Interruption of G Protein-Coupling in CXCR2 Does Not Alter Ligand Binding, but Eliminates Ligand-Activation of GTPγ^{35}S Binding, Calcium Mobilization, and Chemotaxis†

Wei Yang,‡ Wayne P. Schraw,‡ Susan G. Mueller,‡ and Ann Richmond*,‡,§

Department of Cell Biology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-2175, and Veterans Affairs Medical Center, Nashville, Tennessee 37212-2637

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ABSTRACT: CXCR2 is a seven-transmembrane receptor that transduces intracellular signals in response to the chemokines IL-8, MGSA/GRO, and other ELR motif-containing CXC chemokines by coupling to heterotrimeric GTP-binding proteins. In this study, we have mutated two putative G protein-coupling regions of CXCR2 and characterized the effects of these mutations on ligand-activated signal transductions: aspartic acid 89 in the second transmembrane domain and the HRAMR sequence (BBXXB motif, found in the third intracellular loop where B indicates a basic amino acid and X represents any amino acid). The Asp89 was replaced by either asparagine (D89N) or glutamic acid (D89E). For the BBXXB motif, the first two basic amino acids were mutated to two neutral isoleucines (HR-II), or alternatively, two isoleucines were inserted between alanine and methionine (II-insert). When expressed in human embryonic kidney 293 cells, the D89E mutant was localized intracellularly with no detectable cell surface expression. In contrast, D89N, HR-II, and II-insert mutants displayed cell surface expression, with K_d values and expression levels similar to that of the wild-type transfectant. The ability of the mutants to transduce signal was assessed by ligand-stimulated GTPγ^{35}S binding, mobilization of intracellular free Ca^{2+}, and chemotaxis assays. Both D89N and HR-II mutants signaled similarly to a wild-type receptor in all three assays. However, the II-insert mutant exhibited a loss of ligand-stimulated GTPγ^{35}S binding, calcium mobilization, and chemotaxis. Unexpectedly, this receptor underwent ligand-induced sequestration comparable to wild-type CXCR2. These data indicate that Asp89 and the basic amino acids in the third intracellular domain do not play essential roles in ligand-induced signal transduction through CXCR2. However, proper secondary structure and orientation of the third intracellular loop of CXCR2 are essential for ligand-mediated signal transduction but not for receptor sequestration.

Melanoma growth-stimulatory activity (MGSA)/growth-regulated protein (GRO),[1] interleukin-8 (IL-8), and neutrophil-activating peptide-2 (NAP-2) are members of a family of structurally related cytokines which induce chemotaxis and respiratory burst in neutrophils (*1*−*3*). These chemokines belong to the CXC chemokine subfamily in which the first two conserved cysteine residues are separated by an intervening amino acid (*3*). Several chemokine receptors have been cloned which bind MGSA/GRO; these are CXCR2 (formerly called IL-8 RB) (*4*), Duffy antigen receptor for chemokine (DARC), and two herpesvirus-encoded receptors: the herpesvirus saimiri ECRF-3 and the human herpesvirus-8 G protein-coupled receptor (*5*). IL-8 binds to these four receptors as well as CXCR1 (formerly called IL-8 RA), which is a specific receptor for IL-8 (*6*). CXCR2 is a shared receptor that binds to IL-8, MGSA/GRO, NAP-2, ENA-78, and GCP-2.

CXC receptors are members of a superfamily of integral membrane receptors that transduce signals to the interior of the cell through heterotrimeric guanine nucleotide-binding proteins (G proteins). They share a common putative structural topology comprised of seven-transmembrane domains separated by three extracellular and three intracellular regions. The extracellular regions form pockets in which specific ligands can interact or bind. Upon agonist binding, CXC receptors activate G protein-mediated phosphoinositide hydrolysis to generate diacylglycerol and inositol 1,4,5-triphosphate, thereby activating protein kinase and mobilizing Ca^{2+} to initiate a variety of cellular responses (*7*). Receptor activation is followed by receptor phosphorylation and subsequent down-regulation. These events are accompanied by receptor endocytosis and/or recycling of the receptor (*8*).

There are considerable structural and sequence diversities among the seven-transmembrane receptor family to accommodate functional differences. For example, adrenergic and muscarinic receptors have a much larger third intracellular loop and cytoplasmic tail than most peptide receptors. Despite this diversity, G protein-coupled receptors contain some highly conserved amino acid residues. Site-directed

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* Author to whom correspondence should be addressed.

‡ Vanderbilt University School of Medicine.

§ Veterans Affairs Medical Center.

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[1] Abbreviations: CXC chemokine, chemokines with the first two conserved cysteine residues separated by an intervening amino acid; CC chemokine, chemokines with the first two cysteines positioned side by side; D89N, CXCR2 mutant with asparagine-to-aspartic acid substitution at position 89; D89E, CXCR2 mutant with glutamic acid-to-aspartic acid substitution at position 89; HR-II, CXCR2 mutant with two isoleucines substituting for histidine and arginine at positions 241−242; II-insert, CXCR2 mutant with two isoleucines between alanine 243 and methionine 344; HEK-293 cells, human embryonic kidney cell line; CMV, cytomegalovirus; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; FURA-2, fluorescence indicator for free calcium; IL-8, interleukin-8; MGSA/GRO, melanoma growth-stimulatory activity/growth-regulated protein; WT, wild-type; PCR, polymerase chain reaction.

