Ligand-induced Desensitization of the Human CXC Chemokine Receptor-2 Is Modulated by Multiple Serine Residues in the Carboxyl-terminal Domain of the Receptor*

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We have characterized the ligand-enhanced phosphorylation of the CXC chemokine receptor-2 (CXCR2) in a series of clonal 3ASubE cell lines expressing receptors truncated or mutated in the carboxyl-terminal domain. Truncation of CXCR2 by substitution of a stop codon for Ser-342 (342T) or Ser-331 (331T) results in total loss of melanoma growth stimulatory activity/growth-related protein (MGSA/GRO)-enhanced receptor phosphorylation, which cannot be explained based upon altered ligand binding affinity or receptor number. 3ASubE cells expressing 342T or CXCR2 with mutation of Ser-342, 331T, 342T, and 342T to alanine (4A) exhibit strong mobilization of Ca²⁺ in response to ligand (interleukin-8 or MGSA/GRO), with a recovery phase significantly slower than that of cells expressing wild type (WT) CXCR2. In contrast to the WT CXCR2, which is 93% desensitized by 20 nM ligand, the 331T, 342T, and 4A CXCR2 mutants do not undergo significant ligand-induced desensitization, and respond to a second ligand challenge by mobilizing Ca²⁺. The 3ASubE cells expressing CXCR2 with mutation of Ser-346, -347, and -348 to alanine, or with mutation of only one serine in this domain, continue to be phosphorylated in response to ligand and are 60–70% desensitized following the initial ligand challenge. WT CXCR2 phosphorylation and desensitization occur in <1 min, while receptor sequestration is a much later event (30–60 min). However, mutant receptors that are neither phosphorylated nor desensitized in response to ligand are <10% sequestered 60 min following ligand challenge. These data demonstrate for the first time that ligand binding to CXCR2 results in receptor phosphorylation, desensitization, and sequestration and that serine residues 342 and 346–348 participate in the desensitization and sequestration processes.

The CXC chemokine, IL-8, specifically binds the CXC che-

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§The abbreviations used are: CXC chemokine, chemokine with the first two amino acids separated by an intervening amino acid; 3A, CXCR2 mutant with serine to alanine substitutions at positions 346, 347, and 348; 4A, CXCR2 mutant with serine to alanine substitutions at positions 342, 346, 347, and 348; 342T, CXCR2 truncated at the Ser-342 by placing a stop codon at Ser-342; 331T CXCR2 truncated at Ser-331 by placement of a stop codon in place of Ser; 352T, CXCR2 truncated at Ser-352 by placing a stop codon at Ser-352; 293 cells, human embryonic kidney cell line; 293T, human embryonic kidney 293 cells expressing transfected human CXCR2; CC chemokines, chemokines with the first two cysteines positioned side-by-side; CMV, cytomegalovirus; CXCR1, receptor for CXC chemokines formerly referred to as IL-8 receptor A; CXCR2, receptor for CXC chemokines formerly defined as IL-8 receptor B; C5a, complement fragment 5a; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; fMLP, N-formyl-methionyl-leucyl-phenylalanine; FURA-2 fluorescence indicator for free calcium; IL-8, interleukin-8; MGSA/GRO, melanoma growth-stimulatory activity/growth-related protein; PBS, phosphate-buffered saline; TPA, 12-O-tetradecanoylphorbol-13-acetate; WT, wild type.

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(S342A, S346A, S347A, S348A) or groups of serine residues to alanine: 346/6/7/8 (4A) and 342/6/7/8 (4A). Studies with 3ASubE cells expressing these mutant receptors, as compared with 3ASubE cells expressing wild type CXCR2, revealed that serines 342, 346, 347, and 348 of CXCR2 are involved in the desensitization of the receptor to its ligand. However, when these serine residues are mutated to alanine (4A or 3A CXCR2), other serine residues in the carboxyl-terminal domain can be phosphorylated, and the receptor is partially desensitized in response to ligand. In 3ASubE cells, phosphorylation and desensitization of WT CXCR2 occurs within 1 min after ligand treatment, while receptor sequestration requires >30 min. The 342T CXCR2 receptor is not sequestered in response to ligand. These data demonstrate that for CXCR2, specific residues along the carboxyl-terminal domain of the receptor mediate receptor desensitization and sequestration in response to ligand.

