Regulation of Human Interleukin-8 Receptor A: Identification of a Phosphorylation Site Involved in Modulating Receptor Functions†

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Received July 14, 1995; Revised Manuscript Received August 30, 1995®

ABSTRACT: The human type A interleukin-8 receptor (IL-8RA) was modified to express an amino-terminal epitope tag and stably overexpressed in a rat basophilic leukemia cell line (RBL-2H3). This receptor (ET-IL-8RA) displayed functional properties similar to those of the native receptor in neutrophils in that exposure to IL-8 stimulated GTPase activity, phosphoinositide (PI) hydrolysis, intracellular calcium mobilization, and degranulation in a pertussis toxin (PTx) susceptible fashion. IL-8 induced dose- and time-dependent phosphorylation of ET-IL-8RA. Phorbol 12-myristate 13-acetate (PMA) treatment also resulted in phosphorylation of the receptor although to a lesser extent. Staurosporine totally blocked PMA-induced phosphorylation but only partially inhibited IL-8-mediated phosphorylation. Phosphorylation of ET-IL-8RA correlated with its desensitization as measured by GTPase activation and calcium mobilization. To determine the role of phosphorylation in IL-8RA signal transduction, three mutants lacking specific serine and threonine residues located at the C-terminal of this receptor were constructed by site-directed mutagenesis (M1, M2, and M3). The mutated receptors expressed in RBL-2H3 cells displayed pharmacological properties ($K_d \sim 2-2.8$ nM and $B_{max} \sim 3-3.5$ pmol/mg of protein) similar to those of the wild-type ET-IL-8RA. M2 and M3, but not M1, showed a marked decrease in IL-8-induced phosphorylation compared to the wild-type receptor. M2 and M3 but not M1 were resistant to PMA-mediated phosphorylation and desensitization and were also more resistant to homologous desensitization than M1 or ET-IL-8RA. Following exposure to IL-8, M1 and M3 stimulated PI hydrolysis and secretion to the same extent as wild-type IL-8RA. M2, however, showed an ~4- and ~12-fold increase in IL-8-induced PI hydrolysis and secretion, respectively. These data suggest that the IL-8RA is susceptible to phosphorylation and desensitization by a receptor kinase (GRK) and protein kinase C (PKC), respectively. Moreover, the residues modified in M2 and M3 are required for PKC-mediated phosphorylation and desensitization. Interestingly, the M2 cluster appears to participate in the downregulation of IL-8RA-mediated responses.

IL-8[1] is a member of the family of structurally and functionally related chemotactic cytokines called chemokines, which comprise the melanoma-growth stimulating activity (MGSA), neutrophil activating protein 2 (NAP-2), monocyte chemoattractant protein (MCP-1), interferon-inducible protein (IP-10), and platelet factor 4 (PF4) (Baggiolini & Dahinden, 1994; Geiser et al., 1993; Moser et al., 1991). IL-8 belongs to the group of chemokines which preferentially activate neutrophils to induce chemotactic and cytotoxic responses such as exocytosis of lysosomal enzymes and production of superoxide anion (Smith et al., 1992; Snyderman & Uhing, 1992; Walz et al., 1991). These activities are mediated by the interaction of IL-8 with membrane receptors and are inhibited by pertussis toxin (Smith et al., 1992; Brennan, 1993; Horuk, 1994). Molecular cloning studies have identified two IL-8 receptor subtypes in human neutrophils, IL-8RA and IL-8RB (Holmes et al., 1991; Murphy & Tiffany, 1991). Both subtypes have been expressed in mammalian cell lines and bind IL-8 with high affinity (~2 nM). IL-8RA is expressed selectively in neutrophils while IL-8RB is also expressed in monocytes, melanoma cells lines, T-cells, and Jukart cell lines. While IL-8RA is specific for IL-8, the type B can also bind MGSA, GRO, and NAP-2 with high affinity (Horuk, 1994). IL-8 is clearly an important mediator of inflammation, but little is known of the molecular mechanism of its receptor's regulation.

IL-8Rs become desensitized and downregulated upon agonist exposure, resulting in a loss of cellular responsiveness to agonist, followed by a decrease in the number of receptors on the cell's surface (Baggiolini & Dahinden, 1994; Smith et al., 1992). When neutrophils were stimulated by IL-8, both receptor subtypes were desensitized, and no subsequent responses were obtained with any of the other chemokines tested (Geiser et al., 1993; Moser et al., 1991; Walz et al., 1991; Schumacher et al., 1992). In contrast, when neutrophils were exposed to either MGSA, NAP, or GRO, the cells were desensitized to non-IL-8 chemokines but were still responsive to IL-8 (Moser et al., 1991). These observations suggest that the type A IL-8R is specific for IL-8, while the

† This work was supported by National Institutes of Health Grant DE-03738 to R.S.

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® Abstract published in *Advance ACS Abstracts,* October 15, 1995.

[1] Abbreviations: IL-8, interleukin-8; ET-IL-8R, epitope-tagged IL-8 receptor; PTx, pertussis toxin; PMA, phorbol 12-myristate 13-acetate; IP, inositol phosphate; GTPγS, guanosine 5′−3′-*O*-(thiotriphosphate); G protein, GTP-regulatory protein; FITC, fluorescein isothiocyanate; B_{t_2}cAMP, dibutyryl cyclic AMP.

type B receptor is activated and desensitized by IL-8 and related chemokines. Recently, Mueller et al. (1994) demonstrated that IL-8RB undergoes phosphorylation upon stimulation by MGSA and phorbol ester. However, the consequences of such phosphorylation in the receptor-mediated responses were not determined. In the present work we have studied the regulation of IL-8RA-mediated functions. For this purpose an epitope-tagged IL-8RA cDNA was expressed in a rat basophilic leukemia (RBL-2H3) cell line which possesses the machinery to induce cellular responses similar to that of neutrophils. In addition, alanine mutagenesis was used to identify specific IL-8RA residues of the C-terminus important in modulating receptor functions. The studies herein describe the phosphorylation and desensitization of the IL-8RA and the identification of serine/threonine clusters of the C-terminus involved in regulation of the receptor's activity.

MATERIALS AND METHODS

Materials. [^{32}P]Orthophosphate (8500−9120 Ci/mmol), [^{125}I]iodine (17.0 Ci/mg), myo[2-^{3}H]inositol (24.4 Ci/mmol), [^{35}S]GTPγS (1300 Ci/mmol), and [γ-^{32}P]GTP (6000 Ci/mmol) were purchased from DuPont NEN. [^{125}I]IL-8 was obtained from Amersham. IL-8 (monocyte derived) was purchased from Genzyme. Monoclonal 12CA5 antibody was obtained from BabCo. PSV2neo was obtained from American Tissue Culture Collection. Geniticin (G418) and all tissue culture reagents were purchased from Life Technologies, Inc. Protein G−agarose and protease inhibitors were purchased from Boehringer Mannhein. Indo-1 acetoxymethyl ester and pluronic acid were purchased from Molecular Probes. GDP, GTP, GTPγS, and ATP were purchased from Sigma. All other reagents are from commercial sources.

