Role of the Cytoplasmic Tails of CXCR1 and CXCR2 in Mediating Leukocyte Migration, Activation, and Regulation

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*J Immunol* 2003; 170:2904-2911; doi: 10.4049/jimmunol.170.6.2904
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Role of the Cytoplasmic Tails of CXCR1 and CXCR2 in Mediating Leukocyte Migration, Activation, and Regulation

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IL-8 (or CXCL8) activates the receptors CXCR1 (IL-8RA) and CXCR2 (IL-8RB) to induce chemotaxis in leukocytes, but only CXCR1 mediates cytotoxic and cross-regulatory signals. This may be due to the rapid internalization of CXCR2. To investigate the roles of the intracellular domains in receptor regulation, wild-type, chimeric, phosphorylation-deficient, and cytoplasmic tail (C-tail) deletion mutants of both receptors were expressed in RBL-2H3 cells and studied for cellular activation, receptor phosphorylation, desensitization, and internalization. All but one chimeric receptor bound IL-8 and mediated signal transduction, chemotaxis, and exocytosis. Upon IL-8 activation, the chimeric receptors underwent receptor phosphorylation and desensitization. One was resistant to internalization, yet it mediated normal levels of β-arrestin 2 (βarr-2) translocation. The lack of internalization by this receptor may be due to its reduced association with βarr-2 and the adaptor protein-2β. The C-tail-deleted and phosphorylation-deficient receptors were resistant to receptor phosphorylation, desensitization, arrestin translocation, and internalization. They also mediated greater phosphoinositide hydrolysis and exocytosis and sustained Ca²⁺ mobilization, but diminished chemotaxis. These data indicate that phosphorylation of the C-tails of CXCR1 and CXCR2 are required for arrestin translocation and internalization, but are not sufficient to explain the rapid internalization of CXCR2 relative to CXCR1. The data also show that receptor internalization is not required for chemotaxis. The lack of receptor phosphorylation was correlated with greater signal transduction but diminished chemotaxis, indicating that second messenger production, not receptor internalization, negatively regulates chemotaxis. The Journal of Immunology, 2003, 170: 2904–2911.

Materials and Methods

Materials

[³²P]Orthophosphate (8500–9120 Ci/mmol), myo-2-[³H]inositol (24.4 Ci/mmol), [γ⁻³²P]GTP (6000 Ci/mmol), and [³²S]-IL-8 were purchased from DuPont-NEN (Boston, MA). IL-8 (monocyte derived) and growth-related oncogene α were purchased from PeproTech (Rocky Hill, NJ). Geneticin (G418) and all tissue culture reagents were purchased from Life Technologies (Gaithersburg, MD). 12CA5 mAb, protein G-agarose, and protease inhibitors were purchased from Roche (Indianapolis, IN). Anti-human IL-8RA (CXCR1) and IL-8RB (CXCR2) Abs were purchased from BD PharMingen (San Diego, CA). Mouse mAbs against βarr-1 and βarr-2

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Received for publication October 7, 2002. Accepted for publication January 15, 2003.

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1 This work was supported by National Institutes of Health Grants AI38910 (to R.M.R.) and DE03738 (to R.S.).

2 Address correspondence and reprint requests to Dr. Ricardo M. Richardson, Department of Biochemistry, Meharry Medical College, 1005 Dr. D. B. Todd, Jr., Boulevard, Nashville, TN 37208. E-mail address: mrrichardson@mmc.edu

3 Abbreviations used in this paper: PL, phosphoinositide; AP-2β, adaptor protein-2β; βarr-2, β-arrestin 2; C-tail, cytoplasmic tail; CXCR1, IL-8R A; CXCR2, IL-8R B; Dyn I, dynamin I; G protein, GFP, green fluorescent protein; GTP-regulatory protein.
were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal mouse anti-adaptin protein-2β (anti-AP-2β) was obtained from Transduction Laboratories (Lexington, KY). Indo-1 acetoxyethyl ester and pluronic acid were purchased from Molecular Probes (Eugene, OR). PMA, GDP, GTP, and ATP were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents were from commercial sources. cDNAs encoding the chimeric mutants AB5 and BA5 were gifts from Dr. P. M. Murphy (National Institutes of Health, Bethesda, MD). cDNA encoding the tail-deleted mutant of CXCR2, ΔCXCR2 (331T), was provided by Dr. A. Caron (Memorial Sloan-Kettering Cancer Center, New York, NY). The distribution constant (Kd) and binding capacity (Bmax) were determined using GraphPad radioligand binding data analysis (San Diego, CA). For receptor internalization, cells were incubated with ligand for 0–60 min at 37°C. Cells were then washed with ice-cold PBS, and [125I]IL-8 binding (0.1 nM) was conducted as described above.

