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ATBF1 enhances the suppression of STAT3 signaling by interaction with PIAS3☆

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Abstract

ATBF1 was first discovered as a suppressor of AFP expression in hepatocytes. It is present in brain, adult liver, lung, and gastrointestinal tract. Recently, it has been reported that ATBF1 regulates myoblastic differentiation and interacts with v-Myb in regulation of its transactivation. Using the yeast two-hybrid system, we searched for protein–protein interactions to uncover new functions for ATBF1. We present here experimental evidence that ATBF1 is a new regulatory factor for STAT3-mediated signal transduction through its interaction with PIAS3. PIAS3 was thus identified as an ATBF1-binding protein. In co-transfection experiments, the full-length ATBF1 was found to form complexes with PIAS3 in Hep G2 cells. In the luciferase assay, ATBF1 was found to have no influence on STAT3 signaling induced by IL-6 stimulation, but it did synergistically enhance PIAS3 inhibition of activated STAT3. In conclusion, ATBF1 can suppress the IL-6-mediated cellular response by acting together with PIAS3.

ATBF1 was first described as a 306-kDa transcription factor which inhibits α-fetoprotein (AFP) gene expression in the liver [1]. Subsequently, a major isoform of ATBF1 encoding a 404-kDa protein was isolated [2]. In AFP-producing gastric cancer cells (AFP-GC), ATBF1 was deficient or at a very low level [3] resulting in cells of a highly malignant nature. ATBF1 is also expressed in the developmental phase. In adult small intestinal epithelium, ATBF1 regulates amino peptidase N (APN) gene expression, a feature of enterocyte differentiation and maturation of the small intestine of the rat [4]. ATBF1 mRNA levels were high in 13.5-day-old fetal mouse brain and decreased during subsequent development [5]. In P19 murine embryonal carcinoma, ATBF1 was not detected in cells in an undifferentiated state but was expressed during neuronal differentiation induced with retinoic acid [2,6]. Taken together, these observations suggest that ATBF1 suppresses cell proliferation and initiates differentiation. Unexpectedly, the reverse was observed during myoblast differentiation. In the myoblast cell line C2C12, ATBF1 was expressed at high levels in proliferating cells and the expression of its mRNA was suppressed during differentiation [7]. Since ATBF1 is the largest DNA-binding protein known and has a number of motifs involved in transcriptional regulation, it is possible that the functions of ATBF1 vary according to the proteins with which it interacts. The interaction of v-Myb with ATBF1 resulted in an alteration of v-Myb transcriptional activity [8].

We used yeast two-hybrid screening to search for factors that interact with ATBF1, focusing on PIAS3 because of its important role in liver regeneration through controlling STAT3 signaling [9–14]. Recently, the transcription factors GRIP1 [15], SUMO-1 [16], DJ-1 [17], MITF [18], Gfi-1 [19], and IRF-1 [20] have

☆ *Abbreviations:* ATBF1, AT motif binding factor 1; PIAS3, protein inhibitor of activated STAT3; AFP, α-fetoprotein; IL-6, interleukin 6; HA, hemagglutinin; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

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been shown to interact with PIAS or PIAS-like proteins which regulate their transcriptional activities. The aims of this study were to identify proteins which interact with ATBF1 and to analyze its function.

Materials and methods

Plasmids. The bait was constructed in pGBKT7 vector (CLONTECH) by ligation of several PCR-amplified fragments of ATBF1 cDNA [2] indicated in Fig. 1A, using the primers shown in Table 1. For pGADT7-PIAS3 construction (Fig. 1B), three plasmids pGADT7-PIAS3 (N), pGADT7-PIAS3 (C), and pGADT7-PIAS3 (full) were prepared by PCR using the following primers: 5′-CCGGAATT CCCTGTAGGCTCCCCTGGT-3′ and 5′-CCCCCCGGGCTCAA ACCGCTGGCTAGAAG-3′; 5′-CCGGAATTCACTTCTAGCCAG CGGTTTGAG-3′ and 5′-GGGACAGCGAAGTTTCCATA-3′; and 5′-CCGGAATTCGAGCTGGGCGAATTAAAGC-3′ and 5′-GGGA CAGCGAAGTTTCCATA-3′, respectively. A cDNA library from human hepatoma Hep G2 cells was used as a template. PCR products were inserted into pGEM-T Easy (Promega) and each product was made by insertion of *Eco*RI-digested products into *Eco*RI sites of pGADT7 vector (CLONTECH).

