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Protein Inhibitors of Activated STAT Resemble Scaffold Attachment Factors and Function as Interacting Nuclear Receptor Coregulators*

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Jiann-An Tan‡, Susan H. Hall‡, Katherine G. Hamil‡, Gail Grossman§, Peter Petrusz§, and Frank S. French‡¶

From the Laboratories for Reproductive Biology, ‡Departments of Pediatrics and §Cell and Developmental Biology, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599-7500

Protein inhibitor of activated STAT1 (PIAS1) functions as a nuclear receptor coregulator and is expressed in several cell types of human testis. However, the mechanism of PIAS1 coregulation is unknown. We report here that PIAS1 has characteristics of a scaffold attachment protein. PIAS1 localized in nuclei in a speckled pattern and bound A-T-rich double-stranded DNA, a function of scaffold attachment proteins in chromatin regions of active transcription. DNA binding was dependent on a 35-amino acid sequence conserved among members of the PIAS family and in scaffold attachment proteins. The PIAS family also bound the androgen receptor DNA binding domain, and binding required the second zinc finger of this domain. PIAS1 contained an intrinsic activation domain but had bi-directional effects on androgen receptor transactivation; lower expression levels inhibited and higher levels increased transactivation in CV1 cells. Other PIAS family members also had dose-dependent effects on transactivation, but they were in a direction opposite to those of PIAS1. When coexpressed with PIAS1, other PIAS family members counteracted PIAS1 coregulation of androgen receptor transactivation. The interaction of PIAS1 with other members of the PIAS family suggests a transcription coregulatory mechanism involving a multicomponent PIAS nuclear scaffold.

Androgen activation of the androgen receptor (AR)[1] is essential for male sexual development and the initiation and maintenance of spermatogenesis (1–3). AR is a member of the steroid receptor subgroup of the greater family of nuclear receptors that function as transcription factors (4, 5). These receptors have conserved DNA and ligand binding domains that conform to similar three-dimensional structures (6–12), whereas their N-terminal domains are characterized by marked sequence variation (13, 14). Nuclear receptors bind DNA as homo- or heterodimers (6, 15). AR homodimerization is enhanced markedly in the presence of androgen-response element (ARE) DNA and is required for formation of a stable AR·ARE complex (16, 17). Dimerization of AR occurs through a DNA binding domain interface and antiparallel interactions between the N- and C-terminal domains (17, 18). Nuclear receptors regulate the transcription rate of RNA through interactions with coactivators, corepressors, and the general transcription machinery (19–24). Specific genes are regulated through receptor interactions with coregulators and other chromatin remodeling factors (25–35) that control the accessibility of nucleosomal DNA to the transcription complex.

Signal transducers and activators of transcription (STAT) are so named because they serve as signal transducers in the cytoplasm and as activators of gene transcription in the nucleus. PIAS1 was isolated by Liu *et al.* (36) from a human JY112 B cell cDNA library and by Tan *et al.* (37) from a HeLa cell library using yeast two-hybrid screening for STAT1 and AR interacting proteins, respectively. PIAS1 was shown to bind STAT1 and inhibit STAT1 binding to its consensus response element. PIAS1 inhibition of activated STAT1 signaling was demonstrated in cotransfection assays with interferon γ-stimulated 293 cells using a *STAT1* reporter gene (36). In an earlier study (37) we reported that PIAS1 is a transcriptional coactivator with AR and GR but a repressor with progesterone receptor. PIAS1 is expressed predominantly in testis including cell types that express AR and mediate the actions of androgen on spermatogenesis. In addition to PIAS1 that inhibits STAT1, another member of the PIAS family, PIAS3, has been shown to be an inhibitor of STAT3 signaling. PIAS3 mRNA was also abundant in human testis, but unlike PIAS1, it was expressed at similar levels in other organs (38). Other known members of the human PIAS family include PIASxα, PIASxβ, and PIASy. A mutant PIASxβ with deletion of amino acids 1–133 interacted with a homeobox DNA-binding protein, Msx2. This mutant protein, referred to as Miz1, had sequence-specific DNA binding activity and enhanced the DNA binding of Msx2 (36, 39). PIASxα (ARIP3) was also isolated as an AR-interacting protein by two-hybrid screening of a mouse embryo library and was found to be highly expressed in rat testis (40).

Here we report that PIAS family members have characteristics of nuclear scaffold attachment factors (SAF). PIAS family members were bi-directional transcriptional coregulators with AR. In cells where there is expression of more than one family member, our studies suggest that the coregulatory effects of

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¶ To whom correspondence should be addressed: Laboratories for Reproductive Biology, University of North Carolina School of Medicine, Chapel Hill, NC 27599-7500. Tel.: 919-966-0930; Fax: 919-966-2203; E-mail: fsfrench@med.unc.edu.

[1] The abbreviations used are: AR, androgen receptor; STAT, signal transducers and activators of transcription; aa, amino acid; ARE, androgen-response element; GST, glutathione *S*-transferase; MMTV, mouse mammary tumor virus; PSA, prostate-specific antigen; DHT, dihydrotestosterone; SAF, scaffold attachment factors; CBP, CREB-binding protein; E3, ubiquitin-protein isopeptide ligase; SAP, SAF-A/B, Acinus, PIAS domain; SUMO, small ubiquitin-related modifier.

PIAS1 are modulated by interactions with other members of the PIAS family.

EXPERIMENTAL PROCEDURES

Plasmids—Full-length PIAS1 sense and antisense vectors and the mutant PIAS1delF vector with deletion of amino acids 341–536 were described previously by Tan *et al.* (37). Full-length PIASxα, PIASxβ, PIASy, and mouse PIAS3 (mPIAS3) were recovered from digestions of pFLAG-PIASxα, -xβ, -y, and mPIAS3 and cloned into pSG5 to create pSG5-PIASxα, -xβ, -y, and mPIAS3. pFLAG-PIAS vectors were provided by Ke Shuai, UCLA (36). *Bam*HI-Klenow filled in fragments of PIASxα and -xβ derived from pSG-PIASxα and -xβ were cloned into the *Sma*I site of pBDGalCAM to create yeast Gal4 DNA binding domain vectors, pBDGalCAM-PIASxα and -xβ. The same fragments were cloned into the filled in *Xho*I site of pGADGH to create yeast Gal4 activation domain vectors pGADGH-PIASxα and -xβ. A specific probe for PIAS1 was generated by PCR of pGADGH-PIAS1 (37) using 5′ primer (GGTCTAGAGTCTTCCACATCAAGC) and 3′ primer (CAGATCGAATGAACTTGGGAATTC). The PCR product was digested with *Xba*I-*Eco*RI and cloned into the same sites of pBKCMV (Stratagene).

