Trafficking of the HIV Coreceptor CXCR4

ROLE OF ARRESTINS AND IDENTIFICATION OF RESIDUES IN THE C-TERMINAL TAIL THAT MEDIATE RECEPTOR INTERNALIZATION*†

(Received for publication, May 17, 1999, and in revised form, July 23, 1999)

Michael J. Orsini‡, Jean-Luc Parent‡§, Stuart J. Mundell, and Jeffrey L. Benovic¶
From the Department of Microbiology and Immunology, Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

The G protein-coupled chemokine receptor CXCR4 serves as the primary coreceptor for entry of T-cell tropic human immunodeficiency virus. CXCR4 undergoes tonic internalization as well as internalization in response to stimulation with phorbol esters and ligand (SDF-1α). We investigated the trafficking of this receptor, and we attempted to define the residues of CXCR4 that were critical for receptor internalization. In both COS-1 and HEK-293 cells transiently overexpressing CXCR4, SDF-1α and phorbol esters (PMA) promoted rapid internalization of cell surface receptors as assessed by both enzyme-linked immunosorbent assay and immunofluorescence analysis. Expression of GRK2 and/or arrestins promoted modest additional CXCR4 internalization in response to both PMA and SDF. Both PMA- and SDF-mediated CXCR4 internalization was inhibited by coexpression of dominant negative mutants of dynamin-1 and arrestin-3. Arrestin was also recruited to the plasma membrane and appeared to colocalize with internalized receptors in response to SDF but not PMA. We then evaluated the ability of CXCR4 receptors containing mutations of serines and threonines, as well as a dileucine motif, within the C-terminal tail to be internalized and phosphorylated in response to either PMA or SDF-1α. This analysis showed that multiple residues within the CXCR4 C-terminal tail appear to mediate both PMA- and SDF-1α-mediated receptor internalization. The ability of coexpressed GRK2 and arrestins to promote internalization of the CXCR4 mutants revealed distinct differences between respective mutants and suggested that the integrity of the dileucine motif (Ile-328 and Leu-329) and serines 324, 325, 338, and 339 are critical for receptor internalization.

Chemokines comprise a family of between 40 and 50 members that mediate inflammatory responses, chemotaxis, immune cell development, and leukocyte homing (1–3). Their biological functions are mediated through their interaction with their cognate receptors, which are members of the G protein-coupled receptor (GPCR) superfamily. Multiple chemokine receptors have now been shown to act as coreceptors for entry of the human immunodeficiency virus (HIV) into cells (3, 4). The receptor for stromal derived factor-1α (SDF-1α), CXCR4, appears to act as the primary coreceptor for entry of T-cell tropic strains of HIV (5), whereas CCR5, which functions as the receptor for the chemokines RANTES, MIP-1α and MIP-1β, mediates entry of macrophage or M-tropic viruses (6). The simultaneous interaction of HIV envelope protein (gp120) with both the CD4 receptor and a chemokine receptor is required for viral entry, after exposure of a chemokine receptor-binding determinant mediated by the initial interaction of gp120 with CD4 (7–10). However, there are examples of chemokine receptor-dependent but CD4-independent entry of virus (11–14).

Much effort has been directed toward characterizing the interactions between gp120 and chemokine receptors and the role of chemokine receptors in mediating viral tropism (3, 15–18). Entry of HIV appears to be independent of the ability of the chemokine receptor to internalize or signal (19–22). Thus, therapeutic intervention has focused on the disruption of virus-coreceptor interactions, and small molecule inhibitors of this interaction have been identified and characterized (23–26). Recently, it has become apparent that interaction between gp120 or chemokines and chemokine receptors may activate signaling pathways that affect the ability of HIV to replicate (27–31). Furthermore, other molecules that activate HIV transcription or influence cellular gene expression have been shown to modulate HIV infectivity as well as chemokine receptor expression (32–37). In this regard, it has been shown that modulation of chemokine receptor expression on the cell surface correlates with HIV infectivity (38–41). A small molecule agonist of CCR5, aminooxyacetanilide-RANTES, has been reported to interfere with HIV binding to the receptor and also induces receptor internalization (40). To date, this is the only chemokine receptor agonist that appears to be able to block HIV infectivity both by disruption of HIV-coreceptor interaction and by promotion of chemokine receptor internalization.

In contrast to the interaction between HIV and coreceptors that occurs at the cell surface, far less is known regarding the mechanism of CXCR4 or CCR5 trafficking and signaling. An understanding of these mechanisms is needed in order to design effective HIV therapeutics directed against virus-chemokine receptor interactions or that promote clearance of cell surface coreceptors. Importantly, it must be ensured that such therapies do not inappropriately activate signaling pathways that induce activation or replication of existing virus in latently infected cells.

HIV, human immunodeficiency virus; SDF-1α, stromal derived factor-1α; RANTES, regulated and normal T-cell expressed and secreted; PMA, phorbol 12-myristate 13-acetate; HA, hemagglutinin; GPR, G protein-coupled receptor kinase; ELISA, enzyme-linked immunosorbent assay; GFP, green fluorescent protein; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline.

* This work was supported in part by National Institutes of Health Grant GM47419 (to J. L. B.) and National Institutes of Health Training Grant 5-T32-DK07705 (to M. J. O.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Both authors contributed equally to this work.

§ Recipient of a postdoctoral fellowship from the Medical Research Council of Canada.

¶ To whom correspondence should be addressed: Thomas Jefferson University, 233 S. 10th St., Philadelphia, PA 19107, Tel.: 215-503-4607; Fax: 215-923-1098; E-mail: benovic@lac.jci.tju.edu.

The abbreviations used are: GPCR, G protein-coupled receptor; G protein-coupled receptor family; RANTES, regulated and normal T-cell expressed and secreted; PMA, phorbol 12-myristate 13-acetate; HA, hemagglutinin; GPR, G protein-coupled receptor kinase; ELISA, enzyme-linked immunosorbent assay; GFP, green fluorescent protein; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline.
infected cells (29). Internalization and desensitization of an ever-growing number of GPCRs has been shown to require phosphorylation of agonist-activated receptors by a family of proteins known as G protein-coupled receptor kinases (GRKs). This promotes binding of a second family of proteins called arrestins, which act as adaptors between the receptor and components of the endocytic machinery, such as AP-2 and clathrin, both major components of clathrin-coated pits (reviewed in Refs. 42 and 43). This process also promotes dissociation of the receptor from heterotrimeric G proteins, thus terminating receptor signaling. From early endosomes, receptors may then be dephosphorylated and returned to the cell surface for another round of activation or enter a degradative pathway.

The determinants for receptor phosphorylation and internalization of both CCR5 and CXCR4 appear to reside in the C-terminal tail (21, 44). The involvement of GRKs and arrestins in CCR5 internalization and desensitization has been reported (21), and residues in the CCR5 C-tail that are phosphorylated in response to different CCR5 agonists have recently been identified (45). In this study, we sought to characterize the mechanism of CCR5 internalization, examine the role of GRKs and arrestins, and identify determinants in the CXCR4 C-tail that mediate receptor phosphorylation and internalization. We found that both phorbol ester and SDF-1α stimulation promoted CXCR4 internalization in a dynamin-dependent manner but that only SDF-1α-mediated internalization appeared to utilize an arrestin-dependent pathway. In contrast to the 7 serine and threonine residues in the CCR5 C-tail that are phosphorylated in response to different CCR5 agonists (44), only 5 residues in the CXCR4 C-tail may potentially be involved in receptor shut-off. CXCR4 contains 16 serine and threonine residues as well as a dileucine motif. Thus, we created a number of CXCR4 mutants to identify residues in the C-tail that mediate CXCR4 internalization and phosphorylation in response to either phorbol esters or SDF-1α stimulation. This analysis revealed that CXCR4 phosphorylation and internalization is mediated by multiple residues in the C-tail but that the dileucine motif and serines at positions 324, 325, 338, and 339 appear to be the most critical.

