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Activation of Protein Kinase C Enhances the Phosphorylation of the Type B Interleukin-8 Receptor and Stimulates Its Degradation in Non-hematopoietic Cells*

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We have previously characterized the stably transfected, clonally selected human placental cell line, 3ASubE P-3, which overexpresses the type B interleukin-8 receptor (IL-8RB) and responds to the chemokine melanoma growth stimulatory activity (MGSA) with enhanced phosphorylation of this receptor. In work described here, we demonstrate that the MGSA-enhanced phosphorylation of this receptor is mediated via a process involving pertussis toxin-sensitive G proteins. Furthermore, treatment of the 3ASubE P-3 cells with either 12-*O*-tetradecanoylphorbol 13-acetate (TPA) or 1,2-dioctanoyl-*sn*-glycerol (diC_8), two different activators of protein kinase C (PKC), results in a concentration-dependent increase in the phosphorylation of the IL-8RB. Inhibition of PKC, by treatment with staurosporin (50 nM for 2 h), or down-regulation of PKC, by prolonged treatment with TPA (400 nM for 40 h) suppresses the TPA-enhanced receptor phosphorylation, but has no effect on the MGSA-enhanced receptor phosphorylation. These data suggest that the isoforms of PKC that are sensitive to these manipulations may not play a role in mediating the MGSA-enhanced phosphorylation of the IL-8RB. TPA treatment also results in a time-dependent decrease in ^{125}I-MGSA binding to the 3ASubE P-3 cells. A 30-min treatment with 400 nM TPA results in approximately a 50% decrease in binding, whereas a 2-h treatment essentially eliminates specific binding of ^{125}I-MGSA to these cells. The TPA-induced decrease in ^{125}I-MGSA binding is accompanied by enhanced degradation of the IL-8RB, as indicated by Western blot analysis and pulse-chase experiments, suggesting a potential role for PKC as a negative regulator of the IL-8RB. MGSA treatment (50 nM for 2 h) also stimulates receptor degradation in the 3ASubE P-3 cells, indicating that this receptor is down-regulated in response to prolonged exposure to its ligand. In similar studies conducted on the promonocytic cell line, U937, MGSA treatment of the U937 cells resulted in receptor phosphorylation, whereas PKC activation failed to significantly modulate the phosphorylation state of the IL-8RB. Treatment of the U937 cells with MGSA, TPA, or diC_8 resulted in a loss of receptor protein present in these cell types. These data imply that MGSA signaling through the IL-8RB is similar in both the non-hematopoietic and hematopoietic cell types, whereas activation of PKC by TPA or diC_8 elicits different responses in these two distinct cell types.

Chemokines are a group of inflammatory proteins which share several conserved amino acid residues (1). They are subdivided into two families: the C-*X*-C (or α) family, which possess an intervening amino acid between the first two conserved cysteine residues, and the C-C (or β) family, in which the first two conserved cysteine residues are adjacent to one another. Members of the C-*X*-C family are generally chemotactic for neutrophils and to a lesser extent lymphocytes, basophils, and eosinophils. The C-*X*-C chemokines include melanoma growth stimulatory activity (MGSA)[1] (also referred to as GRO), interleukin-8 (IL-8), neutrophil activating protein-2, γ-interferon inducible protein-10, and platelet factor-4. The C-C family members, which are generally chemotactic for monocytes, include macrophage inflammatory protein-1α, macrophage inflammatory protein-1β, monocyte chemotactic protein-1, and RANTES (1).

Various chemokine receptors have been identified and their corresponding cDNAs have been cloned. Two distinct human IL-8 receptors (IL-8R), type A and B (also referred to as type or class I and II, respectively), have been cloned from neutrophil and HL60 cDNA libraries, respectively (2, 3). The names for these receptors has been rather misleading since they bind multiple members of the C-*X*-C family. The type A IL-8R (IL-8RA) binds IL-8 with high affinity and MGSA with low affinity, whereas the type B IL-8R (IL-8RB) binds both MGSA and IL-8 with high affinity and neutrophil activating protein-2 with low affinity (2, 4, 5). Recently, a mouse homolog of the human IL-8 receptors has been cloned (6–8). A C-C chemokine receptor, which binds multiple members of the C-C chemokine family and two monocyte chemotactic protein-1 receptors have also been cloned (9, 10). These various chemokine receptors are all members of the seven transmembrane domain, G protein-coupled receptor family. More recently, the cDNA for the Duffy blood group antigen, which binds IL-8, MGSA, monocyte chemotactic protein-1, and RANTES, has also been isolated (11–13). This is the first reported receptor that binds members of both the C-*X*-C and C-C chemokine families. cDNA analysis of the Duffy antigen, also referred to as the erythrocyte chemokine receptor, predicts that it has seven transmembrane domains but shows very little homology to the other chemokine receptors. Furthermore, this receptor does not appear to couple to G proteins and has been speculated to serve as a "ligand

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[1] The abbreviations used are: MGSA, melanoma growth stimulatory activity; EtOH, ethanol; diC_8, 1,2-dioctanoyl-*sn*-glycerol (8:0); IL-8, interleukin-8; IL-8R, interleukin-8 receptor; PKC, protein kinase C; PVDF, polyvinylidene difluoride; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; DMEM, Dulbecco's modified Eagle's medium.

sink" to regulate circulating chemokine levels (13).

MGSA and IL-8 elicit a variety of common effects on neutrophils (1, 14–17). Presumably, the effects of IL-8 and MGSA on neutrophils are mediated through the type A and B receptors. These two receptors share a 77% amino acid conservation with the amino and carboxyl termini being the most divergent regions between these two receptors (3). Receptor chimera studies have demonstrated that the extracellular amino-terminal domain plays a significant role in the ligand binding specificity of these receptors (18). Specific residues in the third extracellular loop of the IL-8Rs also play an important role in ligand binding (19). These properties distinguish the IL-8Rs from the monoamine-binding G protein-coupled receptors, such as the β-adrenergic receptors, since ligand binding specificity is typically dictated by the transmembrane domains for the other G protein-coupled receptors (20).

MGSA and IL-8, along with their effects on neutrophils, are capable of eliciting both proliferative and non-proliferative responses in a variety of non-hematopoietic cell types (21–28). The receptors through which MGSA and IL-8 transduce their signals in non-hematopoietic cells have not been characterized. Recently, however, the IL-8RA and -B mRNAs have been detected by reverse transcriptase polymerse chain reaction in a variety of MGSA- and IL-8-responsive non-hematopoietic cell types, including primary human keratinocytes (27, 29), melanocyte and melanoma cell lines (29, 30). The expression of these receptors suggests that they may mediate some of the effect(s) of MGSA and IL-8 on non-hematopoietic cells. To assess the mechanism of signal transduction through the IL-8RB, and determine which factors may modulate this signaling in non-hematopoietic cell types, we have previously established a clonally selected, stably transfected human placental cell line, termed 3ASubE P-3, which overexpresses the IL-8RB (29). This receptor is basally phosphorylated in the 3ASubE P-3 cells and this phosphorylation is markedly enhanced upon treatment with MGSA (29). Thus the IL-8RB behaves similarly to several members of the G protein-coupled receptor family in that ligand binding results in receptor phosphorylation. Several members of this receptor family are also subject to modulation by the protein kinase C (PKC) and cAMP-dependent protein kinase A pathways (31). In this report, we have examined the effect of PKC activation on the phosphorylation state of the IL-8RB. We demonstrate that activation of PKC with either the tumor promoter 12-*O*-tetradecanoylphorbol 13-acetate (TPA) or the diacylglycerol analogue 1,2-dioctanoyl-*sn*-glycerol (diC_8) enhanced the phosphorylation of the IL-8RB on serine residues. PKC activation also decreased MGSA binding to the 3ASubE P-3 cells and this was accompanied by a decreased amount of receptor protein present in the cells. PKC does not appear to play a significant role in the MGSA-enhanced phosphorylation of the IL-8RB since neither inhibition nor down-regulation of PKC prevented the MGSA response in these cells. Studies conducted in the promonocytic cell line, U937, revealed that MGSA treatment had effects on receptor phosphorylation and degradation similar to those observed in the 3ASubE P-3 cells. Activation of PKC, however, did not significantly alter the receptor phosphorylation state in the U937 cells although it did stimulate a dramatic decrease in the receptor protein levels. These data demonstrate that receptor responses to ligand binding in hematopoietic cells is similar to that observed in non-hematopoietic cells yet there are differences in regulation in response to activation of PKC by TPA or diC_8 in these two cell types.