Probes specific for PIASxα and -xβ mRNAs were generated by PCR with 5′ primer (GCTCTAGAGCATGTCATCAGATTTGCCAGG) and 3′ primer (CCACAACTAGAATGCAGTG) using pSG5-PIASxα and -xβ as templates. A probe specific for PIASy was generated by PCR with 5′ primer (GCTCTAGAAGGAGCGCAGCTGCA) and 3′ primer (CCACAACTAGAATGCAGTG) with pSG5-PIASy as template. PCR products were digested with *Xba*I-*Bgl*II and cloned into pBKCMV *Xba*I-*Bam*HI site. To create the vector pGBT-PIAS1 amino acids 7–651 of PIAS1 were excised with *Sma*I-*Xho*I and cloned into pGBT8. pGEX-PIAS1-(1–135) and pGEX-PIAS1delSAP (1–166 del 11–45) were constructed by PCR of templates pSG5-PIAS1 and pSG5-PIAS1delSAP with 5′ primer CTCTGAGTCCAAACCGGGCCCCTCTGC and 3′ primer TCTCGAAAGCGCTGACTGTTGTCTGATGC. For cell-free binding assays GST-PIAS1 (amino acids 7–651) was created by digesting pGADGH-PIAS1 (37) with *Spe*I-*Xho*I and cloning the purified PIAS1 into the *Xba*I-*Xho*I site of pGEX-KG. GST-AR (amino acids 544–634) was constructed by PCR of human pCMVhAR and cloning into pGEX-2T *Bam*HI-*Eco*RI sites. All constructs were confirmed by automatic sequencing using a Perkin Elmer model 377 DNA sequencer.

The probasin-luciferase reporter was provided by Dr. R. J. Matusik, Department of Urologic Surgery, Vanderbilt University, and the prostate-specific antigen-luciferase reporter by Dr. M. D. Sadar, British Columbia Cancer Agency, Vancouver, British Columbia, Canada.

Affinity Matrix Assay of PIAS1 Binding to A-T-rich DNA—GST fusion proteins were expressed in *Escherichia coli* BL21 cells as described (41). Scaffold attachment region-like A/T-rich oligonucleotides (AATTCAGAAAATAATAAAATAAAACTAGCTATTTTATATTTTTTC and AATTGAAAAAATATAAAATAGCTAGTTTTATTTTATTATTTTCTG) were synthesized and annealed by heating to 70 °C and cooling slowly to room temperature. Annealed oligonucleotide or *E. coli* DNA was added in excess to glutathione-Sepharose-bound GST or GST-PIAS1 proteins and incubated for 1 h at 4 °C. The mixture was washed several times in 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, and ^{32}P-labeled double-stranded oligonucleotide (~150,000 cpm) was added and the incubation continued for 1 h at 4 °C. After several washes in the same buffer, the samples were counted in a liquid scintillation counter.

Immunohistochemistry—COS7 cells were cultured in two chamber glass slides and transfected with pSG5-PIAS1 as described earlier for AR (42). Immunostaining was performed (43) using a polyclonal antibody raised in rabbit against a glutathione transferase-PIAS1 fusion protein containing 102 C-terminal amino acids (549–650), provided by J. Liao and K. Shuai, UCLA (37).

Yeast Liquid β-Galactosidase Assay—Yeast Y190 cells were used for the liquid β-galactosidase assay. For assay of intrinsic transcriptional activity of PIAS family members, Y190 yeast cells were transformed with the Gal4 DNA binding domain vector (pBDGalCAM) containing PIAS1, the PIAS1 mutant PIAS1delF, PIASxα, or PIASxβ. For two-hybrid protein-protein interactions of PIAS1 with PIASxα or PIASxβ, Y190 cells transformed with the pGBT-PIAS1 and the Gal4 activation domain vector (pGADGH) containing PIASxα or PIASxβ were incubated at 30 °C in 2 ml of selective medium without Trp and Leu. In the case of yeast transformed with the Gal4 DNA binding domain vector alone, medium lacked only Trp and if transformed with the Gal4 activation domain vector alone lacked only Leu. After incubation for 20 h, YPD medium (8 ml) was added, and incubations were continued for 3–4 h at the same temperature. The liquid β-galactosidase assay was performed according to the protocol of CLONTECH Laboratories Inc., Palo Alto, CA.

Transient Cotransfection Assay—Cotransfection assays were performed in triplicate as described (37). In brief, monkey kidney CV1 cells were transfected with the mouse mammary tumor virus-long terminal repeat-luciferase reporter vector, MMTV-luciferase (2.5 μg), prostate-specific antigen, PSA-luciferase (2.5 μg) or probasin-luciferase reporter (5 μg), human androgen receptor (pSG5-hAR) 0.1 μg, and various amounts of pSG5 expression vectors containing PIAS cDNAs. To control for possible DNA effects, CV1 cells were transfected with or without equimolar amounts of the empty pSG5 vector, pSG5-PIAS1 antisense vector, or pSG5-BTG1 that expresses the B cell translocation gene 1 (44). Cells were grown in 6-cm culture dishes and transfected by the $CaPO_4$ method when 70–80% confluent. After 15% glycerol shock for 4 min, the cells were incubated in Dulbecco's modified Eagle's medium-H without phenol red and serum in the presence or absence of 0.1 nM dihydrotestosterone (DHT) for 40 h. Cells were harvested in lysis buffer (Ligand Pharmaceuticals Inc., San Diego, CA), and luciferase activity was measured in a luminometer. Luciferase activity was expressed as mean ± S.D. light units of three replicates and as fold increase in the presence of hormone over background in the absence of hormone. Assay results in each figure are representative of three or more experiments.

In Situ Hybridization—pBKCMV-PIAS1, -xα, -xβ, and -y were used for *in situ* hybridization analysis. Antisense PIAS1-(1587–2101), PIASxα-(1628–1719), PIASxβ-(1628–1866), and PIASy-(1232–1533) RNAs were synthesized and labeled with digoxigenin using the Roche Molecular Biochemicals RNA labeling kit, and *in situ* hybridization of mouse testis was performed as described (45).

AR Binding of ^{35}S-PIAS Proteins—pSG5-PIAS1, -xα, -xβ, and -y (36) vectors were used as DNA templates for *in vitro* synthesis of labeled protein by coupled *in vitro* transcription-translation. Glutathione *S*-transferase (GST)-AR binding assays were performed as described (46). In brief, the above cDNA vectors were incubated with [^{35}S]methionine and reticulocyte lysate from the TnT T7 Quick-coupled Transcription/Translation System kit (Promega), and the labeled proteins were incubated with GST-AR DNA binding domain (amino acids 544–634) glutathione-Sepharose affinity matrix. After incubation and extensive washes, labeled proteins were eluted by boiling in SDS buffer and separated by SDS-PAGE, gel-dried, and exposed to Kodak x-ray film.

PIAS Protein Interaction—Recombinant PIAS family members synthesized and labeled with [^{35}S]methionine as described above were incubated with GST-PIAS1 (amino acids 7–651) coupled to glutathione-Sepharose. The gel was washed several times, and labeled proteins were eluted and processed as above.

Northern Hybridization—Total RNA was extracted from testes of rats at different ages by a modification of the method of Chirgwin *et al.* (47) and Northern hybridizations performed as described (37) using the DNA probes specific for PIAS1, PIASxα, PIASxβ, and PIASy as indicated above. Ribosomal RNA was stained with methylene blue to compare the amounts of sample loaded in each lane. Sample loadings were also checked by hybridization of 18 S rRNA. The cDNA for 18 S rRNA was obtained from Ambion (Austin, TX) and labeled with ^{32}P using a random priming kit (Promega, Madison, WI).

