The Intracellular Signal Transduction Mechanism of Interleukin 5 in Eosinophils: The Involvement of Lyn Tyrosine Kinase and the Ras–Raf-1–MEK–Microtubule-associated Protein Kinase Pathway

By Konrad Pazdrak,* Douglas Schreiber,* Patricia Forsythe,* Louis Justement,‡ and Rafeul Alam*

*From the *Department of Internal Medicine, Allergy Division; and ‡Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, Texas 77555-0762*

Summary

Interleukin 5 (IL-5) regulates the growth and function of eosinophils. The objective of this study was to investigate the intracellular signal transduction mechanism of IL-5 in eosinophils. Purified eosinophils were stimulated with IL-5, and the involvement of various kinases was investigated by immunoblotting, immune complex kinase assay, and in situ denatured/renatured kinase assay. We found that IL-5 induced tyrosine phosphorylation and activation of a number of kinases. Two species of lyn kinases (53 and 56 kD) were present in eosinophils. Both forms were Tyr-phosphorylated and activated rapidly within 1 min. Further, lyn kinase was physically associated with the IL-5β receptor in eosinophils. Ras was studied by immunoprecipitation followed by thin-layer chromatography. Ras bound higher quantities of [α-^{32}P]guanosine 5′-triphosphate upon stimulation with IL-5. Raf-1 kinase showed increased Tyr phosphorylation on immunoblotting and increased activity in the immune complex kinase assay. Two species of MEK (MAP or Erk kinase) (41 and 45 kD) were identified in eosinophils, which underwent autophosphorylation upon stimulation. Microtubule-associated protein (MAP) kinase (p44) was Tyr-phosphorylated on immunoblotting and had increased activity in the immune-complex kinase assay. MAP kinases were also studied after metabolic radiolabeling of the cells with [^{32}P]orthophosphates. IL-5 stimulated phosphorylation of MAP kinases in situ. Thus, we have delineated major components of an important signaling pathway in eosinophils. We believe that one of the signals generated by IL-5 receptor activation is propagated through the lyn–Ras–Raf-1–MEK–MAP kinase pathway.

One of the cardinal features of allergic inflammation and the host defense against parasite is tissue and/or blood eosinophilia (1). The growth, survival, and function of eosinophils are primarily stimulated by IL-5 (2, 3). Mice that are transgenic for IL-5 have remarkable blood and tissue eosinophilia (4). Pretreatment of mice with monoclonal antibodies against IL-5 prevents parasite-induced eosinophilia (5). These studies suggest that IL-5 plays a crucial role in allergic diseases and in host defense against parasites.

The IL-5 receptor is a heterodimer, consisting of α and β subunits. The α chain is specific for IL-5 (6). The β chain is promiscuous and can associate with the α chains of the receptors for IL-3 and GM-CSF. Neither subunit has intrinsic kinase activity. It has been speculated that specific tyrosine kinases associate with the receptor upon ligand binding. IL-3 and GM-CSF have previously been shown to activate p53/p56 lyn kinase (7). The consequence of activation of many tyrosine kinases is the propagation of signals through the Ras–Raf-1–MEK (MAP or Erk kinase)–MAP (microtubule-associated protein) kinase pathway (8, 9). Raf-1 kinase activates NF-κB by directly phosphorylating the inhibitor, IκB (10). MAP kinases activate many cytosolic proteins such as phospholipase C, phospholipase A2, and nuclear transactivating factors such as NFIL-6, c-*fos*, c-*myc*, p62^{TCF}, and others (11). The activation of tyrosine kinases and the involvement of the Ras–Raf-1–MEK–MAP kinase pathway in eosinophils have not been investigated.

Materials and Methods

Percoll was purchased from Pharmacia LKB Biotechnology, Inc. (Piscataway, NJ). Monoclonal antibody against antiphosphotyro-

[1] *Abbreviations used in this paper:* ECL, enhanced chemiluminescence; MAP, microtubule-associated protein; MBP, myelin basic protein; MEK, MAP or Erk kinase.