Construction of Epitope-Tagged IL-8RA. Nucleotides encoding a nine amino acid epitope sequence (YPYDVPDYA) were inserted between the N-terminal initiator methionine and the second amino acid of each cDNA by polymerase chain reaction as described previously (Ali et al., 1993, 1994). The 5′-oligonucleotide, in a 5′ to 3′ order, contained 3 miscellaneous bases, 6 bases encoding a *Hin*dIII site, 3 bases from the 5′ untranslated sequence preceding the ATG codon of the human IL-8RA, 3 bases encoding a methionine, 27 bases encoding the 9 amino acid epitope tag, and 24 bases complementary to the cDNA sequence encoding amino acids 2−9 of IL-8RA. The 3′-oligonucleotide was complementary to the region encoding the sequence C-terminal to a unique *Pst*I site at nucleotide 119. Polymerase chain reaction was carried out with these primers using the human IL-8RA cDNA as a template. The resulting polymerase chain reaction product was digested with *Hin*dIII and *Pst*I and ligated into the plasmid vector pBluescript containing the IL-8RA receptor cDNA and was digested with the same enzymes. The PCR-amplified coding region including the epitope tag was sequenced and transferred into a pRK5 expression vector as a *Cla*I−*Xba*I fragment.

Cell Culture. 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 μg/mL) (Ali et al., 1993).

Transfection. RBL-2H3 cells (1×10^7 cells) were transfected by electroporation with pRK5 containing the receptor cDNAs (21 μg) along with PSV2neo (4 μg) plasmid containing the geneticin-resistant marker (Ali et al., 1993, 1994). Geneticin-resistant cells were selected by subculturing the transfected cells in growth medium supplemented with geneticin (1 mg/mL). Ten days post electroporation, the antibiotic-resistant clones were analyzed for cell surface expression of ET-IL-8RA as follows: Cells were detached by versene treatment, washed twice with serum-free medium, and preincubated with rat IgG (10 μg/mL for 10 min) to saturate Fc receptors present on the surface of RBL-2H3 cells (Alber et al., 1992). The binding was carried out in a total volume of 400 μL of serum-free medium for 60 min at 4 °C using FITC-12CA5 antibody (1 μg/mL). The cells were then washed and analyzed on a Beckton Dickinson FACS cytometer. The top 3% of the cells expressing ET-IL-8RA were subjected to two rounds of sorting, and the resulting mixed cell population was used throughout this study.

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 Earle's modified Eagle's medium supplemented with 20 mM HEPES, pH 7.4, and 10 mg/mL BSA and incubated on ice for 2−4 h in the same medium (250 μL) containing IL-8 (0−100 nM) and [^{125}I]IL-8. Reactions were stopped with 1 mL of ice-cold PBS containing 10 mg/mL BSA and washed four times with the same buffer. Then cells were lysed with 0.1 N NaOH (250 μL) and dried under vacuum, and bound radioactivity was evaluated by counting in a γ counter (Baggiolini et al., 1989). Nonspecific radioactivity bound was determined in the presence of 300 nM unlabeled IL-8.

Phosphoinositide Hydrolysis. RBL-2H3 cells were subcultured overnight in 96-well culture plates (50 000 cells/well) in inositol-free medium supplemented with 10% dialyzed fetal bovine serum and 1 μCi/mL [^{3}H]inositol. Then cells were washed with HEPES-buffered Hank's balanced salt solution (HHBSS) containing 100 mM LiCl and 0.1% BSA and incubated in the same buffer with and without IL-8. Reactions were stopped by addition of 200 μL of chloroform−methanol−4 N HCl (100:200:2). Total [^{3}H]-inositol phosphates in the aqueous phase were separated on a column of Dowex formate (Ali et al., 1993, 1994).

Secretion of β-Hexosaminidase. Cells were seeded as for PI hydrolysis, washed, and incubated with HHBSS for 5 min. Cells were then stimulated with different concentrations of IL-8. The reaction was terminated 10 min after stimulation by placing the plate on ice. Secretion of β-hexosaminidase into the medium was determined by incubating 10 μL of the supernatant of the cell lysate with 10 μL of 1 mM *p*-(nitrophenyl)-*N*-acetyl-β-D-glucosamide in 0.1 M sodium citrate buffer (pH 4.5) at 37 °C for 1 h. At the end of the incubation, 250 μL of a 0.1 M Na_2CO_3−$NaHCO_3$ buffer (pH 4.5) was added. Absorbance was monitored at 400 nM (Ali et al., 1993, 1994).

Calcium Measurement. Cells (3×10^6) were removed, washed with HBBSS, and loaded with 1 μM indo-1-AM 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 IL-8 was measured in a Perkin-Elmer fluorescence spectrophotometer with an excitation wavelength of 410 nM. Maximum and minimum fluorescence was determined in the presence of 0.1% Triton X-100 and of 20 mM Tris-HCl, pH 8.0, and 5 mM EGTA,