RESULTS

PIAS Family Members Contain a Conserved Sequence Found in Proteins That Bind Scaffold Attachment Region DNA—A 35-amino acid N-terminal sequence (aa 11–45) common to PIAS family members and a number of other eukaryotic proteins is referred to as the scaffold attachment factor, SAF box (48), or SAF-A/B, Acinus, PIAS (SAP) domain (49). Secondary structure modeling predicts the sequence forms two amphipathic helices (Fig. 1) with homology to helices 1 and 2 of homeodomain proteins that are known to fold into a hook-like structure with two α-helices separated by a turn (48). Within the conserved sequence is a bipartite distribution of hydrophobic and polar amino acids separated by a region that contains an invariant glycine (49). In contrast to homeodomains that contain three α-helices and bind to strictly defined sequences in the major groove of DNA, SAF box binding to DNA occurs through a cooperative binding mode that recognizes scaffold attachment region DNA through minor groove interactions with multiple clustered adenine (A) tracts (48). In different molecules the SAF box/SAP domain is linked to a diverse set of

FIG. 1. **PIAS family members contain an SAF box also referred to as a SAP domain.** Alignment of conserved sequences in PIAS1, PIASxα, PIASxβ, and PIASy amino acids 11–45 with those of SAF-A (aa 8–42) and SAF-B (aa 31–65), two well characterized scaffold attachment factors. Amphipathic helices within the SAF box sequence are indicated by the enlarged regions (*green*) in a linear diagram at the *top*. Highly conserved amino acids are in *red*.

domains, several of which are known to be involved in pre-mRNA processing (49).

PIAS1 Binds Double-stranded A-T-rich DNA—To learn whether PIAS1 has the DNA binding properties of a scaffold attachment region binding protein, we tested its binding to A-T-rich DNA using an affinity matrix assay with the protein attached to Sepharose beads (Fig. 2). GST or GST-PIAS1 (aa 1–135) were coupled to glutathione-Sepharose beads and incubated with ^{32}P-labeled A/T-rich oligonucleotide using a batch method. Either unlabeled A-T-rich oligonucleotide or *E. coli* DNA was used in competition with the ^{32}P-labeled A-T-rich oligonucleotide to demonstrate specific binding. It has been demonstrated previously (48, 50, 51) that scaffold attachment proteins do not bind *E. coli* DNA. Radioactive A-T-rich DNA bound to GST-PIAS1-Sepharose was more than 300-fold higher than that bound to the GST-Sepharose control, and binding was inhibited by cold-A-T-rich DNA but not by *E. coli* DNA. Deletion of the SAF box/SAP domain (GST-PIAS1 aa 1–166 del 11–45) abolished the binding to A-T-rich DNA demonstrating the potential role of this domain in PIAS1 binding to scaffold attachment region DNA.

PIAS1 Is Localized in Nuclei in a Punctate Distribution—Immunostaining of PIAS1 transfected into COS cells revealed a speckled pattern of localization in nuclei (Fig. 3) similar to that of the scaffold attachment proteins SAF-B (52) and SAF-A (heterogeneous nuclear ribonucleoprotein U) (53). Under the microscope this same pattern of PIAS1 staining in nuclei could be visualized in testis tissue sections by fine adjustment of focusing, but the speckling was not apparent in photographs.

PIAS Family Members Have Negative and Positive Effects on AR Transactivation—Coregulatory effects of the PIAS family with DHT-dependent AR transactivation were analyzed in CV1 cell cotransfection assays with three different luciferase reporter genes, the mouse mammary tumor virus-long terminal repeat (MMTV), the rat probasin gene 5′-flanking region (nucleotides −426 to +28), and the human prostate-specific antigen gene (PSA) (nucleotides −630 to +12). With the MMTV-Luc reporter gene PIAS1 had bi-directional effects on AR-induced transcriptional activity (Fig. 4, *top panel*). At lower amounts (0.01 and 0.05 μg) transfected PIAS1 inhibited DHT-dependent AR transactivation relative to equivalent amounts of the antisense PIAS1 control. At a higher amount (0.5 μg) PIAS1 stimulated a 3-fold increase in DHT-dependent AR transactivation. Similar results were obtained using the probasin-Luc reporter gene (Fig. 4, *middle panel*). There was inhibition with 0.01 μg of PIAS1 and enhancement with 0.5 μg. PIAS1 (0.5 μg) also increased DHT-dependent AR transactivation of the PSA-Luc reporter above the level obtained with equivalent amounts of pSG5PIAS1 antisense control vector or the PIAS1delF mutant control that was shown previously (37) to lack coactivator function (Fig. 4, *bottom*). We were unable to test the inhibitory amounts of PIAS1 (0.01–0.1 μg) with the PSA-LUC reporter because of its lower responsiveness to AR-induced transactivation.

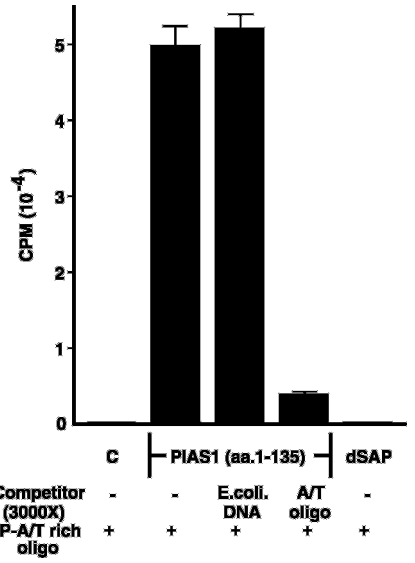

FIG. 2. **PIAS1 binds double-stranded A/T-rich DNA.** Control GST, GST-PIAS1 (aa 1–135), or GST-PIAS1 (aa 1–166 del 11–45), indicated as *dSAP*, were coupled to glutathione-Sepharose beads and incubated with ^{32}P-labeled A/T-rich oligonucleotide using a batch method. Either unlabeled A/T-rich oligonucleotide or *E. coli* DNA was used in competition with the ^{32}P-labeled A/T-rich oligonucleotide to demonstrate specific binding. Beads were washed, and the radioactivity was measured in a liquid scintillation counter. *Error bars* indicate ± S.D. of data from three independent experiments.

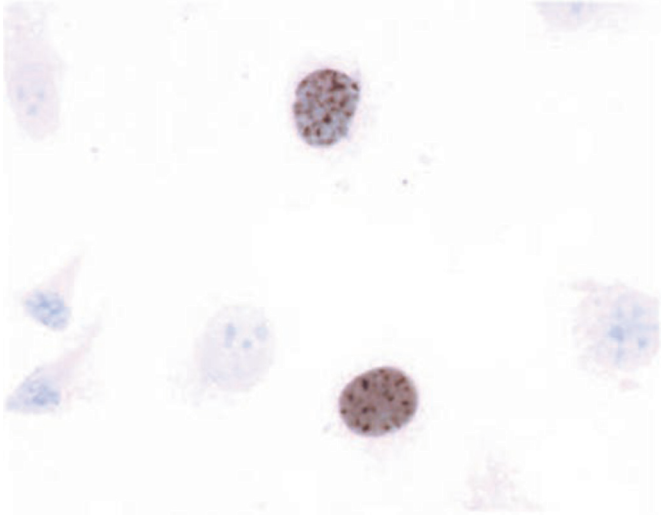

FIG. 3. **Localization of PIAS1 in nuclei.** COS cells cultured on two-chamber microscope slides were transfected with pSG5PIAS1 and incubated for 40 h. Cells were fixed and immunostained for PIAS1 as described under "Experimental Procedures." PIAS1 is indicated by *brownish* staining clustered in dense foci to produce a *stippled* pattern. Nuclei of several non-transfected cells can be seen in the same field of view.