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sine (clone 4G10) was from Upstate Biotechnology, Inc. (Lake Placid, NY). Monoclonal anti-erk1 and -erk2 (p42 and p44, clone ZO33), and antiphosphotyrosine (clone PY20) antibodies were from Zymed Laboratories, Inc. (South San Francisco, CA). Rabbit polyclonal anti-IL-5β receptor, anti-MEK, and anti-Raf-1 antibodies, and goat anti-mouse, anti-rabbit, and anti-rat antibodies conjugated with horseradish peroxidase were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). A mouse monoclonal antibody against Raf-1 was purchased from Oncogene Science, Inc. (Uniondale, NY). Rabbit polyclonal anti-lyn antibody was prepared against the peptide Pro^{36}-Lys^{50} of lyn as described previously (12). Monoclonal anti-Ras (clone 142-24E5) was purchased from American Type Culture Collection (Rockville, MD). Enhanced chemiluminescence (ECL) kit, [γ-^{32}P]ATP, [α-^{32}P]GTP, and [^{32}P]orthophosphates were from Amersham Corp. (Arlington Heights, IL). Histone 1 and myelin basic protein (MBP) were from Sigma Chemical Co. (St. Louis, MO).

Eosinophil Purification. Peripheral blood for eosinophil purification was obtained from subjects with mild to moderate eosinophilia (6–12%). Eosinophils were isolated by sedimentation with 3% hydroxyethyl starch followed by centrifugation on discontinuous Percoll gradients according to Gartner (13) as described previously (14). The band at the interface of 1.090 and 1.100 consisted of eosinophils of 97–99% purity as judged by staining with aniline blue or Wright's staining. Eosinophils were then suspended in RPMI 1640 in tubes coated with 3% human serum albumin.

Preparation of Cytosolic Cell Extract and Immunoprecipitates. Eosinophils were incubated with IL-5 (10^{-10} M, unless otherwise stated) or medium for various periods of time at 37°C. The reaction was terminated by the addition of 1 vol of ice-cold PBS containing 1 mM Na_3VO_4. The cells were pelleted by centrifugation, washed rapidly with PBS/Na_3VO_4, and lysed in a buffer containing 20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 2 mM EGTA, 1 mM PMSF, 1 mM Na_3VO_4, 1% Triton X-100, 2 μg/ml aprotinin, and 1 μg/ml pepstatin. After 10 min on ice, detergent-insoluble materials were removed by centrifugation at 4°C for 30 min at 12,000 *g*. The protein concentration was determined using bicinchoninic acid assay (Pierce Chemical Co., Rockford, IL).

For immunoprecipitations, the cell lysates were precleared by incubating with 25 μl of protein A agarose for 2 h. After removal of the beads, the lysates were incubated with an appropriate antibody (1–5 μg for each sample) and 25 μl of protein A agarose for 4–6 h at 4°C (14). The immunoprecipitates were washed three times with the cold lysis buffer, and, for the kinase assay, twice in the kinase buffer (10 mM Hepes, pH 7.4, 50 mM NaCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$, and 0.1 mM Na_3VO_4).

Immune-Complex Kinase Assay. Aliquots of equal amounts of the immunoprecipitates were assayed for Raf-1 and MAP kinases by measuring the phosphotransferase activity for histone 1 and MBP, respectively, as described previously (15). The kinase activity of the immune complexes was assayed by incubating the immunoprecipitates in 80 μl of kinase buffer with 40 μCi of [γ-^{32}P]ATP (10 mCi/ml) and 20 μl of either histone 1 (5 mg/ml) or MBP (0.5 mg/ml) for 15 min at 25°C. The phosphorylation reaction was terminated by spotting 15-μl aliquots of the assay mixture on a 2 × 2–cm phosphocellulose filter (P81; Whatman, Inc., Clifton, NJ). The filters were then washed 10 times for 5 min in 1% orthophosphoric acid, and the amount of ^{32}P radioactivity was determined by Cerenkov counting.

For the autophosphorylation assay, the immunoprecipitates were suspended in 20 μl kinase buffer with 0.25 mCi/ml [γ-^{32}P]ATP for 60 min at room temperature. The reaction was stopped by washing immunoprecipitates three times with the lysis buffer and by adding the Laemmli's sample buffer. The kinase reaction products were then applied to SDS-PAGE and autoradiography.

Gel Electrophoresis and Immunoblotting. SDS polyacrylamide gels were prepared according to the Laemmli protocol and used for immunoblotting. The concentration of polyacrylamide varied from 7.5 to 12% depending on the molecular weight range of the studied kinases. Equal amounts of protein were used in immunoblotting experiments. All samples were prepared in 2× concentrated Laemmli reducing buffer and boiled for 4 min before application. Gels were blotted onto Hybond membranes (Amersham Corp.) for the ECL system. Blots were developed by washing (three times) and incubating in a blocking buffer containing 5% skim milk in TBST buffer (20 mM Tris base, 137 mM NaCl, made to pH 7.6 with HCl, and 0.05% Tween) for 1 h, followed by incubation in the primary antibodies (0.1 μg/ml) for 1 h. After washing five times in TBST, blots were incubated for 30 min with a horseradish peroxidase conjugated with the secondary antibody (0.1 μg/ml) directed against the primary antibody. The blots were developed with the ECL substrate according to manufacturer protocol. In some experiments, blots were reprobed with other antibodies after stripping in a buffer of 62.5 mM Tris-HCl, pH 6.7, 100 mM 2-mercaptoheptanol, and 2% SDS at 50°C for 30 min.