EXPERIMENTAL PROCEDURES

Cell Culture—The human placental cell line, 3ASubE, had previously been stably transfected with the pRC/CMV mammalian expression vector (Invitrogen) containing the IL-8RB cDNA. Stable transfectants, which were clonally selected, have previously been characterized and demonstrated to bind MGSA (29). One clonally selected stable transfectant termed 3ASubE P-3 was used in these studies. The parental 3ASubE cell line was maintained in 5% fetal bovine serum/MEM, whereas the 3ASubE P-3 clone was maintained in 5% fetal bovine serum, 400 μg/ml G418/MEM. U937 cells, a human promonocytic cell line (32), was routinely cultured in the presence of 10% fetal bovine serum/RPMI and differentiated with 1 μM retinoic acid for 5 days prior to experimentation. All cell types were incubated at 37 °C and 5% CO_2.

In Vivo Phosphorylation of the IL-8RB in 3ASubE P-3 and U937 Cells—In vivo phosphorylation studies were conducted as described previously (29). Briefly, confluent cultures of 3ASubE P-3 cells (35-mm plates) were washed and incubated in serum-free DMEM for 40 h at 37 °C. The medium was replaced with phosphate-free MEM and the cells were incubated for an additional 3 h at 37 °C. After phosphate starvation, cells were incubated in phosphate-free MEM containing 250 μCi/ml [^{32}P]orthophosphate (9000 Ci/mmol; Amersham) for 3 h at 37 °C. MGSA, TPA, or the appropriate vehicle was added directly to the [^{32}P]orthophosphate-containing medium after the 3-h incubation. Prior to use, MGSA, which is stored in 10% acetonitrile, 0.1% trifluoroacetic acid, was lyophilized in the presence of 25 μg of bovine serum albumin, resuspended in the appropriate media and used directly. An equal volume of 10% acetonitrile, 0.1% trifluoroacetic acid was treated in an identical manner and served as the acetonitrile vehicle control. The TPA stock was a 1000-fold concentrate in ethanol (EtOH). Inhibitors, when used, were added to the cells during the 3-h incubation with [^{32}P]orthophosphate. Staurosporin (50 nM), or vehicle (dimethyl formamide), was added to the cells for the final 2 h of the 3-h incubation with [^{32}P]orthophosphate, whereas pertussis toxin (1 μg/ml) or vehicle was added to the cells at the beginning of the 3-h incubation. Pertussis toxin was dialyzed against Tris-buffered saline to remove the phosphate present in its storage buffer prior to use in this assay.

Differentiated U937 cells were washed extensively with phosphate-free MEM then incubated in phosphate-free MEM containing 10% dialyzed fetal bovine serum (phosphate-free) and 1 mCi/ml [^{32}P]orthophosphate, at a density of 3×10^7 cells/ml, for 3 h at 37 °C. Cells were treated with MGSA, TPA, or the corresponding vehicle controls, for the indicated period of time.

Immunoprecipitation of the IL-8RB Receptor—Cells were lysed in either RIPA buffer containing 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 20 mM NaFl, 10 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml leupeptin, or they were lysed in a Triton X-100 buffer containing 1% Triton X-100, 10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA with the above protease inhibitors. Lysates were centrifuged for 15 min at 4 °C in an Eppendorf Microfuge, the supernatant was removed, and either trichloroacetic acid-precipitable counts were determined or protein concentrations were estimated (BCA, Pierce). Lysates containing either an equal number of trichloroacetic acid-precipitable counts (5×10^6 cpm) or an equal amount of protein (200 μg) were incubated with 5 μg of affinity-purified anti-amino-terminal peptide polyclonal antibodies which have previously been demonstrated to be specific for IL-8RB (29). The lysates were rocked at 4 °C for 2 h, followed by precipitation with 30 μl of 1:1 dilution protein A/G-agarose (Pierce) for 1 h at 4 °C. Thereafter, immunoprecipitates were washed three times with ice-cold RIPA buffer and pelleted. Pellets were denatured in 40 μl of 2 × Laemmli sample buffer containing 5% SDS and 10% (v/v) β-mercaptoethanol, electrophoresed through a 9% SDS-polyacrylamide gel, transferred to nitrocellulose, and exposed to autoradiographic film (Hyperfilm, Amersham).

^{125}I-MGSA Binding Assay—^{125}I-MGSA binding assays were conducted as described previously (29). MGSA (1 μg), generously provided by R+D Systems, was iodinated using the chloramine-T method. Routinely, a specific activity of 100 μCi/μg was obtained, assuming a 100% recovery. Cells were seeded at 2×10^5 cells/well in 24-well plates. Forty-eight hours later the binding assay was performed. Prior to the assay, cells were treated with 400 nM TPA, or ethanol as the vehicle control, for 0, 15, 30, 60, or 120 min at 37 °C. Cells were washed once with ice-cold binding buffer (0.1 mg/ml ovalbumin, 30 mM Hepes/DMEM). ^{125}I-MGSA (20–30,000 cpm/well) was added to the cells and they were rocked at 4 °C for 4 h. Cells were washed 3 times with ice-cold binding buffer, the bound ^{125}I-MGSA was eluted with 0.1 N NaOH, 1% SDS, and counted in a γ-counter (Beckman, Gamma 5500). Nonspecific binding was defined as the amount of ^{125}I-MGSA bound to the 3ASubE P-3 cells in the presence of 50 ng/ml unlabeled MGSA, which has previously been demonstrated to eliminate specific binding of ^{125}I-MGSA to these cells (29).

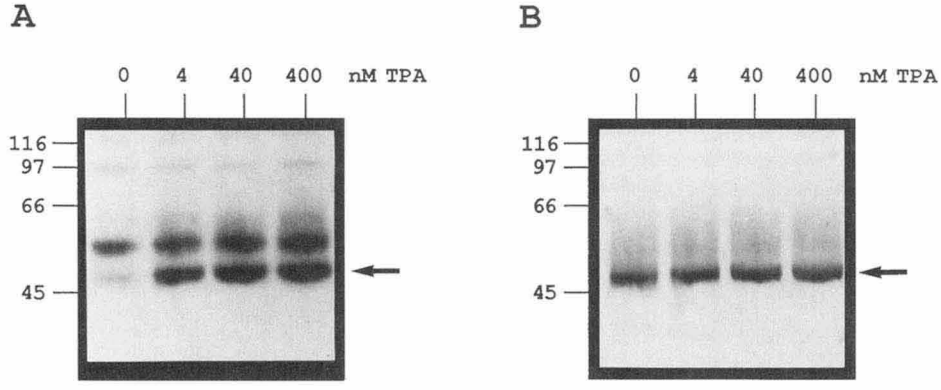


FIG. 1. **TPA treatment enhances the phosphorylation of the IL-8RB in 3ASubE P-3 cells.** 3ASubE P-3 cells were labeled with [^{32}P]orthophosphate as described under "Experimental Procedures" and treated with an ethanol vehicle (0 nM) or increasing concentrations of TPA (4–400 nM) for 10 min at 37 °C. Whole cell lysates were prepared and the IL-8RB was immunoprecipitated from an equal number of trichloroacetic acid-precipitable counts (5×10^6 cpm), using affinity purified anti-amino-terminal peptide polyclonal antibodies which recognize the IL-8RB. Immunoprecipitates were electrophoresed through a 9% SDS-polyacrylamide gel, transblotted onto a nitrocellulose membrane, and exposed to autoradiographic film for approximately 16 h (*A*). After development, the nitrocellulose membrane was analyzed by Western blot analysis, using the above mentioned anti-amino-terminal peptide antibodies. The Western blot was developed using an alkaline phosphatase detection system (*B*). Molecular size standards, in kilodaltons, are shown on the *left*. The position of the IL-8RB is marked by an *arrowhead*.