PI hydrolysis and secretion, and calcium measurement

RBL-2H3 cells were subcultured overnight in 96-well culture plates (50,000 cells/well) in isonol-free medium supplemented with 10% dialyzed FBS and 1 μg/ml [3H]inositol. The generation of inositol phosphates and secretion of β-hexosaminidase were determined as previously reported (18, 20). For calcium mobilization, cells (3 × 10⁶) were removed, washed with HEPES-buffered saline, and loaded with 1 μM Indo-IAM 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. The increase in intracellular calcium in the presence or the absence of ligands was measured as described previously (18, 20).

Chemoattractant assay

RBL-2H3 cells (n = 50,000) were incubated at 37°C with different concentrations of IL-8. Chemoattractant assay was performed 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 PBS, scraped, fixed, and stained. The results are representative of at least three separate experiments.

GTPase activity

Cells were treated with the appropriate concentrations of stimuli, and membranes were prepared as previously described (20). GTPase activity was determined using 10–20 μg of membrane preparations was determined as described previously (20).

Phosphorylation of receptors

Phosphorylation of receptors was performed as described previously (18, 20, 21). RBL cells (5 × 10⁶) expressing the receptors were incubated with [32P]orthophosphate (150 μCi/dish) for 90 min. Then labeled cells were stimulated with the indicated ligands for 5 min at 37°C. Cells were then washed and solubilized in 1 ml of buffer (RIPA) containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS. Cells lysates were immunoprecipitated with specific Abs against the N terminus of CXCR1 or CXCR2, analyzed by SDS-PAGE, and visualized by autoradiography.

Table I. Amino acid sequences of the carboxyl-terminal tail of the wild-type CXCR1 and CXCR2, the chimeric receptors ABt and BA5, the phosphorylation-deficient mutants of CXCR1 and CXCR2 M8-A and M10-B, respectively, and the tail-deleted mutants ΔCXCR1 and ΔCXCR2

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Amino Acid Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-tail CXCR1</td>
<td>NPIIYAFIQGFRHRGLK1LAIHGLK1SKDLPQGRSRP2VGSGSHSTTL</td>
</tr>
<tr>
<td>C-tail CXCR2</td>
<td>NPLIYAFIQGFRHRGLK1LAIHGLK1SKDLPQGRSRP2VGSGSHSTTL</td>
</tr>
<tr>
<td>ABt</td>
<td>NPIIYAFIQGFRHRGLK1LAIHGLK1SKDLPQGRSRP2VGSGSHSTTL</td>
</tr>
<tr>
<td>BA5</td>
<td>NPLIYAFIQGFRHRGLK1LAIHGLK1SKDLPQGRSRP2VGSGSHSTTL</td>
</tr>
<tr>
<td>M8-A</td>
<td>NPIIYAFIQGFRHRGLK1LAIHGLK1SKDLPQGRSRP2VGSGSHSTTL</td>
</tr>
<tr>
<td>M10-B</td>
<td>NPLIYAFIQGFRHRGLK1LAIHGLK1SKDLPQGRSRP2VGSGSHSTTL</td>
</tr>
<tr>
<td>ΔCXCR1</td>
<td>NPIIYAFIQGFRHRGLK1LAIHGLK1SKDLPQGRSRP2VGSGSHSTTL</td>
</tr>
<tr>
<td>ΔCXCR2</td>
<td>NPLIYAFIQGFRHRGLK1LAIHGLK1SKDLPQGRSRP2VGSGSHSTTL</td>
</tr>
</tbody>
</table>

* Underlined serine and threonine residues are potential sites for receptor phosphorylation.
Confocal microscopy studies
Transfected cells were plated overnight onto 35-mm plastic dishes containing a centered, 1-cm, glass-bottom well. Cells were placed in 1 ml of MEM buffered with 20 mM HEPES and treated with IL-8 (100 nM) in the same medium. Images were collected with a laser scanning confocal microscope (LSM-410; Carl Zeiss, New York, NY) (22).

