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Activation of the STAT3/Acute Phase Response Factor Transcription Factor by Interleukin-5*

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The receptor for interleukin-5 (IL-5R) is composed of a unique α chain (IL-5Rα) expressed on eosinophils and basophils, associated with a βc subunit, which is shared by the receptors for IL-3 and granulocyte macrophage-colony stimulating factor. One of the molecular events activated via the IL-5R is the JAK/STAT signaling pathway. Recent reports have shown that IL-5 induces tyrosine phosphorylation of JAK2 followed by the subsequent cell type-specific activation of either STAT1α or STAT5. To identify additional STAT proteins activated by IL-5, we co-transfected the IL-5R with STAT cDNAs in COS cells. We found that IL-5 induces binding of STAT3 to the intercellular adhesion molecule-1 pIRE, and activates STAT3-dependent transcription. Moreover, endogenous STAT3 was tyrosine phosphorylated and activated in human IL-5-stimulated BaF3 cells ectopically expressing the human IL-5R (BaF3/IL5R). These data imply that multiple STAT proteins are involved in gene regulation by IL-5 in a cell type-specific manner. We further demonstrate using C-terminal truncations of the α and βc subunits of the IL-5R that the membrane-proximal regions of both subunits are required for STAT activation. Interestingly, a βc receptor mutant lacking intracellular tyrosine residues is able to mediate STAT3 activation, suggesting that tyrosine phosphorylation of the βc receptor is not essential for STAT3 activation.

Hematopoiesis is tightly regulated by a complex network of stromal interactions and by soluble polypeptide factors named cytokines. IL-3,[1] IL-5, and GM-CSF are cytokines that have diverse effects on the proliferation, differentiation, and activation of blood cells and their precursors (1–3). Whereas IL-3 and GM-CSF also have effects on other hematopoietic lineages (3, 4), the effect of IL-5 in humans is restricted to eosinophils and basophils. IL-5 is essential for eosinophil differentiation (5, 6) and plays an important role in the function of mature eosinophils (7–11). The specific effects of IL-5 on eosinophils and basophils are due to restricted expression of the low affinity IL-5Rα receptor on these cell types (12, 13). The high affinity receptor for IL-5 (IL-5R) is composed of a unique α subunit associated with a βc subunit that is shared with the receptors for IL-3 and GM-CSF (14). The βc subunit is essential for signal transduction (15, 16), but also, the α chain transduces intracellular growth signals (17). Therefore, the common use of the βc subunit by IL-3, IL-5, and GM-CSF explains the partial observed functional redundancy of these cytokines (16, 18). However, postreceptor signal transduction pathways are not well defined and are likely to be composed of both mitogenic and differentiation signals. It is known that the βc subunit, like other cytokine receptors, does not contain intrinsic tyrosine kinase activity (19, 20). However, one of the earliest events to occur after IL-3, IL-5, and GM-CSF stimulation is induction of protein tyrosine phosphorylation (19, 21). This tyrosine phosphorylation is caused by the activation of several cytoplasmic protein tyrosine kinases such as Lyn (22, 23) and c-Fps/Fes (24). Recent studies have demonstrated that the family of Janus kinases (JAKs), containing four members (JAK1, JAK2, JAK3, and TYK2), is associated with different cytokine receptors and is phosphorylated after ligand binding (25). We and others have shown that only JAK2 is activated in response to IL-3, IL-5, and GM-CSF (26–28) and specifically associates with the membrane-proximal region of the β chain (29). The JAK protein-tyrosine kinases activate members of a novel family of transcription factors named signal transducers and activators of transcription (STATs) (25).

STAT proteins were first described as intermediates in the interferon-α/β (IFN-α/β) signaling pathway (30, 31). At present, eight different STATs (STAT1α, STAT1β, STAT2, STAT3, STAT4, STAT5A, STAT5B, STAT6) have been described and are involved in specific cytokine regulation. (31–39). This family of STAT proteins comprises a new class of transcription factors that contain Src homology 2 (SH2), SH3-like domains, and a carboxyl-terminal tyrosine phosphorylation site (25). STAT proteins normally exist as inactive monomers in the cytoplasm, but after phosphorylation on tyrosine, they form homo- or heterodimers, which translocate to the nucleus and bind to specific DNA motifs (40–44). We recently found that STAT3 is also phosphorylated on serine residues (45). This serine phosphorylation seems to be necessary for the regulation of the transactivation potential of STAT3.