PIASxα, PIASxβ, and PIASy (0.5 μg) each inhibited AR induction of MMTV-Luc transcription relative to the pSG5 antisense control, whereas lower amounts (0.01 and 0.05 μg) were either less inhibitory than PIAS1 or had no effect (Fig. 4, *top panel*). In assays with the probasin reporter gene (Fig. 4, *middle panel*) PIASxα, PIASxβ, and PIASy had either no effect or were inhibitory (Fig. 4, *middle panel*). With the PSA reporter gene PIASxα, PIASxβ, and PIASy (0.5 μg) were inhibitory (Fig. 4, *bottom panel*).

PIAS Proteins Interact Directly with AR in Vitro—In affinity matrix assays full-length ^{35}S-PIAS proteins bound glutathione

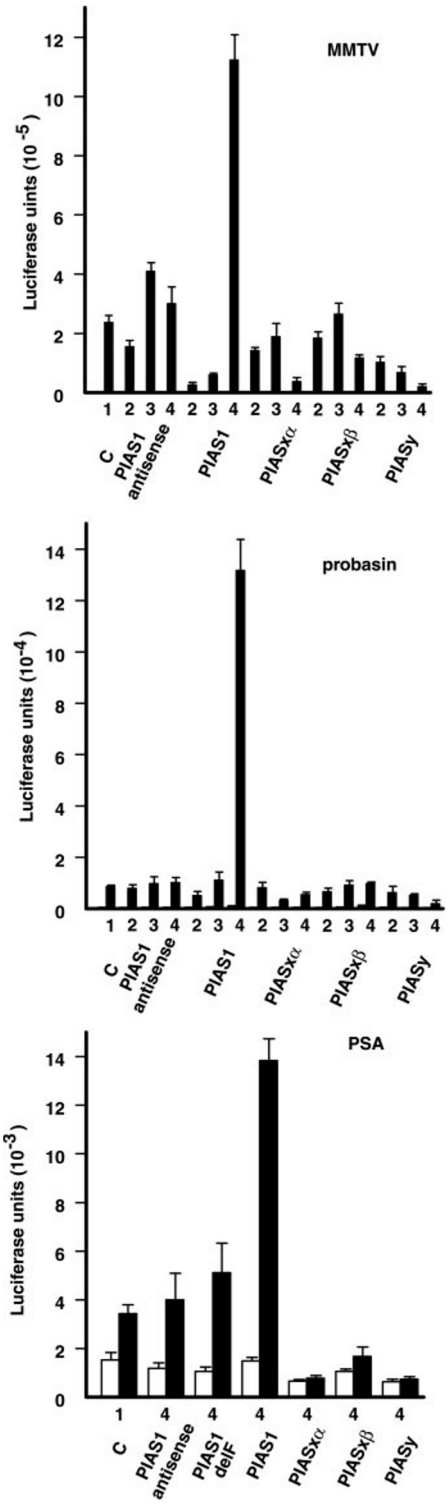

FIG. 4. **Coregulatory effects of PIAS proteins on AR transactivation.** The AR expression vector pSG5hAR (0.1 μg) and a reporter vector (2.5 μg) were cotransfected into CV1 cells in 6-cm dishes with different amounts of pSG5-PIAS1 antisense vector (37) to balance the DNA or pSG5-PIAS expression vector as indicated at the bottom of each bar: *1*, control (*C*) pSG5-AR 0.1 μg + reporter 2.5 μg; *2,* control + 0.01 μg of the indicated expression vector; *3,* control + 0.05 μg of the vector; *4*, control + 0.5 μg of the vector. Cells were incubated in the absence (on the *left* of each *solid bar*) and presence (*solid bars*) of 0.1 nM dihydrotestosterone. The PIAS expression vectors were pSG5PIAS1, pSG5PIAS1delF (full-length PIAS1 with deletion of amino acids 341–537) (37), pSG5PIASxα, pSG5PIASxβ, and pSG5PIASy. The reporter vectors are as follows: *top*, mouse mammary tumor virus (MMTV)-luciferase; *middle*, probasin-luciferase; *bottom*, PSA-luciferase. Assays were performed in triplicate, and *error bars* represent ± S.D.

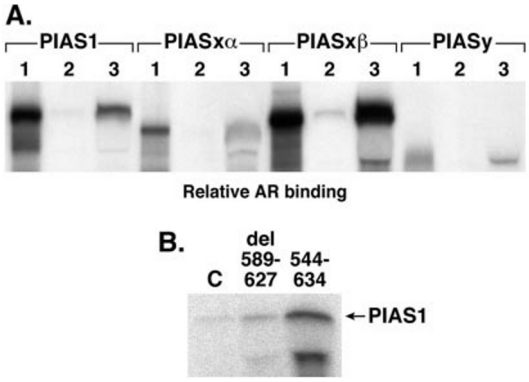

FIG. 5. **PIAS family members bind the AR DNA binding domain.** *A*, binding of full-length [^{35}S]methionine-labeled PIAS proteins (indicated at the *top* of the figure) to GST-AR DNA binding domain (AR amino acids 544–634). *Lane 1*, input of ^{35}S-PIAS protein (10%); *lane 2*, GST control; *lane 3*, ^{35}S-PIAS binding to GST-AR DNA binding domain. *B*, absence of binding of PIAS proteins to a mutant AR DNA binding domain with deletion of 2nd zinc finger amino acids 589–627 as a result of an AR gene exon 3 deletion: *C* is GST control; *del589–627* is the AR-DNA binding domain with deletion of 2nd zinc finger, and *544–634* is the wild-type AR DNA binding domain.

S-transferase (GST) AR (amino acids 544–634). This region of AR includes the entire DNA binding domain and small portions of the N-terminal and hinge regions (14). Each of the proteins PIAS1, PIASxα, PIASxβ, or PIASy bound the AR DNA binding domain region (Fig. 5*A*) suggesting they interact with AR by similar mechanisms. From previous studies we concluded that a sequence within PIAS1 amino acids 1–318 is required for AR binding; however, the precise binding motif remains to be identified (37).

Because the 2nd zinc finger has been implicated in steroid receptor protein-protein interactions, we tested the binding of PIAS1 to the AR DNA binding domain fragment (amino acids 544–634) from which the 2nd zinc finger was deleted. Deletion of the 2nd zinc finger (amino acids 589–627 encoded by AR gene exon 3) resulted in a major decrease in PIAS1 binding relative to its binding to the wild-type AR (Fig. 5*B*).

PIAS1 Has an Intrinsic Activation Function in Yeast Greater Than That of PIASxα or -xβ—Because PIAS1 stimulated AR transactivation to a greater extent than PIASxα or PIASxβ, we compared their intrinsic activation functions in Y190 yeast cells using the Gal DNA binding domain vector (pBDGalCAM) containing PIAS1, PIASxα, PIASxβ, or the PIAS1 mutant PIAS1delF (Fig. 6). In liquid β-galactosidase assays PIAS1 had intrinsic transcriptional activity that was 7 times greater than that of PIASxβ. PIASxα had no activity and there was barely detectable activity with PIAS1delF. Thus the relative intrinsic activation functions of these proteins in yeast are reflective of their coregulator effects in the CV-1 cell cotransfection assay with PIAS1 being the only coactivator member of the family in this system.

