Interleukin-8 Receptors R1 and R2 Activate Mitogen-Activated Protein Kinases and Induce c-fos, Independent of Ras and Raf-1 in Chinese Hamster Ovary Cells

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ABSTRACT: Many of the biological effects of interleukin-8 (IL-8) are realized by binding to the two seven-transmembrane receptors IL-8 R1 and IL-8 R2. IL-8 R1 is activated only by IL-8, while IL-8 R2 is activated by IL-8, GROα, and a few other α chemokines. In addition to the well-known chemoattractant function, IL-8 is also angiogenic and mitogenic. IL-8 R1 and R2 have been shown to interact with $G\alpha_{i2}$ and $G\alpha_{16}$, resulting in the activation of several mitogen-activated protein kinases. We have investigated IL-8 R1 and IL-8 R2 regulated upstream mediators and downstream effects of extracellularly responsive kinase (ERK) signaling pathways by expressing the individual receptors in a heterologous system. Our results demonstrate the following in CHO cells stably expressing either IL-8 R1 or R2 receptors: (a) IL-8 activates ERK and ERK kinases (MEK) through R1. Both IL-8 and GROα activate ERK and MEK through R2, whereas MIP-1α, a β chemokine, does not activate these kinases through either of these receptors. (b) ERK activation is inhibited by pertussis toxin and MEK1 inhibitor. (c) ERK activation is independent of the upstream mediators Ras and Raf-1. (d) The downstream effects of ERK activation result in an increase of c-fos mRNA through both R1 and R2 receptors.

Chemokines are produced by a variety of cell types and consist of over 30 members (reviewed in *1, 2*). Based on the presence or absence of a single amino acid between the first two of the four conserved cysteines, the majority of the chemokines can be classified into α chemokines (CXC) and β chemokines (CC). Some of the α chemokine functions include neutrophil migration, exocytosis of granulocytic enzymes, respiratory burst, cell growth, mitogenesis, tissue remodeling, and repair (*1*). The α chemokine mediated mitogenic signaling pathway has not been sufficiently investigated. Chemokines exert their functions by binding to high-affinity G protein-coupled seven-transmembrane receptors (GPCRs)[1] (*3, 4*).

Ligand-bound receptors activate G proteins by catalyzing the exchange of GDP bound to the α subunit (the inactive state) with GTP (the active state), resulting in dissociation of α-GTP from the $\beta\gamma$ subunits (*5*). Several major intracellular signaling pathways are regulated by both α and $\beta\gamma$ subunits of GPCRs. These include the cAMP/protein kinase A pathway, the mitogen-activated protein (MAP) kinase pathway, and the phosphatidylinositol/calcium/protein kinase C pathway (*6*). The coupling mechanisms of G α subunits to the cAMP/protein kinase A pathways have been well-defined. However, the mechanism by which G proteins activate the MAP kinase pathway is less clearly defined and is an area of intense research (*7*).

MAP kinases are a group of widely distributed serine-threonine kinases which mediate mainly proliferative and mitogenic responses by activating transcription factors (*8*). Although initially all ERK1/2 activation was attributed to $\beta\gamma$ subunits, several lines of evidence emphasize participation of the α subunit (*9*). These include the inability of overexpressed G$\beta\gamma$-sequestering peptide (βARK-1) to inhibit ERK1/2 activation (*10*) and the inhibition of Ras-mediated Raf activation by cAMP which is regulated by the Gα subunit (*11, 12*). The oncogenic constitutively active mutants of $G\alpha_{i2}$ and $G\alpha_s$, termed *gip2* and *gsp*, respectively, activate cell proliferation and transformation (*13*). $G\alpha_o$ and $G\alpha_q$ proteins activate ERKs through protein kinase C (PKC) and intermediary tyrosine kinases (*10, 14*).

Work from several laboratories has established that $\beta\gamma$ subunits mediate the activation of the MAP kinase cascade for a majority of GPCRs. The evidence includes inhibition of ERK1/2 activation by coexpression of G$\beta\gamma$-sequestering peptide (*15, 16*) and disruption of $\beta\gamma$ subunit association through deletions of β subunit (*17*). The $\beta\gamma$ subunits of $G\alpha_i$-coupled receptors have been demonstrated to function through a PI3-kinase/Ras/Raf/Sos/MEK/ERK pathway typical for signaling by receptor protein tyrosine kinases (*18*−*20*), as well as through additional pathways involving PYK2 tyrosine kinase (*21*), and also through the activation of phospholipase C (PLC) (*22*).

In addition to neutrophil chemotaxis, IL-8 effects release of histamine and leukotrienes in basophils, increases Ca^{2+} and release of peroxidase in eosinophils, and effects chemo-

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[1] Abbreviations: cAMP, adenosine 3′-cyclic monophosphate; bDNA, branched DNA; $G_{\alpha\beta\gamma}$, $\alpha\beta\gamma$ subunits of the G protein; GPCR, G-protein coupled receptor; EGF, epidermal growth factor; GRO, growth-related peptide; MGSA, melanoma growth stimulatory activity; ERK, extracellular signal-regulated kinase; IL-8, interleukin 8; MAP kinase, mitogen-activated protein kinase; MEK, MAP or ERK kinase; MEKK, mitogen-activated protein/ERK kinase kinase; MIP-1α, macrophage inflammatory protein 1α; PI3-K, phosphatidylinositol 3-kinase; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; PTX, pertussis toxin.

