β-Arrestin Differentially Regulates the Chemokine Receptor
CXCR4-mediated Signaling and Receptor Internalization, and This
Implicates Multiple Interaction Sites between β-Arrestin and
CXCR4*

(Received for publication, July 26, 1999, and in revised form, November 1, 1999)

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The chemokine receptor CXCR4 has recently been shown to be a co-receptor involved in the entry of human immunodeficiency virus type 1 into target cells. This study shows that coexpression of β-arrestin with CXCR4 in human embryonic kidney 293 cells attenuated chemokine-stimulated G protein activation and inhibition of cAMP production. Truncation of the C-terminal 34 amino acids of CXCR4 (CXCR4-T) abolished the effects of β-arrestin on CXCR4/G protein signaling, indicating the functional interaction of the receptor C terminus with β-arrestin. On the other hand, receptor internalization and the subsequent activation of extracellular signal-regulated kinases were significantly promoted by coexpression of β-arrestin with CXCR4, whereas the C-terminal truncation of CXCR4 did not affect this regulation of β-arrestin, suggesting that β-arrestin can functionally interact with CXCR4 with or without the C terminus. Moreover, β2V54D, the dominant inhibitory mutant of β-arrestin 2, exerted no effects on CXCR4/G protein signaling, but strongly influenced receptor internalization and extracellular signal-regulated kinase activation. Further cross-linking experiments demonstrated that β-arrestin as well as β2V54D could physically contact both CXCR4 and CXCR4-T. Glutathione S-transferase pull-down assay showed that β-arrestin was able to bind efficiently in vitro to both the third intracellular loop and the 34-amino acid C terminus of CXCR4. Taken together, our data clearly establish that β-arrestin can effectively regulate different functions of CXCR4 and that this is mediated through its distinct interactions with the C terminus and other regions including the third loop of CXCR4.

Chemokines are a large family of chemotactic proteins that regulate leukocyte activation, recruitment to sites of inflammation, and many other immune responses (1). Four classes of chemokines have been defined based on the arrangement of the conserved cysteine residues of the mature proteins (2, 3). Chemokines bind to a family of G protein-coupled receptors (GPCRs) that are differentially and widely expressed in blood cells (4). The chemokine receptors CCR5 and CXCR4 have recently been reported to act as major co-receptors (along with CD4) involved in the entry of macrophage trophic and T-cell tropic HIV-1 strains, respectively, into target cells (5–10). In addition, CD4-independent infection by HIV-2 has also been shown to be mediated by CXCR4 (11). Stromal cell-derived factor-1α (SDF-1α), a CXC chemokine, is a specific ligand for the chemokine receptor CXCR4 (5, 12, 13) and was recently found to be able to inhibit CXCR4-mediated HIV-1 infection in vitro (14, 15).

Despite the increasingly prominent role of CXCR4 and SDF-1α in the regulation of HIV infection, little is known about the regulation of signal transduction of CXCR4. The classical mechanism of signal transduction is based on the work of the β2-adrenergic receptor, a well studied model system of the GPCR superfamily. Upon agonist stimulation, the receptors are phosphorylated by cAMP-dependent kinase A, protein kinase C, or G protein-coupled receptor kinase (GRK), followed by binding of an important regulator, arrestin. Arrestin proteins have been shown to play important roles in the desensitization and internalization of the β2-adrenergic receptor and receptor-mediated activation of extracellular signal-regulated kinase (ERK) (16–18). Up to date, six members of the arrestin family have been cloned and characterized: visual arrestin, β-arrestins 1 and 2, X-arrestin, D-arrestin, and E-arrestin (19). Visual arrestin, which was originally named S-antigen, is a highly pathogenic protein and is responsible for induction of experimental autoimmune uveitis, a T-cell-mediated disease (20). Both β-arrestins are ubiquitously expressed, but are found with the highest expression in nervous and lymphatic tissues (21), suggesting a potential important role of β-arrestin in regulating the physiological activity of leukocytes. Accumulating evidence has indicated that β-arrestin is involved in signal transduction of other chemokine receptors such as CXCR1 (22), CCR2B (23), and CCR5, another intensively investigated HIV co-receptor (24).

