ERK1 and ERK2 Activation by Chemotactic Factors in Human Eosinophils Is Interleukin 5-dependent and Contributes to Leukotriene C4 Biosynthesis*

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Eosinophils, the major immune effector cells contributing to allergic inflammation and asthma, are profoundly affected by interleukin (IL) 5 with respect to their differentiation, viability, recruitment, and cytotoxic effector functions. IL-5 enhances eosinophil responsiveness to a variety of chemotactic factors via a process called priming, although the molecular mechanism is unknown. In this study, we report that, following IL-5 priming of eosinophils, chemotactic agents including fMet-Leu-Phe, IL-8, and RANTES, promote vigorous transient activation of ERK1 and ERK2. In contrast, these chemotactic factors stimulate weak or indiscernible ERK activation in unprimed eosinophils. Furthermore, this intracellular marker of priming is selective for IL-5-related cytokines, in that it is observed following exposure to IL-5 and granulocyte macrophage-colony stimulating factor but not to interferon-γ, stem cell factor, tumor necrosis factor α, or IL-4. Interestingly, priming of chemoattractant-induced ERK activation is accompanied by an increase in association of tyrosine-phosphorylated proteins with the adapter protein Grb2. The biological relevance of ERK activation to IL-5 priming is supported by the observation that inhibition of ERK activity by treatment with the MEK inhibitors PD98059 or U0126 inhibited the release of leukotriene C4 stimulated by fMet-Leu-Phe in IL-5-primed eosinophils. These data provide evidence for a previously undescribed fundamental mechanism by which stimulation of IL-5 family receptors induces a rapid phenotypic alteration in the signal transduction pathways of chemotactic receptors, enabling their activation and the capacity of these cells to synthesize LTC4.

Asthma is an inflammatory disease of the airway characterized by pronounced eosinophilia and elevated levels of IL-5.1 The eosinophil, a granulocytic leukocyte important in the immune surveillance of tissues with a mucosal epithelial interface with the environment, is the major inflammatory effector cell in the chronic inflammation associated with allergic disorders and asthma (1, 2). IL-5 is the principal cytokine regulating eosinophils, profoundly affecting their differentiation, viability, recruitment, and cytotoxic effector functions (3). Following in vitro incubation with IL-5, several eosinophil responses to chemotactic factors are augmented via a process called priming. For example, IL-5 priming enhances the capacity of blood eosinophils to undergo fMLP-induced chemotaxis, cytotoxicity, respiratory burst, and release of proinflammatory lipid mediators (4–8). One mechanism by which eosinophils may contribute to the pathogenesis of asthma is through their ability to synthesize and release a specific class of proinflammatory lipid mediators, the sulfidopeptide leukotrienes. These products, arachidonic acid metabolites of the 5-lipoxygenase pathway, are potent bronchoconstrictive agents and promote mucous secretion as well as increased vascular permeability (9).

Among the chemotactic factors to which eosinophils respond are IL-8, RANTES (an acronym for regulated upon activation normal T-cell expressed and secreted), and formyl peptides such as n-formylmethionylleucylphenylalanine (fMLP) (10–12). IL-8 and RANTES are chemokines of the C-X-C and C-C families, respectively (13). The synthetic agent, fMLP, is an analogue of N-formyl peptides that are released by bacteria and from mitochondria of dead eukaryotic cells (14). All three of these chemotactic stimuli have been reported to initiate responses in cells by binding to G-protein coupled receptors (GPCR) that are characterized by seven-membrane spanning domains coupled with heterotrimeric G-proteins. Subsequent intracellular signaling processes, including stimulation of calcium mobilization and phospholipase C activity, are sensitive to inhibition by pertussis toxin, suggesting involvement of either Gαi family of G proteins (15–18).

The intracellular mechanisms by which IL-5 enhances the ability of eosinophils to respond to chemotactic factors are unknown and are the subject of this study. The human IL-5 receptor is a member of the cytokine family of transmembrane proteins and is composed of two receptor subunits, α subunit which is specific for IL-5 and a β-subunit which is identical to β-subunits of the human IL-3 and granulocyte macrophage-colony stimulating factor (GM-CSF) receptors (19). Signal transduction through these receptors in eosinophils is believed to occur via activation of cytoplasmic tyrosine kinases including Jak1 (20), Jak2 (21–23), Lyn (24), and Syk (25). Subsequent intracellular processes encompassing activation of small G-proteins (24), serine-threonine protein kinases (23, 24, 26, 27), phosphatases (28), phosphatidylinositol 3-kinase (27), adapter proteins (29), and transcription factors (22, 30) result in mod-

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The abbreviations used are: IL-5, interleukin 5; GM-CSF, granulocyte macrophage-colony stimulating factor; RANTES, regulated upon activation normal T-cell expressed and secreted; LTC4, leukotriene C4; GPCR, G-protein-coupled receptor; IFN-γ, interferon-γ; ERK, extracellularly regulated kinase; MAP kinase, mitogen-activated protein kinase; fMLP, fMet-Leu-Phe; PMA, phorbol 13-myristate 13-acetate; PAGE, polyacrylamide gel electrophoresis.