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taxis of lymphocytes with or without Ca^{2+} increase. More interestingly, IL-8 and GROα also induce hematopoiesis, induce mitogenesis in melanoma cells, inhibit collagen expression, indicative of tissue repair and remodeling, and induce angiogenesis in rat cornea (*1*). The broad spectrum of physiological effects of chemokines on a wide variety of cells suggests cell type specific functional differences (*23*−*26*). To date, α chemokines have been shown to interact with several receptors including the two major receptors IL-8 R1 and R2. The cloning of IL-8 R1 and R2 receptors has made possible heterologous expression of the individual receptors to examine the distinct role of each receptor in cell signaling. IL-8 R1 binds only IL-8 with high affinity, whereas IL-8 R2 binds several α chemokines, including IL-8 and GROα, with high affinity (*27, 28*). Both R1 and R2 receptors in CHO cells interact with the PTX-sensitive $G\alpha_{i2}$, inhibit cAMP, and mobilize calcium (*29*−*33*). In COS cells, the receptors interact with coexpressed PTX-insensitive $G\alpha_{16}$ and activate PLC-β, resulting in calcium mobilization and increased inositol (*34, 35*). In HEK-293 cells, the receptors interact with endogenous $G\alpha_{i2}$ and coexpressed $G\alpha_{16}$ to effect increase in calcium and inositol phosphates and activation of MAP kinase (*35*−*37*).

We have examined potential mediators and effects of ERK1/2 activation by IL-8 R1 and R2 receptors in CHO cells stably expressing these individual receptors. Our results demonstrate that in these cells IL-8-induced ERK activation is independent of cAMP status, and sensitive to PTX, and MEK inhibitor. Through overexpression of mutant forms of Ras and Raf-1, we demonstrate that ERK activation is independent of these mediators. ERK activation by both receptors leads to c-fos induction.

MATERIALS AND METHODS

Materials. Wortmannin, bisindolylmaleimide I (GF-109203X), U73122, D609, sp-8-Br-cAMP, and SB202190 were from Calbiochem. *B. pertussis* toxin was from List Biological Laboratories. PD098059, antibodies to ERK1/2, and MEK and their phosphorylated forms were from New England Biolabs. Antibodies to Ras, Rac, and v-Raf were from Santa Cruz Biotechnology. Antibody for HA epitope was from Boehringer. Tissue culture reagents were from Life Technologies. bDNA assay reagents for c-fos mRNA quantitation were from Chiron Corp. All other reagents were from Sigma.

Cell Culture and Transfection. CHO cells stably expressing IL-8 R1 and R2 have been described previously (*27, 38*). Cells were maintained in F12-selection medium without hypoxanthine, thymidine, and glycine. Cloning, expression, and purification of IL-8, MIP-1α, and GROα have been previously described (*27, 38*).

Signaling Studies. A total of 0.5×10^6 cells were seeded in 60 mm dishes in F12-selection medium containing 10% heat-inactivated dialyzed serum. Following overnight incubation, the cells were starved with serum-free F12 media, treated with IL-8, MIP-1α, or GROα for varying periods of time.

Treatment with Inhibitors. The cells were incubated for 1 h with Wortmannin, PD098059, and SB202190 prior to treatment with IL-8. For PTX treatment, the cells were seeded and allowed to attach overnight, followed by PTX treatment 3 times at 12 h intervals. The first treatment was in the presence of F12-selection media containing 10% serum, and the last two treatments were in serum-free medium. GF109203X treatment at 2 μM final concentration was for 15 min. D609 was tested at 100 μM and 10 μM final concentrations for 30 min, U73122 was tested at 1 μM and 0.1 μM concentrations for 15 min, and sp-8-Br-cAMP was tested at 500 μM and 50 μM for 5 min. All inhibitor treatments were followed by 10 nM IL-8 treatment for 10 min.

Overexpression of Ras, Raf-1, and Rac1. Eight hours following seeding of 0.5×10^6 cells in 60 mm dishes, they were transfected with 5 μg of $p21^{Ras}$, Rac1, and $p74^{Raf-1}$ kinase cDNA in pCDNA3 vector, with TransIT-LT1 (Mirus), in the presence of Opti-Mem (Life Technologies). Following 16 h of transfection, the media was changed to 10% serum containing F12 selection media for 8 h, and to serum free media for 16 h to starve the cells. IL-8, GROα, and MIP-1α were added at 10 nM concentrations for 10 min.

