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

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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 co-immunoprecipitated, which indicated that other nuclear proteins were not required for the interaction. Deletion analysis mapped the PIAS-interacting domain of TBP to the conserved TBPCORE and the TBP-interacting domain on PIAS1 to a 39-amino acid C-terminal region. Mammals issue seven known PIAS proteins from four piase genes, piase1, piase3, piase, and piasey, 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, TFIIID, and TFIIIB, which function with RNA polymerase I (RNAPI), RNAPII, and RNAPIII, respectively. RNAPII requires TFIIID 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).

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 TFIIID complex and direct TFIIID to the promoter (6, 7). TBP function can also be up- or down-regulated through interactions with BTF1/MOT1 and the NC2o/ab 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 families of PIAS proteins consists of PIAS1, PIAS3, PIASx, and PIASy proteins (12). The piase genes encode two splice variants, PIASx/H ARIP (androgen receptor-interacting protein-3) (13, 14) and PIASx/Myr1 (Mxs-interacting zinc finger-1) (15), the difference being in their C termini. piase3 and piasey also each encode two isoforms, PIAS3/PIAS3b and PIASy/PIASyE6, as a result of alternative splicing. The PIAS3b isoform contains an insertion of 39 amino acids in its N-terminal region and PIASyE6 lacks exon 16 (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 SAP-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 LXXLL 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/Ala). Within the acidic domain, a SIM (1 interaction motif in 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 PIASy. 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: pGBK7T (BD Bioscience) and MP34 (R. Brazas, Duke University). pGBK7T 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 bearing inserts; the placental library contained 10 mg/ml sheared denatured salmon sperm DNA, and 120 μg/ml of 10 mg/ml sheared denatured salmon sperm DNA, and 120 μg/ml 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 bearing inserts; the placental library contained 10 mg/ml sheared denatured salmon sperm DNA, and 120 μg/ml of the bait (Fig. 1A).

**TABLE 1**

Oligonucleotide primer sequences for construction of bait and prey cDNAs

| Name                        | Sequence*                                                                 |
|------------------------------|---------------------------------------------------------------------------|
| TBP-N-start                  | 5'-ttgttttggtttttggtttttc-3'                                              |
| TBP-N-end                    | 5'-ttgttttggtttttggtttttc-3'                                              |
| TBP-C-start                  | 5'-ttgttttggtttttggtttttc-3'                                              |
| TBP-C-end                    | 5'-ttgttttggtttttggtttttc-3'                                              |
| TBP-C160-forward primer     | 5'-tatagtttttggtttttggtttttc-3'                                          |
| TBP-C201-forward primer     | 5'-tatagtttttggtttttggtttttc-3'                                          |
| TBP-C251-forward primer     | 5'-tatagtttttggtttttggtttttc-3'                                          |
| TBP-C210-reverse primer     | 5'-tatagtttttggtttttggtttttc-3'                                          |
| TBP-C263-reverse primer     | 5'-tatagtttttggtttttggtttttc-3'                                          |
| PIASSy-forward primer       | 5'-tatagtttttggtttttggtttttc-3'                                          |
| PIASSy-reverse primer       | 5'-tatagtttttggtttttggtttttc-3'                                          |
| PIAS1-C452-reverse primer   | 5'-tatagtttttggtttttggtttttc-3'                                          |
| PIAS1-C491-reverse primer   | 5'-tatagtttttggtttttggtttttc-3'                                          |
| PIAS1-C562-reverse primer   | 5'-tatagtttttggtttttggtttttc-3'                                          |
| PIAS1-C605-reverse primer   | 5'-tatagtttttggtttttggtttttc-3'                                          |
| PIAS1-N1-forward primer     | 5'-tatagtttttggtttttggtttttc-3'                                          |
| PIAS1-N844-forward primer   | 5'-tatagtttttggtttttggtttttc-3'                                          |
| PIAS1-N555-forward primer   | 5'-atatagtttttggtttttggtttttc-3'                                         |
| PIAS1-N598-forward primer   | 5'-atatagtttttggtttttggtttttc-3'                                         |
| pGAD7T-reverse primer       | 5'-atatagtttttggtttttggtttttc-3'                                         |

* Endogenous protein-coding sequences are in bold and engineered restriction sites are in italics.

