

Granulocyte-macrophage colony-stimulating factor and IL-5 activate mitogen-activated protein kinase through *Jak2* kinase and phosphatidylinositol 3-kinase in human eosinophils

Masaki Hiraguri, MD, Satoshi Miike, MD, Hideki Sano, MD,
Kazuhiro Kurasawa, MD, Yasushi Saito, MD, and Itsuo Iwamoto, MD
Chiba, Japan

Mitogen-activated protein (MAP) kinases are activated by the sequential activation of *Ras*, *Raf*, and MEK (MAP kinase kinase) and regulate a wide variety of cell functions. To determine the kinase cascade for granulocyte-macrophage colony-stimulating factor (GM-CSF)- and IL-5-induced MAP kinase activation in eosinophils, we studied the effect of inhibitors of *Jak2* kinase, tyrosine kinases, phosphatidylinositol 3-kinase, and protein kinase C on GM-CSF- and IL-5-induced MAP kinase activation in human eosinophils. GM-CSF and IL-5 activated 40, 42, and 44 kilodalton MAP kinase isoforms in eosinophils. This was indicated by the electrophoretic mobility shift of the three isoforms of MAP kinase in immunoblotting with anti-MAP kinase antibody and also by in-gel MAP kinase assay. MAP kinase activation was time- and dose-dependent, becoming maximal 3 to 15 minutes after stimulation. A *Jak2* kinase inhibitor AG-490, a tyrosine kinase inhibitor genistein, and a phosphatidylinositol 3-kinase inhibitor wortmannin inhibited GM-CSF- and IL-5-induced MAP kinase activation in eosinophils, whereas a protein kinase C inhibitor staurosporine had a weak inhibitory effect. Furthermore, AG-490 and genistein prevented GM-CSF-induced tyrosine phosphorylation of *Jak2* kinase in eosinophils. Taken together, these results indicate that GM-CSF and IL-5 activate MAP kinases through the signaling pathway of *Jak2* kinase-tyrosine phosphorylated β chain-phosphatidylinositol 3-kinase-*Ras* in eosinophils. (J Allergy Clin Immunol 1997;100:S45-51.)

Key words: Granulocyte-macrophage colony-stimulating factor, IL-5, mitogen-activated protein kinase, *Jak2* kinase, phosphatidylinositol 3-kinase, eosinophils

Eosinophils are the principal effector cells of allergic inflammations such as asthma. Granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-5 are eosinophil-active cytokines that regulate the growth and function of eosinophils. Both cytokines induce the differentiation,^{1,2} activation and chemotaxis,^{3,4} and pro-

Abbreviations used

ATP:	Adenosine triphosphate
GM-CSF:	Granulocyte-macrophage colony-stimulating factor
MACS:	Magnetic activated cell sorter
MAP:	Mitogen-activated protein
MEK:	MAP kinase kinase
PI:	Phosphatidylinositol
PMA:	Phorbol myristate acetate
PVDF:	Polyvinylidene fluoride
SDS:	Sodium dodecyl sulfate
SDS-PAGE:	SDS-polyacrylamide gel electrophoresis
STAT:	Signal transducers and activators of transcription

longation of survival^{5,6} of human eosinophils. The high-affinity receptors of GM-CSF and IL-5 consist of an α chain specific for each cytokine and a common β chain.⁷ Although neither the α nor the β chain has tyrosine kinase activity, stimulation with these cytokines induces tyrosine phosphorylation of several proteins in a variety of cells. It has been shown that IL-5 activates tyrosine kinases *lyn* and *Jak2* in human eosinophils.⁸⁻¹⁰ In addition, *Jak2* kinase activates STAT1,⁹ part of a family of latent transcription factors termed *STAT* (signal transducers and activators of transcription) *proteins*.¹¹ The phosphorylated STAT protein then moves to the nucleus and directs transcription.¹²

Mitogen-activated protein (MAP) kinases are activated in response to various stimuli and regulate a wide variety of cellular phenomena not restricted to mitogenesis, including the activation of protein kinases such as S6 kinase, protein phosphatases such as PP-1, and nuclear transcription factors.^{13,14} It has recently been shown that IL-5 activates MAP kinases in human eosinophils, suggesting an importance of the MAP kinase cascade in eosinophil activation.^{8,10} However, GM-CSF- and IL-5-induced signaling pathways of MAP kinase activation in eosinophils are largely unknown.