We have previously shown that IL-5 activates STAT1α together with an unidentified DNA-binding protein in human eosinophils (27). Therefore, we were interested whether other known STAT proteins can be activated by IL-5. For this purpose, we reconstituted both subunits of the IL-5 receptor together with different STAT cDNAs in COS cells. We demonstrated that besides STAT1α, STAT3 was also activated by IL-5 in these transfected COS cells. In addition, endogenous STAT3 was tyrosine phosphorylated and activated by hIL-5 in BaF3 cells stably expressing the hIL-5R (BaF3/IL5R). Finally we

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[1] The abbreviations used are: IL, interleukin; GM-CSF, granulocyte macrophage-colony stimulating factor; IL-5R, receptor for IL-5; JAK, janus kinase; STAT, signal transducer and activator of transcription; SH domain, Src homology domain; IFN, interferon; hIL, human interleukin; GRR, γ responsive region; ICAM-1, intercellular adhesion molecule-1; pIRE, palindromic IL-6/IFN-γ response element; SIE, Sis-inducible element.

demonstrate, using carboxyl-terminal truncations of both the α and β subunits, that the membrane-proximal region of both subunits are necessary for STAT3 activation.

MATERIALS AND METHODS

Cell Culture, Reagents, and Antibodies—Monkey COS-1 cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% heat inactivated fetal calf serum. A mouse IL-3-dependent pro-B cell line, BaF3, and BaF3 cells expressing the wild type human IL-5 receptor α and β subunits (BaF3/IL5R) were maintained in RPMI 1640 supplemented with 10% Hyclone serum and the appropriate cytokine. Parental BaF3 cells were grown in medium with 10^{-10} M mouse IL-3 (Genzyme, Cambridge, MA), BaF3/IL5R cells were grown in medium with 10^{-10} M hIL-5 (a kind gift of Dr. D. Fattah, Glaxo Group Research, Greenford, United Kingdom) and 1 mg/ml G418 (Life Technologies, Inc.). Anti-phosphotyrosine monoclonal antibody (4G10) was obtained from UBI (Lake Placid, NY). The monoclonal antibody directed against STAT1α/β was purchased from Transduction Laboratories (Lexington, Kentucky). The STAT3 rabbit polyclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, California).

Synthetic Oligonucleotides and Plasmid Construction—Oligonucleotides with the following sequences were used in this study (only the top strands are shown): the human ICAM-1 pIRE, 5′-AGCTTAGGTTTCCGGGAAAGCAC-3′; a mutant pIRE, 5′-AGGCGCGAGGTT**AG**CGG**T-C**AAGCAGCACCGC-3′; the *c-fos* SIEm67, 5′-GTGCATTTCCCGTAAATCTTGTCTACAATTC-3′; and the FcγRI GRR, 5′-AGCTTGAGATGTATTTCCCAGAAAAGA-3′. The 2xpIREtkluc reporter construct has been described by us previously (43); also the pSV-lacZ expression vector has been described previously (46). The reporter plasmids 4xIREtkCAT and 4xGRRtkCAT were constructed by cloning four oligonucleotides containing either the pIRE or GRR in the *Hin*dIII site upstream of the thymidine kinase minimal promoter in pBLCAT2. pSGhSTAT1α was constructed by inserting the cDNA for human STAT1α from pMNC91 (47) into the *Not*I/*Bam*HI sites of pSG513 (48). pSGhSTAT3 was constructed by inserting the human STAT3 cDNA isolated from a human eosinophil cDNA library into PSG513. pSGmSTAT4 was constructed by inserting the cDNA for mouse STAT4 from Rc/CMV-STAT4 (32) into the *Hin*dIII/*Sma*I sites of pSG513. pSGshSTAT5 was constructed by inserting the cDNA for sheep STAT5 (MGF) from pXM-STAT5 (49) into the *Hin*dIII/*Not*I sites of pSG513. pSGhSTAT6 was constructed by inserting the cDNA for human STAT6 (IL-4STAT) from TPU231 (38) into the *Eco*RI/*Xho*I sites of pSG513. pSGhIL5Rα was constructed by inserting the cDNA for the human IL-5α receptor from pBKhIL5Rα (50) into the *Not*I/*Kpn*I sites of pSG513. pSGhIL5Rβ was constructed by inserting the cDNA for the human βc subunit from pSV532 (14) into the *Eco*RI sites of pSG513. For construction of the c-terminal deletions of the IL-5 receptor α and β chains, stop codons where introduced at the indicated amino acids using PCR.

Generation of Stable Transfectants—Plasmid DNAs of the human IL-5Rα and βc containing the neomycin resistance gene were transfected into the BaF3 cells by using electroporation as described previously (21), and transfectants were selected using G418 (1 mg/ml). Expression of the α and βc subunits was verified by PCR analysis and by growth in the presence of hIL-5.

Transient Transfection—For transfection experiments, COS cells were split 1:3 in 6-well plates (Costar), and 2 h later the cells were transfected with 10 μg of supercoiled plasmid DNA by the calcium phosphate coprecipitation technique (51). Following 16–20 h of exposure to the calcium-phosphate precipitate, medium was refreshed, and cells were incubated for 16 h with IL-5. Transfected cells were subsequently harvested for luciferase assay (52) and lacZ determination (53).