EXPERIMENTAL PROCEDURES

Generation of Truncated CXCR2 Mutants—Polymerase chain reaction strategies were employed to generate truncated CXCR2 mutants with stop codons introduced at Ser-342 or Ser-342/346/347/348. Polymerase chain reaction was conducted on the cDNA encoding the entire open reading frame for the CXCR2, which had been subcloned into Blue-Script. The primer pair for each reaction included a common primer for the 5' end of the open reading frame. Unique primers that would introduce the desired stop codons were used for the 3' end and were as follows: ser341/341 (TGTGCGCTTTGTTGGCCGCA; ser342/342T), GCAAGCAGCTTTAACAGGTGTGCGCC; ser342/347 (TTTGTTGGCTCTTACCATCGGCCGCGTGGCC; ser342/342T), GTTGGCTCTTCTGCCGGGTTGGCGCCTCTTCAGGGCAC; ser342/347 (TTTGTTGGCTCTTACCATCGGCCGCGTGGCC; ser342/347T), CTTTGTTGGCTCTTACCATCGGCCGCGTGGCC

Among the multiple polymerase chain reaction-generated fragments were isolated, subcloned into Blue-Script, and sequenced. Once the sequences were shown to be correct, the cDNAs for the truncated receptors were subcloned into the mammalian expression vector pRc/CMV and subsequently transfected into the human placental cell line, 3ASubE, or human embryonic kidney 293 cells.

Site-directed Mutagenesis of the CXCR2—Mutagenesis of specific serine to alanine (Ser → Ala) residues was conducted using the pALTER site-directed mutagenesis system (Promega). The following mutations were synthesized using the indicated primers: ser342alai/S342A), AGACACAGCAGCCGCGCTTTGTTGGC; ser346alai/S346A), TCTTTGTTGGC; ser347alai/S347A), CTTTGTTGGCTCTTACCATCGGCCGCGTGGCC; ser348alai/S348A), GTTGGCTCTTCTGCCGGGTTGGCGCCTCTTCAGGGCAC; ser342/347 (TTTGTTGGCTCTTACCATCGGCCGCGTGGCC; ser342/347T), CTTTGTTGGCTCTTACCATCGGCCGCGTGGCC

Receptor Degradation Studies—Confluent cultures (60-mm dishes) of the 3ASubE transfectants were placed in serum-free DMEM and treated with MGSA (50 nM), TPA (400 nM), or the appropriate vehicle control for 2 h at 37 °C. For some experiments, serum-starved cells were treated with 25 nM MGSA/GRO for 1–6 h. Plates were rinsed on ice with Tris-buffered saline and then scraped in 300 μl of Triton X-100 lysis buffer as described previously (5). The cell lysates were clarified by centrifugation at 15,000 × g for 4 °C for 15 min, the supernatants were transferred to a fresh tube, and protein estimates were performed (BCA, Pierce). Twenty-five micrograms of protein were loaded per lane on a 9% SDS-polyacrylamide gel, framed, transferred onto a nitrocellulose membrane, and analyzed as described above.

Calcium Fluorimetry—Transfected 3ASubE cells expressing truncated Ser → Ala mutants were grown until confluent. Cells were released by a short exposure (1–2 min) to Versine (trypsin/EDTA) and washed once in culture medium containing 5% FBS. Cells were then washed a second time in Krebs-Ringer solution (118 mM NaCl, 4.56 mM KCl, 25 mM NaHCO3, 1.03 mM KH2PO4/1.17 mM glucose, 5 mM HEPES) without Ca2+ or Mg2+ and the cells were incubated for 10 min. Cells were then centrifuged (300 × g, 6 min), and washed once (50 ml) in Krebs-Ringer solution containing Ca2+ and Mg2+ (1 mM). The cells were finally adjusted to 1 × 106 cells/ml, loaded with 1 μM Fura-2 AM, and excited at 380 and 340 nm. Fluorescence was measured continuously for 20 min and the results were expressed as the ratio F340/F380. The response to the agonist was determined by comparing the ratio of fluorescence intensity obtained in the absence of the agonist to the ratio obtained in the presence of the agonist. Each experiment was performed using a single scanning spectrophotometer constructed by the University of Pennsylvania Department of Bioengineering. Data were collected using an IBM model PS-II computer with custom written software provided by the Department of Laboratory Automation, SmithKline Beecham. Data were analyzed using the software program IGOR, which used the following equation to determine free Ca2+.}

\[
\text{Ca}^{2+} \text{nm} = 244 \times \frac{(F - F_{\text{min}})}{(F_{\text{max}} - F)} 
\]  

