Multiple Signaling Pathways of Human Interleukin-8 Receptor A

INDEPENDENT REGULATION BY PHOSPHORYLATION*

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

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

Interleukin-8 (IL-8) receptor A (CXCR1) couples to a pertussis toxin-sensitive G protein to mediate phospholipase Cβ (PLCβ) activation and cellular responses. Responses to CXCR1 are attenuated by prior exposure of neutrophils to either IL-8, a cleavage product of the fifth component of complement (C5a) or N-formylated peptides (formylmethionylleucylphenylalanine, fMLP). To characterize the role of receptor phosphorylation in the regulation of the CXCR1, a phosphorylation-deficient mutant, M2CXCR1, was constructed. This receptor, stably expressed in RBL-2H3 cells, coupled more efficiently to G protein and stimulated enhanced phosphoinositide hydrolysis, cAMP production, exocytosis, and phospholipase D activation, and was resistant to IL-8-induced receptor internalization. The rate and total amount of ligand stimulated actin polymerization remained unchanged, but interestingly, chemotaxis was decreased by ~30% compared with the wild type receptor. To study the role of receptor phosphorylation in cross-desensitization of chemoattractant receptors, M2CXCR1 was coexpressed with cDNAs encoding receptors for either fMLP (FR), C5a (C5aR), or platelet-activating factor (PAFR). Both C5aR and PAFR were cross-phosphorylated upon M2CXCR1 activation, resulting in attenuated guanosine 5′-3′-O-(thio)triphasphate (GTP-γS) binding in membranes. In contrast, FR and M2CXCR1 were resistant to cross-phosphorylation and cross-inhibition of GTP-γS binding by other receptors. Despite the resistance of M2CXCR1 to cross-phosphorylation and receptor/G protein uncoupling, its susceptibility to cross-desensitization of its Ca²⁺ response by fMLP and C5a, was equivalent to CXCR1. Regardless of the enhancement in certain receptor functions in M2CXCR1 compared with the wild type CXCR1, the mutated receptors mediated equivalent PLCβ₂ phosphorylation and cross-desensitization of Ca²⁺ mobilization by FR, C5aR, and PAFR. The results herein indicate that phosphorylation of CXCR1 regulates some, but not all of the receptors functions. While receptor phosphorylation inhibits G protein turnover, PLC activation, Ca²⁺ mobilization and secretion, it is required for normal chemotaxis and receptor internalization. Since phosphorylation of CXCR1 had no effect on its ability to induce phosphorylation of PLCβ, or to mediate class-desensitization, these activities may be mediated by independently regulated pathways.

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‡ To whom correspondence should be addressed: Dept. of Medicine, Duke University Medical Center, Box 3680, Durham, NC 27710. Tel.: 919-684-5332; Fax: 919-684-4390; E-mail: richa021@mc.duke.edu.

1 The abbreviations used are: IL-8, interleukin-8; fMLP, N-formylmethionylleucylphenylalanine; FR, fMLP receptor; C5a, peptide from the fifth component of complement; C5aR, C5a receptor; PMA, phorbol 12-myristate 13-acetate; GTPγS, guanosine 5′-3′-O-(thio)triphasphate; G protein, GTP-regulatory protein; PAF, platelet-activating factor; PAFR, PAF receptor; PLC, phospholipase C.
suggesting a pathway distal to receptor/G protein activation for PLC regulation. Moreover, this IL-8-induced phosphorylation of PLCβ3 may play an important role in class desensitization.

EXPERIMENTAL PROCEDURES

Materials—[^32P]Orthophosphate (8500–9120 Ci/mmol), nyc-2-[3H]inositol (24.4 Ci/mmol), [35S]GTPγS (1300 Ci/mmol), and [γ-32P]GTP (6000 Ci/mmol) were purchased from NEN Life Science Products. 125I-IL-8 labeled-IL-8 was obtained from Amersham Corp. IL-8 (monocyte-derived) was purchased from Genzyme. PAF was from Calbiochem. Gelatin (G418) and all tissue culture reagents were purchased from Life Technologies, Inc. Monoclonal 12CA5 antibody, protein G-agarose, and protease inhibitors were purchased from Boehringer Mannheim. Polyclonal antibody against PLCβ3 was obtained from Santa Cruz Biotechnology. FMLP, Indo-1 aceetoxyethyl ester, and pluronic acid were purchased from Molecular Probes. C5a and 8-4-chlorophenylthio-cAMP, phorbol 12-myristate 13-acetate (PMA), GDP, GTP, GTPγS, and ATP were purchased from Sigma. All other reagents are from commercial sources.