Western Blot Analysis of the IL-8RB—Whole cell lysates (equal protein per lane) or IL-8RB immunoprecipitates were electrophoresed through an 9% SDS-polyacrylamide gel, transblotted onto either a nitrocellulose or polyvinylidene difluoride (PVDF) membrane (Bio-Rad) and subjected to Western blot analysis using either 2 μg/ml of the rabbit anti-peptide antibodies described above or 0.25 μg/ml mouse monoclonal anti-IL-8RB (a kind gift from Richard Horuk and Steven Peiper). Goat anti-rabbit, or rabbit anti-mouse, IgG whole molecule conjugated with alkaline phosphatase (Sigma) was used at a 1:2000 dilution. Blots were developed typically for 10–15 min at pH 9.5 using bromochloroindoyl phosphate (Sigma) and nitro blue tetrazolium (Sigma). Where indicated, the Triton X-100 insoluble pellet, which remains after the microcentrifugation, was washed twice with the Triton X-100 buffer then solubilized in 2 × Laemmli sample buffer described above and subjected to Western blot analysis.

Phosphoamino Acid Analysis—Phosphoamino acid analysis was performed as described by Boyle *et al.* (33). Briefly, the IL-8RB was immunoprecipitated from [^{32}P]orthophosphate-labeled 3ASubE P-3 cells that had been treated with 40 nM TPA for 10 min. The immunoprecipitates were electrophoresed through a 9% SDS-polyacrylamide gel, transblotted onto a PVDF membrane and subjected to autoradiography. After autoradiography, the band corresponding to the IL-8RB was excised from the membrane, incubated in the presence of 6 M HCl for 1 h at 110 °C, lyophilized, then electrophoresed through a cellulose thin layer plate in two dimensions (first dimension was in pH 1.9 buffer, the second dimension was in pH 3.5 buffer). Phosphoserine, phosphothreonine, and phosphotyrosine standards were included in the loading buffer. After electrophoresis, the plates were stained with ninhydrin to detect the position of the phosphoamino acid standards then exposed to autoradiographic film.

[^{35}S]Methionine/Cysteine Labeling of the 3ASubE P-3 Cells—3ASubE P-3 and the parental 3ASubE cell lines were grown to confluence in 35-mm plates. Cells were rinsed twice with phosphate-buffered saline then incubated with cysteine/methionine free-MEM (Life Technologies, Inc.) for 1 h at 37 °C, the culture medium was then replaced with cysteine/methionine-free media containing 100 μCi/ml [^{35}S]cysteine/methionine (Tran^{35}S-label, >1000 Ci/mmol; ICN). Cells were labeled for 6 h at 37 °C. Cells were rinsed and fresh media containing unlabeled cysteine/methionine was added to the cells. Cells were either untreated, or treated with MGSA (50 nM) or TPA (400 nM) for 2 h. Triton X-100 extracts were prepared and the IL-8RB was immunoprecipitated from an equal number of trichloroacetic acid-precipitable counts (2×10^7 cpm), electrophoresed through a 9% SDS-polyacrylamide gel, dried, and exposed to autoradiographic film.

RESULTS

PKC Activation Enhances Phosphorylation of the IL-8RB—Since several members of the G protein-coupled receptor family are targets for phosphorylation by PKC, direct activation studies were conducted to determine if the IL-8RB is also phosphorylated in response to PKC activation. The 3ASubE P-3 cell line is a clonally selected, stably transfected human placental cell line which expresses the IL-8RB, whereas the parental 3ASubE cell line does not express this receptor. Previously we have demonstrated that the IL-8RB expressed in the 3ASubE P-3 cells migrates at approximately 45 kDa on an SDS-polyacrylamide gel (29). 3ASubE P-3 cells were labeled with [^{32}P]orthophosphate, then treated in the presence of increasing concentrations of TPA for 10 min. The IL-8RB was immunoprecipitated with affinity purified anti-amino-terminal peptide polyclonal antibodies which recognize the IL-8RB (29). The immunoprecipitates were electrophoresed, then analyzed by autoradiography and Western blot analysis. TPA treatment of the 3ASubE P-3 cells enhanced the phosphorylation of the IL-8RB (Fig. 1*A*). Under these conditions, 40 and 400 nM TPA were equally effective in stimulating the maximal level of receptor phosphorylation. Treatment of the 3ASubE P-3 cells with 4α-phorbol 12-myristate 13-acetate, the inactive isomer of TPA, had no effect on the phosphorylation of the IL-8RB (data not shown). Typically in our immunoprecipitations of [^{32}P]orthophosphate-labeled 3ASubE P-3 cells, two labeled proteins appear which migrate at approximately 97 kDa and between the 45- and 66-kDa molecular mass markers. We have previously demonstrated that these bands are nonspecifically immunoprecipitated since they can be eliminated by double immunoprecipitations (29). Western blot analysis indicated that approximately equal amounts of the IL-8RB were immunoprecipitated from cells treated with the various concentrations of TPA (Fig. 1*B*). Phosphoamino acid analysis revealed that TPA enhanced the phosphorylation of serine residue(s) on the IL-8RB (Fig. 2). Phosphorylation of the IL-8RB in response to PKC activation was not unique to stable transfectants of the 3ASubE cell line. Similar observations were noted in two other stably transfected non-hematopoietic cell lines, namely the 293 human embryonic kidney epithelial cell line and the Melan-A mouse melanocyte cell line (data not shown).

The effect of the diacylglycerol analogue diC_8, a natural activator of PKC, on the phosphorylation state of the IL-8RB in the 3ASubE P-3 cells was also investigated. [^{32}P]Orthophosphate-labeled 3ASubE P-3 cells were treated in the presence of 20 or 200 μM diC_8 for 10 min. For a comparison, 3ASubE P-3 cells were also treated with 40 nM TPA. Whole cell lysates were prepared and the IL-8RB was immunoprecipitated and analyzed by autoradiography and Western blot analysis. As shown in Fig. 3, diC_8 enhanced the phosphorylation of the IL-8RB in

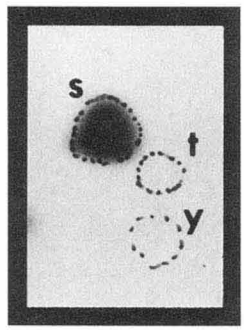


FIG. 2. **TPA treatment enhances the phosphorylation of the IL-8RB on serine residues.** The IL-8RB was immunoprecipitated from 3ASubE P-3 cells which had been labeled with [^{32}P]orthophosphate and treated with 40 nM TPA for 10 min. The immunoprecipitates were electrophoresed through a 9% SDS-polyacrylamide gel, transblotted onto a PVDF membrane, and exposed to autoradiography. The band corresponding to the IL-8RB was excised from the membrane and subjected to phosphoamino acid analysis, as described under "Experimental Procedures." The position of the phosphoamino acid standards have been traced onto the autoradiogram.

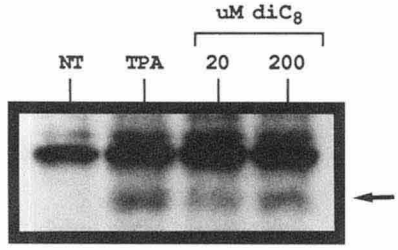


FIG. 3. **DiC$_8$ treatment enhances the phosphorylation of the IL-8RB in 3ASubE P-3 cells.** [^{32}P]Orthophosphate-labeled 3ASubE P-3 cells were treated with either an ethanol vehicle control (not treated, *NT*), TPA (40 nM), or diC$_8$ (20 or 200 μM) for 10 min at 37 °C. Whole cell lysates were prepared and the IL-8RB was immunoprecipitated from an equal number of trichloroacetic acid-precipitable counts (5×10^6 cpm) as described under "Experimental Procedures." Immunoprecipitates were electrophoresed through a 9% SDS-polyacrylamide gel, transblotted onto a nitrocellulose membrane, and exposed to autoradiographic film for approximately 16 h. The position of the IL-8RB is marked by an *arrowhead*.

the 3ASubE P-3 cells. Under the conditions used for these experiments, 200 μM diC$_8$ was more potent than 20 μM diC$_8$ in enhancing receptor phosphorylation. Western blot analysis revealed that approximately equal amounts of the IL-8RB were immunoprecipitated from the treated and untreated samples (data not shown).