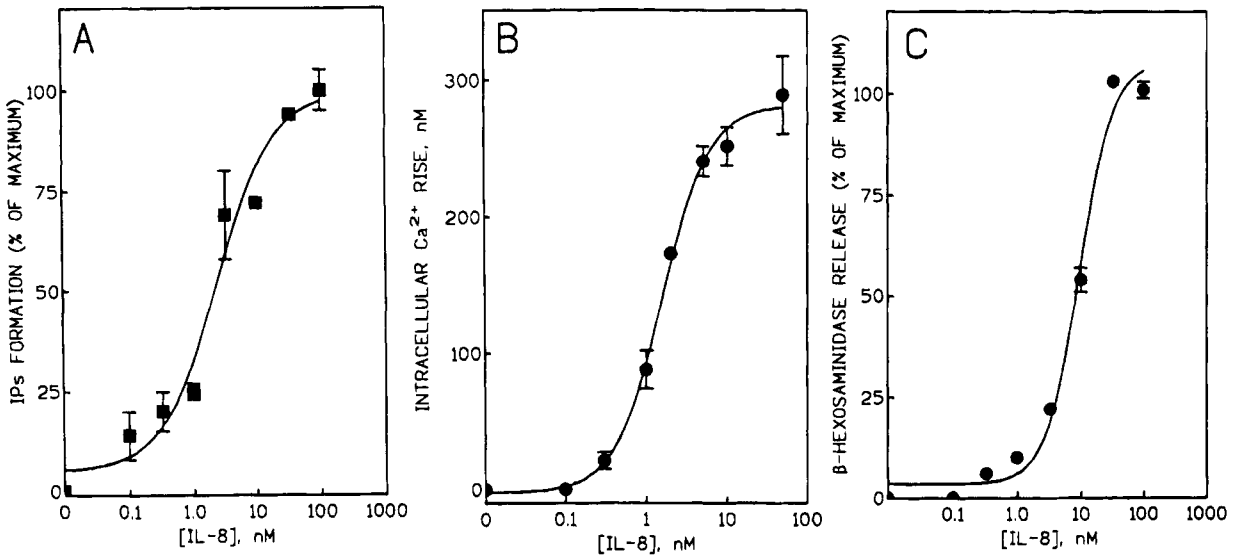

FIGURE 1: Functional characteristics of the ET-IL-8RA expressed in RBL-2H3 cells. For the generation of inositol phosphates ([^{3}H]IPs) (A) and secretion of β-hexosaminidase (C), cells were cultured overnight in the presence of [^{3}H]inositol (1 μCi/mL). Cells were preincubated (10 min, 37 °C) with a HEPES-buffered saline solution containing 10 mM LiCl in a total volume of 200 μL and stimulated with different concentrations of IL-8 for 10 min. Supernatant (10 μL) was removed for the secretion assay, and the rest of the sample was used to determine the release of [^{3}H]IPs. Data are presented as the percentage of maximum stimulation over basal which were 349 ± 4 cpm for PI hydrolysis and 57 ± 2 cpm for secretion. The experiment was repeated four times with similar results. (B) For calcium mobilization cells (2.5×10^6) were loaded with indo-1 and stimulated with different concentrations of IL-8. Data are representative of one experiment performed in duplicate, which was repeated twice with similar results.

respectively. Intracellular calcium concentrations were calculated using the formula: $[Ca^{2+}] = K_d[(F - F_{min})/F_{max} - F]$ (Tomhave et al., 1994).

Iodination of ET-IL-8RA. RBL-2H3 cells (5×10^6) expressing ET-IL-8RA were resuspended in 200 μL of phosphate-buffered saline (PBS) in an iodogen-coated glass tube (100 μg/tube). Carrier-free $Na^{125}I$ (200 μCi) was added to the tube, and the mixture was incubated for 10 min at 4 °C. Cells were removed, washed, lysed, immunoprecipitated with 12CA5 antibody, and analyzed by SDS−polyacrylamide gel and visualized by autoradiography (Ali et al., 1993).

Phosphorylation of ET-IL-8RA. Phosphorylation of ET-IL-8RA was performed as described previously (Ali et al., 1993, 1994). RBL-2H3 cells (2.5×10^6) expressing ET-IL-8RA were subcultured overnight in 60 mm tissue culture dishes. The following day the cells were rinsed twice with 5 mL of phosphate-free Dulbecco's modified Eagle's medium and incubated in the same medium supplemented with [^{32}P]orthophosphate (150 μCi/dish) for 90 min in order to metabolically label the intracellular ATP pool. Then labeled cells were stimulated with the agonist, IL-8 (100 nM), or vehicle for 5−7 min at 37 °C. The reactions were stopped by placing the cells on ice. The cells were washed twice with ice-cold PBS and lysed with cold detergent-containing buffer (1 mL/dish) supplemented with 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 10 μg/mL leupeptin, 1 μg/mL pepstatin, 100 μg/mL 1-chloro-3-(tosylamido)-4-phenyl-2-butanone, 50 μg/mL 1-chloro-3-(tosylamido)-7-amino-2-heptanone, and 10 μg/mL phenylmethanesulfonyl fluoride. The phosphorylated receptors were immunoprecipitated with the 12CA5 antibody, analyzed by SDS electrophoresis, and visualized by autoradiography.

GTPase Activity and GTPγS Binding. Cells were treated with either IL-8 (100 nM) or PMA (100 nM) for 7 min, and membranes were prepared as previously described (Ali et al., 1993, 1994; Tomhave et al., 1994). GTPase activity or [^{35}S]GTPγS binding, using 10−20 μg of membrane preparations, was carried out as described previously (Ali et al., 1993, 1994; Tomhave et al., 1994).

Construction of ET-IL-8RA cDNA Mutants. The IL-8RA cDNA contained a unique *Nsi*I site which is located 95 nucleotides upstream of the sequence encoding the last amino acid of the predicted cytoplasmic tail. All the potential phosphorylation sites are found between the *Nsi*I site and the stop codon. To generate each receptor mutant, two overlapping nucleotide primers were synthesized. The first one in the 5′−3′ direction will contain the *Nsi*I and nucleotide-containing part of the IL-8RA tail replacing serine and threonine residues with alanine. Antisense oligonucleotides with a *Xba*I site were made to the C-terminal end of IL-8RA such that they overlap with one another. Each of the oligonucleotides was annealed; double-stranded DNA was prepared by filling in with the Klenow fragment of DNA polymerase and digested with *Nsi*I and *Xba*I. These individual fragments were then cloned into the epitope-tagged IL-8RA cDNA in the pBluescript vector digested with the same restriction enzymes. All receptor mutants were confirmed by complete sequencing of the altered regions. This DNA was then digested with *Cla*I and *Xba*I, and the fragments containing the mutated receptor were ligated into the pRK5 vector digested with the same restriction enzymes.

RESULTS

Expression and Characterization of ET-IL-8RA in RBL-2H3 Cells. ET-IL-8RA was stably expressed in RBL-2H3 cells, and their pharmacological properties were determined. Competition binding assays using [^{125}I]IL8 and Scatchard analysis (data not shown) revealed that the ET-IL-8RA expressed in RBL-2H3 cells bound IL-8 with a dissociation constant (K_d) of 2.3 ± 0.3 and B_{max} of 3.5 ± 1.1 pmol of receptor/mg of protein. The K_d for IL-8 binding in RBL-2H3 cells is similar to that of the native IL-8R in neutrophils (~1−2 nM) (Horuk, 1994), indicating that the epitope tag

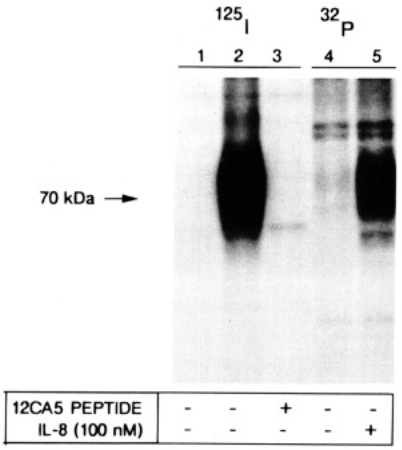

FIGURE 2: Immunoprecipitation of IL-8RA expressed in RBL-2H3 cells. ^{125}I- (lanes 1−3) and ^{32}P- (lanes 4 and 5) labeled RBL-2H3 cells (2.5 × 10^6/60 mm plate) expressing ET-IL-8RA (lanes 2−5) or untransfected RBL-2H3 cells (lane 1) were incubated for 7 min with (lane 5) or without (lanes 1−4) IL-8. Cells were lysed, immunoprecipitated with 12CA5 antibody in the presence (lane 3) and absence of the 12CA5 peptide, and analyzed by SDS−PAGE and autoradiography.

