STAT3β, a Splice Variant of Transcription Factor STAT3, Is a Dominant Negative Regulator of Transcription*

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The 89-kDa STAT3 protein is a latent transcription factor which is activated in response to cytokines (interleukin (IL)-5 and -6) and growth factors (epidermal growth factor). Binding of IL-5 to its specific receptor activates JAK2 which leads to the tyrosine phosphorylation of STAT3 proteins. Here we report the cloning of a cDNA encoding a variant of the transcription factor STAT3 (named STAT3β) which was isolated by screening an eosinophil cDNA library. Compared to wild-type STAT3, STAT3β lacks an internal domain of 50 base pairs located near the C terminus. This splice product is a naturally occurring isoform of STAT3 and encodes a 80-kDa protein. We found by reconstitution of the human IL-5R in COS cells that like STAT3, STAT3β is phosphorylated on tyrosine and binds to the pIRE from the ICAM-1 promoter after IL-5 stimulation. However, STAT3β fails to activate a pIRE containing promoter in transient transfection assays. Instead, co-expression of STAT3β inhibits the transactivation potential of STAT3. These results suggests that STAT3β functions as a negative regulator of transcription.

Stimulation of transcription factors by cytokines or growth factors is an important step in activating specific gene transcription leading to cell growth, differentiation, and many other cellular functions. To activate or repress transcription, transcription factors must be located in the nucleus, bind DNA, and interact with the basal transcriptional machinery. Most of these processes are achieved by phosphorylation of a transcription factor by protein kinases (1, 2). One of the earliest signaling events after cytokine stimulation is the activation of non-receptor protein tyrosine kinases, such as members of the Src and Janus kinase (JAK) families (3). Many individual cytokine receptors are linked to specific members of the JAK family which are activated after ligand binding. The activated JAK kinases phosphorylate and activate a novel family of transcription factors termed signal transducers and activators of transcription (STATs)[1] (4). STAT proteins were first recognized in the interferon-α (IFN-α) and β signaling pathway. IFNα activates a latent cytoplasmic transcription factor complex interferon-stimulated gene factor 3 (5–7). This complex consists of a 48-kDa DNA-binding component, and the tyrosine-phosphorylated proteins STAT1α, STAT1β, and STAT2 (8). By contrast, only STAT1α is tyrosine phosphorylated upon stimulation of cells with IFN-γ (9). Until now, eight members of the STAT family: STAT1α, STAT1β (6, 7), STAT2 (6), STAT3 (10, 11), STAT4 (12, 13), STAT5A, STAT5B (14–16), and STAT6 (17) have been identified and characterized. All the STATs are widely expressed in different cell types and tissues, except for STAT4, which is expressed predominantly in testis and in cells of hematopoietic origin. Phosphorylation on tyrosine of the STAT proteins is required for dimerization, DNA-binding, and the activation of transcription (18, 8). However, STAT1β, which is a splice product of STAT1α and lacks 38 amino acids of the carboxyl terminus, is phosphorylated on tyrosine but is transcriptionally inactive (18). Furthermore, we and others found that H7, which is a serine/threonine kinase inhibitor, blocked the transactivation potential of STAT1 and/or STAT3 (19–22). These results suggest that phosphorylation of serine residues in STAT1 and STAT3 are necessary for the transcriptional activity of these proteins.

Cytokines such as interleukin-3 (IL-3), IL-5, and granulocyte-macrophage colony stimulating factor (GM-CSF) play an important role in hematopoiesis (23–25). Although IL-3 and GM-CSF also have effects on other hematopoietic lineages (25, 26), the actions of IL-5 in humans are restricted to eosinophils and basophils, since the IL-5 receptor (IL-5R) is only expressed on these cell types (27, 28). IL-5 is essential for eosinophil differentiation (29, 30) and plays an important role in functioning of mature eosinophils and basophils (31–35). The IL-5R is composed of a unique α subunit associated with a βc subunit that is identical to those of the receptors for IL-3 and GM-CSF (36). We and others have shown that JAK2 is activated by IL-3, IL-5, and GM-CSF (37, 38), and constitutively associates with the membrane-proximal region of the βc subunit (39). Recently, we have described that STAT3 activity increases in response to interleukin-5 (IL-5) in both BaF3 and COS cells ectopically expressing the hIL-5 receptor (IL-5R) (40). Although STAT3 is tyrosine phosphorylated and activated by IL-5 in these cells, it was only activated to a very low extent in mature eosinophils. Based on this and the observation that multiple STATs are activated by IL-5 in eosinophils (37), we screened an eosinophil cDNA library to identify IL-5 induced novel STAT cDNAs.

Here we report the cloning and characterization of a STAT3 variant which we isolated from the eosinophil cDNA library. This protein (STAT3β) is a truncated form of STAT3 which is

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[1] The abbreviations used are: STAT, signal transducer and activator of transcription; JAK, Janus kinase; IFN, interferon; IL-5, interleukin-5; IL-5R, interleukin-5 receptor; GM-CSF, granulocyte macrophage-colony stimulating factor; IRE, interferon/IL-6-responsive element; pIRE, palindromic IL-6/IFNγ response element; ICAM-1, intercellular adhesion molecule-1; CREM, cAMP-responsive element modulator; S-CREM, short CREM.

probably generated by differential splicing. STAT3 and STAT3β protein are co-expressed in various cell types. We found that STAT3β is rapidly phosphorylated on tyrosine upon IL-5 stimulation of COS cells. However, although STAT3β efficiently binds to the palindromic IL-6/IFNγ response element (pIRE) from the intercellular adhesion molecule 1 (ICAM-1) promoter, it is unable to activate a promoter containing this pIRE element. We also demonstrate that STAT3β is a strong dominant inhibitor of transcription.