Metabolic Labeling of Cells and In Situ Phosphorylation. Eosinophils were metabolically labeled with [^{32}P]orthophosphate according to a previously described method (15). Cells (5 × 10^6/ml) were washed three times with phosphate-free RPMI 1640 and then cultured for 2 h in the phosphate-free medium. Next, the cells were cultured for 1 h in this medium containing 2 mCi of carrier-free [^{32}P]orthophosphates. After three washes, cells were stimulated with IL-5 for various time periods and then lysed with the lysis buffer as above. MAP kinase was immunoprecipitated with an anti-MAP kinase (p42 and p44) monoclonal antibody and separated by SDS-PAGE, as described above, and visualized by autoradiography.

[α-^{32}P]GTP Labeling. Eosinophils were radiolabeled with [α-^{32}P]GTP for studies of Ras according to a previously described method (16). Briefly, 7.5 × 10^6 cells were permeabilized in 350 μl of Meyer's buffer (10 mM $MnCl_2$, 20 mM Mg acetate, 2 mM EGTA, 296 mM $CaCl_2$, 1 mM 2-mercaptoethanol, 40 mM Hepes, and 285 μg/ml α-lysophosphatidylcholine palmitoyl) at 4°C for 3 min. Cells were then incubated for 3 min with 75 μCi of [α-^{32}P]GTP made to 1 μM with unlabeled GTP or ATP. Subsequently, the cells were stimulated with IL-5 for 3 min and then lysed in the lysis buffer for further experiments.

In Situ Denatured/Renatured Kinase Assay. This assay was performed according to a previously described method (17). Briefly, cell lysates were resolved in 10% SDS polyacrylamide gels. After electrophoresis, the gel was blotted onto a polyvinyldifluoride membrane. The proteins were denatured by incubating the membrane with 7 M guanidine HCl at room temperature for 1 h. After washing with 30 mM Tris-HCl (pH 7.4), the kinases were renatured by incubating in 50 mM Tris buffer (pH 7.4) containing 100 mM NaCl, 2 mM dithiothreitol, 2 mM EDTA, 1% BSA, 1 mM sodium orthovanadate, and 0.1% NP-40 at 4°C for 16 h. The membrane was then blocked with 5% BSA, 5% skim milk, and 1 mM sodium orthovanadate in 30 mM Tris-HCl (pH 7.4), and subsequently incubated with 50 μCi/ml [γ-^{32}P]ATP in the presence of 10 mM $MgCl_2$ and 2 mM $MnCl_2$. The membrane was washed and subjected to autoradiography.

Measurement of GTP- and GDP-bound Ras. Cells radiolabeled with [α-^{32}P]GTP were used for the analysis of p21 Ras according to a method described previously (18). Stimulated cells were pelleted and lysed by incubation for 1 h at 4°C in 450 μl of lysis buffer (Tris-buffered saline, pH 7.5, 20 mM $MgCl_2$, 1% Triton X-100,

0.1% SDS, 0.5% deoxycholate, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml α1-antitrypsin, and 10 μg/ml of anti-p21 Ras). Protein G–Sepharose (Sigma Chemical Co.) precoupled to rabbit anti-rat IgG was added to the mixture and incubated for 2 h. The immunoprecipitates were washed 6× with 50 mM Hepes, pH 7.4, 500 mM NaCl, 5 mM $MgCl_2$, 0.1% Triton X-100, and 0.005% SDS.

Guanine nucleotides were eluted from Ras by incubation for 20 min at 68°C in 20 μl solution containing 2 mM EDTA, 2 mM dithiothreitol, 0.2% SDS, 0.5 mM GTP, and 0.5 mM GDP. Aliquots of 5 μl of the eluate were applied to PEI-F cellulose plates (J. T. Baker, Inc., Phillipsburg, NJ) and resolved in 0.75 M KH_2PO_4, pH 3.4. The guanine nucleotides were visualized using a short UV light, and the spots were excised and counted by liquid scintillation. Percent GTP bound to Ras was calculated as GTP/[(1.5 GDP) + GTP].