To determine the kinase cascade for GM-CSF- and IL-5-induced MAP kinase activation in eosinophils, we studied the effect of inhibitors of *Jak2* kinase, tyrosine

From the Department of Internal Medicine, Chiba University School of Medicine.

Supported in part by grants from the Ministry of Education, Science and Culture, Japan and by grants from Sandoz Pharmaceutical Co., Japan. Reprint requests: Itsuo Iwamoto, MD, Department of Internal Medicine, Chiba University School of Medicine, 1-8-1 Inohana, Chiba City, Chiba 260 Japan.

Copyright © 1997 by Mosby-Year Book, Inc.
0091-6749/97 \$5.00 + 0 1/0/85416

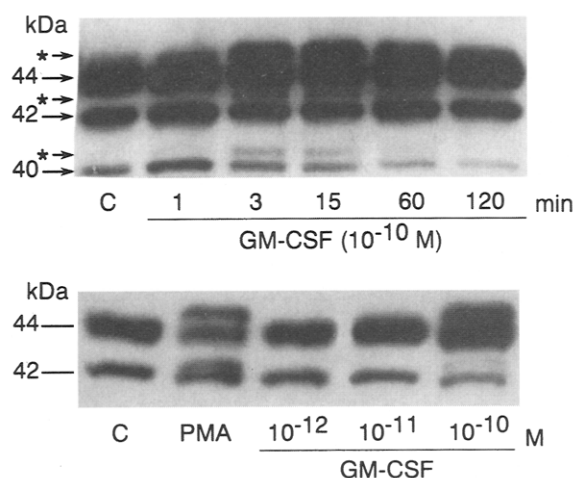


FIG. 1. Time course (upper panel) and dose dependence (lower panel) of GM-CSF-induced activation of MAP kinase in human eosinophils. Upper panel, Human eosinophils were stimulated with GM-CSF (10^{-10} mol/L) at 37°C for 1 to 120 minutes. Cell lysates were immunoblotted with anti-MAP kinase antibody (*erk1-CT*). The position of MAP kinase isoforms at 40, 42, and 44 kDa and their phosphorylated forms (*) are shown by the arrows. Lower panel, Eosinophils were stimulated with GM-CSF (10^{-12} to 10^{-10} mol/L) at 37°C for 3 minutes. Cell lysates were immunoblotted with anti-MAP kinase antibody.

kinases, phosphatidylinositol (PI) 3-kinase, and protein kinase C on GM-CSF- and IL-5-induced MAP kinase activation in human eosinophils. Our results indicate that GM-CSF and IL-5 activate three isoforms of MAP kinases (40, 42, and 44 kilodaltons) in human eosinophils and that *Jak2* kinase, tyrosine kinase, and PI 3-kinase mediate GM-CSF- and IL-5-induced MAP kinase activation in eosinophils.

