Differential Cross-regulation of the Human Chemokine Receptors CXCR1 and CXCR2

EVIDENCE FOR TIME-DEPENDENT SIGNAL GENERATION*

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Ricardo M. Richardson‡‡, Bryan C. Pridgen‡, Bodduluri Haribabu‡, Hydar Ali‡, and Ralph Snyderman‡‡

From the Departments of ‡Medicine and ‡Immunology, Duke University Medical Center, Durham, North Carolina 27710

Neutrophils and transfected RBL-2H3 cells were used to investigate the mechanism of cross-regulation of the human interleukin-8 (IL-8) receptors CXCR1 and CXCR2 by chemoattractants. In neutrophils, Ca\textsuperscript{2+} mobilization by the CXCR2-specific chemokine, growth-related oncogene \(\alpha\) (Gro\(\alpha\)), was desensitized by prior exposure to the chemoattractants N-formylated peptides (fMLP) or a complement cleavage product (C5a). In contrast, growth-related oncogene \(\alpha\) did not desensitize the latter receptors. To investigate this phenomenon, CXCR2 was stably expressed in RBL-2H3 cells and mediated phosphoinositide hydrolysis, Ca\textsuperscript{2+} mobilization, chemotaxis, and secretion. In cells co-expressing CXCR2 and receptors for either C5a (C5aR) or fMLP (FR), CXCR2 was cross-phosphorylated and cross-desensitized by C5a and fMLP. However, neither C5aR nor FR was cross-phosphorylated or cross-desensitized by CXCR2 activation, although CXCR1 did mediate this process. Receptor internalization induced by IL-8 was more rapid and occurred at lower doses with CXCR2 than CXCR1, although both receptors mediated equipotent chemotaxis and exocytosis in RBL. Truncation of the cytoplasmic tail of CXCR2 (331T) prolonged its signaling relative to CXCR2, increased its resistance to internalization, and induced phospholipase D activation. 331T was resistant to homologous phosphorylation and cross-phosphorylation but not cross-desensitization of its Ca\textsuperscript{2+} mobilization by fMLP or C5a, indicating an inhibitory site distal to receptor/G protein coupling. In contrast to CXCR2, stimulation of 331T cross-desensitized Ca\textsuperscript{2+} mobilization by both FR and C5aR. CXCR2 and the mutant 331T induced phospholipase C \(\beta\), phosphorylation to an extent equivalent to that of CXCR1. Taken together, these results suggest that CXCR1 and CXCR2 bind IL-8 to produce a group of equipotent responses, but their ability to generate other signals, including receptor internalization, cross-desensitization, and phospholipase D activation, are very different. The latter phenomena apparently require prolonged receptor activation, which in the case of CXCR2 is precluded by rapid receptor phosphorylation and internalization. Thus, receptors coupling to identical G proteins may trigger different cellular responses dependent on the length of their signaling time, which can be regulated by receptor phosphorylation.


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over, little is known regarding the comparative activation or regulation of these two receptors for IL-8. For this purpose, neutrophils and RBL-2H3 cells stably co-expressing the wild type or a cytoplasmic tail deletion mutant of CXCR2 with receptors for either fMLP or C5a were used to study the mechanism(s) of CXCR2 regulation and cross-regulation. The results presented here demonstrate that responses to CXCR2 can be cross-regulated by either fMLP or C5a. In contrast to CXCR1, CXCR2 did not cross-regulate responses to the other chemotactic receptors. However, deletion of the cytoplasmic tail of CXCR2 delayed its internalization and prolonged signal generation, thus disclosing the requirements for time-dependent activation in certain receptor-mediated signals.

**EXPERIMENTAL PROCEDURES**

**Materials**—[^3]PiOr (orthophosphate (8500–9120 Ci/mmol), myo-[2-3H]inositol (24.4 Ci/mmol), and [γ-[^32]P](GTP (6000 Ci/mmol) were purchased from NEN Life Science Products. 125I-IL-8 was obtained from Amer sham Pharmacia Biotech. IL-8 (monocyte-derived) and GROα were purchased from Genzyme. Genetin (G418) and all tissue culture reagents were purchased from Life Technologies, Inc. Monoclonal 12CA5 antibody, protein G-agarose, and protease inhibitors were purchased from Santa Cruz Biotechnology, Inc. Anti-human IL-8R (CXCR2) antibody was purchased from Pharmingen. Polyclonal antibody against PLCβ, was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). fMLP, indo-1 acetoxymethyl ester, and pluronic acid were purchased from Molecular Probes, Inc. (Eugene, OR). C5a and cpt-cAMP, phorbol 12-myristate 13-acetate (PMA), GDP, GTP, and ATP were purchased from Sigma. All other reagents are from commercial sources. The cDNA encoding the human CXCR2 was kindly provided by Drs. B. Moser and M. Baggioiini.

