Published in final edited form as:
J Biol Chem. 2006 May 5; 281(18): 12260–12269.

Interaction of Protein Inhibitor of Activated STAT (PIAS) Proteins with the TATA-binding Protein, TBP*

Justin R. Prigge‡ and **Edward E. Schmidt**‡,§,1

‡*Veterinary Molecular Biology, Montana State University, Bozeman, Montana 59717*

§*Center for Reproductive Biology, Washington State University, Pullman, Washington 99164*

Abstract

Transcription activators often recruit promoter-targeted assembly of a pre-initiation complex; many repressors antagonize recruitment. These activities can involve direct interactions with proteins in the pre-initiation complex. We used an optimized yeast two-hybrid system to screen mouse pregnancy-associated libraries for proteins that interact with TATA-binding protein (TBP). Screens revealed an interaction between TBP and a single member of the zinc finger family of transcription factors, ZFP523. Two members of the protein inhibitor of activated STAT (PIAS) family, PIAS1 and PIAS3, also interacted with TBP in screens. Endogenous PIAS1 and TBP co-immunoprecipitated from nuclear extracts, suggesting the interaction occurred *in vivo*. *In vitro*-translated PIAS1 and TBP coimmunopreciptated, which indicated that other nuclear proteins were not required for the interaction. Deletion analysis mapped the PIAS-interacting domain of TBP to the conserved TBP_{CORE} and the TBP-interacting domain on PIAS1 to a 39-amino acid C-terminal region. Mammals issue seven known PIAS proteins from four *pias* genes, *pias1*, *pias3*, *piasx*, and *piasy*, each with different cell type-specific expression patterns; the TBP-interacting domain reported here is the only part of the PIAS C-terminal region shared by all seven PIAS proteins. Direct analyses indicated that PIASx and PIASy also interacted with TBP. Our results suggest that all PIAS proteins might mediate situation-specific regulatory signaling at the TBP interface and that previously unknown levels of complexity could exist in the gene regulatory interplay between TBP, PIAS proteins, ZFP523, and other transcription factors.

TATA-binding protein (TBP)[2] functions in transcription initiation by all three nuclear eukaryotic RNA polymerases (1,2). TBP-containing complexes include SL1, TFIID, and TFIIIB, which function with RNA polymerase I (RNAPI), RNAPII, and RNAPIII, respectively. RNAPII requires TFIID for promoter-targeted assembly of the pre-initiation complex (3). TBP is also an essential component of the human SNAPc complex, which functions in transcription initiation at small nuclear RNA genes by both RNAPII and RNAPIII (4), and the yeast SAGA complex (5).

*This work was supported in part by a United States Department of Agriculture (USDA) National Needs Fellowship (to J. R. P.), a March of Dimes Basil O'Connor New Investigator Award, and an appointment to the Montana Agricultural Experiment Station (to E. E. S.), and by grants from the March of Dimes Foundation, the National Science Foundation, the National Institutes of Health, and USDA Formula Funds (to E. E. S.).

[1]To whom correspondence should be addressed: Molecular Biosciences Bldg., Montana State University, Bozeman, MT 59717. Tel.: 406-994-6375; Fax: 406-994-4303; E-mail: eschmidt@montana.edu.

[2]The abbreviations used are: TBP, TATA-binding protein; AD, activation domain; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; PIAS, protein inhibitor of activated signal transducers and activators of transcription proteins; SC, synthetic complete media; Ser/Ac, acidic serine-rich domain; X-α-gal, 5-bromo-4-chloro-3-indolyl α-D-galactopyranoside; ZFP523, mouse zinc finger protein 523; ZNF76, human zinc finger protein 76; DMEM, Dulbecco's modification of Eagle's medium; STAT, signal transducer and activator of transcription; TBP-FL, TATA-binding protein full-length; TBP-N, TATA-binding protein N terminus; TBP-C, TATA-binding protein C terminus; TF, transcription factor; BRF1, B'-related factor 1; RNAP, RNA polymerase; SC-L-W-H, synthetic complete medium lacking leucine, tryptophan, and histidine.

Recruitment of TBP to the core promoter is regulated by both positive and negative factors (3). Some activators of transcription bind TBP or the TBP-associated factor components of the TFIID complex and direct TFIID to the promoter (6,7). TBP function can also be up- or down-regulated through interactions with BTAF1/MOT1 and the NC2*α*/*β* subunits of the NC2 complex (8,9). Recently, ZNF76, the human ortholog of mouse zinc finger protein 523 (ZFP523) and of frog Staf (10), was shown to function via direct interaction with TBP (11). The interaction of ZNF76 with TBP is blocked by PIAS1-dependent sumoylation of ZNF76 (11).

PIAS proteins are found in all eukaryotes. The human and mouse family of PIAS proteins consists of PIAS1, PIAS3, PIASx, and PIASy proteins (12). The *piasx* gene encodes two splice variants, PIASx*α*/ARIP3 (androgen receptor-interacting protein-3) (13,14) and PIASx*β*/Miz1 (Msx-interacting zinc finger-1) (15), the difference being in their C termini. *pias3* and *piasy* also each encode two isoforms, PIAS3/PIAS3*β* and PIASy/PIASyE6^{-}, as a result of alternative splicing. The PIAS3*β* isoform contains an insertion of 39 amino acids in its N-terminal region and PIASyE6^{-} lacks exon 6 (16). In total, seven different PIAS proteins are expressed in mammals, each of which likely differs in which cell types and conditions favor its expression.

PIAS proteins regulate the activities of transcription factors including the signal transducer and activator of transcription (STAT) family of proteins (12,17-20). PIAS proteins have SUMO E3-ligase activity and interaction of PIAS proteins with transcription factors often results in sumoylation of that protein. Ligation of SUMO-1 to most transcription factors represses activity, although the mechanisms that underlie regulation differ (21,22). In addition to sumoylation, PIAS proteins can regulate gene expression by blocking the interaction of a transcription factor with its target DNA, by recruiting co-repressors and co-activators of transcription, and by targeting proteins to nuclear bodies (23).

The conserved N-terminal region of PIAS proteins contains several well characterized domains (16). The SAF-A/B, acinus, PIAS (SAP) domain binds A/T-rich DNA and may be involved in targeting PIAS proteins to the nuclear scaffold (24). The SAP domain encompasses an L*XX*LL motif that is required for transcriptional repression (19). The RING finger-like zinc-binding domain mediates the SUMO-E3-ligase activity of PIAS proteins (18) and binds directly to Ubc9, the SUMO E2 enzyme (25). Most PIAS proteins also contain a PINIT motif, which plays a role in nuclear retention (26).

The C termini of PIAS proteins are more diverse; however, all contain an acidic domain preceded by several serines (Ser/Ac). Within the acidic domain, a SUMO-1 interaction motif (SIM) exists, although deletion of SIM does not abolish PIAS-mediated sumoylation of interacting proteins (18,27). Also, a serine- and threonine-rich region (S/T) is present in the C termini of all PIAS proteins except PIAS*γ*. The function of this region is unknown (16).

Here, we show that mouse TBP interacts with ZFP523, the mouse ortholog of human ZNF76. In addition, we report the novel interaction of TBP with PIAS1, PIAS3, PIASx, and PIASy proteins. The TBP/PIAS interaction is shown to occur between *in vitro* translated proteins, suggesting the interaction is direct, and it is detected between endogenous proteins in nuclear extracts, suggesting it occurs *in vivo*. Our results suggest that PIAS proteins might modulate transcriptional signaling at the TBP interface.

EXPERIMENTAL PROCEDURES

TBP Bait Constructs and cDNA Prey Libraries

Two bait vectors were used for two-hybrid screens and confirmatory interactions: pGBKT7 (BD Bioscience) and MP34 (R. Brazas, Duke University). pGBKT7 places the Gal4 DNA

binding domain upstream of the bait in the fusion protein, whereas MP34 places the DNA binding domain downstream of the bait (Fig. 1A). In both vectors, expression is from the ADH1 promoter, replication uses a 2-μ origin, and the TRP1 marker is used for selection in yeast. The vectors also encode ampicillin resistance for selection in bacteria. cDNA fragments encoding mouse TBP full-length (TBP-FL) and TBP N terminus (TBP-N, amino acids 1–136) were generated by PCR from a plasmid containing the predominant mouse somatic TBP cDNA (28,29) using the following primer sets (Table 1): TBP-FL, TBP-N-start primer and TBP-C-end primer; TBP-N, TBP-N-start primer and TBP-N-end primer. PCR-amplified TBP cDNA fragments were cut with SalI/NotI and ligated into SalI/ NotI-cut MP34. The TBP C terminus (TBP-C) was amplified using TBP-C-start primer and TBP-C-end primer, digested with SalI and NotI, and inserted into a pGBKT7 vector modified by digesting with BamHI, filling with Klenow, and ligating to itself to shift the reading frame one base in the +1 direction, allowing for in-frame insertion of TBP-C into the modified pGBKT7^{+1} vector. All bait clones and vector modifications were verified by sequencing.

The pGADT7 prey plasmid (BD Bioscience) was altered to allow efficient directional cloning of oligo-(dT)-primed cDNA libraries with SalI and NotI 5′ and 3′ linkers, respectively, as follows. The NotI site was cut, filled with Klenow, and re-ligated to kill the site, generating pGADT7$^{\Delta NotI}$. A linker containing internal SalI and NotI sites and 5′ BamHI and 3′ XhoI overhangs was ligated into BamHI/XhoI-cut pGADT7$^{\Delta NotI}$. The modified prey plasmid was named pGADT7^{SN}.

Oligo-(dT)-primed cDNA prey libraries were constructed and inserted into the pGADT7^{SN} vector as follows. Total RNA was extracted and CsCl-purified (30) from embryonic day 10.5 (E10.5) wild-type C57Bl/6J whole pregnant uteri or placentas.[3] In each case, either whole pregnant uteri or placentas were obtained from four pregnant dams to generate a pool of RNA. Poly(A$^+$) mRNA from these samples was purified using Oligo-(dT)$_{25}$ Dynabeads (Dynal Biotech ASA, Oslo, Norway) following the manufacturer's protocols. Each cDNA library was constructed using 2.5 μg of poly(A$^+$) mRNA and the Superscript plasmid system for cDNA synthesis and cloning (Invitrogen), which yields cDNAs containing 5′ SalI and 3′ NotI overhangs. cDNAs were ligated into SalI/NotI-digested pGADT7^{SN}. The whole pregnant uteri library contained $\sim 3.0 \times 10^6$ independent recombinants with 82% bearing inserts; the placental library contained $\sim 2.6 \times 10^6$ independent recombinants with 95% bearing inserts. The average insert size in both libraries exceeded 1 kb (Fig. 1B). Both libraries and all plasmids are freely available on request.