1 The abbreviations used are: IL-5, interleukin 5; GM-CSF, granulocyte macrophage-colony stimulating factor; RANTES, regulated upon activation normal T-cell expressed and secreted; LTC4, leukotriene C4; GPCR, G-protein-coupled receptor; IFN-γ, interferon-γ; ERK, extracellularly regulated kinase; MAP kinase, mitogen-activated protein kinase; fMLP, fMet-Leu-Phe; PMA, phorbol 13-myristate 13-acetate; PAGE, polyacrylamide gel electrophoresis.

ulation of eosinophil phenotypic properties and inflammatory capacity.

In a variety of inflammatory conditions, both IL-5 and chemotactic factors exist simultaneously in the microenvironment (32). This observation, when considered with the divergent roles played by the chemotactic factors and the hematopoietic cytokines in the regulation of eosinophil biology, suggests that intracellular mechanisms exist to integrate the activities of these two different receptor classes. In this report, we show that ERK1 and ERK2 are activated in response to three chemotactic factors in IL-5-primed and GM-CSF-primed blood eosinophils, whereas the level of activity in unprimed eosinophils is weak or undetectable. This characteristic is specific to IL-5 family cytokines since other eosinophil-active cytokines including IL-4, interferon (IFN) γ, stem cell factor, and tumor necrosis factor (tumor necrosis factor-α) do not prime eosinophils for subsequent MIP-1α-stimulated ERK1 and ERK2 activation. Inhibition of eosinophil ERK activity by treatment of the cells with the MEK inhibitors PD98059 or U0126, resulted in inhibition of the ability of the IL-5-primed cells to synthesize LTC4 in response to MIP-1α. This study suggests that cytokine receptor systems are critical regulators in terms of potentiating the ability of GPCR to activate ERK1 and ERK2 in human eosinophils and support a key role for IL-5 as a regulator of multiple intracellular processes that contribute to the inflammatory capacity of the eosinophil in asthma.

**EXPERIMENTAL PROCEDURES**

**Materials—**Reagents for eosinophil preparation included Percoll, which was purchased from Amersham Pharmacia Biotech and anti-CD16-conjugated microbeads which were purchased from Miltenyi Biotechnology (Auburn, CA). IL-4, IL-5, IL-8, RANTES, IFN-γ, tumor necrosis factor-α, and stem cell factor were purchased from R&D Systems (Minneapolis, MN). Sigma was the source for phosphatase inhibitors, PMA and MIP-1α. Protease inhibitor tablets (Complete™) were obtained from Roche Molecular Biochemicals (Indianapolis, IN). We purchased the MEK inhibitor PD98059 or Calbiochem (La Jolla, CA) and U0126 from Promega (Madison, WI). Immunoblotting reagents came from a variety of suppliers including Santa Cruz Biotechnology, Sigma, CA (horseradish peroxidase-conjugated goat anti-rabbit IgG, agarose-conjugated Protein A and anti-Grb2); Kirkegaard & Perry Laboratories, Gaithersburg, MD (Lumiglo™ chemiluminescence substrate reagents); Promega Life Sciences, Madison, WI (anti-Active™ MAPK antisera raised against the dual phosphorylated activation motif of ERK1 and ERK2), and Upstate Biotechnology Co., Lake Placid, NY (antianthropoietin receptor clone 4G10 and anti-ERK1/CT antisera). This latter immunochromogenic was raised against a C-terminal amino acid fragment of rat ERK1 and recognizes human ERK1 with greater affinity than ERK2.

**Isolation of Human Eosinophils from Peripheral Blood—**Blood donors included individuals who were both atopc and non-atopic with eosinophils comprising between 3 and 12% of their peripheral blood leukocytes. Eosinophils were purified from the heparinized peripheral blood of volunteer donors as described previously (29). Briefly, a granulocyte mixture was obtained from the leucocyte buffy coat after centrifugation through a Percoll solution (density 1.090 g/ml) and, after lysis of erythrocytes by hypotonic shocks, the suspension was depleted of neutrophils by incubation with anti-CD16-conjugated microbeads and exposure to a magnetic field. The recovered eosinophils were resuspended, at a concentration of 10^7 cells/ml, in Hank’s balanced salt solution supplemented with 1% human serum albumin. These cell preparations were between 94 and 99% eosinophils as determined by microscopic examination of Wright’s Stained cytofuge preparations.