EXPERIMENTAL PROCEDURES
Plasmid Constructs—Wild-type and mutant CXCR4 receptors were subcloned in-frame into pCDNA3 containing a hemagglutinin (HA) epitope tag at the 5′ end constructed as described previously (46). Wild-type CXCR4 was also expressed in the vector pSR1 (a gift from Robert Doms, University of Pennsylvania) with equivalent results. CXCR4 mutants were constructed by oligonucleotide-directed polymerase chain reaction mutagenesis, using oligonucleotides up to 99 bases long. Polymerase chain reaction products were digested with EcoRI or EcoRI and XhoI and ligated with pCDNA3 cut with the same enzymes. All constructs were sequenced to confirm the correct orientation and to ensure that no undesired mutations were introduced. Constructs used to express arrestin-2, arrestin-3, GRK2, GRK2-K220R, dynamin-1-K44A, arrestin-3-1–320), and arrestin-3-284–409) have been previously described (47, 48).

Transient Transfections and Assay of Receptor Internalization—COS-1 or HEK-293 cells were grown in Dulbecco’s modified Eagle’s medium (Life Technologies Inc.) at 37 °C in a humidified atmosphere containing 95% air and 5% CO₂. Cells were grown to 70–80% confluence and transfected with FuGene-6 reagent (Roche Molecular Biochemicals) according to the manufacturer’s protocol. Receptor internalization was measured as described previously (48, 49). Briefly, cells in 60-mm dishes were transfected for 5 h and then split into 24-well dishes coated with poly-L-lysine (Sigma). The following day, cells were washed once with serum-free Dulbecco’s modified Eagle’s medium and stimulated with the indicated concentrations of phorbol 12-myristate-13-acetate (PMA, Alexis Corp.) or SDF-1α (Feprotech) for the times indicated. Media were aspirated, and cells were fixed for 10 min at room temperature with 3.7% formaldehyde in Tris-buffered saline (TBS). Cells were washed three times with TBS and then blocked for 45 min with 1% bovine serum albumin/TBS. Cells were then incubated for 1 h with a monoclonal antibody directed against the HA epitope (101R, Covance Biologicals) diluted 1:1,000. Cells were washed three times, reblocked for 15 min, and incubated for 1 h with goat anti-mouse alkaline phosphatase-conjugated antibody (Sigma or Bio-Rad) diluted 1:1,000. Cells were washed three times, and antibody binding was visualized by adding 0.25 ml of alkaline phosphatase substrate (Bio-Rad). Development was stopped by removing 0.1 ml of the substrate to a 96-well microtiter plate containing 0.1 ml of 0.4 m NaOH. Plates were read at 405 nm in a microplate reader (Bio-Rad) using Microplate Manager software.

Immunofluorescence Microscopy—To assess redistribution of CXCR4 and arrestin in living cells, HEK-293 cells were transfected with 1 μg of receptor and 0.25 μg of GFP-tagged arrestin-2. Cells were grown on poly-L-lysine-coated glass coverslips and mounted on an imaging chamber (Bioptics, Morton Instrument Corp.) equipped with an inlet port through which media and compounds could be perfused. For these studies, the medium used did not contain phenol red or antibiotics. Cell surface receptors were labeled at 4 °C by incubating cells with rhodamine-conjugated antibody directed against the HA epitope (12CA5, Roche Molecular Biochemicals). Cells were examined by microscopy on a Nikon Eclipse E800 fluorescence microscope using a Plan Fluor 40 × 0.75 objective. Images were collected using QED Camera software and processed with Adobe Photoshop. For confocal microscopy, cells were prepared in the same manner, and images were obtained on a Bio-Rad MRC-Zeiss Axiosver 100 confocal microscope (Hemmelholstede, UK) using a Zeiss Plan-Apo 63 × 1.40 NA oil immersion objective.

Receptor Phosphorylation—HEK-293 cells in 60-mm dishes were transfected with 2.5 μg of the indicated CXCR4 construct. 24 h later, cells were washed twice in PBS and incubated in medium modified Eagle’s medium and then incubated in the same medium for 2 h. Cells were then labeled with 0.2 mCi of [32P]orthophosphate (NEN, 8500–9120 Ci/mmol) for 90 min. Cells were then stimulated with either 1 μM PMA or 100 nM SDF-1α for 10 min. The medium was removed, and cells were washed once with TBS and lysed in 1 ml of ice-cold lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% (v/v) Nonidet P-40 (Igepal CA-630, Sigma), 5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM phenylmethylsulfonil fluoride, 20 μg/ml benzamidine, 10 μg/ml pepstatin, and 10 μg/ml leupeptin). All subsequent steps were performed on ice or at 4 °C. Cells were then sonicated for 20 s using a Fisher model 550 sonic dismembrator. Cell debris was pelleted by centrifugation in an Eppendorf centrifuge at 13,000 × g for 10 min. The supernatant was preclarified by adding 25 μl of a 50% slurry of protein A-agarose beads (Roche Molecular Biochemicals) with gentle rocking for 1 h. The beads were removed by centrifugation, and receptors were immunoprecipitated by adding 4 μl of anti-HA polyclonal antibody (101C, Covance Biologicals) to the supernatant for 1–2 h. 50 μl of protein A-agarose beads were added, and the suspension was incubated at 4 °C overnight. The following day, immune complexes were collected by centrifugation and washed 4 times with ice-cold lysis buffer. Beads were resuspended in 25 μl of sample buffer (62.5 mM Tris, pH 6.5, 10% β-mercaptoethanol, 10% glycerol, 5% sodium dodecyl sulfate, and 0.1 mg/ml bromphenol blue) and incubated at room temperature for 30 min. The immunoprecipitates were resolved on 10% SDS-PAGE gels. The gels were stained with Coomassie Blue, destained, dried, and subjected to autoradiography at 80 °C.