MATERIALS AND METHODS

Eosinophil cDNA Library Construction—Human eosinophils were isolated from two hyper-eosinophilic individuals according to a slight modification (41) of the method described previously (42). The purity of the eosinophils collected was at least 95% as determined by histochemical staining of cytospins with May-Grunwald-Giemsa stain. Viability determined by trypan blue exclusion was more than 98%. Total RNA was extracted from 1×10^8 eosinophils using an Rnaid kit according to the manufacturers instructions (BIO 101 Inc.). Poly(A)$^+$ mRNA was extracted by oligo(dT) affinity purification using Dynabeads Oligo(dT)$_{25}$ (Dynal, Norway). This mRNA was used to construct an *Eco*RI/*Xho*I directional cDNA library in the λ ZAPII vector as described by the manufacturer (Stratagene). The library contained greater than 2×10^6 primary recombinants with a background of less than 10%. The average insert size was approximately 1.5 kilobase and the largest inserts were estimated to be greater than 5 kilobases.

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. Human IL-5 (hIL-5) was a kind gift of Dr. D. Fattah (Glaxo Wellcome, Stevenage). The 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, KY). The STAT3 rabbit polyclonal antibodies K15 (directed against amino acids 626–640) and C20 (directed against amino acids 750–769) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Synthetic Oligonucleotides and Plasmid Construction—Oligonucleotides with the following sequence were used in this study (only the top strand is shown): the human ICAM-1 pIRE, 5′-AGCTTAGGTTTCCGG-GAAAGCAC-3′. The 2xpIREtkluc and pICAM1–339 reporter constructs has been described by Caldenhoven *et al.* (43) and the pSV-lacZ expression vector by Shen *et al.* (44). pSGhIL5Rα was constructed by inserting the cDNA for the human IL-5α receptor from pBKhIL5Rα (45) into the *Not*I/*Kpn*I sites of pSG513. pSGhIL5Rβ was constructed by inserting the cDNA for the human βc subunit from pSV532 (36) into the *Eco*RI sites of pSG513. The expression vectors containing the cDNAs for hSTAT1, mSTAT3, and hSTAT4 were provided by Dr. James E. Darnell, Jr. (7, 11). The hSTAT6 cDNA was provided by Dr. Steven L. McKnight (17). Full-length STAT cDNAs were used for the screening of the cDNA library. The hSTAT3 and hSTAT3β were isolated from the eosinophil cDNA library and cloned into the *Eco*RI site of PSG513.

Transient Transfections—For transfection experiments, COS cells were split 1:3 in 6-well plates (Costar), and 2 h later the cells were transfected with 10–20 μg of supercoiled plasmid DNA by the calcium phosphate coprecipitation technique (46). Following 16–20 h 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 (47) and lacZ determination (48).

Gel Retardation Assay—Nuclear extracts were prepared from unstimulated and IL-5 stimulated COS cells following a previously described procedure (49). 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 (5). 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), 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. Supershift analysis were performed by preincubating 10 μg of nuclear extract with 1 μl (1 μg) of anti-STAT3 antibody for 30 min on ice prior to addition of the binding buffer and ^{32}P-labeled probe.

Immunoprecipitation and Western Blotting—Unstimulated and IL-5

stimulated COS cells were incubated with RIPA 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$_3$VO$_4$, 10 mg/ml aprotinine, 1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin) for 15 min on ice. The lysate was centrifuged to remove DNA 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 three times with lysis buffer, and boiled in 1 × 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. After washing three 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 five times with TBST and immunoprecipitated proteins were visualized with enhanced chemiluminescence (ECL, Amersham). 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.

RESULTS

Isolation of a Short Form of STAT3—In order to identify new STAT proteins expressed in eosinophils that may play a role in IL-5 signaling, an eosinophil cDNA library was screened with a labeled probe containing the full-length STAT cDNAs of hSTAT1, mSTAT3, hSTAT4, and hSTAT6. After low stringency screening of the cDNA library, several positive phage clones were isolated. The inserts of these clones were characterized by Southern hybridization and sequencing. All the isolated clones encode known STAT proteins including hSTAT3, hSTAT4, and hSTAT6. However, one of the STAT3 positive cDNA clones showed a different restriction pattern compared to the wild type STAT3 cDNA. Sequencing of this clone revealed that it is almost identical to STAT3 but lacks an internal part of 50 base pairs, covering nucleotides 2145–2195 near the COOH terminus (Fig. 1*A*). This deletion removes codons 716 to 732 and in addition causes a shift in the open reading frame resulting in the formation of a stop codon after 7 amino acids (Fig. 1*B*). This truncated STAT3 mRNA encodes a protein consisting of the first 715 amino acids of STAT3 plus an additional 7 unique amino acids. Comparison of the amino acids in the carboxyl-terminal region shows that this truncated STAT3 protein contains the tyrosine phosphorylation site at position 704, but lacks the conserved PMSP sequence which is a substrate for a serine kinase (50). Based on published work (7) identifying a splice variant of STAT1 named STAT1β, it is likely that the truncated form of STAT3 results from an alternative splicing event as well. We therefore designated the STAT3 clone with this internal deletion as STAT3β. To determine whether this isoform is not a cloning artifact we performed a polymerase chain reaction experiment with internal primers depicted in Fig. 1*A* on mRNA from the cell types mentioned below. As expected, two polymerase chain reaction products were formed a 300-base pair fragment and a minor 250-base pair fragment (data not shown). Sequencing revealed that these DNA fragments corresponds to the internal regions of STAT3 (300 base pairs) and STAT3β (250 base pairs). Additional evidence that this isoform is not a cloning artifact comes from the independent isolation of this cDNA by Schaefer *et al.* (51), who isolated this cDNA via a two hybrid screen with a N-terminal segment of c-Jun.