on the transfected receptor did not affect ligand binding. Untransfected RBL-2H3 cells showed no specific [^{125}I]IL8 binding, suggesting that RBL-2H3 cells do not express endogenous IL-8R. As shown in Figure 1, ET-IL-8RA, when exposed to IL-8, stimulated phosphoinositide hydrolysis (panel A), Ca^{2+} mobilization (panel B), and secretion (panel C) in a dose-dependent manner in RBL-2H3 cells. The EC_{50} values for phosphoinositide hydrolysis (3.3 ± 0.3 nM), Ca^{2+} mobilization (1.4 ± 0.2 nM), and exocytosis (9.4 ± 0.8 nM) were similar to those of the native receptor in neutrophils (0.2−1, 2.5, and 7 nM, respectively) (Smith et al., 1992).

To determine whether the ET-IL-8RA utilizes a pertussis toxin sensitive G-protein, RBL-2H3 cells expressing the receptors were treated with PTx (200 ng/mL) for 3 h (Ca^{2+} mobilization) or overnight (PI hydrolysis and secretion) and assayed for IL-8-induced responses. Treatment with PTx completely inhibited the ability of IL-8 to stimulate phosphoinositide hydrolysis, Ca^{2+} mobilization, and secretion (data not shown).

Phosphorylation and Immunoprecipitation of ET-IL-8RA in RBL-2H3 Cells. The 12CA5 monoclonal antibody to the nine amino acid peptide tag had been shown to specifically immunoprecipitate epitope-tagged fMLP, C5a, and PAF receptors in RBL-2H3 cells (Ali et al., 1993, 1994). The ability of this antibody to immunoprecipitate the ET-IL-8RA was determined. RBL-2H3 cells expressing the receptors were incubated with either ^{125}I (to label the three tyrosine residues which comprise the epitope tag) or ^{32}P (to metabolically label the cellular pool of ATP) and treated with IL-8 (100 nM). As shown in Figure 2, the 12CA5 antibody, in cells transfected with ET-IL-8RA cDNA, immunoprecipitated a broad iodinated band at ~65−70 kDa (lane 2), which corresponds to the reported molecular mass of the glycosylated form of the IL-8RA (Horuk, 1994). The ~65−70 kDa band did not appear when the immunoprecipitation was performed in the presence of excess 12CA5 peptide (lane 3) or in untransfected RBL-2H3 cells (lane 1). IL-8 stimulation of the ^{32}P-labeled cells also resulted in the phosphorylation of an ~65−70 kDa phosphoprotein (lane 5), not seen in unstimulated cells (lane 4).

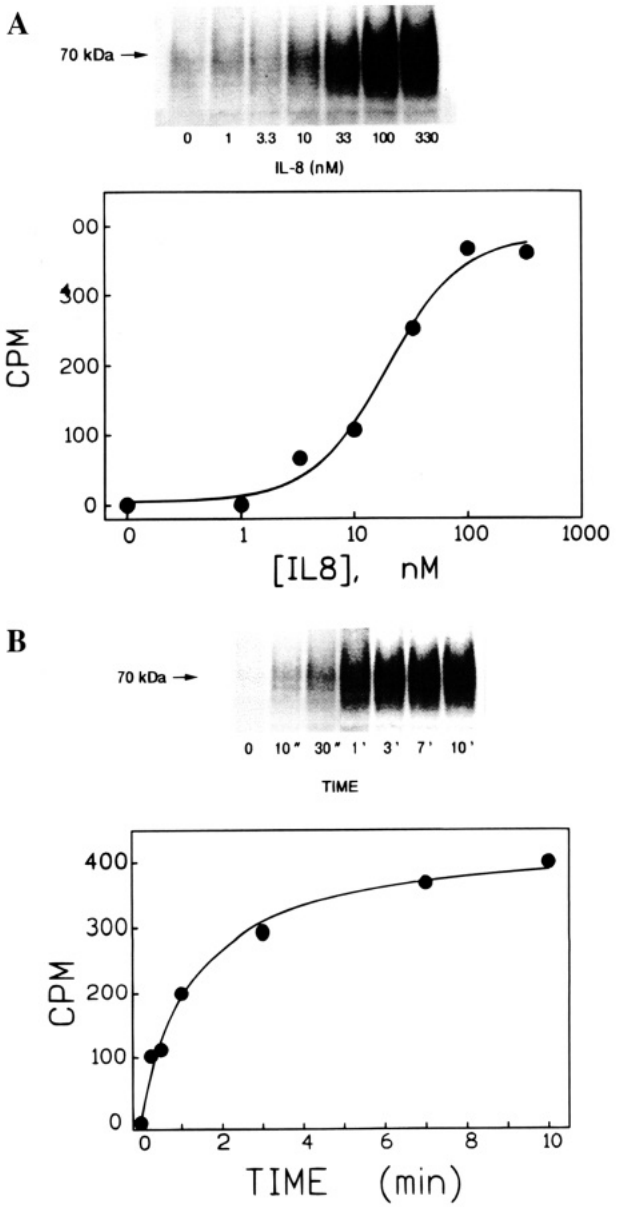

FIGURE 3: Dose- and time-dependent phosphorylation of ET-IL-8RA in RBL-2H3 cells. ^{32}P-Labeled RBL-2H3 cells expressing the ET-IL-8RA were stimulated with either different concentrations of IL-8 for 7 min (A, top) or 100 nM IL-8 for different periods of time (B, bottom). Reactions were stopped by placing samples on ice. Cells were washed with ice-cold PBS, lysed with detergent-containing buffer, and immunoprecipitated with 12CA5 antibody. Samples were separated on 10% SDS−polyacrylamide gel and visualized by autoradiography (upper panels of A and B). The amount of radioactivity per lane was determined by counting excised phosphorylated bands (lower panels of A and B). The results are from a representative experiment that was repeated three times with similar results.