RESULTS
Characterization of PMA and SDF-1α-mediated CXCR4 Internalization and Role of Arrestins—We first determined the parameters for internalization of CXCR4 in response to both heterologous and homologous receptor stimuli. We used both COS-1 and HEK-293 cells for this analysis, since they have been shown to contain different amounts of GRKs and arrestins (50) which affect the extent of receptor internalization (50, 51). Specifically, COS-1 cells appear to contain lower amounts of endogenous GRKs and arrestins that result in inefficient receptor internalization (50). To quantify rapidly the receptor internalization under a variety of conditions, we used an ELISA that measures the level of epitope-tagged cell surface receptors. Thus, internalization is detected as a decrease in cell surface receptor levels compared with non-agonist-treated controls. We first examined CXCR4 internalization in response to its natural agonist, SDF-1α. 100 nM SDF-1α promoted internalization of approximately 40% of cell surface CXCR4 receptors (Fig. 1A). This amount of internalization was achieved by 30 min and did not increase upon longer incubation times.
SDF-1α promoted CXCR4 internalization in COS-1 cells with kinetics and magnitude nearly identical to that observed in HEK-293 cells. CXCR4 is also rapidly internalized in response to phorbol ester stimulation (52, 53). As shown in Fig. 1B, PMA stimulation of HEK-293 cells expressing CXCR4 resulted in internalization of 40–45% of surface receptors after 60 min. Internalization was first detected at 10 nM PMA and was maximal at 1 μM. Time course studies revealed that maximal receptor internalization occurred between 30 and 60 min after PMA addition, similar to the kinetics of SDF-1α-mediated internalization (data not shown). However, PMA-mediated internalization of CXCR4 was less efficient in COS-1 cells. Approximately 20–25% of cell surface CXCR4 was internalized in COS-1 cells in response to PMA (Fig. 1B). Inhibition of protein kinase C activity by treatment of cells with bisindolylmaleimide IX completely abrogated PMA-mediated internalization of CXCR4 but had no effect on SDF-1α-mediated internalization (Fig. 1C). No effect was observed upon treatment of cells with bisindolylmaleimide V, an inactive analog of bisindolylmaleimide IX. Therefore, internalization of CXCR4 in response to PMA appears to be mediated by activation of protein kinase C, whereas protein kinase C activity is not required for CXCR4 internalization in response to SDF-1α.

In contrast to CXCR4, internalization of CCR5 is not significantly promoted by phorbol ester and is relatively inefficient in response to stimulation by CCR5 ligands (21, 53). To compare directly CXCR4 and CCR5 internalization, we examined internalization of CCR5 in response to PMA or RANTES stimulation in HEK-293 cells. As shown in Fig. 2A, only 10–15% of surface CCR5 internalized in response to RANTES. RANTES-mediated CCR5 internalization was not promoted by coexpression of arrestin-2 or arrestin-3 and was modestly promoted by coexpression of GRK2. However, simultaneous overexpression of both arrestin-3 and GRK2 promoted CCR5 internalization in a synergistic manner. These results are similar to those obtained when CCR5 is stimulated by MIP-1β (21). PMA stimulation of CCR5 also did not promote significant internalization and overexpression of GRK2 or arrestins did not promote internalization. However, overexpression of both GRK2 and arrestin-3 promoted a modest but significant increase in CCR5 internalization (Fig. 2B). Taken together, our results indicate that CXCR4 and CCR5 have different requirements for internalization in the same cell type. These differences may result from different affinities of GRKs and arrestins for activated receptors.

We next addressed the question of whether CXCR4 internalization was an arrestin- or dynamin-dependent process. We hypothesized that SDF-1α-mediated activation of CXCR4 would promote receptor phosphorylation by GRKs and recruitment of arrestin. However, PMA may mediate CXCR4 internalization via direct phosphorylation of CXCR4 by protein kinase C, resulting in protein kinase C-mediated activation of...
GRK2 (54, 55). We cotransfected HEK-293 cells with CXCR4 and dynamin-K44A, two different arrestin-3 dominant negative mutants and a dominant negative mutant of GRK2. Dynamin-K44A is deficient in GTP binding and functions to inhibit dynamin-mediated scission of clathrin-coated vesicles from the plasma membrane (56). Arrestin-3-(1–320) lacks the clathrin binding domain and thus appears to act by competing with wild-type arrestin for receptor binding. Conversely, arrestin-3-(284–409) lacks the receptor binding region and thus appears to act by competing with wild-type arrestin for clathrin binding domain and thus appears to act by competing with wild-type arrestin for receptor binding. Alternatively, GRK2-K220R contains a mutation in the GRK2 catalytic domain but retains the ability to bind receptor and has been shown to act as a dominant negative GRK2 (57). As shown in Fig. 3, both arrestin-3-(284–409) and dynamin-K44A inhibited internalization of CXCR4 in response to both PMA (Fig. 3A) and SDF-1α (Fig. 3B). CXCR4 internalization was inhibited 75–90% by dynamin-K44A, whereas arrestin-3-(284–409) inhibited internalization by 50–80%. The greater efficiency of inhibition by dynamin-K44A is consistent with results obtained with other GPCRs (51, 58). In contrast, neither arrestin-3-(1–320) nor GRK2-K220R significantly inhibited internalization of CXCR4 in response to either stimulus (Fig. 3, A and B). The inability of arrestin-3-(1–320) and GRK2-K220R to inhibit CXCR4 internalization might result from a high affinity of endogenous GRKs and arrestins for activated CXCR4, resulting in inefficient competition of GRK2-K220R and arrestin-3-(1–320) for receptor binding. Alternatively, GRK2-K220R and arrestin-3-(1–320) may not efficiently interact with CXCR4.

Immunofluorescence Analysis of CXCR4 and Arrestins—Redistribution of arrestins from the cytosol to the plasma membrane upon receptor stimulation has been demonstrated for a number of GPCRs (59). Arrestins have also been shown to redistribute to clathrin-coated vesicles and colocalize with internalized GPCRs (60, 61). In order to assess redistribution of CXCR4 and arrestins after PMA or SDF stimulation, HEK-293 cells were transfected with CXCR4 and an arrestin-2-green fluorescent protein (GFP) chimera. Cell surface CXCR4 receptors were labeled by incubating cells at 4 °C using rhodamine-conjugated 12CA5 antibody directed against the N-terminal HA epitope. The initial localization of arrestin-2-GFP is shown in A and E, and the initial distribution of CXCR4 is shown in B and F. 100 nM SDF-1α (C and D) or 1 μM PMA (G and H) was added to cells, and redistribution of receptors and arrestin was monitored in real time using the time-lapse acquisition option (QED software) on a Nikon Eclipse E800 fluorescence microscope using a Plan Fluor 40 × 0.75 objective. Images shown were obtained at 20 min after agonist addition and were processed using Adobe Photoshop software.

Fig. 3. Effect of dominant negative dynamin, GRK2, and arrestin-3 on PMA and SDF-1α-mediated CXCR4 internalization. HEK-293 cells were transfected with 2.5 μg of CXCR4 and an equal amount of vector (pcDNA3), pcDNA-dynamin-K44A, pcDNA-arrestin-3-(284–409), pcDNA-arrestin-3-(1–320), or pcDNA-GRK2-K220R. Cells were stimulated with the indicated concentrations of PMA for 60 min (A) or 100 nM SDF-1α for the indicated times (B), and receptor internalization was quantitated by ELISA. All experiments were repeated 4–8 times, and results are expressed as the mean value ± S.E. □, vector; ○, dynamin-K44A; ▽, arrestin-3-(284–409); △, arrestin-3-(1–320); ▽, GRK2-K220R.

