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Lyn Tyrosine Kinase Is Important for IL-5-Stimulated Eosinophil Differentiation

Susan Stafford,* Clifford Lowell,† Sanjiv Sur,* and Rafeul Alam2*

IL-5 plays a pivotal role in growth and differentiation of eosinophils. The signal transduction mechanism of IL-5Rα is largely unknown. We have demonstrated that IL-5 induces tyrosine phosphorylation of IL-5Rα in eosinophils. To identify IL-5Rα-associated tyrosine kinases, we have examined the expression of Src family tyrosine kinases in eosinophils. Among the Src family members, Lyn, Hck, Fgr, and Lck are present in eosinophils, and, among these four kinases, only Lyn is associated with the IL-5Rα under basal conditions. We also confirm the association of Janus kinase (Jak)2 with IL-5Rα. Lyn kinase phosphorylates both IL-5Rα and βcR in vitro. The importance of Lyn kinase for eosinophil differentiation was studied using antisense oligodeoxynucleotides. Lyn antisense oligodeoxynucleotide blocks eosinophil differentiation from stem cells in a dose-dependent manner. The Jak2 inhibitor tyrphostin AG490 also inhibits eosinophil differentiation. The importance of Lyn for eosinophil differentiation was further studied using Lyn knockout mice. The IL-5-stimulated eosinophil differentiation from bone marrow cells is significantly inhibited in Lyn−/− mice as compared with that in control mice. We conclude that both Lyn and Jak2 play an essential role in IL-5Rα signaling, leading to eosinophil differentiation. The effect of Lyn appears to be relatively specific for the eosinophilic lineage. *The Journal of Immunology, 2002, 168: 1978–1983.

The pathologic hallmark of active asthma is eosinophilic inflammation of the airway (1). The increased number of eosinophils in the airways is most likely the result of increased differentiation (2) and prolonged survival of eosinophils (3). Indeed, there is evidence that differentiation of eosinophils is accelerated in subjects following allergic sensitization (2). The differentiation of eosinophils is primarily regulated by IL-5, although other cytokines also contribute to this process (1, 4). In addition, IL-5 causes prolongation of eosinophil survival and primes them for activation (5). Because of these activities, IL-5 is considered to play an important role in the pathogenesis of eosinophilic inflammation in various diseases, including asthma. Indeed, there are reports of increased production of IL-5 in airways from asthmatic patients (6, 7). Experiments with animal models pointed to a pivotal role for IL-5 in eosinophilic inflammation and airway hyperreactivity (8). However, a recent human study with an anti-IL-5 Ab failed to demonstrate clinical efficacy in asthma, although the treatment reduced the eosinophil count in the airway secretion (9). The results, albeit preliminary, suggest that multiple redundant mechanisms are operative in the pathogenesis of asthma. Nonetheless, the role of IL-5 in eosinophilic inflammation remains undisputed.

The IL-5R has two subunits, the ligand-specific α subunit and the βc subunit, which is common to receptors for IL-3 and GM-CSF (10). The signal transduction mechanism of the βcR has previously been studied. βc is associated with Lyn (11, 12), Fes (13), Janus kinase (Jak)31 (14), and Jak2 (15–17) tyrosine kinases. Following receptor oligomerization, the tyrosine kinases are rapidly activated, which leads to receptor phosphorylation and recruitment of cytosolic signaling, including Syk (18), the adapter protein Shc (19), phosphatidylinositol-3-kinase (20), and STAT transcription factors (15, 16, 21, 22). The cytosolic signal is transduced via the Jak-STAT (15, 16) and Ras-mitogen-activated protein (MAP) kinase (12) pathway. Both pathways have been shown to play important roles in IL-5 signaling. The signal transduction mechanism of IL-5Rα subunit is largely unknown. Since IL-5 plays an essential and nonredundant role in eosinophil differentiation, the foregoing function must be attributable to the signaling via the IL-5Rα. Indeed, mice with null mutation for IL-5Rα are unable to increase eosinophil differentiation in response to IL-5 (23, 24).

Tyrosine kinases of the Src family are frequently associated with cytokine receptors and play an important role in generating cytosolic signals (25). The association of Src-type kinases with IL-5Rα has not been previously reported. We investigated the physical association of Src-type kinases with IL-5Rα and examined their biological relevance.