Immunoblotting. After various treatments, the cells were lysed, and the proteins were resolved by SDS−polyacrylamide gel electrophoresis and transferred to an Immobilon (Millipore) nylon membrane. Proteins were detected by immunoblotting with appropriate primary antibodies followed by treatment with alkaline phosphatase-conjugated secondary antibodies. The interaction of proteins with antibodies was visualized by treating the membrane with CDP* reagent (Tropix) and the image captured on Kodak films. The films were scanned on a BioRad phosphoimager to quantitate for protein expression and the phosphorylation status.

Branched DNA (bDNA) Assay To Quantitate Hamster c-fos mRNA. The assay to measure c-fos mRNA levels was performed according to the manufacturer (Chiron Corp.). Briefly, 4×10^4 cells were seeded in 96 well tissue culture plates, in F12-selection media containing 10% serum, and changed to 0.5% serum containing media the following day. After 24 h treatment, the cells were induced with various concentrations of IL-8 for 40 min. The cells were lysed in a solution containing antisense oligonucleotides corresponding to hamster c-fos mRNA (*56*). The antisense oligonucleotides were of three kinds, referred to as Capture extenders (CE), Linker extenders (LE), and Blockers. The 5′ end of the CE hybridizes to various regions of the hamster c-fos mRNA, while the 3′ end hybridizes to generic oligonucleotides coated onto the wells of the 96 well bDNA plate, thus capturing the hamster mRNA on the plate. The 3′ end of LE anneals to various regions of fos mRNA, while the 5′ ends are identical and hybridize to alkaline phosphatase probe. The Blocker oligonucleotides perform two functions: (i) they hybridize to specific regions of c-fos mRNA to open up the secondary structure; and (ii) by hybridizing to problem regions, they prevent nonspecific annealing of CE and LE. Following lysis of cells, the lysate was transferred to a bDNA 96 well plate, and annealed overnight at 53 °C. The plate was washed extensively to remove unhybridized nucleic acids and other nonparticipating cellular components. Lumiphos, the chemiluminescent substrate for phosphatase, is added and light emission measured in a luminometer.

RESULTS

Specificity of ERK1/2 Activation by IL-8, GROα, and MIP-1α and Effect of PTX. IL-8 treatment of neutrophils and of

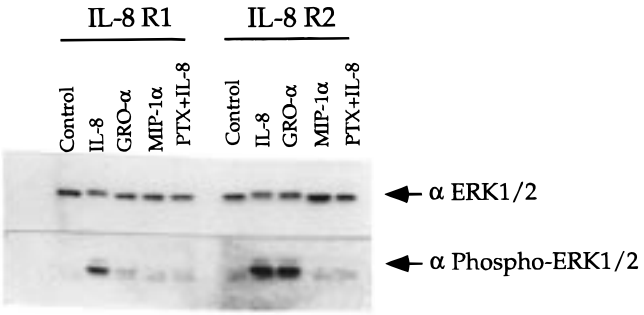

FIGURE 1: Specificity of ERK1/2 activation by IL-8 R1 and R2 receptors expressed in CHO cells. Serum-starved CHO cells stably expressing IL-8 R1 and R2 were treated for 10 min with 10 nM IL-8, 10 nM GROα, and 10 nM MIP-1α as indicated. For PTX treatment, cells were treated 3 times at 12 h intervals with 100 ng/mL PTX and stimulated for 10 min with 10 nM IL-8. ERK1/2 and phospho ERK1/2 activity was monitored by Western analysis.

HEK-293 and Jurkat cells expressing IL-8 receptors results in activation of ERK1/2 (*24*, *25*, *36*, *37*). To study the pathway leading to activation of ERK1/2 by the two well-characterized IL-8 receptors, we have utilized CHO cell lines stably expressing the IL-8 receptors R1 and R2 individually. Both CHO R1 and R2 cells demonstrated IL-8-dependent activation of ERK1/2 (Figure 1). GROα, which selectively interacts with IL-8 R2, activated ERK1/2 efficiently in CHO R2, but not in CHO R1 cells. MIP-1α, a β chemokine, did not activate ERK1/2 in either CHO R1 or R2 cells. IL-8 receptors interact with $G\alpha_i$ and $G\alpha_{16}$ subunits upon activation with IL-8 (*29*, *30*, *34*, *35*). The signaling functions of IL-8 including inhibition of adenylyl cyclase, inositol phosphate turnover, and ERK1/2 activation are sensitive to PTX (*32*, *34*, *36*). As shown in Figure 1, treatment of CHO R1 and R2 cells with PTX resulted in inhibition of ERK1/2 activation, indicating that similar to neutrophils, the receptors in this cell line are signaling through $G\alpha_i$ proteins. Identical results were obtained for the ligand specificity and PTX sensitivity in both CHO R1 and R2 cells for the activation of MEK (Map and ERK kinases), which are upstream activators of ERK1/2 (data not shown). These results demonstrate that CHO R1 and R2 cells are capable of α chemokine-specific, PTX-sensitive signaling.