Fig. 4. Redistribution of CXCR4 and arrestin upon PMA and SDF-1α stimulation. HEK-293 cells were transfected with 1 μg of CXCR4 and 0.25 μg of arrestin-2-GFP and then split onto poly-L-lysine-coated glass coverslips. Prior to stimulation and viewing, coverslips were mounted in a chamber as described under “Experimental Procedures.” For visualization of CXCR4, surface receptors were first labeled at 4 °C using rhodamine-conjugated 12CA5 antibody directed against the N-terminal HA epitope. The initial localization of arrestin-2 GFP is shown in A and E, and the initial distribution of CXCR4 is shown in B and F. 100 nM SDF-1α (C and D) or 1 μM PMA (G and H) was added to cells, and redistribution of receptors and arrestin was monitored in real time using the time-lapse acquisition option (QED software) on a Nikon Eclipse E800 fluorescence microscope using a Plan Fluor 40 × 0.75 objective. Images shown were obtained at 20 min after agonist addition and were processed using Adobe Photoshop software.
occurred to the extent seen after SDF-1α stimulation. The redistribution of arrestin was completely dependent upon CXCR4 expression, since no change in arrestin-GFP localization was observed in cells treated with PMA or SDF-1α that were not transfected with CXCR4 (data not shown). CXCR4 and arrestin-2-GFP that were localized in punctate vesicles also exhibited a significant amount of colocalization with labeled transferrin, suggesting that these molecules are present in early endosomes (data not shown).

Recently, it has been reported that arrestins are recruited to the plasma membrane upon stimulation of cells transfected with β2-adrenergic, angiotensin 1a (AT1a), dopamine D1a, endothelin type A, and neurotensin receptors. However, only in the case of the AT1a and neurotensin receptors was arrestin observed to colocalize with internalized receptors (62). Therefore, we further explored the ability of stimulated CXCR4 to recruit arrestin to the plasma membrane by confocal microscopy. In cells cotransfected with CXCR4 and arrestin-2-GFP, SDF-1α stimulation promoted some redistribution of arrestin to the plasma membrane (Fig. 5, A and B). In contrast, we did not observe any redistribution of arrestin in response to PMA stimulation (Fig. 5, C and D). Even prolonged incubation times (up to 20 min) did not produce any change in the pattern of arrestin distribution (data not shown). The results of our immunofluorescence studies suggest that arrestin is recruited to the plasma membrane upon SDF-1α stimulation of CXCR4 and is colocalized with internalized receptors. In contrast, PMA stimulation of CXCR4 does not appear to promote arrestin recruitment to the plasma membrane or redistribution of arrestin with internalized receptor. Taken together, our results suggest that arrestins play an important role in mediating SDF-1α-promoted CXCR4 internalization. However, the ability of arrestin-3-(284–409) to attenuate PMA-mediated CXCR4 internalization does not appear to be due to direct interference with an arrestin-dependent pathway.

**Mutagenesis of CXCR4 and Internalization of Mutant Receptors**—The C-tail of CXCR4 has been shown to be required for agonist and PMA-mediated CXCR4 internalization (44). A recent study using mink lung epithelial cells (Mv-1-Lu) showed that Ser-324 and Ser-325 and the dileucine motif at amino acids 328 and 329 appear to be involved in phorbol ester- but not SDF-1α-mediated CXCR4 internalization (53). However, a detailed analysis of the residues required for SDF-1α- or phorbol ester-mediated internalization has not been reported. We therefore introduced multiple mutations into the CXCR4 C-tail, focusing on the serine and threonine residues as well as the dileucine motif. The C-tail mutations are depicted schematically in Fig. 6A, and their relative expression levels in HEK-293 cells are shown in Fig. 6B. The expression level and mobility of most of the CXCR4 mutants was comparable to wild-type CXCR4, although the IL3289A CXCR4 receptor migrated with slightly faster mobility compared with wild-type CXCR4 (Fig. 6B). This may be due either to the alteration of two hydrophobic residues or proteolytic degradation. In addition, we did not detect expression of either the H350stop or S34678A CXCR4 mutants by either ELISA or Western blot analysis, except when these constructs were expressed in the presence of dynamin-K44A (data not shown). A possible explanation for this result is that these receptors are poorly expressed but that coexpression of dynamin-K44A inhibits tonic receptor internalization and permits accumulation of detectable steady-state levels of receptor. To circumvent this problem and analyze the role of these residues in receptor internalization, we introduced a stop codon after the glutamic acid residue at position 345 that was expressed comparably to wild-type CXCR4 (Fig. 6B). All CXCR4 receptors used in this study were capable of mediating viral entry into CD4-expressing HEK-293 cells.2

We then determined the ability of the mutant CXCR4 receptors, expressed in either COS-1 or HEK-293 cells, to internalize in response to PMA or SDF-1α. The results of experiments that assessed PMA-mediated internalization of wild-type and mutant CXCR4 receptors in both COS-1 and HEK-293 cells are shown in Fig. 7A. In HEK-293 cells, the PMA-mediated internalization of the S330A CXCR4 receptor was as efficient as wild-type CXCR4, and internalization of E345stop was modestly decreased. The remaining mutant CXCR4 receptors internalized only 40–50% as well as wild-type CXCR4. Notably, no single mutation reduced PMA-mediated internalization greater than 60% compared with wild-type CXCR4, suggesting that multiple residues in the C-tail mediate internalization in

---

2 B. J. Doranz, unpublished observations.
of 6–16 separate experiments. The indicated wild-type or mutant CXCR4 receptors were expressed by transient transfection in HEK-293 or COS-1 cells and challenged with 1 μM PMA (A) or 100 nM SDF-1α (B) for 60 min. Internalization of receptors was quantitated by ELISA. The results are expressed as the percent internalization relative to wild-type CXCR4, which was regarded as 100%. All experiments were performed in triplicate, and results are expressed as the mean value ± S.E. of 6–16 separate experiments. □, HEK-293; ▫, COS-1.

response to PMA. This analysis was repeated in COS-1 cells, in which PMA-mediated CXCR4 internalization is less efficient compared with HEK-293 (Fig. 1B). Mutation of Ser-330 had no effect, and the ST3412A, S344A, and E345stop CXCR4 receptors internalized 40–50% less efficiently than wild-type CXCR4 (Fig. 7A). In contrast, PMA-mediated internalization of the S3389A CXCR4 mutant was almost completely undetectable, whereas internalization of S3245A and IL3289A mutant CXCR4 receptors was significantly reduced. It is possible that since PMA-mediated internalization of wild-type CXCR4 is less efficient in COS-1 cells, mutations in the CXCR4 C-tail have a more profound effect on CXCR4 internalization when expressed in COS-1 cells than in HEK-293 cells.

We performed a similar analysis of the ability of the different CXCR4 receptors to internalize in response to SDF-1α. The results of these experiments in both COS-1 and HEK-293 cells are shown in Fig. 7B. The S330A mutation resulted in an approximately 40% decrease in receptor internalization. In contrast, internalization of the S3245A, IL3289A, and S3389A mutants was reduced between 65 and 90%. Internalization of CXCR4 receptors containing mutations in the distal portion of the C-tail (ST3412A, S344A, and E345stop) was reduced by 50–60%. SDF-1α-mediated internalization of the mutant CXCR4 receptors in COS-1 cells appeared to closely resemble that observed in HEK-293 cells (Fig. 7B). The S330A mutation modestly decreased internalization, and internalization of ST3412A, S344A, and E345stop was reduced by 40–50%. However, mutation of the dileucine motif or the serines at positions 324, 325, 338, and 339 profoundly decreased internalization. Taken together, the dileucine motif and the serines at positions 324, 325, 338, and 339 appear to be critical for both PMA- and SDF-1α-mediated receptor internalization in both COS-1 and HEK-293 cells.