Inhibition, or Down-regulation, of PKC Fails to Block the MGSA-enhanced Phosphorylation of the Type B IL-8R—MGSA has previously been demonstrated to enhance the phosphorylation of the IL-8RB on serine residue(s) (29). Initially, we reported enhanced receptor phosphorylation in response to 5 nM MGSA (29). A more extensive analysis of the concentration dependence of MGSA on receptor phosphorylation revealed that 50 nM MGSA maximally increased receptor phosphorylation in the 3ASubE P-3 cells (Fig. 4). Basal phosphorylation of the IL-8RB is not apparent in this figure due to the shorter exposure time for this autoradiogam (approximately a 5-h exposure for Fig. 4 compared to a 16-h exposure used in Fig. 1). Upon longer exposure of the gel shown in Fig. 4, the basal phosphorylation of the receptor was apparent (data not shown). Since both MGSA and TPA enhanced the phosphorylation of the IL-8RB on serine residue(s), we postulated that PKC may serve as a second messenger for the IL-8RB in response to ligand binding. Studies including PKC inhibition or down-regulation were subsequently conducted to further elucidate a role for PKC in this signaling pathway. 3ASubE P-3 cells were

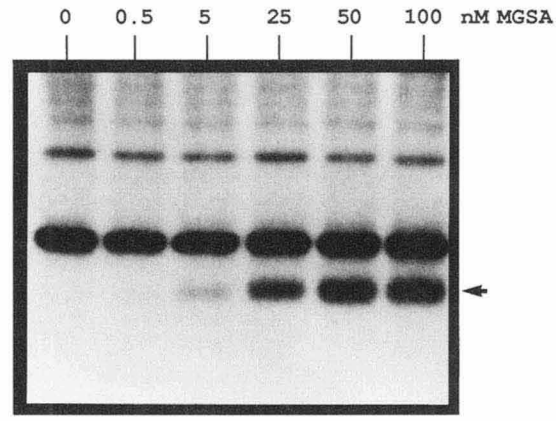


FIG. 4. **Concentration dependence of the MGSA-enhanced phosphorylation of the IL-8RB.** 3ASubE P-3 cells were labeled with [^{32}P]orthophosphate as described under "Experimental Procedures" and treated with an acetonitrile vehicle (0 nM) or increasing concentrations of MGSA (0.5–100 nM) for 10 min at 37 °C. Whole cell lysates were prepared and the IL-8RB was immunoprecipitated as described under "Experimental Procedures." Immunoprecipitates were electrophoresed through a 9% SDS-polyacrylamide gel, dried, and exposed to autoradiographic film for approximately 5 h. The position of the IL-8RB is marked by an *arrowhead*.

pretreated with either dimethylformamide (vehicle control) or 50 nM staurosporin for 2 h, after which the cells were treated with 5 nM MGSA (or an acetonitrile vehicle control) or 400 nM TPA (or an EtOH vehicle control) for 10 min. As demonstrated in Fig. 5, both MGSA and TPA enhanced the phosphorylation of the IL-8RB in cells which had been pretreated with the dimethylformamide vehicle control. Pretreatment with staurosporin suppressed the TPA-enhanced phosphorylation of the IL-8RB, however, it had no apparent effect on the MGSA-enhanced receptor phosphorylation. A similar response was observed in cells which had been pretreated with 400 nM TPA for 40 h to down-regulate PKC. Prolonged TPA treatment suppressed the TPA-enhanced phosphorylation, whereas it had no effect on the MGSA-enhanced phosphorylation of this receptor (Fig. 5). Inhibition of PKC by staurosporin, or down-regulation by prolonged TPA treatment also had no effect on the MGSA-enhanced receptor phosphorylation with 50 nM MGSA (data not shown). Interestingly, the basal level of receptor phosphorylation was greater in the cells which had been pretreated with TPA for 40 h. Similar results were observed in cells treated with 200 nM TPA for 24 h (data not shown). Since neither inhibition nor down-regulation of PKC significantly altered the effect of MGSA on the phosphorylation state of the IL-8RB, PKC isoforms which are sensitive to these manipulations may not play a role in the MGSA-enhanced phosphorylation of this receptor.

Pertussis Toxin Treatment Suppresses the MGSA-enhanced Phosphorylation of the IL-8RB—Previously we have demonstrated that the IL-8RB expressed in the 3ASubE P-3 cells is coupled, at least in part, to pertussis toxin-sensitive G proteins (29). To determine whether the MGSA-enhanced receptor phosphorylation required pertussis toxin-sensitive G proteins, or whether other G proteins participated in this pathway, 3ASubE P-3 cells were treated or not treated with pertussis toxin (1 μg/ml) during the 3-h labeling with [^{32}P]orthophosphate. Cells were then treated in the absence or presence of 5 nM (40 ng/ml) MGSA or 400 nM TPA for 5 min, lysed in RIPA buffer, and the IL-8RB was immunoprecipitated. As demonstrated in Fig. 6, pertussis toxin treatment of the 3ASubE P-3 cells suppressed the MGSA-enhanced phosphorylation of the IL-8RB. These data suggest that pertussis toxin-sensitive G proteins participate in the MGSA-enhanced phosphorylation of

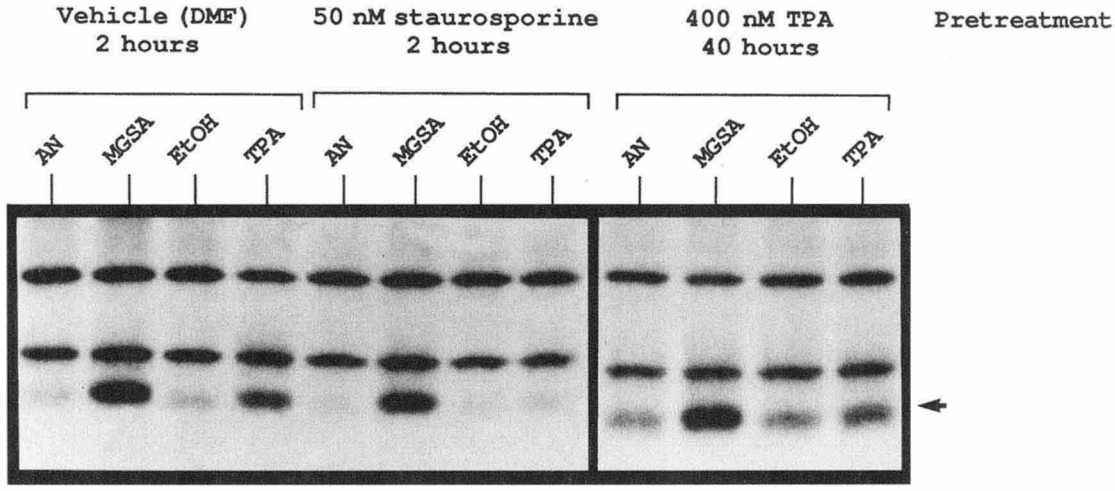
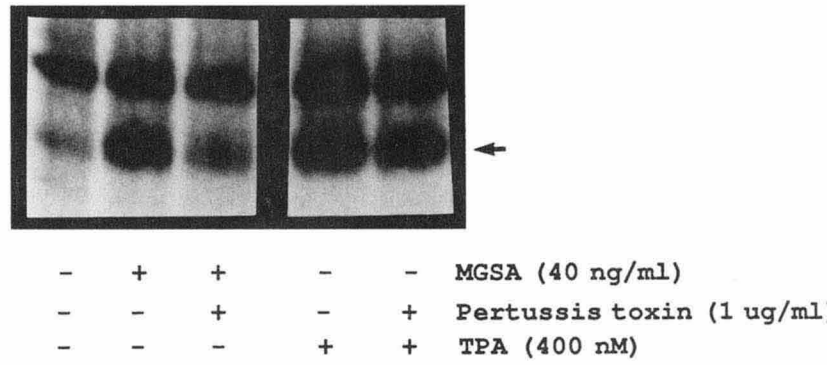


FIG. 5. **PKC inhibition, or down-regulation, does not block the MGSA-enhanced phosphorylation of the IL-8RB.** 3ASubE P-3 cells were labeled with [^{32}P]orthophosphate for 3 h at 37 °C. During the final 2 h of this incubation, cells were treated with dimethylformamide (*DMF*, vehicle control) or 50 nM staurosporin. Alternatively, the 3ASubE P-3 cells were cultured in the presence of 400 nM TPA for 40 h and maintained in TPA during the [^{32}P]orthophosphate labeling period. Once labeled, cells were treated with 5 nM MGSA, or an acetonitrile vehicle control, or with 400 nM TPA, or an ethanol vehicle control, for 10 min at 37 °C. The IL-8RB was immunoprecipitated from whole cell lysates, as described under "Experimental Procedures," electrophoresed through a 9% SDS-polyacrylamide gel, dried, and exposed to autoradiographic film for approximately 8 h. The position of the IL-8RB is indicated by the *arrowhead*.