Materials and Methods

Reagents

Percoll was purchased from Pharmacia (Piscataway, NJ). The mAb against anti-phosphotyrosine (clone 4G10) was obtained from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal anti-IL-5R α and β, anti-Jak2, and Abs against the Src family of tyrosine kinases, Lyn, Hck, Fyn, Fgr, Bk, and Lck, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The Jak2 inhibitor tyrphostin AG490 was purchased from Calbiochem (Carlsbad, CA) and resuspended in DMSO. ECL detection system was purchased from Amersham (Arlington Heights, IL).

Eosinophil purification

Peripheral blood for eosinophil purification was obtained from subjects with mild to moderate eosinophilia (6–12%). Eosinophils were isolated by discontinuous Percoll gradients and by negative selection using anti-CD16

Abbreviations used in this paper: Jak, Janus kinase; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; ODN, oligodeoxynucleotide.
Eosinophils were incubated with IL-5 (10^{-10} M) for various periods of time, and then cells were lysed and immunoprecipitated with an anti-IL-5Rα Ab. The immunoprecipitate was Western blotted with the anti-phosphotyrosine (4G10) Ab (n = 3).

**Preparation of cytosolic cell extracts and immunoprecipitation**
Eosinophils (1–4 × 10^6/ml) were incubated with IL-5 (10^{-10} M) or medium at 37°C for the indicated period of time. The stimulation was terminated by addition of 1 vol of ice-cold PBS containing 1 mM NaN_3, VO_4. The cells were pelleted by centrifugation, washed rapidly with PBS, and lysed in a buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 0.25% sodium deoxycholate, 1 μM PMSF, 1 μM Na_3VO_4, 1 mM NaF, 0.7% Triton X-100, and 1 μg/ml aprotinin, leupeptin, and pepstatin. After incubation on ice for 10 min, the lysates were passed several times through a 26-gauge needle and detergent-insoluble materials were removed by centrifugation at 4°C at 12,000 × g. The protein concentration was determined using bicinchoninic acid assay (Pierce, Rockford, IL). Cell lysates were then resolved on SDS-PAGE and subjected to Western blotting using appropriate Abs according to the method described previously (12). In other experiments, cell lysates were immunoprecipitated with Abs against anti-IL-5Rα and βc and Lyn to study phosphorylation. For this purpose, the cell lysates were precleared by incubation with 20 μl Protein A/G Agarose Plus (Santa Cruz Biotechnology) for 2 h. After removal of the beads, the lysates were incubated with an appropriate Ab and Protein A/G Agarose Plus for 4 h at 4°C. The immunoprecipitates were washed three times with the cold lysis buffer and boiled in the Laemmli sample buffer.

**Gel electrophoresis and immunoblotting**
SDS-polyacrylamide gels were prepared according to the Laemmli protocol and used for immunoblotting. The concentration of polyacrylamide was 7% or 12% depending on the m.w. range of the proteins studied. Gels were blotted onto Hybond membranes (Amersham) for Western blotting using the ECL system. Blots were incubated in a blocking buffer containing 5% BSA in TBST buffer (20 mM Tris-base, 137 mM NaCl, made to pH 7.6, and 0.05% Tween 20) for 1 h, followed by incubation in the primary Ab (0.1 μg/ml) for 1 h. After washing five times in TBST buffer, blots were incubated for 30 min with a HRP-conjugated secondary Ab (0.1 μg/ml) directed against primary Ab. The blots were developed with the ECL substrate according to manufacturer’s protocol. In some experiments, blots were reprobed with another Ab after stripping in a buffer of 62.5 mM Tris-HCl (pH 6.7), 100 μM 2-ME, and 2% SDS at 50°C for 30 min.

**In vitro kinase assay**
Lyn, IL-5Rα, and βc were immunoprecipitated from eosinophils with respective Abs (Santa Cruz Biotechnology). The kinase assay was performed in a buffer containing 20 mM Tris (pH 7.4) 2 mM MgCl_2, 0.5 μM cold ATP, and 2 μCi [γ^32P]ATP for 20 min. The assay was stopped by addition of 6× Laemmli’s buffer. The reactions then were separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and autoradiographed.