**Isolation of Human Neutrophils**—Neutrophil purification (~95% PMNs) was carried out as described previously (7).

**Construction of Epitope-tagged FR, C5aR, CXCR1, and PAFR**—Nucleotides encoding a 9-amino acid hemagglutinin (HA) epitope sequence (YPYDVPDYA) was inserted between the N-terminal initiator methionine and the second amino acid of each cDNA by polymerase chain reaction as described previously (15).

**Cell Culture and Transfection**—RBL-2H3 cells were maintained as monolayer cultures in Earle’s modified Eagle’s medium supplemented with 15% fetal bovine serum, 2 mM glutamine, penicillin (100 units/ml), and streptomycin (100 mg/ml) (15). RBL-2H3 cells (1 × 10⁶ cells) were transfected by electroporation with μgDNAs containing the receptor cDNAs (20 μg), and Geneticin-resistant cells were cloned into single cell by fluorescence-activated cell sorting analysis.

**Radioligand Binding Assays**—RBL-2H3 cells were subcultured overnight in 24-well plates (0.5 × 10⁶ cells/well) in growth medium. Cells were then rinsed with Dulbecco’s modified Eagle’s medium supplemented with 20 mM HEPES, pH 7.4, and 10 mM bovine serum albumin and incubated on ice for 2–4 h in the same medium (250 μl) containing the radiolabeled ligand. Reactions were stopped with 1 ml of ice-cold phosphate-buffered saline containing 10 mg/ml bovine serum albumin and washed three times with the same buffer. Then cells were lysed with 0.1 N NaOH (250 μl) and dried under vacuum, and bound radioactivity was counted (9, 14). Nonspecific radioactivity bound was determined in the presence of 300 nM unlabeled ligand.

**GTPase Activity**—Cells were treated with appropriate concentrations of stimulants, and membranes were prepared as already described (9). GTPase activity using 10–20 μg of membrane preparations were carried out as described previously (9, 16).

**Phosphoinositide Hydrolysis and Calcium Measurement**—RBL-2H3 cells were subcultured overnight in 96-well culture plates (50,000 cells/well) in isosoyt newborn medium supplemented with 10% dialyzed fetal bovine serum and 1 μCi/ml [3H]inositol. The generation of inositol phosphates was determined as reported (15). For calcium mobilization, cells (3 × 10⁵) were removed, washed with HEPES-buffered saline, and loaded with 1 μM indo-1 acetoxymethyl ester in the presence of 1 μM pluronic acid for 30 min at room temperature. Then the cells were washed and resuspended in 1.5 ml of buffer. Intracellular calcium increase in the presence and absence of ligands was measured as described (7, 15).

**Chemotaxis**—RBL-2H3 cells (50,000) were incubated at 37 °C with different concentrations of IL-8. Chemotaxis was assessed in 48-well microchemotaxis chambers, using polyvinylpyrrolidone-free 8-μm pore size membranes. Migration was allowed to continue for 3 h at 37 °C in humidified air containing 5% CO₂. The membrane was removed, and the upper surface was washed with phosphate-buffered saline and scraped, fixed, and stained. The results are represented as mean of number of cells/well (14, 17). The results are representative of three separate experiments.

**Phospholipase D Activation Assay**—Cells were subcultured in 24-well plates (2.5 × 10⁶ cells/well) overnight and labeled with [3H]Hymisric acid (2 μCi/well) in HEPES-buffered saline supplemented with 0.1% bovine serum albumin for 90 min. Cells were then washed and stimulated with IL-8 (100 nM) for 5 min, and phosphatidylethanol formation was measured as described previously (7, 13).