METHODS

Materials

Recombinant human GM-CSF was obtained from the Kirin Co. (Tokyo, Japan), and IL-5 was obtained from the Suntory Co. (Osaka, Japan). A *Jak2* kinase inhibitor, AG-490, was obtained from Mitsubishi Chemical Corp (Tokyo, Japan). Percoll suspension was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Magnetic activated cell sorter (MACS) microbeads conjugated with mouse anti-human CD16 monoclonal antibody were purchased from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany). Ten percent sodium dodecyl sulfate (SDS) gel was purchased from Daiichi Pure Chemicals (Tokyo, Japan). Immobilon polyvinylidene fluoride (PVDF) transfer membrane was purchased from Millipore Corp. (Bedford, Mass.). Rabbit anti-rat MAP kinase polyclonal antibody (*erk1-CT*) that was raised against the carboxy-terminal synthetic peptide of rat *erk1* MAP kinase (Pro333-Pro367), which reacts with 42 kDa and 44 kDa human MAP kinases,^{15,16} was purchased from Upstate Biotechnology (Lake Placid, N.Y.). [γ - ^{32}P]adenosine triphosphate (ATP) was purchased from Amersham Corp. (Buckinghamshire, U.K.). All other reagents were purchased from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise stated.

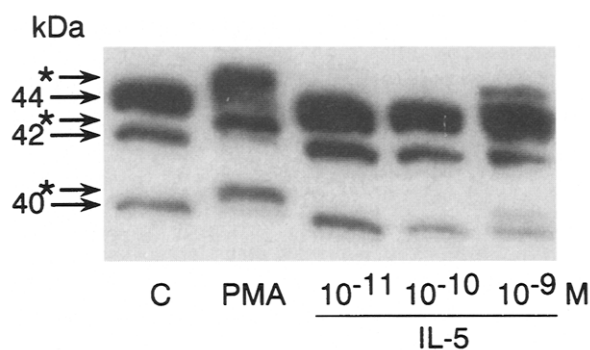


FIG. 2. IL-5 induces MAP kinase activation in human eosinophils. Eosinophils were stimulated with IL-5 (10^{-11} to 10^{-9} mol/L) at 37°C for 3 minutes. Cell lysates were immunoblotted with anti-MAP kinase antibody. PMA, phorbol myristate acetate.

Purification of eosinophils

Human eosinophils were purified from heparinized venous blood of healthy persons by means of Percoll density gradient centrifugation and magnetic cell sorting with MACS anti-CD16 microbeads as described by Hansel et al.¹⁷ Pure eosinophils (purity greater than 98%) were obtained by means of CD16 negative selection and suspended by Hanks balanced salt solution.

Stimulation and treatment of cells

To determine the time course and dose dependence of GM-CSF- and IL-5-induced MAP kinase activation in human eosinophils, eosinophils ($5 \times 10^5/\text{ml}$) were stimulated with GM-CSF or IL-5 (10^{-12} to 10^{-9} mol/L) at 37°C for 1 to 120 minutes. In some experiments, eosinophils also were stimulated with phorbol myristate acetate (PMA; 10 ng/ml) at 37°C for 3 minutes.

To determine whether tyrosine kinases, PI 3-kinase, and protein kinase C are involved in GM-CSF- and IL-5-induced MAP kinase activation in eosinophils, we examined the effect of inhibitors of these kinases on GM-CSF- and IL-5-induced MAP kinase activation in eosinophils. Eosinophils were preincubated with genistein (10^{-7} to 10^{-5} mol/L),¹⁸ wortmannin (10^{-8} to 10^{-6} mol/L),^{19,20} or staurosporine (10^{-10} to 10^{-8} mol/L)^{21,22} at 37°C for 15 minutes and stimulated with GM-CSF (10^{-10} mol/L) or IL-5 (10^{-9} mol/L) at 37°C for 3 minutes.

To determine whether *Jak2* is involved in GM-CSF-induced MAP kinase activation in eosinophils, eosinophils were preincubated with AG-490 (10^{-7} to 10^{-5} mol/L)²³ for 30 minutes at 37°C and stimulated with GM-CSF (10^{-10} mol/L) at 37°C for 3 minutes.