**Eosinophil Stimulation and Preparation of Cell Lysates—**Eosinophils were preincubated at 37 °C with cytokine or control buffer as indicated by each experiment. Following this incubation, the cells were stimulated for various times with chemotactic agents at 37 °C and diluted with ice-cold Buffer A (20 mM Tris, 137 mM NaCl, 1 mM EDTA, 0.1 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM β-glycophosphate and protease inhibitors, pH 7.4). Cells were pelleted, the supernatants discarded, and the cell pellets resuspended in Buffer B (1% Triton X-100, 0.25% deoxycholate, and 0.1% SDS in Buffer A). Following incubation on ice for 10 min, the eosinophil lysates were centrifuged to remove the insoluble material and the supernatants were prepared for immunoblotting or immunoprecipitation.

**Immunoprecipitation—**Eosinophil lysates were precleared for 2 h at 4 °C with agarose-Protein A and incubated with 2 μg of anti-Grb2 antiserum or rabbit IgG for 2 h. The immune complexes were captured with agarose-Protein A and washed with six changes of Buffer B. The agarose beads were resuspended in electrophoresis sample buffer prior to electrophoresis and immunoblotting.

**SDS-PAGE and Immunoblotting—**Eosinophil lysates or immunoprecipitates were diluted with electrophoresis sample buffer and the proteins were resolved on polyacrylamide slab gels. The lanes were loaded with equal amounts of protein. Transfer to polyvinylidene difluoride membrane and immunoblotting were conducted using standard methods. The consistency of protein loading in all lanes was evaluated by staining of the polyvinylidene difluoride membrane with Amido Black following autoradiography. In addition, samples were immunoblotted with control antisera (anti-ERK1/CT or anti-Grb2) to confirm that comparable mass of protein was present in all samples.

**Densitometric Analysis of Chemilumino grams—**Images were converted to a digital format by scanning with Adobe Photoshop 5.0 software or transilluminated digital photography. Relevant area of the images were analyzed by determination of image density with NIH Image software.

**Eosinophil LTC4 Release—**Eosinophils (125 μl of a suspension containing 3.25 × 10^6/ml) were incubated in triplicate with or without MEK inhibitors for 1 h at 37 °C and incubated for an additional hour following dilution with and equal volume of IL-5 (primed eosinophils) or buffer alone (unprimed eosinophils). Cell suspensions were subsequently stimulated for 20 min by an additional volume of control buffer, fMLP (0.1 μM final concentration) or the calcium ionophore A23187 (0.1 μM final concentration) and the supernatants were collected by centrifugation (320 × g for 5 min). The LTC4 content of the supernatants was determined as an assay of immunoreactive LTC4 by enzyme-linked immunosorbent assay (Cayman Chemical Co., Ann Arbor, MI). All supernatants were assayed in duplicate.

**LTC4 Data Summary and Statistical Analysis—**Results of the LTC4 assays on each individual patient were expressed in picogram/ml, and summarized as the mean ± S.D. for triplicate aliquots of cells. To summarize results of LTC4 analysis on multiple patients, the data for each patient were first normalized by expressing LTC4 (picogram/ml) of each cell treatment as a percent of LTC4 generated by control treatment of that patient's eosinophils in the absence of priming or inhibitors. The resulting values (%) of control) for multiple patients were summarized as the mean ± S.E. for graphic presentation. For statistical analysis, un-normalized data (picogram/ml) were logarithmically transformed and analyzed as a 3-way factorial design with individual patients considered as a random effect by S-Plus statistical software. Statistical significance was measured by 95% simultaneous confidence intervals.

**RESULTS**

**Time Course of ERK1 and ERK2 Activation by IL-5—**As previously reported by our laboratory and others (24, 27, 33), IL-5 stimulates the activation of ERK1 and ERK2 in human eosinophils isolated from peripheral blood. The kinetics of this activation is shown in Fig. 1 utilizing, as a measure of ERK1 and ERK2 activation, immunoblotting with anti-Active MAPK antisera. Labeling by this antibody has been demonstrated to closely correlate with kinase activity (34). Immunodetectable levels of active ERK1 and ERK2 reached maximum within 5 to 10 min of stimulation with IL-5. The apparent levels of active ERK1 and -2 in IL-5-stimulated cells was always less than that seen with 50 nM PMA indicating that IL-5 does not maximally stimulate ERK activation in eosinophils. We observed a degree of patient-to-patient variability in the duration of ERK activity following IL-5 stimulation (data not shown), with some patients displaying more prolonged ERK1 and ERK2 activation, whereas in other patients, IL-5 had returned to near pre-stimulation levels by 60 min after addition of IL-5.