Yeast Two-hybrid System

All interactions were tested in *Saccharomyces cerevisiae* strain AH109 (BD Bioscience), which contains the Ade2, His3, and LacZ reporters, each under the control of a different promoter. For library transformations, a culture of AH109 containing the TBP bait construct was grown at 30 °C for 48 h in liquid synthetic complete medium (SC) lacking tryptophan (SC-W) (Q-BIOgene, Irvine, CA). This culture was used to seed 300 ml of 2× yeast extract/peptone/adenine/dextrose at 5×10^6 yeast/ml, which was determined by counting on a hemacytometer. The culture was grown at 30 °C for ~5 h to a density of 2×10^7 yeast/ml. Yeast were collected by centrifugation, washed once with water, and once with 100 mM LiAc and transformed with 14.4 ml of 50% PEG (average M_r 3350), 2.16 ml of 1.0 M LiAc, 0.3 ml of 10 mg/ml sheared denatured salmon sperm DNA, and 120 μg of the cDNA library in pGADT7^{SN}. Single- or two-component transformations of bait and/or prey plasmids into yeast used a standard PEG/LiAc protocol (31).

[3]Animal protocols were approved by the Montana State University Institutional Animal Care and Use Committee.

Two-hybrid screens were performed on SC medium lacking leucine, tryptophan, and histidine (SC-L-W-H). Both two-hybrid screens used TBP-FL as bait to screen either the whole pregnant uteri (screen 1) or placental (screen 2) cDNA prey libraries. Primary transformants were transferred onto a new SC-L-W-H plate and clones that grew well on these plates were transferred to a SC-L-W plate, grown for 48 h at 30 °C, and replica plated to higher stringency selection medium, such as SC-LW-H + 2.5 mM 3-aminotriazole, a competitive inhibitor of the His3 gene product (32) or SC-L-W-H also lacking adenine (SC-L-W-H-A). Yeast were also replica-plated to SC-L-W plates containing X-*α*-gal (Glycosynth, Cheshire, United Kingdom) to identify clones that activated the LacZ reporter gene. Prey plasmids from those clones that grew under higher stringency selection and showed strong LacZ expression were isolated from yeast by glass bead lysis (33). Recovered plasmids were transformed into bacteria, clones were selected, and inserts were sequenced to determine the cDNA identity. Isolated prey plasmids were re-transformed into AH109 with the bait and grown on SC-L-W-H, SC-L-W-H + 2.5 mM 3-AT, and SC-L-W-H-A plates to verify the interaction.

To identify the domain of TBP that interacted with prey proteins, TBP bait constructs in combination with the empty pGADT7 prey plasmid (autoactivation test) or with a prey plasmid containing novel or known TBP interactors were co-transformed into AH109 and plated onto SC-L-W. Individual colonies that grew on SC-L-W plates (containing both the bait and prey plasmid) were grown overnight in yeast extract/peptone/adenine/dextrose at 30 °C, pelleted, and washed three times in sterile water. Yeast pellets were resuspended in 0.5 ml of water and their concentration determined by counting on a hemacytometer. Standard amounts of yeast were plated on SC-L-W-H to verify that bait plasmids were not autoactive or to test interactions of TBP subdomain baits with each prey. Prey plasmids were also tested for autoactivation by co-transforming yeast with prey plasmids and empty bait plasmid.

Clone Isolation and Interaction Tests

PCR primers were designed to allow in-frame insertion of cDNAs into either pGBKT7^{+1} bait plasmid (for TBP deletions) or pGADT7^{SN} prey plasmid (for full-length PIASx*β*, PIASy, and truncated PIAS1 mutants). Reverse transcriptase-PCR amplification-based cloning of PIASx*β* and PIASy used oligo-(dT)-primed first-strand cDNA from either adult C57Bl/6J testis or E10.5 C57Bl/6J placentas, respectively, for PIASy and PIASx and the following primer sets: PIASx*β*, PIASx*β*-forward primer, and PIASx*β*-reverse primer; PIASy, PIASy-forward primer and PIASy-reverse primer (Table 1).

The longest clone of PIAS1 isolated from the two-hybrid screen began at amino acid 6 of the open reading frame. To generate a clone encoding the full-length PIAS1 protein (PIAS1$_{1–651}$), primers PIAS1-N1-forward and pGADT7-reverse were used (Table 1) with the pGADT7^{SN}-PIAS1$_{6–651}$ plasmid clone from the library as template. Construction of truncated TBP-C and PIAS1 cDNAs used plasmid DNA containing either a TBP-FL or pGADT7^{SN}-PIAS1$_{6–651}$, respectively, as the template for PCR amplification. PCR primer sets for amplification of each truncated cDNA were: TBP-C$_{135–263}$, TBP-C-start primer and TBP-C263-reverse primer; TBP-C$_{135–210}$, TBP-C-start primer and TBP-C210-reverse primer; TBP-C$_{201–316}$, TBP-C201-forward primer and TBP-C-end primer; TBP-C$_{251–316}$, TBP-C251-forward primer and TBP-C-end primer; TBP-C$_{160–263}$, TBP-C160-forward primer and TBP-C263-reverse primer; PIAS1$_{6–452}$, T7 primer and PIAS1-C452 reverse primer; PIAS1$_{6–491}$, T7 primer and PIAS1-C491-reverse primer; PIAS1$_{6–562}$, T7 and PIAS1-C562-reverse primer; PIAS1$_{6–605}$, T7 and PIAS1-C605-reverse primer; PIAS1$_{598–651}$, PIAS1-N598-forward primer and pGADT7-reverse primer; PIAS1$_{555–651}$, PIAS1-N555-forward primer and pGADT7-reverse primer; PIAS1$_{484–651}$, PIAS1-N484-forward primer and pGADT7-reverse primer (Table 1). PCR products were digested with SalI/NotI and were inserted into the bait or prey vectors. Four clones of each truncated protein were isolated and separately co-transformed

with either TBP-FL (for PIASxβ, PIASy, and PIAS1 mutants) or PIAS1$_{6–651}$ (for TBP-C mutants) into yeast as above. Yeast were plated onto SC-L-W agar plates and grown at 30 °C. Resultant colonies were suspended in water and streaked onto SC-L-W-H and SC-L-W-H-A to test each protein interaction pair.

Antibodies, Animals, and Nuclear Extract Preparation

Mouse antic-Myc monoclonal antibody was purchased from Amersham Biosciences; mouse anti-FLAG M2 monoclonal antibody was purchased from Sigma; goat anti-PIAS 1 (C-20) and goat anti-PIAS 1/3 (N-18) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); horseradish peroxidase-conjugated donkey anti-rabbit antibodies were purchased from Pierce; horseradish peroxidase-conjugated sheep anti-mouse was purchased from Amersham Biosciences; horseradish peroxidase-conjugated rabbit anti-goat antibody was purchased from Invitrogen. Rabbit-anti-TBP N_C antiserum was raised against recombinant His_6-tagged mouse TBP amino acids 72–135. For this later antiserum, specificity was verified by Western blots using recombinant TBP, wild-type mouse nuclear extracts, and nuclear extracts from mouse cells homozygous for the *tbp*$^{\Delta N}$ mutation (34,35) (described below, mutation eliminates antibody-reactive domain, not shown).

For nuclear extract preparation, young adult male wild-type and *tbp*$^{\Delta N/+}$ mice were used. The *tbp*$^{\Delta N/+}$ mice are heterozygous for a targeted mutation that exchanged the endogenous *tbp* gene for a version that replaced 111 amino acids within the vertebrate-specific N terminus of TBP with two copies of the FLAG epitope tag (34,35). The mutant protein contains the entire TBP_{CORE} region of the protein and Western blots confirm that this FLAG-tagged TBP accumulates to wild-type levels in all tissues (34,35). Heterozygous animals are healthy and fertile (34). Survival of animals homozygous for this mutation to E9.5 (100%), birth (9%), and adulthood (~1%) indicates that the FLAG-tagged protein is a functional replacement for most TBP activities (34).

Nuclear extracts were prepared to procedures described previously (36,37). Briefly, liver and spleen were harvested into ice-cold phosphate-buffered saline, blotted, weighed, minced, and homogenized ice-cold under final conditions of 7.5% (w/v) tissue, 0.5% (w/v) nonfat dry milk, in 1.85 M sucrose, 8.4% (v/v) glycerol, 8.4 mM HEPES, pH 7.6, 12.6 mM KCl, 0.13 mM spermine, 0.42 mM spermidine, 1.7 mM EDTA, 100 μM PMSF, 1× protease inhibitors (Sigma), 5 mM DTT using a motordriven Teflon/glass Dounce homogenizer. Homogenate was layered onto 10-ml cushions of 2.0 M sucrose, 10% glycerol, 10 mM HEPES, pH 7.6, 15 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 2 mM EDTA, 100 μM PMSF, 1× protease inhibitors, 5 mM DTT, and centrifuged in an SW28 rotor at 24,000 rpm for 1 h at 4°C. Pelleted nuclei were resus-pended in 10 mM HEPES, pH 7.6, 100 mM KCl, 10% glycerol, 0.1 mM EDTA, 3 mM $MgCl_2$,10 μM PMSF, 0.1× protease inhibitors, 5 mM DTT. Nuclei were adjusted to 0.3 mg of nucleic acid/ml with dialysis buffer (25 mM HEPES, pH 7.6, 10% glycerol, 40 mM KCl, 0.1 mM EDTA) containing 10 μM PMSF, 0.1× protease inhibitors, 1 mM DTT. While gently mixing, 1/10th volume of 4.0 M $(NH_4)_2SO_4$ was added and tubes were incubated on ice for 1 h. Chromatin was pelleted by centrifugation for 1 h at 40,000 × *g* at 4 °C, in a Ti50 or Ti60 rotor. Supernatant was collected and proteins were precipitated by adding 0.3 g/ml dry $(NH_4)_2SO_4$ and incubating with gentle mixing for 1 h after solid dissolved. Proteins were collected by centrifugation for1hat 40,000 × *g* at 4 °C, in a Ti50 or Ti60 rotor, and were resuspended in dialysis buffer containing 10 μM PMSF, 0.1× protease inhibitors, 1 mM DTT. Nuclear extracts were dialyzed two times for 2 h against dialysis buffer containing 0.2 mM DTT (reduced DTT to preserve antibody disulfides during immunoprecipitations), insoluble material was removed by brief centrifugation, protein concentrations were determined, and aliquots were snap-frozen in liquid nitrogen. Nuclear extracts were verified by electrophoretic mobility shift assays for NF-Y and Oct proteins (data not shown) prior to use in immunoprecipitations.