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mutagenesis, construction of chimeric receptors, and the use of synthetic peptides have suggested that the intracellular portions of the receptors, particularly the second and third intracellular loops, together with the membrane proximal cytoplasmic tail, are the regions that physically interact with G proteins (*9*−*14*). Most of these data, however, are based on the studies of adrenergic and muscarinic receptors; studies on the family of peptide receptors, including CXC receptors, are comparatively more limited. The DRY sequence at the boundary between the third transmembrane domain and second intracellular loop is conserved among adrenergic, muscarinic, dopaminergic, and chemokine receptors. Mutation of this sequence frequently impairs receptor-mediated signaling of CXC chemokine receptors and peptide receptors, suggesting a general role in G protein-activation (*15*). Another highly conserved residue is an aspartic acid residue which is found in the middle in the second transmembrane domain. BBXB or BBXXB (B represents a basic residue, X represents a nonbasic residue) sequences in the third intracellular loop are less well conserved among G protein-coupled receptors; however, they are believed to be important in activating G proteins in some receptor subfamilies (*16*). The Asp89 in the second transmembrane domain and the HRAMR (BBXXB motif) sequence in the third intracellular loop are conserved in CXCR2. In the present study, we explore the roles of these sequences in agonist-induced signal transduction. The ligand-binding profiles and the G protein-mediated signal transduction pathways of these CXCR2 mutants were determined and compared to those of wild-type CXCR2 in stably transfected human embryonic kidney 293 cells. We show here that while ligand binding is retained in the D89N and BBXXB motif mutants, alternation of CXCR2 by insertion of two isoleucine residues in the BBXXB motif prevents ligand-induced G protein-coupling, calcium mobilization, and chemotaxis.

MATERIALS AND METHODS

Cell Culture. A culture of 293 human embryonic kidney cells (HEK-293) was prepared in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. Stable transfectants of HEK-293 cells were selected in the presence of 600 μg/mL Geneticin (G418) (Gibco BRL). All cells were routinely incubated at 37 °C with 5% CO_2.

Construction of Mutant CXCR2. The human CXCR2 cDNA in the mammalian expression vector pRc/CMV (Invitrogen) was used as a template for polymerase chain reaction-mediated mutagenesis (*17*). Two putative G protein-coupling regions of CXCR2 were mutated: (1) a conserved Asp89 in the second transmembrane domain and (2) an HRAMR domain at the carboxyl side of the third intracellular loop. The Asp89 was mutated to either a glutamate residue (D89E), conserving the charge, or an asparagine residue (D89N), removing the charge. Two strategies were used for HRAMR domain mutagenesis: one mutant was designed to replace the first two basic amino acids (HR) with two neutral isoleucine residues (IIAMR), and the other mutant was designed to disrupt the helical structure by inserting two isoleucine residues between the alanine and the methionine (HRIIMR). The PCR was employed to introduce the mutations. Briefly, two overlapping PCR products were generated containing the desired mutation at one end. These products were gel-purified and annealed. Two flanking primers (5′ CXCR2 and 3′ CXCR2 primers) were subsequently used to perform a second round of PCR to generate the full-length receptor. For the first round of PCR involving amplification of the 5′ end of the CXCR2, the primers included a common primer specific for the 5′ end of the CXCR2 receptor. Specific primers for the desired mutations were as follows:

5′ CXCR2:
GCGAAGCCTATGGAGAGTGACAGCTTTGAA

D89E:
AAAGAGTAGCTCGGCCAAGGCTAGGTT

D89N:
AAAGAGTAGGTTGGCCAAGGCTAGGTT

HR-II:
ATGGCAATAATCTTCTGCCCCAT

II-insert:
CGCATAATAATGGCCGGGTGCTTC

For the first round of PCR involving amplification of the 3′ end of CXCR2, the primers included a common primer specific for the 3′ end of the CXCR2 receptor and primers specific for the desired mutations as follows:

3′ CXCR2:
GCGAAGCTTTTAGAGAGTAGTGGAAGTGTG

D89E:
TAGCCTTGGCCGAGCTACTCTTTG

D89N:
TAGCCTTGGCCAACCTACTCTTTG

HR-II:
AGAAGATTATTGCCATGCGGGTC

II-insert:
GGCCATTATTATGCGGGTCATC

The PCR fragments were subcloned into BlueScript and sequenced using Sequenase Version-2 (Amersham) to verify that the desired mutations had been incorporated and to ensure that other mutations had not been introduced by PCR. The mutated cDNAs were then subcloned into the mammalian expression vector pRc/CMV at the *Hind*III site.

Cell Transfections. For stable expression of wild-type and mutant CXCR2, HEK-293 cells cultured to 50% confluence were transfected with 20 μg of receptor cDNA in pRC/CMV vector using the calcium phosphate co-precipitation method (*18*). Transfected cells were selected in the presence of 600 μg/mL G418 for 2−3 weeks. Individual drug-resistant clones were isolated and maintained in 300 μg/mL G418. Clones expressing receptors were identified initially by indirect immunofluorescence staining and were confirmed by Western blot analysis. On the basis of these results, clones selected for complete study were HR-II_G, $D89N_5$, and II-$insert_6$.

Western Blot Analysis. Clonally selected, stable transfectants were grown to confluence on 100 mm culture dishes. Cells were washed once with ice-cold PBS and lysed in RIPA buffer containing 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 10 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 10 μg/mL aprotinin, and 10 μg/mL leupeptin. Lysates were clarified by 10 min centrifugation

at 4 °C at 16000*g* in an Eppendorf microfuge. Cleared supernatants containing approximately 50 μg of protein (estimated by BCA method, Pierce Chemical) were electrophoresed through a 7.5% SDS polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). Membranes were blocked for 1 h at room temperature in 5% milk powder/TBS with 0.05% Tween 20 (TBST). Blots were then incubated at 4 °C overnight with anti-amino terminal CXCR2 peptide antibody (1:1000 dilution) as previously described (*17*). After incubation with antibody, the membranes were washed three times with TBST for 10 min each and incubated at room temperature with a 1:2000 dilution horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Boehringer) or alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG secondary antibody (Sigma). The membranes were subsequently washed three times with TBST and developed with the ECL Western blotting analysis system (Amersham) for HRP conjugates or bromochloroindoyl phosphate (Sigma) and nitroblue tetrazolium (Sigma) for AP conjugates.