FIG. 6. **Pertussis toxin suppresses the MGSA-enhanced phosphorylation of the IL-8RB in 3ASubE P-3 cells.** 3ASubE P-3 cells were labeled with [^{32}P]orthophosphate in the presence of either pertussis toxin (1 μg/ml) or its vehicle for 3 h. Cells were subsequently treated with MGSA (5 nM) or TPA (400 nM) for 5 min at 37 °C. Whole cell lysates were prepared and the IL-8RB was immunoprecipitated from 5×10^6 trichloroacetic acid-precipitable counts/min. Immunoprecipitates were analyzed as described. The position of the IL-8RB is marked by an *arrowhead*.



the IL-8RB. As expected, pertussis toxin had no effect on the TPA-enhanced phosphorylation of the IL-8RB (Fig. 6). However, these data demonstrate that the concentration and duration of the pertussis toxin treatment was not toxic, nor did it nonspecifically inhibit kinases within the cells.

TPA Treatment Concomitantly Reduces both ^{125}I-MGSA Binding to 3ASubE P-3 Cells and IL-8RB Protein Levels—^{125}I-MGSA binding assays were conducted to determine whether IL-8RB phosphorylation in response to TPA was accompanied by a change in ligand binding to this receptor. 3ASubE P-3 cells were treated with TPA (400 nM) for increasing periods of time (0–120 min) prior to the initiation of the binding assay. As shown in Fig. 7*A*, there was a time-dependent decrease in total binding of ^{125}I-MGSA to the TPA-treated 3ASubE P-3 cells. Pretreatment of the 3ASubE P-3 cells with TPA for 30 min resulted in approximately 50% reduction in ^{125}I-MGSA binding, whereas a 2-h pretreatment essentially reduced ^{125}I-MGSA binding to background levels. This effect was transient; ^{125}I-MGSA binding to the 3ASubE P-3 cells treated with 400 nM TPA for 24 h was equivalent to the ^{125}I-MGSA binding to untreated cells (data not shown).

Western blot analysis was conducted to determine if the TPA-induced decrease in ^{125}I-MGSA binding was accompanied by a decrease in the level of IL-8RB protein present in the 3ASubE P-3 cells. Triton X-100 extracts were prepared from 3ASubE P-3 cells which had been treated for increasing periods of time with 400 nM TPA. Western blot analysis of the extracts (50 μg of protein/lane) revealed a time-dependent decrease in the amount of immunoreactive IL-8RB present in the TPA-treated cells (Fig. 7*A*, *inset*). A decrease in the level of IL-8RB protein was notable after a 15-min treatment with 400 nM TPA and it was maximal after a 2-h treatment. The effect of TPA on receptor protein level was also concentration-dependent. Treatment of the 3ASubE P-3 cells for 2 h with either 40 or 400 nM TPA resulted in a significant decrease in the level of the IL-8RB protein present in the whole cell lysates (Fig. 7*B*). Similar to the transient effect of TPA treatment on ligand binding to the 3ASubE P-3 cells, the effect of TPA on receptor protein levels was also transient. Cells treated with 400 nM TPA for 24 h expressed the same level of receptor protein as untreated cells, as determined by Western blot analysis (data not shown). DiC_8 treatment also resulted in a decrease in the IL-8RB protein level in the 3ASubE P-3 cells. Western blot analysis of whole cell lysates (25 μg of protein/lane) prepared from 3ASubE P-3 cells which had been treated with increasing concentrations of diC_8 indicated that a 2-h treatment with 500 μM diC_8 resulted in a decrease in the receptor protein level (Fig. 8). Greater concentrations of diC_8 did not result in an increased reduction of the IL-8RB protein level in the 3ASubE P-3 cells. Under these conditions, the effect of diC_8 was not as potent as the effect observed in response to 40 nM TPA (Fig. 8).

A similar experiment was conducted to determine if MGSA treatment would alter the amount of IL-8RB protein present in the 3ASubE P-3 cells. Cells were treated with increasing concentrations of MGSA (0–50 nM) for 2 h, Triton X-100 extracts of cells were prepared and subjected to Western blot analysis. For