Immunoblotting

After eosinophils were stimulated with GM-CSF or IL-5 (10^{-12} to 10^{-9} mol/L) for an indicated period, the cells were immediately centrifuged with a microcentrifuge and lysed on ice by means of addition of 60 μL of lysis buffer (20 mmol/L Tris-HCl at pH 7.5, 60 mmol/L β -glycerophosphate, 10 mmol/L EGTA, 10 mmol/L MgCl_2 , 10 mmol/L NaF, 2 mmol/L dithiothreitol, 1 mmol/L Na_3VO_4 , 1 mmol/L phenylmethylsulfonyl fluoride, 1% Nonidet P-40, and 5 $\mu\text{g}/\text{ml}$ leupeptin). Cell lysates were boiled with SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (20% glycerol, 2% SDS, 125 mmol/L Tris-HCl at pH 6.8, 100 mmol/L dithiothreitol, and 0.15% bromophenol blue) for 5 minutes and cleared by means of

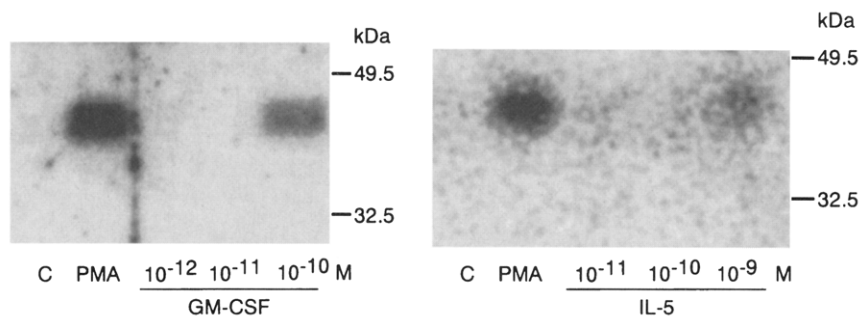


FIG. 3. In-gel kinase assay of GM-CSF- and IL-5-induced MAP kinase activation in human eosinophils. Eosinophils were stimulated with GM-CSF (10^{-12} to 10^{-10} mol/L) or IL-5 (10^{-11} to 10^{-9} mol/L) for 3 minutes, and MAP kinase activity was determined on the basis of ^{32}P incorporation into myelin basic protein, a substrate of MAP kinase. The autoradiograms show that GM-CSF (10^{-10} mol/L) and IL-5 (10^{-9} mol/L) induce phosphorylation of major basic protein at 40 to 44 kd. PMA, phorbol myristate acetate.

centrifugation at 3000 g at 4° C for 15 minutes with a microcentrifuge. Ten micrograms of cell lysate per lane was applied to SDS-PAGE and transferred electrically onto PVDF transfer membranes. The membranes were blocked with phosphate-buffered saline solution containing 0.1% Tween-20 and 5% bovine serum albumin, probed with the anti-MAP kinase antibody (*erk1-CT*), and visualized with an enhanced chemiluminescence system (Amersham).

The tyrosine phosphorylation of *Jak2* kinase was detected by means of immunoprecipitation of cell lysates with anti-*Jak2* antibody and blotting with anti-phosphotyrosine antibody (4G10) (Upstate Biotechnology).

In-gel kinase assay

Activation of MAP kinase was measured by means of an in-gel kinase assay with myelin basic protein as a substrate according to the method of Gotoh et al.^{24,25} In brief, cell lysates were electrophoresed on a 10% SDS-polyacrylamide gel containing 1 mg/ml myelin basic protein at room temperature. After electrophoresis, SDS was removed by means of washing of the gel with 20% isopropyl alcohol in 50 mmol/L Tris (pH 8.0) for 30 minutes and then with 50 mmol/L Tris (pH 8.0) containing 5 mmol/L 2-mercaptoethanol (buffer A) for 30 minutes. The protein in the gel was denatured by means of incubation with buffer A containing 8 mol/L guanidine-HCl for 1 hour at room temperature. The protein was then renatured by means of incubation with buffer A containing 0.04% Tween 40 and 5 mmol/L 2-mercaptoethanol for 3 hours at 4° C. The gel was incubated with 40 mmol/L Hepes (pH 7.5) containing 0.1 mmol/L EGTA, 15 mmol/L MgCl_2 , and 2 mmol/L dithiothreitol (buffer B) for 30 minutes at room temperature. After that, final incubation was performed with buffer B containing cold ATP (15 mmol) and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (25 μCi) for 1 hour. The gel was washed with 5% trichloroacetic acid solution containing 1% sodium pyrophosphate until the radioactivity of the solution became negligible. The gel was dried and then subjected to autoradiography.