Effect of GRK2 and Arrestin Overexpression on Internalization of Mutant CXCR4 Receptors—Since multiple residues in the C-tail appear to mediate CXCR4 internalization, we attempted to define the most critical residues by coexpression of CXCR4 mutants with GRK2 and arrestins. We reasoned that overexpression of GRK2 and/or arrestins would promote internalization of CXCR4 receptors containing mutations in critical residues less efficiently compared with CXCR4 receptors containing mutations in residues that were less important for internalization or could be compensated for by the presence of wild-type residues at other positions. The result of this analysis is depicted in Fig. 8 for selected CXCR4 receptors and is summarized in Table I. Three distinct patterns of internalization were observed. First, overexpression of GRK2, arrestin-2, or arrestin-3 alone had little effect on SDF-1α-mediated internalization of wild-type CXCR4. However, coexpression of GRK2 and arrestin-3 promoted a significant and synergistic increase in receptor internalization in response to SDF-1α (Fig. 8A and Table I). Overexpression of arrestin-2, arrestin-3, or GRK2 alone modestly increased PMA-mediated internalization, and coexpression of GRK2 and arrestin-3 did not significantly enhance internalization compared with expression of either of these proteins alone (Fig. 8A and Table I). A similar effect of GRK2 and arrestin expression was observed with regard to SDF-mediated internalization of CXCR4 receptors containing mutations in the distal portion of the C-tail (ST3412A, S344A, and E345stop). Expression of GRK2, arrestin-2, or arrestin-3 alone had little or no effect on SDF-1α-mediated internalization of these receptors, whereas coexpression of GRK2 and

![Figure 7](image7.png)

**Fig. 7.** Effect of CXCR4 C-tail mutations on PMA and SDF-mediated internalization. The indicated wild-type or mutant CXCR4 receptors were expressed by transient transfection in HEK-293 or COS-1 cells and challenged with 1 μM PMA (A) or 100 nM SDF-1α (B) for 60 min. Internalization of receptors was quantitated by ELISA. The results are expressed as the percent internalization relative to wild-type CXCR4, which was regarded as 100%. All experiments were performed in triplicate, and results are expressed as the mean value ± S.E. of 6–16 separate experiments. □, HEK-293; ▫, COS-1.

![Figure 8](image8.png)

**Fig. 8.** GRK and arrestin coexpression differentially promotes internalization of CXCR4 mutants. HEK-293 cells were transiently transfected with 2.5 μg of wild-type CXCR4 (A), CXCR4(ST3412A) (B), CXCR4(IL3289A) (C), and CXCR4(ST3412A + IL3289A) (D) together with 2.5 μg of pCDNA3 (vector), GRK2, arrestin-2, arrestin-3 constructs, or 1.25 μg each of GRK2 and arrestin-3. Cells were stimulated with 100 nM SDF-1α or 1 μM PMA for 60 min, and receptor internalization was quantitated by ELISA. Experiments were performed in duplicate 4–8 times, and the results are expressed as the mean value ± S.E.
arrestin-3 promoted a synergistic increase in receptor internalization that was nearly equivalent to that of wild-type CXCR4 alone (Fig. 8B and Table I). Similar to wild-type CXCR4, expression of arrestin-2, arrestin-3, or GRK2 alone promoted a modest increase in PMA-mediated internalization of these receptors. In contrast to wild-type CXCR4, coexpression of GRK2 and arrestin-3 synergistically promoted internalization of these receptors in response to PMA. The exception to this pattern was E345stop, which may be related to the observation that PMA-mediated internalization of this receptor is less impaired compared with ST3412A or S344A.

The effect of GRK2 and/or arrestin overexpression on internalization of the IL3289A and S3389A CXCR4 receptors was dramatically different. Internalization of the IL3289A CXCR4 receptor mutant in response to PMA was modestly promoted by GRK2 or arrestin-3 expression, but coexpression of these proteins did not increase internalization further (Fig. 8C and Table I). In contrast, expression of arrestins had no effect on SDF-1α-mediated internalization of IL3289A, whereas GRK2 and GRK2/arrestin-3 modestly promoted internalization (Fig. 8C and Table I). The maximal amount of internalization of IL3289A was significantly less than wild-type CXCR4 in the absence of coexpressed GRK2 and arrestins, suggesting that the dileucine motif is critical for both PMA- and SDF-1α-mediated CXCR4 internalization. A similar effect of GRK2 and arrestin expression was observed with the S3389A CXCR4 receptor mutant, suggesting that these residues are also critical for CXCR4 internalization (Table I). However, the internalization of this mutant receptor in response to SDF-1α was approximately half that for PMA, suggesting that these residues are slightly more important for SDF-1α-mediated internalization.

The S3245A CXCR4 receptor behaved in a manner intermediate between these two patterns. PMA-mediated internalization of S3245A was modestly increased upon coexpression of arrestin-2 and arrestin-3, unaffected by GRK2 expression, and significantly promoted by simultaneous expression of GRK2 and arrestin-3 (Fig. 8D and Table I). In contrast, expression of arrestins or GRK2 had little or no effect on SDF-1α-mediated internalization of S32435A, but coexpression of GRK2 and arrestin-3 promoted internalization, albeit to a lesser extent than with PMA. Therefore, these residues may also be more critical for SDF-1α compared with PMA-mediated internalization. Taken together, the results of this analysis suggest that the

| Transfection | Stimulus | Vector | Arrestin-2 | Arrestin-3 | GRK2 | GRK2 + Arrestin-3 |
|-------------|----------|--------|------------|------------|------|------------------|
| CXCR4 (wild-type) | PMA | 100 ± 1.5 | 114.4 ± 3.0 | 124.7 ± 3.8 | 119.9 ± 4.5 | 130.8 ± 4.0 |
| S3245A | SDF | 100 ± 6.8 | 100.8 ± 4.5 | 110.1 ± 5.6 | 120.3 ± 5.6 | 174.9 ± 5.1 |
| IL3289A | PMA | 59 ± 4.3 | 62.6 ± 4.3 | 73.6 ± 6.1 | 54.0 ± 3.3 | 91.9 ± 3.0 |
| S3389A | E345stop | 52.4 ± 9.3 | 49.0 ± 7.3 | 50.0 ± 6.1 | 39.2 ± 5.1 | 74.9 ± 9.8 |
| ST3412A | IL3289A | 67.5 ± 7.5 | 73.9 ± 11.3 | 42.0 ± 4.2 | 45.9 ± 10.4 | 55.3 ± 6.3 |
| S344A | S3389A | 62.1 ± 3.5 | 65.7 ± 4.8 | 67.7 ± 7.8 | 61.7 ± 5.1 | 70.5 ± 4.5 |
| E345stop | S3389A | 75.5 ± 4.5 | 67.7 ± 4.8 | 77.7 ± 6.2 | 67.6 ± 11.0 | 102.0 ± 3.3 |