Time Course of ERK1/2 Activation by IL-8. In both CHO R1 and R2 cells, a rapid increase in phospho ERK1/2 was observed upon treatment with IL-8. Activation through CHO R1 was maximal by 5 min and decreased rapidly by 15 min, with no further activation up to 45 min. CHO R2 also exhibits rapid activation by 5 min; however, the response decreases slowly over a 45 min period (Figure 2). Prado et al. (*31*) have demonstrated that binding of IL-8 to CHO R1 and R2 cells triggers a dramatically faster internalization of R2 than R1, which could effectively decrease the duration of signaling through R2. Since the biological effects of short-term activation of ERKs are thought to be different from prolonged effects, it is possible that through these variations some distinctions between the functioning of IL-8 R1 versus R2 receptors are manifested.

Effect of Inhibitors of the ERK Signaling Cascade. To characterize the upstream mediators of ERK1/2 activation by IL-8 R1 and IL-8 R2, we examined the effects of several inhibitors. PD098059 inhibits MEK1 which is immediately upstream of ERK1/2 (*39*). As shown in Figure 3, lanes b and c, ERK1/2 activation through R1 and R2 is partially

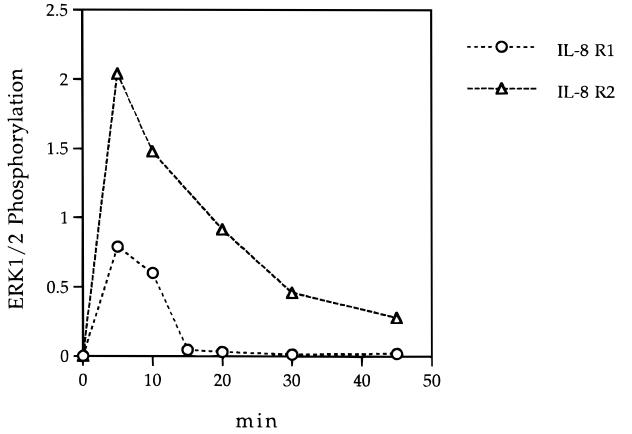

FIGURE 2: Time course of IL-8-dependent ERK1/2 activation in CHO R1 and R2 cells. Serum-starved CHO R1 and R2 cells were treated with 10 nM IL-8 for the indicated periods of time. Data are expressed as ERK1/2 phosphorylation, corrected for the ERK1/2 protein concentration. The value for the phosphorylated ERK1/2 in the control sample was 0.001, indicating a 700- and 2000-fold maximum ERK1/2 activation for R1 and R2, respectively. The data shown are typical of three independent experiments.

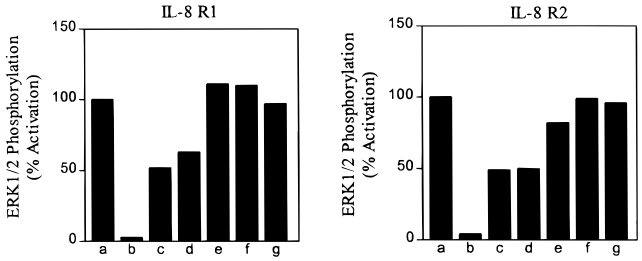

FIGURE 3: Sensitivity of CHO R1 and R2 cells to Wortmannin, PD098059, and SB202190 for activation of ERK1/2. Serum-starved CHO R1 and R2 cells were pretreated with inhibitors for 1 h, prior to induction with IL-8 (10 nM) for 10 min. ERK1/2 activation was quantitated following Western analysis. IL-8 treatment in the absence of inhibitors, in lanes a, is calculated as 100%. Lanes b, c, 50, 5 μM PD098059; d−f, 1, 0.1, 0.01 μM Wortmannin; and g, 100 μM SB202190. Values are data from a representative experiment, which was replicated 3 times with comparable results.

inhibited by PD098059 at 5 μM and completely inhibited at 50 μM concentrations. The complete inhibition with 50 μM PD098059 was confirmed in HEK-293 cells transiently expressing R1 receptor, indicating a role for MEK1 in ERK activation in two different cell types (data not shown). Wortmannin inhibits tyrosine and the serine/threonine kinase group of PI3-kinase enzymes to varying degrees. PI3-kinases function several levels upstream of MEKs (*19*). In both CHO R1 and R2 cells, Wortmannin did not have any significant effect on ERK1/2 activation at 0.1 μM and only 40−50% reduction in ERK activation at 1 μM (lanes d−f). The high concentration of Wortmannin required for inhibiting ERK1/2 activation is indicative of other pathways or of nonspecific effects. IL-8-induced ERK activation in HEK-293 cells expressing R1 receptor was also not inhibited by 0.1 μM concentration of Wortmannin (data not shown). The role of PI3-kinases in neutrophil functions has been controversial, since Wortmannin treatment has been shown to inhibit (*24*, *25*) or not inhibit neutrophil chemotaxis (*26*, *37*). As hypothesized by Neptune and Bourne for IL-8 R2 (*37*), our observations indicate that in CHO R1, CHO R2, and HEK-293 R1 cells, and possibly in other cells, IL-8 receptors have the potential to signal either through a novel PI3-kinase,