In Vitro Translation, Transient Co-transfections, and Co-immunoprecipitations

In vitro transcription/translation reactions were performed using the "TNT" system (Promega, Madison, WI) following the manufacturer's protocols with the plasmid templates and conditions detailed in the figure legends.

For transient transfection/co-immunoprecipitation assays, TBP-FL was inserted into pCMV-HA (BD Bioscience), which fused the HA epitope tag to the N terminus of TBP. $PIAS1_{1–691}$, $PIAS1_{1–452}$, $PIAS1_{6–691}$, $PIAS3_{325–628}$, $BRF1_{264–676}$, or $ZFP523_{202–568}$ were inserted into pCMV-Myc (BD Bioscience), which fused the c-Myc epitope tag to the N terminus of each protein. Human embryonic kidney 293 cells (HEK293) were plated onto 60-mm dishes at ~30% confluence. The next day, cells were washed once with serum-free, drug-free Dulbecco's modification of Eagle's medium (DMEM) (Mediatech, Herndon, VA). Transfection mixes contained 300 μl of serum-free drug-free DMEM, 1 μg of each plasmid, and 10 μg of Novafector (Venn Nova, Inc., Pompano Beach, FL). Transfection mixes were added to washed cells in dishes containing 2 ml of serum-free drug-free DMEM and incubated for 5 h at 37 °C, 7.5% CO_2. After incubation, 2 ml of DMEM containing 20% newborn calf serum (Invitrogen), 4% fetal bovine serum (HyClone, Logan, UT), and 2× antibiotic-antimycotic solution (Mediatech) was added to each plate. Approximately 14 h later, the medium was replaced. At 48 h post-transfection, cells were washed 2 times in ice-cold phosphate-buffered saline and 500 μl of lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.8% deoxycholic acid, 10% glycerol, 1 mM EDTA, 1 mM PMSF, 5 μg/ml leupeptin-pepstatin-aprotinin, 0.15 mM $NaVO_3$, and 1 mM DTT) was added to each plate. Plates were rocked for 15 min at 4 °C to allow cells to detach from the plate. The contents of each plate were transferred to a 1.5-ml tube and placed on ice for 30 min with brief mixing at 5-min intervals. Lysates were clarified at 10,000 × *g* for 20 min at 4 °C. Lysates from each set of plates were pooled and a portion of each lysate (~50 μl) was stored at −80 °C for use as transfection controls. The remaining 950 μl of each lysate was pre-cleared with 20 μl of protein G plus/protein A-agarose (Calbiochem, San Diego, CA) and 5 μl of nonspecific antibody or antiserum for 2 h at 4°C on a rotator. Samples were centrifuged to pellet the agarose and 450 μl of each supernatant was used for immunoprecipitations.

For co-immunoprecipitations, protein samples (either 450 μl of pre-cleared transfected cell lysate, 100 μl of nuclear extract diluted to 1.0 ml with 1× TBS (50 mM Tris, pH 7.5, 150 mM NaCl), or 50 μl of TNT lysates from *in vitro* co-translation reactions diluted to 500 μl with binding buffer (20 mM Tris, pH 7.5, 0.1 M NaCl, 5 mM $MgCl_2$, 10% glycerol, 1 mM PMSF, 5 μg/ml inhibitors) were transferred to tubes containing specific or nonspecific monoclonal antibody (2 μg), specific or nonspecific goat polyclonal antiserum (5 μl), or anti-FLAG M2 resin (40 μl, Sigma), as indicated in the figure legends. Binding reactions were incubated overnight at 4 °C on a rotator and 30–40 μl of protein G plus/protein A-aga-rose was added to each tube (except those containing anti-FLAG m2 resin) and rotated for an additional hour at 4 °C. For transfected cell lysates, the agarose was pelleted at 750 × *g* for 1 min, washed once with ice-cold lysis buffer, four times with the first wash buffer (50 mM Tris, pH 7.5, 0.3 M NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 1 mM PMSF, and 0.15 mM $NaVO_3$), and one time with a final wash buffer that contained the same components as the first wash buffer, except that the NaCl concentration was lowered to 0.1M. For *in vitro* translation immunoprecipitations, washes were performed with binding buffer containing 0.1% IgePAL-CA-630 detergent (Sigma) in place of Triton X-100, and for FLAG immunoprecipitations, washes were performed with 1× TBS containing 0.1% Triton X-100. Pellets were resuspended in 1× loading buffer (38), boiled 5 min, and separated by electrophoresis through a 12% SDS-polyacrylamide gel. Proteins were transferred to supported nitrocellulose, probed with the indicated primary and secondary antibodies, and visualized using Supersignal-West chemiluminescence (Pierce) and x-ray film.

RESULTS

Identification of TBP-interacting Proteins by Yeast Two-hybrid

To identify mouse proteins that interacted with TBP, we screened both a placental cDNA library (screen #1) and a whole pregnant uteri cDNA library (screen #2) with mTBP-FL in the MP34 bait plasmid (Fig. 1). MP34-mTBP-FL did not grow on SC-L-W-H medium in the presence of an empty prey plasmid (Fig. 2, sector 1), confirming that the bait was not autoactive.

In screen 1 (placenta library), $\sim 2.5 \times 10^6$ primary transformants (yeast containing both bait and prey plasmids) (Fig. 1C) were plated onto SC-L-W-H medium. Fifty-eight colonies grew and all were transferred to a SC-L-W-H plate containing X-α-gal. Thirty-seven clones grew well and expressed LacZ. These were transferred to a SC-L-W plate, grown for 48 h, and replica plated to SC-L-W-H-A medium to test for combined expression of both the *HIS3* and *ADE2* reporter genes. In this round of selection, nine clones grew well. After isolating the prey plasmid from each, we re-tested the interactions by co-transforming new yeast with the cDNA-containing prey plasmid and TBP-FL bait plasmid. All nine clones re-grew on SC-L-W-H + X-α-gal medium and all expressed LacZ (not shown).

In screen 2 (whole pregnant uteri library; Fig. 1C), of $\sim 0.6 \times 10^6$ primary transformants, $\sim$100 colonies grew on SC-L-W-H plates, of which 41 were selected for further analysis. These were transferred to SC-L-W-H-A medium containing X-α-gal. Thirteen clones grew well and expressed LacZ following retransformation.

Sequence analysis of the prey cDNAs from screen 1 revealed that seven of the nine clones encoded PIAS1, one clone encoded PIAS3, and one encoded the α-subunit of transcription factor IIA (TFIIA), a known TBP-interacting protein (39-41). Analysis of TBP-interacting prey cDNAs from screen 2 identified three more PIAS1 clones. We also obtained three clones encoding B′-related factor 1 (BRF1), another known TBP interactor (39-42). Additionally, we identified ZFP523, the mouse homologue of a recently identified novel TBP interacting protein, hZNF76 (11). The remaining six clones were all unique, although no obvious physiological connection to TBP was evident. The interactions of TBP with TFIIA, ZFP523, PIAS1, and PIAS3 in the yeast two-hybrid system is shown in Fig. 2.

For verification, the interactions between TBP and proteins identified by two-hybrid screens were tested by co-immunoprecipitation in cell culture. Both PIAS1 and PIAS3 co-precipitated with TBP when co-expressed in HEK293 cells (Fig. 3A). Additionally, ZFP523 and BRF1, both known TBP interactors, co-precipitated with TBP (Fig. 3B).

In Vitro Co-translated PIAS1 and TBP Co-immunoprecipitate

The PIAS/TBP interaction was identified using yeast, which contain homologues of most mammalian transcription machinery components. Verification used transfected human cells, which contain the mammalian machinery. It was possible that, in both systems, PIAS and TBP did not interact directly, but rather, PIAS proteins assembled into a stable TBP-containing complex via interactions with other endogenous TBP-interacting proteins. To distinguish these possibilities, we co-translated the two proteins *in vitro* using a reticulocyte-based system and tested whether PIAS1 and TBP co-immunopreciptated. Immunoprecipitation of either protein pulled-down the other; however, nonspecific antibody did not pull-down either (Fig. 3C). Thus, the PIAS/TBP interaction does not require other nuclear proteins, but rather, is very likely the result of direct PIAS/TBP contacts.

Identification of the TBP-interacting Domain on PIAS1

Sequence analysis of the 10 TBP-interacting PIAS1 clones isolated in the two-hybrid screens revealed that six were of differing lengths. The longest clone encoded amino acids 6 to 651 ($PIAS1_{6–651}$); the shortest encoded amino acids 453 to 651 ($PIAS1_{453–651}$) (Figs. 2 and 4). Other clones encoded amino acids 135–651, 359–651, 430–651, and 439–651 of PIAS1 (Fig. 4B). The single PIAS3 clone encoded PIAS3 amino acids 325–628. Because libraries were oligo-(dT)-primed, all clones used their natural stop codon.

The shortest PIAS1 clone isolated from the two-hybrid screens ($PIAS1_{453–651}$) contained the last 199 amino acids of PIAS1. To more precisely identify the domain that interacted with TBP, we constructed more 5′-truncated versions, which encoded amino acids 484–651, 555–651, and 598–651, and 3′-truncated versions of PIAS1, which encoded amino acids 6–452, 6–491, 6–562, and 6–605 (Fig. 4B). Each PIAS1 mutant was tested for interaction with TBP-FL in the two-hybrid system (Fig. 4A). Only clones containing the region from 453 to 491 interacted with TBP, as indicated by growth on SC-L-W-H selective medium (Fig. 4A). Yeast that contained the $PIAS1_{6–491}$ prey grew less well (Fig. 4A, *bottom panel*, sector *1*), suggesting that this truncation might have weakened but not ablated the TBP interaction domain. Sectors *7–10* are TBP interaction and autoactivation controls for $PIAS1_{6-651}$ and $PIAS1_{453–651}$, respectively. Our data suggest that the TBP interaction domain of PIAS1 requires the 39 amino acids from positions 453 to 491, which includes the Ser/Ac domain (Fig. 4B).