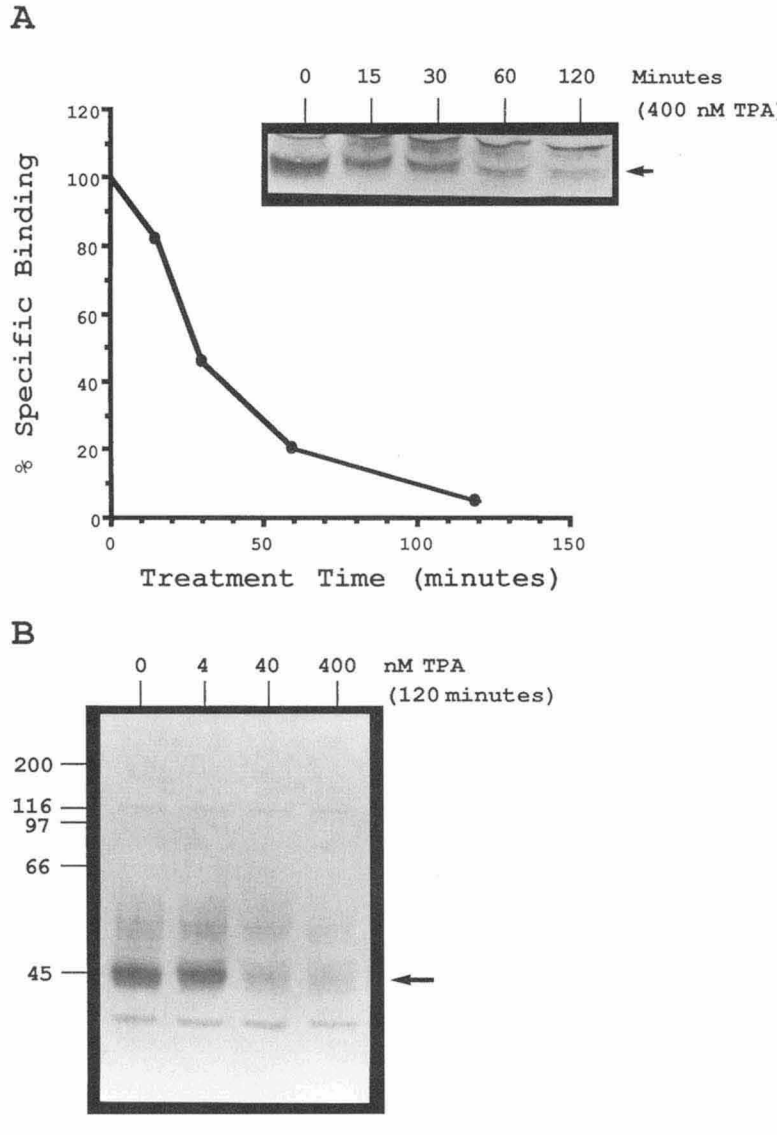


FIG. 7. **TPA treatment of 3ASubE P-3 cells concomitantly reduces both ^{125}I-MGSA binding and IL-8RB protein levels.** *A*, ^{125}I-MGSA binding to TPA-treated 3ASubE P-3 cells. 3ASubE P-3 cells were incubated with TPA (400 nM) or ethanol vehicle, in DMEM for 0, 15, 30, 60, or 120 min prior to the binding assay. ^{125}I-MGSA (20–30,000 cpm/well, specific activity of 100 μCi/μg) was incubated with the 3ASubE P-3 cells in 100 μg/ml ovalbumin, 30 mM Hepes/DMEM for 4 h at 4 °C. Cells were washed and counts were eluted with 0.1 N NaOH, 1% SDS. Nonspecific binding was estimated as the binding of ^{125}I-MGSA in the presence of 50 ng/ml unlabeled MGSA. Each point represents the mean of triplicate determinations (standard deviations were typically less than 10%). *A, inset,* TPA time dependence on receptor protein levels. Triton X-100 extracts were prepared from 3ASubE P-3 cells which had been treated with 400 nM TPA for increasing periods of time. Fifty micrograms of protein per lane was electrophoresed through a 9% SDS-polyacrylamide gel, transblotted onto a PVDF membrane, and subjected to Western blot analysis. The position of the IL-8RB is marked by an *arrowhead*. *B*, TPA concentration dependence on receptor protein levels. 3ASubE P-3 cells were treated with 0, 4, 40, or 400 nM TPA for 2 h prior to extraction with Triton X-100. Molecular size standards in kilodaltons are shown on the *left*. The position of the IL-8RB is marked by an *arrowhead*.

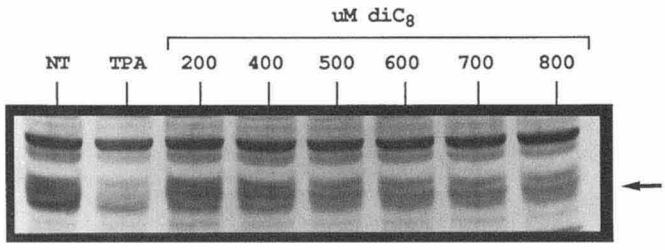


FIG. 8. **DiC$_8$ treatment reduces the IL-8RB protein level in 3ASubE P-3 cells.** Triton X-100 extracts were prepared from 3ASubE P-3 cells which had been treated with an ethanol vehicle control (*NT*), TPA (40 nM), or increasing concentrations of diC$_8$ (200–800 μM) for 2 h at 37 °C. Twenty-five micrograms of protein per lane was electrophoresed through a 9% SDS-polyacrylamide gel, transblotted onto a PDVF membrane, and subjected to Western blot analysis. The position of the IL-8RB receptor is marked by an *arrowhead*.

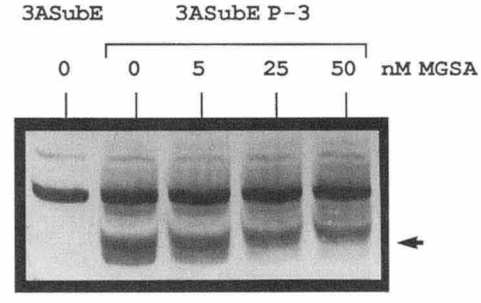


FIG. 9. **MGSA treatment reduces the level of the IL-8RB protein in 3ASubE P-3 cells.** Triton X-100 extracts were prepared from either the parental 3ASubE cell line or the 3ASubE P-3 cells which had been treated with MGSA (0, 5, 25, or 50 nM) for 2 h at 37 °C. Twenty-five micrograms of protein were electrophoresed through a 9% SDS-polyacrylamide gel, and transblotted onto a PVDF membrane. The blot was analyzed as described under "Experimental Procedures." The position of the IL-8RB is marked by an *arrowhead*.

comparison, a Triton X-100 extract of the parental 3ASubE cell line, which does not express the IL-8RB, was included in this Western blot. Similar to TPA, MGSA treatment also resulted in a concentration-dependent decrease in the level of IL-8RB protein in the 3ASubE P-3 cells (Fig. 9). This decrease was detectable after a 2-h treatment with 25 nM MGSA. The IL-8RB present in the lysates prepared from cells treated with the greater concentrations of MGSA migrated at a slower rate, consistent with hyperphosphorylation of this receptor. Under these experimental conditions, the amount of IL-8RB protein present in cells treated with 400 nM TPA was consistently less than the amount present in cells treated with 50 nM MGSA.

The decrease in immunoreactive IL-8RB protein present in the Triton X-100 extracts of TPA- or MGSA-treated 3ASubE P-3 cells could result from the mobilization of this receptor to a

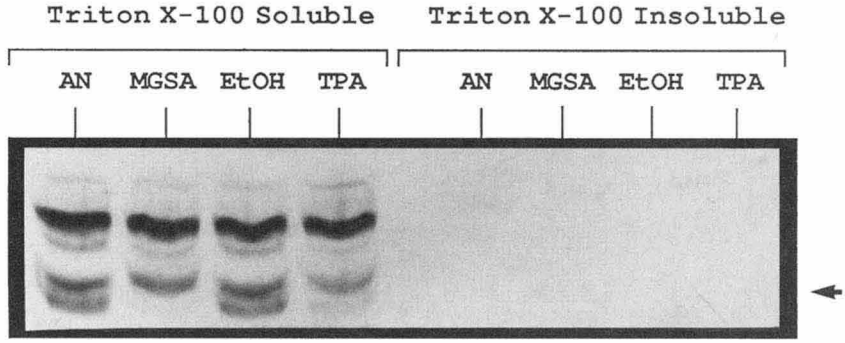


FIG. 10. **TPA or MGSA treatment of 3ASubE P-3 cells does not mobilize the IL-8RB into a Triton X-100 insoluble pool.** 3ASubE P-3 cells were incubated in the presence of 50 nM MGSA (or an acetonitrile vehicle control), or with 400 nM TPA (or an EtOH vehicle control) for 2 h at 37 °C. Triton X-100 extracts were prepared and 25 μg of extract were electrophoresed per lane. Alternatively, the entire Triton X-100-insoluble pellet from 3ASubE P-3 cells was resuspended in 2 × Laemmli buffer containing 5% SDS and 10% β-mercaptoethanol and electrophoresed. Proteins were transblotted and subjected to Western blot analysis as described. The migration of the IL-8RB is indicated by the *arrowhead*.

Triton X-100 insoluble pool. Such mobilization has previously been reported for the fMet-Leu-Phe receptor in response to ligand binding in granulocytes (34). Alternatively, the decrease in receptor protein could be due to an increase in receptor degradation. Two distinct approaches were taken to address the question of receptor mobilization *versus* degradation in response to TPA or MGSA treatment. First, to assess receptor mobilization we examined both the Triton X-100 soluble and insoluble fractions of 3ASubE P-3 cells that had been treated with MGSA (50 nM), TPA (400 nM), or the appropriate vehicle controls for 2 h. Under these conditions, we again detected decreased levels of IL-8RB protein in the Triton X-100 extracts from cells treated with MGSA or TPA, as compared to the cells treated with vehicle, however, no apparent IL-8RB immunoreactivity was detectable in the Triton X-100 insoluble pellets (Fig. 10). A Coomassie stain of a polyacrylamide gel of Triton X-100 insoluble pellets revealed that proteins from the insoluble pellets did migrate through the gel (data not shown). These data suggest that the decrease in IL-8RB immunoreactivity in response to MGSA or TPA treatment is not due to the mobilization of this receptor to a Triton X-100 insoluble pool.

Pulse-chase experiments were conducted to assess whether MGSA or TPA stimulated receptor degradation. The parental cell line, 3ASubE, along with the 3ASubE P-3 cells were metabolically pulsed with [^{35}S]cysteine/methionine (Tran^{35}S-label, ICN) for 6 h then chased in the presence of cold cysteine and methionine in the absence or presence of MGSA (50 nM) or TPA (400 nM) for 2 h. As shown in Fig. 11, a decreased level of the IL-8RB was immunoprecipitated from the MGSA- or TPA-treated cells, as compared to the untreated 3ASubE P-3 cells, suggesting that both MGSA and TPA stimulate receptor degradation. As expected, the IL-8RB was undetectable in the ^{35}S-labeled parental 3ASubE cell line (Fig. 11).