Construction of Epitope-tagged CXCR1, M1CXCR1, and M2CXCR1—Nucleotides encoding a nine-amino acid epitope sequence (YPDVPDYDA) was inserted between the N-terminal initiator methionine and the second amino acid of each cDNA by polymerase chain reaction as described previously (16, 22) for the substitution of serine and threonine residues of the carboxyl terminus of CXCR1 to generate M2CXCR1 was carried out by polymerase chain reaction.

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) (19). RBL-2H3 cells (1 × 10^6) were transfected by electroporation with pCDNA3 containing the receptor cDNAs (20 μg) and geneticin-resistant cells were cloned into single cell by fluorescein-activated cell sorting analysis.

Radioligand Binding Assays—RBL-2H3 cells were subcultured overnight in 24-well plates (0.5 × 10^6 cells/well) in growth medium. Cells were then rinsed with Dulbecco’s modified Eagle’s medium supplemented with 0.5% fetal bovine serum, 10 mM HEPES, pH 7.4, and incubated on ice for 2–4 h in the same medium (250 μl) containing 125I-IL-8. Reactions were stopped with 1 ml of ice-cold phosphate-buffered saline containing 10 mg/ml bovine serum albumin, and washed four times with the same buffer. Then cells were lysed with 0.1 N NaOH (250 μl), dried under vacuum, and bound radioactivity was evaluated by counting in a gamma counter. Nonspecific radioactivity bound was determined with 10 mM unlabeled IL-8. For competitive binding experiments, cells were incubated with 100 nM IL-8 for 0–60 min at 37 °C. After the incubation period, cells were washed three times with ice-cold phosphate-buffered saline and 125I-IL-8 binding was carried out as described above.

GTPase Activity and [35S]GTPγS binding—Cells were treated with appropriate concentrations of stimulants and membranes were prepared as already described (19). GTPase activity and [35S]GTPγS binding using 10–20 μg of membrane preparations were carried out as described previously (7, 19–21).

Phosphoinositide Hydrolysis and Calcium Measurement—RBL-2H3 cells were subcultured overnight in 96-well culture plates (50,000 cells/well) in isoinitol-free medium supplemented with 10% dialyzed fetal bovine serum and 1 μCi/ml [3H]inositol. The generation of inositol phosphates was determined as reported previously (19). For calcium mobilization, cells (3 × 10^6) were loaded with 1 μM Indo 1 aceetoxyethyl 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).

ACTIN POLYMERIZATION AND CHEMOTAXIS—Actin polymerization assays were carried out as described (19). RBL-2H3 cells (50,000) were incubated at 37 °C with different concentration of IL-8. Chemotaxis was assessed in 48-well microchemotaxis chambers, using polyvinylpyrrolidone-free 5-μm pore size membranes. Migration was allowed to continue for 5 h at 37 °C in 5% CO2. The membrane was removed, the upper surface was washed with phosphate-buffered saline and scraped, fixed, and stained. The results are represented as chemotaxis index (mean number of cells per high power field for chemokine dilution/mean number of cells per high power field for medium) (23).

RESULTS

Characterization of M2CXCR1 in RBL-2H3 Cells—M2CXCR1 was previously expressed in RBL-2H3 cells and it was demonstrated that the mutant bound IL-8 with a dissociation constant (Kd) of 2.8 ± 0.7 nM and a B_max of 7792 ± 284 receptors/cell. This was similar to that of wild type CXCR1 expressed in RBL-2H3 cells (Kd, 2.3 ± 0.3 nM; B_max 8532 ± 152 receptors/cell) or the native receptors in neutrophils (1–2 nM) (3, 7), indicating that mutation of the four amino acid residues which comprise the M2 cluster (7) did not affect ligand binding. However, M2CXCR1-mediated inositol phosphates formation and secretion were 5- and 10-fold, respectively, higher than the wild type receptor (7). IL-8-induced GTPase activity in membranes, and cAMP production and phospholipase D activity in intact cells were also greater in cells expressing M2CXCR1 than CXCR1 (data not shown). IL-8-induced actin polymerization was similar for both M2CXCR1 and CXCR1-expressing cells (Fig. 1A). However, cells expressing M2CXCR1 showed a 30% decrease in maximal chemotaxis compared with cells expressing CXCR1 (Fig. 1B). The EC_50 (∼0.1 nM), however, remained unchanged.

M2CXCR1 was more resistant to IL-8-induced internalization than CXCR1 (∼45 versus ∼80% of 125I-IL-8 binding after 60 min) (data not shown). These results are consistent with the ones reported by Prado et al. (24) and indicated that the M2 site play an important role in phosphorylation-mediated down-regulation of the CXCR1.