Studies on CXCR4 in leukocytes have revealed that CXCR4, in response to SDF-1α, can mediate many signaling events such as inhibitory G protein activation, receptor phosphorylation, internalization, inositol phosphate generation, and ERK phosphorylation (25, 26). In this study, we used the well-established HEK 293 cell system to investigate the regulation by β-arrestin of the signaling and internalization of CXCR4. Our...
results demonstrate that β-arrestin effectively regulates the different functions of CXCR4 and, more interestingly, that the regulation by β-arrestin appears to be mediated via at least two distinct interaction sites on CXCR4.

**EXPERIMENTAL PROCEDURES**

** Constructs—** Human wild-type CXCR4 cDNA with an influenza hemagglutinin (HA) epitope at the N terminus and human β-arrestin 1 and 2 cDNA constructs were described (27). The human HA-tagged CXCR4-T (with a truncation of the C-terminal 34 amino acids of CXCR4) cDNA clone and β5V54D (the dominant inhibitory mutant of β-arrestin 2 with valine 54 substituted to aromatic amino acid) cDNA clone were constructed by polymerase chain reaction mutagenesis. All of the above constructs were in pcDNA3 (Invitrogen). The sequence of the CXCR4 third intracellular loop subdomain (amino acids 219–243) was generated by polymerase chain reaction and inserted into pGEX-4T1 (Amersham Pharmacia Biotech). The sequence of the GST-fused CXCR4 C-terminal (the last 34 amino acids in the C terminus) subdomain was inserted into pcDNA3. Authenticity of sequences was confirmed by DNA sequencing. SDF-1α was obtained from PeproTech (London, United Kingdom).

** Transfection and Expression of β-Arrestin and Chemokine Receptors—** HEK 293 cells (American Type Culture Collection) were plated on 60-mm dishes to minimize crowded conditions in modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal bovine serum 20 h before transfection. Transfection was performed using 2.0 μg of chemokine receptor and 2.0 μg of β-arrestin cDNA/1 × 10⁶ cells and the calcium phosphate-DNA coprecipitation method as described (28). The total amount of DNA transfected was kept constant (4 μg of DNA/1 × 10⁶ cells) by addition of pcDNA3. The cells were used 48 h post-transfection. The level of chemokine receptor expression was carefully controlled and monitored by flow cytometry or immunoprecipitation and Western blot analysis. Expression of β-arrestin was monitored by Western blot analysis. The expression levels of both the receptor and β-arrestin were kept consistent in this study.

**cAMP Assay—** Cells were challenged with agonist in the presence of 30 μM forskolin (Sigma) and 500 μM 1-methyl-β-isobutylxantine (Sigma) at 37 °C for 15 min. The reactions were terminated with 1 N HCl. The cells were lysed in 5 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 1 mM EDTA, and 100 mM NaCl, 40 mM GTP (γ-S), and 8 mM EDTA. The reaction mixture was centrifuged at 12,000 × g for 10 min. The supernatants were assayed for [35S]GTPγS binding.

**[35S]GTPγS Binding Assay—** The experiments were performed as described previously (29). Cells were lysed in 5 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 5 mM EGTA at 4 °C, and the lysate was centrifuged at 30,000 × g for 10 min. The membrane pellet was resuspended, and an aliquot (100 μg of protein) was incubated with 1 N HCl. The cells were lysed in 5 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 1 mM EDTA, 100 mM NaCl, 40 μM GTP (γ-S), and 8 mM EDTA. The reaction mixture was centrifuged at 105,000 × g for 30 min, and the supernatants were assayed for [35S]GTPγS binding.

**ERK Phosphorylation and Western Blot Analysis—** Cells were challenged with agonist in the presence of 30 μM forskolin (Sigma) and 500 μM 1-methyl-β-isobutylxantine (Sigma) at 37 °C for 15 min. The reactions were terminated with 1 N HCl. The cells were lysed in 5 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 1 mM EDTA, and 100 mM NaCl, 40 mM GTP (γ-S), and 8 mM EDTA. The reaction mixture was centrifuged at 12,000 × g for 10 min. The supernatants were assayed for [35S]GTPγS binding.