Data analysis

Data are summarized as mean $\pm$ SD. The statistical analysis of the results was performed by means of analysis of variance with Fisher's least significant difference test for multiple comparisons. Probability values less than 0.05 were considered to indicate a statistically significant difference.

RESULTS

GM-CSF and IL-5 activate 40, 42, and 44 kd MAP kinases in eosinophils

GM-CSF activated 40, 42, and 44 kd MAP kinases in human eosinophils. We first examined the activation of MAP kinases by using the mobility shift in immunoblotting, because it has been shown that MAP kinases, when activated, migrate more slowly during gel electrophoresis than do the inactivated forms.²⁶ Stimulation of eosinophils with GM-CSF (10^{-10} mol/L) caused the electrophoretic mobility shift of the three isoforms of MAP kinase in immunoblotting with anti-MAP kinase antibody (*erk1-CT*), the shift of which reached a maximum 3 to 15 minutes after stimulation, declined, and returned to the basal level after 60 to 120 minutes (Fig. 1).

The dose-dependent effect of GM-CSF on MAP kinase activation of eosinophils was examined. Stimulation of eosinophils with GM-CSF (10^{-12} to 10^{-10} mol/L) for 3 minutes induced activation of MAP kinases in a dose-dependent manner with a significant minimum effect at 10^{-10} mol/L (Fig. 1). IL-5 also activated the three isoforms of MAP kinase in a dose-dependent manner with a significant minimum effect at 10^{-9} mol/L (Fig. 2).

In-gel kinase assay with major basic protein as a substrate also showed that GM-CSF induced activation of MAP kinase in eosinophils (Fig. 3). Stimulation of eosinophils with GM-CSF (10^{-10} mol/L for 3 minutes induced phosphorylation of major basic protein at 40 to 44 kd (Fig. 3). Stimulation of eosinophils with IL-5 (10^{-9} mol/L for 3 minutes also induced MAP kinase activation in the in-gel kinase assay (Fig. 3).

Effect of inhibition of Jak kinase, tyrosine kinase, PI 3-kinase, and protein kinase C on GM-CSF- and IL-5-induced MAP kinase activation in eosinophils

Jak2 kinase is essentially involved in GM-CSF-induced MAP kinase activation in eosinophils. Pretreat-

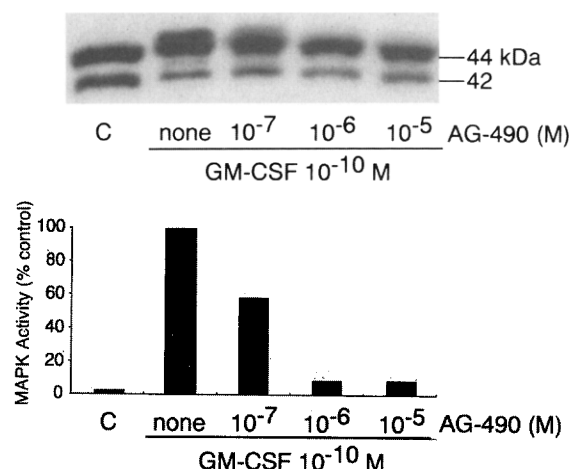


FIG. 4. Effect of *Jak2* kinase inhibitor AG-490 on GM-CSF-induced MAP kinase (MAPK) activation in human eosinophils. Eosinophils were preincubated with or without AG-490 (10^{-7} to 10^{-5} mol/L) for 30 minutes and stimulated with or without GM-CSF (10^{-10} mol/L) for 3 minutes. The data shown are representative of three experiments.