Dose and Time Dependency of IL-8-Induced ET-IL-8RA Phosphorylation in RBL-2H3 Cells. The dose−response of IL-8-induced phosphorylation of ET-IL-8RA was studied by stimulating ^{32}P-labeled RBL-2H3 cells expressing ET-IL-8RA with different concentrations of IL-8. IL-8 induced phosphorylation of ET-IL-8RA in a dose-dependent manner (Figure 3A, upper panel). As determined by Cerenkov counting of the excised phosphorylated band, IL-8 induced phosphorylation of the receptor with an EC_{50} of ~20 nM (Figure 3A, lower panel). Maximum phosphorylation was achieved at ~100 nM. The time−course of phosphorylation was determined using an EC_{100} dose of IL-8 (100 nM). As

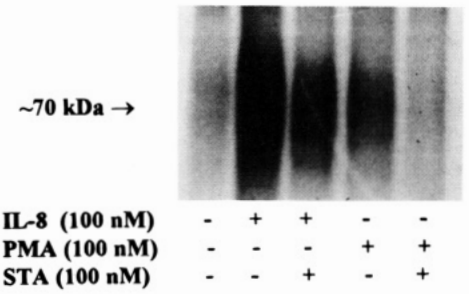

FIGURE 4: Effect of staurosporine on IL-8 and PMA-induced phosphorylation of ET-IL-8RA in RBL-2H3 cells. ^{32}P-Labeled RBL-2H3 cells expressing ET-IL-8RA were incubated with and without staurosporine for 5 min and then stimulated with either IL-8 (lanes 2 and 3), PMA (lanes 4 and 5), or vehicle (lane 1) (lanes from left to right). Cells were lysed, immunoprecipitated with 12CA5 antibody, electrophoresed into 10% SDS−polyacrylamide gel, and autoradiographed. Three other experiments yielded similar results.

shown in Figure 3B (upper panel), phosphorylation of ET-IL-8RA was detectable by 30 s after stimulation, was half-maximal at ~1 min, and was maximal at ~3 min (Figure 3B, lower panel).

Phorbol Ester-Induced Phosphorylation of ET-IL-8RA and Effect of Staurosporine. ^{32}P-Labeled RBL-2H3 cells expressing ET-IL-8RA were stimulated with PMA (100 nM), and phosphorylation of the receptor was noted (Figure 4, lane 4), although to a lesser extent than with IL-8 (Figure 4, lane 2). Phosphorylation induced by PMA was totally inhibited by pretreatment of the cells with the PKC-inhibitor staurosporine (100 nM) (Figure 4, lane 5) whereas IL-8-induced phosphorylation was only partially blocked by staurosporine (Figure 4, lane 3). Stimulation of ET-IL-8RA containing RBL-2H3 cells with up to 10 mM B_{t2}cAMP did not result in phosphorylation of the receptor (data not shown).

Desensitization of ET-IL-8RA-Mediated Functions in RBL-2H3 Cells. The effect of phosphorylation on IL-8-stimulated GTPase activity in membranes from cells pretreated with either IL-8 (100 nM) or PMA (100 nM) was measured. Both IL-8 and PMA pretreatment decreased the ability of the ET-IL-8RA to promote agonist-stimulated GTP hydrolysis with IL-8 causing greater inhibition than PMA (Figure 5A).

Ca^{2+} mobilization was also measured to determine whether ET-IL-8RA phosphorylation affected this process. As shown in Figure 5B, stimulation of indo-1-loaded RBL-2H3 cells with an EC_{100} dose of IL-8 (10 nM) resulted in a rapid and transient increase in intracellular Ca^{2+} mobilization. Response to a second dose of IL-8 was markedly attenuated.

Expression and Characterization of ET-IL-8RA Mutants. The C-terminus of the IL-8RA possesses three clusters of serine and threonine residues. To assess their roles in receptor phosphorylation, three mutants (M1, M2, M3) were constructed by replacing the serine and threonine residues of each one of the clusters with alanine (Table 1). RBL-2H3 cells were transfected, and expression of cell surface receptors was monitored by FACS analysis and characterized as for the ET-IL-8RA. All three mutants displayed pharmacological properties similar to those of the wild-type ET-IL-8RA in RBL-2H3 cells (K_d ~ 2−2.8 nM and B_{max} ~ 3−3.5 pmol/mg of protein) (data not shown). As for the wild type, all three mutated receptors mediated PI hydrolysis (Figure 6A), Ca^{2+} mobilization (Figure 6B), and secretion (Figure 6C) in responses to IL-8 stimulation. M2, however,

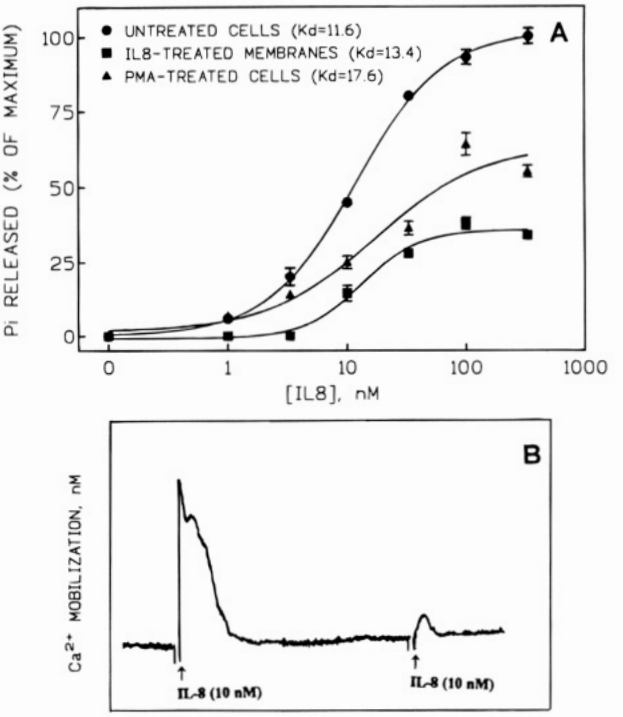

FIGURE 5: Homologous and heterologous desensitization of ET-IL-8RA-mediated GTPase activity and Ca^{2+} mobilization. (A) RBL-2H3 cells expressing the ET-IL-8RA were pretreated with either IL-8 (100 nM), PMA (100 nM), or vehicle. Membranes were prepared and assayed for GTPase activity at different concentrations of IL-8. The data are presented as the 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. (B) RBL-2H3 cells expressing ET-IL-8RA (2.5×10^6 cells) were loaded with indo-1 and stimulated with IL-8 (10 nM). Cells were rechallenged 5 min later with the same concentration of IL-8. This experiment has been repeated four times with similar results.

Table 1: Amino Acid Sequence of the Carboxy-Terminal Portion of the IL-8RA and the Serine and Threonine Residues Replaced with Alanine in Each Mutant

C-tail IL8RA	QNFRHGFLKILAMHGLVSKEFLARHRVTSYTSSSVNVSSNL
M1	QNFRHGFLKILAMHGLVSKEFLARHRVTSYTSSSVNVAANL
M2	QNFRHGFLKILAMHGLVSKEFLARHRVTSYAAAAVNVSSNL
M3	QNFRHGFLKILAMHGLVSKEFLARHRVAAYTSSSVNVSSNL

showed an ~4 and ~12-fold increase in IL-8-mediated PI hydrolysis and secretion, respectively, relative to wild-type ET-IL-8RA or the other mutants (Figure 6A,C).