[^{125}I]MGSA Binding Assay. MGSA/GRO was generously provided by R+D Systems and Repligen Corp. [^{125}I]MGSA was purchased from DuPont NEN at a specific activity of 272 μCi/μg. Binding assays were performed as previously described with some modifications specific for HEK-293 cells (*8*). Cells were plated onto 100 mm dishes 24 h before the binding assay to obtain about 90% confluence at the time of the experiment. Cells were trypsinized and washed once with medium. The cells were then allowed to recover in 10 mL of serum-containing medium in a polypropylene tube at 37 °C for 2−3 h. Cells were incubated in ice-cold binding buffer (1 mg/mL ovalbumin in DMEM) for 30 min, then binding buffer containing [^{125}I]MGSA (20 000−30 000 cpm) and various concentrations of unlabeled MGSA were added to the cells and they were gently rocked at 4 °C for 2 h. Cells were washed with the binding buffer, and the bound [^{125}I]MGSA was counted in a γ-counter (Beckman, Gamma 5500). Each assay was repeated a minimum of two times in triplicate. Scatchard analysis of binding data was determined using the LIGAND program.

GTPγ^{35}S Binding Assay. Membranes for GTPγ^{35}S binding assay were prepared as described previously (*17*). Briefly, cells were scraped and washed once in ice-cold PBS. Cell pellets were resuspended in 10 mL of PBS buffer containing 200 mM sucrose and homogenized with 20 strokes of a Dounce homogenizer. The homogenates were centrifuged at 500*g* for 10 min to remove the unbroken cells and nuclei. The cleared supernatants were overlaid onto a 15 mL 40% sucrose cushion and centrifuged at 35000*g* for 1 h. The plasma membranes were then collected at the interface and centrifuged at 40000*g* for 30 min. The membranes were resuspended in PBS, and the protein concentrations were determined (BCA, Pierce). Typically, membranes containing 5 μg of protein were used for each reaction. The reaction, which was initiated by the addition of membrane to the reaction mixture, was allowed to proceed at 37 °C for 10 min. Reactions were terminated by the addition of ice-cold wash buffer (50 mM Tris, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA). The reaction mixtures containing membrane-bound GTPγ^{35}S were then filtered through 0.45 μm Gelman A/E fiberglass filters which were subsequently washed three times with wash buffer. The amount of GTPγ^{35}S bound to membrane on the filter was determined by scintillation counting (Beckman, LS 3801).

Indirect Immunofluorescence. Cells were grown on 10 mm coverslips (Fisher) and were fixed and permeabilized with −20 °C methanol for 4 min for intracellular staining or with 3.7% paraformaldehyde for cell surface staining. The cells were incubated with anti-amino terminus CXCR2 antibody (1:100 dilution) for 30 min at room temperature. After three washes, goat anti-rabbit antibody conjugated to FITC (Sigma) was added at 1:200 dilution for 30 min. After the final three washes, the specimens were mounted on a glass slide with Mowiol solution (Polyscience, Inc.). Cells were examined with an epifluorescence microscope (Carl Zeiss, Inc.), using a filter set selective for FITC. Photographs were taken using a CCD camera and processed by Adobe Photoshop software.

Chemotaxis Assay. Chemotaxis assays were performed on HEK-293 cells expressing wild-type or mutant CXCR2 as described (*8*) using a 96-well chemotaxis chamber (Neuroprobe, Inc.). The lower compartment of the chamber was loaded with 360 μL/well of 1 mg/mL ovalbumin/DMEM containing MGSA/GRO at 0−500 ng/mL. The polycarbonate membrane (10 μm pore size, Neuroprobe, Inc.) was coated on both sides with human collagen IV (Sigma) for 2 h at 37 °C. Before chemotaxis experiments, cells were removed from culture dishes by trypsinization, then allowed to recover during a 2−3 h incubation at 37 °C in 10% FBS/DMEM in suspension. Cells were washed in chemotaxis buffer (DMEM + 1 mg/mL ovalbumin), and then placed into the upper chamber in 250 μL of chemotaxis buffer at $(0.5-1.25) \times 10^6$ cells/well. The chambers were incubated 5 h at 37 °C in 5% CO_2/air. Thereafter, the filter was stripped to remove cells on the upper surface, fixed, and stained with a DiffQuik kit (Baxter). Evaluation of the relative chemotactic index was performed by counting the mean number of cells migrating to the underside of the filter in five high-power fields (×400) and expressing this relative to the mean number of cells migrating to ovalbumin alone.

Calcium Fluorimetry. Transfected HEK-293 cells expressing wild-type or mutant CXCR2 were grown on duplicate 100 mm tissue culture dishes until confluent. Cells were removed from dishes with trypsin/EDTA, washed once with culture medium, and then incubated 1.5−3 h at 37 °C in culture medium to allow recovery. Cells were washed twice with Krebs Ringer buffer (KRB) containing 118 mM NaCl, 4.56 mM KCl, 25 mM $NaHCO_3$, 1.03 mM KH_2PO_4, 5 mM Hepes, and 11.1 mM glucose without Ca^{2+} or Mg^{2+}. Cells were subsequently resuspended at $(2-4) \times 10^6$ cells/mL and incubated with 2 μM FURA-2 for 30 min in a final volume of 10 mL. Cells were diluted with an equal volume of Krebs Ringer buffer without Ca^{2+}/Mg^{2+} and incubated for additional 10 min. Cells were then centrifuged and resuspended in Krebs Ringer buffer containing Ca^{2+}/Mg^{2+}, recentrifuged, and resuspended in Ca^{2+}/Mg^{2+} containing Krebs Ringer buffer at a density of 1×10^6 cells/mL. Experiments were performed using a Perkin-Elmer LS50 fluorimeter with a stirred, heated (37 °C) cuvette. Cells were temperature equilibrated for 5 min and treated with placebo over which time the basal line spectrum was observed for 4 min. Immediately afterward, the same cell suspension was treated with 5 nM IL-8 or 10 nM MGSA/GRO. Initial dose-dependent studies (1−10 nM ligand) demonstrated that this concentration was optimal for the cells studied. Data curves are presented as the ratio of the excitation (340 nm/380 nm)

maxima of FURA-2, 510 nm emission relative to time. Each experiment was repeated three times on different days with similar results for each clone.

