RAPID COMMUNICATION

Interleukin-5 Signaling in Human Eosinophils Involves JAK2 Tyrosine Kinase and Stat1α

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Signaling by a wide variety of cytokines, including interferons, interleukins, and growth factors, involves activation of JAK kinases and Stat (signal transducers and activators of transcription) proteins. At present, not much is known about the molecular mechanisms by which interleukin-5 (IL-5) exerts its diverse biologic effects. Human eosinophils are one of the most important target cells for IL-5 and were used here to study IL-5 signaling in a primary human cell. IL-5 induced rapid and transient tyrosine phosphorylation of JAK2. Moreover, IL-5 induced at least two DNA-binding complexes, using nuclear extracts from normal human eosinophils and the IL-6/interferon-γ response element of the ICAM-1 promoter (ICAM-1 pIRE) in an electromobility shift assay. From supershift experiments it was concluded that one DNA-binding complex contained Stat1α, probably as a homodimer. Both DNA-binding complexes were inhibited by a phosphotyrosine antibody (4G10), suggesting that tyrosine phosphorylation is required for complex formation. IL-3 and granulocyte-macrophage colony-stimulating factor induced, similar to IL-5, two DNA-binding complexes in human eosinophils, including Stat1α. These data show for the first time that molecular mechanisms of IL-5 signaling in human eosinophils involve members of the JAK kinase family as well as members of the Stat family.

HUMAN EOSINOPHILS play an important role in the pathogenesis of allergic diseases such as allergic asthma.1 From in vitro experiments it has been established that eosinophil functioning, as well as expression of cell surface proteins, can be induced or increased by several cytokines such as interleukin-2 (IL-2), IL-3, IL-4, IL-5, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor-α (TNF-α), or interferon-γ (IFN-γ).2-8 IL-5 is of special interest, because it is the terminal differentiation factor for eosinophils.9 Several lines of evidence suggest that eosinophil functions are also modulated in vivo. For instance, the phenotype of eosinophils isolated from the peripheral blood of allergic asthmatic subjects resembles the IL-5-primed (preactivated) phenotype in a chemotaxis assay.9,10 Eosinophils obtained from a bronchoalveolar lavage (BAL) from allergen-challenged allergic asthmatics have a changed expression of multiple adhesion molecules, indicating an activated phenotype.12 IL-5 is a rather selective cytokine, because the IL-5 receptor is expressed on a limited number of hemopoietic cells, i.e., eosinophils, basophils, and some B cells.13 High-affinity receptors for IL-5, IL-3, and GM-CSF are composed of a unique α-subunit and a common β-subunit.14-17 The β-subunit is important for cytokine signaling and these cytokines, as one might expect, have similar effects on eosinophils.17

Tested on a variety of cells, IL-5, IL-3, and GM-CSF have several effects, such as tyrosine phosphorylation of multiple cellular substrates, including the β-subunit of the IL-5/IL-3/GM-CSF receptors and Shc, and activation of p21 ras and MAP kinase.2,16-21 Some tyrosine kinases have been identified that are activated by IL-3 and GM-CSF, e.g., the proto-oncogene product c-fps/s and lyn kinase.22 JAK2, a member of the Janus family of tyrosine kinases (JAK kinases), is also activated by IL-3 and GM-CSF.26,27 JAK kinases play a central role in signaling by several cytokines, including interferons, interleukins, and growth factors.27 The JAK kinase family comprises at least four members, i.e., JAK1, JAK2, JAK3, and TYK2.27 Activation of JAK kinases is important for the subsequent activation of members of the Stat (signal transducers and activators of transcription) family, a rapidly expanding family comprising Stat1α, Stat1β, Stat2, Stat3, Stat4, and mammary gland factor-28-30 IFN-α-induced activation of the interferon-stimulated gene factor-3 complex (composed of Stat1α, Stat1β, Stat2, and p48) requires JAK1 and TYK2, whereas activation of Stat1α by IFN-γ is dependent on JAK1 and JAK2.32

In this study, we have investigated whether members of the JAK kinase family are involved in IL-5 signaling. Furthermore, it was tested if IL-5 could induce DNA-binding complexes, containing proteins that possibly share homology with members of the Stat family. We found that JAK2 was phosphorylated on tyrosine upon IL-5 treatment of eosinophils. Using the IL-6/IFN-γ response element of the ICAM-1 promoter (ICAM-1 pIRE),33 we found that IL-5, IL-3, and GM-CSF induced at least two DNA-binding complexes. One of these DNA-binding complexes contained Stat1α.