**Results**

**[35S]GTPγS Binding Assay—** The experiments were performed as described previously (29). Cells were lysed in 5 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 5 mM EGTA at 4 °C, and the lysate was centrifuged at 30,000 × g for 10 min. The membrane pellet was resuspended, and an aliquot (100 μg of protein) was incubated with 1 N HCl. The cells were lysed in 5 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 1 mM EDTA, 100 mM NaCl, 40 μM GTP (γ-S), and 8 mM EDTA. The reaction mixture was centrifuged at 105,000 × g for 30 min, and the supernatants were assayed for [35S]GTPγS binding into agonist and nonagonist, [35S]GTPγS was in the presence of agonist and nonagonist. The reaction was terminated by dilution in cold phosphate-buffered saline and filtered through GF/C filters under vacuum. Bound radioactivity was determined in duplicate by liquid scintillation spectrophotometry. Basal binding was determined in the presence of agonist, and non-specific binding was determined in the presence of 10 μM non-radioactive GTP-S. Data were averaged from several independent measurements, and percentage of stimulation was calculated as follows: 100 × (cpmagonist − cpmnonagonist)/(cpmagonist + cpmnonagonist).

**Adenylyl cyclase activity** was measured in agonist and nonagonist, [35S]GTP-S was in the presence of agonist and nonagonist. The reaction was terminated by dilution in cold phosphate-buffered saline and filtered through GF/C filters under vacuum. Bound radioactivity was determined in duplicate by liquid scintillation spectrophotometry. Basal binding was determined in the presence of agonist, and non-specific binding was determined in the presence of 10 μM non-radioactive GTP-S. Data were averaged from several independent measurements, and percentage of stimulation was calculated as follows: 100 × (cpmagonist − cpmnonagonist)/(cpmagonist + cpmnonagonist).

**Immunoprecipitation and Cross-linking Experiments—** The immunoprecipitation experiment was performed as described (27, 30). HEK 293 cells grown on a 60-mm culture dish were lysed in 0.8 ml of immunoprecipitation buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.4% digitonin) containing protease inhibitors on ice for 45 min. The lysate was centrifuged at 12,000 × g for 30 min, and the supernatants were incubated with 12CA5 monoclonal antibodies (0.5 μg) and protein A-Sepharose (Life Technologies, Inc.) on ice for 4 h. After washing with immunoprecipitation buffer, the immunocomplexes absorbed onto protein A-Sepharose were eluted in SDS-polyacrylamide gel electrophoresis sample buffer (50 mM Tris-HCl, pH 7.4, 9%, SDS, 50 mM dithiothreitol, 10% glycerol, 0.1% bromphenol blue), and the presence of CXCR4 was detected by Western blot analysis using 12CA5 monoclonal antibodies.

The cross-linking was performed using disuccinimidyl suberate (Pierce) following the manufacturer’s instructions. In brief, HEK 293 cells were suspended in phosphate-buffered saline containing 10 mM Hepes, pH 7.4, and stimulated with SDF-1α (10 nM) at 37 °C for 15 min. The cells were then incubated with disuccinimidyl suberate (0.1 mM) at room temperature for 30 min in a total volume of 400 μl. The reaction was terminated by addition of cold Tris-HCl, pH 7.4, to a final concentration of 10 mM and incubation for an additional 15 min. The samples were analyzed by immunoprecipitation and Western blotting. The existence of a physical complex of β-arrestin and chemokine receptors was detected by anti-β-arrestin antibodies.