$F_{\text{max}}$ is the maximum fluorescence (in the presence of 1 mM free Ca2+), and $F_{\text{min}}$ is the minimum fluorescence in the presence of EGTA (5 mM). The constant, $K_{c}$, is the dissociation constant for the binding of Ca2+ to Fura-2 and Ca2+. Generally, cells (2 ml) were allowed to reach 37 °C for 5 min prior to stimulation with MGSA/GRO (Peprotech) or IL-8 at the indicated concentration. The fluorescence was monitored continuously for the specified time. Once none of the clones reached prestimulatory levels of Ca2+ within the time frame of the experiment (5 min), the time taken to remove 80% of the mobilized Ca2+ was used as a measure of Ca2+ removal ($t_{\text{rem}}$). For the purpose of comparison for ligand binding, we first performed to determine the concentration of ligand that would provide similar Ca2+ mobilization in each of the clones. Based upon these results, clones expressing WT, 331T, 342T, 4A, and S342A CXCR2 were stimulated with IL-8 concentrations of 5–10 nM, 0.33–1 nM, 0.33–1 nM, 1–3.3 nM, and 3.3–10 nM, respectively. Because of the variability between experiments and the requirement to compare each clone with a similar Ca2+ mobilization response, all measurements were performed in triplicate and the results were expressed as the mean ± S.E. of the mean.
zation, the concentration of IL-8 used to achieve that Ca<sup>2+</sup> change is given as a range. The data were analyzed by the Kruskall-Wallis test and the paired Student's t test.

In the desensitization experiments, cells (6 ml total, at 1 × 10<sup>6</sup> cells/ml, 37 °C) were stimulated with 20 nM MGSA/GRO, IL-8 (stimulated), or buffer (control) for 5 min before being washed three times with 15 ml of Krebs-Ringer buffer with Ca<sup>2+</sup> and Mg<sup>2+</sup> (1 mM). Cells were finally resuspended at (1 × 10<sup>6</sup> cells/ml) and kept on ice until needed.

Cells were allowed to warm to 37 °C for 5 min before the second stimulus of IL-8 or MGSA/GRO (5 nM) as described previously. The data were analyzed by both the paired Student's t test and the Kruskall-Wallis test. To measure the time course of desensitization, clones expressing wild type and the 342T CXCR2 were stimulated with MGSA/GRO or IL-8 (20 nM) for 30 s to 30 min before being washed, resuspended, and stimulated with a second concentration of IL-8 (5 nM), and the maximum Ca<sup>2+</sup> mobilization was calculated. The data were analyzed by the Wilcoxon rank-sum test.

**Effects of Mutation of Carboxyl Terminus of CXCR2 on Receptor Sequestration**—To compare the time course of internalization of the wild-type CXCR2 receptor to that of the 342T mutant CXCR2 (4), confluent, serum-starved 3ASubE cells expressing WT or 342T CXCR2 were pretreated with saturating concentrations of MGSA/GRO or IL-8 (100 nM) for 1, 5, 15, 30, or 60 min at 37 °C. Free ligand was removed with three ice-cold (0.5 ml) washes of binding buffer (1 mg/ml ovalbumin in DMEM), and the receptor remaining at the cell surface was monitored by indirect immunodetection. Briefly, the cells were incubated with 2 μg of antibody to the NH<sub>2</sub>-terminus of CXCR2 in 250 μl of binding buffer for 2 h at 4 °C, excess antibody was removed by washing with 1 ml of binding buffer at 4 °C, and then 250 μl of 125<sup>I</sup>-labeled goat anti-rabbit IgG (2 × 10<sup>6</sup> cpm, specific activity 300 μCi/ml) was added and incubated for 30 min at 4 °C. Excess antibody was then aspirated; cells were washed twice with 1 ml of binding buffer at 4 °C, lysed in 0.5 ml of lysis buffer (1% SDS, 0.1 N NaOH); and radioactivity determinations were made by γ counting. Nonspecific binding was estimated by preblocking the NH<sub>2</sub>-terminal antibody with a 20-fold molar excess of peptide to which the antibody was generated before adding the antibody to the cells. The data exhibited a normal distribution and were analyzed by the paired t test as well as by the nonparametric Wilcoxon rank-sum test.

**Chemotaxis Assay**— Chemotaxis assays were performed on 293 cells expressing WT or mutant CXCR2 according to the protocols of Ben-Baruch et al. (9) with modification. The 96-well chemotaxis chamber (Neuroprobe Inc., Cabin John, MD) was used, and the lower compartment of the chamber was loaded with 360-μl aliquots of 1 mg/ml ovalbumin/DMEM or MGSA/GRO diluted in ovalbumin/DMEM (chemotaxis buffer). The polycarbonate membrane (10-μm pore size) was coated on both sides with 20 μg/ml human collagen type IV for 2 h at 37 °C and stored overnight at 4 °C. The cells were removed by trypsinization and incubated in 10% FBS/DMEM for 1.5 h at 37 °C to allow restoration of receptor, washed in chemotaxis buffer, and then placed into the upper chamber in the ovalbumin medium. The chambers were incubated for 4.5–6 h at 37 °C in humidified air with 5% CO<sub>2</sub>, and then the filter was removed, washed, fixed, and stained with Diff-Quik kit. Quantitation of relative chemotaxis indices was by densitometric scanning of the stained filter. Briefly, the filter was scanned using an Epson ES 1200C densitometric scanner and the Adobe Photoshop software. Digitized images were subsequently scanned using the Molecular Dynamics PhosphorImager. Volumes were integrated and normalized to a value of 1 for the control of each set.