Cross-activation and Cross-phosphorylation of M2CXCR1—The ability of M2CXCR1 to cross-phosphorylate and cross-desensitize chemoattractant receptors was determined. Cells expressing CXCR1 and cells coexpressing M2CXCR1 and receptors for either fMLP (M2CXCR1-FR) or PAF (M2CXCR1-PAFR) were stimulated with either IL-8 (100 nM), fMLP (1 μM), PAF (100 nM), or PMA (100 nM). As shown in Fig. 2, CXCR1 (lane 2, ∼70 kDa), FR (lane 6, ∼65 kDa), and PAFR (lane 11, ∼45 kDa) were homologously phosphorylated by their ligands. CXCR1 and PAFR were also phosphorylated by PMA (lanes 3 and 10, respectively) but not FR (lane 7) and M2CXCR1 (lanes 7 and 10). PAFR was cross-phosphorylated by M2CXCR1 activation (lane 9). Both M2CXCR1 (lanes 6 and 11) and FR (lane 5) were resistant to cross-phosphorylation. Some homologous phosphorylation of M2CXCR1 was detected with longer exposure of the autoradiogram (7) (data not shown).

Cross-desensitization of M2CXCR1—Ca2+ mobilization was measured to determine the relationship between cross-phosphorylation and cross-desensitization of receptor-mediated cellular responses. Ca2+ mobilization in response to an EC_50 dose of either fMLP (100 nM; M2CXCR1-FR cells), PAF (10 nM; M2CXCR1-PAFR cells) or IL-8 (10 nM; M2CXCR1-FR and M2CXCR1-PAFR cells) was homologically desensitized by a
CXCR1 Phosphorylation and Desensitization

Phagocytic leukocytes respond to inflammatory mediators such as IL-8, fMLP, and C5a by migrating to sites of inflammation where they may exert their cytotoxic activities (25). Despite the presence of two receptors for IL-8 in neutrophils (CXCR1 and CXCR2), certain cellular responses to IL-8 are lower in magnitude compared with fMLP and C5a. For example, IL-8 is a weaker stimulator of exocytosis and respiratory burst (26). IL-8 has also been shown to be less effective than fMLP and C5a in stimulating phosphoinositide hydrolysis and mitogen-activated protein kinase activation in neutrophils (27). The results presented here indicate that responses to IL-8 are modulated by specific phosphorylation sites in the cytoplasmic tail of the receptor as well as a site downstream of G protein activation. A phosphorylation-deficient receptor mutant (M2CXCR1) more effectively activated G protein and was more resistant to agonist-mediated desensitization and internalization. Despite the ability of this mutant to up-regulate phosphoinositide hydrolysis, cAMP production, and phospholipase D activation and secretion, actin polymerization was not affected, and chemotaxis in response to IL-8 was actually di-

**FIG. 1.** Characteristics of CXCR1 and M2CXCR1 induced actin polymerization and chemotaxis in response to IL-8. A, for actin polymerization, RBL cells (1 x 10^6 cells/tube) were treated with or without IL-8 (100 nM) for 1 min. Cells were then permeabilized, fixed, stained, and analyzed by fluorescein-activated cell sorting. The experiment was repeated twice with similar results. B, chemotactic response to IL-8 was measured as described under “Experimental Procedures.” The results are representative of one of four experiments performed in triplicate.

**FIG. 2.** Cross-phosphorylation of chemoattractant receptors. 32P-Labeled RBL-2H3 cells (3 x 10^6/60-mm plate) expressing the wild type CXCR1 or the mutant M2CXCR1 with either FR (M2CXCR1-FR) or PAFR (M2CXCR1-PAFR) were incubated for 5 min with or without stimulants as shown. Cells were lysed, immunoprecipitated with 12CA5 antibody and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The results are from a representative experiment that was repeated three times.
minished compared with wild type CXCR1 (Fig. 1). These results may distinguish events which are important for leukocyte recruitment from those for cytotoxic functions. Cytoskeletal rearrangement and chemotaxis which are early events in inflammation occur via pathways which require lower doses of agonist to reach maximum responses. Cytotoxic activation such as exocytosis of lysosomal enzymes and superoxide production require higher doses of ligand (25). The molecular basis for these observations may be provided by the findings described here. Prevention of receptor phosphorylation which resulted in enhanced G protein activation at all ligand doses was associated with enhanced cytotoxic activity yet diminished chemotaxis and unchanged cytoskeletal actin assembly. This can be inferred to indicate that cytotoxic activity requires higher G protein turnover than chemotaxis. The finding that chemotaxis to IL-8 decreased in M2CXCR1 cells may be interpreted in two ways. First, that receptor desensitization and/or internalization may be required for normal gradient detection. However, this contention is not supported by the recent findings that chemotaxis may be regulated via receptor phosphorylation-independent pathways (28, 29). Second, since maximal cell migration occurs at doses of agonist lower than those required for Ca\textsuperscript{2+} mobilization or cAMP production (25), it is possible that the pathway(s) regulating chemotaxis is/are sensitive to second messenger levels and that the up-regulation of phospholipase C activation mediated by M2CXCR1 plays a negative regulatory role in sensing chemical gradients. Supporting this contention is that truncation of the carboxyl tail of the monocye chemoattractant protein 1 (MCP1) receptor (CCR2B) which enhanced receptor mediated Ca\textsuperscript{2+} mobilization and cAMP production, diminished chemotaxis in response to MCP1 (29). In addition, chemotaxis of leukocytes was enhanced in PLC\textsubscript{b2} deficient mice in which phosphoinositide hydrolysis, Ca\textsuperscript{2+} mobilization and superoxide production was decreased (30).