Expression of PIAS1, PIASxα, PIASxβ, and PIASy Genes in Testis—We reported earlier (37) that PIAS1 mRNA is expressed at a relatively high level in human testis, and PIAS1 protein is localized by immunohistochemical staining in nuclei of androgen/AR-regulated peritubular myoid cells and Sertoli cells. In addition there was staining of developing germ cells throughout the seminiferous tubular epithelium. Similar distribution of PIASxα protein in mouse testis (referred to as ARIP3) was reported by Moilanen *et al.* (40). To localize the expression of other PIAS genes in testis, we performed *in situ* hybridization using specific probes for PIAS1, PIASxα, PIASxβ, and PIASy mRNAs based on sequences reported by Liu *et al.* (36) (Fig. 7). In the sexually mature mouse, there was staining of PIAS1 and other family members in cytoplasm throughout

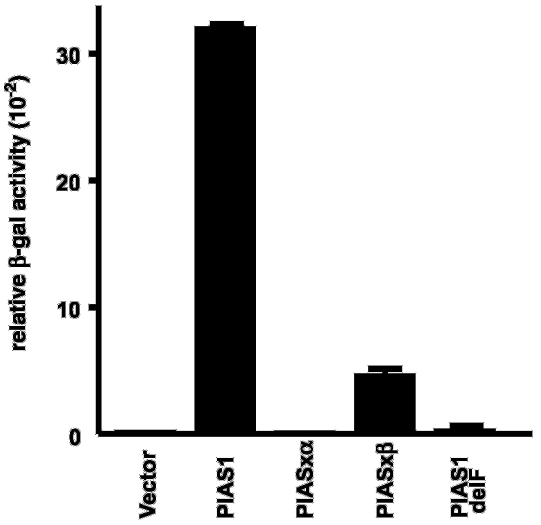

FIG. 6. **Intrinsic activation functions of PIAS1 and PIASxβ.** The Gal DNA binding domain vector pBD-GalCAM containing either full-length PIAS1, PIASxα, PIASxβ, or a PIAS1 mutant with deletion of amino acids 341–537 (PIAS1delF) was used to transform yeast Y190. Yeast were plated and colonies were picked on selective medium lacking Trp. β-Galactosidase (β-*gal*) units were determined in a liquid assay using the substrate *o*-nitrophenyl β-D-galactopyranoside. Relative β-galactosidase activity represents units measured above the empty vector background.

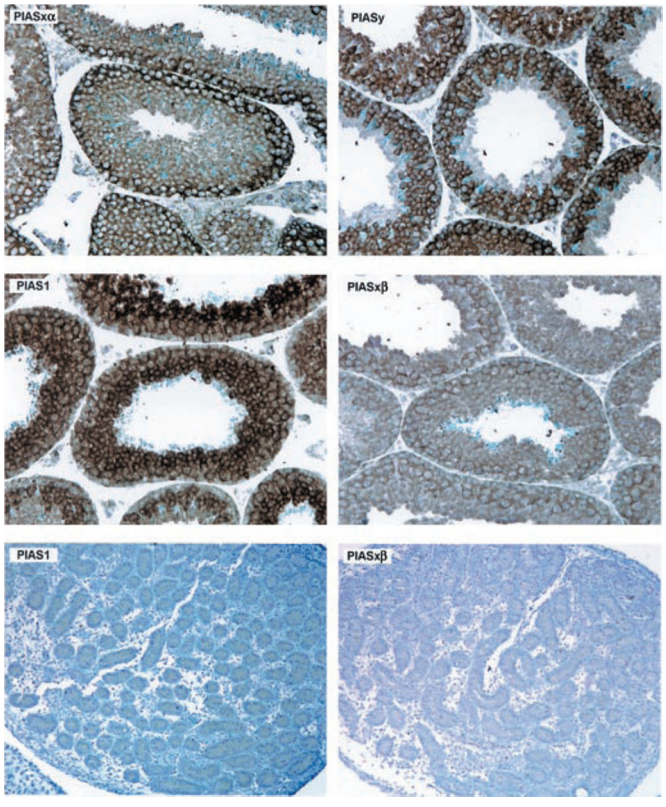

FIG. 7. **Expression of PIAS genes in mouse testis.** *In situ* hybridization of PIAS mRNA in adult mouse testis was performed using probes specific for the different family members, PIAS1 (nucleotides 1587–2101), PIASxα-(1628–1719), PIASxβ-(1628–1866), and PIASy-(1232–1533). In the *two bottom panels* are *in situ* hybridizations of 3-day-old mouse testis with PIAS1 and PIASxβ. PIASxα and PIASy were also negative in 3-day-old mouse testis.

the germinal epithelium, but regional differences in the intensity of staining were noted. PIAS1 staining was darker near the central region associated with round spermatids. In contrast, there was more PIASxα in the peripheral layers of cells that

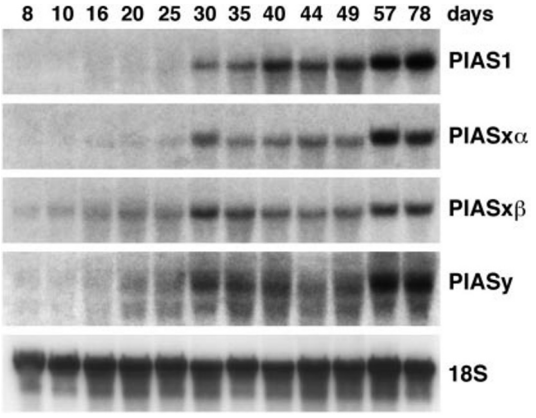

FIG. 8. **Age-dependent expression of PIAS mRNA in rat testis.** Northern hybridization of PIAS mRNAs from rat testis during sexual development was performed as described (37). Total RNA (10 μg per lane) was isolated from rat testes at the ages indicated and hybridized with ^{32}P-PIAS probes containing sequences specific for the different family members as shown in Fig. 7. RNA sample loadings are indicated by a representative hybridization of 18 S rRNA shown at the *bottom*.

appeared to include Sertoli cells, spermatogonia, and early spermatocytes. PIASxβ was similar to PIASxα but was less intense and tended to be more evenly distributed throughout the germinal epithelium. PIASy mRNA staining was somewhat darker in the mid-region of the epithelium. Variable staining among different tubules suggested the expression of PIAS genes is dependent on the stage of spermatogenesis.

In the 3-day-old mouse testis there was little or no staining of mRNAs for PIAS1, PIASxβ (Fig. 7, *bottom panel*), PIASxα, or PIASy (not shown). However, by 12 days of age all family members were detected with PIAS1 > PIASxα > PIASy > PIASxβ (not shown).

In rat testis the different PIAS family members were expressed similarly during development although PIASxβ and PIASy appeared somewhat earlier than did PIASxα or PIAS1 as shown by Northern hybridization of total RNA using specific probes (Fig. 8). mRNA levels were detected in prepubertal rats and increased in intensity with age consistent with expression in Sertoli cells as well as spermatogenic cells.