Oligo-(dT)-primed cDNA prey libraries were constructed and inserted into the pGAD7T-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 placenta 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'-Sal and 3' NotI overhangs. cDNAs were ligated into SalI/NotI-digested pGAD7T-SN. The whole pregnant uteri library contained ~3.0 × 106 independent recombinants with 82% bearing insertions; the placental library contained ~2.6 × 106 independent recombinants with 95% bearing insertions. 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 yeast reporter (Table 1). TBP bait constructs were 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/potassium/ adenine/dextrose at 5 × 107 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 × 109 yeast/ml. Yeast were collected by centrifugation

3 Animal protocols were approved by the Montana State University Institutional Animal Care and Use Committee.
PIAS/TBP Interaction

Two-hybrid screens were performed on SC medium lacking uracil, tryptophan, and histidine (SC-L-W-H). Both two-hybrid screens used TBP-FL as bait to screen either the whole pregnant uterus (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-L-W-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-a-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 pGBK7T7 prey plasmid (autoduction 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 pGBK7T7 + 1 bait plasmid (for TBP deletions) or pGBK7T7SN prey plasmid (for full-length PIASxβ, PIASy, and truncated PIAS1 mutants). Reverse transcriptase-PCR amplification-based cloning of PIASyβ and PIASy was used (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% (v/v) tissue, 0.5% (v/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 motor-driven 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 resuspended in 10 mM HEPES, pH 7.6, 100 mM KCl, 0.1% glycerol, 0.1 mM EDTA, 3 mM MgCl2, 100 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 (NH4)2SO4 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 (NH4)2SO4 and incubating with gentle mixing for 1 h after solid dissolved. Proteins were collected by centrifugation for 1 h at 40,000 × g at 4 °C, in a TI50 or TI60 rotor, and were resuspended in dialysis buffer containing 10 μM PMSF.
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 upstream of TBP. TBP-C was expressed from pGBK7, which fused the Gal4 DNA binding domain downstream of TBP. N and C designate the vertebrate-specific N terminus and the pan- eukaryotic C terminus of TBP (TBPcore), respectively. B, two prey libraries were constructed and inserted into pGADT7, 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 HindIII-cut phospho-ate-buffered saline plasmid markers; lane “x” 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 uter library for interacting proteins. TBP-interacting prey library clones were subsequently identified by sequencing.

0.1X 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-immuno-precipitations—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, Pias1, Pias3, BRF1, and ZFP523 were inserted into pCMV-Myc (BD Bioscience), which fused the c-Myc epitope tag to the N terminus of TBP. 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 modified 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 Novapector (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. After incubation, 2 ml of DMEM containing 20% newborn calf serum (Invitrogen), 4% fetal bovine serum (HyClone, Logan, UT), and 2X 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-pestatin-aprotinin, 0.15 mM NaVO₃, 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 into 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 antisera 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 1X 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 MgCl₂, 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-agarose 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 NaVO₃), 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 0.1 M. In for 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.

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 plasmid combinations that were contained within each yeast clone are indicated. Sector SC-L-W-H action may be weaker than the TFIIA/TBP interaction because growth of yeast on the sector grew well under all conditions tested. In sector H-A, Sector 12264 between TFIIA and TBP, and served as a positive control because TFIIA is known to interact with TBP (40, 41). Sector SC-L-W-H control supplied with the Matchmaker III system encoding non-interacting bait and prey proteins. Sector 3 was a negative 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 sector. SC-L-W-H-H showed the interaction of PIAS1 and PIAS3, respectively, with TBP. Both the TFIIA-TBP interaction was scored as strongly positive. As a positive control, a BRF1 clone isolated from our yeast two-hybrid screens using TFIIA-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 sector SC-L-W-H + 2.5 mM 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.
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. mTBP-FL grew on SC-L-W-H medium in the presence of an empty bait plasmid (not shown), confirming that the prey plasmids 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 autoactive. 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 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.