Understanding molecular events underlying cross-desensitization of receptors was facilitated by the availability of M2CXCR1. Phosphorylation of unoccupied receptors by second messenger-dependent kinases activated via different receptors appear to account for cross-desensitization at the level of R/G protein coupling (12). Our previous studies with FR have revealed that receptor cross-desensitization can also occur independently of receptor phosphorylation and G protein uncou-

**Fig. 3.** Cross-desensitization of M2CXCR1-mediated Ca\textsuperscript{2+} mobilization by fMLP and PAF. Double-transfected RBL-2H3 cells (3 × 10\textsuperscript{6} cells/assay) expressing the mutant receptor M2CXCR1 and receptor for either FR (M2CXCR1-FR) or PAFR (M2CXCR1-PAFR) were loaded with Indo-1 and treated with (open symbols) or without (closed symbols) an EC\textsubscript{100} dose of either IL-8 (10 nM), fMLP (100 nM), or PAF (10 nM). Cells were rechallenged 3 min later with a second dose of ligand and peak of intracellular Ca\textsuperscript{2+} mobilization was determined. The data are from a representative experiment performed in triplicate that was repeated twice with similar results.

**Fig. 4.** CXCR1- and M2CXCR1-mediated PLC\textsubscript{b} phosphorylation. A, RBL-2H3 cells expressing CXCR1 or M2CXCR1 were 32P-labeled and stimulated for 5 min with either IL-8 (100 nM), PMA (100 nM), or 8-(4-chlorophenylthio)-cAMP (1 mM). Cells were lysed, immunoprecipitated with anti-PLC\textsubscript{b} antibody and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. B, the amount of radioactivity per lane was determined by counting excised phosphorylated bands. The results are from a representative experiment that was repeated three times.
and involve modification of downstream effector(s) and, perhaps, different accessory proteins.

Another question addressed in this study is the role of second messenger production in receptor cross-regulation. M2CXCR1 induced greater G protein turnover, phosphoinositide hydrolysis and cAMP production than CXCR1. However, M2CXCR1 activation by IL-8 did not result in greater cross-phosphorylation or cross-desensitization of Ca\(^{2+}\) mobilization in response to either FR, PAFR, or C5aR (22–30%) compared with CXCR1 (20–30%) (12, 21). These results suggest that cross-desensitization of receptor-mediated Ca\(^{2+}\) mobilization occurs via pathways which are independent of receptor phosphorylation or rate of second messenger production measured in the present work. Supporting that contention is that phosphorylation of PLC\(\beta_3\), which is thought to be one of the target effectors for class-desensitization (16, 17), is mediated by both CXCR1 and M2CXCR1 to the same extent (Fig. 4).

This work provides evidence for independent mechanisms for CXCR1 receptor mediated chemotactic versus cytotoxic functions of phagocytic leukocytes. Whereas secretion was enhanced by removal of specific phosphorylation sites in the cytoplasmic tail of the receptor, chemotaxis and receptor internalization were inhibited by loss of receptor phosphorylation. In contrast, IL-8 mediated actin polymerization, PLC\(\beta_3\) phosphorylation, receptor cross-phosphorylation and cross-desensitization of Ca\(^{2+}\) response were not affected. Moreover, the lack of receptor phosphorylation did not affect cross-desensitization of or by M2CXCR1 at the level of Ca\(^{2+}\) mobilization. These data further underscore the presence of downstream effector(s) of receptor class-desensitization which appear to be regulated independently of receptor phosphorylation and enhanced G protein turnover.

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