Differential Effects of MGSA and TPA Treatment on the IL-8RB Expressed in U937 Cells—Experiments were conducted on the promonocytic cell line, U937, as an initial attempt to compare the signaling by MGSA through the IL-8RB, and the effect of PKC activation on this receptor in non-hematopoietic cell types *versus* hematopoietic cell types. Previous studies have demonstrated that U937 cells, differentiated with retinoic acid, express the endogenous IL-8 receptors (35). U937 cells were treated with 1 μM retinoic acid for 5 days prior to experimentation. Differentiated U937 cells were labeled with [^{32}P]orthophosphate, then treated in the absence or presence of MGSA (50 nM) for 10 min or TPA (40 nM) for 1–10 min. Whole cell lysates were prepared, the IL-8RB was immunoprecipitated, electrophoresed, transferred onto a nitrocellulose membrane, then analyzed by autoradiography and Western blot analysis. MGSA treatment of the U937 cells enhanced the

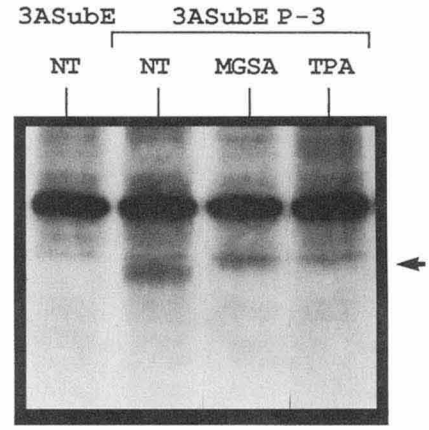


FIG. 11. **TPA or MGSA treatment of the 3ASubE P-3 cells results in degradation of the IL-8RB.** The parental 3ASubE cell line, or the IL-8RB expressing 3ASubE P-3 cells were pulse labeled with [^{35}S]cysteine/methionine as described under "Experimental Procedures." Cells were then chased with unlabeled cysteine and methionine for 2 h in the absence or presence of MGSA (50 nM) or TPA (400 nM). Triton X-100 extracts were prepared and the IL-8RB was immunoprecipitated from an equal number of trichloroacetic acid-precipitable counts (2×10^7), electrophoresed through a 9% SDS-polyacrylamide gel, dried, and subjected to autoradiography. The migration of the IL-8RB is indicated by the *arrowhead*.

phosphorylation of the IL-8RB (Fig. 12*A*). Phosphoamino acid analysis revealed that, similar to the 3ASubE P-3 cells, MGSA enhanced the phosphorylation of serine residues on the IL-8RB expressed in the U937 cells (data not shown). There was no evidence for either threonine or tyrosine phosphorylation on this receptor. Unlike the effect observed for MGSA, TPA treatment, under these conditions, did not appear to modulate the phosphorylation state of the IL-8RB expressed in U937 cells (Fig. 12*A*). Western blot analysis of the immunoprecipitates, using a mouse monoclonal anti-IL-8RB antibody, indicated that approximately equal levels of receptor protein were immunoprecipitated from the treated and untreated U937 cells (Fig. 12*B*). Greater concentrations of TPA (400 nM) also failed to modulate the IL-8RB phosphorylation status (data not shown). Similarly, treatment with diC_8 (200 μM) or 1-oleoyl-2-acetyl-*sn*-glycerol (200 μM), another diacylglycerol analogue, for 10 min failed to alter the phosphorylation state of this receptor in the U937 cells (data not shown).

The effect of MGSA treatment and PKC activation on the level of IL-8RB protein present in the differentiated U937 cells was also examined. U937 cells were treated with MGSA (50 nM), TPA (4 or 40 nM), diC_8 (10–100 μM), or the appropriate vehicle control for 2 h at 37 °C. Whole cell lysates were pre-

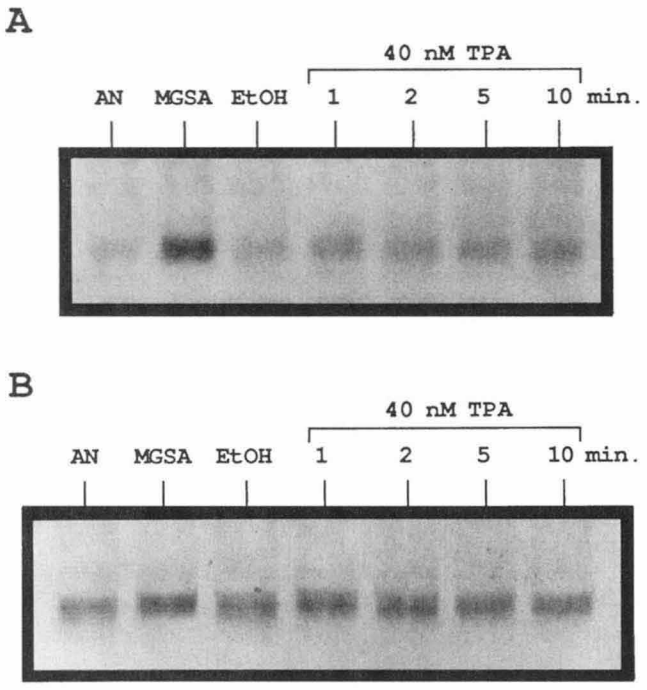


FIG. 12. **MGSA, but not TPA, treatment enhances the phosphorylation of the IL-8RB in U937 cells.** Differentiated U937 cells were labeled with [^{32}P]orthophosphate as described under "Experimental Procedures" and treated with an acetonitrile vehicle control (*AN*), MGSA (50 nM) for 10 min, or TPA (40 nM) for 1–10 min at 37 °C. Whole cell lysates were prepared and the IL-8RB was immunoprecipitated from an equal number of trichloroacetic acid-precipitable counts (5×10^6 cpm). The immunoprecipitates were electrophoresed through a 9% SDS-polyacrylamide gel, transblotted onto a nitrocellulose membrane, and exposed to autoradiographic film for approximately 16 h (*A*). After development, the nitrocellulose membrane was analyzed by Western blot analysis, using a mouse monoclonal antibody against the IL-8RB (*B*).

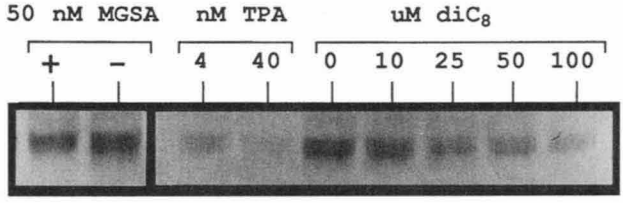


FIG. 13. **MGSA, TPA, or diC_8 treatment reduces the level of the IL-8RB protein present in U937 cells.** U937 cells were treated in the absence (–) or presence (+) of MGSA (50 nM), TPA (4 or 40 nM), diC_8 (10–100 μM), or an EtOH vehicle control (0 μM) for 2 h at 37 °C. Cells were washed and whole cell lysates were prepared using a Triton X-100 buffer. The protein content of the lysates were estimated (BCA, Pierce) and 200 μg of protein was immunoprecipitated using 5 μg of affinity purified anti-amino-terminal rabbit polyclonal antibody. The immunoprecipitates were electrophoresed through a 9% SDS-polyacrylamide gel, transblotted, then subjected to Western blot analysis using the mouse monoclonal antibody.

pared, the IL-8RB was immunoprecipitated using the rabbit anti-amino-terminal peptide antibody, and the immunoprecipitates were subjected to Western blot analysis using the above mentioned mouse monoclonal antibody. Results from this experiment (Fig. 13) indicated that MGSA treatment resulted in a modest decrease in the IL-8RB protein level, whereas TPA or diC_8 treatment significantly reduced the level of IL-8RB protein in the U937 cells.