Gel Retardation Assay—Nuclear extracts were prepared from unstimulated and stimulated COS and BaF3 cells following a previously described procedure (54). Oligonucleotides were labeled by filling in the cohesive ends with [α-^{32}P]dCTP using Klenow fragment of DNA polymerase I. Gel retardation assays were carried out according to published procedures with slight modifications (30). Briefly, nuclear extracts (10 μg) were incubated in a final volume of 20 μl, containing 10 mM HEPES, pH 7.8, 50 mM KCL, 1 mM EDTA, 5 mM $MgCl_2$, 10% (v/v) glycerol, 5 mM dithiothreitol, 2 μg of poly(dI-dC) (Pharmacia Biotech Inc.), 20 μg of bovine serum albumin, and 1.0 ng of ^{32}P-labeled ICAM1-pIRE oligonucleotide for 20 min at room temperature. In competition experiments, extracts were incubated for 5 min with the indicated molar excess of unlabeled oligonucleotide prior to the addition of labeled oligonucleotide. Identification of the different DNA binding proteins was performed by preincubating nuclear extracts for 30 min on ice with antisera before addition of the probe.

Immunoprecipitation and Western Blotting—Unstimulated and IL-5 stimulated BaF3 cells were incubated with lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 5 mM EDTA, Na_3VO_4, 10 μg/ml aprotinine, 1 mM phenylmethylsulfonylfluoride, 1 mM leupeptin) for 15 min on ice. The lysate was centrifuged to remove nuclei and cellular debris. The cell lysates were incubated with the anti-STAT3 polyclonal antibody for 1 h at 4 °C. Immune complexes were then precipitated with protein A-Sepharose for 1 h at 4 °C, washed 3 times with lysis buffer, and boiled in 1.0 × Laemmli's sample buffer. The proteins were electrophoresed on a SDS-polyacrylamide gel and transferred to nitrocellulose membrane. After blocking in TBST (150 mM NaCl, 10 mM Tris, pH 8.0, 0.3% Tween 20) with 5% bovine serum albumin, the membrane was either incubated with the anti-phosphotyrosine (4G10) monoclonal antibody or with the polyclonal anti-STAT3 antibody. Between the incubation with 4G10 and STAT3 antibodies, the membrane was stripped with 1% SDS, 30 mM Tris, pH 8.0, 50 mM β-mercaptoethanol for 2 × 15 min at 55 °C. After washing 3 times with TBST, the membrane was incubated for 1 h with peroxidase-conjugated rabbit anti-mouse or swine anti-rabbit antibodies, respectively. In both cases, the membrane was washed 5 times with TBST, and immunoprecipitated proteins were visualized with enhanced chemiluminescence (ECL, Amersham Corp.).

RESULTS

Reconstitution of the IL-5 Receptor in COS Cells Results in STAT3 Activation by IL-5—We have previously shown that IL-5 treatment of human eosinophils activates the binding of multiple protein complexes to the pIRE from the ICAM-1 promoter (27). We also demonstrated that one of these complexes contained STAT1α (p91) by using STAT1α specific antibodies. However, the identity of the other DNA-binding proteins activated by IL-5 remained unknown. One approach to define whether IL-5 can also activate other known members of the STAT family is to reconstitute STAT activation by IL-5 in COS cells. We therefore transiently transfected these cells with expression vectors encoding the human IL-5Rα and IL-5Rβ subunits in combination with expression vectors encoding the different STAT members. These were co-transfected together with a reporter construct containing 2 pIRE sequences coupled to the thymidine kinase (tk) promoter and the luciferase (luc) gene (2xpIREtkluc). We previously identified this pIRE sequence from the ICAM-1 promoter to bind IL-6- and IFN-γ-induced DNA-binding proteins (43). We analyzed STAT activation by IL-5 through the measurement of luciferase activity. As can be seen in Fig. 1, COS cells transfected with the pSG5 expression vector without STAT cDNAs showed an IL-5-dependent 2-fold increase in luciferase activity, which is probably due to endogenous STATs in COS cells. Overexpression of hSTAT1α resulted in an IL-5-dependent 2-fold increase of luciferase activity compared with cells transfected with pSG5. Surprisingly, IL-5 also activated hSTAT3, which resulted in a 14-fold increase of luciferase activity. As a control for ligand specificity, we transfected COS cells with the cDNA for STAT3 and treated the cells with IFN-γ. We observed that STAT3 was not activated by IFN-γ, whereas IFN-γ was able to activate STAT1α.[2]

Furthermore, we observed no effect of IL-5 on luciferase activity after cotransfection of the cDNAs encoding for STAT4, STAT5, and STAT6 (Fig. 1). These results show that IL-5 can activate the transactivation potential of both STAT1α and STAT3 in COS cells.

Since DNA-binding is a prerequisite for transactivation, we tested nuclear extracts from untreated and IL-5-stimulated COS cells transfected with STAT3. Indeed, when COS cells were stimulated with IL-5 for 30 min, a single DNA binding complex is induced using the ICAM-1 pIRE as a probe (Fig. 2).

[2] E. Caldenhoven and R. de Groot, unpublished observation.