**GST Pull-down Assay—** The GST pull-down experiment was performed as described (31). GST and GST fusion proteins with the CXCR4 third loop (GST-TL) were expressed in Escherichia coli BL21(DE3). GST fusion proteins with the CXCR4 C terminus (GST-C) were expressed in HEK 293 cells due to protein degradation when expressed in bacteria. All of the fusion proteins were purified using glutathione-Sepharose 4B (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. The cytosolic fractions from HEK 293 cells expressing β-arrestin 2 and β5V54D were incubated with −5 μg GST fusion proteins bound to the glutathione resin in 100 μl buffer containing 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride for 1 h at 4 °C. The resin was washed three times with 0.2 ml of the same buffer and then eluted with SDS-polyacrylamide gel electrophoresis sample buffer and applied to a denaturing 10% polyacrylamide gel. The proteins retained on the resin were detected by anti-GST and anti-β-arrestin antibodies, respectively.

**Statistical Analysis—** Data were analyzed by Student’s t test for comparison of independent means, with pooled estimates of common variances.

**Results**

Previous studies on CXCR4 have revealed that signaling and internalization of CXCR4 are regulated by phosphorylation-dependent and -independent mechanisms, respectively, and that truncation of the last 34 amino acids in the C terminus of CXCR4 (CXCR4-T) causes partial loss of phosphorylation of the receptor (26). The current study was undertaken to test the possible role of β-arrestin in the signaling and internalization mediated by CXCR4 or by the same truncated receptor (CXCR4-T) in HEK 293 cells. Both receptors, with HA tags at their amino termini, were well expressed on the cell surface as determined by fluorescence-activated cell sorting using anti-HA 12CA5 monoclonal antibodies (Fig. 1A). Expression of CXCR4 and CXCR4-T was further confirmed by Western blot analysis with 12CA5 monoclonal antibodies after immunopre-
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Fig. 1. Expression of CXCR4 and CXCR-T in HEK 293 cells. A, flow cytometric analysis of surface expression of receptors in pcDNA3-transfected HEK 293 cells (dashed line) and in HEK 293 cells expressing HA-tagged human CXCR4 (thick line) or CXCR4-T (thin line) with anti-HA 12CA5 monoclonal antibodies and fluorescein isothiocyanate-labeled goat anti-mouse antibodies; B, Western blot analysis of immunoprecipitated receptors from cells expressing CXCR4 (lane 1) or CXCR4-T (lane 2) with anti-HA 12CA5 monoclonal antibodies as described under “Experimental Procedures”; C, Western blot analysis of expression of β-arrestins (β-arrestin 1, lane 2) and 2 (lane 3) expressed in HEK 293 cells compared with endogenous β-arrestin in pcDNA3-transfected cells (lane 1) with anti-β-arrestin antibodies. Data are representative of several independent experiments.

To investigate the effect of cotransfected β-arrestin on CXCR4 internalization in HEK 293 cells, we measured CXCR4 internalization following a 30-min exposure to 10 nM SDF-1α by flow cytometry after fluorescence staining of the cell-surface receptor with 12CA5 monoclonal antibodies (Fig. 3A). When expressed alone in HEK 293 cells, CXCR4 was internalized ~20%. Internalization of the receptor was modestly enhanced by coexpression of β-arrestin 1, but was significantly increased up to ~40% by coexpression of β-arrestin 2 (Fig. 3A). Moreover, cotransfection of β-arrestin 1 or 2 with a G protein-coupled receptor kinase (GRK2) produced a synergistic increase in CXCR4 internalization, indicating that β-arrestin-enhanced CXCR4 internalization can be facilitated by GRK phosphorylation. The function of β-arrestin in CXCR4-mediated ERK activation was also investigated (Fig. 3B). It has been reported that SDF-1α treatment can activate ERK via CXCR4 (25). Our results show that a 10-min exposure to SDF-1α led to an increase in ERK phosphorylation ~3–4-fold above the control level (without SDF-1α stimulation) in cells transfected with CXCR4 (Fig. 3B). Under such conditions, coexpression of either
β-arrestin resulted in a dramatic increase in SDF-1α-induced ERK phosphorylation to ~6–7-fold above the control level (Fig. 3B), whereas the basal expression of ERK was unchanged in the same cells (data not shown). Thus, in contrast to the negative regulation by β-arrestin of CXCR4-mediated G protein activation, β-arrestin exerted a remarkably positive effect on CXCR4 internalization and receptor-mediated ERK activation.