**RESULTS**

**Effects of Serine Mutation on Ligand-induced CXCR2 Phosphorylation**—We have previously demonstrated that the CXCR2 expressed in nonhematopoietic cells is phosphorylated on serine residue(s) in response to either MGSA/GRO or phorbol ester treatment in a time- and concentration-dependent manner (4, 5). There are eight serine residues within the carboxyl-terminal domain of CXCR2 that may serve as potential phosphorylation sites (Table I). As an initial attempt to localize the site(s) of serine phosphorylation on this receptor, truncated receptors were generated by introducing stop codons at Ser-331, -342, and -352. These truncations were designed to remove the 342T CXCR2 expressed in nonhematopoietic cells is phosphorylated on serine residue(s) in response to either MGSA/GRO or phorbol ester treatment in a time- and concentration-dependent manner (4, 5). There are eight serine residues within the carboxyl-terminal domain of CXCR2 that may serve as potential phosphorylation sites (Table I). As an initial attempt to localize the site(s) of serine phosphorylation on this receptor, truncated receptors were generated by introducing stop codons at Ser-331, -342, and -352. These truncations were designed to remove all eight serine residues from the carboxyl-terminal domain, the final five serine residues, or the final serine residue, respectively. The truncated receptor constructs were subcloned into the pRe/CMV mammalian expression vector and transfected into the human placental 3ASubE cell line or the human embryonic kidney 293 cell line. Neither of these cells naturally expresses CXCR2 or exhibits specific binding for 125<sup>I</sup>-MGSA/GRO (9).

Selected clones of 3ASubE cells, expressing the WT CXCR2 or various truncated forms of CXCR2, were characterized for ligand binding and ligand-induced receptor phosphorylation. Clones were also analyzed to determine whether these receptors are phosphorylated in response to TPA, an activator of protein kinase C. As demonstrated in Fig. 1, both the wild-type CXCR2 and the 352T receptor were phosphorylated in response to 10-min treatment with either MGSA/GRO (5 nM) or TPA (400 nM), while the 331T and 342T receptors were not phosphorylated in response to either of these treatments (Fig. 1). In contrast, the binding of 125<sup>I</sup>-MGSA/GRO was not impaired in the 3ASubE cells expressing CXCR2 truncation mutants relative to cells expressing WT CXCR2 (data not shown). Western blot analysis of the immunoprecipitates indicated that approximately equal amounts of CXCR2 protein were immunoprecipi-
tated from the treated and untreated samples (data not shown). Similar observations were made when the in vivo phosphorylation assay was conducted using higher concentrations of MGSA/GRO (50 nM) (data not shown). These results suggested that the potential sites of receptor phosphorylation include serine residues at position 342, 346, 347, and/or 348.

**Effects of Ser → Ala Mutagenesis on Ligand-induced Receptor Phosphorylation in 3ASubE Cells**—Serine to alanine mutations were then made at individual serine residues (Ser-342 → Ala (S342A), Ser-346 → Ala (S346A), Ser-347 → Ala (S347A), Ser-348 → Ala (S348A)) and at multiple serine residues (Ser-346, -347, and -348 (3A) or Ser-342, -346, -347, and -348 (4A)) (Table I). Stable 3ASubE transfectants expressing these Ser/Ala mutations were generated. These clones were characterized with regard to MGSA/GRO binding and MGSA/GRO ligand-induced receptor phosphorylation. Four clones expressing CXCR2 exhibited reduced basal receptor phosphorylation as compared with WT: 4A, S342A, S346A, and S348A (Fig. 2 and Table II). The ligand-induced phosphorylation was diminished 25–75% in the clones expressing mutant receptor (Table II). However, when these data were normalized to receptor number, we found that the ligand-induced receptor phosphorylation in the mutant clones was comparable with that of clones expressing the WT receptor (Table II). The ligand dissociation constant ($K_d$) for each mutant and WT CXCR2 was in the 2–4 nM range (Table II). These results suggested that multiple serine residues were phosphorylated in response to MGSA/GRO, and altering individual serine residues did not eliminate ligand-induced receptor phosphorylation. Replacement of as many as four of the serine residues with alanine (4A) did not diminish the overall -fold increase for ligand-induction of phosphorylation. (Table II and Fig. 2). Similar results were observed for the two different 4A mutant clones studied. Thus, mutation of the serine residues, which are the apparent natural substrates for the ligand-activated serine kinase for CXCR2, did not eliminate ligand-induced receptor phosphorylation.