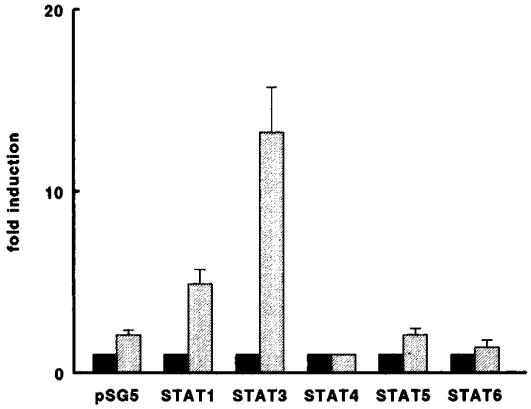

FIG. 1. **Transactivation by STAT3 is induced in response to hIL-5.** Monkey COS cells were transiently transfected by calcium phosphate precipitation with 2 μg of 2xIREtkluc reporter construct, 0.5 μg of hIL-5Rα cDNA, 0.5 μg of hIL-5Rβ cDNA, 4 μg of pSG5 (*lane 1*), 4 μg of hSTAT1α cDNA (*lane 2*), 4 μg of hSTAT3 cDNA (*lane 3*), 4 μg of mSTAT4 cDNA (*lane 4*), 4 μg of shSTAT5 cDNA (*lane 5*), 4 μg of hSTAT6 cDNA (*lane 6*) and 2 μg of a β-galactosidase expression vector (pSV-lacZ) as a control for transfection efficiency. 1 day after transfection, cells were incubated for 16 h with IL-5. The cells were then harvested, and β-galactosidase and luciferase activities were determined. Luciferase activities were normalized to β-galactosidase activities. -Fold induction of luciferase activity in IL-5 treated (*gray bars*) compared with untreated control cells (*black bars*) is shown. Values represent the averages of four different experiments ± S.E. IL-5 clearly enhances IL-6/IFN-γ response element activation by STAT1α and STAT3.

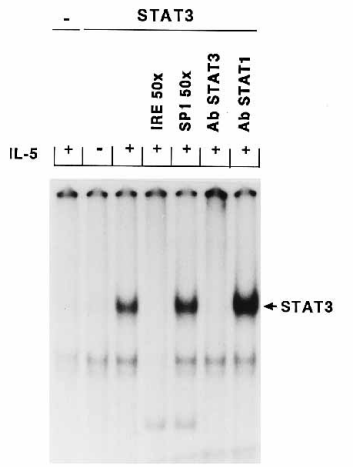

FIG. 2. **hIL-5 induces a DNA binding complex containing STAT3.** The hIL-5 receptor and STAT3 were expressed in COS cells. These cells were either untreated or treated for 30 min with IL-5, after which nuclear extracts were prepared. Nuclear extract were assayed for binding to the ^{32}P-labeled ICAM-1 pIRE in bandshift experiments. For competition experiments, extracts were preincubated for 5 min with a 50-fold molar excess of unlabeled oligonucleotide as indicated. For identification of the STAT proteins, the extracts were incubated with either anti-STAT1α, anti-STAT3 antibodies, or nonimmune serum for 30 min before the addition of the ^{32}P-labeled ICAM-1 pIRE. IL-5 clearly induces binding of STAT3 to the ICAM-1 pIRE.

This is a specific binding complex because it could be competed with an excess of unlabeled pIRE, whereas a nonspecific competitor (SP1) was unable to compete for binding. To confirm that this DNA-binding protein was STAT3, we preincubated nuclear extracts with antibodies against STAT3. As shown in Fig. 2, this antibody against STAT3 binds to the DNA-protein complex and is therefore not able to migrate into the gel. In contrast, a control preincubation with STAT1α antibodies had no effect on this DNA-protein complex. These results indicate that IL-5 activates STAT3 binding to the ICAM-1 pIRE.