The third intracellular loop and carboxyl terminus have previously been implicated in GPCR interaction with cytosolic proteins such as GRK and arrestin (31–36). It has recently been demonstrated that truncation at different sites in the CXCR4 C terminus causes total loss of receptor phosphorylation, desensitization, and internalization (25, 37). To access the contributions of the C terminus of CXCR4 to the regulation by β-arrestin, either the receptor (CXCR4-T) or β-arrestin (β2-arr2) were coexpressed with CXCR4 or CXCR4-T (with the carboxyl-terminal 34 amino acids removed) was constructed. The results show that truncation of the CXCR4 C terminus strongly attenuated receptor phosphorylation, desensitization, and inhibition of adenyl cyclase (Fig. 4, A and B), which indicated that the C-terminal structure of CXCR4 is crucial for β-arrestin to regulate receptor/G protein coupling. In contrast, the same truncation of the CXCR4 C terminus did not significantly affect the enhancement role of β-arrestin in SDF-1α-stimulated receptor internalization and ERK activation (Fig. 5, A and B). This suggests that the C-terminal domain of CXCR4 is not essential for β-arrestin to regulate receptor internalization, and it also implies that some regions of CXCR4 other than the C terminus would be subject to this regulation.

The β-arrestin 2 mutant β2V54D is known to prevent GPCR targeting to clathrin-coated pits and subsequently to inhibit internalization of GPCRs (17). The current study revealed that coexpression of β2V54D exhibited little effect on the receptor signaling of CXCR4 or CXCR4-T (Fig. 4, A and B), but dramatically decreased SDF-1α-stimulated internalization of both receptors (Fig. 5A). Coexpression of β2V54D also significantly impaired SDF-1α-induced ERK activation by 40 and 50% in cells expressing CXCR4 and CXCR4-T, respectively (Fig. 5B), indicating a critical role of β-arrestin-promoted CXCR4 internalization in ERK activation, as in the case of the β2-adrenergic receptor. Our data thus demonstrate that β-arrestin regulation of two important signaling functions of CXCR4, namely G protein activation and receptor internalization, is mediated by at least two distinct interactions since the CXCR4/β-arrestin interactions can be separately disrupted by the mutation of either the receptor (CXCR4-T) or β-arrestin (β2V54D).

The direct physiological interaction between β-arrestin and CXCR4 was further accessed using a cross-linking assay that has been successfully applied in the study of the β2-adrenergic receptor (18). When coexpressed with CXCR4 in HEK 293 cells, either β-arrestin 2 or β2V54D could be coprecipitated with the HA-tagged receptor after exposure of the cells to SDF-1α and the covalent cross-linking agent (Fig. 6A). Both arrestins were also shown to be cross-linked to CXCR4-T in an agonist-dependent manner (Fig. 6B) at even higher levels compared with wild-type CXCR4. Our data indicate that β-arrestin can effectively interact with CXCR4 whether the C terminus of the receptor is present or not, thus further supporting the existence of multifunctional interactions via at least two distinct sites.
between β-arrestin and CXCR4.

To define the concrete sites on CXCR4 contributing to its interaction with β-arrestin, a GST pull-down assay was employed. The results show that β-arrestin 2 expressed in HEK 293 cells bound effectively to both GST-C and GST-TL fusion proteins, but not to the control GST proteins (Fig. 7). Interestingly, the β-arrestin 2 mutant βV54D interacted with GST-TL as efficiently as its wild-type counterpart, whereas it bound much less to GST-C compared with wild-type β-arrestin. The lack of interaction of βV54D with GST-C in this assay agreed well with the results showing that the β-arrestin 2 mutant did not affect CXCR4 signaling and that it blocked the receptor internalization of both CXCR4 receptors with or without the C terminus. Thus, our data provide clear evidence that β-arrestin can directly interact with the chemokine receptor CXCR4 via at least two distinct sites, the third loop domain and the C terminus, and that different interactions of β-arrestin with CXCR4 play differential functional roles in the regulation of CXCR4 by β-arrestin.