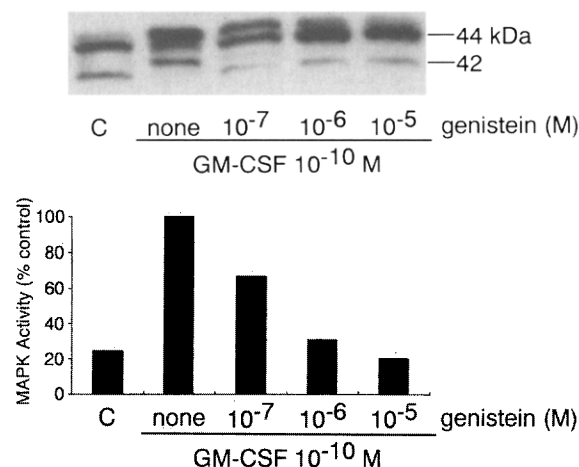


FIG. 5. Effect of tyrosine kinase inhibitor genistein on GM-CSF-induced MAP kinase (MAPK) activation in human eosinophils. Eosinophils were preincubated with or without genistein (10^{-7} to 10^{-5} mol/L) for 15 minutes and stimulated with or without GM-CSF (10^{-10} mol/L) for 3 minutes. The data shown are representative of four experiments.

ment with AG-490 (10^{-5} mmol/L), a selective inhibitor of *Jak2* kinase,²³ completely inhibited GM-CSF (10^{-10} mmol/L)-induced MAP kinase activation in eosinophils ($n = 3$ experiments) (Fig. 4), as indicated by the inhibitory effect on GM-CSF-induced electrophoretic mobility shift of MAP kinases in immunoblotting with anti-MAP kinase antibody. AG-490 also prevented GM-CSF-induced tyrosine phosphorylation of *Jak2* kinase itself in eosinophils (data not shown).

A tyrosine kinase inhibitor genistein (10^{-7} to 10^{-5} mol/L)¹⁸ inhibited GM-CSF-induced MAP kinase activation in eosinophils with almost complete inhibition at 10^{-5} mol/L ($n = 4$) (Fig. 5), although the GM-CSF receptor itself has no intrinsic tyrosine kinase activity.⁷ Genistein (10^{-5} mol/L) also prevented GM-CSF-induced tyrosine phosphorylation of *Jak2* kinase (data not shown).

PI 3-kinase also is involved in GM-CSF-induced MAP kinase activation in eosinophils. Pretreatment with wortmannin (1 μ mol/L), a selective PI 3-kinase inhibitor,^{19,20} completely inhibited GM-CSF-induced kinase activation in eosinophils ($n = 4$) (Fig. 6). IL-5 (10^{-9} mol/L)-induced MAP kinase activation in eosinophils also was prevented by genistein and wortmannin ($n = 3$) (data not shown).

In contrast, pretreatment with staurosporine (10 nmol/L), the most potent inhibitor of protein kinase C,^{21,22} decreased GM-CSF-induced MAP kinase activation in eosinophils by only 36% ($n = 4$, $p < 0.001$) (Fig. 7). On the other hand, stimulation with PMA (10 ng/ml) for 3 minutes, which directly activates diacylglycerol-responsive protein kinase C,²⁷ induced activation of the three isoforms of MAP kinase in eosinophils. Pretreatment with staurosporine (10 nmol/L) completely inhibited

ited PMA-induced MAP kinase activation in eosinophils (data not shown).