**Preincubation with IL-5 Increases MIP-1α-induced ERK1 and ERK2 Activation in Human Eosinophils—**Following priming with IL-5, eosinophil responsiveness to MIP-1α is enhanced with respect to chemotaxis, superoxide anion production, and LTC4 release (4–7). To investigate the potential molecular mecha-
nisms associated with priming, we determined if ERK activation is a feature of fMLP-induced signaling in eosinophils and if IL-5 priming affects the ability of eosinophils to stimulate ERK1 and ERK2 activity following fMLP exposure. In these experiments, eosinophils were primed with control buffer or 1 nM IL-5 for 1 h and then stimulated with 100 nM fMLP for various times. In the absence of IL-5 priming, immunoblotting with anti-Active MAPK antisera did not detect dually phosphorylated ERK1 or ERK2 in eosinophil lysates, either in the presence or absence of fMLP stimulation (Fig. 2A, lanes 1–4). However, stimulation by PMA induced a very strong response in these unprimed cells (Fig. 2A, lane 5) indicating that the MAP kinase pathway was intact and responsive to other factors. Interestingly, priming of the eosinophils with IL-5 now allowed for fMLP treatment to cause a rapid increase in ERK1 and ERK2 activity (Fig. 2A, lanes 7 and 8). This activation of ERK1 and ERK2 in IL-5-primed eosinophils was transient in that the signal had returned to basal levels within 15 min of fMLP addition (Fig. 2A, lane 9). Immunoblotting of the same samples with anti-ERK1 and ERK2 antisera confirmed equivalent protein loading in the samples (Fig. 2B). Enhancement of fMLP-stimulated ERK1 and ERK2 activity by IL-5 priming was observed in the eosinophils of at least 15 different blood donors. These data suggest that activation of upstream elements of the MAP kinase pathway is one of the mechanisms by which IL-5 modulates fMLP-induced responses in eosinophils.

**Kinetics and Dose Response of IL-5 Priming of fMLP-stimulated ERK1 and ERK2 Activity**—To further characterize these priming responses, the time course (Table I) and dose response (Table II) of ERK1 and ERK2 activation following priming and fMLP stimulation were determined. Stimulation of IL-5-primed eosinophils with fMLP for 2 min resulted in a further increase of ERK1 and ERK2 activity for priming times tested (Table I). Enhancement of ERK activation following fMLP stimulation was evident following 2 min of priming by IL-5, increased during the course of the incubation and was still clearly evident following 60 min of priming. These data (Table I) demonstrate that the intracellular processes mediating IL-5 priming of this fMLP response is initiated rapidly and therefore, in all probability, not wholly dependent on IL-5-mediated regulation of transcriptional processes.

The priming of ERK1 and ERK2 activation by fMLP was dependent on the concentration of IL-5 (Table II). Increased ERK1 and ERK2 activation was evident following priming with 1 to 1000 pM IL-5. This range of IL-5 concentrations mirrors those reported to promote the priming of fMLP-stimulated eosinophil activation including LTC4 generation, superoxide release, and chemotaxis (4–8). Priming with fMLP Does Not Enhance ERK1 and ERK2 Activation in Response to IL-5—To confirm that the increase in ERK1 and ERK2 activity in IL-5 primed eosinophils was not simply due to an additive effect of two stimuli, a reverse priming protocol was employed. Eosinophils were primed for 1 h with control buffer, 1 nM IL-5 or 100 nM fMLP and subsequently stimulated with control, IL-5, fMLP, or PMA (as a positive control). As shown in Fig. 3, unprimed (Cont.) eosinophils did not respond to fMLP with vigorous ERK activation whereas, in IL-5-primed eosinophils, ERK activation was evident (compare Fig. 3, lane 2 versus lane 6). However, treating eosinophils with fMLP first did not enhance responsiveness to IL-5. The modest increase in ERK1 and ERK2 activation seen following a 5-min stimulation with IL-5 in fMLP-primed eosinophils (Fig. 3, lane 11) was similar to that observed in unprimed cells (Fig. 3, lane 3). These data are consistent with a model in which selected signaling processes must first be activated by IL-5 to enable chemotactic factors to stimulate ERK1 and ERK2.