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), ~2.5 × 10⁶ 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 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 ~0.6 × 10⁶ primary transformants, ~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 ZFPS23, the mouse homologue of a recently identified novel TBP interacting protein, z2NF76 (11). The remaining six clones were all unique, although no obvious physiological connection to TBP was evident. The interac-
tion of TBP-C is required for the interaction with PIAS1. This suggested that an extended conformation of TBP-C is required for interaction with PIAS1.

**FIGURE 5.** PIAS/TBP Interaction

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 to 651, 359 to 651, 430 to 651, and 439 to 651 of PIAS1 (Fig. 4B). The single PIAS3 clone encoded PIAS3 amino acids

**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^{−/+} mice. A, ΔN-TBP co-immunoprecipitates with PIAS1. Nuclear extracts from wild-type (+/+ or heterozygous (ΔN/) 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 (ΔN-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-immunoprecipitated with endogenous FLAG-tagged TBP_{core} from heterozygous but not wild-type nuclear extracts; the nonspecific antibody did not bring down PIAS1.
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 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 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. Sections 7–10 are TBP interaction and autoactivation controls for PIAS1_{453–651} and PIAS3_{325–628}, 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).

FIGURE 7. Alignment of mouse PIAS1, PIAS3, PIASx, 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, with the greatest amino acid identity occurring within the Ser/Asp domain. PIASy contains a similar, albeit more extended Ser/Asp 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.
Our results indicated that PIAS1 could interact with the TBP

type and heterozygous (34, 35).

We also constructed truncated TBP CORE mutants to further define
the domain of TBP that interacted with PIAS1. Only one of our mutants,
TBP-C135–263, encoding approximately the first two-thirds of the TBP-CORE,
allowed growth with PIAS16–651 (Fig. 5B, 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 nor-
mal 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


tag located on the loci of mouse 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 mem-
bers. 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 and PIASy were isolated by reverse
transcriptase-PCR and were inserted into the two-hybrid prey vector
(pGADT7SN) 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
co-expressed with the empty bait plasmid (sectors 2 and 4, respectively).

FIGURE 8. Interaction of PIASx and PIASy with TBP. Two-hybrid assays were per-
formed as in previous figures. PIASx16–612 and PIASy1–507 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).

DISCUSSION

TBP is a central player in transcription initiation whose activity is
controlled by interactions with activators, repressors, and other tran-
scriptional 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 tran-
scription machinery.

The interaction between TBP and PIAS proteins involves the con-
served 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 char-
acterized, the C-terminal region is less well understood (21). The C-ter-
ninal regions of individual PIAS proteins have been shown to mediate
protein–protein interactions with some regulators (21). However, align-
ment 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 inter-
acting 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,
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.