IL-8 and Phorbol Ester-Induced Phosphorylation of ET-IL-8RA Mutants. The ET-IL-8RA mutants were analyzed for their ability to undergo IL-8- and PMA-mediated phosphorylation. RBL-2H3 cells expressing the mutant receptors were ^{32}P labeled and subjected to stimulation by either IL-8 (100 nM) or PMA (100 nM). The autoradiogram in Figure 7 shows that IL-8 induced phosphorylation of all three receptor mutants (upper panel). M2 and M3 were phosphorylated to a lesser extent than M1 and ET-IL-8RA (lower panel). However, PMA phosphorylated M1, but not M2 or M3, relative to control ET-IL-8RA (upper and lower panels). As for ET-IL-8RA, staurosporine pretreatment (100 nM) totally blocked PMA-induced phosphorylation but partially inhibited IL-8-induced phosphorylation of the mutant M1.

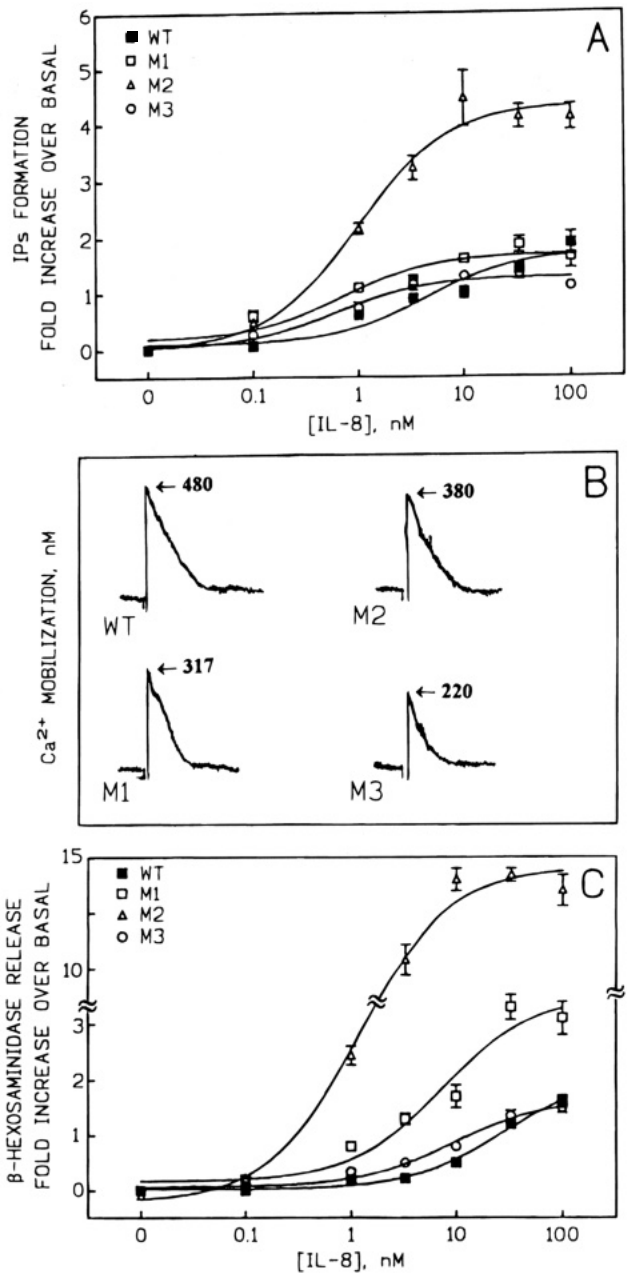

FIGURE 6: Functional characteristics of ET-IL-8RA phosphorylation-deficient mutants. RBL-2H3 cells expressing M1, M2, and M3 mutants and the wild-type ET-IL-8RA were cultured overnight in the presence of [^{3}H]inositol (1 μCi/mL). Phosphoinositide hydrolysis (A) and β-hexosaminidase release (C) were determined as described in the legend to Figure 1. Data are represented as x-fold stimulation over basal. Results shown are representative of one of six experiments performed in triplicate. (B) Calcium mobilization was measured in RBL-2H3 cells (2.5×10^6 cells) expressing mutants of wild-type ET-IL-8RA, which were loaded with indo-1 and stimulated with IL-8 (10 nM). Traces are representative of two independent experiments.

Staurosporine had no effect on IL-8-mediated phosphorylation of either M2 or M3 mutants (data not shown).

Desensitization of ET-IL-8RA Mutants. To assess the effect of the site-specific mutations on the desensitization of IL-8R-mediated cellular responses, we analyzed the ability of the mutated receptors to stimulate [^{35}S]GTPγS binding to G protein in membranes from cells pretreated with either IL-8 (100 nM) or PMA (100 nM). IL-8 pretreatment decreased by 50−75% the ability of wild-type and mutants of ET-IL-8RA to stimulate [^{35}S]GTPγS binding, compared to untreated cells (Figure 8A). PMA pretreatment caused

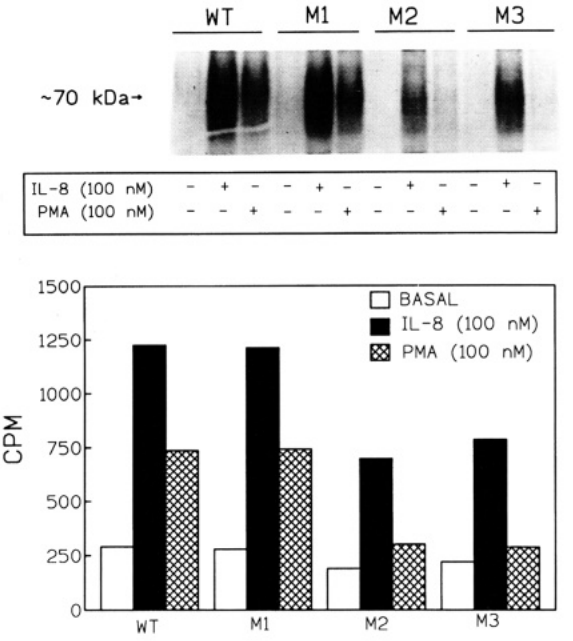

FIGURE 7: Homologous and heterologous phosphorylation of ET-IL-8RA mutants in RBL-2H3 cells. ^{32}P-Labeled RBL-2H3 cells expressing ET-IL-8RA mutants were stimulated with either IL-8 (100 nM), PMA (100 nM), or vehicle. Cells were lysed, immunoprecipitated with 12CA5 antibody, electrophoresed into 10% SDS−polyacrylamide gel, and autoradiographed. The results are from a representative experiment that was repeated four times.

an ~40% and ~50% desensitization of ET-IL-8RA and M1-stimulated [^{35}S]GTPγS binding, respectively (Figure 8A). M2 and M3 were resistant to PMA-mediated desensitization. Ca^{2+} mobilization in response to a second dose of IL-8 (10 nM) was desensitized by prestimulation of the cells with a first dose of IL-8 (Figure 8B). While M1 was desensitized to an extent similar to that of the wild-type receptor (~60% and ~68%, respectively), M2 and M3 were more resistant to desensitization (~32% and 49%, respectively).