Results

We studied the presence and activation of lyn kinase in eosinophils by immunoprecipitation and immunoblotting using a purified polyclonal rabbit antibody (Fig. 1, *left*). A 56-kD protein band and a faint 53-kD band were precipitated and blotted by the antibody that disappeared with the preincubation of the antibody with the immunizing peptide. In parallel experiments, the immunoprecipitate was immunoblotted with a monoclonal antiphosphotyrosine antibody (Fig. 1, *right*). IL-5 increased Tyr phosphorylation of both p56 and p53 lyn kinases within 1 min. An additional protein band (~62 kD) coprecipitated with the lyn kinases and stained with the antiphosphotyrosine antibody. The identity of the band at this time is unknown. The activation of lyn kinase was investigated by measuring autophosphorylation of the renatured kinase (Fig. 2). For this purpose, whole-cell lysate was subjected to the denatured/renatured kinase assay in situ. A 56-kD protein was found to autophosphorylate strongly within 1 min in the eosinophilic lysate. The band was identified as lyn kinase upon immunoblotting with the anti-lyn antibody. To further confirm the activation, lyn kinase was immunoprecipitated, and autophosphorylation of the immune

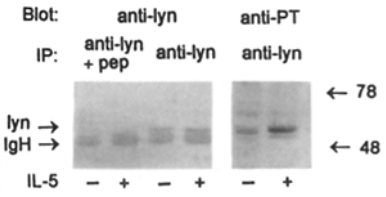


Figure 1. Immunoblotting of lyn kinase. Eosinophils were activated with IL-5 for 1 min and then immunoprecipitated (*IP*) with anti-lyn antibody. The membranes were then immunoblotted either with anti-lyn (*left*) or antiphosphotyrosine (*anti-PT*) antibodies (*right*). The blots were developed with the ECL detection system. In parallel experiments, the antibody was preincubated with the immunizing peptide (*pep*). In the presence of the immunizing peptide, the lyn-specific bands disappeared on the left panel. The bottom band on the left panel is rabbit IgH. The right panel shows two bands of phosphorylated lyn kinases (53 and 56 kD). A 62-kD Tyr-phosphorylated protein coprecipitated with lyn kinases, and its identity is unknown. Results of one of three experiments are shown.

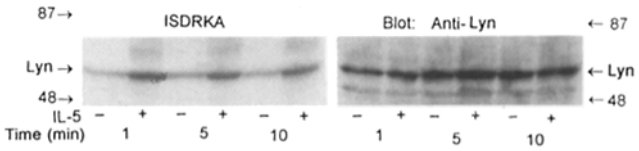


Figure 2. In situ denatured/renatured assay (*ISDRKA*) of lyn. Eosinophil lysates were electrophoresed, blotted, and denatured/renatured as described in Materials and Methods. The blot was then incubated with [γ-^{32}P]ATP. A major band of 56 kD showed strong autophosphorylation. The membrane was then immunoblotted with anti-lyn antibody. The 56-kD band was identified as lyn kinase.

complex kinase was performed. We found that lyn kinase obtained from IL-5–stimulated eosinophils underwent autophosphorylation (Fig. 3).

The physical association of lyn kinase with the IL-5β receptor was investigated by coprecipitation studies. For this purpose, eosinophil lysates were immunoprecipitated with the anti-lyn antibody followed by immunoblotting with an anti-IL-5β receptor antibody. We found that the IL-5β receptor coprecipitated with lyn kinase (Fig. 4, *right*). The membrane was stripped and reprobed with antiphosphotyrosine antibody (Fig. 4, *middle*) and then with anti-lyn antibody (Fig. 4, *left*). The middle panel demonstrated that lyn kinase was tyrosine-phosphorylated upon IL-5 stimulation. The left panel established the presence of two molecular mass forms of lyn (53 and 56 kD) in eosinophils.

The src-type Tyr kinases such as lyn kinase activate many downstream signaling molecules, including a group of proteins that exchange GDP for GTP on Ras. The latter exists on inactive GDP-bound and active GTP-bound forms (19). We studied the effect of IL-5 on the GTP binding of Ras. Eosinophil lysates were immunoprecipitated with a monoclonal anti-Ras antibody, and the bound GTP and GDP were eluted and resolved by TLC (Fig. 5). The percentage of Ras-bound GTP/total nucleotides (GDP + GTP) was 30 ± 2.5% in the IL-5–stimulated cells as compared to 19 ± 3.5% in unstimulated cells (n = 3). Thus, there was a 50% increase in the GTP binding after IL-5 stimulation, and the difference was significant (P <0.05, paired Student's t test).

