

Identification of a Motif in the Carboxyl Terminus of CXCR2 That Is Involved in Adaptin 2 Binding and Receptor Internalization[†]

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Received July 17, 2000; Revised Manuscript Received October 19, 2000

ABSTRACT: Agonist treatment of cells expressing the chemokine receptor, CXCR2, induces receptor phosphorylation and internalization through a dynamin-dependent mechanism. In the present study, we demonstrate that a carboxyl terminus-truncated mutant of CXCR2 (331T), which no longer undergoes agonist-induced phosphorylation, continues to undergo ligand-induced internalization in HEK293 cells. This mutant receptor exhibits reduced association with β -arrestin 1 but continues to exhibit association with adaptin 2 α and β subunits. Replacing Leu320-321 and/or Ile323-Leu324 with Ala (LL320,321AA, IL323,324AA, and LLIL320,321,323,324AAAA) in wild-type CXCR2 or 331T causes little change in ligand binding and signaling through Ca^{2+} mobilization but greatly impairs the agonist-induced receptor sequestration and ligand-mediated chemotaxis. The LL320,321AA, IL323,324AA, and LLIL320,321,323,324AAAA mutants of CXCR2 exhibit normal binding to β -arrestin 1 but exhibit decreased binding to adaptin 2 α and β . These data demonstrate a role for the LLKIL motif in the carboxyl terminus of CXCR2 in receptor internalization and cell chemotaxis and imply a role for adaptin 2 in the endocytosis of CXCR2.

The chemokine receptor, CXCR2,¹ is a member of a superfamily of G protein-coupled seven-transmembrane receptors (GPCRs) that transduce intracellular signals via heterotrimeric guanine nucleotide-binding proteins (G proteins). Upon stimulation by agonists, such as interleukin 8 (IL-8) or melanoma growth-stimulatory activity (MGSA)/growth-regulatory protein (GRO), CXCR2 activates a series of G protein-mediated events, including phosphatidylinositol hydrolysis, to generate inositol 1,4,5-trisphosphate and diacylglycerol, as well as mobilization of intracellular free Ca^{2+} to initiate a series of cellular responses (1). In addition, CXCR2 mediates cell chemotaxis, a distinct function of chemokine receptors (2). Like many other types of GPCRs, CXCR2 undergoes a dynamic trafficking between the cell surface and the intracellular compartments (3). Such trafficking may be involved in both transmission and termination

of the receptor signals and may play an important role in mediating cell chemotaxis. For CXCR2, the most remarkable trafficking process is agonist-induced receptor internalization.

Agonist-induced phosphorylation of the carboxyl terminus by G protein-coupled receptor kinases (GRKs) has been proposed to be necessary for the desensitization and internalization of many GPCRs (4–7). Clathrin-coated pits have been demonstrated to play a central role in GPCR sequestration (8, 9). β -arrestins, which interact with the phosphorylated form of agonist-stimulated GPCRs to desensitize their signaling, might act as adaptor proteins between the receptor and clathrin to initiate clathrin-mediated internalization (10–14). In a word, for many GPCRs, receptor phosphorylation has been suggested to be required for the receptor internalization, and β -arrestins have been proposed to play an important role in this process. Although β -arrestins have been shown to bind to clathrin with high affinity (15), they are not constitutively associated with clathrin-coated vesicles (16), nor do they promote clathrin-coat assembly (17). These studies imply that some other adaptor proteins are also involved in this process. In fact, recent studies on the β_2 -adrenergic receptor (β_2 -AR), a prototypic GPCR, have shown that adaptin 2 (AP-2) functions as a clathrin adaptor for the endocytosis of β_2 -AR (18).

Our previous studies have demonstrated that carboxyl-terminal phosphorylation is necessary for the desensitization of CXCR2 (19), and dynamin, a component of clathrin-mediated endocytosis, is essential for CXCR2 internalization and resensitization (3). However, the receptor domains that might be involved in the receptor sequestration are still poorly defined. Our preliminary data show that a carboxyl-terminal-truncated receptor (331T), which loses agonist-

[†] This research was supported by a Department of Veterans Affairs Career Scientist Award (A.R.), National Institutes of Health Grant CA34590 (A.R.), and Vanderbilt Cancer Support Grant CA68485.

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¹ Abbreviations: CXCR2, receptor for CXC chemokines formerly defined as interleukin 8 receptor B; IL-8, interleukin 8; MGSA/GRO, melanoma growth-stimulatory activity/growth-regulatory protein; GPCRs, G protein-coupled receptors; G proteins, guanine nucleotide-binding proteins; β_2 -AR, β_2 -adrenergic receptor; AP-2, adaptin 2; HEK293 cells, human embryonic kidney 293 cells; RBL-2H3 cells, rabbit basophilic leukemia cells; DSP, dithiobis(succinimido propionate); GRKs, G protein-coupled receptor kinases; FITC, fluorescein isothiocyanate; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

induced phosphorylation, still undergoes agonist-induced internalization in human embryonic kidney 293 (HEK293) cells but not in 3ASubE cells, although binding of the truncated receptor to β -arrestin 1 was severely impaired. These data suggest that additional motifs in CXCR2 might associate with other adaptor proteins to facilitate receptor endocytosis.

A highly conserved carboxyl-terminal Leu-Leu motif has been demonstrated to involve the endocytosis of various membrane proteins (20–22), including G protein-coupled β_2 -AR (23). A Leu-Leu motif has been proposed to act as a binding site for adaptor proteins, such as AP-2, that are required for intracellular protein trafficking (24). In addition, a Leu-Ile motif has been reported to be important for the endocytosis of the leukemia inhibitory factor receptor (25). However, it has never been directly demonstrated that mutation of the dileucine residues eliminates the association of AP-2 with GPCRs. Comparable Leu-Leu, Leu-Ile, and Ile-Leu motifs are also present in the carboxyl terminus of CXCR2, suggesting a potential role of these domains in the internalization of CXCR2. In this paper, we report that the LLKIL motif in the carboxyl terminus of CXCR2 is required for association of the receptor with AP-2 and is essential for the receptor sequestration as well as for ligand-mediated chemotaxis.

MATERIALS AND METHODS

Generation and Expression of Mutant CXCR2. Plasmids encoding wild-type, the truncated (331T), and the phosphorylation-deficient mutant (4A) of CXCR2 were generated in the laboratory previously (19). Mutagenesis of specific leucine or isoleucine to alanine (L \rightarrow A or I \rightarrow A) residues was conducted by use of the GeneEditor site-directed mutagenesis system (Promega). The following mutations were generated with the indicated primers: LL320,321AA, ATGTATAGCTAGAATCTTTGCGGCTCCATGGCGAAA-CTTCTG; IL323,324AA, CAAGCCATGTATAGCGGCGCCCTTGAGGAGTCC; LLIL 320,321,323,324AAAA, CAAGCCATGTATAGCGGCGCCCTTTGCGGCTCCGTGGCGAAACTT. Receptor mutants were screened by restriction analysis. Once the mutations were confirmed, the cDNAs encoding the open reading frames for CXCR2 mutants were subcloned into the pRc/CMV expression vector.

Cell Culture and Transfections. HEK293 cells and rat basophilic leukemia (RBL-2H3) cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 1:100 dilution of penicillin/streptomycin (BioWhittaker) at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. They were transfected with wild-type or mutant CXCR2 in the presence of LipofectAMINE plus reagent (Life Technologies, Inc.). Stably transfected cells were selected with 560 μ g/mL Geneticin (G418) and evaluated for receptor expression by ¹²⁵I-MGSA/GRO binding.