![Fig. 1. Time course if IL-5 stimulation of ERK1 and ERK2 activity.](image)

**FIG. 1.** Time course if IL-5 stimulation of ERK1 and ERK2 activity. Eosinophils were incubated for various times with control buffer (lanes 1, 3, 5, 7, 9, and 11), 1 nM IL-4 (lanes 2, 4, 6, 8, 10, and 12), or 80 nM PMA for 10 min (lane 13). The cells were lysed, prepared for SDS-PAGE, and immunoblotted with antisera specific for the dual phosphorylated form of ERK1 and ERK2 (anti-Active MAPK) which labels pTEpY of enzymatically active ERK1 and ERK2.

<table>
<thead>
<tr>
<th>Time of priming incubation</th>
<th>Increase in fMLP-stimulated ERK1 and ERK2 activation following IL-5 priming</th>
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<tbody>
<tr>
<td>Unprimed</td>
<td>0</td>
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<tr>
<td>2 min</td>
<td>24</td>
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<tr>
<td>5 min</td>
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<td>30 min</td>
<td>90</td>
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<tr>
<td>60 min</td>
<td>80</td>
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**TABLE I**

<table>
<thead>
<tr>
<th>Time of priming incubation with 1 nM IL-5</th>
<th>Increase in fMLP-stimulated ERK1 and ERK2 activation following IL-5 priming</th>
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<tr>
<td>Unprimed</td>
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<td>2 min</td>
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<td>60 min</td>
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**ERK Activation by Chemoattractants in Eosinophils is IL-5-dependent**

The Priming of fMLP-stimulated ERK1 and ERK2 Activity Is Not Diminished by IL-5 Removal Prior to fMLP Stimulation—To determine if IL-5 and fMLP need to be present simultaneously to promote optimal ERK1 and ERK2 activation, eosinophils were primed for 1 h with control buffer or IL-5. An additional aliquot of the same cell suspension was incubated with IL-5 for 45 min and then quickly pelleted and washed before resuspending in control buffer. As presented in Fig. 4, subsequent stimulation with fMLP showed that the eosinophil responsiveness to fMLP was undiminished by removing the IL-5 following priming (compare Fig. 4, lane 5 versus lane 8). These data suggest that IL-5 priming induces a phenotypic change in eosinophil function that persists after IL-5 removal. Therefore, the IL-5 need not be continuously present to enhance ERK activation following fMLP stimulation.

**MEK Inhibitors PD98059 and U0126 Reduce ERK Activation by fMLP in IL-5-primed Eosinophils**—We have shown that priming of eosinophils with IL-5 enhances the ability of eosinophils, subsequently stimulated with fMLP, to activate the MAP kinases ERK1 and ERK2 (Figs. 2–4). A key question remaining is whether this intracellular indicator of priming is relevant to the known proinflammatory processes by which IL-5-primed eosinophils contribute to the pathology of asthma and allergic diseases. Eosinophil production of sulfidopeptide leukotrienes is enhanced by priming with IL-5 and these mediators directly contribute to the bronchial hyperresponsiveness and increased vascular permeability characteristic of asthma (7, 9). To determine if production of LTC4 by IL-5-primed eosinophils is regulated by ERK1 and ERK2 activity, we inhibited ERK activation by preincubating eosinophils with either of two pharmacological agents that antagonize the activity of MEKs, the upstream activators of ERK1 and ERK2. These MEK inhibitors, PD98059 and U0126, are chemically unrelated and have been previously characterized by numerous studies (35–38) to effectively reduce the activity of MEK1 and MEK2. Preincubation of eosinophils with 50 μM PD98059 (Fig. 5, lanes 5–8) or 30 μM U0126 (Fig. 5, lanes 9–12) effectively decreased the activation of ERK1 and ERK2 stimulated by fMLP in IL-5 primed eosinophils (Fig. 5, lane 4 versus lanes 1, 2, and 3). Dose response experiments demonstrated that these dosages were the lowest concentrations that effectively and consistently inhibited ERK activity in fMLP stimulated IL-5-primed eosinophils. The figure presented (Fig. 5) is one of five nearly identical separate experiments. The observed inhibition of ERK activity was not due to cytotoxic effects of incubation with these agents because eosinophils were >90% viable, as evaluated by trypan blue exclusion. Furthermore, following treatment with these inhibitors, cells were still capable of activating ERK1 and ERK2 when exposed to a different stimulus, namely 50 nM PMA (data not shown).

**MEK Inhibitors PD98059 and U0126 Decrease LTC4 Release from IL-5-primed, fMLP-stimulated Eosinophils**—The effect of ERK1 and ERK2 inhibition was evaluated with respect to the release of immunoreactive LTC4 from IL-5-primed, fMLP-stimulated eosinophils (Fig. 6, A and B). As previously reported (7), and as demonstrated in Fig. 6A, priming of eosinophils with IL-5 enhanced the release of LTC4 following fMLP stimulation. Furthermore, our data in Fig. 6A demonstrate that preincubation of eosinophils with 50 μM PD98059 significantly reduced LTC4 release by fMLP-stimulated, IL-5-primed eosinophils (p < 0.05, n = 6, Fig. 6A). Under these priming conditions, 50 μM PD98059 is an effective inhibitor of ERK1 and ERK2 activity (Fig. 5).