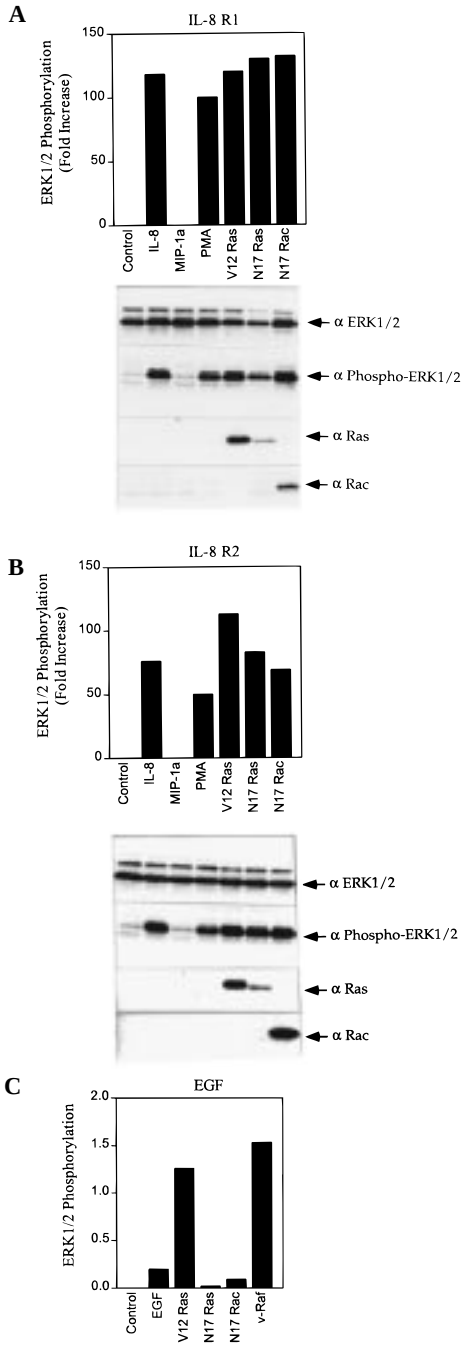

FIGURE 4: Role of Ras and Rac1 in ERK1/2 activation by IL-8 and EGF. CHO R1 (A) and R2 (B) cells were transfected for 16 h with the respective cDNA constructs in pcDNA3 vectors, starved overnight, and treated with 10 nM IL-8. Treatments for nontransfected cells include control lanes with no IL-8 treatment, IL-8-denoted lanes indicate treatment with 10 nM IL-8, MIP-1α denoted lanes with 10 nM MIP-1α, and PMA denoted lanes with 100 nM PMA. All treatments were for 10 min. CHO R1 and R2 cells transfected with V12-Ras, N17-Ras, and N17-Rac were treated with 10 nM IL-8 for 10 min. Western analysis was performed with the indicated antibodies. (C) CHO R1 cells transfected with V12-Ras, N17-Ras, N17-Rac, and v-Raf and the nontransfected CHO R1 cells were treated with 100 nM EGF for 10 min to analyze activation of ERK1/2 through endogenous EGF receptor. Control cells did not receive EGF. Similar results were obtained for EGF treatment of CHO R2 cells. The graphs represent the ratio of phosphorylated ERK1/2 to nonphosphorylated ERK1/2 calculated for the fold increase over untreated control. The data shown are typical of two independent transfection experiments.

which is less sensitive to Wortmannin, or through a PI3-kinase-independent pathway, which then proceeds through MEK1/2 to activate ERK1/2. SB202190 is a specific inhibitor for the p38 group of MAP kinase (*40*) and as expected did not inhibit ERK1/2 activation (lanes g).

Pathways Leading to ERK1/2 Activation: Role of Ras. In HEK-293 R1 and R2 cells, treatment with IL-8 results in association of the receptors with the PTX-sensitive $G\alpha_{i2}$ subunit (*29, 30*). To date, all of the $G\alpha_i$ signaling pathways leading to ERK activation have been shown to proceed through Ras and Raf (*5*). Therefore, we examined the involvement of Ras and Rac1 on IL-8-mediated ERK1/2 activation in CHO R1 and R2 cells. As shown in Figure 4A, CHO R1 and, Figure 4B, CHO R2 cells transfected with dominant-negative mutant form of $p21^{Ras}$ (N17-Ras) did not attenuate ERK1/2 activation, suggesting that Ras might not play a role in IL-8 signaling in these cells. The expression of constitutively active V12-Ras also did not enhance IL-8-mediated ERK1/2 activity, confirming that ERK1/2 activation in these cells is a Ras-independent phenomenon. Rac1 is a downstream mediator of Ras signaling, which regulates the non-ERK1/2 MAP kinase pathway (*41*). Accordingly, expression of a dominant-negative form of Rac1 (N17-Rac1) did not inhibit ERK1/2 activation. In a parallel set of experiments, CHO R1 and R2 cells were treated with epidermal growth factor (EGF) to activate ERK1/2 through the endogenous EGF receptor. As shown in Figure 4C, EGF induced moderate activation of ERK1/2, which was completely inhibited in the presence of N17-Ras, and potentiated in the presence of V12-Ras and v-Raf in CHO R1 cells. These observations confirmed that the mutant forms of the proteins are functionally effective in CHO R1 and R2 cells. In summary, through overexpression of mutant forms of $p21^{Ras}$, we demonstrate that IL-8-induced ERK1/2 activation in CHO R1 and R2 cells is independent of Ras.