STAT3β Is Expressed in Different Cell Types—To investigate the existence of the STAT3β protein and to establish its tissue distribution, we performed a STAT3 immunoprecipitation from different cell types. For this purpose, cell lysates were prepared from U937, HL-60, BaF3 cells, eosinophils, COS cells, and COS cells transfected with the cDNA encoding for STAT3β (COS/STAT3β). The proteins were precipitated with a specific STAT3
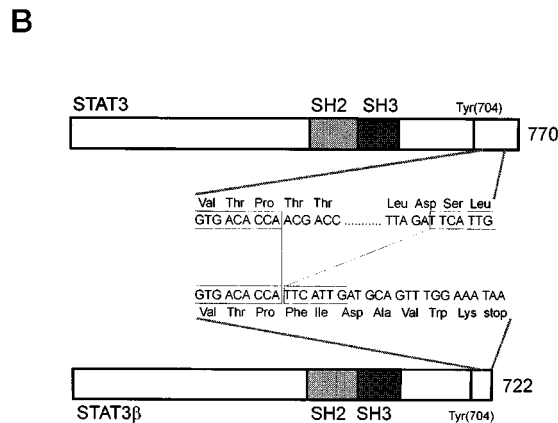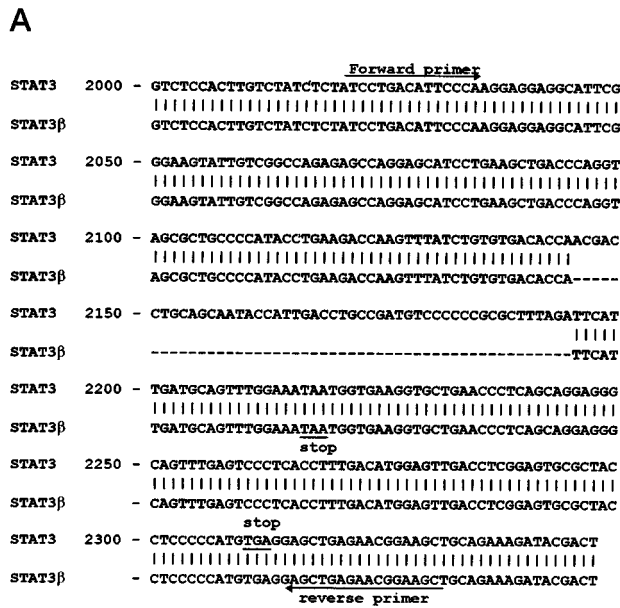
A

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                          Forward primer
STAT3   2000 - GTCTCCACTTGTCTATCTCTATCCTGACATTCCCAAGGAGGAGGCATTCG
               ||||||||||||||||||||||||||||||||||||||||||||||||
STAT3β         GTCTCCACTTGTCTATCTCTATCCTGACATTCCCAAGGAGGAGGCATTCG

STAT3   2050 - GGAAGTATTGTCGGCCAGAGAGCCAGGAGCATCCTGAAGCTGACCCAGGT
               ||||||||||||||||||||||||||||||||||||||||||||||||||
STAT3β         GGAAGTATTGTCGGCCAGAGAGCCAGGAGCATCCTGAAGCTGACCCAGGT

STAT3   2100 - AGCGCTGCCCCATACCTGAAGACCAAGTTTATCTGTGTGACACCAACGAC
               ||||||||||||||||||||||||||||||||||||||||||||||
STAT3β         AGCGCTGCCCCATACCTGAAGACCAAGTTTATCTGTGTGACACCA-----

STAT3   2150 - CTGCAGCAATACCATTGACCTGCCGATGTCCCCCCGCGCTTTAGATTCAT
                                                            |||||
STAT3β         --------------------------------------------TTCAT