For many GPCRs, agonist activation results in phosphorylation of receptors by GRKs (42). However, involvement of arrestins in GPCR internalization in the absence of receptor phosphorylation has been reported (63, 64). In the case of CXCR4, phosphorylation could occur by several mechanisms, such as SDF-1α-mediated receptor activation and phosphorylation by GRKs, direct phosphorylation of receptor by protein kinase C as a result of phorbol ester stimulation, or phosphorylation by GRK2 as a result of its activation via phorbol ester-mediated activation of protein kinase C (54, 55). The serines at positions 344, 346, 347, and 348 are potentially good candidates for phosphorylation by GRK2, since they are C-terminal to an acidic residue (65). In contrast, the serines at positions 324, 325, 338, and 339 conform more closely to potential protein kinase C phosphorylation sites (66). Therefore, we analyzed wild-type and mutant CXCR4 receptors to determine the extent of receptor phosphorylation and to identify residues that were important for SDF-1α or PMA-mediated CXCR4 phosphorylation. To do this, HEK-293 cells were transfected with the different CXCR4 receptors, metabolically labeled with [32P]orthophosphate, and stimulated with PMA or SDF-1α for 10 min. HA-tagged receptors were immunoprecipitated, and phosphorylation of receptors was visualized by autoradiography. Upon PMA stimulation, phosphorylation of wild-type and all mutant CXCR4 receptors was increased by 2-3-fold (Fig. 9A). However, the CXCR4 receptors IL3289A and E345stop were phosphorylated to a lesser extent compared with wild-type CXCR4, whereas receptors containing the S330A, ST3412A, and S344A mutations were phosphorylated to a somewhat greater extent. Thus, receptor phosphorylation in response to PMA stimulation does not appear to closely correlate with the extent of internalization. The extent of receptor phosphorylation in response to SDF-1α stimulation is shown in Fig. 9B. Stimulation of wild-type CXCR4 by SDF-1α resulted in an approximately 2-fold increase in receptor phosphorylation after 10 min. However, the IL3289A, S3389A, and E345stop CXCR4 receptors did not appear to be significantly phospho-
in response to PMA or SDF-1.

The indicated constructs were transfected into HEK-293 cells. 24 h later, cells were washed and incubated for 2 h in serum and phosphate-free medium. The cells were metabolically labeled with [32P]orthophosphate (0.2 mCi/ml) for 90 min and then stimulated with 100 nM SDF-1α or 1 μM PMA (B) for 10 min. Cells were lysed, and HA-tagged CXCR4 receptors were immunoprecipitated and separated by SDS-PAGE as described under "Experimental Procedures." The dried gel was then subjected to autoradiography at -70 °C. The autoradiograms shown are representative of three independent experiments.

**DISCUSSION**

The chemokine receptor CXCR4 plays an important role in B-cell development as well as inflammation and cell migration (3, 67). In addition, this chemokine receptor is the major coreceptor for entry of T-cell tropic human immunodeficiency viruses. In this study, we have characterized the trafficking of CXCR4 in two different cell types. Internalization of CXCR4 in response to PMA or SDF-1α stimulation was inhibited by dominant negative forms of both dynamin-1 and arrestin-3. However, only SDF-1α appeared to promote CXCR4 internalization via an arrestin-dependent pathway, as assessed by immunofluorescence analysis of arrestin recruitment and redistribution after CXCR4 activation. In addition, we have identified residues in the C-tail of CXCR4 that mediate both receptor phosphorylation and internalization. The integrity of the dileucine motif and serines 324, 325, 338, and 339 appeared to be the most critical. Mutations at other positions in the C-tail also appeared to play a role in receptor internalization, but reduced internalization as a result of mutation of other serine and threonine residues could largely be compensated for by overexpression of GRKs and arrestins.

We noted differences between CXCR4 internalization and internalization of the other major coreceptor for HIV, CCR5. Although CCR5 is phosphorylated in response to PMA (45), this does not result in efficient receptor internalization (53), a result confirmed in this study. Similarly, CCR5 internalization was also inefficient in response to RANTES, one of its endogenous ligands, in agreement with a previous study using MIP-1α (21). However, RANTES-mediated CCR5 internalization was synergistically promoted by coexpression of both GRK2 and arrestin-3, whereas PMA-mediated internalization was modestly promoted. In contrast, CXCR4 internalization was efficient in the absence of GRK2 and arrestin overexpression in both HEK-293 and COS-1 cells. COS cells contain lower levels of endogenous GRKs and arrestins, and internalization of both α- and β-adrenergic receptors in COS cells is inefficient in the absence of overexpressed arrestins (47, 50, 51, 68). However, SDF-1α-mediated internalization of CXCR4 in COS cells was equivalent to that observed in HEK-293 cells, although PMA-mediated internalization was less efficient. Taken together, these observations suggest that the affinity of GRKs and arrestins present in the same cell types for agonist-activated CXCR4 may be higher than for the CCR5 and adrenergic receptors. Biochemical studies will be needed to understand better the determinants of GRK and arrestin affinity and specificity at the level of both receptor and effector.

CXCR4 was internalized in response to stimulation with PMA and SDF-1α as assessed by immunofluorescence of labeled cell surface receptors. However, we failed to observe either arrestin recruitment to the plasma membrane or redistribution of arrestin with internalized CXCR4 after PMA stimulation, suggesting that PMA-mediated CXCR4 internalization is not an arrestin-dependent process. It is possible that arrestin interaction with CXCR4 activated by PMA may be very transient and thus was not detected under our conditions. Interestingly, both PMA- and SDF-mediated CXCR4 internalization was inhibited by coexpression of dynamin-K44A and dominant negative arrestin-3. Expression of dynamin-K44A, which lacks the ability to bind GTP, almost completely ablated internalization in response to both stimuli. Arrestin-3-(284–409), which competes for clathrin binding, inhibited internalization by approximately 50%. Inhibition of receptor internalization by dynamin-K44A occurs at a very early step in endocytosis, i.e. pinching off of clathrin-coated pits. Inhibition of internalization by arrestin-3-(284–409), which encompasses the clathrin binding domain, is predicted to occur by competition with endogenous arrestins for clathrin binding. Thus, expression of arrestin-3-(284–409) may sequester clathrin and potentially inhibit endocytosis by either arrestin-dependent or -independent pathways. It has recently been reported that arrestin-3 may also interact with the AP-2 β-adaptin subunit (69). Thus, it is conceivable that expression of the arrestin C-tail may sequester clathrin, AP-2, or other proteins with which they interact.

In contrast to PMA, SDF-1α-mediated CXCR4 internalization does appear to be mediated by a pathway involving arrestin. Stimulation of CXCR4 by SDF-1α promoted redistribution of arrestin to the plasma membrane. Arrestin also appeared to colocalize with internalized CXCR4, similar to the colocalization of arrestin observed with internalized AT1a and neurotensin receptors (62). Dynamin-K44A and arrestin-3-(284–409) significantly inhibited SDF-1α-mediated internalization. Im-
importantly, this effect was seen in concert with the endogenous cellular complement of GRKs and arrestins. This is significant, since it has previously been shown that internalization of the AT1a receptor in the absence of overexpressed wild-type arrestins, which is equally efficient in both COS-7 and HEK-293 cells, appears to be dynamin- and arrestin-independent (70). Neither arrestin-3(1–320) nor GRK2-K220R had a significant effect on CXCR4 internalization. However, the function of both of these dominant negative proteins is dependent upon their ability to bind to receptor (48, 57). If GRKs and arrestins have a higher affinity for activated CXCR4, inhibition of internalization by these dominant negative proteins might be inefficient. Alternatively, these mutant proteins may have an intrinsically low affinity for CXCR4. Indeed, previous studies have shown very modest effects of dominant negative GRK2 mutants in concert with other receptors (57, 71, 72). Moreover, the affinity of the GRK2-K220R protein for the β2-adrenergic receptor has been reported to be 10-fold lower than wild-type GRK2 in the presence of Gβγ subunits (57).