Because chemical enzyme inhibitors may affect cellular processes unrelated to their intended targets (39), we confirmed our observations, that ERK1 or ERK2 activity appears necessary for LTC4 synthesis stimulated by fMLP in IL-5-primed eosinophils, by using a chemically unrelated inhibitor of the same enzymes, namely U0126. As demonstrated in Fig. 6B, the LTC4 release in fMLP-stimulated, IL-5-primed eosinophils was effectively inhibited by pretreatment of the eosinophils with 30 μM U0126. These data are representative of experiments on three separate eosinophil preparations, all of which showed that U0126 caused 55–90% inhibition of LTC4 release from fMLP stimulated, IL-5-primed eosinophils.

Our observations of LTC4 release in human eosinophils concur with the results reported by Takeguti et al. (7) with respect to the large degree of patient-to-patient variability in responsiveness to fMLP stimulation. All subjects tested showed increased responsiveness to fMLP following IL-5 priming. Whereas, many individuals did not release significant amounts of LTC4 from unprimed eosinophils following stimulation with fMLP, other individuals (as shown in Fig. 6B, for example) released large amounts of LTC4 even in the absence of priming. Among those individuals, LTC4 release from unprimed eosinophils was also inhibited by pretreatment with MEK inhibitors. In primed and unprimed eosinophils, the inhibition of LTC4 synthesis by PD98059 or U0126 is not likely to be due to nonspecific effects on LTC4 biosynthetic enzymes because, in all experiments, eosinophils stimulated with the calcium ionophore A23187 (0.1 μM) released at least 1000 pg/ml immuno-

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**TABLE II**

Dose response of IL-5 used for priming of ERK1/2 activation in fMLP-stimulated eosinophils

Eosinophils were incubated with various concentrations of IL-5 for 1 h, stimulated with fMLP for 2 min, and the cell lysates analyzed as described in Table I. The value for 0 pm IL-5 is defined to be 0% because the density units from these lanes are subtracted from all other treatments.

<table>
<thead>
<tr>
<th>IL-5 concentrations used for priming (1 h)</th>
<th>Increase in fMLP-stimulated ERK1 and ERK2 activity by IL-5 priming</th>
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<tr>
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<td>Experiment 1</td>
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<tr>
<td>0</td>
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<tr>
<td>1</td>
<td>13</td>
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<tr>
<td>10</td>
<td>17</td>
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<tr>
<td>100</td>
<td>57</td>
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<tr>
<td>1000</td>
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a ND, not determined.

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**Fig. 3.** fMLP priming does not enhance IL-5-stimulated ERK1 and ERK2 activity. Eosinophils were primed for 1 h with control buffer (lanes 1–4), 1 nM IL-5 (lanes 5–8), or 100 nM fMLP (lanes 9–12). Cells were then stimulated with control buffer (lanes 1, 5, and 9), 100 nM fMLP (lanes 2, 6, and 10), 1 nM IL-5 (lanes 3, 7, and 11), or 80 nM PMA (lanes 4, 8, and 12). Eosinophils were lysed and samples were immunoblotted with anti-Active MAPK antiseria with lysates of equal numbers of cells loaded in each lane. Immunoblotting of the same samples with anti-ERK1-CT confirmed equal mass of ERK protein in all samples (not shown).
reactive LTC₄ in the presence of either inhibitor (data not shown). Therefore, these data suggest that ERK1 or ERK2 activity contributes to the biosynthesis of LTC₄ by IL-5-primed, fMLP-stimulated eosinophils.

**Additional Chemoattractants, IL-8, and RANTES Enhance ERK1 and ERK2 Activity in IL-5-primed Eosinophils**—We next determined if the priming of ERK1 and ERK2 activation by IL-5 was limited to fMLP-stimulated responses. The fMLP receptor belongs to the class of seven-transmembrane receptors coupled to heterotrimeric G-proteins (GPCR). Eosinophils respond to a variety of stimuli that bind to these GPCRs, including RANTES and IL-8, members of the C-C and C-X-C chemokine families, respectively. Previous studies reported that, as with fMLP, IL-5 priming enhances the ability of eosinophils to respond to both of these factors (4, 11). To determine if these chemokines alter the activity of eosinophil ERKs and if preincubation with IL-5 modulates IL-8-induced or RANTES-induced ERK activation, eosinophils were primed and stimulated with fMLP or chemokines for 2 min. ERK1 and ERK2 activation in primed eosinophils was evident following treatment with fMLP (Fig. 7A), IL-8 (Fig. 7B), and RANTES (Fig. 7C) but was not detectable in unprimed cells (compare lanes 2 and 3 in each panel of Fig. 7). The enhancement of IL-8- or RANTES-induced ERK1 and ERK2 activity was observed for IL-5-primed eosinophils of all of the 4 separate eosinophil preparations examined. The kinetics of IL-8 and RANTES activation of eosinophil ERKs was similar to that observed in fMLP-stimulated primed eosinophils (Fig. 2A), being maximal at 2 min following stimulation and returning to basal levels by 15 min (data not shown). Therefore, like fMLP receptors, C-C chemo-