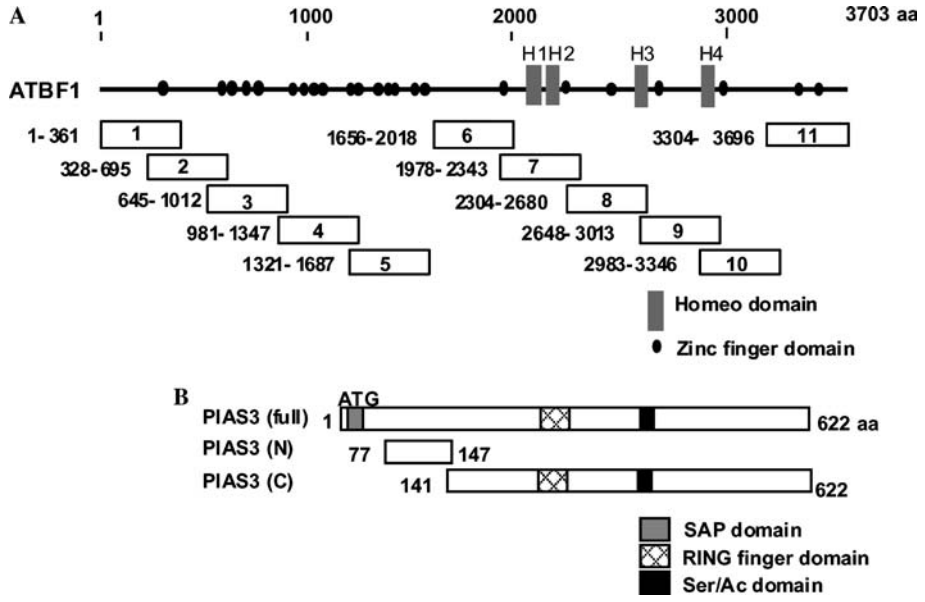

Fig. 1. (A) Structure of ATBF1. Schematic representation of the ATBF1 molecule segments in yeasts in the form of myc fusion proteins. Rectangles, homeodomains I-IV; solid circles, zinc finger motifs. (B) Schematic representation of various PIAS3 constructs. PIAS3 contains a putative chromatin-binding SAP (SAF-A/B, Acinus, and PIAS) domain, a RING finger domain, and a Ser/Ac (serine- and acidic amino acid-rich) domain.