¹²⁵I-MGSA/GRO Binding Assay. Binding assays were performed on HEK293 cells stably expressing wild-type or mutant CXCR2 as described previously (19).

Receptor Internalization Assay. The acid-wash technique (26) was used to determine the kinetics of MGSA/GRO-induced internalization of CXCR2. HEK293 cells stably

expressing wild-type or mutant CXCR2 or RBL-2H3 cells transiently transfected with wild-type or mutant CXCR2 were grown confluent on 24-well plates, which were precoated with 0.1 mg/mL poly-L-lysine (Sigma, MW 30 000–70 000) for 1 h and washed once with distilled water before use. Cells were incubated at 4 °C in 0.5 mL of serum-free DMEM containing 75 nCi/mL ¹²⁵I-MGSA/GRO for 1 h. The medium was subsequently removed, 1 mL of ice-cold serum-free DMEM was added carefully into each well, aspirated, then another 1 mL aliquot of ice-cold serum-free DMEM was added prior to incubation at 37 °C for the indicated time. The medium was removed and the cells were incubated with 1 mL of ice-cold 0.2 M acetic acid with 0.5 M NaCl for 6 min. After the incubation, the cells were washed once with 1 mL of ice-cold serum-free DMEM and lysed in 1 mL of 1% SDS with 0.1 N NaOH (lysis solution). The radioactive cell lysates were then counted in a γ -counter (Gamma 5500, Beckman). Total cell surface receptor binding was measured after incubation with ¹²⁵I-MGSA medium, followed by washing the cells with ice-cold serum-free DMEM and lysing with the lysis solution. Nonspecific binding was measured by adding ice-cold 0.2 M acetic acid with 0.5 M NaCl after incubation with ¹²⁵I-MGSA in binding medium. Calculation of the percentage of internalized receptor was performed as described before (26).

Confocal Microscopy. Confocal microscopy was performed on a Zeiss LSM-410 laser scanning microscope with a 40 \times 1.3 oil immersion lens. HEK293 cells stably expressing wild-type or the mutant CXCR2 were grown on 10-mm cover slips (Fisher). The cells were treated with or without MGSA/GRO (100 ng/mL) for 5 or 30 min, fixed with methanol for 5 min, and then washed three times with ice-cold PBS. CXCR2 was visualized by incubating fixed cells with an affinity-purified CXCR2 polyclonal rabbit antibody (27), diluted to a final concentration of 2 μ g/mL in phosphate-buffered saline (PBS) supplemented with 0.1% bovine serum albumin (BSA) for 30 min at room temperature, and then incubating with anti-rabbit fluorescein isothiocyanate- (FITC-) conjugated second antibody (diluted 1:100) (Molecular Probes). CXCR2 fluorescent signals were collected by use of single line excitation (488 nm).

Immunoprecipitation and Western Blot. Cells were serum-starved overnight in DMEM containing 0.5% fetal bovine serum (FBS). Cells were treated with or without MGSA/GRO (100 ng/mL) for different times (1, 2, 5, 10, and 20 min), washed three times with ice-cold PBS, and lysed in 1 mL of lysis buffer (RIPA) containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS). The cell debris was removed by centrifugation for 4 min at 13 000 rpm in an Eppendorf microcentrifuge. The supernatant was precleared for 1 h to reduce nonspecific binding by addition of 40 μ L of protein A/G agarose (Pierce). After removal of the protein A/G agarose by centrifugation in an Eppendorf microcentrifuge at 3000 rpm for 1 min, the cleared supernatant was collected and 10 μ L of anti-CXCR2 antibody was added for overnight precipitation at 4 °C. Protein A/G (40 μ L) was then added and the mixture was incubated at 4 °C for 2 h. The protein A/G-antibody-antigen complex was then collected by washing three times with ice-cold RIPA buffer. The final pellet was resuspended in 50 μ L of SDS sample buffer containing 5% β -mercaptoethanol and heated

Table 1: Design of Mutations of the Carboxyl Terminus of CXCR2^a

wt	IYFIGQKFRHGLLKILAIHGLISKDSLPKDSRPSFVGSSSGHTSTTL
LL320,321AA	IYFIGQKFRHGAALKILAIHGLISKDSLPKDSRPSFVGSSSGHTSTTL
IL323,324AA	IYFIGQKFRHGLLKAAAIAHGLISKDSLPKDSRPSFVGSSSGHTSTTL
LLIL320,321,323,324AAAA	IYFIGQKFRHGAAKAAAIAHGLISKDSLPKDSRPSFVGSSSGHTSTTL
331T	IYFIGQKFRHGLLKILAIHGLI
(331T) LL320,321AA	IYFIGQKFRHGAALKILAIHGLI
(331T) IL323,324AA	IYFIGQKFRHGLLKAAAIAHGLI
(331T) LLIL320,321,323,324AAAA	IYFIGQKFRHGAAKAAAIAHGLI

^a The carboxyl-terminal truncation of CXCR2 was produced by introducing a stop codon at Ser331, and the position for the mutations of the Leu-Leu and Ile-Leu to Ala in the LLKIL motif in the carboxyl terminus of CXCR2 are shown.

to 50 °C for 10 min. A 20 μ L aliquot was electrophoresed on an SDS–10% polyacrylamide gel, and the proteins on the gel were transferred to nitrocellulose membranes (Bio-Rad) as previously described (26). Coprecipitated β -arrestin 1 or AP-2 was detected by probing the Western blot with specific monoclonal mouse antibodies against AP-2 α (Transduction Laboratories, no. A43920), AP-2 β (Transduction Laboratories, no. A35620), and β -arrestin 1 (Santa Cruz, no. sc-6388), respectively.

In Vivo Phosphorylation Assay. Receptor phosphorylation assays were performed as described previously (3). In brief, the transiently transfected cells were replated on 100-mm plates 1 day after the transfection. On the following day, after incubation in serum- and phosphate-free medium for 1 h, cells were metabolically labeled by incubating with [³²P]orthophosphate (100 μ Ci/mL) (Dupont NEN) in the same medium at 37 °C for 2 h. Cells were stimulated with or without agonists and then lysed in RIPA buffer. Receptors were immunoprecipitated as described above with a specific antibody. The immunoprecipitates were electrophoresed through an SDS–10% polyacrylamide gel and transferred to a nitrocellulose membrane (Bio-Rad). The phosphorylated receptors were then detected by autoradiography, and the amount of receptor immunoprecipitated was determined by immunodetection after Western blotting, with use of a monoclonal antibody against CXCR2 to ensure equal loading.

Chemotaxis Assay. Chemotaxis assays were performed on stably transfected HEK293 cells as described previously (3). Briefly, a 96-well chemotaxis chamber (Neuroprobe Inc) was used, and the lower compartment of the chamber was loaded with 360 μ L aliquots of 1 mg/mL ovalbumin/DMEM (chemotaxis buffer) with or without IL-8 diluted in the chemotaxis buffer (5–200 ng/mL). Polycarbonate membranes (10 μ m pore size) were coated on both sides with 20 μ g/mL human collagen type IV, incubated for 2 h at 37 °C, and then stored at 4 °C overnight. To prepare cells for chemotaxis assay, they were removed by trypsinization and incubated in 10% FBS/DMEM for 2 h at 37 °C to allow time for restoration of receptors. The cells were washed with chemotaxis buffer and then loaded into the upper chamber in the chemotaxis buffer. The chamber was incubated for 4 h at 37 °C in humidified air with 5% CO₂, then the membrane was removed, washed, fixed, and stained with a Diff-Quik

kit. Cell chemotaxis was quantified by counting the number of cells present in 10 microscope fields (20 \times objective).