STAT3   2200 - TGATGCAGTTTGGAAATAATGGTGAAGGTGCTGAACCCTCAGCAGGAGGG
               ||||||||||||||||||||||||||||||||||||||||||||||||||
STAT3β         TGATGCAGTTTGGAAATAATGGTGAAGGTGCTGAACCCTCAGCAGGAGGG
                             stop
STAT3   2250 - CAGTTTGAGTCCCTCACCTTTGACATGGAGTTGACCTCGGAGTGCGCTAC
                |||||||||||||||||||||||||||||||||||||||||||||||||
STAT3β        - CAGTTTGAGTCCCTCACCTTTGACATGGAGTTGACCTCGGAGTGCGCTAC
                          stop
STAT3   2300 - CTCCCCCATGTGAGGAGCTGAGAACGGAAGCTGCAGAAAGATACGACT
                ||||||||||||||||||||||||||||||||||||||||||||||||
STAT3β        - CTCCCCCATGTGAGGAGCTGAGAACGGAAGCTGCAGAAAGATACGACT
                       reverse primer
```

B

FIG. 1. **Nucleotide and amino acid sequence of the COOH-terminal regions of human STAT3 and STAT3β.** *A*, STAT3 and STAT3β cDNAs were isolated from a human eosinophil cDNA library, subcloned, and sequenced. As can be observed, STAT3β contains an internal deletion of 50 nucleotides. The positions of the stop codons used in STAT3 and STAT3β and the primers used for the detection of STAT3 and STAT3β mRNA are indicated. *B*, schematic representation of the amino acid sequences of STAT3 and STAT3β around the internal deletion. Positions of the Src homology 2 (SH2), SH3, and tyrosine phosphorylation (Tyr[704]) domains are indicated. Due to the deletion in STAT3β, the reading frame is switched and a stop codon is generated 7 amino acids downstream of the internal deletion.

A

B

C

FIG. 2. **Expression and tyrosine phosphorylation of the STAT3β protein.** *A*, whole cell extracts from COS (*lane 1*), COS transfected with STAT3β (*lane 2*), BaF3 (*lane 3*), HL-60 (*lane 4*), U937 (*lane 5*), and eosinophils (*lane 6*) were monitored for the presence of STAT3 and STAT3β by immunoprecipitation with a STAT3 specific antibody (amino acids 629–640) followed by Western blotting with the same antibody. The protein of about 89 kDa represents STAT3, while the smaller protein (80 kDa) is STAT3β. STAT3 and STAT3β are co-expressed in different cell types, although at different ratios. *B*, COS cells were transfected with the IL-5R α and β cDNAs together with the pSG5 expression vector (*lanes 1 and 2*), STAT3 (*lanes 3 and 4*), or STAT3β (*lanes 5 and 6*). 48 h after transfection, cells received IL-5 for 15 min (*lanes 2, 4, and 6*), after which cells were lyzed and STAT3 was immunoprecipitated. The blot was first probed with an anti-phosphotyrosine antibody (*upper panel*), stripped, and probed with the STAT3 antibody (*lower panel*). IL-5 causes a strong increase in tyrosine phosphorylation of both STAT3 and STAT3β. The faster migrating bands observed in *lanes 3* and *4* (*lower panel*) are sometimes observed, and are probably the result of degradation of STAT3. *C*, whole cell extracts from COS cells transfected with STAT3 (*lanes 1 and 5*), or STAT3b (*lanes 2 and 6*), U937 (*lane 3 and 7*), and HL-60 cells (*lanes 4 and 8*) were immunoprecipitated with a STAT3 antibody (629–640) (*lanes 1–4*) or a STAT3 antibody (amino acids 750–769) (*lanes 5–8*) and blotted with STAT3 antibody (629–640). The endogenously expressed 80-kDa protein is likely to be STAT3β.

antibody directed against residues 626–640 and immunoblotted with this STAT3 antibody. Fig. 2*A* shows that untransfected COS cells express only a low amount of the 89-kDa STAT3 protein. However, overexpressing STAT3β in COS cells results in the appearance of an 80-kDa protein which comigrated with an endogenously expressed 80-kDa protein in the other cell types analyzed with the STAT3 antibody. The expression of this 80-kDa protein varies between the different cell types studied (Fig. 2*A*). This 80-kDa protein was also found in blood monocytes, lymphocytes, and neutrophils (data not shown). To ascertain that the endogenously expressed 80-kDa protein was STAT3β, we precipitated the proteins with a STAT3 antibody directed against amino acids 750–769, a domain lacking in the STAT3β protein. Using this antibody we detect only the 89-kDa wild type STAT3 protein in COS/STAT3, COS/STAT3β, U937, and HL-60 cells (Fig. 2*C, lanes 5–8*). When proteins from these cells were precipitated with STAT3 antibodies directed against residues 629–640, two
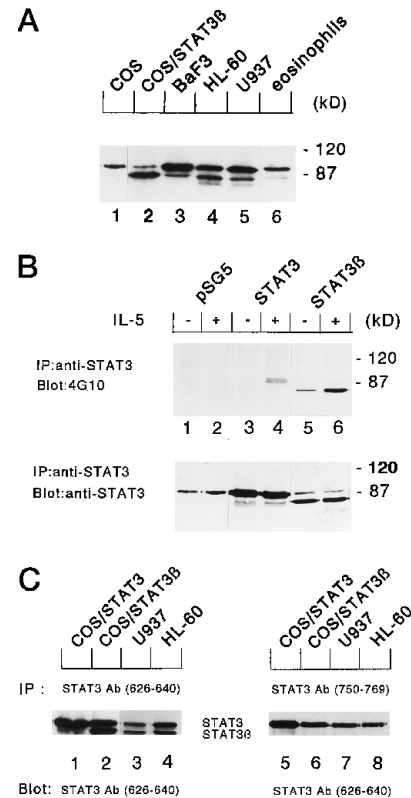
STAT3 proteins appeared of 89 and 80 kDa, except for the COS/STAT3 cells which shows only the wild type STAT3 (Fig. 2*C, lanes 1–4*). We therefore conclude that the endogenously expressed 80-kDa protein in primary cells as well as in the cell lines is likely to be STAT3β since it is not recognized by an antibody against the N terminus which is lacking from STAT3β.