![Fig. 2. MGSA-induced phosphorylation of serine to alanine mutants of CXCR2.](image)

**TABLE II**

Quantitation of the phosphorylation of WT and serine to alanine mutant CXCR2 in response to MGSA

| Clone     | Receptors/cell ($\times 10^6$ range) | $K_d$ (range) | Mean basal phosphorylation | $n$ | MGSA-stimulated phosphorylation/basal | Fold induction/receptors per cell |
|-----------|------------------------------------|---------------|----------------------------|-----|--------------------------------------|----------------------------------|
| WT        | 6.7 (9.7–3.6)                      | 3.4 (3.2–3.5) | 1.00                       | 3   | 11.6 ± 1.5                           | 1.7                              |
| 3A        | 2.6 (1.8–3.4)                      | 3.7 (3.5–4.0) | 1.64                       | 4   | 5.8 ± 0.8                            | 2.2                              |
| 4A        | 0.6 (0.8–0.5)                      | 3.0 (2.8–3.2) | 0.68                       | 6   | 3.2 ± 0.9                            | 5.3                              |
| S342A     | 4.3 (4.5–4.0)                      | 3.5 (2.2–4.8) | 0.79                       | 3   | 6.4 ± 0.8                            | 1.5                              |
| S346A     | 2.4 (2.4–2.3)                      | 1.9 (1.8–2.0) | 0.80                       | 3   | 5.7 ± 2.0                            | 2.4                              |
| S347A     | 3.0 (3.3–2.8)                      | 2.5 (2.2–2.8) | 1.00                       | 4   | 7.4 ± 0.70                           | 2.5                              |
| S348A     | 2.8 (4.1–1.5)                      | 2.4 (2.0–2.7) | 0.73                       | 2   | 8.45 (8.4–8.5)                       | 3.0                              |

**Effects of Receptor Truncation on Receptor Degradation**—In 3ASubE cells, phorbol ester-induced phosphorylation of CXCR2 has been demonstrated to be accompanied by receptor downregulation and degradation (5). MGSA/GRO treatment (50 nM for 2 h) of 3ASubE cells expressing WT CXCR2 also resulted in phosphorylation and partial degradation of the receptor (5). As an initial approach to determine whether receptor phosphorylation is required for receptor degradation in response to these stimuli, the effect of MGSA/GRO (50 nM) or TPA treatment (400 nM) for 2 h at 37 °C on the degradation of 331T, 342T, and 352T CXCR2 was examined in 3ASubE cells. As we have previously demonstrated, Western blot analysis of the lysates prepared from TPA-treated 3ASubE cells expressing WT CXCR2 revealed that continuous TPA treatment resulted in a significant decrease in immunoreactive CXCR2. MGSA/GRO treatment of 3ASubE cells expressing 352T also decreased CXCR2 protein. Neither MGSA/GRO nor TPA treatment of 3ASubE clones expressing 331T or 342T produced a decrease in CXCR2 protein (Fig. 3A). In response to MGSA/GRO, the reduction in CXCR2 was less striking, although all of the receptor exhibited the reduced electrophoretic mobility that is indicative of the phosphorylated form of the receptor (Fig. 3A). However, MGSA/GRO treatment (25 nM for 6 h) of 3ASubE cells expressing WT CXCR2 produces a ~50% diminution in the immunoreactive CXCR2 (Fig. 3B). Thus, the carboxyl-terminal domain of CXCR2 appears to be required for the receptor degradation that usually accompanies ligand-induced receptor phosphorylation. Furthermore, the degradation of CXCR2 that occurs in response to TPA is more extensive than that which occurs in response to MGSA/GRO (Fig. 3A).
Calcium Mobilization in 3ASubE Cells—To determine the requirement for serine phosphorylation in signal transduction, Ca\(^{2+}\) mobilization and desensitization were investigated in 3ASubE clones expressing WT or mutant CXCR2. All 3ASubE clones studied mobilized Ca\(^{2+}\) in response to IL-8 comparable with that observed in neutrophils or differentiated HL60 cells (EC\(_{50}\), 2–5 nM), except that the maximum Ca\(^{2+}\) response was diminished in magnitude. The transfected 3ASubE cells expressing WT CXCR2 responded to IL-8 (5 nM) with an increase in intracellular Ca\(^{2+}\), which peaked approximately 25 s following agonist addition (Fig. 4). The level of intracellular Ca\(^{2+}\) returned to baseline rapidly. 3ASubE clones expressing 331T or 4A CXCR2 exhibited a markedly slower removal of ligand mobilized Ca\(^{2+}\) with a t\(_{1/2}\) of 179 and 135 s, respectively, while the clone expressing WT CXCR2 required only 71 s to remove the mobilized Ca\(^{2+}\) (Fig. 4 and Table III). These data suggest that serines 342-, 346-, 347-, and 348- participate in the regulation of Ca\(^{2+}\) homeostasis. 3ASubE clones expressing 3342A CXCR2 exhibited removal of Ca\(^{2+}\), which was not significantly different from the WT. Since intracellular Ca\(^{2+}\) as determined by FURA-2 in the presence or absence of EGTA (5 mM) has both a component of influx and efflux (data not shown), we cannot rule out the possibility that the various mutants differ in the removal of influxed versus effluxed Ca\(^{2+}\).