It was reported recently by Schlegel *et al.* (54) that PIASxα mRNA was not detected in mouse and rat Leydig cells and Sertoli cells isolated by centrifugal elutriation but only in spermatogonia, primary spermatocytes, and round spermatids. Moreover PIASxα mRNA, although present in testes of men with normal spermatogenesis, was not detected in infertile men with the Sertoli cell only syndrome. We did not detect PIASxα by Northern hybridizations of total RNA from cultured Sertoli cells of 18-day-old rats, although under the same conditions PIAS xβ mRNA was abundant and PIASy was a weaker band (results not shown). However, this difference may reflect the immaturity of the cultured Sertoli cells. As shown in the above developmental study in rat testis (Fig. 8), at 16 and 20 days of age PIASxβ and PIASy mRNAs were more abundant than PIASxα.

Coregulatory Effects of PIAS1 on AR Are Modulated by Coexpression of PIASxα, PIASxβ, or PIASy—Because some members of the PIAS family are coexpressed in AR-regulated cells of testis, we asked if the bidirectional regulatory effects of PIAS1 on AR transactivation were altered by coexpression with other proteins of the PIAS family (Fig. 9). Cotransfection assays were performed in CV1 cells using pSG5hAR and MMTV-luciferase. In the presence of a low amount of transfected PIAS1 (0.05 μg), DHT-dependent AR-induced luciferase activity was markedly inhibited. This inhibition by PIAS1 was attenuated by cotransfection of an equal amount (0.05 μg) of PIASxα, PIASxβ, or

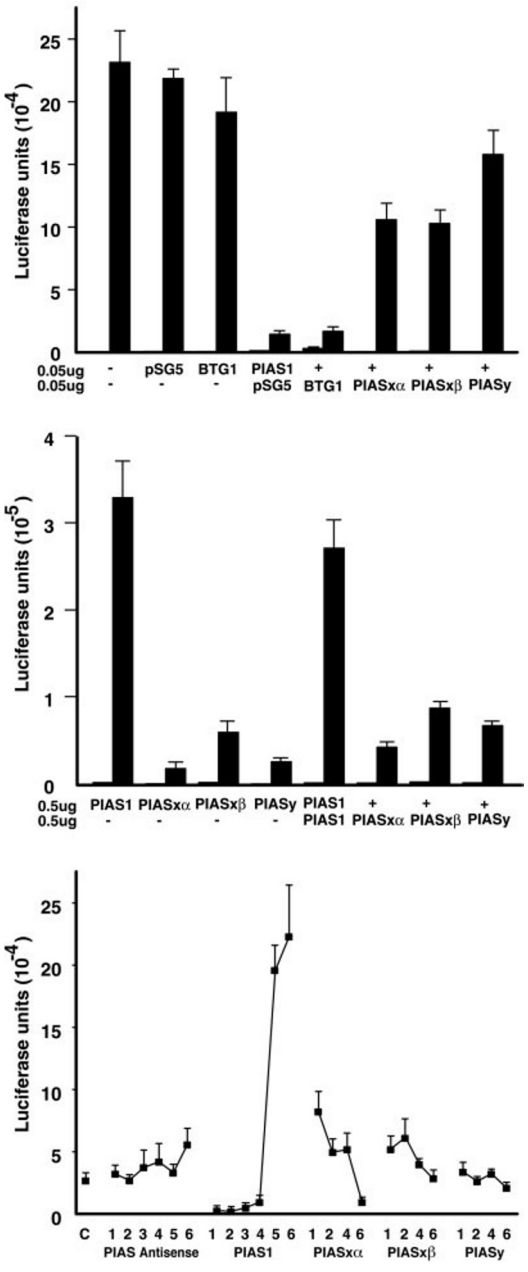

FIG. 9. **AR coregulator effects of PIAS1 are modulated by PIASxα, PIASxβ, or PIASy.** AR expression vector pSG5hAR (0.1 μg) and reporter vector MMTV-Luciferase (2.5 μg) were cotransfected into CV1 cells in 6-cm dishes, and the effect of PIAS1 on AR transactivation was tested in combination with PIASxα, PIASxβ, or PIASy. Cells were incubated in the absence (shown on the *left* of each *solid bar*) or presence (*solid bars*) of 0.1 nM dihydrotestosterone. *Top panel*, the lower amount of PIAS1 transfected (0.05 μg) inhibited AR transactivation. PIAS1 (indicated by + sign) inhibition was attenuated by cotransfection with 0.05 μg of PIASxα, PIASxβ, or PIASy. DNA was balanced with equimolar amounts of pSG5 empty vector or pSG5-BTG1. Neither vector alone inhibited AR transactivation. *Middle panel*, cells were transfected with pSG5hAR, MMTV-luciferase, and pSG5PIAS vectors as above. The 10-fold higher amount of PIAS1 (0.5 μg) increased AR transactivation, whereas equal amounts of PIASxα, PIASxβ, or PIASy reduced or caused no change in AR transactivation (shown on the *left* side of *panel*). The PIAS1-induced increase in luciferase units was not changed by cotransfection of an additional 0.5 μg of PIAS1 but was inhibited by 0.5 μg of PIASxα, PIASxβ, or PIASy (shown on the *right*). *Bottom panel*, dose-response curves for antisense PIAS1, PIAS1, PIASxα, PIASxβ, and PIASy. Assays were performed as above with transfections of increasing amounts of pSG5 expression vector DNA (indicated by numbers *1–6*); *C* (control), pSG5AR + MMTV-luciferase alone; *1*, control + 0.025 μg; *2*, control + 0.05 μg; *3*, control + 0.075 μg; *4*, control + 0.1 μg; *5*, control + 0.25 μg; and *6*, control + 0.5 μg. The points represent activity measured in the presence of 0.1 nM dihydrotestosterone. Assays were performed in triplicate, and *error bars* represent ± S.D.

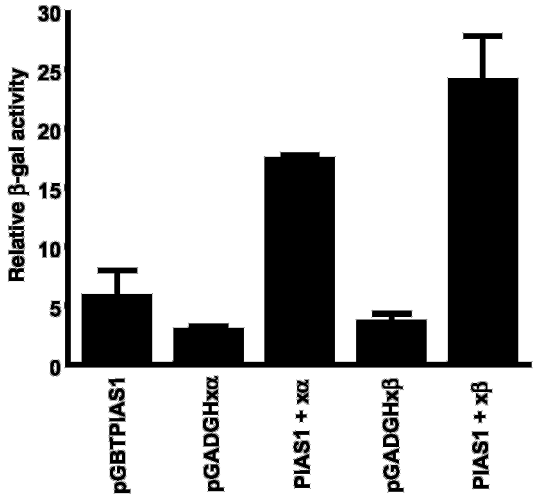

FIG. 10. **Binding of PIAS1 to PIASxα and PIASxβ in a yeast two-hybrid assay.** PIAS1 was cloned into the Gal DNA binding domain vector, pGBT8. PIASxα and PIASxβ were cloned into the Gal activation domain vector, pGADGH. Y190 yeast were transformed with the individual vectors and with combinations of PIAS1 + PIASxα and PIAS1 + PIAS xβ. The yeast liquid β-galactosidase (*β-gal*) assay was performed as described under "Experimental Procedures."