Table 1
Oligonucleotides used for PCR to encode sequences of ATBF1 cDNA

Name	Amino acid	Nucleotide	Sequence
1	1–361	670–1752	5′-GGAATTCACCATGGAAGGCTGTGACTC-3′ 5′-CGGGATCCGGGGCCTATGAGGTTAGCTG-3′
2	328–695	1653–2753	5′-GGAATTCGGGATCGGCAAAGACAAG-3′ 5′-CGGGATCCTGCTTCTCCTTCATGTGTGC-3′
3	645–1012	2602–3702	5′-GGAATTCAAATGCGACACGGTCCTG-3′ 5′-ACGCGTCGACTTTCAAATCTCCGCTTTGCT-3′
4	981–1347	3610–4707	5′-TGCCATGGTGGGGGACTCATACCAGTG-3′ 5′-ACGCGTCGACTTTCAAATCTCCGCTTTGCT-3′
5	1321–1687	4630–5728	5′-GGAATTCAAGCAGCCTGAAACCTCAGA-3′ 5′-CGGGATCCCAGTGGGCACTTGGCTTAGTA-3′
6	1656–2018	5635–6721	5′-GGAATTCGTGAGCACCAGTGGCAGTAA-3′ 5′-CGGGATCCTCTCGAGCTGTTTGAAAGGAA-3′
7	1978–2343	6600–7697	5′-GGAATTCGGAGAGAACCTGGAAAAGC-3′ 5′-CGGGATCCTCAAAGATGCGCTGAAACAC-3′
8	2304–2680	7580–8706	5′-GGAATTCCAGGGAGAGGGCAAAGAT-3′ 5′-CGGGATCCCACCTCGTGTGCAATGTGAT-3′
9	2648–3013	8611–9706	5′-GGAATTCACCATCACACCGGAACAACT-3′ 5′-CGGGATCCAATGCTTGGCCATGCTTAAC-3′
10	2983–3346	9615–10,706	5′-GGAATTCGACATTGGACTGCCAAAGA-3′ 5′-CGGGATCCCTGTAGGGGAACAGGCCTTC-3′
11	3304–3696	10,578–11,754	5′-GGAATTCCTCACAAGCCAGTTCCTTC-3′ 5′-CGGGATCCGGTCAGACCACTGTCCTTGG-3′

The expression vectors for mammalian cells were constructed with Myc-tag and HA-tag in pCI Mammalian Expression Vectors (Promega). The full-length ATBF1 cDNA and its 5′-partial fragment sequences (670–3351 bp) were cloned into pCI-Myc vector termed pCI-Myc-ATBF1 (full) and pCI-Myc-ATBF1 (1–893), respectively. The full-length PIAS3 cDNA was cloned into pCI-HA vector termed pCI-HA-PIAS3 (full).

Yeast two-hybrid experiments. Yeast transformation and two-hybrid screening and assays were performed according to the instructions for the MATCHMAKER two-hybrid system (CLONTECH). Yeast AH109 was transformed with pGBKT7-ATBF1 fragments and the Human Brain MATCHMAKER cDNA library (CLONTECH). Approximately 1×10^6 yeast transformants were plated on 50×15 cm plates containing synthetic medium lacking tryptophan, leucine, histidine, and adenine and containing X-α-galactoside. Plates were incubated at 30 °C for 7 days. Eleven X-α-galactosidase positive colonies were selected. Plasmids containing human cDNAs were isolated, and the cDNA inserts were sequenced and rescued in *Escherichia coli* DH10B. To confirm the interaction of the bait plasmid and the cDNA library, the constructs were remade using pGADT7 vectors because cDNA libraries were constructed in pACT2 vectors.

In vitro coimmunoprecipitation. The TNT Quick Coupled Transcription/Translation System (Promega) was used. Redivue L-[^{35}S]Methionine was purchased from Amersham. Three plasmids pGADT7-PIAS3 (N), pGADT7-PIAS3 (C), and pGADT7-PIAS3 (full), and several ATBF1 fragment-inserted pGBKT7 vectors were used. A mixture of Master Mix, [^{35}S]methionine, and DNA template (bait or library candidate) was incubated at 30 °C for 90 min. Bait and library candidate proteins were mixed for 1 h at room temperature and then incubated with c-Myc monoclonal antibody-agarose beads (CLONTECH) or Anti-HA Affinity Matrix (Roche) for 1 h at room temperature on a rotating apparatus. Immunoprecipitates were washed extensively with PBS (150 mM NaCl, 10 mM potassium phosphate, pH 7.4), dissolved in Laemmli buffer, and placed in boiling water for 5 min. The samples were separated by 10% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and subjected to autoradiography.