To determine if mutation or truncation of the receptor was associated with desensitization to ligand, FURA-2-loaded clones were treated with buffer, 20 nM IL-8, or 20 nM MGSA/GRO. Ligand was removed by repeated washing, and the clones were treated a second time with a lower concentration (5 nM) of either MGSA/GRO or IL-8. As expected, WT clones were substantially desensitized by the first addition of IL-8, with the second addition (noted by the arrow on the chart) promoting a Ca\(^{2+}\) mobilization response that was only 7.1% of the original response (Fig. 5, A and B). In contrast, the clones expressing the 3A (Fig. 5, C and D) and 331T (Table III) truncation mutants failed to be desensitized by the high concentration of ligand (p < 0.05) and exhibited a second response to ligand that was equivalent to the original response (Fig. 5 and Table III). Clones expressing the 4A mutant of CXCR2 exhibited a second calcium response, which was 79% of the initial response (only 21% desensitization). The clones expressing the 3A mutant or the single Ser → Ala mutants were 60–70% desensitized (Fig. 5, E and F, and Table III). These data strongly suggest that the

![Figure 3](image-url)

**Fig. 3.** Effect of MGSA or TPA treatment on CXCR2 phosphorylation and degradation. A, confluent cultures of 3ASubE P-3 clones expressing WT or mutant CXCR2 were stimulated with MGSA (50 nM), TPA (400 nM), or vehicle alone for 2 h at 37 °C. 25 μg of protein from clarified Triton X-100 lysates were loaded per lane and electrophoresed through a 9% SDS-polyacrylamide gel, and Western blot analysis was performed with polyclonal antibody to NH\(_{2}\)-terminal CXCR2 and visualized by alkaline phosphatase-conjugated secondary antibody as described under “Experimental Procedures.” A, vehicle alone; M, MGSA treatment; T, TPA treatment. B, confluent cultures of 3ASubE P-3 cells expressing wild type CXCR2 were exposed to MGSA/GRO (25 nM) for the time periods indicated as described under “Experimental Procedures” and analyzed by Western blot to determine the receptor degradation over time with treatment of saturating concentrations of ligand. The density of the bands representing CXCR2 were determined by densitometric scanning for three independent experiments, and the quantitation of these scans is shown in the histogram as mean ± S.D.

![Figure 4](image-url)

**Fig. 4.** Time course of Ca\(^{2+}\) mobilization in 3ASubE cells expressing WT and mutant CXCR2. 3ASubE cells expressing WT, 331T, or 4A CXCR2 were loaded with FURA-2, stimulated with IL-8, and analyzed by Western blot to determine the receptor degradation and visualized by alkaline phosphatase-conjugated secondary antibody as described under “Experimental Procedures.” To compare the time course for removal of Ca\(^{2+}\) mobilized in response to ligand, each of the clones was stimulated with a concentration of ligand that would result in similar maximum of Ca\(^{2+}\) mobilization, and the fluorescence was monitored for 180–210 s. The clones expressing WT, 331T, and 4A CXCR2 were stimulated with 5.0, 0.33, and 0.33 nM IL-8, respectively. This figure shows a representative experiment. Each of the clones was studied six times, with the variability described in Table IV. Sec, seconds.
residues beyond Ser-342 are responsible for desensitization of the receptor to its ligand, and serine residues 342, 346, 347, and 348 appear to be involved. Statistical analysis of the data comparing ligand-induced desensitization of mutant CXCR2 with WT CXCR2 demonstrated the mutant clones were significantly different from WT in their desensitization properties (Table III).

For comparison, 3ASubE cells expressing WT and 342T CXCR2 were treated with 20 nM MGSAGRO for 5 min, and then cells were washed and then warmed and stimulated with a second concentration of MGSAGRO (5 nM) as described under “Experimental Procedures.” The experiment was performed in triplicate. The percentage of response remaining with the second stimulus of MGSAGRO (WT CXCR2, 18.3%; 342T CXCR2, 79.8%) was not remarkably different from that which occurred in response to IL-8 (WT CXCR2, 7.2%; 342T CXCR2, 100.8%).