**Fig. 4. Enhanced ERK activation in IL-5-primed eosinophils is present if the IL-5 is removed by washing prior to fMLP stimulation.** Eosinophils were primed with control buffer (lanes 1–3) or with 1 nM IL-5 (lanes 4–6) for 1 h. Alternatively, eosinophils were incubated with 1 nM IL-5 for 45 min, the cells pelleted and washed and resuspended in control buffer for a further 5-min incubation (lanes 7–9). The eosinophils were then stimulated with control buffer (lanes 1, 4, and 7) or 100 nM fMLP (lanes 2, 5, and 8) or 80 nM PMA (lanes 3, 6, and 9). The cells were lysed and the lanes of an SDS-PAGE gel were loaded with samples of 2.5 × 10⁶ eosinophils. The resulting blot was probed with anti-Active MAPK antisera. Immunoblotting of the same samples with anti-ERK1-CT confirmed equal mass of ERK protein in all samples (not shown).

**Fig. 5. Preincubation of eosinophils with MEK inhibitors PD98059 or U0126 decreases ERK1 and ERK2 activation by fMLP in IL-5-primed eosinophils.** Eosinophils were preincubated at 37 °C in the presence of control buffer (lanes 1–4), 50 μM PD98059 (lanes 5–8), or 30 μM U0126 (lanes 9–12) for 1 h and primed for an additional hour with control buffer (lanes 1, 2, 5, 6, 9, and 10) or 1 nM IL-5 (lanes 3, 4, 7, 8, 11, and 12). Cells were subsequently stimulated with buffer (odd numbered lanes) or 100 nM fMLP (even numbered lanes) for 2 min and prepared for immunoblotting with anti-Active MAPK antisera. Immunoblotting of the same samples with anti-ERK1-CT confirmed equal mass of ERK protein in all samples.

**DISCUSSION**

Eosinophils are the major immune effector cells contributing to allergic inflammation and asthma. Following exposure to IL-5, the eosinophil responsiveness to a variety of chemotactic factors is markedly enhanced; this phenomenon is generally referred to as priming (4–6, 8). The process of priming is likely highly relevant to the cytotoxic capacity of the eosinophil, e.g. it enables the cells to remain in a quiescent state until provoked by the two distinct stimuli. In a number of inflammatory con-
ERK Activation by Chemoattractants in Eosinophils is IL-5-dependent

**Fig. 6.** A, pretreatment with 50 μM PD98059 inhibits LTC4 release stimulated by 100 nM fMLP in IL-5-primed eosinophils. Eosinophils were preincubated with 50 μM PD98059 or control buffer for 1 h, primed for 1 h with 1 nM IL-5 or control buffer, stimulated with 100 nM fMLP for 20 min and assayed for immunoreactive LTC4 as described under “Experimental Procedures.” The data shown following stimulation with 100 nM fMLP (dark bars) or control buffer (open bars) were normalized as % of control within each experiment and summarized as the mean ± S.E. for data on six patients. LTC4 release from IL-5-primed eosinophils stimulated with fMLP was significantly attenuated by preincubation of the eosinophils with 50 μM PD98059 (p < 0.05, n = 6). B, pretreatment with 30 μM U0126 inhibits LTC4 release stimulated by 100 nM fMLP in IL-5-primed eosinophils. Eosinophils were preincubated with 30 μM U0126 and/or IL-5 exactly as described for Fig. 5 and triplicate samples were stimulated with 100 nM fMLP (dark bars) or control buffer (open bars) for 20 min. Cell supernatants were analyzed for LTC4, as described under “Experimental Procedures.” The values of triplicate treatments were averaged and are presented as the mean ± S.D. for each cell treatment category. This experiment is representative of data on three separate eosinophil preparations.