**DISCUSSION**

Although it has been reported that β-arrestin can functionally regulate many chemokine receptors such as CCR5, CCR2B, and CXCR1 (23, 24), it was not known until this study was performed whether β-arrestin could play similar functional roles in CXCR4 signal transduction. Our results have demonstrated that β-arrestin is critically involved in the regulation of the most studied signal pathways mediated by CXCR4, a chemokine receptor of considerable interest due to its role as both chemoattractant receptor and HIV co-receptor. Since CXCR4 is widely expressed in human blood neutrophils and lymphocytes (38), in which β-arrestin expression is also very high (21), the functional regulation of CXCR4 by β-arrestin, as revealed by this study, should play an important role in CXCR4- and other chemokine receptor-mediated immunological responses. Moreover, it can be reasonably speculated that β-arrestin may be also functionally involved in the process of HIV infection due to its regulation of the two HIV co-receptors, CXCR4 and CCR5 (24).

It has been reported that truncation of the C terminus of CXCR4 leads to higher receptor-mediated activities in inositol phosphate formation and in the induction of sustained calcium fluxes as compared with wild-type CXCR4 (26). It has been suggested that these phenomena are due to loss of down-regulatory control to the mutant receptor. The current results that β-arrestin can effectively regulate CXCR4/G protein signaling of CXCR4, but not that of CXCR4-T, indicate that the inhibitory regulation of CXCR4 by β-arrestin could be the hypothesized down-regulatory control and partly explain the previous observation.

For most members of the GPCR family, agonist-stimulated phosphorylation of GPCR occurs at serine and threonine in the intracellular domains of the C terminus and/or the third intra-

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**Fig. 5.** β-Arrestin regulation of receptor internalization and ERK activation in CXCR4 and CXCR4-T. HEK 293 cells were co-transfected with CXCR4 or CXCR4-T plus pcDNA3 or β-arrestin 2 (β-ar2) or βV54D cDNA. A, 48 h after transfection, cells were incubated without (control) or with 10 nM SDF-1α for 30 min at 37 °C, and receptor internalization was determined by flow cytometry. B, transfected cells were incubated without (basal) or with 10 nM SDF-1α for 15 min at 37 °C, and the phosphorylation of ERK was determined by Western blot analysis using phospho-specific p44/p42 mitogen-activated protein kinase antibodies. *, p < 0.05 compared with cells transfected with receptors alone. Data are means ± S.E. of three independent experiments.

**Fig. 6.** Cross-linking of β-arrestin to SDF-1α-stimulated CXCR4 or CXCR4-T. HEK 293 cells were cotransfected with CXCR4 (A) or CXCR4-T (B) plus β-arrestin 2 (β-ar2) or βV54D, and the expression levels of β-arrestin 2 and βV54D (lower panel) were detected by anti-β-arrestin antibodies as compared with endogenous β-arrestin in HEK 293 cells (lane 1). The cells were incubated without or with 10 nM SDF-1α for 15 min at 37 °C, and β-arrestin-receptor complexes were stabilized by covalent cross-linking with disuccinimidyl suberate (DSS). After cross-linking, HA-tagged CXCR4 (A) or CXCR4-T (B) was immunoprecipitated and detected by Western blot analysis using 12CA5 monoclonal antibodies (upper panel), and the existence of the β-arrestin/βV54D-receptor complex was detected by anti-β-arrestin antibodies (middle panel). Results are representative of three experiments with similar results.
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FIG. 7. Interaction of β-arrestin with GST fusion proteins of the third intracellular loop and C terminus of CXCR4. GST fusion proteins and GST (each 5 μg) bound to glutathione affinity matrix were incubated with β-arrestin 2 (β-arrestin 2) or β, V54D overexpressed in HEK 293 cells, and the expression levels of β-arrestin 2 and β, V54D were detected by anti-β-arrestin antibodies (lower panel) as compared with endogenous β-arrestin in HEK 293 cells (lane 7). After washing, the proteins retained on the matrix were detected by Western blot analysis using anti-GST antibodies (upper panel) and anti-β-arrestin antibodies (middle panel). Results are representative of three experiments with similar results.