Calcium Mobilization Assay. HEK293 cells stably expressing wild-type or mutant CXCR2 were grown until confluent. Cells were released by shaking, collected by centrifugation at 300g for 6 min, and washed with Hanks' buffer containing 5 mM HEPES. Cells were resuspended at 2×10^6 cells/mL and incubated with 2.5 μ M Fluo-3 (Molecular Probes) for 30 min at 37 °C. After incubation, the cells were washed once with Hanks' buffer containing 5 mM HEPES and 2 mM Ca²⁺. The cells were finally adjusted to 2×10^6 cells/mL. Ca²⁺ mobilization experiments were performed as described previously (19). In the desensitization experiments, cells were incubated with 20 nM IL-8 or buffer for 5 min, washed three times with Hanks' solution, resuspended and kept on ice until the time for second ligand treatment. Cells were then warmed at 37 °C and stimulated with 5 nM IL-8. Calcium mobilization was measured as described before (19). The time taken to recover 80% of the mobilized Ca²⁺ ($t_{0.80}$) was used as a measure of Ca²⁺ removal.

RESULTS

Expression and Signaling Properties of Wild-Type or Mutant CXCR2. Plasmid DNA expression vectors encoding wild-type human CXCR2, or the mutants produced by truncating the carboxyl-terminal domain at Ser331 and/or replacing either Leu320–321 or Ile323–Leu324 or both to Ala (LL320,321AA, IL323,324AA, and LLIL320,321,323,324AAAA) (Table 1) were transfected and stably expressed in HEK293 cells. Saturation binding assays with [¹²⁵I]-MGSA/GRO were performed and the ligand dissociation constant (K_d) and the maximal binding sites (B_{max}) were calculated with the LIGAND program. The K_d and B_{max} for the mutant receptors were similar to that for the wild-type receptor ($p > 0.05$, Student's *t*-test, Table 2). These data indicate that the mutations did not significantly affect the ligand-binding properties of the receptors.

To monitor the biological function of wild-type and mutant receptors, we compared the calcium mobilization in response to agonist treatment by calcium fluorometry. The transfected HEK293 cells stably expressing wild-type CXCR2 responded to MGSA (100 ng/mL) with increased mobilization of

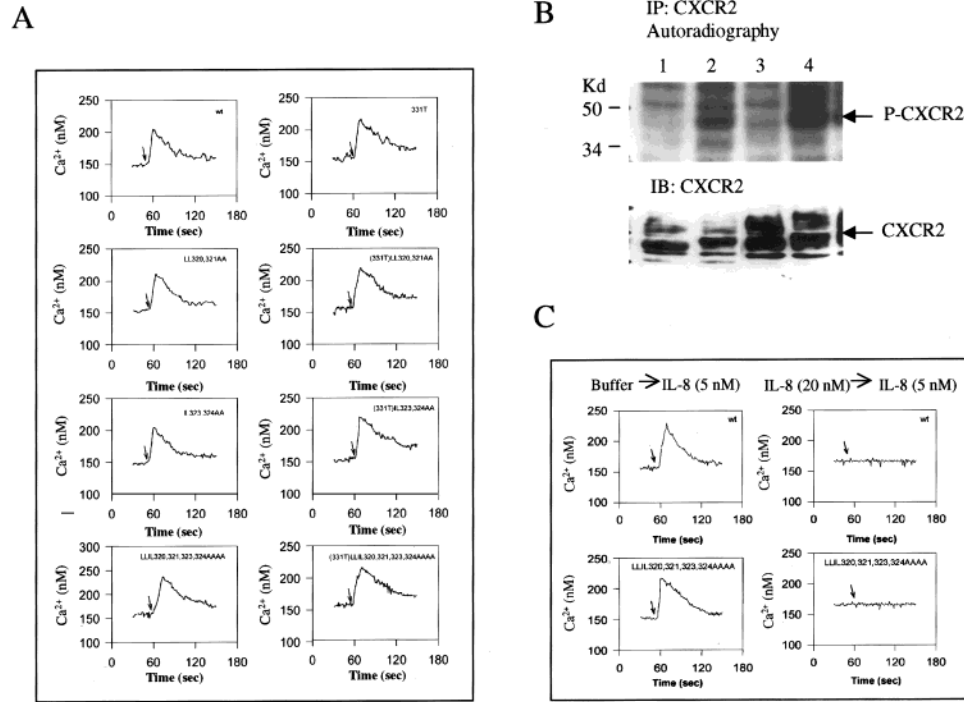


FIGURE 1: Signaling, phosphorylation, and desensitization of wild-type and mutant CXCR2 in HEK293 cells. (A) HEK293 cells stably expressing wild-type or mutant CXCR2 were loaded with Fluo-3, stimulated with MGSA/GRO, and analyzed for ability to mobilize intracellular free Ca^{2+} . The arrow indicates the addition of ligand. Data shown are representative of at least three independent experiments. (B) Cells expressing wild-type (lanes 1 and 2) or the LLIL/AAAA mutant (lanes 3 and 4) CXCR2 were metabolically labeled with [^{32}P]orthophosphate and then stimulated without (lanes 1 and 3) or with (lanes 2 and 4) MGSA/GRO (100 ng/mL). The cell lysates were immunoprecipitated with a rabbit polyclonal anti-CXCR2. Proteins were separated by SDS-10% PAGE and transferred to a nitrocellulose membrane. Phosphorylated proteins were detected by autoradiography (upper panel). The membrane was blotted with a mouse monoclonal anti-CXCR2 to confirm the receptor expression (lower panel). (C) Cells expressing wild-type or the mutant CXCR2 (indicated) were preincubated with buffer (left panel) or 20 nM IL-8 (right panel) and then stimulated with 5 nM IL-8. The maximum calcium mobilization was measured. The arrow indicates the addition of ligand. Data shown are representative of at least three independent experiments.