Immunoprecipitation, gel electrophoresis, and Western blotting. Cells were disrupted in lysis buffer containing 50 mM Tris (pH 8.0), 1 mM EDTA, 120 mM NaCl, 0.5% Triton X-100, 10% glycerol, and protease inhibitor cocktail (Roche). After 10 min on ice, the solutions were incubated at 4 °C for 2 h with continuous rocking. c-Myc monoclonal antibody–agarose beads were used to immunoprecipitate ATBF1-Myc protein. Immunoprecipitates were washed extensively, dissolved in Laemmli buffer, and placed in boiling water for 5 min. Samples were separated by SDS–PAGE on 10% gels and transferred to PVDF membranes. After blocking with 1% BSA in TBST (20 mM Tris (pH 8.0), 150 mM NaCl, and 0.01% Triton X-100) for 1 h at room temperature, anti-c-Myc immunoprecipitates were probed with 2 μg/ml anti-c-Myc monoclonal antibody, followed by goat anti-mouse antibody conjugated with horseradish peroxidase. After extensive washing with TBST, membranes were treated with enhanced chemiluminescence reagent (Amersham–Pharmacia Biotech). STAT3 antibody was purchased from Santa Cruz.

Synthetic oligonucleotides and plasmid construction for luciferase assay. The following oligonucleotides were used to obtain the two binding sites for STAT proteins, previously designated A (TTACCAG AA) and B (GTCCGAGAA) [21]; 5′-GATCTGTATTACCAGA AATTATCATCTGGTCATTTCCAGTCCGAGAACAGA-3′ and 5′-GATCTCTGTTCTCGGACTGGAAATGACCAGATGATAATTT CTGGTAATACA-3′. After annealing these oligonucleotides, we ligated these double-stranded forms to make multiple copies of the STAT-binding elements. Plasmid p3 × (ACT − (A + B)) svLUC (p3svLUC) was obtained by insertion of three copies of the STAT-binding elements with *Bgl*II ends into the *Bgl*II site of PicaGene Promoter Vector 2 (Fig. 4A).

Cell culture and transient transfection. Hep G2 hepatoma cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS) and antibiotics. Twenty-four hours after seeding, transient transfections were carried out in 12-well plates or 10 cm-diameter dishes, as indicated, using TransIT-LT1 (Mirus Corporation). The cells were incubated with DNA containing the reporter and expression plasmids indicated in the figure legends. After culturing for 24 h, the cells were kept in media without FBS for 18 h, treated with 5–10 ng of recombinant human IL-6 (rhIL-6) (Bioscience) per ml for 4 h, and harvested with 1× Lysis Buffer (Promega). Luciferase activities were measured using a Luminometer (LUMAT LB9597).

Statistical analysis. Statistical analysis was done by ANOVA. Results are expressed as means ± SD for three or more individual experiments.

Results

Interaction of ATBF1 C termini and PIAS3 in the yeast two-hybrid system

The yeast two-hybrid system was used to isolate protein(s) interacting with the ATBF1 C-terminus. We screened for four types of ATBF1 fragments. The bait plasmids, pGBKT7-ATBF1 (# 6, 9, 10, and 11) contained partial ATBF1 cDNA, 5635–6721, 8611–9706, 9615–10,706, and 10,578–11,754, fused in-frame to the GAL4 DNA-binding domain (Fig. 1A). The library proteins were prepared from the cDNA library (human brain) fused in-frame to the GAL4 activation domain in pACT2. Two types of plasmids were co-transformed into yeast AH109 and transformants were selected on plates lacking tryptophan (Trp), leucine (Leu), histidine (His), and adenine (Ade). Eleven colonies were obtained and cDNA-containing plasmids were isolated from them and sequenced. Among the candidates of clones interacting with pGBKT7-ATBF1 (#11), one clone was PIAS3 cDNA lacking the 147 amino acids of the N-terminus. The 3′ end of the cDNA could not be determined because the reverse sequence was not clear. Hence, we tried to confirm the interaction using various portions of PIAS3 for two-hybrid screening. Three kinds of HA-tagged-PIAS3 fusion proteins were made using the pGADT7 vector (Fig. 1B). ATBF1 (#11) interacted with pGADT7-PIAS3(C) and pGADT7-PIAS3 (full) but not with pGADT7-PIAS3 (N) or reverse pGADT7-PIAS3 (C) (Fig. 2B). The same assays were performed for each fragment of ATBF1 and the full-length PIAS3. Only ATBF1 (#11) and full-length ATBF1 interacted with the full-length PIAS3 and ATBF1 (#8) interacted weakly (Fig. 2A).