PIAS1 and PIAS3 Interact with the TBP_{CORE}

To determine whether the PIAS clones that were isolated in two-hybrid screens interacted with the conserved C-terminal TBP_{CORE} or the vertebrate-specific N terminus (43), we co-transformed yeast with the following baits and preys: TBP-N/$PIAS1_{453–651}$, TBP-C/$PIAS1_{453–651}$, TBP-N/$PIAS1_{6–651}$, TBP-C/$PIAS1_{6–651}$, TBP-N/$PIAS3_{325–628}$, and TBP-C/$PIAS3_{325–628}$. Growth of all clones on SC-L-W verified the presence of both the bait and the prey plasmid. As controls, we tested the interactions of TBP-N or TBP-C with TFIIAα (Fig. 5A) (40,41). Neither the TBP bait clones nor the PIAS and TFIIA prey clones were autoactive (Fig. 5A), confirming the validity of the assay. We found that all PIAS clones and the TFIIA clone interacted with the TBP_{CORE} (indicated by growth on SC-L-W-H in Fig. 5A, *right panels*), but not with the TBP N terminus (Fig. 5A).

We also constructed truncated TBP_{CORE} mutants to further define the domain of TBP that interacted with PIAS1. Only one of our mutants, TBP-$C_{135–263}$, encoding approximately the first two-thirds of the TBP-$_{CORE}$, allowed growth with $PIAS1_{6–651}$ (Fig. 5A, sector *1*).

Association of Endogenous PIAS1 and TBP in Mouse Nuclear Extracts

Our results indicated that PIAS1 could interact with the TBP_{CORE} both *in vivo* under conditions of overexpression (two-hybrid and transient transfection) and in reticulocyte extracts. To gain insights into whether endogenous PIAS1 and TBP proteins in normal cells might also interact, we tested whether we could detect physical association of the endogenous proteins in mouse nuclear extracts. Mice bearing a targeted mutation at the *tbp* locus that replaces most of the vertebrate-specific N-terminal domain with two copies of the FLAG tag ($tbp^{\Delta N}$ mice) provided a unique resource in which a functional TBP_{CORE} expressed at wild-type levels is epitope-tagged (34,35).

Nuclear extracts were prepared from livers and spleens of adult wild-type and heterozygous ($tbp^{\Delta N/+}$) mice. Immunoprecipitation using anti-PIAS1 antibody brought down TBPΔN protein from heterozygous nuclear extracts; absence of an anti-FLAG antibody-reactive band in wild-type extracts confirmed the specificity of the FLAG antibody/tag combination (Fig. 6A). Nuclear extracts were then immunoprecipitated with the anti-FLAG antibody or a negative

control antibody and Western blots were probed with anti-PIAS1 antibody (Fig. 6B). The anti-FLAG antibody, but not the control antibody, brought down PIAS1 protein from heterozygous but not wild-type extracts. These results indicated that a portion of the endogenous PIAS1 in mouse liver and spleen nuclear extracts was complexed with TBPΔN.

TBP Interacts with PIASx and PIASy

Amino acid alignment of all known isoforms of PIAS proteins from all four *pias* genes (16) revealed that all shared the TBP interaction domain (Fig. 7 and data not shown) identified here (Fig. 4, A and B). The remainder of the C-terminal region shows little identity between family members. In fact, PIASy does not contain ~100 amino acids that are found in the other PIAS family members. Based on the amino acid conservation in the TBP interaction domain between all PIAS proteins, we wished to determine whether proteins from the *piasx* and *piasy* genes also functionally interacted with TBP. Full-length cDNAs for PIASx*β* and PIASy were isolated by reverse transcriptase-PCR and were inserted into the two-hybrid prey vector ($pGADT7^{SN}$) to test for interaction with either MP34-TBP-FL or the empty bait plasmid in two-component two-hybrid assays. Both proteins interacted strongly with TBP-FL; neither prey was autoactive when tested with empty bait (MP34) vector (Fig. 8).

In conclusion, our data suggest that TBP interacts with ZFP523 and with all members of the PIAS family of proteins through the conserved TBP_{CORE}. Using PIAS1 as a family representative, we show that that PIAS/TBP interaction involves a conserved 39-amino acid region within the PIAS C-terminal region and very likely occurs through direct contacts between PIAS and TBP proteins. Moreover, using nuclear extracts from mice bearing an epitope-tagged TBP_{CORE}, we present evidence suggesting that the endogenous proteins interact as well. These results lend insights into the gene-regulatory interplay between TBP, ZNF523, PIAS proteins, and other transcriptional regulators, as discussed below.

DISCUSSION

TBP is a central player in transcription initiation whose activity is controlled by interactions with activators, repressors, and other transcriptional regulatory proteins. In agreement with the recently reported interaction between TBP and human ZNF76 (11), we show that mouse ZFP523, which shares 92% amino acid identity with ZNF76, interacts with TBP (Figs. 2 and 3B). The clone of ZFP523 we isolated in our screen was a partial cDNA that encoded the last 366 amino acids of the 568-amino acid protein. This clone contained the entire GARD domain (amino acids 362–444), which is the region of the human protein shown to interact with TBP (11).

Most of the TBP-interacting clones isolated in our two-hybrid screens encoded PIAS1 or PIAS3 proteins (Fig. 2). Previously, regulation of gene transcription by the PIAS family proteins has been shown to occur via interactions with factors that are upstream of TBP in the transcriptional initiation process, some of which have also been shown to directly interact with TBP. These include the general co-activator CBP/p300 (44), the transcriptional factors MSX2 (15), p53 (25,45), p73 (46), and others. To our knowledge, this is the first report of a direct interaction between PIAS proteins and a component of the basal transcription machinery.

The interaction between TBP and PIAS proteins involves the conserved C-terminal core of TBP and a C-terminal 39-amino acid region found in common between PIAS1, PIAS3, PIASx, and PIASy proteins. Although the functions of the more highly conserved N-terminal region of PIAS proteins, including its SUMO E3-ligase activity, are well characterized, the C-terminal region is less well understood (21). The C-terminal regions of individual PIAS proteins have been shown to mediate protein-protein interactions with some regulators (21). However, alignment shows that only a region within the TBP-interacting domain described here (Fig.

4B) is shared among all seven PIAS proteins (Fig. 7). Our interpretation is that all PIAS proteins share the property of interacting directly with TBP; the remainder of the PIAS C-terminal domains determine the interactions that distinguish the activities of each family member from the others.

Another activity shared by all PIAS proteins is that they function as SUMO E3-ligases through amino acids in the conserved N terminus (12,18,24,27). PIAS1-dependent sumoylation of the ZNF76 prevents ZNF76 from interacting with TBP (11). Our data suggest that the interaction of the PIAS1 C terminus with TBP could also influence the interaction of ZNF76 with TBP. The localization of SUMO E3-ligase activity and TBP-binding activity to opposite ends of PIAS proteins suggests that PIAS proteins might “dock” at TBP and sumoylate transcription factors at the promoter. Further investigation of the interactions between TBP, PIAS proteins, and transcription factors like ZNF76 will be required to determine which interactions are cooperative, which are antagonistic, and how these proteins interact at the TBP interface to regulate the expression of specific target genes.

Acknowledgments

We thank O. Lucas for assistance, M. Jutila and R. Brazas for reagents, M. White for discussions, and K. Daughenbaugh for reading the manuscript.

REFERENCES

1. Cormack BP, Struhl K. Cell 1992;69:685–696. [PubMed: 1586947]
2. Hernandez N. Genes Dev 1993;7:1291–1308. [PubMed: 8330735]
3. Pugh BF. Gene (Amst.) 2000;255:1–14. [PubMed: 10974559]
4. Mittal V, Cleary MA, Herr W, Hernandez N. Mol. Cell. Biol 1996;16:1955–1965. [PubMed: 8628262]
5. Timmers HT, Tora L. Trends Biochem. Sci 2005;30:7–10. [PubMed: 15653319]
6. Albright SR, Tjian R. Gene (Amst.) 2000;242:1–13. [PubMed: 10721692]
7. Naar AM, Lemon BD, Tjian R. Annu. Rev. Biochem 2001;70:475–501. [PubMed: 11395415]
8. Klejman MP, Pereira LA, van Zeeburg HJ, Gilfillan S, Meisterernst M, Timmers HT. Mol. Cell. Biol 2004;24:10072–10082. [PubMed: 15509807]
9. Pereira LA, Klejman MP, Timmers HT. Gene (Amst.) 2003;315:1–13. [PubMed: 14557059]
10. Myslinski E, Krol A, Carbon P. J. Biol. Chem 1998;273:21998–22006. [PubMed: 9705341]
11. Zheng G, Yang YC. J. Biol. Chem 2004;279:42410–42421. [PubMed: 15280358]
12. Schmidt D, Muller S. Cell Mol. Life Sci 2003;60:2561–2574. [PubMed: 14685683]
13. Moilanen AM, Karvonen U, Poukka H, Yan W, Toppari J, Janne OA, Palvimo JJ. J. Biol. Chem 1999;274:3700–3704. [PubMed: 9920921]
14. Sturm S, Koch M, White FA. J. Mol. Neurosci 2000;14:107–121. [PubMed: 10854042]
15. Wu L, Wu H, Ma L, Sangiorgi F, Wu N, Bell JR, Lyons GE, Maxson R. Mech. Dev 1997;65:3–17. [PubMed: 9256341]
16. Shuai K, Liu B. Nat. Rev. Immunol 2005;5:593–605. [PubMed: 16056253]
17. Shuai K. Oncogene 2000;19:2638–2644. [PubMed: 10851063]
18. Kotaja N, Karvonen U, Janne OA, Palvimo JJ. Mol. Cell. Biol 2002;22:5222–5234. [PubMed: 12077349]
19. Liu B, Gross M, ten Hoeve J, Shuai K. Proc. Natl. Acad. Sci. U. S. A 2001;98:3203–3207. [PubMed: 11248056]
20. Liu B, Liao J, Rao X, Kushner SA, Chung CD, Chang DD, Shuai K. Proc. Natl. Acad. Sci. U. S. A 1998;95:10626–10631. [PubMed: 9724754]
21. Johnson ES. Annu. Rev. Biochem 2004;73:355–382. [PubMed: 15189146]
22. Melchior F, Hengst L. Nat. Cell Biol 2000;2:E161–E163. [PubMed: 10980713]
23. Liu B, Yang R, Wong KA, Getman C, Stein N, Teitell MA, Cheng G, Wu H, Shuai K. Mol. Cell. Biol 2005;25:1113–1123. [PubMed: 15657437]