Next, we studied the Raf-1, MEK, and MAP kinase activity in eosinophils. Raf-1 and MAP kinases were immunoprecipitated from IL-5–stimulated cells. The immunoprecipitates were assayed for Raf-1 and MAP kinase activity. IL-5 caused nearly 300% increase in the activity of the kinases

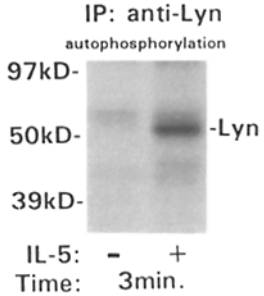


Figure 3. Immunoprecipitation of lyn kinase and autophosphorylation. Lyn kinase was immunoprecipitated (*IP*) with the anti-lyn antibody as in Fig. 1. The immune complex kinase was incubated with [γ-^{32}P]ATP in the kinase buffer and then electrophoresed and autoradiographed. Lyn kinase obtained from IL-5–stimulated eosinophils underwent autophosphorylation in vitro.

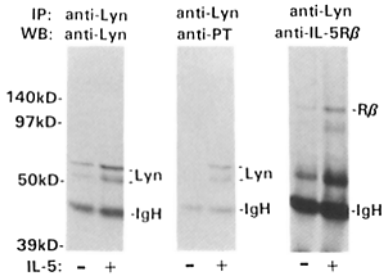


Figure 4. Physical association of IL-5β receptor with lyn kinase. Eosinophil lysates were immunoprecipitated (*IP*) with the anti-lyn antibody and then Western blotted (*WB*) with an anti-IL-5β receptor antibody (*right*). The membrane was stripped and reprobed successively with the antiphosphotyrosine (*middle*) and anti-lyn antibody (*left*). The IL-5β receptor coprecipitated with lyn kinase (53 and 56 kD) obtained from unstimulated and stimulated eosinophils. Further, lyn kinase was tyrosine-phosphorylated upon IL-5 stimulation. Results of one of two experiments are shown.

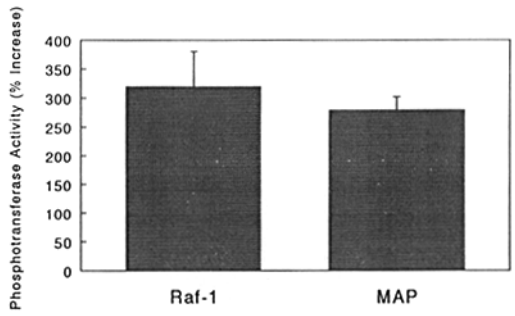


Figure 6. Raf-1 and MAP kinase activity of immunoprecipitates. Eosinophils were incubated with or without IL-5 (10^{-10} M) for 3 min (Raf-1 kinase) and 15 min (MAP kinase). The lysates were immunoprecipitated with monoclonal antibodies against Raf-1 and MAP kinases. The immunoprecipitates were assayed for kinase activity using histone 1 (Raf-1) and MBP (MAP kinase) in the presence of [γ-^{32}P]ATP. The phosphotransferase activity was expressed as percent increase over the baseline of unstimulated cells. n = 6 for Raf-1, and n = 5 for MAP kinase.

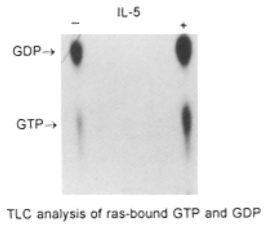
(Fig. 6). The activity of Raf-1 was detectable within 1 min, reaching the peak by 3 min (Fig. 7). In contrast, MAP kinase activation occurred at a slower pace, reaching the peak at 20 min. The optimal concentration of IL-5 for stimulation of Raf-1 kinase was 10^{-11}–10^{-10} M, which is in accord with other biologic activities of this cytokine on eosinophils (data not shown). The tyrosine phosphorylation of Raf-1 kinase was detected in IL-5–stimulated eosinophils by immunoprecipitation followed by immunoblotting with antiphosphotyrosine antibodies (Fig. 8, *left*). In parallel experiments, the immunoprecipitate was immunoblotted with a polyclonal anti-Raf-1 antibody, which demonstrated that equal amounts of the protein were analyzed (Fig. 8, *right*). The specificity of the antibody was determined by preincubating with the immunizing peptide, which caused disappearance of the band.