In vitro interaction of ATBF1 and PIAS3

To confirm the ATBF1-PIAS3 interaction, the TNT coupled Reticulocyte Lysate System was used. As shown in Fig. 3A, ATBF1 (#11) bound efficiently to pGADT7-PIAS3 (C), but not to the HA-tag alone. pGADT7-PIAS3 (C) did not bind to myc-tag alone. Attempts to

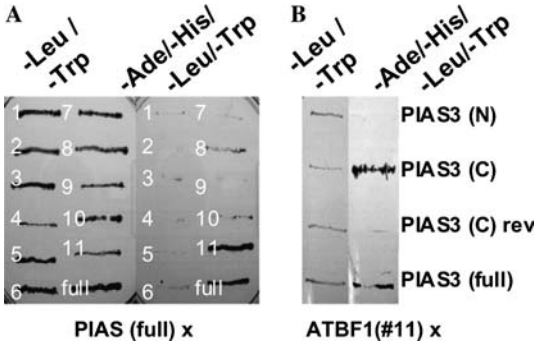

Fig. 2. Biological assay for interaction of several ATBF1 and PIAS3 fragments. Yeast AH109 cells were transformed with expression constructs of several fragments of ATBF1 and PIAS3 (A), and with expression constructs for ATBF1 (#11) and for one of the following proteins: pGADT7-PIAS3 (C), pGADT7-PIAS3 (N), reverse pGADT7-PIAS3 (C), and pGADT7-PIAS3 (full) (B). Established clones were first streaked on a plate without tryptophan, leucine, histidine, and adenine. Then, the same wire loop was streaked on another plate without tryptophan and leucine. Growth on the plate without tryptophan and leucine indicates the presence of the two expression constructs and growth on the plate without tryptophan, leucine, histidine, and adenine indicates the presence of the two expression constructs and interaction of the expressed recombinant proteins. Only ATBF1 (#11) and ATBF1 (full-length) could interact with PIAS3. ATBF1 (#11) could interact with PIAS3 (C) and PIAS3 (full) but could not interact with PIAS3 (N) or reverse PIAS3 (C).

construct the HA-tagged full-length PIAS3 and Myc-tagged full-length ATBF1 in this system were not successful.

ATBF1-PIAS3 interaction in vivo

To document the direct binding of transiently expressed full-length ATBF1 and PIAS3 proteins in Hep G2 cells, co-expression of Myc-tagged ATBF1 and HA-tagged PIAS3 was used. The cell lysates were immunoprecipitated with monoclonal anti-Myc antibody and immunocomplexes were resolved by SDS–PAGE. Western blots were then developed with anti-STAT3 antibody. As shown in Fig. 3B, STAT3 was detected in anti-Myc immunoprecipitates from lysates of cells which were expressed with Myc-tagged full-length ATBF1 and HA-tagged PIAS3 in an IL-6 activated condition. STAT3 was not detected in anti-Myc immunoprecipitates from lysates of cells expressed with only ATBF1 or PIAS3 or in those of cells in which IL-6 was not activated. These results revealed that ATBF1 could not bind to STAT3 directly but did bind via PIAS3. As reported previously, only phosphorylated STAT3 can interact with PIAS3, and as a result, STAT3 can only be detected when there is IL-6 activation. ATBF1 and PIAS3 can interact both with and without IL-6 activation (Fig. 3C).