MATERIALS AND METHODS

Reagents. Ficoll-paque, Percoll, and poly(dI-dC) were obtained from Pharmacia (Uppsala, Sweden). Recombinant human IL-5 was a kind gift of Dr D. Fattah (GLAXO Group Research, Greenford, UK). Recombinant human IL-3 and GM-CSF were obtained from Genzyme (Cambridge, MA). Oligonucleotides used in this study were the ICAM-1 pIRE (5'-agcgctagTTTCCGGGAAAacg-3') and a mutant ICAM-1 pIRE (5'-agcgctagTTAGCCGGTCAAacg-3').
caccgc-3'). All other reagents were reagent grade. Cell incubations were performed in HEPES buffer, containing 132 mmol/L NaCl, 6.0 mmol/L KCl, 1.0 mmol/L CaCl₂, 1.0 mmol/L MgSO₄, 1.2 mmol/L potassium phosphate, 20 mmol/L HEPES, 5 mmol/L glucose, and 0.5% human serum albumin (HSA; wt/vol), pH 7.4.

**Antibodies.** Antiphosphotyrosine monoclonal antibody (MoAb; mouse IgG2b, hybridoma 4G10), anti-JAK2, and anti-TYK2 polyclonal rabbit antiserum were obtained from UBI (Lake Placid, NY). Anti-Stat91 (Statα) polyclonal rabbit antiserum was raised against the 36 COOH-terminal amino acids unique to Statα and was a kind gift of Dr C. Schindler (Columbia University, NY).

**Isolation of human eosinophils.** Blood from healthy volunteers was kindly provided by the Bloodbank of Utrecht, The Netherlands. Mixed granulocytes were isolated from the buffy coat of 500 mL blood anticoagulated with 0.4% trisodium citrate (pH 7.4) as described before.3² The granulocyte fraction was centrifuged for 20 minutes at room temperature over Percoll (density, 1.084 g/mL; layered on percoll with a density of 1.1 g/mL) to reduce the number of neutrophils. The eosinophil enriched fraction was collected from the percoll 1.084/1.1 interface. Eosinophils were subsequently isolated by the method essentially as described by Hansel et al.3⁵ This isolation procedure makes use of the fact that, in marked contrast to neutrophils, eosinophils lack the epitope on FcγR.

**Preparation of nuclear extracts.** Nuclear extracts were prepared from eosinophils, which were treated with IL-5 (5 × 10⁻⁴ mol/L). These extracts were used for an EMSA with the [³²P]-ICAM-1 pIRE. IL-5 induced at least two DNA-binding complexes, which are indicated as c1 and c2 (Fig 2). The appearance of the DNA-
IL-5 (min)

C 1 5 10 30

probed with anti-PTyr

reprobed with anti-JAK2

JAK2

Fig 1. IL-5 induces tyrosine phosphorylation of JAK2. Human eosinophils (10 x 10^6 cells/mL) were treated with buffer (C) or IL-5 (5 x 10^{-10} mol/L) for the indicated times at 37°C. Subsequently, JAK2 was immunoprecipitated and used for Western blot with an anti-PTyr MoAb (4G10) followed by ECL (upper panel). Blots were reprobed with the JAK2 antiserum to check for immunoprecipitation efficiency (lower panel). The position of JAK2 (130 kD) is indicated. The figure is representative of three independent experiments.

binding complexes was optimal after 15 minutes and decreased to basal levels after 2.5 hours.

The c1 and c2 complexes were specific, as shown in Fig 3. Formation of the c1 and c2 complexes was completely inhibited in the presence of excess (50- or 100-fold) unlabeled wild-type oligonucleotide. Excess mutant oligonucleotide (5'-aggcgcgaggTTAGCGGTCAAgcagcaccgc-3') did not affect the c1 and c2 complex formation with the ^32P-labeled wild-type probe, even at 100-fold excess (Fig 3).