Tyrosine Phosphorylation and DNA Binding of STAT3β—We have previously shown that STAT3 becomes tyrosine phosphorylated after IL-5 treatment in both BaF3 and COS cells (40). Since STAT3β still retains the tyrosine residue necessary for STAT3 activation, we were interested whether STAT3β is phosphorylated on tyrosine after IL-5 stimulation. To test this prediction we co-transfected COS cells with expression vectors encoding both subunits of the IL-5 receptor (IL-5Rα and IL-5Rβ), together with STAT3 or STAT3β. We immu-
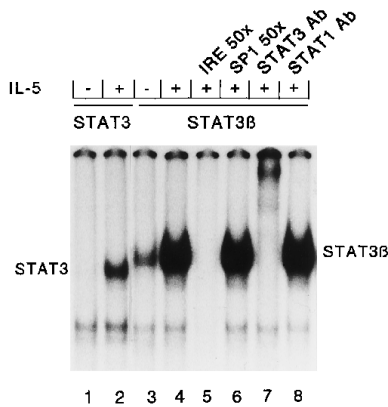
FIG. 3. **Both STAT3 and STAT3β bind to DNA after activation by IL-5.** The hIL-5 receptor and STAT3 or STAT3β were expressed in COS cells. These cells were either untreated or treated for 30 min with IL-5, after which nuclear extracts were prepared. Nuclear extract were assayed for binding to the ^{32}P-labeled ICAM-1 pIRE in band shift experiments. For competition experiments, extracts were preincubated for 5 min with a 50-fold molar excess of unlabeled oligonucleotide as indicated. For supershift analysis, the extracts were incubated with either anti-STAT1α or anti-STAT3 antibodies for 30 min before the addition of the ^{32}P-labeled ICAM-1 pIRE. IL-5 clearly induces binding of STAT3 and STAT3β to the ICAM-1 pIRE.

noprecipitated STAT3 from unstimulated and IL-5 stimulated COS cells and tyrosine phosphorylation was then monitored by Western blotting using the anti-phosphotyrosine antibody 4G10. Fig. 2B shows that both wild-type STAT3 and STAT3β are phosphorylated on tyrosine after IL-5 stimulation. However, although the expression of STAT3β protein is less then wild-type STAT3, the amount of tyrosine phosphorylation is higher. In addition, even in the absence of IL-5 signaling, we detected some basal level tyrosine phosphorylation of STAT3β, which we have never observed with STAT3.

Tyrosine phosphorylation of STAT proteins leads to dimerization, translocation to the nucleus, and binding to specific binding sites on the DNA. We therefore tested the ability of STAT3β to bind DNA and compared this with wild-type STAT3. COS cells expressing the IL-5R and either wild-type STAT3 or STAT3β were treated with IL-5 for 15 min and nuclear extracts were prepared. When these nuclear extracts were assayed in a gel retardation assay for binding to a ^{32}P-labeled ICAM-1 pIRE, an increase in STAT3 and STAT3β binding was observed after IL-5 treatment (Fig. 3). Unexpectedly, however, the STAT3 complex migrated faster than the STAT3β complex. The STAT3β complex is specific because an anti-STAT3 antibody produced a supershift, while STAT1 antiserum had no effect on this complex. Furthermore, the DNA binding activity of STAT3β is higher than STAT3, which is probably due to the higher amount of tyrosine-phosphorylated STAT3β proteins observed (Fig. 2B). In addition, we observed some basal level DNA binding activity by STAT3β in unstimulated cells, which is in agreement with the observed tyrosine phosphorylation in unstimulated cells (Fig. 2B). We can conclude that both wild-type STAT3 and STAT3β are tyrosine phosphorylated after IL-5 stimulation which results in an increase in DNA binding of both proteins.

STAT3β Acts as a Dominant Transcriptional Repressor—To compare the transcriptional activity of STAT3 with STAT3β, we transiently transfected COS cells with expression vectors for the IL-5R, wild-type STAT3, or STAT3β together with a luciferase reporter construct, containing two copies of the pIRE from the ICAM-1 promoter. Transfection of the wild-type STAT3 cDNA shows an IL-5 dependent 15-fold increase in luciferase activity (Fig. 4A). By contrast, transfection of STAT3β gave almost no increase in luciferase activity after
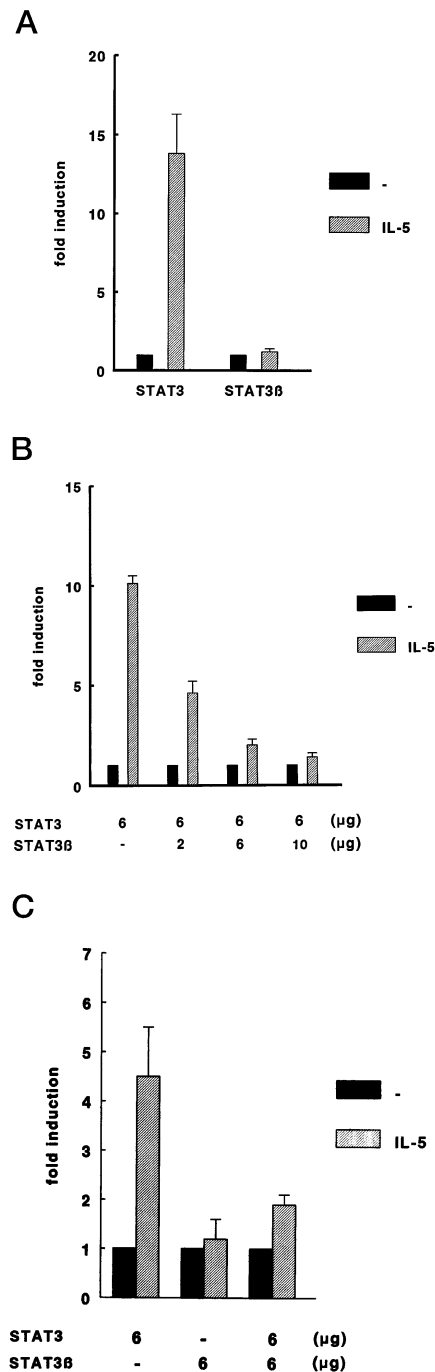

FIG. 4. **STAT3β is a dominant negative regulator of transcription.** A, COS cells were transfected with the IL-5R, a pIRE containing luciferase reporter construct and STAT3 or STAT3β. 24 h post-transfection, cells were stimulated for 16 h with IL-5 (10^{-10} M), after which transcriptional activation was measured by assaying for luciferase activity. Fold induction represents luciferase activity in IL-5-treated cells compared to untreated cells, and is the mean of three independent experiments. STAT3β is unable to support IL-5 induced activation of the pIRE reporter construct. B, COS cells were transfected as described in A with different amounts of STAT3 and STAT3β as indicated. Low amounts of STAT3β already significantly decrease trans-activation by STAT3, while high amounts of STAT3β completely inhibit STAT3-mediated transcriptional activation. C, COS cells were transfected as described in A and B. We used the ICAM-1 promoter as a luciferase reporter construct (pIC-339luc). STAT3β is also transcriptionally inactive on the natural ICAM-1 promoter.