cellular loop (32–35, 39, 40). The cytoplasmic tail of CXCR4 contains 18 serine/threonine residues, among the highest in the chemokine receptors. Truncation of its C terminus is reported to cause nearly total loss of receptor phosphorylation in rat basophilic leukemia cells stably expressing CXCR4 while producing no change in CXCR4 internalization (26). The current study reveals that β-arrestin significantly augments SDF-1α-induced internalization of both CXCR4 and CXCR4-T in HEK 293 cells, indicating that the regulation of receptor internalization by β-arrestin is not critically dependent on phosphorylation of the receptor C terminus. Of interest, the synergistic increase in receptor internalization by coexpression of β-arrestin with GRK2 shown in this report for CXCR4 and in an earlier study for CCR5 (24) implies that chemokine receptor internalization can be facilitated by GRK at least under the conditions of overexpression. It has demonstrated that CXCR4 undergoes significant spontaneous endocytosis and that recycling of internalized receptors is not efficient (41). Our data show that overexpression of β-arrestin exerted little effect on CXCR4 spontaneous endocytosis while remarkably increasing receptor recycling to the membrane (data not shown). Further study to investigate the role of β-arrestin in CXCR4 internalization and recycling is to be continued.

The dominant inhibitory mutant of β-arrestin 2 (β, V54D) is known to prevent GPCR targeting to clathrin-coated pits and subsequent receptor internalization (17). In the current study, overexpression of the same β-arrestin 2 mutant led to a dramatic decrease in SDF-1α-induced internalization of both CXCR4 and CXCR4-T. SDF-1α-stimulated phosphorylation of ERK via both receptors was also inhibited by β, V54D. Taken together, our data demonstrate that β-arrestin-facilitated receptor internalization plays a crucial role in CXCR4-mediated ERK activation, as in the well-known case of the β2-adrenergic receptor (18, 42). It has been demonstrated that opioid-mediated ERK activation is not dependent on N- or C-terminal peptide internalization (43), suggesting that receptor internalization-dependent ERK activation may not be universal and may have some receptor specificity. It is also worth noting that β-arrestin 2 induced a more robust increase in CXCR4 internalization than did β-arrestin 1, although they appeared equal in enhancing CXCR4-mediated ERK activation. However, β-arrestins 1 and 2 were equivalent in the regulation of CXCR4 internalization in the presence of overexpressed GRK2, suggesting that regulation by β-arrestin 1 more likely depends on CXCR4 phosphorylation.

β-Arrestin has been reported to interact with GPCRs at their third intracellular loop (31) or C terminus (36, 44), according to the localization of relevant phosphorylation sites and the sizes and amino acid sequences of both domains (45). However, no evidence has emerged that β-arrestin can functionally interact at two distinct domains of GPCRs simultaneously. This study has demonstrated that β-arrestin efficiently interferes with both receptor/G protein coupling, which is mediated by the CXCR4 C terminus, and receptor internalization, which is independent of the C terminus. The regulation of CXCR4 internalization by β-arrestin seems likely to be mediated by its interaction with the third intracellular loop and/or other intracellular domain(s) such as the second loop of the receptor. Our results from in vivo cross-linking and in vitro GST pull-down assays provide direct evidence of a physical interaction between β-arrestin and two distinct sites of CXCR4, namely the C terminus and the third intracellular loop. Moreover, this study further establishes that the distinct interaction of β-arrestin with different domains of CXCR4 produces the differential regulation of receptor functions by β-arrestin. Thus, it is feasible both in theory and in practice to block or to enhance one of the regulatory functions of β-arrestin for receptors by purposefully modifying the structures of β-arrestin or the receptors it regulates.

Acknowledgments—We thank Shumin Xin, Professor Yaling Huang, XuMing Zhang, and PeiHua Wu for assistance and Dr. Lan Ma for helpful discussion. We especially thank Dr. Kun Ling for technical expertise and helpful discussion.

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