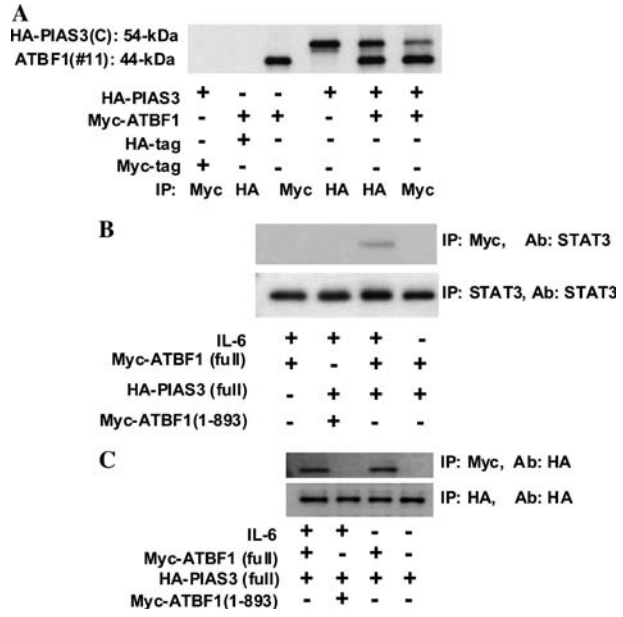

Fig. 3. Interaction of ATBF1 and PIAS3. (A) Binding of ATBF1 (#11) and PIAS3 (C) in vitro. A TNT coupled Reticulocyte Lysate System was used. Bound proteins were analyzed on 10% SDS–PAGE, followed by Western blotting. ATBF1 (#11) bound efficiently to PIAS3 (C), but not to the HA-tag alone. PIAS3 (C) could not bind solely to Myc-tag. The full-length PIAS3 (1.9-kb) and ATBF1 (11-kb) failed to express in this system. PIAS3 interaction in Hep G2 cells. Cell extracts were isolated from cells co-transfected with Myc-ATBF1 (full) or Myc-ATBF1 (1-893) and HA-PIAS3 (full) expression plasmids. Detergent-extracted proteins were immunoprecipitated with anti-Myc antibody or anti-STAT3 antibody (B) and with anti-Myc or anti-HA antibody (C). Immunoprecipitates were separated on 10% SDS–PAGE and analyzed by Western blotting using anti-STAT3 antibody (B) or anti-HA antibody (C). The expression level of endogenous STAT3 (B, lower lane) was determined in cell extracts from Hep G2 cells by Western blotting using the anti-STAT3 antibody. The expression level of HA-PIAS3 transfectants (C, lower lane) was determined in cell extracts from Hep G2 cells by Western blotting using the anti-HA antibody. All data shown are representative of several independent experiments.

Synergistic effect of interaction of ATBF1 and PIAS3 on the STAT3 signaling

Three copies of the STAT-binding elements were linked to a SV40 promoter driving transcription of the luciferase gene (p3svLUC) (Fig. 4A) which can be activated in Hep G2 cells by treatment with IL-6 without STAT3 over-expression. In the presence of IL-6, p3svLUC was activated about 15-fold compared with values obtained without IL-6. Co-transfection of PIAS3 expression constructs resulted in a reduction of IL-6-mediated p3svLUC transcription in a concentration-dependent manner, confirming earlier reports [22] (Fig. 4Ba). In contrast, ATBF1 expression had no effect on the luciferase activity (Fig. 4Bb). To assess whether synergy between ATBF1 and PIAS3 in Hep G2 cells occurs via STAT3 signaling, ATBF1 and PIAS3 were co-transfected with the reporter gene. Although ATBF1