**Phosphorylation of Receptors and PLCβ**—Phosphorylation of receptors or PLCβ was performed as described previously (9, 15, 18). RBL-2H3 cells (2.5 × 10⁶) expressing the receptors were incubated with [32P]orthophosphate (150 μc/dish) for 90 min. Then labeled cells were stimulated with the indicated ligands for 5 min at 37 °C. Cell lysates were immunoprecipitated with specific antibodies against the N terminus of CXCR2, the HA epitope tag (12CA5), and the PLCβ2 analyzed by SDS electrophoresis; and visualized by autoradiography.

**Two-dimensional Peptide Mapping**—PLCβ phosphorylation was carried out as described above, transferred to a nitrocellulose filter and autoradiographed. Phosphoprotein bands corresponding to PLCβ were cut, washed, and digested with t-1-tryosidase-2-phenylthethyl chloromethyl ketone-treated trypsin, and two-dimensional peptide mapping was performed as described (19, 20).

**RESULTS**

**Cross-desensitization of CXCR2 in Neutrophils**—To study the cross-desensitization of CXCR2 by chemotactic receptors in neutrophils, intracellular Ca²⁺ mobilization elicited by GROα was used as a measure of CXCR2 activation. As shown in Fig. 1, response to an EC100 dose of GROα (10 nM) was desensitized by prior exposure of the cells to an EC100 dose of either C5a (10 nM) or fMLP (10 nM) but not PAF (10 nM). GROα pretreatment did not affect intracellular Ca²⁺ mobilization in response to fMLP, C5a, or PAF.

**Expression and Characterization of CXCR2 in RBL-2H3 Cells**—To further study the cross-regulation of CXCR2, RBL-2H3 cells stably expressing different combinations of receptors were generated. Ligand binding studies of all receptors except CXCR2 in RBL cells were previously determined and found to be similar to native receptors in neutrophils (9, 13). CXCR2 bound both IL-8 (Kd = 2.4 ± 1.3 nM; Bmax = 8460 ± 232 receptors/cell) and GROα (Kd = 1.9 ± 0.9 nM; Bmax = 7885 ± 637 receptors/cell) with similar affinities. The Kd were similar to that of CXCR2 expressed in 3ASubE cells (3.1 nM) (10), HEK 293 cells (4 nM) (21) or the native receptors in neutrophils (~1–2 nM) (26). Upon IL-8 (Fig. 2) or GROα (data not shown) activation, CXCR2 stimulated dose-dependent chemotaxis (Fig. 2C), PI hydrolysis (Fig. 2A), secretion (Fig. 2D), and peak of intracellular Ca²⁺ mobilization (Fig. 2B). IL-8 (Fig. 2A, B, C, D) and GROα (data not shown) mediated CXCR2 responses were equipotent to those of CXCR1 (Fig. 2A, B, C, D) in RBL-2H3 cells expressing similar number of receptors.
fMLP-mediated Ca$^{2+}$ mobilization (Table I), although Ca$^{2+}$ response to a second dose of IL-8 was inhibited by 90% (data not shown). Similar results were obtained with GRO$\alpha$ instead of IL-8 (data not shown).

GTPase activity in membranes was measured to further study the cross-desensitization of CXCR2. Pretreatment of CXCR2-C5aR cells with IL-8 (100 nM), C5a (100 nM), or PMA (100 nM) resulted in desensitization (50–60%) of IL-8-induced GTPase activity in membranes (Fig. 3). Treatment of the cells with either C5a or PMA, but not IL-8, resulted in a ~40% desensitization of C5a-mediated GTPase activity.

Cross-phosphorylation of CXCR2—To determine whether cross-desensitization of CXCR2 correlated with its cross-phosphorylation, $^{32}$P-labeled cells were stimulated with IL-8 (100 nM), C5a (100 nM), or fMLP (1 $\mu$M). The cell lysates were immunoprecipitated first with a specific antibody directed against the N terminus of CXCR2 and then with the 12CA5 antibody specific for the HA epitope tag expressed at the N-terminus of CXCR2. The results were representative of one experiment performed in triplicate. The experiment was repeated twice with similar results.