DISCUSSION

IL-8 is thought to play a central role in the inflammatory responses of phagocytic cells, and given the broad array of biological events initiated by this chemokine, its receptor is likely to be tightly regulated. To define the molecular mechanism involved in the regulation of the type A IL-8 receptor, it was modified to facilitate immunoprecipitation and expressed in a biologically relevant mast cell line, RBL-2H3. As is the case for the fMLP, C5a, and PAF chemoattractant receptors, the IL-8RA expressed in RBL-2H3 stimulated phosphoinositide hydrolysis, Ca^{2+} mobilization, and exocytosis in a dose-dependent fashion (Figure 1), with affinities which mirror those obtained for the native IL-8 receptors in neutrophils (Horuk, 1994). In addition, inhibition of ET-IL-8RA-induced responses by preincubation of the cells with pertussis toxin suggests that these receptors couple to PTx-sensitive G proteins, presumably G_i, as does the native IL-8RA in neutrophils.

The molecular events underlying desensitization of receptors are poorly understood, but for the G protein-coupled receptors it appears that covalent modification in the cytoplasmic domains by protein phosphorylation plays an important role (Hausdorff et al., 1990; Hosey, 1992). Two types of such phosphorylation have been described: agonist-induced (homologous) phosphorylation and agonist-inde-

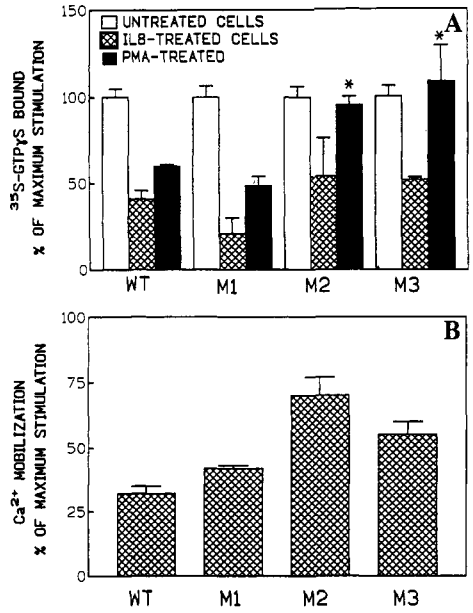

FIGURE 8: Homologous and heterologous desensitization of ET-IL-8RA mutants. (A) RBL-2H3 cells expressing M1, M2, and M3 or wild-type ET-IL-8RA were incubated with either IL-8 (100 nM) or PMA (100 nM) for 7 min. Membranes were prepared and assayed for IL-8- (100 nM) stimulated [^{35}S]GTPγS binding. The data shown are the means of triplicates from a representative experiment which was repeated three times. The values are represented as the percentage of maximum stimulation, which was defined as the maximal amount over basal of [^{35}S]GTPγS bound to control membranes (untreated cells) after 10 min of reaction. Basal activities were ~0.2−0.3 pmol of [^{35}S]GTPγS bound/mg of protein. Maximum stimulations were 0.25 ± 0.008 (WT), 0.19 ± 0.010 (M1), 0.2 ± 0.011 (M2), and 0.17 ± 0.012 (M3) pmol of [^{35}S]GTPγS bound/mg of protein. *, $P < 0.05$ compared to control. (B) RBL-2H3 cells (2 × 10^6 cells/assay) expressing M1, M2, and M3 mutants and the wild-type ET-IL-8RA were loaded with indo-1 and stimulated with IL-8 (10 nM). Cells were rechallenged 5 min later with the same concentration of IL-8. Data are represented as the percentage of maximum stimulation of the response obtained with the first dose of agonist. This experiment has been repeated twice with similar results.

pendent phosphorylation (heterologous) (Hausdorff, 1990). Homologous phosphorylation is mediated by a family of G protein-coupled receptor-specific kinases (GRK) that specifically phosphorylate the activated form of the receptors, resulting in homologous desensitization (Hausdorff, 1990; Palczewski & Benovic, 1991). Heterologous phosphorylation is mediated by second messenger-activated protein kinases such as protein kinase A (PKA) and protein kinase C (PKC) (Ali et al., 1994; Hausdorff, 1990; Strader et al., 1989; O'Dowd et al., 1988; Ligget & Lefkowitz, 1994). The IL-8RA expressed in RBL-2H3 cells undergoes phosphorylation following exposure to IL-8 or PMA but not B_{t2}cAMP (Figure 4). Measurement of [^{35}S]GTPγS binding and GTPase activity in membranes as well as Ca^{2+} mobilization in whole cells (Figures 5 and 8) indicated that phosphorylation of IL-8RA by either IL-8 or PMA was correlated with desensitization, suggesting that phosphorylation promotes receptor desensitization (Hausdorff, 1990; Hosey, 1992). Together with the ability of staurosporine to partially and completely inhibit IL-8- and PMA-mediated phosphorylation, respectively, these results also indicated that the IL-8RA is similar to the C5aR and PAFR but not the fMLPR in that it is regulated by an agonist-independent process mediated by PKC in addition to an agonist-dependent process presumably mediated by a GRK. The fMLPR is resistant to PKC-mediated phosphorylation (Ali et al., 1993, 1994). In contrast to the PAFR (Ali et al., 1994), B_{t2}cAMP did not result in phosphorylation of ET-IL-8RA, indicating that this receptor along with fMLPR and C5aR is not regulated by a PKA-mediated process. These different phosphorylation profiles among chemoattractant receptors suggest that while mediating similar biological responses, *viz.* chemotaxis, exocytosis, and respiratory burst, their functions are regulated by shared and unique mechanisms.