Table 2: Binding Parameters for [^{125}I]-MGSA/GRO in Membranes from Cells Expressing Wild-Type or Mutant Human CXCR2^a

Receptor	K _d (nM)	B _{max} (sites/cells) × 10 ⁴
wt	1.03 ± 0.24	2.14 ± 0.27
LL320,321AA	1.04 ± 0.17	2.19 ± 0.31
IL323,324AA	1.56 ± 0.11	2.47 ± 0.26
LLIL320,321,323,324AAAA	1.17 ± 0.14	2.11 ± 0.46
331T	1.68 ± 0.27	2.24 ± 0.48
(331T) LL320,321AA	1.18 ± 0.15	2.23 ± 0.29
(331T) IL323,324AA	1.82 ± 0.34	2.42 ± 0.39
(331T) LLIL320,321,323,324AAAA	1.78 ± 0.27	2.21 ± 0.11

^a Binding parameters were determined from ligand binding saturation experiments (15–150 pM) with the nonlinear regression program LIGAND as described under Materials and Methods. Each value represents the mean ± SEM of three different experiments.

intracellular free Ca^{2+} (Figure 1), while the control HEK293 cells stably transfected with vector DNA did not exhibit the agonist-induced calcium mobilization response (data not shown). The level of intracellular free Ca^{2+} nearly returned to the baseline within 60 s, with a $t_{0.80}$ of 54.66 ± 3.28 s. Similar levels of increase in intracellular free Ca^{2+} were observed in the cells expressing the LL320,321AA, IL323,324AA, and LLIL320,321,323,324AAAA mutants of CXCR2 ($t_{0.80}$ 54.33 ± 3.84 s, 49.33 ± 1.76 s, and 57.66 ± 4.04 s, respectively). HEK293 cells expressing the truncated CXCR2 (331T) exhibited a similar increase in intracellular free Ca^{2+} in response to agonist treatment, but the free calcium was sequestered more slowly and did not fully return to the baseline ($t_{0.80}$ 70.00 ± 2.31 s) as compared to the cells

expressing wild-type CXCR2 ($t_{0.80}$ 54.66 ± 3.28 , $p < 0.02$, Student's t -test). The LL320,321AA, IL323,324AA, and LLIL320,321,323,324AAAA mutations of 331T did not affect the potency of the calcium response or alter the slower return of the mobilized Ca^{2+} to baseline exhibited by 331T without LL/IL mutations ($t_{0.80}$ 77.00 ± 1.15 s, 76.23 ± 2.33 s, and 83.66 ± 8.76 s, respectively, as compared to 70.00 ± 2.31 s, $p > 0.2$, Student's t -test) (Figure 1). Calcium mobilization mediated by CXCR2 has been shown to be pertussis toxin- (PTX-) sensitive, suggesting coupling to a G_i/G_o protein (28). In the present study, both wild-type and the LLIL/AAAA mutant CXCR2-mediated calcium mobilization was blocked by PTX pretreatment, indicating that mutation of the LLKIL motif does not change the receptor/G protein coupling (data not shown). Thus, these data indicate very similar signaling properties among the LL/AA and/or IL/AA mutants of either wild-type CXCR2 or CXCR2-331T in HEK293 cells.

To determine whether mutation of the LLKIL motif affects the receptor phosphorylation, the transfected HEK293 cells were metabolically labeled with [^{32}P]orthophosphate and then stimulated with MGSA/GRO. Both the wild-type and the LLIL/AAAA mutant receptor were phosphorylated to a similar extent in response to agonist treatment (Figure 1B). Receptor desensitization was determined by calcium mobilization assay. Fluo-3 loaded cells stably expressing wild-type or the LLIL/AAAA mutant CXCR2 were incubated with buffer or 20 nM IL-8. Ligand was removed by repeated washing, and the cells were treated with 5 nM IL-8

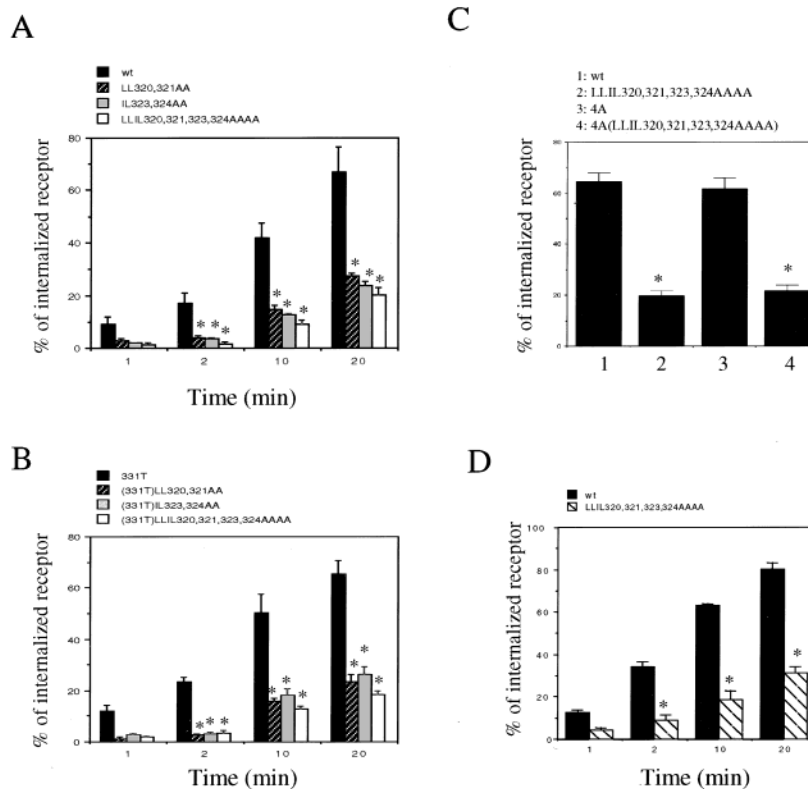


FIGURE 2: Agonist-induced internalization of wild-type or mutant CXCR2 in HEK 293 cells (A–C) and in RBL-2H3 cells (D). Cells stably expressing CXCR2 or mutants were preincubated with binding buffer containing 75 nCi of 125 I-MGSA/GRO for 1 h at 4 °C. Unbound 125 I-MGSA/GRO was removed by washing at 4 °C. The cells were warmed to 37 °C for the times indicated (A, B, D) or for 20 min (C). 125 I-MGSA/GRO remaining at the cell surface was removed by acid wash (0.2 M acetic acid, pH 2.5, containing 0.5 M NaCl), and the internalized 125 I-MGSA/GRO was then quantitated on a γ -counter (Beckman). Nonspecific binding was measured by acidic washing before the cells were warmed. Percentage of internalization was calculated as internalized cpm divided by the total specific cpm bound prior to incubation at 37 °C. Values represent the mean \pm SEM of five different experiments performed in duplicate. The data were analyzed by Student's paired *t*-test. Statistical significance is indicated by an asterisk (*p* < 0.05).

for the second time. As expected, both the wild-type and the LLIL/AAAA mutant CXCR2 were desensitized by the first addition of IL-8 (Figure 1C). These data indicate that mutation of the LLKIL motif does not change the receptor phosphorylation and desensitization.

Agonist-Induced Internalization of Wild-Type or Mutant CXCR2. HEK293 cells stably expressing wild-type or mutant CXCR2 were incubated at 4 °C for 1 h with DMEM containing 75 nCi of 125 I-MGSA/GRO per well. The medium was aspirated and the cells were washed with ice-cold DMEM, after which the medium was replaced with ice-cold DMEM, and cells were incubated at 37 °C for the indicated times. As shown in Figure 2A, wild-type CXCR2 underwent internalization quickly and in a time-dependent manner. About 70% of the cell surface receptors were internalized within 20 min, whereas only about 20–30% of the LL320,321AA, IL323,324AA, and LLIL320,321,323,324AAAA mutants of full-length CXCR2 were sequestered upon exposure to MGSA/GRO. The truncated CXCR2 (331T) was sequestered similarly upon agonist treatment compared to the wild-type receptor (Figure 2B). Replacing Leu320–321 and Ile323–Leu324 to Ala in 331T caused a reduction in internalization to about 50% relative to 331T. Maximum loss of receptor internalization was observed in the cells expressing the LLIL320,321,323,324AAAA mutant of 331T (Figure 2B). Furthermore, a phosphorylation-deficient mutant (4A), in which Ser352 and 356–358 were replaced by Ala (19), with or without mutation of the LLKIL

motif, was tested for the agonist-induced internalization. 4A exhibited a similar internalization as the wild-type receptor, and mutation of the LLKIL motif (LLIL320,321,323,324AAAA) in the carboxyl terminus of 4A greatly impaired the receptor internalization (Figure 2C).