24. Okubo S, Hara F, Tsuchida Y, Shimotakahara S, Suzuki S, Hatanaka H, Yokoyama S, Tanaka H, Yasuda H, Shindo H. J. Biol. Chem 2004;279:31455–31461. [PubMed: 15133049]
25. Kahyo T, Nishida T, Yasuda H. Mol. Cell 2001;8:713–718. [PubMed: 11583632]
26. Duval D, Duval G, Kedinger C, Poch O, Boeuf H. FEBS Lett 2003;554:111–118. [PubMed: 14596924]
27. Sachdev S, Bruhn L, Sieber H, Pichler A, Melchior F, Grosschedl R. Genes Dev 2001;15:3088–3103. [PubMed: 11731474]
28. Ohbayashi T, Schmidt EE, Makino Y, Kishimoto T, Nabeshima Y, Muramatsu M, Tamura T. Biochem. Biophys. Res. Commun 1996;225:275–280. [PubMed: 8769130]
29. Sumita K, Makino Y, Katoh K, Kishimoto T, Muramatsu M, Mikoshiba K, Tamura T. Nucleic Acids Res 1993;21:2769. [PubMed: 8332475]
30. Schmidt EE, Schibler U. J. Cell Biol 1995;128:467–483. [PubMed: 7532171]
31. Gietz RD, Schiestl RH, Willems AR, Woods RA. Yeast 1995;11:355–360. [PubMed: 7785336]
32. Fields, S.; Johnston, M. Yeast Methods for Analyzing Proteins from Other Organisms. Academic Press; San Diego, CA: 1993.
33. Hoffman CS, Winston F. Gene (Amst.) 1987;57:267–272. [PubMed: 3319781]
34. Hobbs NK, Bondareva AA, Barnett S, Capecchi MR, Schmidt EE. Cell 2002;110:43–54. [PubMed: 12150996]
35. Schmidt EE, Bondareva AA, Radke JR, Capecchi MR. J. Biol. Chem 2003;278:6168–6174. [PubMed: 12471023]
36. Gorski K, Carneiro M, Schibler U. Cell 1986;47:767–776. [PubMed: 3779841]
37. Wuarin J, Schibler U. Mol. Cell. Biol 1994;14:7219–7225. [PubMed: 7523861]
38. Laemmli UK. Nature 1970;227:680–685. [PubMed: 5432063]
39. DeJong J, Bernstein R, Roeder RG. Proc. Natl. Acad. Sci. U. S. A 1995;92:3313–3317. [PubMed: 7724559]
40. Geiger JH, Hahn S, Lee S, Sigler PB. Science 1996;272:830–836. [PubMed: 8629014]
41. Tang H, Sun X, Reinberg D, Ebright RH. Proc. Natl. Acad. Sci. U. S. A 1996;93:1119–1124. [PubMed: 8577725]
42. Wang Z, Roeder RG. Proc. Natl. Acad. Sci. U. S. A 1995;92:7026–7030. [PubMed: 7624363]
43. Bondareva AA, Schmidt EE. Mol. Biol. Evol 2003;20:1932–1939. [PubMed: 12885957]
44. Swope DL, Mueller CL, Chrivia JC. J. Biol. Chem 1996;271:28138–28145. [PubMed: 8910428]
45. Schmidt D, Muller S. Proc. Natl. Acad. Sci. U. S. A 2002;99:2872–2877. [PubMed: 11867732]
46. Munarriz E, Barcaroli D, Stephanou A, Townsend PA, Maisse C, Terrinoni A, Neale MH, Martin SJ, Latchman DS, Knight RA, Melino G, De Laurenzi V. Mol. Cell. Biol 2004;24:10593–10610. [PubMed: 15572666]

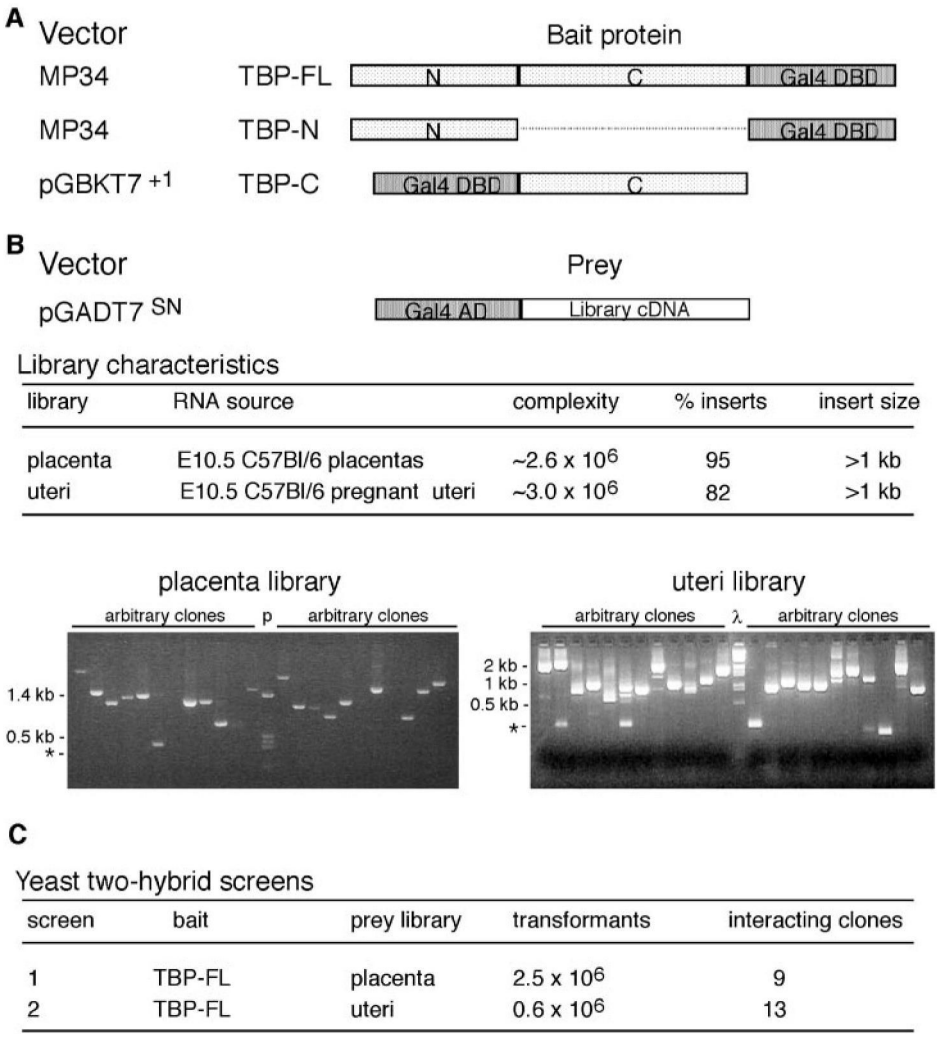
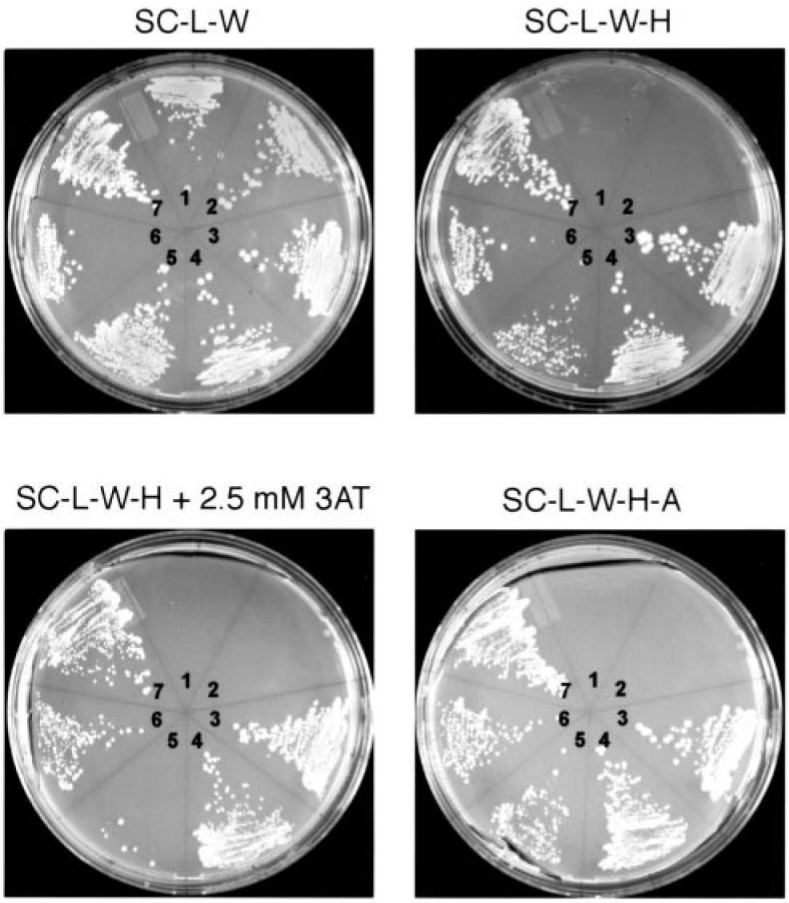


FIGURE 1. Yeast two-hybrid bait constructs, prey libraries, and screens

A, bait constructs. TBP-FL and TBP-N were expressed from the MP34 plasmid, which fused the Gal4 DNA binding domain downstream of TBP. TBP-C was expressed from pGBKT7^{+1}, which fused the Gal4 DNA binding domain upstream of TBP. *N* and *C* designate the vertebrate-specific N terminus and the paneukaryotic C terminus of TBP (TBP_{CORE}), respectively. *B*, two prey libraries were constructed and inserted into pGADT7^{SN}, which fused prey cDNAs downstream of the Gal4 AD. Characteristics of each library are indicated. *Below* is shown PCR analysis of arbitrary clones from each library using a primer pair that spans the multiple cloning site of the vector. Lane "*p*" contained HinfI-cut phosphate-buffered saline^{+} plasmid markers; lane "λ" contained HindIII/EcoRI-cut λ-phage DNA markers. Landmark band sizes are indicated at *left* of gels; *asterisks* denote the size of the PCR product arising from empty prey vector. *C*, the results of the two yeast two-hybrid screens performed are shown. In both screens, TBP-FL was used as bait to screen either the placental or pregnant uteri library for interacting proteins. TBP-interacting prey library clones were subsequently identified by sequencing.