**FIG. 1.** Cross-desensitization of CXCR2-mediated intracellular calcium mobilization in human neutrophils. Human neutrophils were loaded with the calcium indicator indo-1 and exposed to a first EC$_{10}$ dose (10 nM) of GRO$\alpha$ and either fMLP (A), C5a (B), or PAF (C). Cells were rechallenged 3 min later with a second dose of ligand as indicated. Traces are representative of three experiments.

**FIG. 2.** Functional characterization of CXCR2 relative to CXCR1 expressed in RBL-2H3 cells. A, for the generation of $[^{3}H]$inositol phosphates, cells were cultured overnight in the presence of $[^{3}H]$inositol (1 $\mu$Ci/ml). Cells were preincubated (10 min, 37 °C) with a HEPES-buffered saline containing 10 mM LiCl in a total volume of 200 ml and stimulated with different concentrations of IL-8 for 10 min. $[^{3}H]$inositol phosphate released was determined as described under “Experimental Procedures.”

This data was corrected for basal and represented as total cpm. The experiment was repeated four times with similar results. B, for intracellular calcium mobilization, RBL cells (3 $\times$ 10$^6$) were loaded with indo-1 and IL-8 (10 nM) stimulated Ca$^{2+}$ mobilization was measured. Representative tracings of five experiments are shown. C, chemotactic response to IL-8 was measured as described under “Experimental Procedures.” The results are representative of one of four experiments performed in triplicate. D, for secretion, 10 $\mu$l of the supernatant for PI hydrolysis was removed, and $\beta$-hexosaminidase released was measured. The results were represented as percentage of total $\beta$-hexosaminidase in the cells. The experiment was repeated four times with similar results. For IL-8-induced internalization, RBL-2H3 cells (0.5 $\times$ 10$^6$ cells/well) expressing CXCR2 or CXCR1 were either treated with IL-8 (100 nM) at different times (E) or with different concentrations of IL-8 for 30 min (F), washed, and assayed for $^{125}$I-IL-8 binding. The values are presented as percentage of total, which is defined as the total amount of $^{125}$I-IL-8 bound to control (untreated) cells. The experiment was repeated twice with similar results.
Cross-regulation of CXCR2

RBL-2H3 cells (3 x 10^6 cells/assay) expressing CXCR2-C5aR or CXCR2-FR were loaded with indo-1 and stimulated with IL-8 (10 nM), C5a (10 nM), or fMLP (100 nM). Cells were rechallenged 3 min later with a second dose of the indicated ligand, and peak intracellular Ca^{2+} mobilization was determined. Data are the means ± S.E. of three different experiments.

### Table I
Cross-desensitization of CXCR2-mediated Ca^{2+} mobilization by C5a and fMLP RBL-2H3 cells

| Cells/treatment | Peak Ca^{2+} mobilization | Cross-desensitization % |
|-----------------|---------------------------|-------------------------|
| CXCR2-C5aR      |                           |                         |
| IL-8 → C5a      | 450 ± 27 → 494 ± 36       | 0                       |
| C5a → IL-8      | 484 ± 32 → 187 ± 10       | 60                      |
| CXCR2-FR        |                           |                         |
| IL-8 → fMLP     | 471 ± 17 → 486 ± 29       | 3                       |
| fMLP → IL-8     | 501 ± 11 → 134 ± 7        | 72                      |

**Fig. 4.** Cross-phosphorylation of chemoattractant receptors.

32P-Labeled RBL-2H3 cells (3 x 10^6/60-mm plate) expressing CXCR2 with either C5aR (CXCR2-C5aR) or FR (CXCR2-FR) were incubated for 5 min with or without stimulants as shown. Cells were lysed and immunoprecipitated first with a CXCR2-specific antibody (Anti-CXCR2) and second with 12CA5 antibody specific for the HA epitope tag (Anti-HA) expressed at the amino terminus of C5aR and FR and then analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The results are from a representative experiment that was repeated three times.

**Fig. 5.** Effect of staurosporine on cross-phosphorylation of CXCR2.

32P-Labeled RBL-2H3 cells were incubated with and without staurosporine for 5 min and then stimulated with IL-8 (lanes 3 and 4), C5a (lanes 5 and 6), or [P] (lanes 7 and 8). Cells were lysed, immunoprecipitated with anti-CXCR2 (A) and then 12CA5 (B) antibodies, electrophoresed into 10% SDS-polyacrylamide gel, and autoradiographed. Three other experiments yielded similar results.