**Murine bone marrow cell culture**
In vitro liquid culture was performed as described elsewhere (26). OVA-sensitized BALB/c mice were sacrificed, and the femurs were removed. The bone marrow cavity was flushed with saline to obtain cells. The bone marrow cells (5 × 10^7 cells/ml) were suspended in IMDM. These cells were incubated with and without the inhibitors for 30 min at 37°C, followed by further culture in the presence of 1 ng/ml murine IL-3 and 6 ng/ml murine IL-5 plus 10% FCS for 1 wk. After harvesting, the total cell count was obtained and the remaining cells were used for cytopsin prep-

**FIGURE 1.** Phosphorylation of IL-5Rα by IL-5 stimulation. Purified eosinophils were incubated with IL-5 (10^{-10} M) for various periods of time, and then cells were lysed and immunoprecipitated with an anti-IL-5Rα Ab. The immunoprecipitate was Western blotted with the anti-phosphotyrosine (4G10) Ab (n = 3).

**FIGURE 2.** Expression of Src family kinases in eosinophils. Purified eosinophils were Western blotted for the presence of Hck, Lyn, Lck, Fgr, and Fyn using polyclonal Abs. Hck, Lyn, Lck, and Fgr were readily detectable. Fyn was not detectable in eosinophils (Eos) but was present in mononuclear cells (MNC) (n = 2).

**FIGURE 3.** Coprecipitation of IL-5Rα with Lyn tyrosine kinase. Purified eosinophils were stimulated with IL-5 (10^{-10} M) for 3 min and then lysed and immunoprecipitated separately with Abs against IL-5Rα and Lyn. The immunoprecipitates were then Western blotted with Abs against Lyn and IL-5Rα, respectively (n = 3).

**Antisense ODNs**
Two 15-mer Lyn sense and antisense oligodeoxynucleotides (ODN) were synthesized by Operon Technologies (Alameda, CA) based on previously published sequence information (18). These ODN do not match any other cDNA in the GenBank Database by basic local alignment search tool analyses. They have been shown to decrease the expression of Lyn in human eosinophils by one group (18), and this observation was confirmed in our laboratory (27). Sequences used were as follows: antisense Lyn (CATATT TCCGCGTGC) and sense Lyn (CGAGCGGGAAATATGC). The ODNs were phosphorothioate modified and resuspended in sterile H_2O at 100 μM concentration.

**Statistical analysis**
Results were expressed as mean ± SD. Data were analyzed for statistical significance using ANOVA and Student’s t test.

**Results**
**Tyrosine phosphorylation of IL-5Rα**
The signal transduction mechanism of IL-5Rα is largely unknown. IL-5Rα has a short cytoplasmic tail, which consists of ~55 amino acid residues. The cytoplasmic tail has one tyrosine residue. We investigated whether IL-5Rα underwent tyrosine phosphorylation upon ligand binding. Eosinophils were stimulated with IL-5, and the lystate was Western blotted with an anti-phosphotyrosine Ab. IL-5 induced tyrosine phosphorylation of the α receptor (Fig. 1). IL-5Rα has previously been reported to be differentially glycosylated and appears as multiple bands on Western blotting in the molecular mass range of 60–85 kDa. We have observed predominantly two bands (Fig. 1) upon Western blotting of eosinophil lysates.
As expected, the pellet, but not the supernatant, showed the presence of the βcR (Fig. 4). This supernatant, which does not contain βc, was then immunoprecipitated with an anti-IL-5Rα Ab. The immunoprecipitate (pellet II) was separated from the supernatant (supernatant II). Pellet I, pellet II, and supernatant II were Western blotted with the anti-Lyn Ab. The Western blot of pellet I confirms our previous report that a fraction of Lyn is associated with βc, and this association is increased following IL-5 stimulation of cells. The Western blot of pellet II suggests that IL-5Rα is associated with Lyn under basal conditions in the absence of βc. This physical association is modestly reduced after IL-5 stimulation. The Western blot of supernatant II suggests that a fraction of Lyn is not associated with either receptor subunit and is most likely associated with other receptors. In a next step, we examined the association of Hck with IL-5Rα under basal conditions (Fig. 3). Stimulation of eosinophils with IL-5 for a short period of time (3 min) did not increase the association of Lyn with IL-5Rα.

