Requirement of Lyn and Syk Tyrosine Kinases for the Prevention of Apoptosis by Cytokines in Human Eosinophils

By Shida Yousefi,* Daniel C. Hoessli,† Kurt Blaser,* Gordon B. Mills,§ and Hans-Uwe Simon*

From the * Swiss Institute of Allergy and Asthma Research, University of Zurich, CH-7270 Davos, Switzerland; and the Department of Pathology, University of Geneva, CH-1211 Geneva 4, Switzerland; and the § Division of Medicine, University of Texas, MD Anderson Cancer Center, Houston, Texas 77030

Summary

In allergic diseases, the cytokines interleukin (IL)-5 and granulocyte/macrophage colony-stimulating factor (GM-CSF) are upregulated and have been proposed to cause blood and tissue eosinophilia by inhibition of eosinophil apoptosis. We demonstrate herein, in freshly isolated human eosinophils, that the IL-3/IL-5/GM-CSF receptor β subunit interacts with cytoplasmic tyrosine kinases to induce phosphorylation of several cellular substrates, including the β subunit itself. The Lyn and Syk intracellular tyrosine kinases constitutively associate at a low level with the IL-3/IL-5/GM-CSF receptor β subunit in human eosinophils. Stimulation with GM-CSF or IL-5 results in a rapid and transient increase in the amount of Lyn and Syk associated with the IL-3/IL-5/GM-CSF receptor β subunit. Lyn is required for optimal tyrosine phosphorylation and activation of Syk. In contrast, Syk is not required for optimal tyrosine phosphorylation and activation of Lyn. These data suggest that Lyn is proximal to Syk in a tyrosine kinase cascade that transduces IL-3, IL-5, or GM-CSF signals. Compatible with this model, both Lyn and Syk are essential for the activation of the antiapoptotic pathway(s) induced through the IL-3/IL-5/GM-CSF receptor β subunit in human eosinophils.

Materials and Methods

Antibodies. Anti-Lyn and anti-Syk mAbs as well as RC-20 Ab were obtained from Transduction Lab. (Lexington, KY). Polyclonal rabbit Abs against Syk and Lyn as well as anti-IL-3/IL-5/GM-CSF receptor β subunit were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). mAb against anti-phosphotyrosine (pTyr)1, clone 4G10, was from Upstate Biotechnology, Inc. (Lake Placid, NY). Goat anti-mouse horseradish peroxidase (HRP)–labeled second Ab was obtained from Amersham International (Amersham, Bucks, UK). Anti-CD16 mAb microbeads were from Miltenyi Biotec (Bergisch-Gladbach, Germany). FITC-conjugated and cross-absorbed F(ab)’2 fragments of goat anti-rabbit IgG were purchased from Jackson ImmunoResearch Labs., Inc. (West Grove, PA).

Abbreviations used in this paper: HRP, horseradish peroxidase; ITAM, immunoreceptor tyrosine-based activation motif; pTyr, phosphotyrosine.
Eosinophil Purification. Eosinophils were purified as previously described (6, 12, 13). Briefly, PBMC were separated from peripheral blood of patients with moderate eosinophilia (6–14%) by ficoll-hypaque (seromed-fakola ag, basel, switzerland) centrifugation. the remaining cell population, mainly granulocytes and erythrocytes, were treated with erythrocyte lysis solution (155 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM EDTA, pH 7.3). the resulting granulocyte population contained mainly neutrophils. To isolate eosinophils, the granulocyte population was incubated with anti-CD16 mAb microbeads. CD16-positive neutrophils were depleted by passing the granulocytes through a magnetic cell separation system (MACS®, miltenyi biotec) with column type C and an attached 21-gauge needle in the field of a permanent magnet. CD16-negative eosinophils were collected and used for experiments. the purity was controlled by staining with Diff-Quik (Baxter, düdingen, switzerland) and light microscopy as well as, in some cases, by flow cytometry. the resulting cell population contained 99% eosinophils.