**Fig. 6.** Effect of staurosporine on cross-phosphorylation of CXCR2.

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Cross-desensitization of CXCR2-mediated Ca^{2+} mobilization by C5a and fMLP RBL-2H3 cells

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| CXCR2-FR        |                           |                         |
| IL-8 → fMLP     | 471 ± 17 → 486 ± 29       | 3                       |
| fMLP → IL-8     | 501 ± 11 → 134 ± 7        | 72                      |

**Fig. 3.** Homologous desensitization and cross-desensitization of CXCR2-mediated GTPase activity. Double transfected RBL-2H3 cells expressing CXCR2 and C5aR (CXCR2-C5aR) were treated with IL-8 (100 nM), C5a (100 nM), or PMA (100 nM) for 5 min. Membranes were prepared and assayed for agonist-stimulated GTP hydrolysis. The data shown are the means of three different experiments performed in triplicate. The data are presented as percentage of control, which is the net maximal stimulation obtained with untreated cells. Data shown are representative of one of three experiments performed in triplicate.

terminus of FR and C5aR (15). As shown in Fig. 4, CXCR2 was homologously phosphorylated by IL-8 (A and B, lanes 2) and cross-phosphorylated by C5a (A, lane 3) and fMLP (B, lane 3). Two forms of CXCR2 were observed, a slow (~70-kDa) and a fast migrating (~45-kDa) form. Similar results were obtained with three different antibodies directed against the N terminus of the CXCR2. C5aR (A, lane 6, ~42 kDa) and FR (B, lane 6, ~65 kDa) were homologously phosphorylated by their ligands. No significant cross-phosphorylation of C5aR (A, lane 5) by IL-8 was observed. FR is resistant to that process (12, 13).

Dose-response of C5a-mediated cross-phosphorylation of CXCR2 was also studied. Both forms of CXCR2 were cross-phosphorylated to a similar extent by activation of C5aR (data not shown). Half-maximal concentration was ~10 nM C5a, and maximal cross-phosphorylation was obtained at ~33 nM.

**Effect of Staurosporine on CXCR2 Cross-phosphorylation—**

The involvement of protein kinase C on C5a-mediated cross-phosphorylation of CXCR2 was studied. As shown in Fig. 5A, IL-8-induced phosphorylation of CXCR2 was not affected by staurosporine (lanes 3 and 4), whereas cross-phosphorylation by C5a (lanes 5 and 6) and heterologous phosphorylation by PMA (lanes 7 and 8) were markedly inhibited. As expected, C5aR phosphorylation (Fig. 5B) by PMA was completely inhibited by staurosporine (lane 7 versus lane 8), whereas only the fast form of C5a-mediated phosphorylation was blocked (lanes 5 and 6) (15).

**Co-expression, Characterization, and Cross-desensitization of 331T in RBL-2H3 Cells—**

In order to assess the role of receptor phosphorylation on CXCR2-mediated cellular responses, a phosphorylation-deficient CXCR2 mutant, 331T, in which the carboxyl terminus has been truncated by placing a stop codon at serine 331 (10) was co-transfected into RBL-2H3 cells with either C5aR (331T-C5aR) or FR (331T-FR). Stable transfectants were generated, and single cell cloning was utilized to isolate double transfectants. As was the case in 3ASubE cells, the mutant 331T expressed in RBL cells bound 125I-IL-8 with a Kd of 2.0 ± 0.1 nM, similar to that of CXCR2 (2.4 ± 0.8 nM). However, 331T was more active than CXCR2 in mediating PI hydrolysis (Fig. 6C). As reported previously (10), 331T mediated a sustained Ca^{2+} mobilization (Fig. 6D) and was resistant to IL-8-induced receptor internalization relative to CXCR2 (5 versus 95% for 331T and CXCR2, respectively, after 30 min of reactions) (Fig. 6E). IL-8 caused no PLD activity in CXCR2 cells, whereas it showed an ~1.7-fold increase over basal level in 331T cells (Fig. 6F).