Pathways Leading to ERK1/2 Activation: Role of Raf. Raf kinases are one of the upstream activators of MEK-1/2, and $\beta\gamma$ subunits of G proteins have been shown to interact with Raf-1 kinase (*42*). Therefore, we examined the involvement of Raf-1 in IL-8-dependent activation of ERK1/2 by overexpression of dominant-negative and constitutively active forms of Raf. The carboxy-terminal catalytic domain of Raf-1 has been truncated (NΔ-Raf) to generate a dominant-negative form of Raf coding for 278 aa of the amino-terminus (*20*). The amino terminal regulatory domain has been deleted to obtain a constitutively active 328 aa carboxy-terminal coding form, the v-Raf. The IL-8-dependent activation of ERK1/2 in CHO R1 and R2 cells was not affected by the overexpression of mutant forms of Raf-1 kinase (Figure 5). In parallel experiments, ERK activation by EGF was inhibited by overexpression of NΔ-Raf, and potentiated by v-Raf, indicating that the Raf mutant proteins were effective (Figure 5). These results through overexpression of specific individual proteins suggest that depending on the available intracellular signaling proteins, IL-8 R1 and R2 receptors can utilize upstream activators of MEK1/2 other than Raf-1. It is possible that other Raf family members including MEK kinases (MEKK) and B-Raf might play a role in the activation of MEK1/2 (*24, 43*).

Alternative Pathways for ERK1/2 Activation: Role of PLC, PKC, and PKA. In COS cells expressing R1 and R2 receptors and HEK-293 cells expressing R1 receptors, IL-8 treatment results in activation of PLC and increased inositol turnover (*34, 35*). In addition, $\beta\gamma$ subunits of $G\alpha_i$ proteins

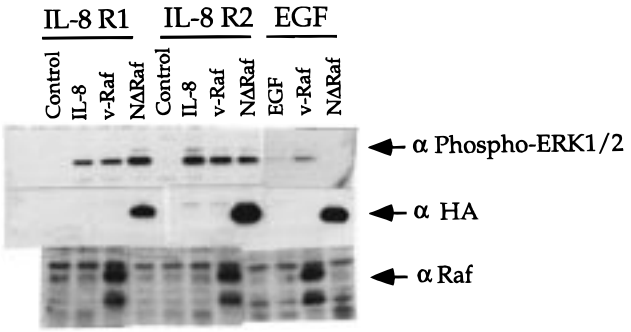

FIGURE 5: Role of Raf-1 kinase in IL-8 R1 and R2 mediated ERK1/2 activation. CHO R1 and R2 cells were transfected for 16 h with HA epitope-tagged NΔ-Raf and untagged v-Raf cDNA constructs in pcDNA3 vectors, starved overnight, and treated with 10 nM IL-8 for 10 min. Control lanes received no treatment; IL-8-denoted lanes received 10 nM IL-8 for 10 min. v-Raf- and NΔ-Raf-denoted lanes were transfected with the respective cDNA constructs and were treated with 10 nM IL-8 for 10 min. In the EGF-marked panel, all lanes received 100 nM EGF, while lanes marked v-Raf and NΔ-Raf were transfected with the respective cDNA constructs. For Western analysis, pertinent strips were excised and reacted with the respective antibodies to determine the expression level of transfected cDNA. The data shown are typical of two independent transfection experiments.

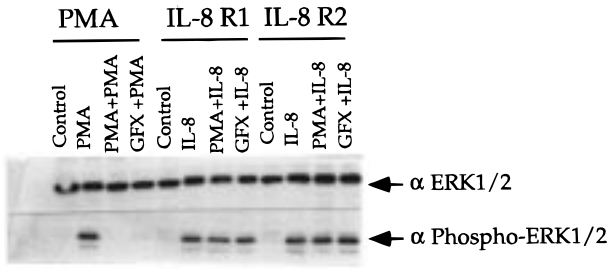

FIGURE 6: Role of PKC in IL-8 R1 and R2 signaling. CHO R1 cells were induced with 100 nM PMA for 15 min with or without 20 h pretreatment with 100 nM PMA, and 2 μM GF109203X for 15 min. CHO R1 and R2 cells were treated with 10 nM IL-8 for 10 min, with or without 20 h pretreatment with 100 nM PMA, and 2 μM GFX for 15 min. The data shown are typical of two independent experiments.