**Effects of Mutation of the Carboxyl-terminal Domain of CXCR2 on the Time Course of Receptor Desensitization and Sequestration**—To compare the time course of desensitization of the WT CXCR2 to that of the 342T CXCR2, we examined the time course for recovery following ligand induction of CXCR2 desensitization in 3ASubE clones expressing these receptors. Desensitization of WT CXCR2 occurred within 1 min after ligand treatment and remained >70% desensitized after 20 min. In contrast, the 342T CXCR2 underwent only 18.5% desensitization by 5 min and was almost fully recovered after 30 min. (Table IV). To determine whether desensitization occurred as a result of receptor internalization, we monitored the time course for CXCR2 sequestration in 3ASubE cells expressing mutant or WT CXCR2. After a 15-min incubation with saturating concentrations of ligand (37 °C), approximately 25% of the WT receptors were sequestered (Table V). By 30 min, approximately 30% of the WT receptors were sequestered, and by 60 min, 33% were sequestered (Table V). IL-8 was about 10–15% more efficient than MGSAGRO in the WT receptor sequestration assay, p < 0.01 (Table V). In contrast, for 3ASubE cells expressing the 342T mutant, nearly 100% of the immunoreactive receptor remained at the membrane after treatment with saturating concentrations of ligand for up to 30 min. At 60 min, 92% of the receptor remained at the membrane (Table V). IL-8 was not more efficient than MGSAGRO in sequestering 342T CXCR2 in 3ASubE cells. (Table V). This was the expected result, based upon the failure of these receptors to phosphorylate or desensitize in response to ligand. The failure to sequester 100% of CXCR2 was probably due to the dynamics of the display of receptors at the cell membrane at 37 °C, where new receptors would be cycling to the membrane to replace those that were sequestered.

**MGSAGRO-induced Chemotaxis of 293 Cells Expressing WT and 342T CXCR2**—To determine whether loss of ligand-induced CXCR2 phosphorylation, desensitization, and sequestration resulted in a chemokine receptor that exhibited loss of function, we examined the ability of 293 cells and 3ASubE cells expressing WT and 342T CXCR2 to chemotax toward a gradient of MGSAGRO. We did not observe chemotaxis in response to MGSAGRO in the 3ASubE cells expressing WT or 342T CXCR2 using filters coated with collagen type I, fibronectin, or...
Matrigel or filters with no coating (data not shown). However, 293 cells expressing both WT and 342T CXCR2 exhibited chemotaxis toward MGSAGRO, and the cells expressing 342T CXCR2 showed a chemotaxis response that at the higher concentrations (40 ng/ml) was equivalent to that of cells expressing WT CXCR2 (data not shown). Thus, disruption of the carboxy-terminal domain of CXCR2 involved in phosphorylation, desensitization, and sequestration, does not ablate the chemotactic functions of this receptor.

**DISCUSSION**

There is now considerable evidence that ligand binding to transmembrane-spanning receptors stimulates receptor phosphorylation on the carboxyl-terminal domain of the receptor and that this event coincides with receptor desensitization, sequestration, and in some instances degradation or recycling (3–8). Both CC and CXC chemokine receptors are phosphorylated in response to ligand binding, and multiple serine or threonine residues are phosphorylated (4, 10, 11). Richardson et al. (11) have shown that the phosphorylation of CXCR1 is accompanied by receptor desensitization. CXCR2 expressed in CHO cells is reported to be internalized more rapidly than CXCR1, and MGSAGRO induces this internalization more slowly than does IL-8 (12). Moreover, truncation of CXCR1 eliminates ~60% of the ligand-induced internalization of the receptor (12). In contrast, neutrophil CXCR1 and CXCR2 are both rapidly down-modulated by ligand (5 min), although considerably more ligand is required to down-modulate CXCR1 than CXCR2 (3). CXCR2 recycles more slowly than the CXCR1; moreover, only about 40% of the CXCR2 recycles during a 3-h culture period (3), while CXCR1 fully recovers 1.5 h after treatment with the IL-8 ligand in neutrophils. Our studies with 3ASubE cells show that desensitization and phosphorylation of WT CXCR2 occurs within 30–60 s after ligand treatment, while sequestration occurs much more slowly, requiring 30–60 min. Thus, CXCR2 clearance in 3ASubE cells is different from that reported for CHO cells (12). We have also observed that after 6 h of treatment with 25 nM MGSAGRO, 3ASubE cells expressing WT CXCR2 exhibit only ~50% reduction in the total receptor detected by Western blot, suggesting that either only a percentage of the receptors that bind ligand undergo degradation after sequestration or, alternatively, newly translated CXCR2 rapidly replenishes the receptor pool. A recent study of the N-formyl peptide receptor demonstrates a similar dynamic state of receptor sequestration, counterbalanced by the reappearance of receptors at the membrane (13).