Immunoprecipitation. Eosinophils (2–5 × 10⁷/ml) were stimulated in RPMI 1640 in the absence of FCS with GM-CSF or IL-5 (30 ng/ml) or medium for the indicated times at 37°C. the reaction was stopped by the addition of ice-cold PBS containing 0.5 mM Na₃VO₄. Cells were immediately pelleted at 4°C and lysed with 1 ml of ice-cold 0.5% Triton X-100 lysis buffer (50 mM Tris-HCl, pH 7.4, 25 mM KCl, 5 mM MgCl₂, 1 mM EGTA, 1 mM Na₃VO₄, 10 µg/ml pepstatin A, 18 µg/ml aprotinin, 1 mM PMSF, 1 µg/ml leupeptin, and 1 mM benzamidine) on ice for 10–15 min. Insoluble material was removed by centrifugation at 4°C for 15 min at 15,800 g. Cell lysates were precleared with 100 µl of Pansorbin (Calbiochem-Novabiochem Corp., San Diego, CA; 10% vol/vol solution of fixed Staphylococcus aureus Cowan I, prewashed three times in lysis buffer without proteinase inhibitors) at room temperature for 1 h or at 4°C overnight. lysate supernatants were further incubated with 50 µl of packed Sepharose 4B (Pharmacia, uppsala, sweden) coupled to BSA at room temperature for 1 h. Supernatants were transferred to 25–30 µl protein g–Sepharose (Pharmacia) that had been cross-linked with a specific antikinase Ab or 4G10 mAb overnight at 4°C and rinsed four times with lysis buffer. tubes were rotated at 4°C overnight and immunoprecipitates were washed four times with cold lysis buffer. the proteins were eluted by adding Laemmli sample buffer plus 0.1 M dithiothreitol and heating to 95°C for 5 min before gel electrophoresis. in other experiments, immunoprecipitates were subjected to an in vitro kinase assay.

Cell Electrophoresis and Immunoblotting. Electrophoresis was conducted in 10–12% SDS polyacrylamide gels, and the separated proteins were transferred to a polyvinylidene difluoride filter (Millipore, basel, switzerland). the filters were blocked at 37°C for 30 min (RC-20 experiments) or at room temperature for 1 h (antikinase mAb experiments) in blocking solution (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20, and 5% BSA; Sigma, Buchs, Switzerland). filters were incubated in 1% BSA blocking solution with HRP-labeled RC-20 Ab or primary antikinase mAb at room temperature for 1–2 h. Binding of antikinase mAb was detected by additional incubation of the filters with goat anti-mouse HRP-labeled second Ab at room temperature for 1 h. Blots were developed by an enhanced chemiluminescence technique (ECL kit; Amersham International) according to the manufacturer’s instructions.

In Vivo Kinase Assay. Kinase activity of anti-pyr and anti-Lyn immune complexes was assayed by incubating in 100 µl kinase buffer (10 mM 1,4-piperazinediethane-sulfonic acid, pH 7.1, 10 mM MgCl₂) with 5 µCi of γ-[³²P]ATP (Amersham International) at 30°C for 10 min. the phosphorylation reaction was stopped by washing the immunoprecipitates with ice-cold 0.5% Triton X-100 lysis buffer, and the kinase reaction products were resolved by SDS-PAGE and autoradiography.

Fluorescence Microscopy. Intracellular staining of Src family kinases has been described (14). Briefly, purified eosinophils (10⁶) were seeded on poly-L-lysine-coated glass coverslips at 37°C for 10 min. Cells were stimulated with GM-CSF or IL-5 (30 ng/ml) or medium at 37°C for 30 min. Cells were fixed with 3% paraformaldehyde solution, and permeabilized with PBS containing 0.15% Triton X-100 for 5 min. Nonspecific staining was inhib-
edited by incubation with 20% normal goat serum for 30 min. Cells were then labeled with rabbit anti-Lyn Ab, followed by FITC-conjugated and cross-adsorbed F(ab') 2 fragments of goat anti-rabbit IgG, each for 45 min. The coverslips were mounted in 90% (vol/vol) glycerol/100 mM Tris-HCl, pH 8.0, and examined on an Axiovert fluorescence microscope (Zeiss, Oberkochen, Germany) using a ×100 oil immersion lens. Kodak Elite 400 film (Eastman Kodak, Rochester, NY) was used for photography.

**Antisense Oligodeoxynucleotides.** Eosinophils (10^6/ml) were cultured in RPMI 1640 plus 10% FCS (complete culture medium) in the presence of phosphorothioate oligodeoxynucleotides (MWG-Biotech, Münchenstein, Germany) at 10 μM. Sequences used were as follows: antisense Lyn (15) CATATTTCGGCTCG; sense Lyn, CGAGCGGGAAATATG; antisense Syk, CATGCTTCAGGGGCGC; sense Syk, CCGGGCGTGAGATC; antisense Fes (16), CAGCTCGGAGAGAAGCC; and sense Fes, GGCTTCTTTCGAGCGT. Antisense Syk was designed based on the recently published human Syk cDNA (17). For immunoprecipitation, cells were washed extensively to remove FCS before cell stimulation.