In addition to HEK293 cells, the effect of the LLKIL motif in the receptor internalization was also tested in RBL-2H3 cells transiently transfected with wild-type or the LLIL/AAAA mutant CXCR2. Wild-type CXCR2 exhibited a time-dependent endocytosis in response to agonist-treatment. In contrast, the internalization of the LLIL/AAAA mutant was significantly decreased (Figure 2D).

Receptor internalization was also visualized by confocal microscopy. Cells expressing the wild-type or the mutant form of CXCR2 were treated with or without MGSA/GRO, fixed with methanol, and the cellular localization of wild-type or mutant CXCR2 was detected by indirect immunofluorescence using affinity-purified rabbit polyclonal antibody to CXCR2 and FITC-conjugated anti-rabbit antibody. As shown in Figure 3, the wild-type receptors were located sharply at the plasma membrane (Figure 3A). Upon stimulation with 100 ng/mL MGSA/GRO for 30 min, a large proportion of the receptors became visible in discrete accumulations in the interior of the cells (Figure 3B). Like the wild-type CXCR2, the LLIL320,321,323,324AAAA mutants of the full-length CXCR2 were located on the cell membrane under control conditions (Figure 3C), indicating normal cell surface delivery. However, after agonist treat-

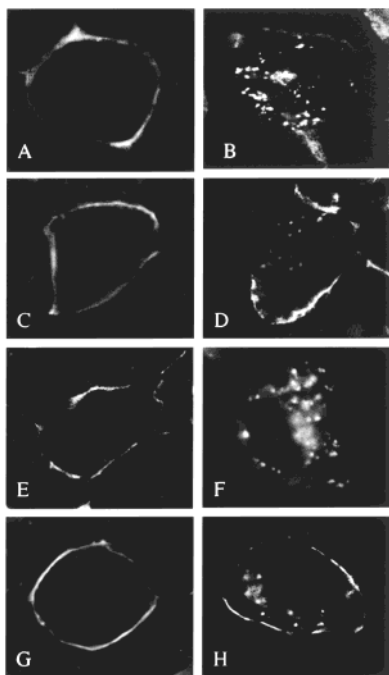


FIGURE 3: Subcellular distribution of CXCR2 and mutants in HEK293 cells. Cells expressing wild-type (A–D) or LLIL320,321,323,324AAAA mutant CXCR2 (E–H) were incubated with (B, D, F, and H) or without (A, C, E, and G) 100 ng/mL MGSA/GRO for 20 min and then fixed, and the receptors were visualized with specific antibodies. Shown are confocal microscopic images of results representative of four separate experiments.

ment, only a small proportion of the fluorescence became visible in the cytosol, whereas most of the fluorescence remained on the cell surface (Figure 3D). The truncated receptor (331T) and the LLIL/AAAA mutants of 331T were also located at the plasma membrane in the absence of agonist (Figure 3E, G). Interestingly, after MGSA/GRO treatment, a large proportion of the truncated receptors (331T) were translocated to the cytoplasm (Figure 3F), whereas most of the LLIL/AAAA mutants of 331T remained on the cell surface (Figure 3H). These data are compatible with the data obtained by radioligand binding.

Agonist-Induced Chemotaxis Mediated by Wild-Type or Mutant CXCR2. Chemokine receptors mediate cell chemotaxis. Receptor internalization has been postulated to play a role in the receptor-mediated chemotaxis (3). To determine whether the mutations in the carboxyl-terminal domain of CXCR2 affect agonist-induced chemotaxis, chemotactic response was assessed in HEK293 cells stably transfected with wild-type or LL320,321AA, IL323,324AA, or LLIL320,321,323,324AAAA mutant forms of CXCR2. As shown in Figure 4A, cells expressing the wild-type receptor exhibited an IL-8 concentration-dependent chemotactic response, with a peak migration occurring at a concentration around 10–50 ng/mL IL-8. The chemotactic responses were significantly reduced in the cells expressing the LL/AA and/or IL/AA mutants of CXCR2. HEK293 cells expressing the truncated CXCR2 (331T) also migrated in a concentration-dependent manner in response to IL-8 stimulation, while the maximal response was reduced about 20% compared with the wild-type CXCR2. The chemotactic response was decreased 50–70% in the cells transfected with LL320,321AA, IL323,324AA, and LLIL320,321,323,324AAAA mutants of CXCR2–331T (Figure 4B) ($p < 0.05$, Student's t -test).

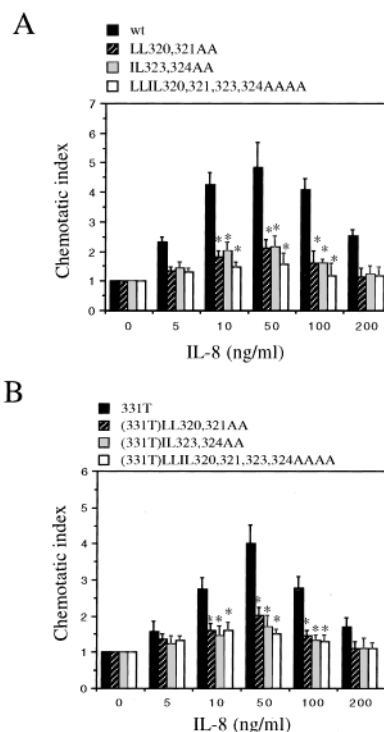


FIGURE 4: IL-8 induced chemotaxis of HEK293 cells stably expressing CXCR2 or mutant receptors in HEK293 cells. Cells stably expressing wild-type CXCR2 or the LL and/or IL mutant receptors (A) or cells stably expressing 331T or the LL and/or IL mutants of 331T (B) were compared for chemotaxis in response to IL-8 stimulation as described under Materials and Methods. Values represent the mean \pm SEM of three different experiments. The data were analyzed by Student's paired t -test. Statistical significance is indicated by an asterisk ($p < 0.05$).