DISCUSSION

In this study, we showed that GM-CSF and IL-5 activate three isoforms (40, 42, and 44 kD) of MAP kinase through a kinase cascade of *Jak2* kinase, tyrosine kinase, and PI 3-kinase in human eosinophils. We found that GM-CSF and IL-5 activated 40, 42, and 44 kD MAP kinase isoforms in human eosinophils, as indicated with the electrophoretic mobility shift of the three isoforms of MAP kinase in immunoblotting with anti-MAP kinase antibody (Figs. 1 and 2) and with the in-gel MAP kinase assay (Fig. 3). MAP kinase activation was time- and dose-dependent, becoming maximal 3 to 15 minutes after stimulation (Fig. 1). We also found that *Jak2* kinase inhibitor AG-490, tyrosine kinase inhibitor genistein, and PI 3-kinase inhibitor wortmannin inhibited GM-CSF- and IL-5-induced MAP kinase activation in eosinophils (Figs. 4 through 6), whereas a protein kinase C inhibitor staurosporine had a weak inhibitory effect (Fig. 7). Furthermore, AG-490 and genistein were found to prevent GM-CSF-induced tyrosine phosphorylation of *Jak2* kinase itself in eosinophils. Taken together, these results indicate that GM-CSF- and IL-5-induced MAP kinase activation in eosinophils is mediated through a signaling pathway of *Jak2* kinase-tyrosine phosphorylated β chain-PI 3-kinase-*Ras*.

MAP kinases are serine and threonine kinases that are activated in many cell types in response to various stimuli through tyrosine kinase receptors, cytokine receptors, and G protein-coupled receptors. They are believed to be key signal transduction intermediates for regulation of a wide variety of cell functions.^{13,14} MAP kinases have been shown to phosphorylate and activate

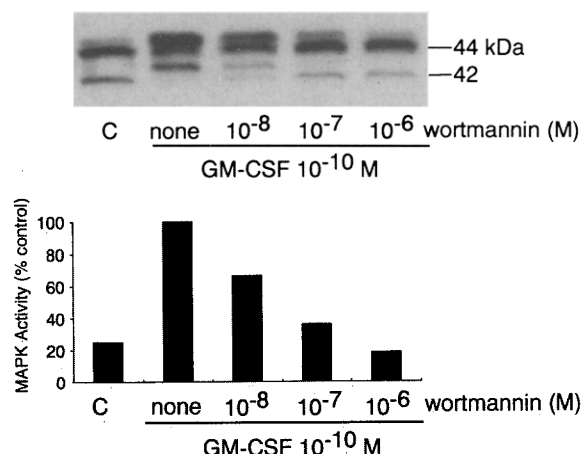


FIG. 6. Effect of a PI 3-kinase inhibitor wortmannin on GM-CSF-induced MAP kinase (MAPK) activation in human eosinophils. Eosinophils were preincubated with or without wortmannin (10^{-8} to 10^{-6} mol/L) for 15 minutes and stimulated with or without GM-CSF (10^{-10} mol/L) for 3 minutes. The data shown are representative of four experiments.

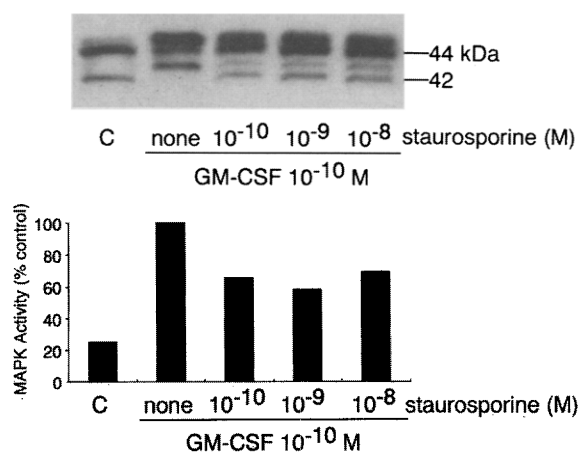


FIG. 7. Effect of a protein kinase C inhibitor staurosporine on GM-CSF-induced MAP kinase (MAPK) activation in human eosinophils. Eosinophils were preincubated with or without staurosporine (10^{-10} to 10^{-8} mol/L) for 15 minutes and then stimulated with or without GM-CSF (10^{-10} mol/L) for 3 minutes. The data shown are representative of four experiments.