Coimmunoprecipitation and immunoblotting
RBL cells stably expressing the receptors were treated with or without IL-8 (100 nM) for 2 min, washed three times with ice-cold PBS, and solubilized in 1 ml of RIPA. The lysates were precleared for 1 h by addition of 20 μl of protein G–agarose beads. Supernatants were immunoprecipitated with 15 μl of anti-CXCR1 or anti-CXCR2 and protein G–agarose beads for 2 h. The beads were then washed with three times with 1 ml of ice-cold RIPA and immunoprecipitates were resolved by 10% SDS-PAGE, transferred to nitrocellulose membrane, and probed with mouse mAbs against βar2, AP-2β, CXCR1, or CXCR2. Abs were detected with HRP-conjugated sheep anti-mouse Ab (Amersham Pharmacia Biotech, Piscataway, NJ) and ECL (Amersham Pharmacia Biotech).

Results
Expression and characterization of wild-type and mutant CXCR1 and CXCR2 in RBL-2H3 cells
To determine the role of the C-tails of CXCR1 and CXCR2 in IL-8-mediated leukocyte functions, four chimeric receptors, ABt, BAt, MA5-A, and MA10-B, two phosphorylation-deficient mutants, M8-A and MA10-B, and two carboxyl tail-truncated mutants, ΔCXCR1 and ΔCXCR2, were made (Table I and Fig. 1) (17). ABt, BAt, MA5-A, MA10-B, ΔCXCR1, and ΔCXCR2, expressed in RBL cells (Fig. 1, B and C), bound IL-8 with affinities (Table II) similar to those of the wild-type CXCR1 and CXCR2. AB5 did not bind IL-8 or melanoma growth-stimulating activity, although surface expression of the receptor could be measured by FACS analysis (Table II and Fig. 1B).

Upon IL-8 activation CXCR1, CXCR2, ABt, BAt, MA5-A, MA10-B, ΔCXCR1, and ΔCXCR2 stimulated dose-dependent PI hydrolysis (Fig. 2, A and B), secretion of β-hexosaminidase (Fig. 2, C and D) and chemotaxis (Fig. 2, E and F). Cellular responses to the chimeric receptors BAt and MA5-A (Fig. 2, B, D, and F) were equipotent to those of CXCR2, whereas ABt (Fig. 2, A, C, and E) induced greater PI hydrolysis and secretion, but lower chemotaxis, relative to CXCR1. The phosphorylation-deficient M8-A and MA10-B and the tail-deleted mutants, ΔCXCR1 and ΔCXCR2, mediated much greater PI hydrolysis and secretion (Fig. 2, A–D), but ∼50% lower chemotaxis (Fig. 2, E and F) relative to CXCR1 and CXCR2.

ABt, BAt, MA5-A, MA10-B, ΔCXCR1, and ΔCXCR2 stimulated a rapid and transient increase in free intracellular Ca2+ mobilization in response to IL-8 (Fig. 3). However, Ca2+ responses to M8-A, MA10-B, ΔCXCR1, ΔCXCR2, and ABt were more sustained than those of CXCR1, CXCR2, BAt, and MA5.

Four other chimeras, exchanging the N terminus of CXCR1 for that of CXCR2 (ABn and BAn) and exchanging transmembrane domain 4 and extracellular loop 2 (ATCB and BCTA), were constructed. BAn did not express. ABn expressed in RBL cells bound IL-8 (Kd = 1.9 ± 0.38) and mediated Ca2+ mobilization, PI hydrolysis, and secretion as well as CXCR1 (data not shown). ATCB and BCTA expressed, but did not bind ligand.