IL-5 stimulation. An explanation for this could be that the region which is absent from STAT3β contains a domain important for transactivation. We further determined the effect of

STAT3β on transactivation mediated by STAT3. We tested this by co-transfection of an increasing amount of STAT3β expression vector together with a constant amount of STAT3 expression vector. The total amount of DNA was kept constant by adding the pSG5 vector. In this experiment we see that transactivation by STAT3 is already inhibited by low amounts of STAT3β (Fig. 4B). Western blotting indicated that STAT3β expression did not alter the level of STAT3 (data not shown). These results implicate that STAT3β can act as a dominant negative regulator of STAT3-mediated transcription. To make sure that this dominant effect of STAT3β also occurs on a natural promoter we used the ICAM-1 promoter containing the IRE in transient transfections experiments. COS cells were transfected with this reporter construct together with STAT3, STAT3β, or a combination of both STAT proteins and stimulated with IL-5. We found that although STAT3 is an activator of the ICAM-I promoter, STAT3β is transcriptionally inactive on the ICAM-1 promoter and acts as a dominant negative regulator of STAT3-mediated transcription. (Fig. 4C).

Since all STAT proteins form homo- or heterodimers after phosphorylation on tyrosine, a mechanism for the inhibition could be that STAT3 and STAT3β form heterodimers which are unable or less able to mediate transactivation. To investigate this, we prepared nuclear extracts from IL-5-treated COS cells transfected with STAT3 and increasing amounts of STAT3β. We assayed these nuclear extracts for binding to the ICAM-1 pIRE and performed a long run gel retardation to resolve the different complexes. As we already observed, the affinity of STAT3β homodimers (Fig. 5A, lane 6) to the pIRE is higher then the binding of STAT3 homodimers (lane 1). Interestingly, an intermediate complex C2 was observed when STAT3β is co-transfected together with STAT3, which is likely to consist of a STAT3/STAT3β heterodimer. To resolve all three DNA-binding complexes, lanes 2–7 were exposed less than lane 1. We also identified the components in these complexes using two specific STAT3 antibodies, one directed against residues 629–640 supershifting both STAT3 and STAT3β (Fig. 5B, lanes 2 and 5), the other against residues 750–769 which recognized only STAT3 (lanes 3 and 6). We further show that the STAT3 (750–769) antibody which recognizes only STAT3 supershifted only the STAT3/STAT3β heterodimer and had no effect on the STAT3β homodimer (lane 9). These results clearly demonstrate that STAT3 and STAT3β form heterodimeric DNA-binding complexes.

DISCUSSION

STAT proteins are a rapidly expanding family of transcription factors that transduce short-term cytoplasmic signals elicited by polypeptide growth factors and cytokines into long-term changes in gene expression (52). Here, the cloning and characterization of a novel isoform of the STAT3 transcription factor is reported, which is named STAT3β in analogy with STAT1α/STAT1β. Although STAT3β is phosphorylated on tyrosine upon IL-5 stimulation and binds efficiently to the pIRE from the ICAM-1 promoter, it fails to support pIRE-driven transcription in IL-5-stimulated cells. Moreover, STAT3β is an efficient dominant negative regulator of STAT3-mediated transcription.

Differential splicing in the STAT family is not unprecedented. Schindler *et al.* (7) have shown that the STAT1 gene encodes at least two different proteins, STAT1α and STAT1β, that are generated by alternative splicing. Like STAT3β, STAT1β can be phosphorylated on tyrosine but fails to activate transcription (18). However, whether STAT1β can act as a dominant negative regulator of STAT1α was never investigated. The splicing event occurs at a highly homologous position in STAT1 and STAT3 (22, 52), suggesting that STAT1 and STAT3 might have a conserved exonic organization as was