protein kinases such as the ribosomal protein S6 kinase, protein phosphatases such as PP-1, cytoplasmic phospholipase A2, and nuclear transcription factors such as *c-myc* and *c-jun*.^{13, 14, 28} The signaling pathways that trigger MAP kinase activation are initiated variously by tyrosine kinases, protein kinase C, or G proteins, and the resulting sequential activation of *Ras*, *Raf*, and MEK (MAP kinase kinase) has been proposed as the main pathway for activation of MAP kinase.²⁹⁻³¹ Until now, however, the signaling pathways of GM-CSF- and IL-5-induced MAP kinase activation in eosinophils have not been elucidated, although previous studies showed that IL-5 activates MAP kinases in human eosinophils.^{8, 10}

We showed that *Jak2* kinase is essentially involved in GM-CSF- and IL-5-induced MAP kinase activation in eosinophils. It has been shown that *Jak2* kinase constitutively associates with the β chain of GM-CSF and IL-5 receptors in hematopoietic cells and that the binding of GM-CSF or IL-5 to its receptor causes *Jak2* kinase activation, which in turn induces the tyrosine phosphorylation of the β chain.³²⁻³⁵ Our findings of the crucial involvement of *Jak2* kinase in the GM-CSF- and IL-5-induced MAP kinase activation are consistent with a recent report by Watanabe et al.³⁶ that *Jak2* kinase activation is an initial and essential event that mediates GM-CSF-induced intracellular signal transduction. Jucker and Feldman³⁷ reported that when the β chain is tyrosine phosphorylated, PI 3-kinase is activated through the interaction with an adapter protein that has bound specifically to the tyrosine phosphorylated β chain.

In addition to *Jak2* kinase inhibition, the inhibitory effect of genistein on the GM-CSF- and IL-5-induced MAP kinase activation may be exerted by inhibition of cytoplasmic tyrosine kinases such as *lyn*, the activation of which may induce phosphorylation and activation of the receptor β chain and PI 3-kinase. It is still controversial

whether genistein can inhibit the activation of *Jak2* kinase.^{18, 38, 39} It has been shown that IL-5 stimulation causes activation of *lyn* kinase in eosinophils.⁸ In addition, the p60src tyrosine kinase has been shown to activate *Ras*.⁴⁰

We also showed that PI 3-kinase is an important signaling molecule for mediating GM-CSF- and IL-5-induced activation of MAP kinase in eosinophils. It has already been shown that GM-CSF and IL-5 induce activation of PI 3-kinase in human myeloid cells.⁴¹⁻⁴³ PI 3-kinase has been shown to be a cytoplasmic signaling protein and to associate physically with activated receptors that contain the tyrosine kinase domain⁴⁴⁻⁴⁷ and with activated cytokine receptors, including GM-CSF and IL-5 receptors.⁴³ Although the function of PI 3-kinase is still unknown, several reports have suggested the importance of PI 3-kinase in the signal pathway of cell proliferation.⁴⁸⁻⁵⁰ It has been shown that PI 3-kinase associates with tyrosine-phosphorylated proteins through the binding of src homology 2 (SH2) domains present in the 85 kd subunit of PI 3-kinase to tyrosine-phosphorylated YXXM motif present in many receptor tyrosine kinases and certain cellular substrates.^{51, 52} Therefore, because the β chain of GM-CSF and IL-5 receptors has no YXXM motif in the cytoplasmic domain,⁷ PI 3-kinase may associate with the β chain indirectly, possibly through other tyrosine-phosphorylated proteins that have YXXM motifs.³⁷

It has been shown that PI 3-kinase directly activates *Ras* and causes diverse *Ras*-dependent cellular activation, including *Raf* activation.^{53, 54} It also has been suggested that PI 3-kinase is important to form a functional association between *Ras*, guanosine triphosphatase activating protein, and PI 3-kinase in cell signaling.⁵⁵ Therefore, taken together with our findings, it is suggested that PI 3-kinase probably acts on *Ras* for