**Fig. 7.** Three chemotactic factors, fMLP, IL-8, and RANTES stimulate the activity of ERK1 and ERK2 in IL-5-primed eosinophils. Eosinophils were primed with 1 nM IL-5 (lanes 3 and 4) or control buffer (lanes 1 and 2) for 1 h and subsequently stimulated for 2 min with control buffer (lanes 1 and 2) or chemotactic factors (lanes 2 and 3) 100 nM fMLP (panel A), 10 nM IL-8 (panel B), or 10 nM RANTES (panel C). Cells were lysed, prepared for SDS-PAGE, and immunoblotted with anti-Active MAPK. Immunoblotting of the same samples with anti-ERK1-CT confirmed equal mass of ERK protein in all samples (not shown).
patients exhibited the ability to weakly activate ERK1 and ERK2 following fMLP stimulation (Figs. 5, 7, and 8) even in the absence of cytokine priming. Regardless of the patient, however, our study demonstrated that the level of ERK1 and ERK2 activation stimulated in eosinophils by fMLP was enhanced by priming of the eosinophils with IL-5-family cytokines.

The immediate downstream effectors of ERK1 and ERK2 in eosinophils are unknown and are the subject of current analysis. This report reveals that incubation of eosinophils with inhibitors of MEK1 and MEK2, the upstream activators of ERK1 and ERK2, results in the inhibition of ERK activation in IL-5 primed (Fig. 5) and fMLP-induced LTC4 release in IL-5-primed eosinophils (Figs. 6, A and B). Since a rate-limiting substrate for LTE4 synthesis is generated by the activity of cytoplasmic phospholipase A2, a previously characterized ERK1 and ERK2 substrate (42), this may represent a critical pathway by which ERK1 and ERK2 may modulate the cytoxic effector functions in the eosinophil. Additional intracellular ERK substrates, characterized in other cell systems, include cytoskeletal proteins (43), transcription factors (44, 45), and other kinases (46). These molecules, in turn, may also contribute to the stimulation of chemotaxis, cytotoxic effector functions, release of lipid mediators, or alterations of gene expression; processes by which the eosinophil contributes to the pathogenesis of asthma.

One issue concerning IL-5 priming of ERK activation by chemotactic factors involves the precise molecular mechanisms at play. IL-5 family receptors and chemotactic GPCR utilize several receptor-proximal intracellular processes to transmit signals to downstream effectors. Chemotactic factors stimulate the activation of isoforms of the G-protein-coupled receptor kinases in many cell systems and these enzymes may be responsible for the rapid desensitization of agonist-occupied receptors (47). The transient nature of ERK activation observed following stimulation by fMLP (Fig. 2), IL-8, and RANTES (data not shown) may be an indication that G-protein-coupled receptor kinase isoforms are present in eosinophils and activated in response to chemotactic factors. One study has suggested that activation of MAP kinase pathways following stimulation of GPCRs may be mediated, in part, by the activity of G-protein-coupled receptor kinases (48).

Several intracellular enzymes are activated by both IL-5 and chemotactic receptors. Both cytokine and chemokine receptor classes are known to stimulate PKC isoforms, phosphatidylinositol 3-kinase activity, Lyn tyrosine kinase, and the adapter proteins Shc and Grb2. A previous study in neutrophils demonstrated that the GM-CSF priming of ERK activation by fMLP was not inhibitable by wortmannin suggesting that, in a related cell system, phosphatidylinositol 3-kinase was probably not a point of integration enabling ERK activation by chemotactic factors in primed cells (49). In many receptor systems, the adapter protein Grb2 facilitates activation of the ERK pathway. This is accomplished through recruitment of proteins that promote the accumulation of the GTP-bound (and active) form of Ras. Our observation that, in primed eosinophils, fMLP enhances the association of tyrosine-phosphorylated proteins with Grb2 suggests that Grb2 may be an upstream activator of this enhanced responsiveness to chemotactic factors. One model by which this interaction could be envisioned is suggested by the studies of Daub et al. (31). In investigating the activation of tyrosine kinases by GPCR in rat-1 cells, a mechanism for ligand-independent transactivation of receptor tyrosine kinases was implied. The results suggested that growth factor receptors may be utilized as downstream mediators of GPCR and, through intracellular cross-talk, the signaling pathways of the growth factor receptors can be stimulated by ligands of GPCR. In an analogous model, it is conceivable that during the priming of eosinophils, IL-5 or GM-CSF receptors act as a scaffold for the assembly of signaling molecules which can subsequently be utilized by GPCR for the activation of ERK1 and ERK2, perhaps through the participation of Grb2. The validity of this model is currently being evaluated. Insights offered by such experiments may further elucidate the molecular mechanisms through which IL-5 family cytokines contribute to the pathobiology of asthma through cross-talk among diverse signaling pathways.

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