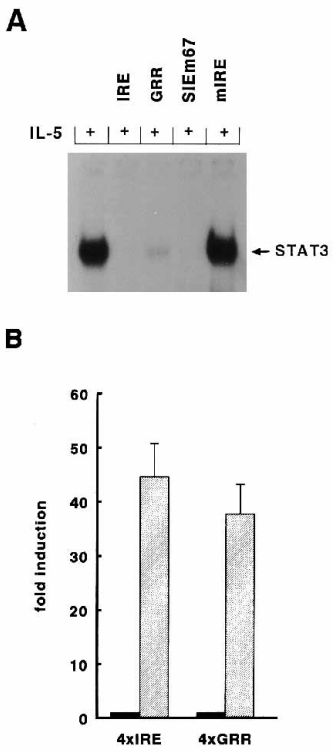

FIG. 3. **hIL-5 induced STAT3 activity is not restricted to the ICAM-1 pIRE.** *A*, COS cells expressing the hIL-5 receptor and STAT3 were stimulated with IL-5 for 30 min. Nuclear extracts were prepared and incubated with a ^{32}P-labeled ICAM-1 pIRE. For competition experiments, extracts were pre-incubated for 5 min with a 50-fold molar excess of unlabeled oligonucleotide as indicated. IL-5-induced STAT3 can bind to other STAT binding sites. *B*, COS cells were transient transfected with the IL-5Rα, IL-5Rβ, and STAT3 cDNAs together with either a 4xIREtkCAT or a 4xGRRtkCAT reporter construct. 1 day after transfection, cells were stimulated with IL-5 and harvested after 16 h. Chloramphenicol acetyltransferase assays were performed with β-galactosidase-normalized samples, and -fold induction was calculated relative to untreated cells. Values represent the averages of three different experiments ± S.E. IL-5 induces activation of both IRE- and GRR-containing promoters by STAT3.

We further characterized the DNA-binding specificity of the IL-5-induced STAT3 complex. These experiments were performed with nuclear extracts from COS cells transfected with the IL-5R and STAT3 and treated with IL-5 for 30 min. The ICAM-1 pIRE was used as a radioactive probe, and as competitors we used the IFN-γ activating site elements from the c-fos (SIEm67) (40) and the FcγRI promoter (GRR) (41). Fig. 3*A* shows that competition with a 50-fold molar excess of either the pIRE or SIEm67 oligonucleotides completely inhibited binding of STAT3 to the ICAM-1 pIRE, whereas a mutant ICAM-1 pIRE was unable to compete for binding. Competition with the GRR sequence resulted in a decrease of STAT3 binding to the pIRE, suggesting that the pIRE is a better binding site for STAT3 than the GRR. We also tested the transcriptional activity of STAT3 mediated on the GRR sequence. We therefore inserted four copies of the GRR sequence in front of a tkCAT reporter plasmid. This 4xGRRtkCAT reporter construct was transfected in COS cells together with cDNAs encoding the IL-5Rα and βc subunits and STAT3. As shown in Fig. 3*B*, overexpression of the IL-5R and STAT3 in the presence of IL-5 resulted in a 40-fold induction of luciferase activity mediated via the GRR sequence. The increase in luciferase activity is comparable with the IL-5-induced transactivation of a 4xpIREtkCAT reporter construct. Taken together, these results show that IL-5 is capable of activating STAT3 in COS

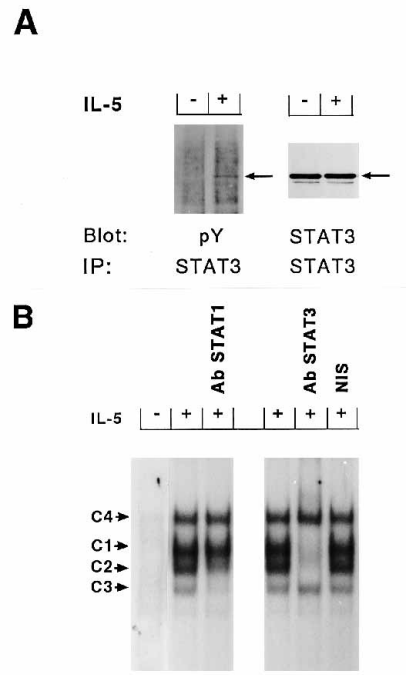

FIG. 4. **Tyrosine phoshorylation and activation of STAT3 by hIL-5 in BaF3/IL5R cells.** BaF3 cells stably expressing the hIL-5 receptor (BaF3/IL5R) were untreated or treated with hIL-5 for 15 min. *A*, endogenous STAT3 was immunoprecipitated from these BaF3/IL5R cells with a monoclonal antibody specific against STAT3. Immunoprecipitates were analyzed by Western blotting with the phosphotyrosine-specific antibody 4G10 (*left panel*). The membrane was stripped and reprobed with the STAT3 specific antibody (*right panel*). hIL-5 clearly induces phosporylation of STAT3 on tyrosine residues. *B*, nuclear extracts from these BaF3/IL5R cells were analyzed in a bandshift assay using the ICAM-1 pIRE as a probe. The extracts from hIL-5-stimulated cells were incubated with either anti-STAT1α, anti-STAT3 antibodies, or nonimmune serum. The hIL-5-induced pIRE binding complex contains both STAT1α and STAT3.

cells.

IL-5 Induces Tyrosine Phosphorylation and DNA Binding of STAT3 in BaF3/IL5R Cells—Since the results described above were obtained using transiently transfected cells overexpressing STAT3, we next addressed the question of whether endogenous STAT3 could be activated by hIL-5. For this purpose, we used the IL-3-dependent murine pro B-cell line BaF3, which we stably transfected with the human IL-5Rα and βc subunits (BaF3/IL5R). Since tyrosine phosphorylation of STAT proteins is rapidly induced after cytokine stimulation, we immunoprecipitated STAT3 from unstimulated and hIL-5-stimulated BaF3/IL5R cells. Tyrosine phosphorylation of STAT3 was then monitored by Western blotting using the antiphosphotyrosine antibody 4G10. As shown in Fig. 4*A*, stimulation with hIL-5 resulted in phosphorylation of the STAT3 protein.