sector	bait	prey
1	TBP-FL	empty vector
2	pLAM C	SV40 T-Ag
3	p53	SV40 T-Ag
4	TBP-FL	TFIIAα
5	TBP-FL	ZFP523
6	TBP-FL	PIAS1$_{6-651}$
7	TBP-FL	PIAS3$_{325-628}$

FIGURE 2. Yeast two-hybrid screens for proteins that interact with TBP

Bait and prey plasmid combinations that were contained within each yeast clone are indicated. Sector designations correspond to those on plates. Growth of yeast on SC-L-W indicated that all clones contained both the bait and prey plasmid. Sector *1* is a negative "autoactivation" control showing that yeast bearing only the TBP bait vector and an empty prey plasmid (no insert) did not grow on SC-L-W-H, SC-L-W-H + 2.5 m$_M$ 3-aminotriazole, or SC-L-W-H-A. Sector *2* was a negative interaction control that was supplied with the Matchmaker III system encoding non-interacting bait and prey proteins. Sector *3* was a positive interaction control supplied with the Matchmaker III system, and accordingly, yeast in this sector grew well under all conditions tested. In sector *4*, the interaction of TFIIA and TBP is shown. Growth of this clone under all conditions tested indicated a strong interaction between TFIIA and TBP, and served as a positive control because TFIIA is known to interact with TBP (40,41). Sector *5* shows the interaction of ZFP523 with TBP. This interaction may be weaker than the TFIIA/

TBP interaction because growth of yeast on the SC-L-W-H + 2.5 m_M 3-AT plate was reduced. However, in comparison to negative controls in sectors *1* and *2*, the TBP/ZFP523 interaction was scored as strongly positive. Sectors *6* and *7* show the interaction of PIAS1 and PIAS3, respectively, with TBP. Both the PIAS clones interacted strongly with TBP as compared with control interactions in sectors *3* and *4*.

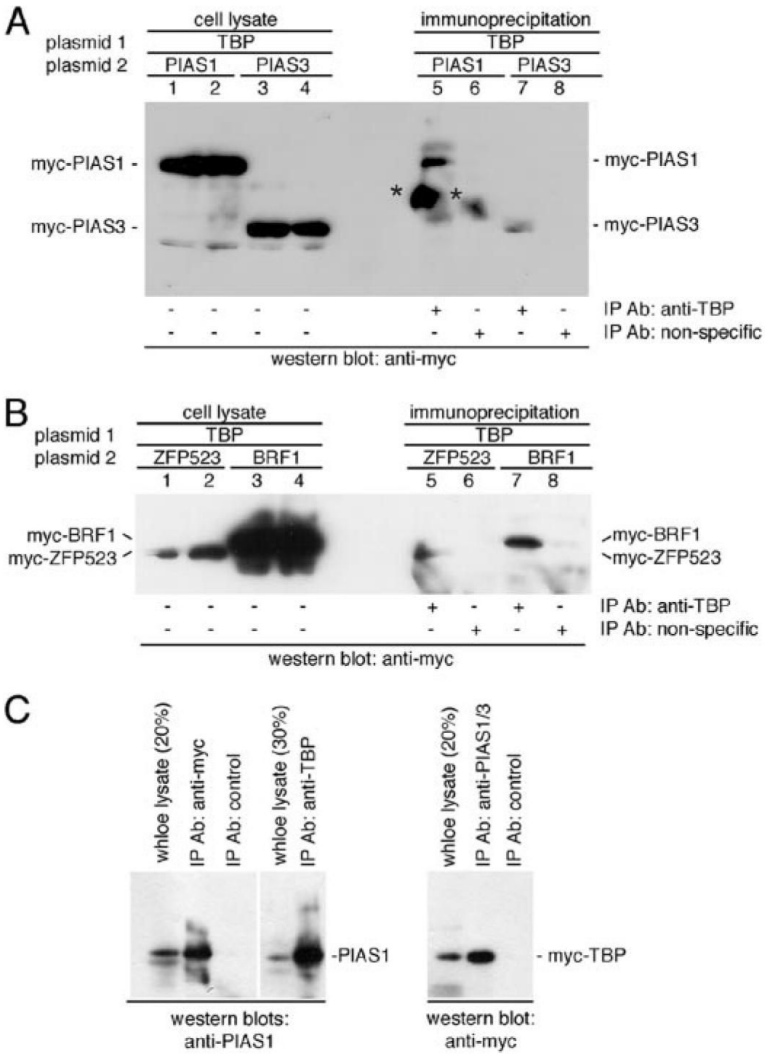


FIGURE 3. Co-immunoprecipitation assays

A, PIAS/TBP interactions in co-transfected cells. HEK293 cells were co-transfected with pCMV-HA-TBP-FL and pCMV-Myc-PIAS1 or pCMV-HA-TBP-FL and pCMV-Myc-PIAS3, as indicated. Whole cell lysates (*lanes 1–2*, PIAS1; *lanes 3–4*, PIAS3) or immunoprecipitated samples (*lanes 5–6*, PIAS1; *lanes 7–8*, PIAS3) were assayed using Western blots and anti-Myc antibody. PIAS1 and PIAS3 co-precipitated with TBP in the presence of the anti-TBP antibody (*lanes 5* and *7*, respectively), but not with the nonspecific antibody (*lanes 6* and *8*). *Asterisks* designate nonspecific cross-reactive materials. *B*, TBP-ZFP523 and TBP-BRF1 interactions in co-transfected cells. ZFP523 co-precipitatated with TBP using the anti-TBP antibody (*lane 5*), but not with the nonspecific antibody (*lane 6*). As a positive control, a BRF1 clone isolated from our yeast two-hybrid screens using TBP-FL as bait was included, because BRF1 is known to directly interact with TBP (42). BRF1 co-precipitated with TBP in the presence of the anti-TBP antibody (*lane 7*), but not with the nonspecific antibody (*lane 8*). *C*, *in vitro* translated TBP and PIAS1 interact in reticulocyte lysates. Transcription/translation reactions were performed in standard 50-μl T_NT reactions charged with 1.0 μg each of NotI-linearized pGBKT7^{+1}-TBPFL (encodes Myc-tagged full-length mouse TBP protein) and NotI-linearized pGADT7^{SN}-PIAS1$_{6–651}$. Immunoprecipitations (*IP*) were performed with anti-Myc, anti-TBP-N_C, anti-PIAS1/3 (N18),

or a matched negative control antibody, as indicated. Western blots were loaded with immunoprecipitate or whole reticulocyte lysate corresponding to the indicated percent of the amount of extract used in the immunoprecipitation and were probed with the indicated antibody. PIAS1 co-precipitated with TBP using either the anti-Myc or anti-TBP-N_C antibody (*left blots*); TBP co-precipitated with PIAS1 using the anti-PIAS1/3 antibody.

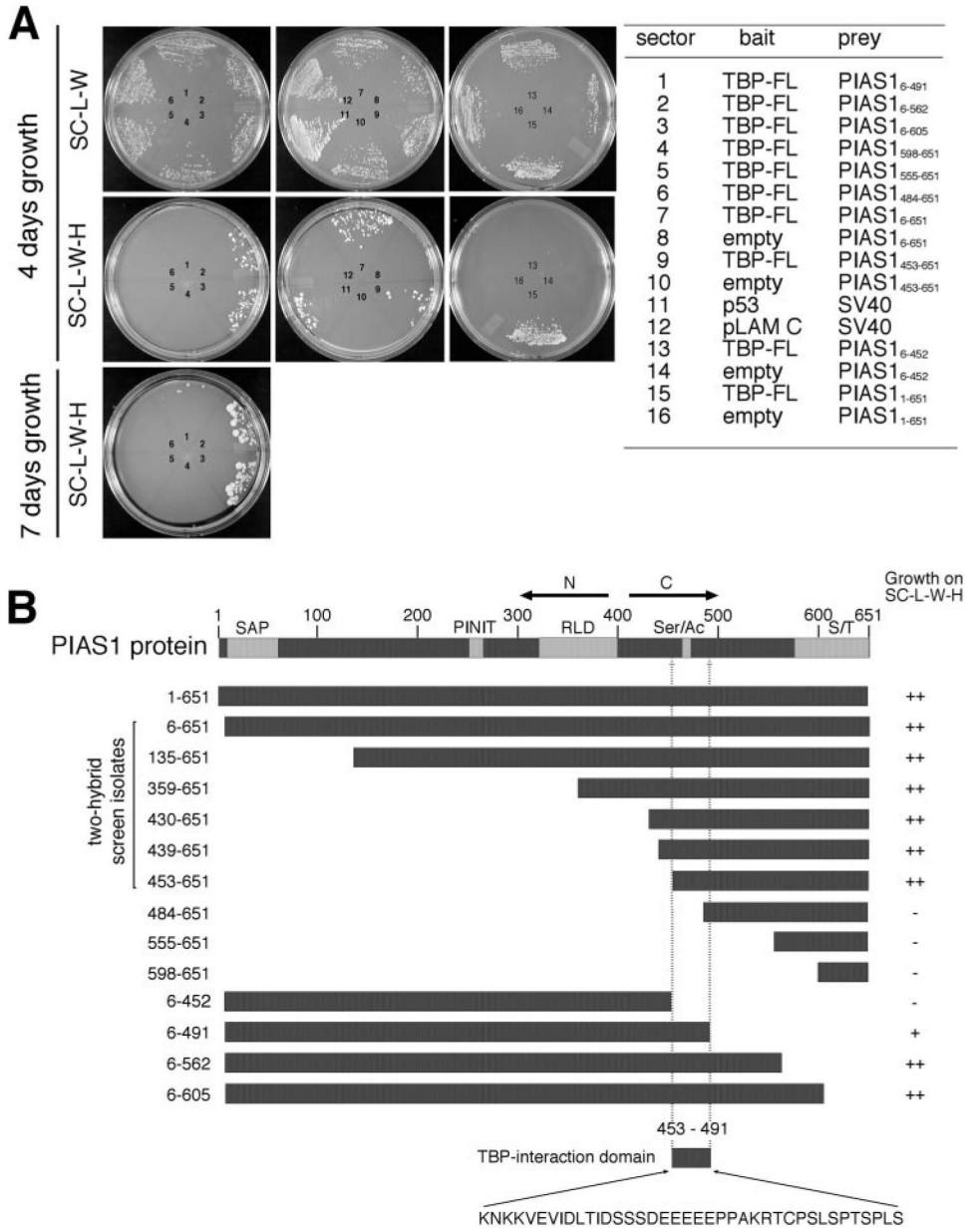
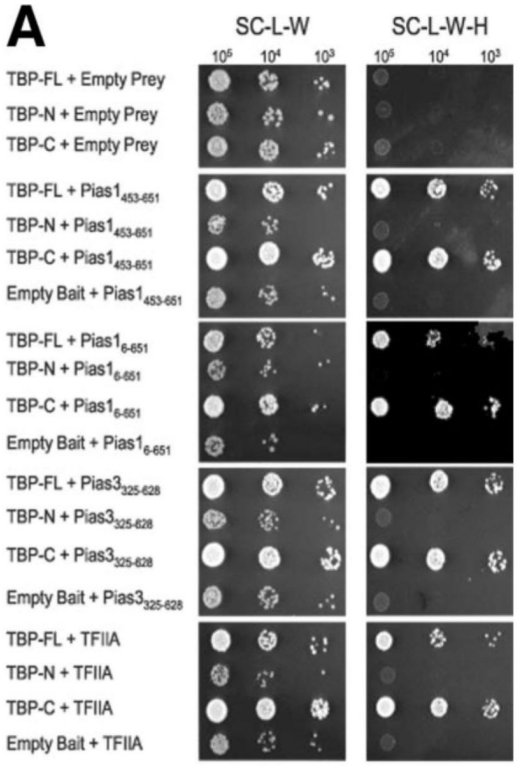
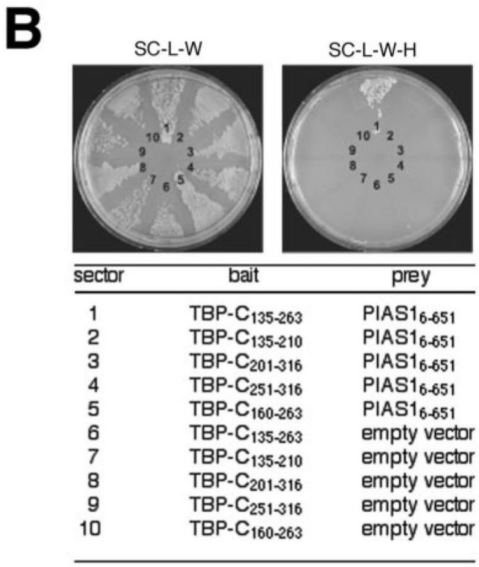