331T was resistant to phosphorylation by IL-8 (Fig. 7, lanes 2 and 8) and cross-phosphorylation by either C5a (lanes 3 or fMLP (lane 9). C5aR (lane 6) and FR (lane 12) were homologously phosphorylated by C5a and fMLP, respectively. FR was resistant to cross-phosphorylation (lane 11), whereas IL-8 stimulation of the receptor mutant 331T resulted in C5aR cross-phosphorylation (lane 5). Despite the absence of receptor phosphorylation, 331T-me-
emediated Ca\(^{2+}\) mobilization was cross-desensitized by pretreatment of the double transfectant cells with either C5a (53%) or fMLP (55%) (Table II). However, in contrast to the wild type CXCR2, pretreatment of cells with a first dose of IL-8 diminished Ca\(^{2+}\) mobilization mediated by either C5aR or FR (Table II). Both PMA and the cAMP analog cpt-cAMP inhibited Ca\(^{2+}\) mobilization in response to 331T (71 and 62%, respectively). Ptx pretreatment also inhibited Ca\(^{2+}\) responses to both the mutant 331T and the wild type CXCR2 (data not shown).

**DISCUSSION**

Regulation of chemoattractant receptors in leukocytes plays a critical role in inflammatory processes and host defense (24). Recent studies demonstrating myriad chemokines and chemokine receptors and a relationship between these and infectivity of human immunodeficiency virus and other viruses have spawned renewed interest in the regulation of chemoattractant receptors. This laboratory has previously reported that motility versus cytotoxic responses of leukocytes to chemoattractants utilized sequential but distinct pathways (24–26). The motility responses were mediated by low doses of chemoattractant and correlated with signals generated rapidly (peak = 30 s). Cytotoxic responses required higher doses (~20–50% higher) of chemoattractant and were correlated with signals that peaked...
Cross-regulation of Ca2⁺ mobilization in cells expressing 331T-C5aR and 331T-FR

RBL-2H3 cells (3 x 10⁶ cells/assay) expressing 331T-C5aR or 331T-FR were loaded with indo-1 and stimulated with either IL-8 (10 nM), C5a (10 nM), or fMLP (100 nM). Cells were rechallenged 5 min later with a second dose of the indicated ligand, and the peak of intracellular Ca²⁺ mobilization was determined. Cells were also pretreated with either PMA (100 nM) or cpt-cAMP (1 mM) for 5 min, and Ca²⁺ mobilization in response to IL-8 was determined. Data are the means ± S.E. of three different experiments.

| Cells/treatment | Peak Ca²⁺ mobilization | Cross-desensitization |
|-----------------|------------------------|-----------------------|
| 331T-C5aR       | 581 ± 23 → 324 ± 9     | 49                    |
| IL-8 → C5a      | 636 ± 11 → 274 ± 6     | 53                    |
| C5a → IL-8      | 517 ± 3 → 305 ± 19     | 38                    |
| 331T-FR         | 493 ± 12 → 233 ± 15    | 55                    |
| IL-8 → fMLP     | 0 → 150 ± 16           | 71                    |
| fMLP → IL-8     | 0 → 196 ± 4            | 62                    |

Recent studies from this laboratory (18, 28) and others (29) have indicated that phosphorylation of PLCβ upon receptor activation may be responsible for the downstream inhibition of receptor-mediated cellular responses. The peptide chemotactant receptors, including CXCR2, couple to Ptx-sensitive G proteins to activate PLCβ via Gβγ (30, 31). Of the four known PLCβ isozymes (PLCβ₁-PLCβ₄) only PLCβ₁ is expressed in RBL-2H3 cells (18, 28). Thus, it could be reasoned that the inability of CXCR2 to cross-desensitize responses to other chemotactant receptors reflects its inability to mediate PLCβ₁ phosphorylation. However, upon stimulation with an EC₁₀₀ dose of IL-8, the wild type CXCR2 and the mutant 331T mediated PLCβ₁ phosphorylation to an extent similar to that of CXCR1, ~2-fold over basal level (Fig. 8) (14). Another explanation could be that CXCR2 mediated PLCβ₁ phosphorylation at sites different from the other chemotactant receptors. Two-dimensional peptide mapping, however, showed that both CXCR2 and C5aR mediated PLCβ₁ phosphorylation at the same domains (data not shown). In addition, 331T, which cross-desensitizes responses to FR and C5aR, mediated PLCβ₁ phosphorylation to the same extent as CXCR2 (Fig. 8). Taken together, these results suggest that phosphorylation of PLCβ may not be the only mechanism of cross-desensitization at downstream sites. Modification of other molecules such as the regulator of G protein signaling or Gβγ, which alter PLCβ activity (32, 33),

