs. 1nM PMA, * = 0.0114; and vehicle vs. 10 nM PMA, ** < 0.0001. (C) cAMP levels were determined in HeLa cells transfected with pGlo22-cAMP sensor plasmid, as described in Experimental Procedures. Cells were pre-treated with either CXCL13 (10 nM, 100 nM or 1000 nM) or vehicle for 30 min at 37°C and the pulsed for 10 min with 10 nM CXCL12 at 25°C. Luminescence recordings were made as described in Experimental Procedures. Bars represent the average desensitization of CXCL12-induced inhibition of cAMP levels from 3 independent experiments ± SD. CXCL12 promoted inhibition of cAMP in cells pretreated with 1 nM, 10 nM or 1000 nM CXCL13 was 119% ± 31%, 49% ± 7% and 18% ± 11%, respectively, compared to vehicle. Data were analyzed by a one-way ANOVA, followed by Tukey’s post hoc test. The adjusted P values compared to vehicle for 1nM CXCL13, * = 0.5024; 10 nM CXCL13, ** = 0.0135; and 1000 nM CXCL13, *** = 0.0008.
Figure 1. Caballero et al.
Figure 2. Caballero et al.
Figure 3. Caballero et al.
**Figure 4.**

**A**

|          | CXCR4-YFP | pSer324/325 | MERGED |
|----------|-----------|-------------|--------|
| **DMSO** | ![](image1) | ![](image2) | ![](image3) |
| **CXCL12** | ![](image4) | ![](image5) | ![](image6) |
| **Bis** | ![](image7) | ![](image8) | ![](image9) |
| **CXCL12** | ![](image10) | ![](image11) | ![](image12) |

**B**

- **CXCL12:**
  - DMSO: p = 0.0321
  - BisI: p = 0.9803

Caballero et al.
**Figure 5. Caballero et al.**

(A) HEK293 cells were treated with DMSO or BisI in the presence or absence of CXCL12 and PMA. Western blots show the levels of HA-CXCR4 and Actin.

(B) Graph showing the percentage of HA-CXCR4 degraded under different conditions.

(C) HeLa cells were treated similarly. Western blots show the levels of CXCR4 and Actin.

(D) Graph showing the percentage of CXCR4 degraded under different conditions.

(E) Western blots showing the levels of FLAG-Ub, HA-CXCR4, and Lysate in the presence or absence of BisI.

(F) Graph showing the fraction of CXCR4 ubiquitination under different conditions.
Figure 6. Caballero et al.
Figure 7. Caballero et al.
Figure 8. Caballero et al.
Figure 9. Caballero et al.
Heterologous regulation of CXCR4 lysosomal trafficking
Adriana Caballero, Sarah A. Mahn, Mudassir S. Ali, M. Rose Rogers and Adriano Marchese

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