Like many members of the G protein-coupled receptor family such as β-adrenergic receptor and rhodopsin, IL-8R possesses a serine/threonine-rich cytoplasmic tail (Table 1). This region has been shown to contain specific sites for phosphorylation-mediated receptor regulation (O'Dowd et al., 1988; Ligget & Lefkowitz, 1994; Hargrave & Hamm, 1994). To determine whether that is also the case for IL-8RA, alanine-scanning mutagenesis was used to substitute specific serine and threonine of that region (Prossnitz et al., 1993; Moro et al., 1993; Hereld et al., 1994; Hunyady et al., 1994). PKC primarily phosphorylates serine and threonine flanked by basic residues, while G protein-coupled receptor kinase (GRK) phosphorylates serines and threonines adjacent to acidic residues (Oronato et al., 1991). However, on the basis of the sequence analysis, the seven serines and two threonines found in the IL-8RA cytoplasmic tail are arranged in three clusters (Table 1), making it difficult to delineate sequence motifs for either PKC or GRK. To overcome this problem, each one of the three clusters was mutated separately. The mutant receptors expressed in RBL-2H3 cells bound ligand with properties similar to those of the native IL-8RA (K_d ~ 2−2.8 nM). However, M3 was somewhat less efficient in stimulating peak intracellular Ca^{2+} mobilization (Figure 6B) relative to M1, M2, or ET-IL-8RA. The reason for that is not clear as M3-mediated agonist stimulation of [^{35}S]GTPγS binding, PI hydrolysis, and exocytosis was similar to that of wild-type ET-IL-8RA (Figures 6A,C and 8A). Interestingly, both M2 and M3 were resistant to PMA-mediated phosphorylation and desensitization, whereas M1, like the wild-type IL-8RA, was phosphorylated and desensitized by both IL-8 and PMA (Figures 7 and 8). Taken together, these results indicate that PKC-mediated phosphorylation and desensitization of the ET-IL-8RA may occur at the serines and threonines comprising clusters 2 and 3 of the receptor's carboxy terminus. Why modification of either M2 or M3 resulted in a lack of PKC-induced phosphorylation and desensitization is not understood. The four amino acid residues (three serines, one threonine) mutated in M2 are separated from the two residues (one serine, one threonine) which comprise M3 by a single tyrosine (Table 1). Therefore, it is possible that mutation of the residues of either cluster results in a conformational change which makes the other one inaccessible to the kinase. IL-8-induced phosphorylation of both M2 and M3 was also decreased by ~50% relative to ET-IL-8RA or M1. This decrease may be explained by the lack of the PKC-mediated phosphorylation of the receptor or the fact that the residues modified in M2 or M3 may also contain GRK substrates. Supporting the former hypothesis is the fact that staurosporine which partially inhibited IL-8-induced phosphorylation of ET-IL8RA and M1 had no effect on M2 and M3 phosphorylation.

Phosphorylation has been shown to mediate receptor desensitization, internalization, and/or downregulation (Haus-

dorff, 1990; Hosey, 1992). Removal of specific phosphorylation sites at the cytoplasmic domains of G protein-coupled receptors impaired the ability of the receptor to undergo phosphorylation-mediated regulation and prolong, but not enhance, receptor-mediated cellular responses (Hausdorff, 1990). An unexpected finding of the studies presented here was that the M2 mutations resulted in a 4−12-fold enhancement of receptor-mediated functions as well as resistance to desensitization (Figures 6A,B and 8). The precise mechanism of enhancement in M2 receptor function is not clear but may be explained in several ways. First, IL-8RA like other members of this family of receptors transduces cellular signals by interacting and activating G proteins. It is possible that the modification of cluster 2 could have caused the receptor to increase the turnover of the associated G protein, resulting in an enhancement of receptor-mediated responses. Against this hypothesis is the fact that the rate of M2-mediated [^{35}S]GTPγS binding to membranes is similar to that of the wild-type ET-IL-8RA (0.2 ± 0.011 and 0.25 ± 0.008 pmol of [^{35}S]GTPγS bound/mg of protein, respectively) (Figure 8A). Second, IL-8RA reconstituted in COS-7 cells coupled to either Gi or Gq ($G\alpha_{14}$ and $G\alpha_{16}$) to stimulate PI hydrolysis by activating PLCβ_2 (Gi) or PLCβ_1 ($G\alpha_{14}$ and $G\alpha_{16}$), respectively (Wu et al., 1993). As both Gi and Gq are present in RBL-2H3 cells (Hirasawa et al., 1995), it is possible that the alanine substitution resulted in a switch of the G protein coupled to the IL-8RA from Gi to the pertussis toxin-resistant Gq. This is unlikely since PTx pretreatment of M2 transfected RBL-2H3 completely abolished its responses (data not shown). Third, IL-8RA like many members of the G protein-coupled receptor family undergoes basal phosphorylation in the absence of agonist stimulation, thereby controlling its ability to be fully activated (Ali et al., 1994; Richardson et al., 1993). Therefore, one (or more) of the residues mutated may be a basal phosphorylation site, which, upon removal, increases the ability of the receptor to stimulate cellular responses. Indeed, basal phosphorylation was slightly decreased for both M2 and M3 (Figure 7 and three other experiments not shown). However, no enhancement was observed for M3-mediated cellular responses. Fourth, IL-8 has been shown to be a weaker mediator of the respiratory burst and secretion in neutrophils compared to the other peptide chemoattractants, fMLP and C5a (Baggiolini et al., 1992). It has recently been shown that a proline-rich motif of the cytoplasmic domain of the β-adrenergic receptors may be responsible for attenuating agonist-promoted adenylyl cyclase activity (Green & Ligget, 1994). Removal of this motif resulted in an enhancement in the ability of the receptor to mediate cAMP accumulation (Green & Ligget, 1994). Likewise, the M2 motif may play a constraining role in mediating PI hydrolysis and secretion. Substitution of these residues with alanine may result in the removal of this constraint motif and, therefore, cause an increase in IL-8RA-mediated PI hydrolysis and exocytosis. Overall, a complete understanding of the enhancement mechanism of the M2-mediated responses will require additional specific receptor mutants.

The studies herein demonstrate that the IL-8RA undergoes agonist-dependent and agonist-independent phosphorylation presumably by a receptor-specific kinase (GRK) and protein kinase C, respectively. Both mechanisms of phosphorylation appear to result in desensitization of the IL-8RA. In addition, two segments of the carboxy terminus of the IL-8RA have been identified to be necessary for PKC-mediated phosphorylation and desensitization. Interestingly, substitution of the four serines and single threonine, which form cluster 2 of the carboxy terminus, resulted in a dramatic increase in IL-8RA-induced phosphoinositide hydrolysis and exocytosis but not Ca^{2+} mobilization, suggesting that this cluster regulates the extent of receptor activation as well as desensitization. The methodology and data described herein should allow a more rapid identification of the molecular mechanisms regulating the type A IL-8 receptor.

ACKNOWLEDGMENT

We are grateful to Drs. B. Moser and M. Baggiolini for the gift of cDNA encoding the human IL-8 receptor A.

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BI9516135