3ASubE cells expressing wild type and mutant CXCR2 exhibit only subtle differences in the time course for desensitization or receptor sequestration produced by IL-8 and MGSAGRO. However, larger differences in IL-8-versus MGSAGRO-induced calcium signaling in 293 cells expressing transfected CXCR2 were recently observed by Damaj et al. (21), who suggested that MGSAGRO elicits a stronger influx of Ca2+ than does IL-8. Differences in IL-8 versus MGSAGRO induction of sequestration of CXCR2 were observed by Prado et al. in transfected CHO cells (11). The basis for the different results between these two studies and our study is unclear but could reflect differences in the specific activity or potency of the ligand preparations or differences in the manner in which the various cell types process the receptors. More in keeping with our own findings, Ahuja et al. (22, 23) reported similar profiles of calcium mobilization for IL-8 and MGSAGRO proteins in stably transfected 293 cells expressing CXCR2, although MGSAGRO was unable to totally desensitize receptors to a second weaker response to IL-8.

The β-adrenergic receptors are phosphorylated along the carboxyl tail by β-adrenergic receptor kinase and then assume a conformation that allows association with an arrestin (14). This association with arrestin is thought to be necessary for both desensitization and for sequestration. In the case of the β2-adrenergic receptor, tyrosine phosphorylation of Tyr-326 is involved in facilitation of sequestration of this receptor (14). We observe no tyrosine phosphorylation of CXCR2 in response to ligand or phorbol-ester stimulation. While some investigators have reported that sequestration is not dependent upon phos-
of the receptor desensitization as well as receptor sequestration results in diminution important in the desensitization process, and loss of amino acid CXCR2 along the carboxyl-terminal domain appears to be initiated by ligand (11). Like the CXCR1, phosphorylation of phosphorylation may also be important, and phorbol-ester regulation of receptor phosphorylation appears to differ from that from CXCR1 in RBL-2H3 leukemia cells, where threonine phosphorylation of CXCR2 studied here revealed only serine phosphorylation of CXCR2 (4, 5). Moreover, phosphoamino acid analysis of the mutant forms of CXCR2 phosphorylation, others have shown that overexpression of β-adrenergic receptor kinase-1 can facilitate the sequestration of the M2-muscarinic receptor. Moreover, for the M2 receptor, desensitization has been shown to proceed in the absence of sequestration (15, 16). A number of seven-transmembrane G protein-coupled receptors show loss of both phosphorylation and sequestration when the carboxyl-terminal domain is deleted (17–19). Our studies on CXCR2 phosphorylation, desensitization, and sequestration demonstrate that the loss of four specific serine residues involved in ligand-stimulated receptor phosphorylation results in markedly diminished receptor desensitization and loss of receptor sequestration but no loss of ligand-induced chemotaxis. However, we are unable to assign significance to any one specific phosphorylation site regarding a role for desensitization or sequestration. Multiple sites appear to be phosphorylated. A similar cluster of serine and threonine residues regulates CXCR1 desensitization in the RBL-2H3 leukemia cell line (11). However, it is important to note that desensitization does not always require receptor phosphorylation. Richardson et al. (20) have recently shown that activation of an epitope-tagged fMLP receptor can cross-desensitize and cross-phosphorylate both the epitope-tagged C5a receptor and CXCR1. C5a and IL-8 also cross-desensitized the epitope-tagged fMLP receptor based upon Ca2+ mobilization and phosphoinositide hydrolysis, but this is not accompanied by phosphorylation of the epitope-tagged fMLP receptor. Richardson et al. (20) concluded that these data indicate that activation of phospholipase C, independent of receptor G protein coupling, might be involved in cross-desensitization in the absence of receptor phosphorylation (20).

We have observed only serine phosphorylation of CXCR2 (4, 5). Moreover, phosphoamino acid analysis of the mutant forms of CXCR2 studied here revealed only serine phosphorylation (data not shown). Truncation and serine mutants of CXCR2 that exhibited a loss of ligand-induced CXCR2 phosphorylation also exhibited a loss of TPA-induced CXCR2 phosphorylation. Thus, in 3ASubE cells CXCR2 is regulated very differently from CXCR1 in RBL-2H3 leukemia cells, where threonine phosphorylation may also be important, and phorbol-ester regulation of receptor phosphorylation appears to differ from that initiated by ligand (11). Like the CXCR1, phosphorylation of CXCR2 along the carboxyl-terminal domain appears to be important in the desensitization process, and loss of amino acid residues involved in this phosphorylation results in diminution of the receptor desensitization as well as receptor sequestration in response to ligand. Moreover, these two receptors appear to exhibit differences in the time course of sequestration and recycling (3). These events are likely to be important in the regulation of the inflammatory response, where an acute response to injury is required to quickly turn on and gradually shut off in an environmental milieu where excess ligand may persist for some time.

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