Desensitization of intracellular Ca2+ mobilization and GTPase activity
Ca2+ mobilization in response to a second dose of IL-8 (100 nM) was desensitized by prestimulation of the cells with the first dose of IL-8 (Table III). Responses to ABt, BAt, and MA5 were desensitized to an extent similar to that of the wild-type receptor (∼90%), whereas M8-A, MA10-B, ΔCXCR1, and ΔCXCR2 were more resistant to desensitization (41, 36, 43, and 49%, respectively). GTPase activity in membranes was measured to further study desensitization of receptor-mediated G protein activation. Pretreatment of CXCR1, CXCR2, ABt, BAt, and MA5 cells with either

Table II. Ligand binding affinity of wild-type and mutant CXCR1 and CXCR2 expressed in RBL-2H3 cells

<table>
<thead>
<tr>
<th>Receptor</th>
<th>KD (nM)</th>
<th>Bmax (receptors/cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCR1</td>
<td>1.2 ± 0.54</td>
<td>7113 ± 156</td>
</tr>
<tr>
<td>Abt</td>
<td>7.8 ± 3.11</td>
<td>7310 ± 337</td>
</tr>
<tr>
<td>MA5</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>M8-A</td>
<td>4.3 ± 1.52</td>
<td>6555 ± 577</td>
</tr>
<tr>
<td>ΔCXCR1</td>
<td>3.1 ± 1.10</td>
<td>6830 ± 451</td>
</tr>
<tr>
<td>ΔCXCR2</td>
<td>1.02 ± 0.94</td>
<td>7881 ± 210</td>
</tr>
<tr>
<td>BA1</td>
<td>2.20 ± 1.37</td>
<td>8030 ± 733</td>
</tr>
<tr>
<td>MA5</td>
<td>2.80 ± 0.30</td>
<td>6732 ± 252</td>
</tr>
<tr>
<td>MA10-B</td>
<td>1.33 ± 0.62</td>
<td>6308 ± 665</td>
</tr>
<tr>
<td>ΔCXCR2</td>
<td>1.90 ± 0.71</td>
<td>7763 ± 512</td>
</tr>
</tbody>
</table>

*values for wild-type and mutant receptors. ND, not determined.

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IL-8 (100 nM) or PMA (100 nM) resulted in desensitization (45–60%) of IL-8-induced GTPase activity in membranes (Fig. 4). IL-8 or PMA pretreatment had no effect on M8-A-, M10-B-, H9004CXCR1-, and H9004CXCR2-mediated GTPase activity. Desensitization of receptor-mediated GTPase activity was lower and more sensitive to receptor phosphorylation than that of Ca²⁺ mobilization. This difference probably indicates that the desensitization of Ca²⁺ mobilization occurs at two levels: receptor/G protein coupling via receptor phosphorylation and modification of phospholipase C activity, whereas desensitization of GTPase activity occurs only at the level of receptor/G protein coupling (23).

Receptor phosphorylation

To study receptor phosphorylation, 32P-labeled cells expressing CXCR1, CXCR2, ABt, BA5, M8-A, M10-B, ΔCXCR1, or ΔCXCR2 were stimulated with either IL-8 (100 nM) or PMA (100 nM) for 5 min. The cell lysates were immunoprecipitated with specific Ab directed against the N terminus of either CXCR1 or CXCR2. As shown previously (8, 20) CXCR1 migrated as a single band of ~70 kDa, whereas two forms of CXCR2 were observed: a slow (~70 kDa) and a fast (~45 kDa) migrating form. IL-8 and PMA mediated phosphorylation of ABt as well as CXCR1 (Fig. 5A, lanes 5 and 6 vs lanes 2 and 3). Phosphorylation of BA5 by IL-8 and PMA was also similar to that of CXCR2 (Fig. 5B, lanes 5 and 6 vs lane 2 and lanes 7 and 8 vs lane 3). M8-A (Fig. 5A, lanes 7–9), M10-B (Fig. 5B, lanes 10–12), and the C-tail-deleted mutants, ΔCXCR1 and ΔCXCR2 (data not shown), were resistant to receptor phosphorylation.