We have previously reported that Lyn is also associated with βcR (12). Thus, coprecipitation of Lyn with IL-5Rα could be the result of coprecipitation of IL-5Rα with βc. To address this concern, we first performed immunoprecipitation of stimulated and nonstimulated eosinophils with an anti-βc Ab. The immunoprecipitating pellet (pellet I) was separated from the supernatant (supernatant I), and both were Western blotted for the presence of βc.

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5Rα under basal conditions. There is only modest increase in IL-5Rα binding to Jak2 following IL-5 stimulation of eosinophils.

**Lyn kinase and Jak2 are important for eosinophil differentiation**

The differentiation of eosinophils from stem cells occurs stepwise. Lineage-committed stem cells initially require IL-3 for their proliferation. Subsequent stimulation with IL-5 leads to the differentiation of eosinophils. Previous studies have shown that a combination of IL-3 and IL-5 stimulates eosinophilopoiesis in vitro (29).

We have used an in vitro liquid culture system using bone marrow cells from mouse according to the method described previously (26). In this model, allergic sensitization of mice significantly increases the sensitivity of bone marrow stem cells to IL-5. The percentage of in vitro differentiated eosinophils increases from ~10% in nonsensitized mice to 30% in sensitized mice. We have studied the importance of Lyn kinase and Jak2 for eosinophil differentiation using bone marrow cells from sensitized mice. Murine bone marrow cells were incubated with IL-3 and IL-5 for 1 wk, followed by cytopsin preparations for Wright-Giemsa staining. Murine eosinophils were recognized by the ring-shaped nucleus and the presence of eosinophilic granules. The presence of major basic protein-containing granules in bone marrow-derived eosinophils was confirmed by immunocytochemical staining in a previous publication (26). The total cell and eosinophil counts after 1 wk were 46 ± 6 and 14 ± 2 × 10⁴ cells, respectively (n = 3). Approximately 32% of the total cells were of eosinophilic lineage.

To block Lyn kinase, we used two strategies. First, we used a Lyn antisense ODN, which blocks the expression of Lyn kinase, but not Jak2, in eosinophils (27). Tyrphostin AG490 was used to block Jak2 (30). The Lyn antisense ODN and AG490 blocked eosinophil differentiation from stem cells in a dose-dependent manner (Fig. 8, A and B). The Lyn antisense ODN did not affect proliferation of other cells in the culture (Fig. 8C). However, AG490 showed a tendency to inhibit growth of all lineages in the culture, which is in agreement with the previous finding that Jak2 is essential for hemopoiesis in general (31, 32).

**Eosinophil differentiation is impaired in Lyn knockout mice**

Next we have examined the differentiation of eosinophils in Lyn knockout mice. Lyn knockout mice do not have any developmental abnormalities (33). However, they show some dysregulation of Ig synthesis, implying a regulatory role of Lyn kinase in this process (34, 35). Furthermore, they have impaired mast cell degranulation.

We have immunized Lyn knockout mice and control mice (C57/B6) with OVA to stimulate eosinophilopoiesis in vivo. Bone marrow cells were collected for eosinophil differentiation studies according to the method described previously. Eosinophil differentiation was stimulated by culturing them in IL-3 and IL-5 for 8 days. Eosinophil differentiation was significantly reduced (10.2 ± 2 vs 27 ± 2.2, n = 6, p < 0.01) in Lyn knockout mice as compared with that in control mice (A). The total number of cells (mostly of monocytic lineage) in the culture was not affected (B).
IL-5, as described above. The IL-5-induced differentiation of eosinophils was strikingly inhibited in Lyn−/− mice as compared with that in control mice (10 ± 2 vs 27 ± 2, p < 0.01, Fig. 9). The total cell count in bone marrow cultures from knockout and control mice was similar. The noneosinophilic cells are mostly of monocytic lineage, indicating that Lyn is not essential for their growth.

Discussion

The IL-5Rα subunit plays an essential role in IL-5 signaling and eosinophil differentiation. The significance of Src family kinases in IL-5Rα signaling has not been previously investigated. We show that eosinophils express four different Src family kinases, Hck, Lyn, Fgr, and Lck. Among these four tyrosine kinases, only Lyn is physically associated with IL-5Rα, and this association occurs independent of its association with βc following IL-5 stimulation. Lyn kinase is able to phosphorylate IL-5Rα in vitro. Lyn is important for IL-5-induced eosinophil differentiation from bone marrow stem cells. We have confirmed a previous report that Jak2 is associated with IL-5Rα. Jak2 is also important for eosinophil differentiation.