Association of Wild-Type or Mutant CXCR2 with β -Arrestin 1 and AP-2 in HEK293 Cells. β -Arrestins and AP-2 have been demonstrated to be involved in the sequestration of β_2 -AR by forming a complex with clathrin (15, 18). To assess whether the mutations of CXCR2 affect the association of the receptors with the adaptor proteins, HEK293 cells were transiently cotransfected with β -arrestin 1 together with wild-type or mutant CXCR2. The cells were treated with or without 100 ng/mL MGSA/GRO for 0, 1, 2, 5, 10, and 20 min, lysed, and immunoprecipitated with an anti-CXCR2 antibody as described under Materials and Methods. The immunoprecipitate was separated on an SDS–10% polyacrylamide gel under reducing conditions and transferred to a nitrocellulose membrane, and coprecipitated adaptor proteins were detected by immunodetection of the Western blot. As shown in Figure 5A, agonist treatment induced time-dependent association of wild-type CXCR2 with β -arrestin 1. Maximal association occurred by 5 min, followed by dissociation. The truncated CXCR2 (331T) exhibited an impaired association with β -arrestin 1 (Figure 6A), while mutations of Leu320–321 and or Ile323–Leu324 in the full-length CXCR2 did not significantly affect the receptor association with β -arrestin 1 (Figure 7A, compare lane 2 with lanes 4, 6, and 8). The LLIL/AAAA mutant CXCR2 bound a similar amount of β -arrestin 1 as the wild-type receptor over the entire time course tested (Figure 7B), suggesting a modest effect of the LLKIL motif on β -arrestin 1 binding. Interestingly, the CXCR2 receptor also undergoes agonist-dependent association with AP-2 in a time-dependent manner with peak association at 5 min, but the association is

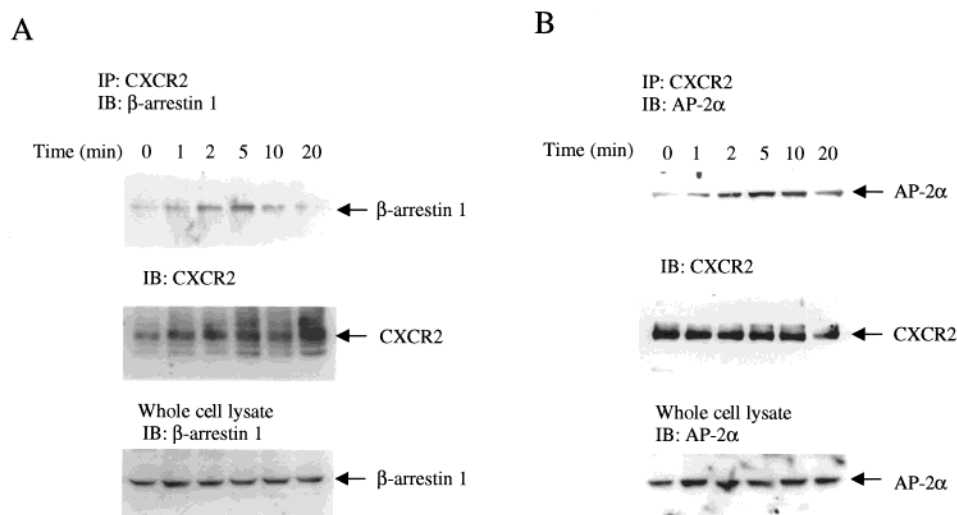


FIGURE 5: Time-dependent association of CXCR2 with β -arrestin 1 or AP-2. HEK293 cells transiently cotransfected with β -arrestin 1 together with CXCR2 (A) or stably expressing CXCR2 (B) were treated with 100 ng/mL MGSA/GRO for the times indicated. The cell lysates were immunoprecipitated with a rabbit polyclonal anti-CXCR2 antibody, and the immunoprecipitate was resolved on reducing SDS-PAGE and transferred to nitrocellulose membrane, and coimmunoprecipitated β -arrestin 1 or AP-2 α was detected with specific mouse monoclonal antibodies (0.20 and 0.25 μ g/mL), respectively, then developed by ECL. Portions representing 5% of the whole-cell lysates (40 μ g of protein) were immunoblotted with β -arrestin 1 (0.2 μ g/mL) or AP-2 α antibodies (0.25 μ g/mL), respectively, to show equal loading. Results shown are representative of three separate experiments.

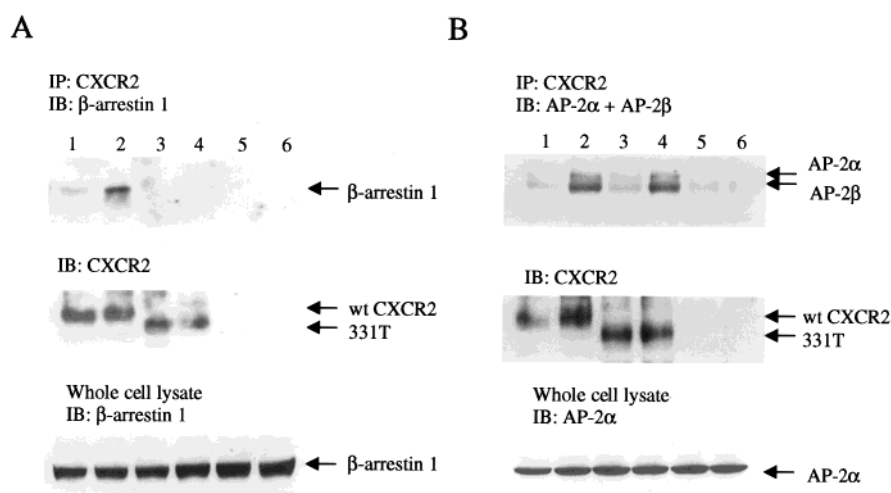


FIGURE 6: Coimmunoprecipitation of wild-type or 331T CXCR2 with β -arrestin 1 or AP-2 in HEK293 cells. (A) HEK293 cells transiently cotransfected with β -arrestin 1 together with wild type (lanes 1 and 2) or 331T (lanes 3 and 4) or vector (lanes 5 and 6) were treated without (lanes 1, 3, and 5) or with (lanes 2, 4, and 6) 100 ng/mL MGSA/GRO for 5 min and immunoprecipitated with an anti-CXCR2 antibody (2 μ g/mL) as described in the caption for Figure 5. Immunoprecipitates were subjected to reducing SDS-PAGE, and coprecipitated β -arrestin 1 was detected after Western blotting by probing with a specific mouse monoclonal antibody (0.20 μ g/mL). (B) HEK293 cells stably expressing wild-type CXCR2 (lanes 1 and 2) or 331T (lanes 3 and 4) or vector (lanes 5 and 6) were treated without (lanes 1, 3, and 5) or with (lanes 2, 4, and 6) MGSA/GRO and immunoprecipitated with an anti-CXCR2 antibody as described in the caption for Figure 5. Immunoprecipitates were subjected to reducing SDS-PAGE, and coprecipitated AP-2 α and β subunits were detected after Western blotting by probing with specific mouse monoclonal antibodies (0.25 μ g/mL). Portions representing 5% of the whole-cell lysates (40 μ g of protein) were immunoblotted in parallel to shown the expression of these proteins. Results are representative of three separate experiments where similar results were obtained.

maintained longer than that of CXCR2 with β -arrestin 1 (Figure 5B). When wild-type CXCR2 (Figure 6B, lanes 1 and 2) or the truncated receptor (331T) (Figure 6B, lanes 3 and 4) was immunoprecipitated with CXCR2 antibody and probed with anti-AP-2 α and AP-2 β antibodies, respectively, we observed that the α and β subunits of AP-2 bind equivalently to wild-type and 331T CXCR2, though the binding of 331T to β -arrestin 1 (Figure 6A, lanes 3 and 4) is compromised in comparison with the wild-type receptor (Figure 6A, lanes 1 and 2). Lanes 5 and 6 of Figure 6A,B show the same analysis from HEK293 cells not transfected with CXCR2. In contrast, mutations of the LL and/or IL

motifs blocked the association of the mutant receptors with AP-2 (Figure 7C, compare lane 2 with lanes 4, 6, and 8). The LLIL/AAAA mutant CXCR2 exhibited impaired association with AP-2 α over the entire time course tested (Figure 7D). These data suggest the involvement of the LLKIL motif in the association of CXCR2 with AP-2.