Receptor Internalization Assay. The acid/buffer wash technique similar to that described by Haigler et al. (*19*) was used to determine kinetics of MGSA/GRO-induced internalization of WT and mutant CXCR2. Cells were trypsinized and recovered in DMEM + 10% FBS for 2 h at 37 °C. Cells were then incubated in ice-cold binding buffer for 30 min. Binding and internalization experiments were performed in duplicate 1.5 mL Eppendoff microcentrifuge tubes with 1 mL cell suspensions (5×10^5 cells/tube). Following incubation with [^{125}I]MGSA/GRO in ice-cold binding buffer, the cells were washed once with ice-cold binding buffer to remove the unbound [^{125}I]MGSA/GRO. Prewarmed (37 °C) binding buffer was added to the cells for indicated time periods to allow the receptor internalization. The cells were then treated with 1 mL of acetic acid (0.2 M, pH 2.5) containing 0.5 M NaCl for 6 min at 4 °C to strip the uninternalized [^{125}I]MGSA/GRO. Subsequently the cells were washed once with 1 mL of the same solution, and the remaining radioactivity was determined by a γ-counter. Nonspecific binding was determined by measuring the binding in the presence of excess unlabeled MGSA/GRO (200 ng/mL). Internalization of the receptor was calculated as the ratio of specific binding after acid wash and specific binding after binding buffer wash. The experiments were repeated at least two times for each clone.

RESULTS

Effects of D89 and BBXXB Mutations on Receptor Expression and Ligand Binding. We constructed several CXCR2 mutants where two putative G protein-coupling regions were selectively mutated to determine the effect of each of these mutations on G protein-mediated signal transduction. These two selected regions were Asp89 in the second transmembrane domain and the HRAMR (BBXXB) motif in third intracellular loop. Both are believed to play important roles in G protein-coupled signaling based on the information available from other seven-transmembrane receptor families. The Asp89 was mutated to either a glutamate residue (D89E) or an asparagine residue (D89N). For the HRAMR motif, either the two basic HR residues were mutated to two neutral isoleucine residues (HR-II) or two isoleucines were inserted between residues A and M (II-insert) to break the helix structure. It is possible that these mutations can affect the receptor folding and processing, giving rise to a receptor which is not expressed on the cell surfaces. Therefore, we tested this by using indirect immunofluorescence staining to confirm the cell surface expression of the mutant receptors. Subsequently, ligand-binding analyses were performed to determine whether the mutations affected the ligand-binding properties of the mutated receptors.

Using previously established methods, the mutant as well as wild-type CXCR2 were transfected into HEK-293 cells which naturally have no detectable CXCR2 expression. Twenty-four hours after transfection, cells were fixed in 3.7% paraformaldehyde for cell surface staining using anti-amino terminal antibody which recognizes the extracellular epitope of CXCR2. Like the cells transfected with wild-type receptor, the cells transfected with D89N, HR-II, and II-insert mutants showed cell surface immunofluorescence

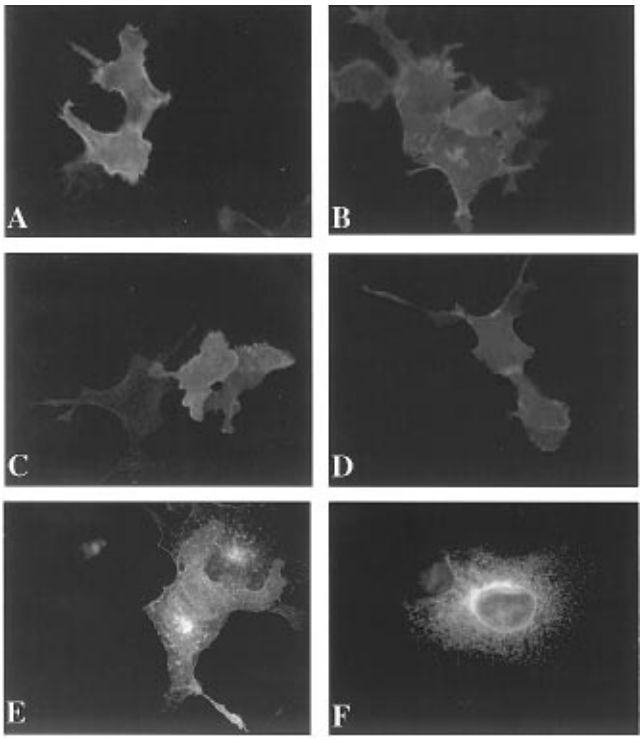

FIGURE 1: Subcellular localization of WT and mutant CXCR2. HEK-293 cells were transfected with plasmids encoding WT (A, E), II-insert (B), HR-II (C), D89N (D), and D89E (F) receptors using the calcium phosphate method. Twenty-four hours after transfection, cells were either fixed with 3.7% paraformaldehyde for the cell surface staining (A−D) or 100% methanol for intracellular staining (E,F). The receptors were visualized with anti-amino terminal CXCR2 rabbit polyclonal antibody followed by FITC conjugated anti-rabbit secondary antibody.

(Figure 1A−D). However, no surface fluorescence was detected for cells transfected with D89E mutant (data not shown). Interestingly, when cells were fixed and permeabilized with 100% methanol for intracellular staining, the D89E mutant stained with a pattern characteristic of the typical endoplasmic reticulum pattern. For all other mutants, the intracellular staining was perinuclear, Golgi-like staining, which was similar to that of the wild-type receptor (Figure 1E,F). These results suggest that substitution of aspartic acid 89 with glutamic acid in the second transmembrane domain of CXCR2 alters the three-dimensional structure of the receptor in such a way as to interfere with its post-translational transportation and sorting.