**Determination of Eosinophil Death.** Cell death of eosinophils was assessed by flow cytometry as previously described (6). Eosinophils (10^6/ml) were cultured in the presence or absence of phosphorothioate oligodeoxynucleotides (10 μM) and GM-CSF (30 ng/ml) for 24 h. The relative amounts of dead cells were determined by uptake of 1 μM ethidium bromide which was analyzed by flow cytometry in an EPICS XL (Coulter Corp., Hialeah, FL).

**Determination of Eosinophil Apoptosis.** Oligonucleosomal DNA fragmentation, a characteristic feature of eosinophil apoptosis, was analyzed by agarose gel DNA electrophoresis as previously described (6). Eosinophils (10^6/ml) were cultured in the presence of phosphorothioate oligodeoxynucleotides (10 μM) and GM-CSF (30 ng/ml) for 24 h. Cells were washed with PBS at room temperature. Cell pellets were resuspended at 2 × 10^6/ml in cell lysis buffer (10 mM EDTA, 50 mM Tris-HCl, pH 8.0, containing 0.5% (wt/vol) N-lauroylsarcosine and 0.5 mg of proteinase K/ml) and incubated at 50°C for 1 h. RNase A (250 μg/ml) was added and incubated at 50°C for another hour. The solution was extracted twice with phenol, buffered with 0.1 M Tris-HCl, pH 7.4, followed by two chloroform/isoamyl alcohol (24: 1) extractions. The DNA solution was brought to 2.5 volumes by the addition of Tris/EDTA (TE) buffer, pH 8.0, and centrifuged at 15,800 g at room temperature for 20 min to separate nucleosomal DNA. The supernatants, containing fragmented DNA, were pre-

![Figure 2](image_url)

**Figure 2.** Lyn is physically associated with the IL-3/IL-5/GM-CSF receptor β subunit and activated after GM-CSF or IL-5 stimulation of eosinophils. (A) Eosinophils were stimulated with GM-CSF for the indicated times. The cell lysates were immunoprecipitated with an anti-IL-3/IL-5/
cipitated in two volumes of ethanol at −70°C for 24 h. The DNA precipitates were recovered by centrifugation at 15,800 g for 1 h. After drying, DNA was dissolved in TE buffer and stored at 4°C. DNA was mixed with 10× loading buffer (0.25% bromophenol, 0.1 M EDTA, pH 8.0, 1% SDS, and 20% Ficoll 400) before loading into wells of a 1.5% agarose gel containing 0.5 μM ethidium bromide. Electrophoresis was carried out in 90 mM Tris base, 90 mM boric acid, and 2 mM EDTA, pH 8.0. After electrophoresis, gels were visualized by ultraviolet light.

Statistical analysis. Statistical analysis was performed by using the Mann-Whitney U test. A probability value of <0.05 was considered statistically significant.

Results and Discussion

We have recently shown that granulocyte apoptosis is regulated by tyrosine phosphorylation (6). To address which tyrosine kinases might be involved in the antiapoptotic pathway mediated by the IL-3/IL-5/GM-CSF receptor β subunit, anti-ptyr immunoprecipitates were examined for the presence of protein kinase activity. In vitro kinase assays performed with anti-ptyr immunoprecipitates from human eosinophils, incubated with and without GM-CSF, revealed activation-dependent phosphorylation of 40-, 56-, 72-, and 92-kD proteins (Fig. 1 A). Furthermore, as demonstrated in Fig. 1 B, the IL-3/IL-5/GM-CSF receptor β subunit is a substrate for the receptor-activated kinase(s). In addition, a series of tyrosine-phosphorylated proteins ranging from 50 to 80 kD inducibly coimmunoprecipitated with the IL-3/IL-5/GM-CSF receptor β subunit (Fig. 1 B). These coimmunoprecipitating proteins were best detected within 3 min after GM-CSF or IL-5 stimulation. Intracellular Src and Syk family kinases have been previously shown to associate with and act as signal transducers for a number of different surface receptors that lack an intracellular catalytic domain (18). This implies that the prominent protein bands at 56 and 72 kD, which become tyrosine phosphorylated and associate with the IL-3/IL-5/GM-CSF receptor β subunit after activation, could represent Src or Syk family kinases.