PIASy but was not influenced by cotransfection of the same amount of control vector DNA (Fig. 9, *top panel*). Thus coexpression of other PIAS family members counteracted the low dose inhibitory effect of PIAS1 on AR transactivation.

Similarly, the stimulation of AR transactivation by cotransfection with a 10-fold larger amount of PIAS1 (0.5 μg) was offset by cotransfection of an equal amount (0.5 μg) of PIASxα, PIASxβ, or PIASy. Luciferase levels obtained with each of these family members in combination with PIAS1 approached the levels obtained with PIASxα, PIASxβ, or PIASy alone. In contrast, when 0.5 μg PIAS1 was cotransfected with an equal amount of the same vector, PIAS1 (0.5 μg), luciferase activity was unchanged (Fig. 9, *middle panel*).

The dose-response effect of PIAS1 on DHT-dependent AR transactivation was quite different from PIASxα, PIASxβ, or PIASy in this system. With the PIAS1 there was inhibition of AR transactivation at the lower doses of expression vector (0.025–0.1 μg) and a steep transition to stimulation of transactivation between 0.1 and 0.25 μg. On the other hand PIASxα, PIASxβ, and PIASy caused either slight stimulation or had no effect at the lower doses and were inhibitory at the higher doses (Fig. 9, *bottom panel*).

PIAS1 Interacts Directly with Other Members of the PIAS Family—Protein-protein interactions of PIAS1 with PIASxα and PIASxβ were analyzed in the yeast two-hybrid system. Y190 yeast cells were transformed with pGBT-PIAS1, that expresses a Gal DNA binding domain-PIAS1 fusion protein together with the vector pGADGH that expresses the Gal activation domain fused to either PIASxα or PIASxβ. Cells were incubated overnight at 30 °C in selective medium, and β-galactosidase activity was measured in the liquid assay (37) (Fig. 10). The β-galactosidase activity of PIAS1 + PIASxβ was 4–6-fold higher than with either vector alone. Similarly the activity with PIAS1 + PIASxα was 3–4-fold higher than either PIAS1 or PIASxα alone indicating that PIAS1 interacts with PIASxβ and PIASxα.

Binding was also examined in affinity matrix assays using GST-PIAS1 (aa 7–651) coupled to glutathione-Sepharose and recombinant ^{35}S-PIAS proteins synthesized *in vitro* (Fig. 11). In these assays ^{35}S-PIAS1, PIASxα, PIASxβ and PIASy each bound to GST-PIAS1, whereas binding to GST-glutathione-Sepharose was negligible. The results indicated that PIAS1 can

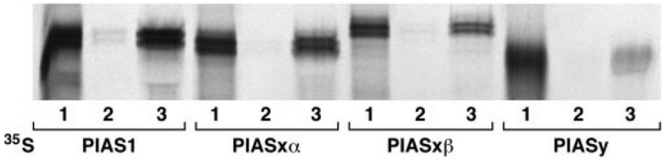

FIG. 11. **Binding of PIAS1 to itself and to other PIAS family members in an affinity matrix assay.** [^{35}S]Methionine-labeled PIAS proteins were synthesized *in vitro* and incubated with GST-PIAS1 coupled to glutathione-Sepharose (*lane 3*) or with the control GST-glutathione Sepharose (*lane 2*). Assays were performed as described under "Experimental Procedures." Input (*lane 1*) was 10% of radioactivity loaded.

self-associate or form multimers with other members of the PIAS family.

DISCUSSION

In earlier studies (37) we found that PIAS1 is a nuclear receptor coregulator that increases transcriptional activity of the ligand-activated AR. Different regulatory functions have been reported for PIAS proteins as we discuss below; however, a common mechanism to explain these multiple actions has not yet been identified. Herein we report that PIAS1 has characteristics of a scaffold/matrix attachment region binding protein (48, 55). It contains a SAF box or SAP domain conserved in this family of proteins (48, 49) and binds double-stranded A-T-rich DNA. Like other S/MAR-binding proteins such as SAF-A (51, 53) and SAF-B (50, 52, 56), PIAS1 was localized in nuclei in clusters that formed a speckled pattern. S/MAR binding scaffold attachment factors SAF-A, also known as heterogeneous nuclear ribonucleoprotein U (53), and SAF-B (50, 56) have been reported to interact with steroid receptors. SAF-B bound the estrogen receptor and in transient transfection assays inhibited estrogen receptor transactivation in a dose-dependent manner (57). Similarly, SAF-A inhibited transactivation of the glucocorticoid receptor (53, 58). Binding of both SAF proteins involved the receptor DNA binding domain and hinge regions. SAF-A and SAF-B are ubiquitous, abundant nuclear proteins. SAF-B colocalizes and interacts with a subset of serine/arginine-rich processing factors. It binds also to RNA polymerase II and may serve as an assembly point for the formation of a "transcriptosomal" complex (52). Whereas steroid receptor transactivation was inhibited by overexpression of SAF-A in transient transfection assays, it was suggested that this may have resulted from a change in the receptor to SAF-A ratio, which converted a positive into a negative effect on transcription (53).

An additional finding in our study was that PIAS1 had a striking concentration-dependent biphasic effect on AR transactivation. At lower expression levels in CV1 cells it inhibited but at higher levels it enhanced AR transactivation. In an earlier report (37) we demonstrated that at a still higher concentration PIAS1 coactivation was reduced. This biphasic effect of concentration was similar to the effect on signaling observed with a kinase scaffold protein. In signaling through a scaffold too much or too little of any component may decrease the output of the pathway (59). PIAS1 contains a RING-finger like domain (amino acids 325–382) (36, 37, 60) that is conserved among members of the PIAS family (36). It has been proposed that RING domains can self-assemble into macromolecular scaffolds that attach other regulatory molecules (61). Self-association of PIAS1 would be consistent with its speckled pattern of localization in nuclei.

In contrast to PIAS1, other members of the PIAS family, PIASxα, PIASxβ, or PIASy at lower expression levels in CV1 cells had smaller coregulator effects on AR transactivation that were somewhat variable but at higher levels inhibited AR transactivation. Thus with this assay system there are distinct differences in AR coregulatory effects within the PIAS family. Whether these differences relate to different scaffold properties or to other functions remains to be determined.

Because some PIAS family members are coexpressed with AR in androgen-regulated cells of the seminiferous tubules, there is a potential for interactions between AR and the different PIAS proteins. In CV-1 cell assays PIASxα, PIASxβ, and PIASy counteracted the effects of PIAS1 on AR transactivation, both the inhibition at lower PIAS1 levels and the enhancement at higher levels were partially reversed. The direct interaction of PIAS family members with PIAS1 in yeast and in cell-free assays indicated that coregulator functions of PIAS1 can be modulated by formation of heteromers with other members of the PIAS family. Androgen-activated AR has a strong tendency to form homodimers in the presence of androgen-response element DNA (16) making it likely that PIAS proteins interact with AR homodimers in nuclei. Our results suggest that PIAS1 can form multimers through self-association or with other PIAS family members. This ability of PIAS family members to associate may relate to the self-assembly properties of their RING domains and suggests that they can form scaffolds containing the different family members.