Subsequent to tyrosine phosphorylation, STATs dimerize and translocate to the nucleus were they bind to specific DNA sequences. Therefore, nuclear extracts from untreated and hIL-5-stimulated BaF3/IL5R cells were incubated with a ^{32}P-labeled pIRE oligonucleotide and analyzed in a bandshift assay. Fig. 4*B* shows that stimulation with hIL-5 for 15 min results in the formation of four binding complexes. The same complexes were observed in mouse IL-3-treated parental BaF3 cells.[2] To determine the identity of the DNA binding proteins induced by hIL-5, we preincubated nuclear extracts with antibodies against STAT1α and STAT3. We found that anti-STAT1α antibodies diminished complex C2 and C3, whereas anti-STAT3 antibodies blocked the formation of complex C1 and C2 (Fig. 4*B*). The upper complex C4 was not affected by either STAT1α or STAT3 antibodies. We conclude from these data that in BaF3/IL5R cells, hIL-5 activates both STAT1α and STAT3, which bind both as homo- and heterodimers to the ICAM-1 pIRE.

The Membrane Proximal Region of the α Subunit and the Box1 Region of the βc Subunit Are Required for STAT3 Activation—The high affinity receptor for IL-5 is composed of a ligand-specific α subunit and a βc subunit that plays an important role in transducing signals to the cytoplasm (17, 21). Previously, it was demonstrated that JAK2 is constitutively associated with the βc subunit (29). In particular, the membrane proximal region, which contains the box1 motif, which is also present in other cytokine receptors, is required for JAK2 activation. Furthermore it has been noticed that a specific tyrosine-based motif in the gp130 receptor is involved in STAT3 activation (55). Since the βc subunit lacks such a specific tyrosine based motif, we were interested in determining which region of the receptor is necessary for STAT3 activation. For this purpose, we generated a series of cytoplasmic truncations of the βc subunit. A schematic representation of these constructs is depicted in Fig. 5*A*. We transfected COS cells with these βc subunit deletion mutants in combination with the wild type IL-5α subunit, STAT3, and the reporter plasmid 2xpIREtkluc (Fig. 5*B*). We found that the βc, β826, β762, β627, and β516 each were able to mediate STAT3 activation in response to IL-5. However, the transactivation potential of construct β516 was diminished with 50%, indicating that the region between 516 and 627 is necessary for optimal STAT activation. By contrast, no STAT3 activation was observed when the most truncated β456 receptor mutant was expressed. Consistent with these data was the observation that the internal deletion mutant β457–543 was not capable of activating STAT3 in response to IL-5. Another internal deletion mutant, β518–543, which still contains box2, clearly activated STAT3. These results suggests that the region between amino acids 452 and 516 containing box1 is required for both JAK2 and STAT3 activation, whereas the region between amino acids 543 and 627 plays an additional role in STAT3 activation. We further conclude from these experiments that tyrosine phosphorylation of the βc receptor is not necessary for STAT3 activation, since the β516 receptor deletion mutant is still able to activate STAT3, although this truncated receptor contains no intracellular tyrosine phosphorylation sites.

We next examined whether the cytoplasmic domain of the hIL-5Rα is involved in STAT3 activation. A schematic representation of the constructed carboxyl-terminal α subunit mutants α405, α390, and α366 is shown in Fig. 5*A*. Transfection of COS cells with these truncated α receptor mutants together with the wild type βc subunit shows that only mutant α366 was unable to mediate STAT3 activation (Fig. 5*C*). This region between amino acids 366 and 390 contains a proline-rich region, which has some similarity with the box1 region in other receptors. Furthermore, it has been shown that this region is necessary for JAK2 activation, although JAK2 is not associated with the α subunit (17, 29). Taken together, we found that the cytoplasmic proline-rich region of IL-5Rα and the box1 region of the βc subunit are essential for STAT3 activation.

DISCUSSION

Our results demonstrate that besides STAT1α and STAT5 (27, 36, 37), STAT3 also plays a role in IL-5 signal transduction. This extends the list of cytokines that are capable to activate the STAT3 protein (32, 56, 57). STAT3 was originally identified as the acute phase response factor, which is activated in response to IL-6 and plays a role in the activation of the acute phase response genes (58). Later on it became clear that this protein shows homology (40–50%) to STAT1α/β (32) and