have been shown to interact with PLC-β isoforms (*22*). However, treatment of CHO R1 and R2 cells with D609 and U73122 inhibitors of PLC family members did not inhibit IL-8-mediated ERK1/2 activation (data not shown), suggesting that some of the PLC isoforms may not play a role in ERK activation. IL-8 initiated neutrophil chemotaxis, and phagocytosis is inhibited by PKC inhibitors (*44*). Chronic stimulation with PMA, which depletes cells of diacylglycerol-regulated PKCs, has been used extensively to implicate PKCs in response to agonists (*5*). In CHO R1 and R2 cells, chronic pretreatment with PMA abrogated ERK1/2 activation by acute treatment with PMA (Figure 6). Pretreatment with the PKC inhibitor GF109203X completely inhibited ERK activation by acute treatment with PMA. IL-8-dependent ERK1/2 activation in CHO R1 and R2 cells was not inhibited by chronic pretreatment with PMA (Figure 6). Accordingly, pretreatment with GF109203X also had no effect on IL-8-mediated ERK1/2 activation (Figure 6). This suggests that PMA-depletable and GF109203X-inhibitable forms of PKC may not be playing a critical role in IL-8-dependent ERK1/2 activation. The possible role of the PTX-sensitive $G\alpha_i$ subunit through its inhibition of adenylyl cyclase was examined. CHO R1 and R2 cells pretreated with sp-8-Br-cAMP, a metabolically stable form of cAMP, did not inhibit IL-8-mediated ERK1/2 activation (data not shown), ruling out a critical role for the Gα subunit-regulated PKA. In summary, studies with inhibitors of the PLC and PKC pathway suggest that many of the common isoforms of these enzymes might not be the upstream mediators of IL-8-dependent ERK activation. The results also suggest an active role for $\beta\gamma$ subunits, since cAMP levels do not influence ERK activation.

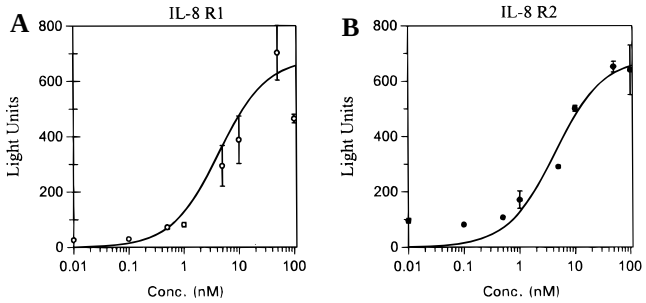

FIGURE 7: bDNA analysis of IL-8-dependent hamster c-fos mRNA increase in CHO R1 and R2 cells. Cells were seeded in a 96 well plate and the following day starved in 0.5% serum-containing media for 24 h. The cells were then treated with increasing concentrations of IL-8 and incubated for 40 min, and lysed in a solution including the antisense oligonucleotides for hamster c-fos mRNA. The lysate was transferred to a bDNA assay plate and handled according to the manufacturer's protocol. The signal was measured in terms of light units generated by alkaline phosphatase release of chemiluminescent product. The data show the fold increase in light units over the untreated sample. The data are the mean ± SD of a representative experiment, and the results were replicated 3 times.

Effect of IL-8 Concentration on Increase in c-fos mRNA. The downstream effects of ERK1/2 activation include translocation of ERK1/2 to the nucleus and phosphorylation of transcription factors such as TCF/Elk-1, which in turn increase c-fos transcription (*8*). We have utilized the branched DNA (bDNA) technology to quantitate changes in endogenous c-fos mRNA levels upon stimulation with IL-8. As shown in Figure 7A, B, treatment of CHO R1 and R2 induces a 20-fold increase in c-fos mRNA with an EC_{50} of 6.68 ± 2. 5 nM for R1 and 2.3 ± 0. 84 nM for R2. This is comparable to the reported receptor binding affinities with IL-8 of 4 and 0.4 nM for R1 and R2 (*27*, *28*) and EC_{50} values of 3.1 and 0.4 nM for cAMP inhibition by R1 and R2, respectively (*32*). Increase in c-fos through a diverse array of reagents results in cell proliferation and/or differentiation (*5*).

DISCUSSION

Besides neutrophils, IL-8 receptors have been detected on monocytes, basophils, peripheral blood lymphocytes, erythrocytes, melanocytes, and melanoma cells (*1*). The varied effects of IL-8 on different tissues suggest functional variations dictated by the repertoire of endogenous signaling molecules. To understand the possible mediators of IL-8 signaling via IL-8 R1 and R2, we have used CHO cells stably expressing IL-8 receptors R1 and R2 to study the signaling pathways of ERK1/2 activation. We aimed at understanding the functional differences of the receptors through independent heterologous expression of the two receptors. Both receptors in neutrophils and HEK-293 cells signal through the PTX-sensitive $G\alpha_i$ subunit and also through the coex-

pressed PTX-insensitive $G\alpha_{16}$ in HEK-293 and COS cells. CHO cells have endogenous $G\alpha_{i2}$ (*32*) and have been used to demonstrate signaling pathways initiated by $\beta\gamma$ subunits of $G\alpha_i$-interacting $\alpha1$ β adrenergic, somatostatin, and 5-HT_{1A} receptors (*9*, *18*, *45*, *46*). These studies suggest that CHO cells expressing R1 and R2 provide a good heterologous system to examine signaling by individual receptors.