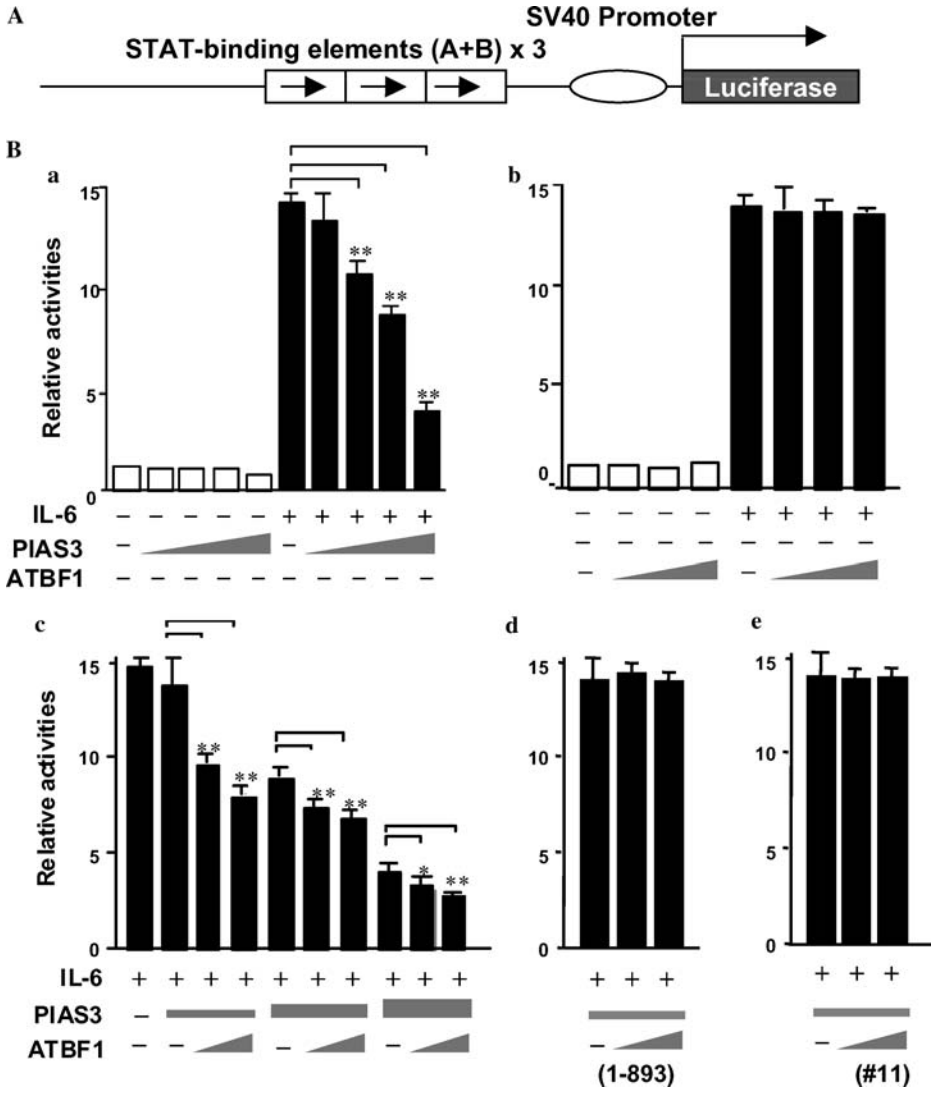

Fig. 4. Functional synergism between ATBF1 and PIAS3. (A) Three STAT-binding elements (A + B) were cloned in front of the SV40 promoter followed by the luciferase reporter gene (p3svLUC). (B) Hep G2 cells were transfected with p3svLUC, and pCI-Myc-ATBF1 or pCI-HA-PIAS3 or both. The results are presented as induction of luciferase activity (arbitrary light units) over control levels in triplicate experiments and the error bars represent standard deviations. In the presence of IL-6, the luciferase reporter could be activated about 15-fold compared with values obtained without IL-6 stimulation and was inhibited by the transfection of PIAS3 in a concentration-dependent manner (0.1, 0.3, 0.6, and 1.1 μg of pCI-HA-PIAS3) (a), but not by ATBF1 (0.3, 0.6, and 1.1 μg of pCI-Myc-ATBF1) (b). $^{**}P < 0.01$ compared with mock pCI vector alone. In the presence of PIAS3 (0.1, 0.6, and 1.1 μg pCI-HA-PIAS3), IL-6 stimulation was inhibited by ATBF1 in a concentration-dependent manner (0.3, 1.1 μg pCI-Myc-ATBF1) (c). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with pCI-PIAS3 vector alone. There were no inhibitions by ATBF1(#11) and ATBF1(1–893) in the presence of PIAS3 (d,e).