DISCUSSION

The detection of the IL-8RA and B mRNAs, by reverse transcriptase-polymerase chain reaction, in a variety of MGSA- or IL-8-responsive non-hematopoietic cell types suggests that these receptors may play a role in eliciting the MGSA or IL-8 responses (29, 30). To investigate the mechanism of signal transduction through the IL-8RB and determine what factors may modulate signaling through this receptor in non-hematopoietic cell types, we have previously established a stably transfected cell line, termed 3ASubE P-3, which overexpresses this receptor (29). In this report we have demonstrated that activation of PKC enhanced the phosphorylation of the IL-8RB on serine residue(s) in the 3ASubE P-3 cells. This response was not unique to the 3ASubE cell line since PKC activation in two other stably transfected non-hematopoietic cell lines which overexpress the IL-8RB also resulted in enhanced phosphorylation of this receptor. Thus the phosphorylation of the IL-8RB which is mediated by PKC may be a generalized phenomena throughout non-hematopoietic cell types. Receptor phosphorylation in response to PKC activation has been reported for several members of the G protein-coupled receptor family, including the α_1- and β-adrenergic receptors (31). Since PKC and MGSA enhanced the phosphorylation of serine residue(s) on the IL-8RB, and furthermore, since PKC has been implicated in the signaling pathway of IL-8 in both neutrophils and lymphocytes (36–38), experiments were conducted to elucidate a role for PKC in the MGSA-enhanced phosphorylation of the IL-8RB. Under conditions where the TPA-enhanced phosphorylation of the IL-8RB was suppressed, either by staurosporin or prolonged exposure to TPA, MGSA still increased the phosphorylation of this receptor. Thus, PKC may not be involved in the MGSA-enhanced phosphorylation of this receptor. Alternatively, MGSA may elicit its signal through PKC isoform(s) not inhibited by staurosporin or down-regulated by TPA under our experimental conditions, such as PKCϵ or PKCζ (39). The sites of receptor phosphorylation mediated by either PKC or MGSA have not been determined. Based upon the proposed membrane topology of the IL-8RB, there are 8 candidate serine residues in the cytoplasmic carboxyl tail of the receptor which may be target(s) for the PKC- or MGSA-mediated phosphorylation. There are no serine residues in the other intracellular domains of the IL-8RB. Studies are currently underway to identify which serine residue(s) are phosphorylated.

Similar to the stable transfectants, MGSA treatment also enhanced the phosphorylation of the IL-8RB in U937 cells, a promonocytic cell type which expresses the endogenous IL-8RB. In contrast to our observations in the non-hematopoietic cell types, we were unable to detect TPA- or diC_8-enhanced phosphorylation of the IL-8RB in U937 cells. One explanation for the difference in receptor phosphorylation in response to PKC activation in the stable transfectants *versus* the U937 cells is that regulation of receptor phosphorylation by PKC is different in monocytic cells *versus* the three different non-hematopoietic stable transfectants which we have examined. It is conceivable that the effect of PKC activation on receptor phosphorylation in non-hematopoietic cell types is mediated through a second kinase which is activated by PKC. If such a kinase is absent in U937 cells, this may explain the lack of IL-8RB phosphorylation in response to PKC activation. *In vitro* kinase assays should be useful to determine whether PKC is capable of directly phosphorylating the IL-8RB.

Recently it has been reported that stimulation of granulocytes with TPA inhibits the IL-8 induced intracellular calcium mobilization (40). The response to TPA was rapid, requiring only a 5-min preincubation with TPA to prevent the IL-8 effect on calcium. It was proposed that TPA treatment of the granulocytes may elicit its effect through receptor phosphorylation, internalization, or shedding. Receptor phosphorylation and internalization, in response to PKC activation, has been reported for several members of the seven-transmembrane domain, G

protein-coupled receptor family, including the α_1- and β-adrenergic receptors (41, 42), the angiotensin II receptor (43), and the bombesin receptor (44). Although our experiments with the 3ASubE P-3 and other stably transfected non-hematopoietic cell lines would be consistent with receptor phosphorylation playing a role in the desensitization response to PKC activation of the granulocytes, the U937 experiments described here do not support this hypothesis. Recently it has been demonstrated that PKC activation enhances the phosphorylation of $G_{\alpha i2}$, resulting in an attenuated inhibition of the adenylyl cyclase pathway (45). Since IL-8 and MGSA mediate their effects on neutrophils via pertussis toxin-sensitive G proteins, and furthermore, since it has been demonstrated that both the IL-8RA and B can couple to $G_{\alpha i2}$ (46), the ability of PKC to attenuate signaling by this route must also be considered as a means of desensitizing granulocytes to IL-8.

The effects of PKC activation on MGSA binding and receptor protein levels in the 3ASubE P-3 and U937 cells appears to be in striking contrast to effects described for the adrenergic receptors. PKC activation is associated with phosphorylation and desensitization of the α_1- and β-adrenergic receptors, however, it has not been reported to alter the agonist binding characteristics of these receptors (41, 42). In contrast, we have shown that TPA treatment of the 3ASubE P-3 and U937 cells results in a rapid decrease in the amount of receptor protein present in these cells. Furthermore, down-regulation, or degradation, of the adrenergic receptors requires agonist occupation of the receptor (31). The effect of PKC activation on the IL-8RB protein levels in the 3ASubE P-3 and U937 cells indicates that receptor occupancy is not necessary for the degradation of this receptor. Although the PKC-mediated degradation of the IL-8RB is novel with respect to G protein-coupled receptors, PKC has previously been demonstrated to stimulate the shedding of several different cytokine and homing receptors, including the IL-6, the tumor necrosis factor-α and -β, and the $gp90^{MEL-14}$ receptors (47–50). Whereas it is unlikely that a seven-transmembrane domain receptor would be shed in response to PKC activation, the overall effect would be similar for receptor degradation and shedding; both would render the cell insensitive to a subsequent cytokine challenge. Thus activation of PKC via different extracellular signals may initiate a negative feedback loop for responses to various cytokines, whereby their respective receptors are either degraded or shed by the cell.

One caveat regarding the effect of PKC activation on the IL-8RB protein level in U937 cells is that a single application of TPA induces the differentiation of U937 cells to macrophages (reviewed in Ref. 51). Thus one could argue that the effect of TPA on the IL-8RB is a nonspecific effect mediated by the differentiation process. In an attempt to discern a direct effect of PKC on the IL-8RB protein in U937 cells, we restricted our studies to 2 h, typically U937 differentiation occurs upon exposure to TPA for 3–5 days. Also the effect of two natural PKC activators, diC_8 and 1-oleoyl-2-acetyl-*sn*-glycerol, on the IL-8RB protein level was investigated. Single application of either of these two PKC activators fails to induce U937 differentiation (52, 53). Our studies demonstrate that a single 2-h treatment of the U937 cells with 25 μM diC_8 resulted in a decrease in the IL-8RB protein. 1-Oleoyl-2-acetyl-*sn*-glycerol treatment also elicited a modest decrease in the level of the IL-8RB protein present in the U937 cells; the magnitude of this effect was comparable to the effect observed in response to treatment with MGSA (data not shown). Thus under conditions where one would not anticipate that the U937 cells would be differentiated, we have observed that treatment with either PKC activators or MGSA decreased the total amount of IL-8RB protein present in these cells.

Reconstitution experiments have demonstrated that the IL-8RA and -B couple to both pertussis toxin-sensitive G proteins ($G_{\alpha i2}$ and $G_{\alpha i3}$) and pertussis toxin-insensitive G proteins ($G_{\alpha 14}$, $G_{\alpha 15}$, $G_{\alpha 16}$) (46). Since pertussis toxin treatment suppresses the MGSA-enhanced phosphorylation of the IL-8RB, it is inferred that this receptor couples to endogenous $G_{\alpha i}$ family member(s) in the 3ASubE P-3 cells and that activation of the kinase(s) which mediate the MGSA-enhanced phosphorylation of the IL-8RB is mediated, at least in part, through the pertussis toxin-sensitive G protein family. Candidate kinase(s) which may play a role in this phosphorylation include members of the recently described G protein-coupled receptor kinase family (54). Investigations into the potential role of these kinase(s), in response to MGSA, are currently underway.

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