DISCUSSION

Carboxyl-terminal phosphorylation has been reported to be important for the desensitization and internalization of CXCR2 in 3ASubE cells (19), but it does not appear to be necessary for the receptor sequestration in HEK293 cells,

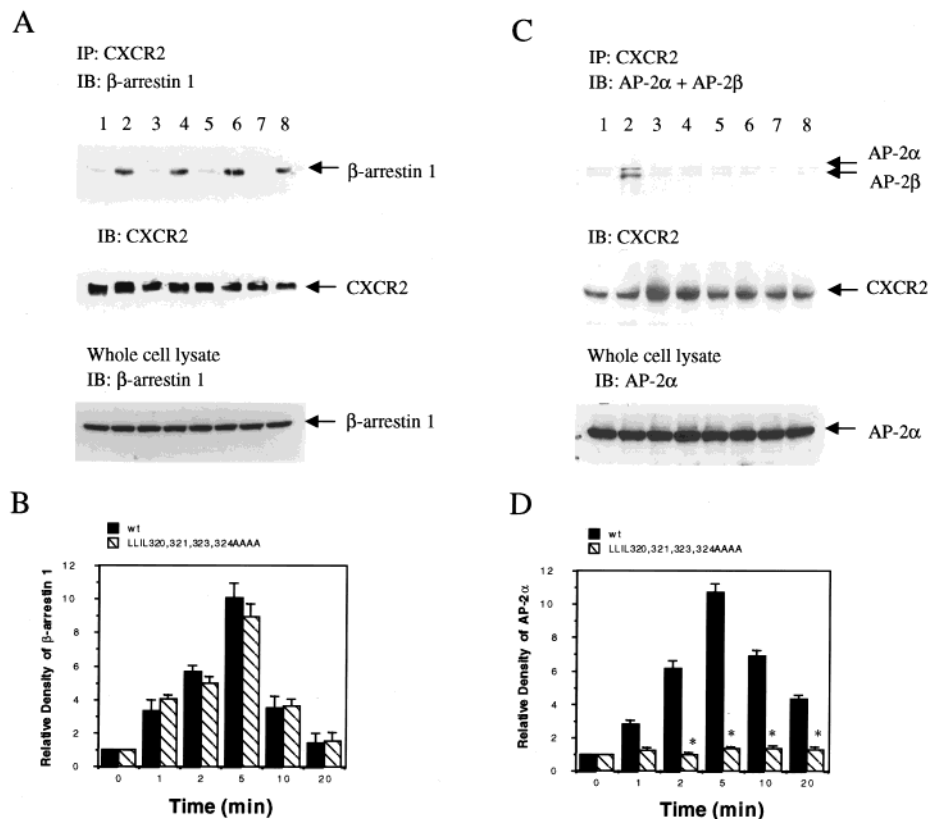


FIGURE 7: Coimmunoprecipitation of wild-type or the LL/AA and/or the IL/AA mutant CXCR2 with β -arrestin 1 or AP-2 in HEK293 cells. (A) HEK293 cells transiently cotransfected with β -arrestin 1 together with wild-type (lanes 1 and 2) or the LL/AA (lanes 3 and 4) or the IL/AA (lanes 5 and 6) or the LLIL/AAAA (lanes 7 and 8) mutant CXCR2 were treated without (lanes 1, 3, 5, and 7) or with (lanes 2, 4, 6, and 8) 100 ng/mL MGSA/GRO and immunoprecipitated with an anti-CXCR2 antibody as described in the caption for Figure 5. Coprecipitated β -arrestin 1 was detected with a specific mouse monoclonal antibody. (B) HEK293 cells transiently cotransfected with β -arrestin 1 together with wild-type or the LLIL/AAAA mutant CXCR2 were treated with 100 ng/mL MGSA/GRO for different times indicated. Coprecipitated β -arrestin 1 was detected with a specific mouse monoclonal antibody. The density of the bands representing β -arrestin 1 from three independent experiments was quantified by densitometric scanning. (C) HEK293 cells stably expressing wild-type CXCR2 (lanes 1 and 2) or the LL320,321AA (lanes 3 and 4) or the IL323,324AA (lanes 5 and 6) or the LLIL320,321,323,324AAAA (lanes 7 and 8) mutant CXCR2 were treated without (lanes 1, 3, 5, and 7) or with (lanes 2, 4, 6, and 8) MGSA/GRO (100 ng/mL) for 5 min and immunoprecipitated with an anti-CXCR2 antibody as described in the caption for Figure 5. Immunoprecipitates were subjected to reducing SDS-PAGE, and coprecipitated AP-2 α and β subunits were detected after Western blotting by probing with specific mouse monoclonal antibodies, respectively. Portions representing 5% of the whole-cell lysates (40 μ g of protein) were immunoblotted in parallel to shown the expression of these proteins. Results are representative of three separate experiments where similar results were obtained. (D) HEK293 cells stably expressing wild-type CXCR2 or the LLIL/AAAA mutant CXCR2 were treated with MGSA/GRO (100 ng/mL) for the time indicated and immunoprecipitated with an anti-CXCR2 antibody. The density of the bands representing AP-2 α from three independent experiments was quantified by densitometric scanning. The data were analyzed by Student's paired *t*-test. Statistical significance is indicated by an asterisk ($p < 0.05$).

since deletion of all the carboxyl-terminal phosphorylation sites does not affect the mutant receptor endocytosis (29). Here we show a critical role for the LLKIL motif in the carboxyl terminus of CXCR2 in the receptor internalization. Mutation of either Leu-Leu or Ile-Leu to Ala results in a remarkable reduction in the mutant receptor internalization. Such Leu-Leu as well as Ile-Leu motifs are present in multiple membrane-bound proteins and have been shown to play a role in protein trafficking (20–23). Membrane-distal Leu-Leu motifs are involved in mediating direct transport of proteins from the trans-Golgi network to the endosomes, while membrane-proximal Leu-Leu motifs in addition to phosphorylated Ser/Thr residues are potentially involved in mediating trafficking from the plasma membrane to endosomes (30). The present data demonstrate that both LL320,321 and IL323,324 are required for the receptor sequestration, so it is postulated that the LLKIL motif in the carboxyl terminus of CXCR2 is an internalization motif of the receptor.

The Leu-Leu motif has been shown to bind to the AP-2–clathrin–adaptor protein complex (24), an intrinsic component of the endocytic machinery (31). Studies on the endocytosis of integral membrane proteins such as receptor tyrosine kinases have demonstrated that the heterotetrameric AP-2 protein complex is involved in the assembly of clathrin-coated pits and functions as an adaptor by linking receptors directly to the clathrin lattice (31–33). The α -subunit of AP-2 binds to clathrin, and association of clathrin-bound AP-2 with receptors has been implicated in receptor movement into clathrin-coated pits. These clathrin-coated pits are subsequently trafficked into early endosomes through interaction with the GTPase dynamin (34, 35). The β -subunit of AP-2 also interacts with clathrin and is able to promote clathrin lattice assembly (36). The μ -subunit of AP-2 recognizes the tyrosine-based internalization signals within the cytoplasmic domain of some receptors (37), while the function of the σ chain remains unclear. Both α - and β -subunits of AP-2 are shown to interact with CXCR2 in

the present study. The carboxyl-terminal phosphorylation sites do not appear to be required for the agonist-dependent binding of the receptor to AP-2, since the carboxyl-terminal-truncated receptor (331T) binds an almost equal amount of AP-2 in response to agonist stimulation compared to wild-type CXCR2. Our data demonstrate that the LLKIL motif in the carboxyl terminus of CXCR2 is involved in association of the receptor with AP-2 α and AP-2 β . Mutation of either the Leu-Leu or the Ile-Leu to Ala results in significant reduction of binding of the receptor to AP-2 α or AP-2 β . While the association of CXCR2 with AP-2 is similar to that reported for the β 2-AR by Laporte et al. (18), different methods were used in the two studies and subtle differences were observed. In the Laporte study a two-hybrid screen demonstrated the β 2-AR binds only to AP-2 β and not AP-2 α (18), while in our study CXCR2 coimmunoprecipitates with both α and β subunits of AP-2.