To characterize the ability of the mutant CXCR2 receptors to transduce signals, HEK-293 cells were stably transfected with these mutant receptors and G418-resistant clones were selected and screened by immunofluorescence. None of the D89E clones showed detectable receptor expression. For the remaining mutants, at least four independent clones were selected and expanded. The level of receptor expression was first estimated by Western blotting where equal amounts of protein were loaded on the SDS−polyacrylamide gel prior to transfer (data not shown). The clones exhibiting the highest level of expression, II-insert$_6$, HR-II$_G$, and D89N$_5$, were then further characterized for MGSA/GRO binding using Scatchard analysis. The ligand dissociation constant (K_d) and number of receptors per cell were calculated using the LIGAND program. The K_d for mutant CXCR2 was similar to that of wild-type receptor (1.2−1.9 nM, Table 1). The similar dissociation constant between the wild-type and

Table 1: MGSA/GRO Binding for Cells Transfected with WT and Mutant CXCR2[a]

cell line	receptors/cell (10^4)	K_d (nM)
wild-type	6.98 ± 0.54	1.37 ± 0.11
$D89N_5$	2.12 ± 0.25	1.17 ± 0.10
D89E	ND	ND
HR-II_G	4.05 ± 0.34	1.88 ± 0.21
II-$insert_6$	4.03 ± 0.48	1.88 ± 0.15

[a] Scatchard analysis of MGSA/GRO binding properties of cells transfected with wild-type and mutant CXCR2. Clones of HEK-293 cells were characterized for the ligand binding of ^{125}I-MGSA/GRO. The K_d and number of expressed receptors were calculated from two separate experiments performed in triplicate using the LIGAND program as described in Materials and Methods.

mutant receptors suggested that the introduced mutations did not disturb the ligand-binding properties or overall conformation of the receptors. The cells expressing wild-type CXCR2 exhibited the greatest number of binding sites/cell (6.98×10^4 receptors/cell). The average number of receptors/cell for cells expressing the $D89N_5$ mutant was about 3-fold less (2.12×10^4 receptors/cell) than that of the wild-type transfectant. The receptor expression levels for the II-$insert_6$ and HR-II_G clones were similar (4×10^4 receptors/cell) and about 1.5-fold lower than wild-type. This slightly lower level of receptor expression in mutant clones reflects the clonal variation and does not appear to result from the mutations introduced. The 1.5- to 3-fold reduction in receptor numbers is unlikely to affect the efficiency of ligand-induced signal transduction.

Effect of Receptor Mutation on GTPγ^{35}S Binding in Response to MGSA/GRO Stimulation. It has been reported previously that MGSA/GRO stimulated a 1.7-fold increase in GTPγ^{35}S binding in neutrophil membrane preparations (*20*). We also have demonstrated that MGSA/GRO increased the GTPγ^{35}S binding to the membranes prepared from HEK-293 and 3ASubE cell lines stably expressing wild-type CXCR2 by approximately 2-fold. These effects were partially blocked by pretreatment of cells with pertussis toxin (*17*). Thus, the data indicate that a pertussis toxin-sensitive G protein, possibly Gαi, is involved in receptor signal transduction. Therefore the GTPγ^{35}S binding assay was used to test whether the mutations introduced into CXCR2-altered G protein-coupling. Membranes prepared from the HEK-293 transfectants were treated in the absence or presence of 100 ng/mL MGSA/GRO for 10 min, after which the membrane-bound GTPγ^{35}S was determined for each clone. Compared to untreated membrane, MGSA/GRO stimulated about a 1.85-fold increase in the level of GTPγ^{35}S binding to membrane preparations from cells transfected with wild-type receptor (Figure 2). The increase in GTPγ^{35}S binding observed for membranes prepared from $D89N_5$ and HR-II_G cells were 1.74-fold and 1.62-fold, respectively (Figure 2). Considering that the receptor expression levels of both mutants is slightly lower than that of wild-type transfectants, the reduced increase of GTPγ^{35}S binding in the membranes prepared from $D89N_5$ and HR_G as compared to that of wild-type receptor is expected. In contrast, the membrane prepared from II-$insert_6$ cells exhibited no increase in GTPγ^{35}S binding with MGSA/GRO treatment (1.08-fold over untreated control) (Figure 2). This level is equivalent to that of membrane from untransfected parental HEK-293 cells. Repeated experiments with three other II-insert clones also failed to show any increase of GTPγ^{35}S binding to the membrane preparations after MGSA/GRO stimulation. These data suggest that while D89N and HR-II mutant receptors are still functionally coupled to G proteins, the ligand-stimulated G protein-coupling of the II-insert receptor is absent or at least markedly reduced.

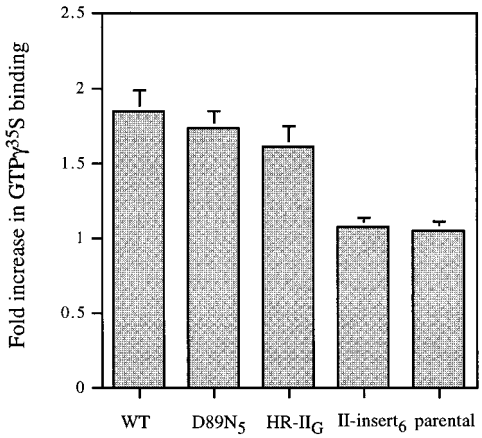

FIGURE 2: MGSA/GRO-induced GTPγ^{35}S binding in membranes prepared from HEK-293 cells transfected with mutant and WT CXCR2. GTPγ^{35}S binding assays were performed using membrane purified by sucrose gradient centrifugation. The binding assays were performed as described in Materials and Methods. Results are expressed as the -fold increase in GTPγ^{35}S binding detected in the MGSA/GRO-treated membranes over the untreated control. The data represent the mean ± SE of four different experiments with triplicate determinations.