Our results further underscore the differences between PMA- and SDF-1α-mediated CXCR4 endocytosis. Moreover, they suggest that the use of dominant negative arrestins, regardless of their mechanism of action, should not be the sole criteria used to determine if GPCR internalization is mediated by an arrestin-dependent pathway. Studies from several groups have noted differences in CXCR4 trafficking depending on the cell type studied and the receptor stimulus. CXCR4 internalization in response to phorbol ester appears to be mediated by clathrin-coated vesicles, and CXCR4 was reported to be recycled to the cell surface after phorbol ester removal (52). In contrast, SDF-1α or gp120 stimulation of CXCR4-expressing U937, HeLa, and CEM cells appeared to be clathrin-dependent, but CXCR4 appeared to enter a degradative pathway (73). However, removal of RANTES appears to result in significant recycling of internalized CCR5 (40). We are currently investigating the cellular localization of CXCR4 at different times after agonist addition, and we are attempting to develop a quantitative assay for receptor recycling. This analysis should reveal whether the differences observed in arrestin recruitment correlate with delivery of the receptor to distinct populations of endosomes that are involved in receptor recycling or degradation and whether differences in the affinity of arrestins for activated receptors play a role in this process.

 Trafficking of both CXCR4 and CCR5 appears to require the serine/threonine-rich C-tail (21, 44). GRKs mediate phosphorylation of agonist-activated receptors on serine and threonine residues (74). CXCR4 is unique in that it, unlike other HIV coreceptors, contains a dileucine motif similar to that identified in the γ subunit of the CD3 receptor and the CD4 receptor. The phosphorylation of one or both of the serine residues in CD3 or CD4 is required for the dileucine motif to function as an endocytic signal (75–77). The mutation of individual serines, serine/serine or serine/threonine pairs, and the dileucine motif revealed residues that are likely to be important for CXCR4 internalization. A considerable amount of plasticity was observed with regard to the requirements for PMA-induced internalization. In HEK-293 cells, no single mutation resulted in greater than a 60% decrease in CXCR4 internalization. Since PMA-induced internalization of CXCR4 was less efficient in COS-1 cells, the effect of the C-tail mutations in this cell line was more revealing. Mutation of the two serines at positions 338 and 339 almost completely eliminated CXCR4 internalization, and mutation of the two serines at positions 324 and 325 or the dileucine motif severely reduced internalization. The effect of C-tail mutations on SDF-1α-mediated internalization was similar in both HEK-293 and COS-1 cells. Internalization of CXCR4 receptors containing the mutations S3245A, S3389A, and IL3289A was significantly impaired. Mutation of the serine/threonine pair at positions 341 and 342, the serine at position 344, or deletion of the C-terminal seven amino acids reduced SDF-1α-mediated internalization by approximately 50–60%. Together, these results suggest that the dileucine motif, SSLKIL, and serines 338 and 339 are the most critical for both PMA- and SDF-mediated CXCR4 internalization.

However, it is clear that multiple residues in the C-tail play a role in CXCR4 internalization. Therefore, we attempted to discern the relative importance of different residues by attempting to rescue CXCR4 internalization by overexpression of GRK2 and arrestins. Thus, if the effect of certain mutations could be compensated for by the presence of wild-type residues at other sites, overexpression of GRKs and arrestins might be expected to promote internalization of these receptors. Conversely, if internalization of certain mutant CXCR4 receptors was not promoted or was promoted inefficiently, this would indicate residues that were potentially more important for receptor internalization. The result of this analysis yielded three distinct patterns of receptor internalization. Similar to wild-type CXCR4, internalization of the CXCR4 mutants ST3412A, S334A, and E345stop was modestly promoted by overexpression of GRK2 or arrestins alone but promoted in a synergistic manner by simultaneous expression of GRK2 and arrestin-3. In contrast, internalization of the CXCR4 mutant receptors S3389A and IL3289A was modestly promoted by expression of GRK2 or arrestin-3 alone, depending on the stimulus. However, simultaneous expression of GRK2 and arrestin-3 had no additional effect, and the maximal amount of receptor internalization in the presence of overexpressed GRK2 and/or arrestin-3 was significantly less compared with the other mutant CXCR4 receptors. The behavior of the CXCR4 mutant S3245A was intermediate. GRK2 and arrestin-3 promoted PMA-mediated internalization of S3245A in a synergistic manner to levels similar to wild-type receptor alone. The coexpression of GRK2 and arrestin-3 synergistically promoted SDF-mediated internalization, albeit to a lesser extent than with PMA. These data suggest that Ser-324 and Ser-325 might play a greater role in CXCR4 internalization mediated by SDF-1α. Therefore, although multiple residues appear to be involved in PMA- and SDF-mediated CXCR4 internalization, the serine residues at positions 338 and 339, and the dileucine motif SSLKIL appear to be the most critical.

SDF-1α and PMA both promoted phosphorylation of CXCR4 in HEK-293 cells, although the extent of phosphorylation was modest. PMA stimulation promoted phosphorylation of all of the mutant receptors to some extent, although phosphorylation of the IL3289A, S3389A, and E345stop CXCR4 mutant receptors was less than wild-type CXCR4 or the remaining mutants. This observation is possibly the result of CXCR4 phosphorylation by either protein kinase C-mediated GRK2 activation or direct phosphorylation of the receptor by protein kinase C. However, phosphorylation of CXCR4 by PMA stimulation, at least in HEK-293 cells, does not appear to be a significant determinant for receptor internalization. In contrast, SDF-1α stimulation promoted significantly less phosphorylation of the IL3289A and S3389A CXCR4 receptors. Interestingly, expression of GRK2, but not arrestin, modestly rescued internalization of these receptors, and coexpression of GRK2 and arrestin-3 did not promote greater internalization than GRK2 alone. This suggests that the IL3289A and S3389A receptors may retain a phosphorylation site(s) for GRK2 but interact poorly with arrestin. Thus, the ability of CXCR4 to undergo phosphorylation in response to SDF-1α stimulation appears to at least partially correlate with receptor internalization. Although the
E345stop receptor was not significantly phosphorylated in response to SDF-1α, internalization of E345stop was not decreased to the same extent as IL3289 and S3389A. Thus, it is possible that internalization of the E345stop CXCR4 receptor is less dependent upon its ability to be phosphorylated. Moreover, internalization of E345stop was significantly promoted by overexpression of GRK2 and arrestin-3, suggesting that other sites in the C-tail can partially compensate for the absence of these residues.

Our results suggest that internalization of CXCR4 is mediated by multiple serines in the C-tail as well as the dileucine motif. This partially contrasts with a recent study by Signoret et al. (53) who reported that the SSLXIL motif was required for phorbol ester- but not SDF-1α-mediated internalization of CXCR4. This difference might readily be explained by differences in receptor trafficking or in the levels of endogenous GRKs and arrestins related to the use of different cell types (Mv-1-Lu cells compared with COS-1 and HEK-293 cells in this study) or the methods used to assess receptor internalization.