The suggestion for the involvement of AP-2 α and AP-2 β in the internalization of CXCR2 in HEK293 cells is supported by the fact that mutations of LL320,321 and/or IL323,324 to Ala impair the receptor association with AP-2 α and AP-2 β and prevent the receptor sequestration. Because the same treatment of the cells with agonist induces endocytosis of the receptors to endosomes (3), we postulate that the association of the receptors with AP-2 occurs in endosomes. Our data suggest that β -arrestins are modestly involved in the endocytosis of CXCR2 in HEK293 cells, since truncation of the carboxyl-terminal phosphorylation sites does not block receptor internalization but does markedly reduce the association of the receptor with the overexpressed β -arrestin 1. In contrast, mutation of the Leu-Leu and/or Ile-Leu in the LLKIL motif of CXCR2 reduces the receptor internalization without altering the association of β -arrestin 1 with the receptor. Moreover, the endogenous expression of β -arrestin 1 is not detectable by Western blot analysis, and CXCR2 does not coimmunoprecipitate with the endogenous β -arrestin 1 (data not shown). Overexpressing dominant negative β -arrestin 1 does not block CXCR2 internalization in HEK293 cells (data not shown). However, it is not easy to exclude the potential role of β -arrestins in the internalization of CXCR2 in other cell types. In fact, the previous study has shown that truncation of the carboxyl-terminal phosphorylation sites impairs the receptor internalization in 3AsubE cells (19), implying the possibility of a greater involvement of β -arrestins in the receptor endocytosis in this cell line. In addition, recent studies on CXCR1 have shown that IL-8 stimulation promotes CXCR1 sequestration in RBL-2H3 cells, but the receptor internalization in HEK293 cells requires cotransfection of GRK2 and β -arrestins (38). These data suggest that HEK293 cells do not express a sufficient amount of endogenous β -arrestins for regulation of CXCR1 internalization. However, Feniger-Barish et al. (39) observe internalization of both CXCR1 and CXCR2 in HEK293 cells in the absence of overexpression of GRK or β -arrestin. It is possible that the HEK293 cells from the two labs have different levels of β -arrestin. Our data confirm that very low endogenous levels of β -arrestin are expressed in HEK293 cells. Moreover, we demonstrate that CXCR2 contains the LLKIL motif that is able to bind endogenous AP-2 as an adaptor protein. This internalization motif is retained in the carboxyl-terminal truncation mutant of CXCR2 and thus facilitates ligand-mediated receptor inter-

nalization. It is implied that RBL and 3AsubE cells express reduced amounts of AP-2 and are unable to internalize the truncated receptor, though they can internalize the wild-type receptor through interaction with β -arrestin. Thus, it appears that not only are there cell type differences in expression of adapter proteins that facilitate receptor endocytosis, but receptors can utilize more than one type of adapter protein to ensure internalization as well.

Zhang et al. (13) also show that activation of the β 2-AR is associated with the translocation of β -arrestin from the cytosol to the plasma membrane in HEK293 cells. Moreover, Orsini et al. (40) have demonstrated that HEK293 cells expressing CXCR4 respond to SDF-1 ligand by redistributing arrestin 2 from the cytosol to clathrin-coated vesicles, where a large portion of the arrestin 2 colocalizes with the internalized CXCR4. In their study, mutation of either the isoleucine/leucine residues or serine residues in the carboxyl-terminal domain of CXCR4 markedly reduced ligand-induced receptor internalization, but overexpression of arrestin-3 and GRK2 would partially compensate with the reduction in receptor internalization of the receptor mutants. Thus the data of Orsini et al. (40) support our conclusions that both dileucine motifs and phosphorylated serine residues are important in the recruitment of adaptins or arrestins to the receptor to facilitate receptor sequestration. In the absence of the dileucine residues, which are involved in the recruitment of adaptins, the binding of arrestins can partially facilitate receptor internalization. It might be argued that since β -arrestins associate endogenously with AP-2 β (18), our data could be interpreted to show that the association of AP-2 with CXCR2 is indirect through association with β -arrestin 1 and not direct through association with CXCR2. However, since CXCR2-331T shows a loss of ligand-induced β -arrestin 1 association but retention of association with AP-2, this seems unlikely. Moreover, the LL and/or IL mutations result in a loss of AP-2 association with CXCR2, but retention of β -arrestin association with the receptor in coimmunoprecipitation experiments. These data clearly show that the LLKIL motif is involved in AP-2 association with CXCR2, independent of the binding of β -arrestin 1.

One of the most important functions of chemokine receptors is receptor-mediated chemotaxis. Because inhibition of agonist-induced receptor endocytosis impairs the cell chemotaxis, it has been suggested that receptor internalization and recycling to the cell membrane may provide an on-off mechanism for the receptor-mediated chemotaxis or may be required for detection/response to the chemokine concentration gradient (3, 19). The amino acid residues 317–324 in the carboxyl terminus of CXCR2 have been shown to be essential for receptor-mediated chemotaxis in response to IL-8 (41). The present data further demonstrate that the carboxyl-terminal internalization motif LLKIL within residues 317–324 is involved in the receptor-mediated chemotaxis. It has been suggested that failure to desensitize or internalize the receptor may increase the length and strength of second messenger, and this conceptually could have an effect on chemotaxis (42). Our calcium mobilization data argue that blocking the internalization of CXCR2 by mutating the LLKIL motif does not alter calcium desensitization but does block chemotaxis. However, we cannot rule out the possibility that other second messenger signals are increased and this might play a negative regulatory role in chemotaxis.

In conclusion, the LLKIL motif in the carboxyl terminus of CXCR2 is essential for the receptor internalization and receptor-mediated chemotaxis in HEK293 cells, and AP-2, which binds to this motif, may be involved in the receptor internalization by interacting with both the receptor and clathrin. This is the first demonstration of a role for AP-2 in chemokine receptor internalization and chemotaxis, and our data provide a possible mechanism for cell-specific differences in the internalization of chemokine receptors based upon cell to cell differences in the availability of these adapter proteins. Moreover, internalization of receptors may vary depending upon the presence or absence of AP-2 binding motifs.

ACKNOWLEDGMENT

We thank Jinming Yang and Dingzhi Wang in our laboratory for helpful discussion, Yingchun Yu for excellent technical assistance, Ben Johnston for editorial assistance, and the Vanderbilt-Ingram Cancer Center Confocal Microscopy Lab for technical assistance with the confocal microscopy. We are indebted to Repligen Corporation for generously supplying the MGSA ligand used for these studies.

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BI001661B