MEK is a ser/thr kinase that activates MAP kinases. At least two molecular mass forms of MEK (41 and 45 kD) have been identified (19). We studied MEK by immunoprecipitation followed by autophosphorylation in the kinase assay (Fig. 9). Three proteins of molecular masses of ~41, 42, and 45 kD underwent autophosphorylation upon IL-5 stimulation. The same membrane was immunoblotted with the anti-MEK antibody. The IgH chains of the precipitating antibody, which has a molecular mass similar to that of MEK, overshadowed the MEK bands. We therefore performed immunoblotting of the whole-cell lysate. The lysate was first subjected to the denatured/renatured kinase assay in situ. Two proteins, p41 and p45, showed significant autophosphorylation. The membrane was next immunoblotted with the anti-MEK antibody. Both p41 and p45 stained with the antibody.

MAP kinases were studied using a monoclonal antibody that recognizes the p42 and p44 species. Cell lysates were first immunoblotted with the antiphosphotyrosine antibody. Among many other bands, a 44-kD band showed strong Tyr phosphorylation upon stimulation with IL-5 (Fig. 10). The blot was stripped and reprobed with the anti-MAP kinase antibody. The antibody stained a single band, which aligned with the 44-kD phosphoprotein band on the antiphosphotyrosine immunoblot. In another set of experiments, eosinophils were metabolically radiolabeled with [^{32}P]orthophosphate and stimulated with IL-5. MAP kinase was immunoprecipitated, subjected to SDS-PAGE, and autoradiographed. A 44-kD MAP kinase and a 54-kD kinase were immunoprecipitated and showed phosphorylation predominantly upon stimulation with IL-5 (Fig. 11).

Discussion

We demonstrated that IL-5 induced tyrosine phosphorylation and activation of multiple protein kinases in eosinophils.



TLC analysis of ras-bound GTP and GDP

Figure 5. TLC analysis of GTP and GDP. Eosinophils were stimulated with IL-5, and then Ras was immunoprecipitated using a monoclonal antibody. The nucleotides were eluted and analyzed by TLC. Results of one of three experiments are shown.

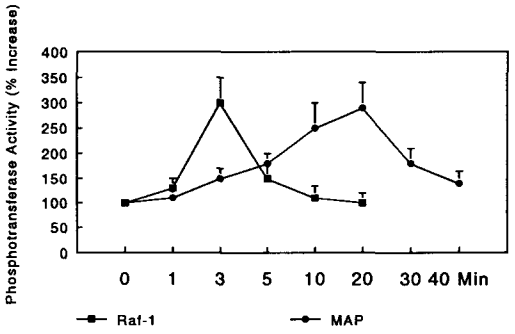


Figure 7. Kinetics of Raf-1 and MAP kinase activity. Eosinophils were stimulated with IL-5 for different time periods. The immunoprecipitates were assayed for Raf-1 and MAP kinases by measuring the phosphotransferase activity (cpm) for histone 1 and MBP, respectively. n = 4.

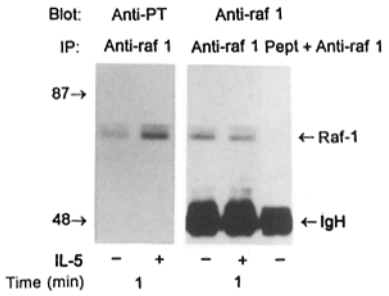


Figure 8. Immunoblotting of Raf-1 kinase. Eosinophils were immunoprecipitated (*IP*) with a polyclonal anti-Raf-1 antibody. The membranes were then immunoblotted either with antiphosphotyrosine (*anti-PT*, *left*) or anti-Raf-1 (*right*) antibodies. The blots were developed with the ECL detection system. In parallel experiments, the antibody was preincubated with the immunizing peptide (*pept*). In the presence of the immunizing peptide, the Raf-1–specific band disappeared on the right panel, indicating the specificity of the band. The bottom thick band is rabbit IgH. The left panel shows Tyr-phosphorylated Raf-1 kinase band. One of three similar experiments are shown.

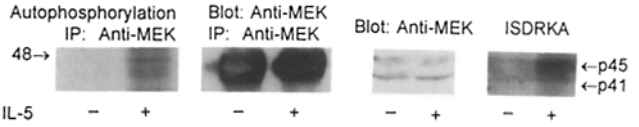


Figure 9. Immune complex kinase assay and in situ denatured/renatured kinase assay (*ISDRKA*) of MEK. MEK was immunoprecipitated and assayed for autophosphorylation. Three proteins (p41, p42, and p45) underwent autophosphorylation. The membrane was subsequently immunoblotted with an anti-MEK antibody. The thick visible band is likely due to IgH chains that comigrated with MEK. In the next set of experiments, eosinophil lysates were electrophoresed, blotted, and subjected to ISDRKA. Two proteins of molecular masses of 41 and 45 kD autophosphorylated. The membrane was then immunoblotted with the anti-MEK antibody, which positively stained the bands of p41 and p45.