To characterize the members of the Src and Syk family that could associate with the IL-3/IL-5/GM-CSF receptor β subunit in human eosinophils, β chain immunoprecipitates were examined for the presence of Lyn and Syk, which have been implicated in signaling through the G-CSF receptor in neutrophils (19). As shown in Fig. 2 A, a small but significant amount of Lyn coimmunoprecipitated with the IL-3/IL-5/GM-CSF receptor β subunit in nonstimulated eosinophils. It is unlikely that the physical association between the receptor β chain and Lyn was due to in vivo activation of eosinophils since we observed similar results in eosinophils from normal control individuals (not shown). Moreover, Lyn was rapidly and transiently recruited to the receptor after stimulation of eosinophils with GM-CSF (Fig. 2 A) or IL-5 (not shown). After exposure to either GM-CSF or IL-5 (Fig. 2 B), Lyn also demonstrated a time-dependent increase in tyrosine kinase activity as assessed by autophosphorylation in in vitro kinase assays. Moreover, activation of Lyn occurred somewhat faster if cells were
stimulated with IL-5 compared to GM-CSF. These data are in agreement with a very recent study describing physical association of Lyn with the IL-3/IL-5/GM-CSF receptor β subunit in eosinophils (20). Immunolocalization revealed a uniform intracellular staining of Lyn in nonstimulated eosinophils. GM-CSF or IL-5 induced changes in both eosinophil shape and in the distribution of Lyn (Fig. 2 C). Together, these results suggest that Lyn is associated with the IL-3/IL-5/GM-CSF receptor β subunit and activated after exposure of human eosinophils to GM-CSF or IL-5.

Similar to Lyn, a small but significant amount of Syk coimmunoprecipitated with the β chain in nonstimulated eosinophils (Fig. 3 A). Syk was also recruited to the receptor 3–5 min after stimulation of the cells with GM-CSF (Fig. 3 A) or IL-5 (not shown). Moreover, induced tyrosine phosphorylation of Syk was observed after GM-CSF or IL-5 stimulation, suggesting activation of Syk (Fig. 3 B). Similar to the Lyn activation experiments, Syk seems to be activated faster by IL-5 compared to GM-CSF. In addition, increasing amounts of Syk coimmunoprecipitated with Lyn in a time-dependent manner (Fig. 3 C). These results suggest a signaling complex for IL-3, IL-5, or GM-CSF in resting human eosinophils composed of the IL-3/IL-5/GM-CSF receptor β subunit, Lyn, and Syk. After ligand binding, additional Lyn and Syk are recruited to the signaling complex. The complex may contain additional tyrosine kinases as the IL-3/IL-5/GM-CSF receptor β subunit has been shown to associate with the Fes tyrosine kinase (21) and members of the Jak tyrosine kinase family (22, 23) in eosinophils and other cell lineages. Whether all of these interactions also occur with the IL-3/IL-5/GM-CSF receptor β subunit in eosinophils requires further exploration.

To examine the possibility of a potential sequential activation of Lyn and Syk by the IL-3/IL-5/GM-CSF receptor β subunit in eosinophils, we determined the effect of decreasing the level of expression of each of the kinases on the activation of the other kinase. Since freshly isolated eosinophils are terminally differentiated cells that undergo spontaneous apoptosis in vitro, the use of antisense oligodeoxynucleotides is the most practical method to specifi-
cally alter Lyn and Syk levels. Antisense oligodeoxynucleotides corresponding to the Lyn tyrosine kinase previously have been used to inhibit Lyn protein expression in B-lineage lymphoma cells (15). As demonstrated in Fig. 4, A and C, eosinophils exposed to an optimal dose of phosphorothioate-derivatized Lyn or Syk antisense oligodeoxynucleotides for 5–6 h expressed little or no detectable Lyn or Syk protein, respectively, whereas Lyn or Syk sense oligodeoxynucleotides did not alter Lyn or Syk protein levels. GM-CSF failed to activate Syk in eosinophils lacking Lyn expression as assessed by ptyr immunoblotting (Fig. 4 B). In contrast, GM-CSF–induced activation of Lyn as assessed by in vitro autophosphorylation assays was not altered in eosinophils that lacked functional Syk (Fig. 4 D). Sense oligodeoxynucleotide treatments did not alter Syk or Lyn activation. Lyn expression is therefore a prerequisite for Syk activation but Syk is not required for Lyn activation in human eosinophils. Thus, Lyn may be proximal to Syk in a cascade initiated by the IL-3/IL-5/GM-CSF receptor β subunit.