FIGURE 4. Identification of the PIAS1 domain required for interaction with TBP

A, interaction of truncated PIAS1 clones with TBP. Yeast containing the indicated bait and prey plasmids (listed at *right*) were grown on SC-L-W (*top panel*), which selects for the presence of the bait and prey plasmids, and SC-L-W-H (*middle* and *bottom panels*), which selects for the interaction between bait and prey. In sectors *1–10* and *13–16*, all baits were expressed from the MP34 plasmid and all truncated PIAS1 preys expressing the indicated amino acids were expressed from the pGADT7^{SN} plasmid. Clones in sectors *11* and *12* were positive and negative controls, respectively, supplied with the Matchmaker III system. All 4 isolates of PIAS1$_{6–491}$ (clone 1) grew at a slower rate and lower density compared with the more robustly growing clones in sectors *2* and *3*. Therefore, the *bottom panel* shows the same plate photographed above that had been allowed to grow for an additional 3 days to reveal growth of the more slowly growing clone in sector *1*. Sectors *7* and *9* were the longest and shortest PIAS1 clones, respectively, that were isolated directly from yeast two-hybrid screens

using TBP-FL as bait. Sectors *8* and *10* were controls confirming that $PIAS1_{453–651}$ and $PIAS1_{6–651}$, respectively, were not autoactive. The absence of growth of clones 4–6 and 13 on SC-L-W-H suggested that these clones lacked the TBP interaction domain. Four independent clones of all PIAS1 truncations were tested and each gave the same result; one representative set is shown. None of the truncated PIAS1 fragments tested were able to grow when supplied with the empty bait plasmid (not shown), confirming that the prey plasmids were not auto-active. *B*, deletion analysis of the TBP interaction domain of PIAS1. The full-length PIAS1 protein is represented at the *top*, including the location of known domains. PIAS1 amino acids expressed by each cDNA clone in $pGADT7^{SN}$ are shown at the *left*. The six different PIAS1 clones isolated from two-hybrid screens as TBP-interacting proteins are indicated. Approximate relative two-hybrid growth rates with the TBP-FL bait vector are represented by ++, +, and −, at the *right*. Results suggested that the TBP-interacting region of PIAS1 was between amino acids 452 and 492, which included the Ser/Ac domain.





sector	bait	prey
1	TBP-C135-263	PIAS16-651
2	TBP-C135-210	PIAS16-651
3	TBP-C201-316	PIAS16-651
4	TBP-C251-316	PIAS16-651
5	TBP-C160-263	PIAS16-651
6	TBP-C135-263	empty vector
7	TBP-C135-210	empty vector
8	TBP-C201-316	empty vector
9	TBP-C251-316	empty vector
10	TBP-C160-263	empty vector

FIGURE 5. PIAS1 and PIAS3 interact with the TBP_{CORE}

A, TBP-FL, -N, and -C baits were tested for autoactivation and for two-hybrid interactions with short (amino acids 453–651) and long (amino acids 6–651) clones of PIAS1 and with a clone of PIAS3 (amino acids 325–628). As controls, interactions of the TBP-FL and the N- and C-terminal TBP subdomains with TFIIA, a known TBP_{CORE} interactor (40,41), are shown. Serial dilutions of yeast containing the plasmid combinations indicated were spotted onto SC-L-W (*left column*) and SC-L-W-H (*right column*) plates. Growth of clones on the SC-L-W plate verified that yeast contained both the bait and prey plasmid. In the *top right panel*, the absence of growth indicated that none of the TBP bait clones were autoactive when supplied with the empty prey plasmid. The *bottom right panel* is a control showing that TFIIA interacted with

TBP-FL and TBP-C, but not with TBP-N. *Right panels 2–4* show that all PIAS clones interact with both TBP-FL and TBP-C, but not with TBP-N. The *bottom rows* of *right panels 2*, *3*, *4*, and *5* are autoactivation controls for each prey plasmid, none of which grew on SC-L-W-H, and thus were not autoactive when supplied with the empty bait plasmid. *B*, truncated versions of the TBP_{CORE} were constructed to more accurately identify the subdomain that is required for interaction with $PIAS1_{6–651}$. Only the $TBPC_{135–263}$ protein (sector *1*), encoding approximately the first two-thirds of TBP-C, was able to maintain the interaction with PIAS1. This suggested that an extended conformation of TBP-C is required for the interaction with PIAS1.

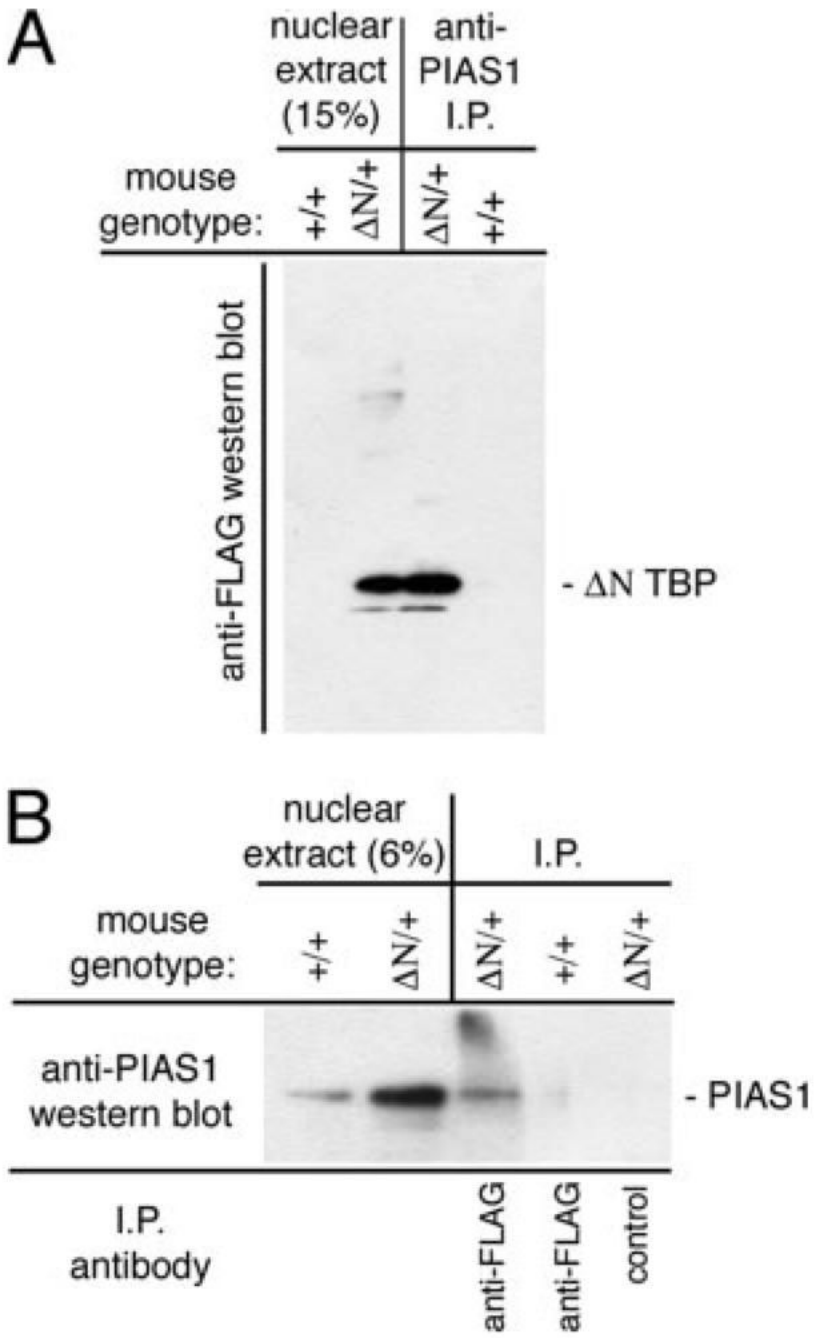


FIGURE 6. Endogenous PIAS/TBP interaction

Co-immunoprecipitation (*IP*) of endogenous PIAS1 and TBP from mouse nuclear extracts were performed with the indicated antibodies on nuclear extracts prepared from livers and spleens harvested from wild-type or $tbp^{\Delta N/+}$ mice. *A*, ΔN-TBP co-immunoprecipitates with PIAS1. Nuclear extracts from wild-type (+/+) or heterozygous (Δ*N*/+) mice were analyzed by Western blotting with anti-FLAG antibody before or after immunoprecipitation with anti-PIAS1 antibody. Results showed that the ΔN/+ nuclear extracts contained a single major anti-FLAG-reactive protein (Δ*N-TBP*) and that this protein co-immunoprecipitated with PIAS1. *B*, PIAS1 co-immunoprecipitates with ΔN-TBP. Nuclear extracts were immunoprecipitated with anti-FLAG or an irrelevant negative control mouse monoclonal antibody and probed

Western blots with goat anti-PIAS1 (C20) antibody. Results showed that a portion of the endogenous PIAS1 co-precipitated with endogenous FLAG-tagged TBP_{CORE} from heterozygous but not wild-type nuclear extracts; the nonspecific antibody did not bring down PIAS1.