Among the activated kinases were lyn, Raf-1, MEK, and MAP kinases. Further, we showed that IL-5 increased the binding of GTP to Ras. This is the first demonstration of the presence of lyn, Ras, Raf-1, MEK, and MAP kinases in eosinophils. Lyn kinase was activated rapidly within 1 min, suggesting that it may be the very first kinase to be activated by the IL-5 receptor. As mentioned previously, the IL-5 receptor (α and β subunits) does not have intrinsic kinase activity. We found that lyn kinase was physically associated with the IL-5β receptor in unstimulated eosinophils. We speculate that the receptors undergo dimerization/oligomerization upon ligand binding, which may lead to the conformational changes in the receptor-associated lyn kinase and its activation.

Lyn kinase, an src-type protein tyrosine kinase, has previously been detected in various hematopoietic cells, including B cells (20) and neutrophils (21). It associates with the nonphosphorylated secretory Ig receptor in B cells (20). A common tyrosine-containing motif (D/E-X7-D/E-X2-Y-X3-L-X7-Y-X2-L/I) that appears to mediate this binding has been identified in many receptors (e.g., B cell receptor) (22). The cytoplasmic domain of neither the α nor β chain of the IL-5 receptor contains this motif. Recently, specific regions of the β receptor that are responsible for signaling have been identified (23). For example, a region spanning leu^{626}–ser^{763} was found to be important for activation of the Ras–Raf-1–MAP kinase pathway, whereas a membrane proximal region upstream of glu^{517} was essential for induction of c-*myc* and pim-1.

Kinases play a crucial role in the intracellular signal transduction mechanism. Many kinases require Tyr phosphorylation for enzymatic activity. We investigated the Tyr phosphorylation of several kinases using antiphosphotyrosine immunoblotting. Further, kinase activity was measured by (*a*) immune complex kinase assay; (*b*) autophosphorylation; (*c*) denatured/renatured kinase assay in situ; and (*d*) in situ phosphorylation. The substrates used for Raf-1 and MAP kinase assays were not specific but have been widely used for this purpose (24–26). We believe that a combination of the above-mentioned kinase assays and immunoblotting provides a strong indication that these kinases are involved in the IL-5 signaling pathway. In some experiments, we noticed some degree of spontaneous phosphorylation of the kinases (e.g., MAP kinases). The causes of such spontaneous phosphorylation could include the activation of cells during in vitro manipulations and/or basal enzyme activity needed for sustaining normal cellular function.

Ras belongs to the family of low molecular weight G-binding proteins that play a crucial role in intracellular signal transduction. The inactive GDP-bound Ras protein is activated through the exchange of GDP for GTP by a number of GDP exchange proteins such as sos or c-vav (for review see reference 27). The latter proteins are usually phosphorylated by Tyr kinases and form multimeric complexes along with some adapter proteins such as GRB2 (28). We did not study the guanine nucleotide exchange factors. However, we found that Ras obtained from IL-5–stimulated eosinophils bound more GTP than that derived from unstimulated cells, suggesting indirectly that a guanine nucleotide exchange factor(s) was activated. The binding of GTP to Ras causes translocation of Raf-1 to the membrane and phosphorylation at the ser^{621}

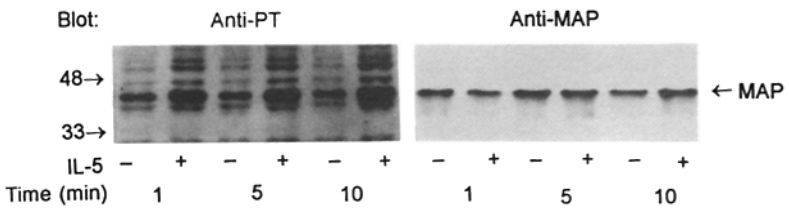


Figure 10. Immunoblotting of MAP kinase. Eosinophils were incubated with (+) and without (−) IL-5, and the lysate was electrophoresed and immunoblotted with antiphosphotyrosine antibody (*anti-PT*). The blot was then stripped and reblotted with a monoclonal anti-MAP kinase (p42 and p44) antibody. A single band of 44-kD protein was stained with the antibody that aligned with the strongest band on the antiphosphotyrosine immunoblot. The results of one of three similar experiments are shown.