Internalization of CXCR1, CXCR2, ABt, BA5, ΔCXCR1, and ΔCXCR2

IL-8 (100 nM) induced a time-dependent internalization of CXCR1 and CXCR2 (Fig. 6). In agreement with previous reports (10, 15, 16) CXCR2 internalized more quickly than CXCR1 (~95 vs ~50% for CXCR2 and CXCR1, respectively, after 60 min). BA5 internalized as well as the wild-type CXCR2 (~90% after 60 min; Fig. 6B), whereas ABt showed a marked decrease in internalization relative to CXCR1 (~10% after 60 min; Fig. 6A). The phosphorylation-deficient mutants, M8-A, ΔCXCR1, and ΔCXCR2, were resistant to internalization, whereas M10-B showed ~20% internalization in response to IL-8 (Fig. 6, A and B). MGSA also induced an ~15% internalization of M10-B vs ~90% for CXCR2, BA5, and BA5 after 60 min (data not shown).
Role of βarr in IL-8-mediated internalization of CXCR1 and CXCR2

RBL cells predominantly express βarr-2 (12). To determine the mechanisms of internalization of CXCR1 and CXCR2, the receptors were stably coexpressed in RBL cells along with GFP-tagged βarr-2 (GFP intensity, <10^3). Fluorescence microscopy was used to study the time course of IL-8-mediated receptor clearance from the cell surface. Upon exposure to IL-8, CXCR1, CXCR2, ABt, BAt, and BA5, but not M8-A, M10-B, ΔCXCR1, or ΔCXCR2, induced rapid translocation of βarr-2 to the cell membrane (Fig. 7 and data not shown). However, arrestin-mediated internalization of the receptors into coated vesicles was shown for CXCR1, CXCR2, BAt, and BA5, but not ABt (Fig. 7, A and B). Vesicle formation and internalization were more rapid for CXCR2, BAt, and BA5 (~12 min) relative to CXCR1 (~35 min).

Association of ABt with βarr-2 and adaptin-2β

Since ABt translocated arrestin as well as CXCR1, it was determined whether the chimeric receptor bound βarr. RBL cells stably expressing CXCR1 and ABt (Table II) were stimulated with IL-8 (100 nM) for 2 min, and cell lysates were immunoprecipitated with anti-CXCR1 and immunoblotted with anti-βarr-2. As shown in Fig. 8A, IL-8 stimulation increased βarr-2 (~50 kDa) association to CXCR1 (lanes 1 and 2), but not ABt (lanes 2 and 4). AP-2β was shown to be important for CXCR2 internalization (24–28). Since ABt expresses the C-tail of CXCR2, its role in ABt internalization was also assessed. CXCR1 and ABT RBL cells were treated as described above, immunoprecipitated with anti-CXCR1, and immunoblotted with anti-AP-2β. As shown in Fig. 8B, IL-8 treatment induced AP-2β (~102 kDa) association to CXCR1, but not ABt.

Role of Src, Dyn I, and AP-2β in ABt internalization

The tyrosine kinase Src, the GTPase protein Dyn I, and AP-2β are known to be important for arrestin-dependent internalization (24–26). To assess their roles in ABt internalization, GFP-tagged Src, Dyn I, or AP-2β was overexpressed in RBL expressing ABT. Cells (GFP intensity, ~10^3) were collected and assayed for receptor internalization. As shown in Fig. 9A, AP-2β, but not Src or Dyn I, increased receptor internalization to an extent similar to that of CXCR1. Overexpression of AP-2β had no effect on IL-8-mediated internalization of M8-A, M10-B, ΔCXCR1, or ΔCXCR2 (Fig. 9B).

Discussion

CXCR1 and CXCR2 bind IL-8 to mediate leukocyte chemotaxis as well as desensitization and internalization of the receptors. Nonetheless, these receptors have a substantially different capacity to activate phospholipase D and the cytotoxic function of leukocytes. Previous data suggest that these difference may relate to the different rates of receptor internalization as a consequence of C-tail phosphorylation (10, 20). The present studies were designed to define, more specifically, the determinants of the difference in activation between CXCR1 and CXCR2. The current data obtained with the phosphorylation-deficient and tail-truncated receptors or receptor chimeras indicate that the receptor’s C-tails are necessary.