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REFERENCES
1. Cormack, B. P., and Struhl, K. (1992) Cell 69, 685–696
2. Hernandez, N. (1993) Genes Dev. 7, 1291–1308
3. Pugh, B. F. (2000) Gene (Amst.) 255, 1–14
4. Mittal, V., Cleary, M. A., Herr, W., and Hernandez, N. (1996) Mol. Cell. Biol. 16, 1955–1965
5. Timmers, H. T., and Tora, L. (2005) Trends Biochem. Sci. 30, 7–10
6. Albright, S. R., and Tjian, R. (2000) Gene (Amst.) 242, 1–13
7. Naar, A. M., Lemon, B. D., and Tjian, R. (2001) Annu. Rev. Biochem. 70, 475–501
8. Kleiman, M. P., Pereira, L. A., van Zeeburg, H. J., Gilfillan, S., Meisterernst, M., and Timmers, H. T. (2004) Mol. Cell. Biol. 24, 10072–10082
9. Fields, S., and Johnston, M. (1993) Yeast Methods for Analyzing Proteins from Other Organisms, Academic Press, San Diego, CA
10. Schmidt, E. E., and Schibler, U. (1995) J. Biol. Chem. 268, 6168–6174
11. Hofman, C. S., and Winston, F. (1987) Gene (Amst.) 57, 267–272
12. Smutka, K., Ratoj, K., Uchimoto, T., Muramatsu, M., Nabeshima, Y., Muramatsu, M., and Timmers, H. T. (2003) J. Biol. Chem. 278, 13137–13146
13. Mylinska, E., Krol, A., and Carbon, P. (1998) J. Biol. Chem. 273, 21998–22006
14. Zheng, G., and Yang, Y. C. (2004) J. Biol. Chem. 279, 42410–42421
15. Schmidt, D., and Muller, S. (2003) Cell Mol. Life Sci. 60, 2561–2574
16. Moilanen, A. M., Karvonen, U., Poukka, H., Yan, W., Toppuri, J., Janne, O. A., and Palvimo, J. J. (1999) J. Biol. Chem. 274, 3700–3704
17. Sarna, S., Koch, M., and White, R. A. (2000) J. Mol. Neurosci. 14, 107–121
18. Liu, B., Gross, M., ten Hoeve, J., and Shuai, K. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 3203–3207
19. Liu, B., Liao, J., Rao, X., Kushner, S. A., Chung, C. D., Chang, D. D., and Shuai, K. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 10626–10631
20. Johnson, E. S. (2004) Annu. Rev. Biochem. 73, 355–382
21. Swope, D. L., Mueller, C. L., and Chrivia, J. C. (1996) Science 272, 7219–7225
22. Schmidt, E. E., Bondareva, A. A., Radke, J. R., and Capecchi, M. R. (2003) J. Biol. Chem. 278, 6168–6174
23. Schibler, U. (1996) Biochem. Biophys. Res. Commun. 225, 275–280
24. Schmidt, D., and Muller, S. (2002) FEBS Lett. 554, 111–118
25. Schibler, U. (1995) Cell 128, 467–483
26. Schmidt, E. E., and Schibler, U. (1995) J. Biol. Chem. 270, 10593–10610
27. Schmidt, E. E., Bondareva, A. A., Barnett, S., Capecchi, M. R., and Schmidt, E. E. (2002) Cell 110, 43–54
28. Schibler, U. (1996) Biochem. Biophys. Res. Commun. 225, 75–80
29. Sumita, K., Makino, Y., Katoh, K., Kishimoto, T., Muramatsu, M., Nabeshima, Y., Muramatsu, M., and Timmers, H. T. (2003) Nucleic Acids Res. 21, 2769
30. Durand, J., Bernstein, R., and Roeder, R. G. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 3313–3317
31. Geiger, J. H., Hahn, S., Lee, S., and Sigler, P. B. (1996) Science 272, 830–836
32. Tang, H., Sun, X., Reinberg, D., and Ehrlich, R. H. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1119–1124
33. Wang, Z., and Roeder, R. G. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7026–7030
34. Bondareva, A. A., and Schmidt, E. E. (2003) Mol. Biol. Evol. 20, 1932–1939
35. Geiger, J. H., Sun, X., Reinberg, D., and Ehrlich, R. H. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1119–1124
36. Wang, Z., and Roeder, R. G. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7026–7030
37. Bondareva, A. A., and Schmidt, E. E. (2003) Mol. Biol. Evol. 20, 1932–1939
38. Dyer, D. L., Mueller, C. L., and Chrivia, J. C. (1996) J. Biol. Chem. 271, 28138–28145
39. Geiger, J. H., Hahn, S., Lee, S., and Sigler, P. B. (1996) Science 272, 830–836
40. Tang, H., Sun, X., Reinberg, D., and Ehrlich, R. H. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1119–1124
41. Wang, Z., and Roeder, R. G. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7026–7030
42. Bondareva, A. A., and Schmidt, E. E. (2003) Mol. Biol. Evol. 20, 1932–1939
43. Dyer, D. L., Mueller, C. L., and Chrivia, J. C. (1996) J. Biol. Chem. 271, 28138–28145
44. Schmidt, D., and Muller, S. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 2872–2877
45. Munarriz, E., Barcaroli, D., Stephanou, A., Townsend, P. A., Maisse, C., Terrinoni, A., Neale, M. H., Martin, S. J., Latchman, D. S., Knight, R. A., Melino, G., and De Laurenzi, V. (2004) Mol. Cell. Biol. 24, 10593–10610