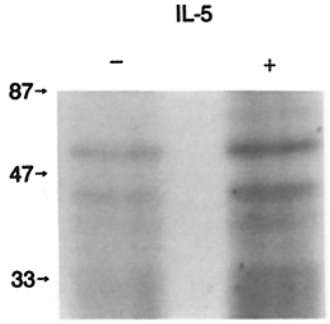


Figure 11. In situ phosphorylation of MAP kinases. Eosinophils were metabolically radiolabeled with [^{32}P]orthophosphates and then stimulated with IL-5. The cell lysates were immunoprecipitated with an anti-MAP kinase antibody, subjected to SDS-PAGE, and autoradiographed. The p44 MAP kinase as well as a p54 kinase were phosphorylated in situ.

residue (29). There are suggestions that Raf-1 requires additional Tyr phosphorylation for adequate activation (30). The latter is likely to be mediated by Tyr kinases.

The current model of signaling pathways that involves Raf-1 predicts that Raf-1 activates MAP kinase kinase or MEK by phosphorylating at ser^{218} and ser^{222} (31). We found that two species of MEK (p41 and p45) were activated in eosinophils. Raf-1 is not the only molecule that activates MEK. It can be activated independently of Raf-1 by MEK kinase, mos, and protein kinase C (32, 33). We did not investigate the latter kinases in this study. MEK directly phosphorylates MAP/erk kinases. The family of MAP kinases represents an important point of convergence for many cytosolic signaling pathways. The importance of MAP kinases stems from the fact that they activate multiple cytosolic proteins and nuclear factors (11). Recent studies suggest that stress-activated protein kinases are structurally and functionally related to MAP kinases (34). There exist multiple molecular mass forms of MAP kinases: p42, p44, p45, p54, and p62. MEK phosphorylates MAP kinases at the thr^{183} (p42 MAP) or thr^{202} (p44) residues (for review see reference 11). MAP kinases require additional phosphorylation on Tyr^{185} (p42) or Tyr^{204} (p44) for optimal activation. We used a monoclonal antibody that recognizes the p42 and p44 MAP kinases. We consistently found the presence of the p44 MAP kinase in eosinophils by immunoblotting. However, an ~54-kD kinase was precipitated by the antibody when cells were metabolically labeled with orthophosphates. The 54-kD kinase could represent an antigenically cross-reactive MAP kinase species that has been previously described (11). Alternatively, the p44 MAP kinase might have coprecipitated an unidentified kinase.

The Ras–Raf-1–MAP kinase pathway has been extensively studied in many proliferating cell lines and has been implicated in cell growth and proliferation. Its role in nonproliferating cells such as eosinophils is unknown. The activation of lyn (21) and MAP kinases (35) has recently been demonstrated in neutrophils, suggesting that these kinases may also be involved in processes other than cellular proliferation. IL-5 prolongs the survival of eosinophils by delaying apoptosis. Further, IL-5 primes eosinophils for many functions. We speculate that Raf-1 and MAP kinases may be involved in the repressor mechanism of apoptosis. IL-5 up-regulates the transcription of genes for a number of cytokines in eosinophils. The Ras–Raf-1–MAP kinase pathways could also contribute to the transcription of such early activation genes.

There have been very few studies to elucidate the signal transduction pathway of IL-5 in eosinophils. One study reported Tyr phosphorylation of a number of proteins by IL-5 in eosinophils, but the identity of the proteins was not investigated (36). In another study, IL-5 activated MAP kinases in the MC-9 mast cell line (25). While this manuscript was in preparation, the activation of several kinases by IL-5 in a mouse B cell line was reported (37). The authors found that IL-5 induced tyrosine phosphorylation of Btk and Jak2, the SH2- and SH3-containing adaptor proteins, shc, and the GDP exchange factor, c-vav. However, IL-5 did not phosphorylate lyn, phospholipase C-γ, and GTPase-activating protein. This is in contrast with our finding of lyn activation in eosinophils. Thus, it appears that the same cytokine may use different tyrosine kinases in the signaling process depending on the cell type.

In this study we have delineated major components of an important signaling pathway in eosinophils. It is likely that many other IL-5 signaling pathways exist. The future task is to define the role of various signaling pathways in evoking specific cellular responses. Eosinophils and IL-5 are intimately involved in the pathogenesis of allergic and parasitic diseases (38). An understanding of the intracellular signal transduction mechanism of IL-5 in eosinophils may help design novel therapeutic modalities for the above-mentioned disorders.

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Address correspondence to Dr. Rafeul Alam, Department of Internal Medicine, Allergy Division, Rt-0762, The University of Texas Medical Branch, Galveston, TX 77555-0762.

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