Table III. Desensitization of Ca^{2+} mobilization elicited by the first dose of IL-8

<table>
<thead>
<tr>
<th>Cells</th>
<th>1st dose</th>
<th>2nd dose</th>
<th>% Desensitization</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCR1</td>
<td>540 ± 41</td>
<td>52 ± 13</td>
<td>90</td>
</tr>
<tr>
<td>ABt</td>
<td>626 ± 27</td>
<td>61 ± 7</td>
<td>90</td>
</tr>
<tr>
<td>M8-A</td>
<td>601 ± 55</td>
<td>351 ± 33</td>
<td>41</td>
</tr>
<tr>
<td>ΔCXCR1</td>
<td>567 ± 41</td>
<td>322 ± 27</td>
<td>43</td>
</tr>
<tr>
<td>CXCR2</td>
<td>471 ± 32</td>
<td>0 ± 0</td>
<td>100</td>
</tr>
<tr>
<td>BAt</td>
<td>507 ± 66</td>
<td>47 ± 11</td>
<td>91</td>
</tr>
<tr>
<td>BA5</td>
<td>523 ± 21</td>
<td>26 ± 17</td>
<td>95</td>
</tr>
<tr>
<td>M10-B</td>
<td>558 ± 39</td>
<td>359 ± 51</td>
<td>36</td>
</tr>
<tr>
<td>ΔCXCR2</td>
<td>495 ± 27</td>
<td>253 ± 33</td>
<td>49</td>
</tr>
</tbody>
</table>

RBL-2H3 cells (5 x 10^6 cells/assay) expressing wild-type or mutants CXCR1 and CXCR2 were loaded with Indo-1 and stimulated with IL-8 (10 nM). Cells were rechallenged 3 min later with a second dose of IL-8 (10 nM), and peak intracellular Ca^{2+} mobilization was determined. Data are the means ± SE of three different experiments.
for receptor phosphorylation and desensitization, but are not sufficient to explain the different rate of internalization of CXCR1 vs CXCR2. This contention is based on the following observations. 1) ABt, the CXCR1 chimera expressing the cytoplasmic tail of CXCR2, underwent phosphorylation and desensitization equivalent to CXCR1, but was resistant to receptor internalization. 2) BAt and BA5, the CXCR2 chimeras expressing the C-tail of CXCR1, internalized as rapidly as native CXCR2. These results indicate that at least two signals mediate the internalization of the IL-8R: a phosphorylation-dependent signal in which the C-tail of the receptor is required in addition to a temporal/rate signal that is independent of the tail.

In other studies CXCR2 expressed in human embryonic kidney 293 (HEK 293) cells was reported to internalize via a phosphorylation/arrestin-independent mechanism (28). Binding of the adaptor protein AP-2 to the carboxyl-terminus, leucine-rich motif LLKIL was required for the phosphorylation-independent internalization of the receptor (28). The authors suggested that AP-2 could cause the rapid internalization of CXCR2 relative to CXCR1. Arguing against this hypothesis is the finding reported herein that CXCR1 bound AP-2β as well as CXCR2 upon IL-8 activation (Fig. 8B and data not shown). In addition, the chimeras BAt and BA5, which express the carboxyl tail of CXCR1 internalized as well as the wild-type CXCR2 (Fig. 6B).

Of interest is that the ABt chimera, despite its resistance to receptor internalization, induced arrestin translocation to the cell membrane as well as CXCR1 (Fig. 7A). Arrestin translocation and binding to the phosphorylated receptor are thought to be the initial requirement for internalization (29). The inability of arrestin to internalize the phosphorylated and desensitized receptor may be explained in several ways. First, it could be that the chimeric receptor was being recycled to the cell surface at a faster rate than the wild-type CXCR1, thus reducing the internalized fraction of the phosphorylated/arrestin bound receptor into clathrin-coated vesicles. Recycling of the receptor, however, would require rapid receptor dephosphorylation and resensitization. No decrease in receptor phosphorylation or the extent of desensitization of GTPase activity was observed in membranes from cells treated with IL-8 or PMA for up to 30 min (data not shown). Second, a conformational change in the C-tail of the receptor could have caused a decrease in the affinity of the phosphorylated receptor for arrestin. The resulting disassociation of the arrestin from the arrestin/receptor complex would thus prevent targeting of the receptor to the clathrin-coated vesicles. Supporting this hypothesis is that upon activation by IL-8, the amount of βarr-2 coimmunoprecipitated with ABt was less than that of CXCR1 (Fig. 8A). Third, several adaptor proteins, including the tyrosine kinase Src, the GTPase protein Dyn I, and AP-2, are shown to be critical for receptor endocytosis (16, 25, 26). Thus, it is possible that the complex of phosphorylated receptor/arrestin lost its affinity for the adaptor proteins, resulting in a failure of receptor internalization. Indeed, the amount of AP-2β coimmunoprecipitated with ABt was less than that of CXCR1 (Fig. 8B). However, while overexpression of AP-2β increases the ability of IL-8 to induce ABt internalization, Src or Dyn I had no effect (Fig. 9).