Calcium Mobilization through Wild-Type and Mutant CXCR2. To further examine the signal transduction pathway of mutant CXCR2, mobilization of intracellular free Ca^{2+} in response to MGSA/GRO or IL-8 treatment was measured using FURA-2 fluorescence. Wild-type CXCR2 responded strongly to 5 nM IL-8 stimulation with an increase in intracellular Ca^{2+} concentration, which peaked approximately 30 s following treatment (Figure 3). MGSA/GRO treatment (10 nM) produced a similar Ca^{2+} mobilization which also peaked around 30 s after ligand addition (data not shown). These observations were consistent with our previous report using 3ASubE P3 cells (*8*). The $D89N_5$ and the HR-II_G clones showed similar Ca^{2+} mobilization responses to IL-8 and MGSA/GRO treatment compared to wild-type receptor clones. Both the magnitude of response and the time required to remove ~80% of the mobilized calcium were identical to that of cells expressing wild-type CXCR2. However, II-$insert_6$ clone failed to mobilize calcium in response to both IL-8 (5 nM) and MGSA/GRO (10 nM) (Figure 3). Similar results were observed for four different II-insert clones in two to three different experiments, demonstrating that the failure to respond to ligand observed in the II-insert expressing clones was not due to clonal variation. These data suggest that the II-insert mutant receptor is defective in the signaling pathway which leads to calcium mobilization.

Chemotaxis of Wild-Type and Mutant CXCR2. Ligand-stimulated CXCR2-mediated chemotaxis is a direct functional test for receptor signal transduction. We used chemotaxis assays to examine the ability of HEK-293 cells expressing wild-type and mutant CXCR2 to chemotaxis toward a gradient of MGSA/GRO. We observed a MGSA/GRO concentration-dependent chemotactic response in cells expressing wild-type receptor, with a peak migration occurring at a concentration around 20 ng/mL MGSA/GRO (10−

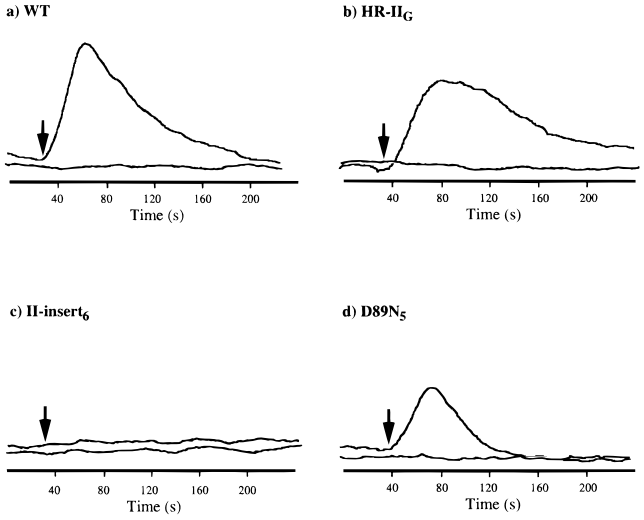

FIGURE 3: Calcium mobilization in response to IL-8 of cells stably expressing mutant and WT receptors. Representative response when wild-type, D89N, HR-II, and II-insert cells were treated with 5 nM IL-8 at the time point indicated by the arrow. Cells were loaded with 2 mM FURA-2 as described in Materials and Methods after allowing cells to recover after trypsinization. Spectra were obtained during 4 min runs to which buffer control (lower curve) and 5 nM IL-8 (upper curve) were added at 30 s (arrow). Relative fluorescence is shown on the ordinate and time (seconds) on the abscissa. Representative experiments are shown in which values obtained on the same day are presented. The experiment was repeated three times on different days with similar results.

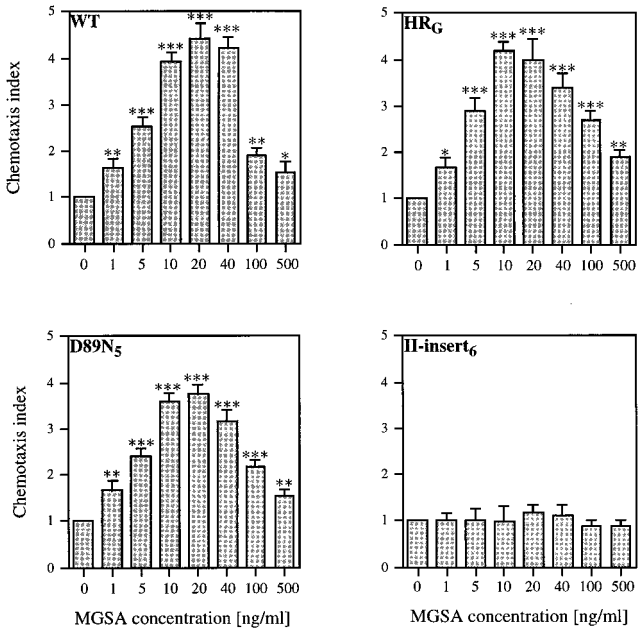

FIGURE 4: Effect of HR-II, II-insert, and D89N mutations of CXCR2 on MGSA/GRO-stimulated chemotaxis of HEK-293 cells stably expressing wild-type or mutant receptors. HEK-293 cells stably expressing wild-type (WT) or mutant receptors at similar levels on the cell surface were compared for chemotaxis in response to MGSA/GRO stimulation as described in Materials and Methods. Values in this figure represent the means ± SE of three independent experiments performed on different days. The data were analyzed using Student's paired *t*-test, *p < 0.05, **p < 0.005, ***p < 0.001.