Supershift analysis of the IL-5- and IFN-γ-induced DNA-binding factors. In marked contrast to IL-5, which induced at least two ICAM-1 pIRE binding complexes, IFN-γ induced a single DNA-binding complex (Fig 4). To characterize the proteins binding to the ICAM-1 pIRE, we performed supershift analysis of the ICAM-1 pIRE/protein complexes with antibodies raised against Stat proteins. As shown in Fig 4, the complex that was induced by IFN-γ was clearly supershifted by an antiserum specifically directed against Stat1α, indicating that this ICAM-1 pIRE/protein complex contained Stat1α. An MoAb directed against an epitope that is present in Stat1α and Stat1β also supershifted this complex (Fig 4). These results are in agreement with those of other studies, demonstrating that IFN-γ induces a Stat1α homodimer.32

When the same experiments were performed with ICAM-1 pIRE/protein complexes induced by IL-5 (30 minutes of treatment), the faster migrating complex (c2) of the doublet was supershifted with the MoAb against Stat1α/β, although the signal intensity of the complex decreased. Inhibition of the IFN-γ-induced DNA-binding complex by this antibody cannot be observed in this figure because of overexposure of the film. At much shorter exposure times, the IFN-γ-induced DNA-binding complex and the IL-5-induced c2 complex were similarly inhibited by the anti-Stat1α/β MoAb (data not shown). The Stat1α-specific antiserum supershifted the c2 complex induced by IL-5 to the top of the lane, similar to the supershift of the IFN-γ-induced DNA-binding complex (Fig 4). Isotype antibody controls for anti-Stat1α/β MoAb (mouse IgG1) and the Stat1α-specific antiserum (nonimmune rabbit antiserum) had no effect on both IL-5- and IFN-γ-induced DNA-binding complexes (results not shown). These data indicate that Stat1α is induced by IL-5 in human eosinophils. The c2 complex comigrates with the IFN-γ-induced

Fig 2. IL-5 treatment of human eosinophils leads to transient induction of ICAM-1 pIRE binding activity. Human eosinophils were isolated from the peripheral blood of healthy volunteers and incubated (10 x 10^6 cells/mL) with buffer (control, lane 1) or IL-5 (5 x 10^{-10} mol/L) for the indicated times at 37°C (lanes 2 through 6). Nuclear extracts were prepared as described. Nuclear extracts (5 μg/ lane) were incubated with a ^32P-labeled oligonucleotide (5’-agcttagg-TTTCGGAAAGcacc-3’) encoding a palindromic IFN/IL-6 response element from the ICAM-1 promotor (pIRE). IL-5 induced two clear DNA binding complexes designated as c1 and c2. The data are representative of three additional experiments.
Competitor

wild type  
mutant

50x  100x  50x  100x

C1  C2

Fig 3. Specificity of the IL-5-induced ICAM-1 pIRE binding complexes. IL-5-induced DNA-binding complexes are shown in lane 1 and are designated c1 and c2. Nuclear extracts were incubated with 50- or 100-fold excess unlabeled wild-type oligonucleotide (lanes 2 and 3) or mutant oligonucleotide (5'-aggcgcgaggtTAGCGGTCAAgcagcaccgc-3'; lanes 4 and 5) 10 minutes before incubation with the 32P-labeled ICAM-1 pIRE. The data are representative of three additional experiments.

Stat1α homodimer, further suggesting that complex c2 contains a Stat1α homodimer. Interestingly, the slower migrating complex (c1) was partially inhibited by both antibodies, suggesting that this complex contains a Stat1α-related protein or Stat1α as part of a heterodimer. Possibly, the anti-Stat1α and -Stat1α/β MoAbs recognize an epitope that is masked in a putative heterodimer complex, explaining the partial, rather than total, inhibition of the c1 complex.