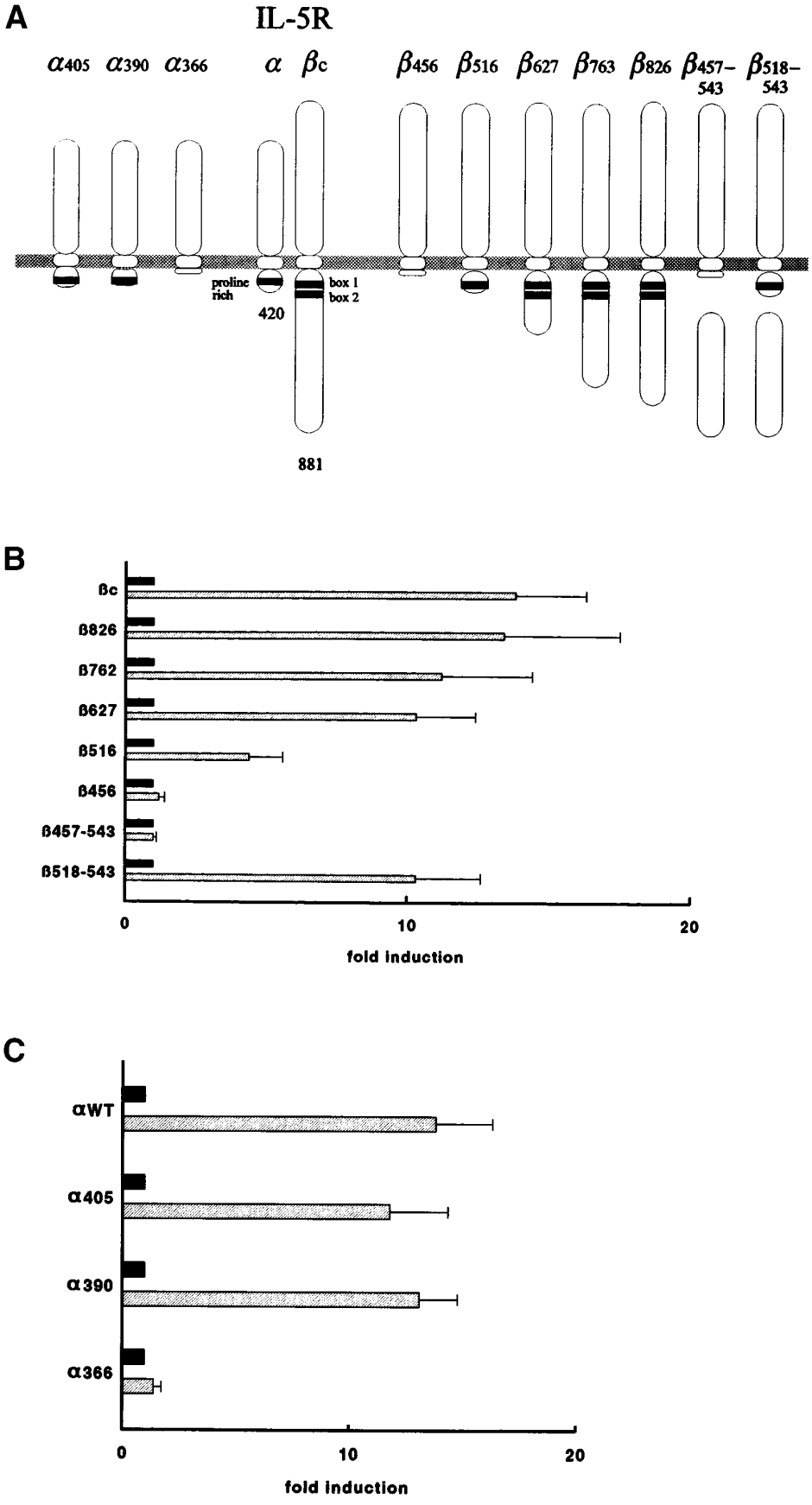

FIG. 5. **Analysis of c-terminally truncated hIL-5 receptor mutants for STAT3 activation.** *A*, schematic representation of the wild type and carboxyl-terminal deletions mutants of the hIL-5 receptor α and βc subunits. The box1/2 homology regions are shown for the βc chain. A proline-rich region of homology between the α subunits of receptors for GM-CSF, IL-3, and IL-5 is also shown. *B*, COS cells were transiently transfected with the wild type hIL-5Rα together with the wild type human βc chain or one of the βc chain deletion mutants. Furthermore, the expression vectors for STAT3 and a β-galactosidase and the 2xpIREtkluc reporter plasmid were co-transfected. 1 day after transfection, cells were

was therefore renamed STAT3.