40 ng/mL). Also the cellular migration response followed a typical bell-shaped curve in which the chemotaxis was inhibited at higher concentrations of MGSA/GRO (Figure 4). This is in agreement with previous data using the same cell line (*8*, *21*). HR-II$_G$ and D89N$_5$ cells responded to all ligand concentrations tested, and the magnitude of migration

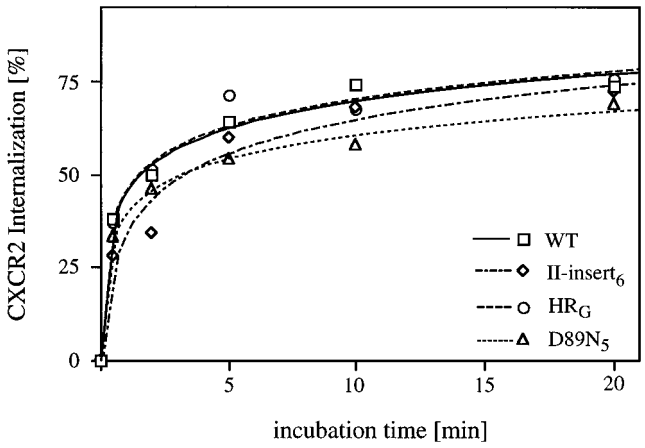

FIGURE 5: The MGSA/GRO-induced WT and mutant CXCR2 internalization. Stable 293 cells expressing WT (□), II-insert$_6$ (◇), HR-II$_G$ (○), and D89N$_5$ (△) were preincubated with binding buffer containing the [^{125}I]MGSA/GRO for 30 min at 4 °C. Unbound [^{125}I]-MGSA/GRO was removed by washing at 4 °C. The cells were warmed to 37 °C for the indicated time period (also see Materials and Methods). [^{125}I]MGSA/GRO remained at cell surface and was removed with acetic acid (0.2 M, pH 2.5) containing 0.5 M NaCl, and the internalized [^{125}I]MGSA/GRO was then counted by a γ-counter. Data are presented as mean (n = 2) from one representive experiment out of three. Binding to duplicate samples varied generally by less than 10%.

toward MGSA/GRO was identical to that seen with the cells expressing wild-type receptor. In contrast, cells expressing the II-insert mutant receptor (II-insert$_6$ clone) did not migrate in response to MGSA/GRO at any concentration tested. Again, multiple clones of each transfectant were tested, and the experiments were repeated at least twice for each clone. These data suggest that the interruption of the third intracellular loop BBXXB motif results in a mutant CXCR2 which is uncoupled from G proteins and is therefore unable to exhibit ligand-induced chemotactic response.

Effect of Mutations on MGSA/GRO-Induced Receptor Internalization. The MGSA/GRO-dependent CXCR2 internalization was analyzed using [^{125}I]MGSA/GRO to induce and determine the rate of CXCR2 internalization. After an incubation of approximately 30 s at 37 °C, MGSA/GRO induced about 35% wild-type CXCR2 internalization. The maximum internalization of the receptors was reached in 5−10 min (Figure 5). The kinetics of agonist-induced internalization of II-insert$_6$, HR-II$_G$, and D89N$_5$ clones were similar to that of the WT cells. The fact that II-insert mutant receptor was internalized normally suggests that the G protein-coupling is not required for ligand-induced CXCR2 endocytosis.

DISCUSSION

Two classes of human IL-8 receptor, CXCR1 and CXCR2, have been identified by expression cloning (*4*, *6*). Both receptors are G protein-coupled seven-transmembrane receptors. The fact that these two receptors share more than 98% identity in their three intracellular domains suggests that similar, if not identical, signal transduction mechanisms operate for both receptors. Extensive studies have been performed to identify biologic functions, ligand-binding specificities, and the intracellular signaling events for these receptors. The structural and functional importance of the carboxyl-terminal domain in signal transduction has been examined in chemotaxis assays of HEK-293 cells expressing

progressively truncated CXCR2 (*21*). This study suggested that the membrane proximal part of carboxyl-terminal domain consisting of amino acids 317−324 was required for receptor-mediated chemotaxis (*21*). However, it was unclear whether this region was involved in G protein-coupling. Recently, Damaj et al. (*15*) identified the residues involved in coupling both CXCR1 and CXCR2 to $G_i\alpha 2$. By co-immunoprecipitation and calcium mobilization assays, four residues (Y136, L137, I139, V140) in the second-intracellular loop and one residue in the third intracellular loop (M241) were shown to be crucial for $G_i\alpha 2$ coupling.

In the present study we have further investigated the importance of regions in the second transmembrane domain (Asp89) and third intracellular loop (BBXXB) in the G protein-mediated signal transduction of CXCR2. The selection of these two regions was based on their conservation among G protein-coupled receptors. The roles of the unique Asp89 and BBXXB sequences were analyzed in mutant receptors by functional assays. The cell surface expression of mutant and wild-type CXCR2 receptors in HEK-293 cells was verified by cell surface immunofluorescence staining and ligand-binding assays. Of the four mutants generated, one appeared not to be functionally expressed at the cell surface (D89E), whereas the other three mutants were expressed on the cell surface and bound ligand. Scatchard analysis of MGSA/GRO binding indicates that cells transfected with wild-type, D89N, HR-II, and II-insert mutant receptors bind MGSA/GRO with similar and high affinity. The K_d of these receptors ranged from 1.17 to 1.88 nM, consistent with previous reports (*8*). The number of receptors per cell was slightly less for clones expressing mutant receptor as compared to wild-type receptor. Functional analyses using GTPγ^{35}S binding, calcium mobilization, and chemotaxis revealed that one of the mutant receptors, the II-insert, showed a markedly impaired response to ligand stimulation in all three assays.