Yang et al. (13) reported that agonist-stimulated receptor internalization is essential for chemokine receptor-mediated chemotaxis. However, the ABt chimera that is resistant to internalization showed only a modest decrease in receptor-mediated chemotaxis relative to the wild-type receptor (Figs. 2 and 3). Interestingly, the modest decrease in chemotaxis correlated with an increase in receptor-mediated PI hydrolysis, exocytosis, and sustained Ca2+ mobilization (Fig. 2). In addition, the phosphorylation-deficient receptors, which mediated greater activation of PI hydrolysis and Ca2+ mobilization, displayed lower chemotaxis responsiveness relative to native receptors (Fig. 2). Thus, it is likely that second messenger production rather than receptor internalization plays a negative feedback regulatory role in chemotaxis. Supporting this contention is that mutations of the formylpeptide receptor, which abolished receptor internalization, did not reduce fMLP-mediated chemotaxis. However, the ABt chimera that is resistant to internalization showed only a modest decrease in receptor-mediated chemotaxis relative to the wild-type receptor (Figs. 2 and 3). Interestingly, the modest decrease in chemotaxis correlated with an increase in receptor-mediated PI hydrolysis, exocytosis, and sustained Ca2+ mobilization (Fig. 2). In addition, the phosphorylation-deficient receptors, which mediated greater activation of PI hydrolysis and Ca2+ mobilization, displayed lower chemotaxis responsiveness relative to native receptors (Fig. 2). Thus, it is likely that second messenger production rather than receptor internalization plays a negative feedback regulatory role in chemotaxis. Supporting this contention is that mutations of the formylpeptide receptor, which abolished receptor internalization, did not reduce fMLP-mediated.
chemotaxis (30, 31). Furthermore, mutations of chemoattractant receptors, including CXCR1, CXCR4, CCR1, CCR2, and protease-activated receptor-1, which enhanced cellular responses (i.e., PI hydrolysis, Ca\(^{2+}\)/H\(_{11003}\) mobilization, and secretion), diminished chemotaxis (20, 21, 32–34). These studies further undermine the requirement for receptor endocytosis in chemotaxis and indicate that endocytosis and chemotaxis are two independent processes that are probably regulated by different pathways.

The data herein indicate that the cytoplasmic tails of CXCR1 and CXCR2 are necessary for IL-8-mediated receptor phosphorylation, desensitization, and internalization. However, the resistance of ABt to IL-8–induced internalization suggests that receptor phosphorylation and arrestin translocation, while necessary, are not sufficient for receptor internalization. Furthermore, the ability of BAT and BA5, which express the C-tail of CXCR1, to internalize as rapidly as the wild-type CXCR2 indicates that another motif(s) or specific conformational changes beyond the C-tails of the receptors modulate their rates of internalization, lengths of signaling, and, thus, biological activities. Despite its resistance to internalization, ABt mediated chemotaxis as well as CXCR1 and CXCR2.

Thus, receptor internalization is not required for directional migration. Since M8-A, M10-B, ΔCXCR1, and ΔCXCR2 induced greater cellular responses (i.e., PI hydrolysis, Ca\(^{2+}\)/H\(_{11003}\) mobilization),
but decreased chemotaxis, these data further underscore the roles of these second messengers in down-regulating chemotaxis.

Acknowledgments
We are grateful to Drs. Ann Richmond, Philip M. Murphy, Marc G. Caron, and Robert J. Lefkowitz for the gifts of cDNAs.

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