The studies presented here demonstrate that STAT3 is activated in response to IL-5 both at the DNA-binding and transcription-activation level. We performed these experiments in COS cells by reconstitution of the IL-5R together with several members of the STAT family. We found that IL-5 induces specifically the transactivation potential of STAT1α and STAT3 mediated via the pIRE. Although we were not able to detect an effect of IL-5 to activate the transcriptional potential of STAT5, we found an IL-5-induced increase in DNA-binding of STAT5 to the GRR.[2] The latter finding is in line with recent studies, which demonstrated an increase in DNA-binding but not in transactivation mediated by mSTAT5 in response to growth hormone and erythropoietin in COS cells (37). This means that probably cell type-specific factors play an additional role in the transactivation of the STAT proteins. We reported recently that serine phosphorylation is necessary to mediate STAT3 transactivation (45). Because IL-5 plays a restricted role in hematopoiesis, it can be envisaged that cell type-specific serine/threonine kinases are necessary to activate the transactivation potential of some STAT proteins. A recent report showed induced tyrosine phosphorylation of STAT6 by IL-3 in murine IL-3-dependent myeloid DA-3 cells (39). Although IL-3 and IL-5 use the common βc subunit, we found no activation of STAT6 by IL-5 after overexpression of STAT6 in COS cells. This observation also supports the hypothesis that cell type-specific factors are required for STAT activation. The involvement of STAT3 in IL-5 signaling is further supported by the observation that STAT3 was tyrosine phosphorylated in BaF3/IL5R cells after treatment with hIL-5. The tyrosine phosphorylation of STAT3 is necessary for binding of the STAT3 protein to the ICAM-1 pIRE as can be seen in Fig. 4*B*. We found that hIL-5 induced four DNA-binding complexes in these BaF3/IL5R cells. Using different STAT antibodies, we identified complex C1 as a STAT3 homodimer, complex C2 as a STAT1α/STAT3 heterodimer, and C3 as a STAT1α homodimer, whereas C4 was not supershifted by both antibodies. Recently a similar DNA binding pattern was found when human breast carcinoma T47D cells were treated with IL-6. However, they identified the C4 complex as a multimeric complex composed of STAT3, a 91-kDa (not STAT1α), and a 46-kDa protein (59). In conclusion, IL-5 activates both STAT1α and STAT3 in COS and BaF3 cells. However, the previously described unidentified DNA-binding protein induced by IL-5 in mature human eosinophils was not recognized by a STAT3 antibody.[2] The activation of different STAT proteins by a single cytokine such as IL-5 provides the cell with a mechanism to activate different subsets of target genes depending on the cellular context. For example, STAT3 might play a role in the precursor cells of the eosinophil. This hypothesis is presently tested by differentiating hematopoietic stem cells from cord blood with IL-5 to mature eosinophils.

Signal transduction by the IL-5 receptor is rather poorly characterized. The cytoplasmic domain of the βc subunit is well characterized in the context of the GM-CSF receptor and contains two distinct regions that are responsible for different signals (19). A membrane proximal region of the approximately 60 amino acids is essential for induction of the *c-myc* and *pim*-1 genes as well as for inducing mitogenesis. A distal region of 140 amino acid residues is required for activation of Ras, Raf-1, mitogen-activated protein kinase and p70 S6 kinase. Recently, it has been shown that a cytoplasmic portion containing box1 of the βc subunit is required for JAK2 activation. Moreover, JAK2 is constitutively associated with the membrane proximal region of the βc chain (29). Deletion analysis of the receptors for growth hormone, erythropoietin, and the interleukin-6 receptor signal transducing protein gp130, showed an absolute requirement of a six amino acid P*XX*P*X*P sequence in box1 for JAK kinase association (60). Furthermore, a proline-rich region in the IL-5α receptor that resembles box1 in the βc subunit is also important for JAK2 activation (17), although JAK2 is not associated with this region (29). However, not much is known about the involvement of the IL-5R in the activation of STAT proteins. Our results using a series of c-terminally truncated deletion mutants of the α and β chain demonstrate that the membrane-proximal region of both subunits are required for STAT activation. Recently, the involvement of a specific tyrosine-based motif in the gp130 receptor has been described in the phosphorylation of STAT3 (55). However, receptor deletion mutant β516 contains no tyrosine phosphorylation sites but was still able to activate STAT3. This means that tyrosine-phosphorylation of the βc subunit is not important for STAT3 activation. The same observation was previously shown for STAT1α activation accomplished via the growth hormone receptor (61). An alternative mechanism could be that the SH2 domain of the STAT3 protein binds to the tyrosine-phosphorylated JAK kinase, or that the SH3 domain of STAT3 binds to the proline-rich box1 region of the βc subunit. Such a mechanism can be explained by the requirement of the membrane-proximal region of the βc subunit for both JAK and STAT activation. This suggests that the precise mechanism by which STAT proteins are activated probably depends on the association of the different JAK kinases to cytokine receptors and on specific binding sites for STAT proteins on the cytokine receptor. Therefore, further studies are required to determine the precise molecular mechanism by which STAT proteins are activated via the βc subunit of the IL-5 receptor.

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incubated for 16 h with IL-5. The cells were then harvested, and β-galactosidase and luciferase activities were determined. Luciferase activities were normalized to galactosidase activities. -Fold induction of luciferase activity in IL-5-treated (*gray bars*) compared with untreated control (*black bars*) cells is shown. Values represent the averages of five different experiments ± S.E. The region between 456 and 516 is necessary for STAT3 activation by IL-5. *C,* transfections were performed with the wild type βc chain together with the the wild type α subunit or one of the α chain deletion mutants as described above. The region between 366 and 390 is necessary for STAT3 activation by IL-5.

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