In previous studies, the highly conserved Asp residue in the second transmembrane domain has been demonstrated to be involved in signal transduction. Mutation of this Asp residue to either Asn or Glu in adrenergic, luteinizing hormone, angiotension II, formyl peptide, and some CC chemokine receptors caused loss of G protein-coupling (*22*−*24*). In addition, the ligand-binding affinity was either unaffected or reduced. The functional role of this residue in the CXCR family however is unknown. Here, we mutated this conserved Asp to either an Asn or a Glu residue. Our results indicate that for the D89N mutant, G protein-coupling has not been eliminated or suppressed. In response to MGSA stimulation, this receptor still enhanced GTPγ^{35}S binding to the membrane and mediated calcium mobilization and chemotaxis. These data suggest that a structural difference may exist between CXCR2 and other seven-transmembrane receptors regarding the conserved second transmembrane Asp residue. Surprisingly, when the same Asp residue was mutated to a Glu residue, the resultant mutant receptor appeared to be localized intracellularly. Although no structural data on seven-transmembrane receptors are available, evidence suggests that the transmembrane domains interact with each other. The proper interactions are essential for both ligand binding and G protein-coupling (*25*, *26*).

A large body of evidence indicates the involvement of the intracellular loops, particularly the third loop, in G protein-activation and selection of the second message system. Third intracellular loops of different G protein-coupled receptors vary extensively in size and primary sequence. The α and β adrenergic and muscarinic receptors have a third intracellular loop with a size of over 100 amino acids, while many newly cloned G protein-coupled receptors, including most chemokine receptors, have a much smaller third intracellular loop. For adrenergic and muscarinic receptors, experimental data suggested that the amino-terminal and carboxyl-terminal portions of third intracellular loops played a significant role in G protein-coupling and -activation (*27*−*30*). Secondary structure analysis predicts these portions of the loop may form amphiphatic α-helix structures which might represent a common structural motif in receptor−G protein interaction. Previous studies revealed the consensus BBXXB sequence in the third intracellular loop to be crucial for G protein-coupling. It was first shown that this sequence was involved in the insulin-like growth factor II/mannose-6 phosphate receptor $G\alpha_i$ protein-mediated signal transduction. A sequence of 14 amino acids containing this domain was found to be able to activate $G\alpha_i$ protein. This effect was blocked by a specific antibody to the same region (*31*). This sequence is also presented in muscarinic, α2 adrenergic, and dopaminergic receptors and is capable of directly activating G proteins (*16*). However, others found that lipophilic rather than charged amino acids in the carboxyl-terminal of the third loop are required for receptor to signal (*32*), and that the amphipathic α-helical structure does not appear to be critical for G protein-activation (*33*). These results indicated that the third intracellular loop junction may either directly interact with the G protein or serve as a hinge region that controls the proper orientation of third loop and G protein interaction.

One BBXXB sequence exists at the carboxyl-terminus of the third intracellular loop of CXCR2 with the amino acid sequence HRAMR. To determine its roles in receptor−G protein interaction, we either eliminated the positive charge by mutating HR to II or we disrupted the secondary structure of this region by inserting two isoleucines between alanine and methionine. Cells expressing HR-II mutant receptor could still respond to ligand and transduce the G protein-coupled signals equivalent to cells expressing wild-type receptor. These data suggested that the positively charged basic amino acids themselves are not absolutely required to activate G protein-signaling for CXCR2. This result is in good agreement with that of Damaj et al. (*15*). Mutation of the corresponding region in CXCR1 did not result in any effect on the IL-8-induced calcium mobilization and G_i protein interaction (*15*). By site-directed mutagenesis, Prossnitz et al. demonstrated that the charged and polar residues in the third intracellular loop of *N*-formyl peptide receptor are not required for G protein interaction (*34*). On the other hand, the basic-rich region in the third intracellular loop is highly critical for α2 adrenergic and muscarinic receptor interaction with its cognate G proteins (*27*, *35*, *36*). These data suggest that chemokine receptors may use different sequences and mechanisms for G protein-recognition and -coupling. More importantly, our findings demonstrate that interruption of the putative secondary structure of the BBXXB motif by inserting two isoleucine residues results in loss of ligand-stimulated increase in GTPγ^{35}S binding. Moreover, ligand failed to stimulate either calcium mobilization or chemotaxis in the cells expressing II-insert CXCR2. These results imply that the carboxyl-terminal region of the

third intracellular loop may direct the orientation of this region to create a G protein interaction pocket. Other amino acids in the third intracellular loop, such as methionine, have also been implicated to be involved in G protein-coupling (*15*). Our results demonstrate that these amino acids need to be placed in a proper secondary structure to effectively couple to G proteins. Current G protein-coupled receptor theory suggests that only after ligand binding, with its consequent conformational change, can the receptors bind G protein with high affinity. The ligand-unoccupied form, called R, has low affinity for the G protein. However, upon ligand binding, the ligand−receptor complex switches from an inactive intermediate form (LR) to an activated form (LR*) (*34*). By this model, the II-insert mutant may be defective in the ligand-stimulated conformational change to activated complex (LR*).

It is well-known that ligand binding causes translocation of G protein-coupled receptors from cell surface to an intracellular vesicular compartment, probably endosomes (*37*). This agonist-induced endocytosis or sequestration has been suggested to play a major role in the resensitization of receptors (*38*). Like the majority G protein-coupled receptors examined to date, the cytoplasmic carboxyl-terminal of CXCR2 is essential for receptor internalization (*8*). However, some reports also suggest that the third cytoplasmic loop of the Hm1 muscarinic cholinergic receptor is involved in the receptor internalization (*39*). Our finding that the ligand-induced internalization of II-insert mutant showed kinetics similar to that of WT receptor indicates endocytosis is a G protein-independent process. Recently, Bock et al. (*40*) demonstrated that neither signal transduction by pertussis toxin-sensitive G protein in rat basophilic leukemia cells nor signal transduction by pertussis toxin-resistant G proteins in HEK-293 cells is required for C5a-induced internalization of the receptor.

In conclusion, our results show that proper conservation of secondary structure near the carboxyl-terminal region of the third intracellular loop of CXCR2 is required for optimal G protein-coupling and subsequent ligand-activated calcium mobilization and chemotaxis. In contrast, the conserved Asp in the second-transmembrane domain is not critical for CXCR2 signaling. To our knowledge, these are novel observations for this CXC receptor family.

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