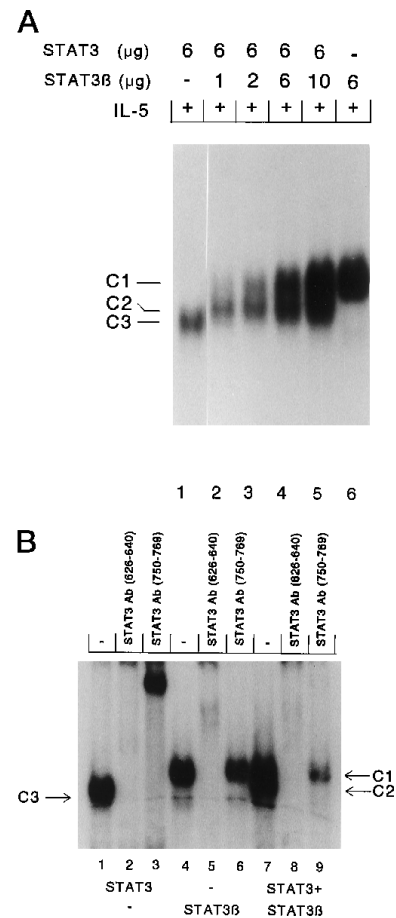

FIG. 5. **Heterodimerization between STAT3 and STAT3β.** *A*, COS cells were transfected with the IL-5R and different amounts of STAT3 and STAT3β. 48 h after transfection, cells were treated with IL-5 for 15 min and DNA binding activity was monitored using a ^{32}P-labeled pIRE. Co-expression of STAT3 (*C3*) and STAT3β (*C1*) results in the formation of a heterodimeric complex (*C2*). *B*, nuclear extracts from COS cells transfected with STAT3, STAT3β, or STAT3/STAT3β, stimulated for 15 min with IL-5 were preincubated with two different STAT3 antibodies STAT3 Ab (629–640) and STAT3 Ab (750–769). DNA binding activity was monitored using a ^{32}P-pIRE. These data clearly show the formation of STAT3β homodimers and STAT3/STAT3β heterodimers.

previously demonstrated for STAT1 and STAT2 (53). Verification of this hypothesis, however, awaits deciphering of the precise exonic structure of the STAT3 gene.

It was previously suggested that besides tyrosine phosphorylation, serine phosphorylation might play a crucial role in gene regulation by STAT proteins. The serine/threonine kinase inhibitor H7 was shown to be able to block transcriptional regulation by STAT1 and STAT3 (19, 20). Similarly, Boulton *et al.* (22) reported an H-7 sensitive phosphorylation of STAT3, but not STAT1. In addition, it was shown that serine phosphorylation might be necessary for DNA binding by STAT3 homodimers, but not by STAT1 homo- or STAT1/STAT3 heterodimers (21). In this paper we have shown that STAT3β is efficiently phosphorylated on tyrosine upon IL-5 stimulation (Fig. 2B), which leads to a strong increase in DNA binding by STAT3β (Fig. 3). However, in contrast to STAT3, STAT3β is unable to mediate transactivation via a pIRE containing promoter. Examination of the amino acids deleted from STAT3β shows the presence of a large number of serine and threonine residues, of which serine 727 is conserved between STAT1α, STAT3, STAT4, and STAT5, but not STAT1β. Interestingly, this serine lies within a highly conserved PMSP sequence that was previously shown to be a microtubule-associated protein

kinase recognition sequence (50). The importance of this serine was very recently shown by Wen *et al.* (20), who showed that this serine is inducibly phosphorylated in both STAT1α and STAT3. Furthermore, when they mutated this serine to alanine (STAT1αS), trans-activation was decreased 5-fold, although STAT1αS was still able to support an IFNγ-induced 5-fold increase in transcription, whereas STAT1β is completely inactive in this system (20). Similarly, mutation of serine 727 in STAT3 also decreased transactivation about 2.5-fold, although the mutant was still able to support an 8-fold induction of INFγ activation site-mediated transcription (20). The lack of serine 727 in STAT3β explains some, but not all of the results presented in this paper. STAT3β is completely unable to mediate transcriptional activation in COS cells (Fig. 4*A*), whereas STAT3 with the 727 mutation is still a relatively good transactivator in U3A cells (20). This might be caused by cell type-specific differences between COS and U3A. Alternatively, there might be more phosphorylated residues present in the region that is deleted from STAT3β which contribute to transcriptional activation. Finally, the basal level tyrosine phosphorylation (Fig. 2*B*) and DNA binding (Fig. 3) observed with STAT3β in unstimulated COS cells represents a striking difference with both STAT3–727 and STAT1β. Although we do not have any experimental data to support this hypothesis, this observation suggests the presence of a domain in the COOH-terminal 55 amino acids of STAT3 that is somehow able to block STAT3 phosphorylation by JAK kinases in unstimulated cells.

The observation that STAT3β is transcriptionally inactive is in contrast with data published recently by Schaefer *et al.* (51). They have identified STAT3β via a two-hybrid system as a protein capable of binding to the NH₂-terminal part of the c-Jun protein. Furthermore, they have shown that STAT3β is transcriptionally active on the α₂-macroglobulin promoter in the absence of added cytokines. This constitutive transactivation potential is consistent with our own results showing a constitutive tyrosine phosphorylation of STAT3β. The opposite effects found on the transactivation potential of STAT3β can be due to the promoter targets used in both studies. We have used an artificial reporter containing only STAT binding sites and the natural ICAM-I promoter, while the α₂-macroglobulin promoter used by Schaefer *et al.* (51) contains a STAT binding site closely linked to a Jun binding site. Occupation of the Jun binding site by members of the Jun/Fos family might somehow alter the transcription activation potential of STAT3β. Opposite effects by a single transcription factor on different promoters has also been described for hormone receptors which can either activate or repress gene transcription depending on the promoter (54). Further experiments are required for deciphering the different roles that STAT3β might have in transcriptional regulation.