We next addressed the question of whether phosphorylation of tyrosine residues is important for the IL-5--induced proteins to bind to the ICAM-1 pIRE. As shown in Fig 4, preincubation of an MoAb (4G10) directed against PTyr inhibits the formation of the c1/c2 complexes, suggesting the importance of tyrosine phosphorylation for DNA binding. Similar results were found with another anti-PTyr MoAb, PY20 (data not shown). Others have shown that phosphorylation of a single tyrosine residue (Tyr701) in Stat1α is necessary for nuclear translocation and DNA-binding.29 When the MoAb was saturated with soluble phosphotyrosine, thereby inhibiting the binding of the MoAb, the inhibition of the formation of ICAM-1 pIRE/Stat complexes was eliminated.

Effect of GM-CSF and IL-3 on the induction of ICAM-1 pIRE/Stat complexes in human eosinophils. GM-CSF (10-8...
GM-CSF primes eosinophil function at picomolar concentrations.\(^5\) Again, in supershift experiments, it is shown that the faster migrating complex c2 supershifted with the antisera specific for Stat1\(\alpha\) and the MoAb directed against the epitope found in both Stat1\(\alpha\) and Stat1\(\beta\). These findings indicate that IL-3 and GM-CSF can induce Stat1\(\alpha\) in normal human eosinophils. The upper complex c1 was partially inhibited by both antibodies, suggesting that this complex also contains a Stat1\(\alpha\)-related protein or Stat1\(\alpha\) as a heterodimer.

It should be noted that donor variation existed with respect to the signals of the c1 and, especially, the c2 complex. However, the c1 and c2 complexes were found in the majority of the experiments and the c2 complex was always supershifted with the anti-Stat1\(\alpha\) and anti-Stat1\(\alpha/\beta\) antibodies.

In marked contrast to this study, others have shown that, in monocytes, U937 cells, and Ly6e cells, IL-3 and GM-CSF induce a single complex that is neither supershifted nor inhibited by anti-Stat1\(\alpha\) antibodies but is able to bind to GAS oligonucleotides.\(^6\)\(^,\)\(^7\) However, it is reported that IL-3 induces tyrosine phosphorylation of Stat1\(\alpha\), although signals were rather low.\(^8\) It was found that IL-5 induced a DNA-binding complex in human basophils; however, no supershift experiments were performed to further analyze this complex.\(^9\) The reason why Stat1\(\alpha\) DNA-binding activity is not induced by IL-3 and GM-CSF in the aforementioned studies is not clear, but might reflect a difference in binding affinity and/or specificity of Stat proteins for the oligonucleotides used in those studies and ours, ie, the GAS element of the Ly6E promotor and the IFN-\(\gamma\) response region of the Fc\(\gamma\)RII promotor versus the ICAM-1 pIRE.\(^10\)\(^,\)\(^11\) Alternatively, the different cell types possibly determine the pattern of Stat proteins (and/or other transcription factors as well) that are induced by IL-3 and GM-CSF.

IL-3, GM-CSF, and IFN-\(\gamma\) induce the expression of several molecules on the cell surface of eosinophils, such as the IL-2 receptor p55 chain, CD4, and Fc\(\gamma\)RIII.\(^33\)\(^,\)\(^34\) ICAM-1 expression is markedly induced by TNF-\(\alpha\) together with IFN-\(\gamma\), GM-CSF, IL-3, or IL-5.\(^5\)\(^,\)\(^6\) In the case of the ICAM-1 gene, a Stat-binding element has been identified.\(^35\) Characterization of other genes encoding cell surface proteins induced by IL-5, IL-3, GM-CSF, or IFN-\(\gamma\) possibly shows Stat binding sequences in the promotor region as well. This would provide a molecular basis for regulation of these genes upon addition of these cytokines to human eosinophils.

In conclusion, IL-5 signaling in human eosinophils involves tyrosine phosphorylation of JAK2 and the induction of DNA-binding complexes containing Stat1\(\alpha\) and, possibly, a Stat-related factor.

NOTE ADDED IN PROOF

After the submission of this manuscript, Sato et al (J Exp Med 180:2101, 1994) reported that IL-5 induces activation of JAK2 tyrosine kinase in an mIL-5–dependent murine early B-cell line (Y16).

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