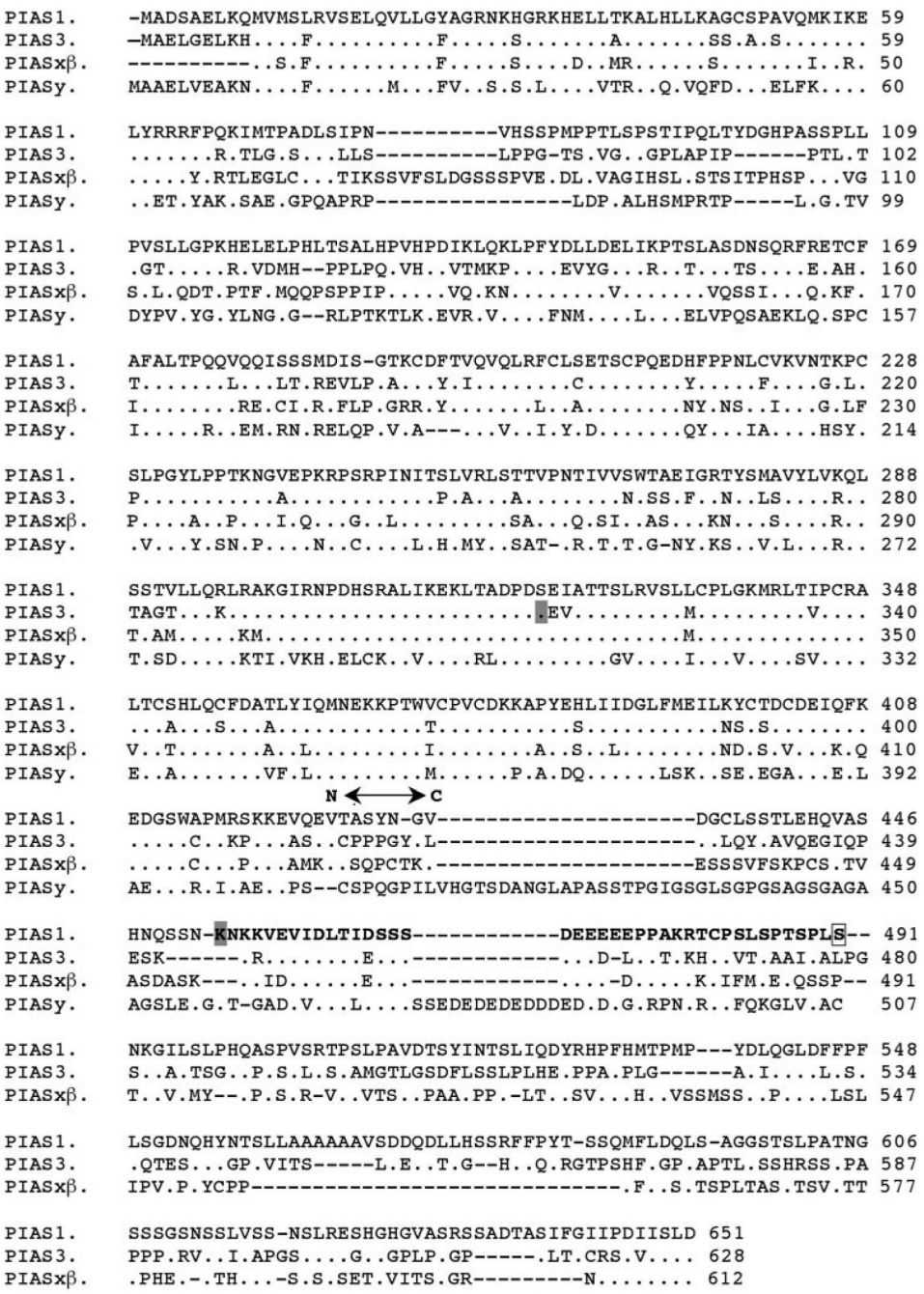
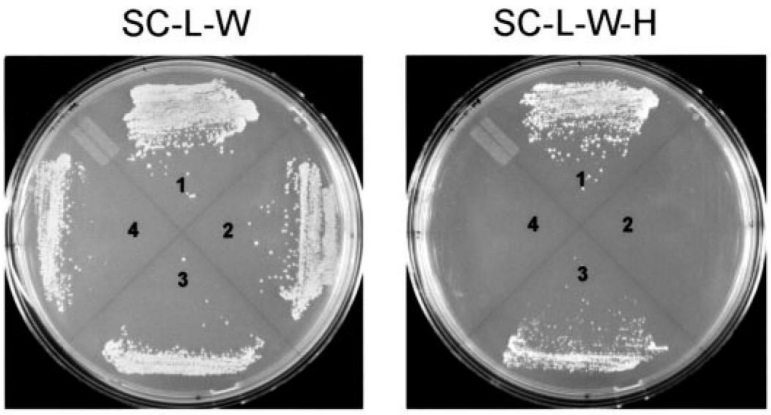


FIGURE 7. Alignment of mouse PIAS1, PIAS3, PIASx*β*, and PIASy proteins

At the *top* is indicated the full amino acid sequence of mouse PIAS1. For other family members, *dots* represent amino acids that are identical to PIAS1. Comparison of the PIAS protein sequences revealed that the deduced TBP-interacting domain of PIAS1 (denoted in *bold*) is highly conserved in PIAS3 and PIASx*β*, with the greatest amino acid identity occurring within the Ser/Ac domain. PIASy contains a similar, albeit more extended Ser/Ac domain (27). *Shaded* amino acids represent the first amino acid encoded by the shortest 5′-truncated PIAS1 clone ($PIAS1_{453–651}$) that interacted with TBP and the single PIAS3 clone ($PIAS3_{325–628}$) that was isolated in yeast two-hybrid screens described above. The *boxed* amino acid in PIAS1 represents the last amino acid encoded by the most 3′-truncated mutant ($PIAS1_{6–491}$) that

interacted with the TBP and defined the C-terminal boundary of the deduced TBP interaction domain of PIAS1.



sector	bait	prey
1	TBP-FL	PIASx
2	empty	PIASx
3	TBP-FL	PIASy
4	empty	PIASy

FIGURE 8. Interaction of PIASx and PIASy with TBP

Two-hybrid assays were performed as in previous figures. PIASx$\beta_{1–612}$ and PIASy$_{1–507}$ interacted with TBP-FL by yeast two-hybrid analysis (sectors *1* and *3*, respectively) and neither was autoactive when co-expressed with the empty bait plasmid (sectors *2* and *4*, respectively).

TABLE 1

Oligonucleotide primer sequences for construction of bait and prey cDNAs

Name	Sequence[a]
TBP-N-start	5′-atc*gtcgac***tatggaccagaacaacagccttcca**-3′
TBP-N-end	5′-tat*gcggccgccc***agagctctcagaagctggtgt**-3′
TBP-C-start	5′-tat*gtcgac*caccatgg**cgagctctggaattgtaccgcag**-3′
TBP-C-end	5′-tat*gcggccgc***gtggtcttcctgaatccctttaa**-3′
TBP-C160-forward primer	5′-at*ctcgag***cattgcacttcgtgcaagaaatgctg**-3′
TBP-C201-forward primer	5′-tat*gtcgac*caccatg**gccaagagtgaagaacaatcc**-3′
TBP-C210-reverse primer	5′-tat*gcggccgc***gctagtctggattgttcttcactc**-3′
TBP-C251-forward primer	5′-tat*gtcgac*caccatg**gtgctgacccaccagcagttc**-3′
TBP-C263-reverse primer	5′-tat*gcggccgc***tctggctcatagctactgaact**-3′
PIASxβ-forward primer	5′-tat*gtcgac*catgaat**atggtttctagttttagggtttc**-3′
PIASxβ-reverse primer	5′-tat*gcggccgc*ctttagtccaaagagatgatgtc-3′
PIASy-forward primer	5′-tat*gtcgac*gctagtggccaag**atggc**-3′
PIASy-reverse primer	5′-tat*gcggccgc***tcagcacgcgggcaccaggcct**-3′
PIAS1-C452-reverse primer	5′-tat*gcggccgc*ctcgaggg**tatttgaggactggttgtggg**-3′
PIAS1-C491-reverse primer	5′-tat*gcggccgc***acttagtggtgacgtaggagacag**-3′
PIAS1-C562-reverse primer	5′-tat*gcggccgc***tagcagggaggtgttgtaatg**-3′
PIAS1-C605-reverse primer	5′-tat*gcggccgc***gttggtggctggcagagatgtgct**-3′
PIAS1-N1-forward primer	5′-tat*gtcgacc***atggcggacagtgcggaactaaagcaaatggttatgagc**-3′
PIAS1-N484-forward primer	5′-tat*gtcgac***cctgtctcctacgtcaccactaagt**-3′
PIAS1-N555-forward primer	5′-tat*gtcgac***gcattacaacacctccctgctag**-3′
PIAS1-N598-forward primer	5′-tat*gtcgac***gagcacatctctgccagccaccaa**-3′
pGADT7-reverse primer	5′-gaaagaaattgagatggtgcac-3′

[a] Endogenous protein-coding sequences are in bold and engineered restriction sites are in italics.