The observed dominant negative effect of STAT3β over STAT3 might be caused by two different mechanisms. One possibility might be that STAT3β homodimers have a higher affinity for the pIRE, and therefore occupy these sites on the DNA even when STAT3 is more abundantly expressed. Indeed, we have observed that while STAT3β expression was 2-fold lower in transfected COS cells (Fig. 2*B*), DNA binding by STAT3β was 2–3-fold higher compared to STAT3 (Fig. 3). This is likely to be caused by the higher extent of tyrosine phosphorylation observed in STAT3β (Fig. 2*B*). On the other hand, since dimerization of STAT proteins is required for the formation of transcriptionally active DNA binding complexes (52), heterodimerization between STAT3 and STAT3β might be involved in the dominant negative effect. In Fig. 5 we show that STAT3 and STAT3β indeed form a heterodimer. Moreover, even a small amount of STAT3β is sufficient to disrupt all the

STAT3 homodimers and drive them into STAT3/STAT3β heterodimers. However, at this ratio between STAT3 and STAT3β (6:2 μg), we still observe transcriptional activation of a pIRE containing plasmid (Fig. 4*B*), although it is 50% less compared to STAT3 alone. This suggests that the STAT3/STAT3β heterodimer is able to support IL-5 induced transcription, albeit with a lower efficiency than the STAT3 homodimer. Taken together, the observed dominant negative effect of STAT3β is likely to be caused by a combination of transcriptionally inactive STAT3β homodimers with high DNA binding activity and STAT3β/STAT3 heterodimers which are weak transcriptional activators. The generation of transcriptional activators and repressors from the same gene is not unprecedented (55). mTFE3, a murine transcription factor involved in the activation of immunoglobulin heavy chain transcription, is turned into a repressor by a splicing event that removes part of the activation domain (56). Similarly, a splicing event removing part of the activation domain of FosB results in the expression of ΔFosB, an inhibitor of Fos/Jun transcriptional activity (57), while splicing out the activation domains of CREMτ generates CREMα, which, despite the fact that it retains the protein kinase A phosphoacceptor site, is an efficient antagonist of cAMP-induced transcription (58, 59). The use of alternative initiation codons can also be a mechanism to generate activators and repressors from the same mRNA, as was reported for liver-enriched activator protein and liver-enriched inhibitory protein (60) and for CREM and S-CREM (61). In this paper we report that an alternative splicing event removing 55 amino acids from the COOH terminus of STAT3 generates STAT3β, an efficient repressor of STAT3-mediated transcription. Although it is likely that the removal of serine 727 contributes to this switch from activator to repressor, the precise molecular mechanism awaits further mutational analysis of STAT3, since the nature of the activation domain of STAT3 remains to be elucidated. As was described previously for mTFE3, FosB, CREM, and liver-enriched activator protein (55), we found that the activator STAT3 and the repressor STAT3β are co-expressed in a wide variety of cell types (Fig. 2*A*), although the ratio between the two differs between cell types studied. Cell type-specific differences in the ratio between STAT3 and STAT3β could lead to differences in the response to IL-5 or other activators of STAT3. It would therefore be interesting to determine the mechanism by which the ratio between STAT3 and STAT3β is modulated in a cell type-specific manner. Another challenge will be to find physiological processes in which the ratio between STAT3 and STAT3β is altered in a temporal or spatial fashion due to signal transduction dependent alternative splicing.

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