Tyrosine kinases of the Src family are associated with many cytokine and growth factor receptors. Previous studies have implied an important role of Src-type kinases in a variety of cellular functions, including cell growth, differentiation, proliferation, adhesion, locomotion, and apoptosis (reviewed in Ref. 25). Specifically, in vitro studies have implicated an important role of the kinases in myeloid cell growth and differentiation. For example, Lyn is associated with receptors for B cell Ag receptor (36), erythropoietin receptor (37), FcγR (38), FcεR (39), FcεR (40), and c-Kit (41). In Lyn−/− mice, differentiation of erythrocytes, granulocytes, macrophages, and mast cells is not impaired (33–35), suggesting that erythropoietin, GM-CSF, and c-Kit signaling are unaltered. In Lyn−/− mice, basal production of neutrophils is mildly increased, whereas the B cell number is decreased by 50% (34). Basal eosinophilopoiesis appears normal in Lyn−/− mice, as reflected by the normal eosinophil count in the peripheral blood (~3% in both Lyn−/− and control mice). However, no specific information regarding IL-5-induced differentiation of eosinophils in Lyn−/− mice is available in the published literature.

Our results suggest that Lyn−/− mice have impaired IL-5-induced eosinophilopoiesis. The results imply that basal eosinophilopoiesis is regulated by multiple factors. It has been shown that the commitment of stem cells to granulocytic lineages is stochastic and independent of growth factors. Once committed, eosinophil progenitors are regulated by a multitude of positive and negative factors, including IL-3, IL-5, GM-CSF, IL-6, TGF-β, corticosteroids, CC chemokines, and others (1). This is confirmed by the observation that basal eosinophil differentiation in IL-5Rα (21) and βc (42) knockout mice is reduced but not eliminated. For example, peripheral blood eosinophil count in IL-5Rα−/− mice is reduced by <50% (23).

Lyn exerts a positive signaling effect by phosphorylating the immunoreceptor tyrosine-based activation motif of the B cell Ag receptor and a negative signaling effect by phosphorylating the immunoreceptor tyrosine-based inhibitory motif of the FcγRII (38). In Lyn−/− mice, the negative signaling in B cells is affected more than the positive signaling, which results in increased Ab synthesis (32–35). c-Kit signaling is unimpaired, and mast cell differentiation is normal. However, mast cell activation through FcεRI is attenuated (33, 35). These observations suggest that the downstream signaling effect of Lyn is cell and receptor specific. Lyn is redundant for downstream signaling by erythropoietin, IL-3, and GM-CSF receptors but is important for signaling through FcγRII and FcεRI. Our results suggest that Lyn is also important for IL-5Rα signaling in eosinophil progenitors. Interestingly, a subpopulation of mouse B cells, the so-called B1 cells, responds to CD38 ligation and IL-5 with cell proliferation and IgG1 synthesis. This effect is significantly abrogated in Lyn−/− mice, suggesting a pivotal role of Lyn in IL-5R signaling in B cells (43).

The signaling pathways downstream of Lyn kinase that lead to eosinophil differentiation are unknown. We have previously shown that extracellular signal-regulated kinase (ERK)1/2 and p38 MAP kinases are important for eosinophil differentiation from stem cells (26). Antisense inhibition of Lyn blocks the activation of ERK1/2 in eosinophils (27), indicating that the latter kinases are coupled to the Lyn signaling pathway. Whether p38 MAP kinases are linked to Lyn kinase in eosinophils is unknown. The activation of ERK1/2 in Lyn knockout mice has previously been examined. ERK1/2 activation is impaired in mast cells but enhanced in B cells from Lyn−/− mice (34). We were unable to study the activation of MAP kinases in purified eosinophils from Lyn−/− mice because of severely impaired production of eosinophils. Given that antisense Lyn ODN block ERK1/2 activation in eosinophils (27), it is likely that ERK1/2 activation is impaired in Lyn−/− eosinophil progenitor cells and results in reduced eosinophil production.

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