Since IL-5 and GM-CSF are upregulated in diseases associated with blood and tissue eosinophilia (1–6), we determined whether Lyn and Syk are required for the prevention of eosinophil apoptosis by GM-CSF and IL-5. The ability of antisense oligodeoxynucleotides to specifically decrease the expression of Lyn and Syk (Fig. 4, A and C) allowed exploration of the role of Lyn and Syk in the prevention of apoptosis. Antisense oligodeoxynucleotides were added to freshly isolated eosinophils 6 h before the cells were exposed to GM-CSF or IL-5. As shown in Fig. 5, A and C, Lyn and Syk antisense oligodeoxynucleotides both blocked the ability of GM-CSF or IL-5 to prevent eosinophil death. As previously reported, GM-CSF prevented DNA fragmentation, a characteristic feature of apoptotic cells, in control eosinophils (6). In contrast, GM-CSF was unable to prevent DNA fragmentation in eosinophils treated with Lyn or Syk antisense oligodeoxynucleotides (Fig. 5, B and D), indicating that both Lyn and Syk are required for GM-CSF to prevent apoptosis. In contrast, Fes antisense oligodeoxynucleotides failed to block cytokine-mediated prevention of cell death (not shown), although antisense to Fes had effects in other systems (16), supporting the specificity of the effects of Syk and Lyn antisense oligodeoxynucleotides. Lyn and Syk sense oligodeoxynucleotides had no effect, further arguing that the effects of Lyn and Syk antisense oligodeoxynucleotides specifically resulted from decreases of Lyn and Syk protein levels. In addition, Lyn and Syk antisense or sense oligodeoxynucleotides had no influence on the viability of eosinophils in the absence of GM-CSF (Fig. 5, A and C), emphasizing the lack of toxic effects of the oligodeoxynucleotides. These observations further imply that decreased expression of Lyn or Syk alone is not sufficient to accelerate the ongoing process of eosinophil apoptosis. Taken together, our data suggest that both tyrosine kinases, Lyn and Syk, are essential for the antiapoptotic signaling pathway induced by activation of the IL-3/IL-5/GM-CSF receptor β subunit.

A number of different kinases have been demonstrated to

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associate with transmembrane receptors through conserved SH3 and SH2 domains which constitutively bind to proline-rich domains and inductively to phosphotyrosine-based motifs, respectively. Lyn contains both SH2 and SH3 domains, whereas Syk contains two SH2 domains in the absence of a SH3 domain. In addition to these widely distributed protein-protein interaction domains, the unique domains of two additional src family kinases, Lck and Fyn, associate with CD4/8 and CD3, respectively (24, 25). Lck has also been demonstrated to associate with the IL-2 receptor β chain through its kinase domain (26). Thus, Lyn and Syk contain a number of candidate domains for interaction with the IL-3/IL-5/GM-CSF receptor β subunit or with each other.

The constitutive binding of Lyn to the IL-3/IL-5/GM-CSF receptor β subunit may be through the interaction of SH3 domains of Lyn with proline-rich sequences found within the IL-3/IL-5/GM-CSF receptor β subunit (27). This could then lead to phosphorylation of tyrosine motifs in the IL-3/IL-5/GM-CSF receptor β subunit which would create unique tyrosine-phosphorylated motifs that could further recruit Lyn and Syk through their SH2 domains.

The SH2 domains of Syk and its closely related ZAP-70 family member have been demonstrated to bind to the tyrosine-phosphorylated but not to the nonphosphorylated immunoreceptor tyrosine-based activation motif (ITAM) located in the cytoplasmic tails of IgM, high affinity IgE receptor, and TCR complexes (28, 29). The ITAM motif consists of a repeated YxxL motif separated by seven or eight amino acids (28, 29). Although a consensus ITAM motif has been recently described in the intracellular domain of this receptor.

Regardless of the mechanism of the physical association of Lyn and Syk with the IL-3/IL-5/GM-CSF receptor β subunit in human eosinophils, Lyn appears to act proximally in a tyrosine kinase cascade that regulates the association and activation of Lyn and Syk by the IL-3/IL-5/GM-CSF receptor β subunit in human eosinophils. An intact cascade appears to be required for the antiapoptotic signaling pathway induced by activation of the cells by GM-CSF or IL-5 and may play a role in the pathophysiology of diseases in which high levels of GM-CSF or IL-5 are present. Further definition of intracellular signaling mechanisms activated by the IL-3/IL-5/GM-CSF receptor β subunit in human eosinophils may facilitate the design of new drugs to normalize the dysregulated apoptosis of eosinophils in allergy and asthma.

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Address correspondence to Dr. Hans-Uwe Simon, Swiss Institute of Allergy and Asthma Research, University of Zurich, Obere Strasse 22, CH-7270 Davos, Switzerland.

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