alone had no effect on luciferase activity, there was a synergistic response when cells co-expressed ATBF1 and PIAS3 (Fig. 4Bc). Synergistic response was not observed by either ATBF1 (#11) or ATBF1 (1–893) co-transfected with PIAS3 (Figs. 4Bd and e). These data showed that ATBF1 had a synergistic effect on the inhibition of STAT3 activity through interaction with the PIAS3 protein.

Discussion

The aim of this work was to identify proteins interacting with ATBF1, a 404-kDa member of the family of transcription factors containing both homeodomain and zinc finger motifs [2]. Its function as a transcription factor is likely regulated by various modifications and interactions with other factors. In fact, ATBF1 binds to the activation domain of v-Myb oncoprotein [8]. ATBF1 binding to v-Myb caused suppression of v-Myb transcriptional activator activity. We prepared eleven truncated fragments of about 1 kb of ATBF1 cDNA for use as bait constructs to screen the cDNA library for yeast two-hybrid screening. We chose the 6, 9, 10, and 11th fragments as the bait because they contained proline-rich regions and because the 11th fragment interacted with Myb protein. Unexpectedly, we failed to

detect the v-Myb gene in positive clones from the first screening. This may have been caused by differences in the cDNA library or in selection of the bait. Interaction between ATBF1 and v-Myb was found with a cDNA library derived from HeLa cells, while our screening was performed using a cDNA library derived from cells of a normal adult human brain. Furthermore, our screening was performed with ATBF1 as bait, not v-Myb.

We confirmed that the eleven clones encoded in-frame fragments of specific genes. Among the positive clones identified by DNA sequencing, PIAS3 was chosen for further analysis because it is an important protein in several activation pathways via STAT family signaling [14,23]. The physical interaction of the two proteins was confirmed with immunoprecipitation experiments using in vitro transcribed protein as well as lysates from co-transfected cells. The binding site specificity was examined using truncated fragments of ATBF1 and PIAS3. Not only the C-terminal fragment but also full-length PIAS3 bound efficiently to the fragment encoding C terminus of ATBF1. The 141-amino acid sequence at the N-terminus of PIAS3 was not involved so the interaction domain would be located at the C-terminal side of PIAS3. On the other hand, full-length PIAS3 bound to the 11th fragment and to full-length ATBF1 protein efficiently. It did not bind to most fragments and only weakly to the 8th fragment, indicating that PIAS3 interacted with the terminal end of ATBF1.

PIAS3 binds to activated tyrosine-phosphorylated STAT3 dimers and prevents DNA binding of the complex [22]. The manner in which this is achieved is not known. Although ATBF1 alone had no effect on IL-6-induced STAT3 activation in Hep G2 cells, overexpression of ATBF1 and PIAS3 synergistically suppressed IL-6 stimulation of the STAT3-activated promoter in Hep G2 cells. There are two possible explanations for this observation. First, ATBF1 and PIAS3 bound to activated STAT3 may inhibit its DNA binding ability. Second, the formation of a tertiary complex of ATBF1, PIAS3, and STAT3 may interfere with the recruitment of a co-activator without affecting the DNA binding activity of STAT3. The exact mechanism cannot be established from the present data.

STAT3, a necessary mediator for transducing signals from IL-6, results in the production of acute phase reactants. Our data revealed that ATBF1 enhances the inhibitory effect of PIAS3 against IL-6 signaling in Hep G2 cells. We are currently investigating the physiological significance of this novel modification in signal transduction.

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