The method we use to assess receptor internalization employs a common epitope tag rather than an antibody directed against the receptor itself. Other groups have detected cell surface receptors with labeled antibodies directed against CXCR4 (12G5) after acid stripping of the SDF-1α used to stimulate receptor internalization (52, 53). This could potentially be problematic since SDF-1α can compete for 12G5 binding (20, 22). In the dileucine motifs present in the CD4 and CD3 γ receptors, phosphorylation of a proximal serine residue(s) is required for the motif to function as an endocytic signal (77, 78). In this regard, it is interesting that mutation of the iso-leucine/leucine pair in CXCR4 abolishes SDF-1α- and reduces PMA-mediated phosphorylation even though the proximal serine residues are present. The CXCR4 IL3289 mutant also appeared to run slightly faster than wild-type CXCR4 or the other mutants in reducing SDS-PAGE gels (Fig. 5). Therefore, these residues may confer a conformational structure that affects receptor phosphorylation and internalization. Dileucine motifs have also been shown to mediate interaction with the AP-1 and AP-2 clathrin adaptor complexes (77, 78). In light of the recent report that arrestin-3 may also bind to AP-2 (69), this suggests that arrestin-mediated CXCR4 endocytosis may be regulated at that point as well.

In light of the recent report that phorbol esters can promote endocytosis of certain CD4 receptors, our results suggest that regulation of CXCR4 endocytosis is likely to be mediated by multiple mechanisms. However, further studies will clearly be required to elucidate the potential contribution of different components of the endocytic pathway, as well as the ultimate fate of internalized receptors.

Although multiple chemokine receptors appear to be capable of mediating HIV entry, a recent report suggests that CXCR4 and CCR5 are obligate for viral entry despite the presence of other coreceptors that are capable of mediating HIV entry (79). This suggests that CXCR4 and CCR5 are the most important targets for antiviral therapy. In contrast to aminooxypentane-RANTES (40), there is no report of a small molecule receptor agonist or antagonist of CXCR4 that induces receptor down-modulation. The bicyclam compounds (23, 25, 26) and Alx-40C (24) have both been shown to inhibit SDF-1α-CXCR4 interaction. However, they do not appear to promote receptor internalization themselves but are capable of selectively inhibiting SDF-1α-mediated CXCR4 internalization.3 Individuals homozygous for a 32-base pair deletion in CCR5 that results in the lack of functional or surface-expressed CCR5 are largely protected from HIV infection but suffer no obvious detrimental effects (80, 81). In contrast, studies on mice in which the CXCR4 or SDF-1α genes have been knocked out reveal an important role for this chemokine-chemokine receptor pair in B-cell lymphopoiesis, myeloopoiesis, migration of cerebellar neurons, vascular development, and cardiogenesis (82, 83). Thus, strategies aimed at inhibiting the natural function of SDF-1α or CXCR4 will need to consider their apparently vital role in immune system development. However, compounds that could induce clearance of cell surface CXCR4 in addition to blocking virus interaction would be very attractive. The ability to block surface CXCR4 expression may also reduce the possibility that signaling as a result of interaction of gp120, chemokines, or other molecules present during HIV infection with chemokine receptors could activate replication of latent viruses or otherwise increase cellular transcriptional activity (27, 29, 31, 84). Moreover, this might also prevent infection by X4 viruses that emerge later in HIV infection. A better understanding of CXCR4 trafficking and signaling will be required to understand how these processes may affect HIV entry and replication and to evaluate the function and effectiveness of compounds designed to influence HIV-coreceptor interaction.

Acknowledgments—We thank the members of the Kimmel Cancer Center Nucleic Acid Facility for oligonucleotide synthesis and DNA sequencing; Drs. Benjamin Doranz and Robert Doms at the University of Pennsylvania for CXCR4 and CCR5 constructs; and Richard Horuk at Berlex Biosciences for valuable advice and discussions.

REFERENCES

1. Luster, A. D. (1998) N. Engl. J. Med. 338, 436–445
2. Nagasawa, T., Hiroto, S., Tachibana, K., Takakura, N., Nishikawa, S., Kitamura, Y., Yoshida, N., Kikutani, H., and Kishimoto, T. (1996) Nature 382, 635–638
3. Proudfoot, A. E., Wells, T. N., and Clapham, P. R. (1999) Biochem. Pharmacol. 57, 451–463
4. Littman, D. R. (1998) Cell 93, 677–680
5. Bleul, C. C., Farzan, M., Choe, H., Parolin, C., Clark-Lewis, I., Sodroski, J., and Springer, T. A. (1996) Nature 382, 829–833
6. Deng, H., Liu, R., Eilmeier, W., Choe, S., Unutmaz, D., Burkhard, M., Di Marzo, F., Marmor, S., Min, B. Y., Hill, C. M., Cuk, L., Peiper, S. C., Schall, T. J., Littman, D. R., and Landau, N. R. (1996) Nature 381, 661–666
7. Lapham, C. K., Ouyang, J., Chandrasekhar, B., Nguyen, N. Y., Dimitrov, D. S., and Golding, H. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 602–605
8. Tolkola, A., Dragic, T., Arthos, J., Binley, J. M., Olson, W. C., Allaway, G. P., Cheng-Mayer, C., Robinson, J., Maddon, P. P., and Moore, J. P. (1996) Nature 384, 184–187
9. Ulgonini, S., Moulard, M., Mondor, I., Barois, N., Demandolz, D., Hoxie, J., Breilet, A., Alizon, M., Davoust, J., and Sattentau, Q. J. (1997) J. Immunol. 159, 3000–3008
10. Wu, L., Gerard, N. P., Wyatt, R., Choe, H., Parolin, C., Ruffing, N., Borsettii, A., Cardoso, A. A., Desjardins, E., Newman, W., Gerard, C., and Sodroski, J. (1998) Nature 384, 179–183
11. Barre-Sinoussi, F., Wang, Q. F., O’Leary, J., Baleaux, F., Amara, A., Hoxie, J. A., Zolla-Paleari, S., and Gorny, M. K. (1998) J. Virol. 72, 2500–2504
12. Edinger, A. L., Mankowski, J. L., Doranz, B. J., Margulies, B. J., Lee, B., Rucker, J., Sharron, M., Hoffman, T. L., Berson, J. F., Zink, C. M., Hirsch, V. M., Clements, J. E., and Doms, R. W. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 14742–14747
13. Emdes, M. J., Clapham, P. R., Marsh, M., Ahuja, M., Turner, J. D., McKnight, A. H., Thomas, J. F., Stoeckenius-Haggarty, B., Choe, S., Vance, P. J., Wells, T. N., Power, C. A., Sutterwala, S. S., Doms, R. W., Landau, N. R., and Hoxie, J. A. (1996) Cell 87, 745–756
14. Martin, K. A., Wyatt, R., Farzan, M., Choe, H., Marcon, L., Desjardins, E., Robinson, J., Sodroski, J., Gerard, C., and Gerard, N. P. (1997) Science 278, 1470–1473
15. Doranz, B. J., Rucker, J., Yi, Y., Smyth, R. J., Samson, M., Peiper, S. C., Farzan, M., and Doms, R. W. (1998) J. Virol. 72, 1149–1156
16. Lu, Z., Berson, J. F., Chen, Y., Turner, J. D., Zheng, T., Sharron, M., Jenks, M. H., Wang, Z., Kim, J., Rucker, J., Hoxie, J. A., Peiper, S. C., and Doms, R. W. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 6426–6431
17. Rucker, J., Edinger, A. L., Sharron, M., Samson, M., Lee, B., Berson, J. F., Yi, Y., Margulies, B., Collman, R. G., Doranz, B. J., Parmentier, M., and Doms, R. W. (1997) J. Virol. 71, 8999–9007
18. Rucker, J., and Doms, R. W. (1998) AIDS Res. Hum. Retroviruses 14, Suppl. 3