| Table II
| Cross-desensitization of Ca²⁺ mobilization in cells expressing 331T-C5aR and 331T-FR |
|-----------------|-----------------------------------|
| Cells/treatment | Peak Ca²⁺ mobilization | Cross-desensitization |
| 331T-C5aR       | 581 ± 23 → 324 ± 9     | 49                    |
| IL-8 → C5a      | 636 ± 11 → 274 ± 6     | 53                    |
| C5a → IL-8      | 517 ± 3 → 305 ± 19     | 38                    |
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| IL-8 → fMLP     | 0 → 150 ± 16           | 71                    |
| fMLP → IL-8     | 0 → 196 ± 4            | 62                    |

**FIG. 8.** CXCR2- and 331T-mediated PLCβ₁ phosphorylation. RBL-2H3 cells expressing wild type CXCR2, the mutant 331T, or CXCR1 were [³²P]-labeled and stimulated for 5 min with IL-8 (100 nM). Cells were lysed, immunoprecipitated with anti-PLCβ₁ antibody, and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The results are from a representative experiment that was repeated three times.
may play an important role in the cross-desensitization process.

The observation that CXCR2 phosphorylation by either IL-8 (Fig. 5) or GROα (34) is resistant to inhibition by staurosporine suggests that phosphorylation of the receptor is predominantly by a GRK-dependent mechanism rather than through activation of second messenger-dependent kinases. IL-8-mediated CXCR1 phosphorylation was partially inhibited by staurosporine, indicating both protein kinase C- and GRK-dependent mechanisms (19). Both CXCR1 and CXCR2 undergo phosphorylation-mediated internalization and/or down-regulation upon IL-8 stimulation. However, CXCR2 internalized more rapidly and recovered more slowly than CXCR1 (~35% recovery of CXCR2 versus 100% of CXCR2, after 90 min) (22, 23). Thus, differential phosphorylation between CXCR1 and CXCR2 may provide a molecular basis for their different rate of internalization/down-regulation and resensitization as well as generation of a cross-desensitizing signal. Supporting that contention is the observation that CXCR2 phosphorylation by either IL-8 and CXCR2, respectively, after 30 min; Fig. 6E) and generated greater and longer signals than CXCR2 (i.e. GTPase activity, PI hydrolysis, Ca2+ mobilization, PLCβ activation) (Fig. 6), cross-desensitizes Ca2+ mobilization to FR and C5aR (Table II). Taken together, these data may indicate that the ability to generate a cross-desensitizing signal may depend on the exact and length of activation of the receptor, which in the case of CXCR2 is prevented by rapid phosphorylation of the carboxyl terminus followed by receptor internalization. Additionally, prolonged signal generation in 331T was accompanied by activation of PLD. In other studies, it was suggested that PLD activation, which results in the delayed (peak in 2–5 min) formation of large quantities of diacylglycerol, is correlated with activation of the respiratory burst in neutrophils (34). Thus, the nature of the signals triggered by chemoattractant receptors appears to be governed by the length of receptor activation.

In summary, these data indicate that despite the ability of both CXCR1 and CXCR2 to initiate equivalent responses in leukocytes (i.e. PI hydrolysis, Ca2+ mobilization, PLCβ phospho-phylation, chemotaxis, and exocytosis) their ability to generate others including PLD activation, receptor cross-phosphorylation, and cross-desensitization are vastly different. These results, together with the inability of CXCR2 to mediate PLD activation and superoxide anion production in neutrophils, support the hypothesis that the ability of chemoattractant receptors to mediate cytotoxic activation requires more prolonged receptor activation than that required for motility-related responses. This distinction appears to be determined by phosphorylation sites on the receptors’ cytoplasmic tail, explaining the previously noted hierarchy among chemoattractant receptors to activate cytotoxic responses and receptor cross-regulation (24). Overall, a complete understanding of the signaling properties of CXCR2 relative to CXCR1 receptor will require specific mutation of the carboxyl terminus of the receptor.

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