Based on the currently known signaling pathways for $G\alpha_i$ (*47*), a $\beta\gamma$ subunit-dependent, PI3-kinase-dependent, Ras-mediated activation of ERK1/2 was anticipated. However, in the present study we have obtained some unusual results. The IL-8-dependent activation of ERK1/2 is (a) sensitive to PTX, (b) independent of PI3-kinase, as shown by lack of inhibition by Wortmannin, (c) independent of Ras, as shown with mutant forms of Ras, (d) independent of Raf-1, as shown with mutant forms of Raf, (e) dependent on MEK activation, since it is inhibited by MEK-1 inhibitor, and (f) effective in induction of c-fos. This suggests several diverging and converging pathways:

(i) The PTX-sensitive activation of ERK implicates receptor signaling through $G\alpha_i$ or $G\alpha_o$ subunits. In CHO cells expressing platelet activating factor receptor and M_1 muscarinic acetylcholine receptor, Ras-independent ERK activation has been reported (*10*, *48*). For both these receptors, a $G\alpha_o$ subunit-dependent PKC-mediated pathway was postulated (*10*). Our results parallel the above observations in terms of PTX-sensitive, Ras-independent activation of ERK1/2 by IL-8, which might suggest a role for $G\alpha_o$. However, we do not believe that $G\alpha_{i/o}$ subunits play an active role for ERK activation by IL-8, since ERK1/2 activation is not inhibited either by stable analogues of cAMP or by GF109203X, an inhibitor of PKC.

(ii) The $\beta\gamma$ subunits of the PTX-sensitive $G\alpha_i$ have been shown to interact with and activate PLC-β2 and -β3 isoforms (*22*). This pathway would be PTX-sensitive because of the participation of $G\alpha_i$ and independent of Ras through the PLC pathway. In CHO R1 and R2 cells ERK activation is not inhibited by pretreatment with the PLC inhibitors D609 and U-73122, indicating that some forms of PLC are not involved.

(iii) The inability of dominant-negative Raf-1 to inhibit IL-8-mediated ERK activation suggests a role for B-Raf and other Raf-like molecules including the MEKKs. MEKK1 and MEKK3 have been shown to activate ERK through MEK (*43*, *49*). Additional kinases, which have been proposed to function as MEKKs, include germinal center kinase (GCK), p21-activated kinase (PAK), TGF β-activated kinase (TAK), tumor progression locus-2 (Tpl-2), mixed lineage kinase (mlk-3), and apoptosis signal regulating kinase 1 (ASK1) (*49*). Recently, a Ras- and Raf-1-independent, Rap- and B-Raf-mediated ERK activation has been described in PC12 cells (*50*). In addition to Raf-1 kinase (*42*), PLC-β2 and -β3 isoforms (*22*), and the PAK family of kinases (*51*), the $\beta\gamma$ subunits of G proteins also interact with several other tyrosine kinases in mammalian cells (*14*), some of which could be regulated by Ca^{2+} (*52*).

(iv) IL-8 induced an increase of fos mRNA by both CHO R1 and R2, confirming the physiologically relevant downstream effect of ERK activation.

(v) GPCRs have been shown to couple to different classes of G proteins depending on cell type, thus controlling a wide spectrum of signaling cascades. The recent observation regarding the ability of PTX-insensitive $G\alpha_s$ to regulate phosphorylation of the GPCR to activate PTX-sensitive $G\alpha_i$ has brought in an additional degree of complication in GPCR signaling (*53*). Accordingly, it could be postulated that in CHO cells expressing IL-8 receptors, the PTX-sensitive $G\alpha_i$-initiated signal could influence $G\alpha_q$-mediated signaling to activate a PI3-kinase, Ras, and Raf-1 independent pathway. This hypothesis is compatible with the observation that in HEK-293 and in COS cells R1 and R2 receptors have been shown to interact with the $G\alpha_{16}$ subunit which signals via the PLC pathway (*34*, *35*).

Our observations with CHO R1 and R2 examining possible mediators in the activation of ERKs are consistent with the recent reports by Neptune et al. (*37*) and Arai et al. (*54*) describing a PTX-sensitive $\beta\gamma$ subunit-mediated chemotaxis. While it is unlikely that cell proliferation, phagocytosis, and chemotaxis mediating pathways, typical for neutrophils, are present in CHO cells (a fibroblast cell line) or in HEK-293 cells (an epithelial cell line), the PTX sensitivity and ERK activation in these cell lines suggest a physiologically relevant signaling pathway. Although the specific role for activated ERKs in IL-8 signaling has been unclear, the presence of viral analogues of IL-8 receptors which are constitutively active and lead to cell proliferation (*55*) indicates a less understood but critical role for ERKs.

G protein-mediated signaling can be looked upon as a highly complex, organized signaling network that allows some cross-talk, owing to diverging and converging transaction steps. The realization that the GPCR signaling pathways are intertwined with and influence growth factor and cytokine-activated signaling pathways has added an additional dimension of complexity.

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