Kotaja *et al.* (62) reported that the coregulatory effects PIASxα, referred to as androgen receptor interacting protein 3 (ARIP3), PIASxβ, and PIAS3 are influenced by cell type and the reporter gene enhancer/promoter in transient cotransfection assays. By using a simple ARE$_2$TATA-LUC reporter, PIASxα was a coactivator with AR in HepG2 cells, and the coactivator activity was stronger in HepG2 cells than in HeLa cells. However, with the more complex probasin gene enhancer/promoter, PIASxα was a repressor of AR transactivation in HeLa cells, and with this same reporter gene in HepG2 cells PIASxα had little effect. Their results suggest that other cellar factors can have a major influence on PIAS coregulation.

In previous studies (37) we demonstrated it is the N-terminal region of PIAS1 that mediates androgen-dependent binding to the AR DNA binding domain and among the PIAS family there is sequence similarity that would suggest a common N-terminal site for AR interaction. N termini of PIAS family members contain an L*XX*LL motif (amino acids 19–23) (36, 37). L*XX*LL motifs of p160 coregulators interact with activation function 2 domains (63–65) in the C-terminal region of nuclear receptors. However, in AR the hydrophobic cleft within the C-terminal region that forms activation function 2 is the interaction site for the N/C interaction mediated by a F*XX*LF motif in the AR N terminus (41, 66). The AR N-terminal region binds p160 coactivators independent of L*XX*LL motifs (67, 68). At present we have no evidence that L*XX*LL motifs of PIAS family members are involved in PIAS binding to the AR.

Gross *et al.* (69) reported that the L*XX*LL motif in PIASy is required for suppression of AR transactivation but not for PIASy binding to the AR DBD. More recently Liu *et al.* (70) observed that PIASy bound activated STAT1 and inhibited transcriptional activation of a *STAT1* reporter gene without affecting STAT1 binding to DNA. The L*XX*LL motif was required for PIASy inhibition of transcription but not for PIASy binding to STAT1. On the basis of these results it was suggested that the L*XX*LL motif enables PIASy to function as an adaptor protein to link STAT1 to a transcriptional corepressor. In our experiments with PIAS1, mutating the L*XX*LL motif to L*XX*AA altered the dose-response curve with AR but did not abolish either the corepression at low doses or coactivation at higher doses (data not shown).

Whereas PIAS1 inhibited the binding of activated STAT1 to its DNA-response element (36, 71), PIAS1 does not likely in-

hibit AR binding to androgen-response element DNA under conditions where it acts as a coactivator. PIAS1 enhancement of AR transcriptional activation requires DNA binding to androgen-response element DNA and is abolished by mutations in the AR DNA binding domain (1, 2, 72). Involvement of the AR second zinc finger motif in AR binding of PIAS proteins is of interest because it was predicted earlier that the nuclear receptor DNA binding domain is a site of interaction with regulatory proteins (6, 73) and has functional surfaces for protein interactions (74). Several regulatory factors interact directly with the DNA binding domain or DNA binding domain and hinge region of AR and other nuclear regulatory proteins (40, 75–81).

The intrinsic transcriptional activity of PIAS1 and to a lesser extent PIASxβ suggests they contain an activation function resulting from enzyme activity or binding to another coregulator. Our assays in yeast were in agreement with the results of Kotaja *et al.* (62) in HeLa cells and HepG2 cells in that intrinsic transcriptional activity of PIAS1 was greater than that of PIASxα. In PIAS1 this activation function was dependent on a sequence within amino acids 341–536, the region deleted in the nonfunctional mutant PIAS1delF (37). Within the activation function domain (aa 341–536) are cysteine residues predicted to form a RING-finger like domain described above and an acidic domain (36, 37, 60). The RING finger-like sequence is conserved in the PIAS family (36). However, in the C-terminal half of the activation domain there is sequence variation that could account for the different AR coregulator activities of PIAS family members.

As scaffold attachment factors PIAS family members may control the regulatory functions of numerous proteins and mediate cross-talk between different signaling pathways. For example, when activated STAT1 binds to response element DNA it recruits CBP/p300 (82). Thus PIAS1 binding of STAT1 (71) might prevent STAT1 recruitment of CBP/p300 thereby making CBP/p300 more available for binding to other transcription factors. Precedent for this idea was reported with γ-interferon and JAK/STAT signaling during macrophage development (83). On the other hand, binding of PIAS1 by nuclear receptors (37, 76) or other proteins could increase activated STAT1 signaling by removing PIAS1 inhibition. STAT3 signaling was enhanced by PIAS3 binding to the zinc finger protein Gfi-1 (84). Similarly, AR transactivation was positively regulated by PIASxα binding to the nuclear protein DJ-1 (85). Binding to DJ-1 counteracted PIASxα inhibition of AR transactivation.

An increasing number of PIAS interactions with other proteins are being identified. Originally PIAS1 was referred to as the Gu-binding protein (86), in that it bound Gu RNA helicase II. The Gu-binding protein was localized throughout the nucleoplasm, whereas Gu RNA helicase was confined to nucleoli (87). Although the full significance of Gu RNA helicase II binding remains to be determined, ATP-dependent helicases are involved in a variety of transcription-related processes (see Ref. 37 and references therein).

PIAS1 also has sequence similarity to a K^+ channel-associated protein, KChAP (88), and PIAS3 has even greater homology with KChAP (89). Although KChAP localizes in nuclei (88–90), it interacts with both α and β subunits of K^+ channels, and when expressed in *Xenopus* oocytes, it increases the amounts of specific K^+ channel α subunit proteins at the cell surface.

The mouse homologue of PIASxα (mDIP) binds the p67 isoform of mouse disabled 2 (mDab2) (91). mDab2, especially the p67 isoform, is highly expressed in differentiating murine F9 embryonal carcinoma cells following treatment with retinoic acid. The N-terminal phosphotyrosine interacting domain of mDab2 interacts with the C-terminal region of mDIP.

In *Drosophila melanogaster*, homologues of PIAS proteins are expressed by alternative splicing from the *zimp* gene, which is an allele of *Suppressor of Position-Effect Variegation SU-(var)2-10* (92). SU(var)2-10 proteins colocalize with nuclear lamin in interphase and are present in some polytene chromosome telomeres. *SU(var)2-10* is essential for embryo development. The SAP domain may link specific chromosome regions to the nuclear lamina. One model holds that SU(var)2-10 protein isoforms function in a variety of transcription regulation complexes together with chromosome-bound transcription factors controlling different cellular responses (93).

PIAS family members can function as E3 ligases for small ubiquitin-related modifier (SUMO) conjugation. E3 ligase activity resides in the RING-related motif. Yeast E3-like factors Siz 1 and Siz 2 have homology with the RING motif of PIAS (94). PIAS1 was reported to bind p53 as well as the ubiquitin carrier protein ligase Ubc9 and to promote SUMO conjugation of p53 (60). PIASy was also an E3 ligase for the transcription factor, lymphoid enhancer factor 1 (LEF1), a nuclear mediator of Wnt signaling. PIASy repressed LEF1 induced transcription and sequestered LEF1 in nuclear bodies. The targeting to nuclear bodies was dependent on the RING domain of PIASy. However, PIASY-mediated localization of LEF1 in nuclear bodies appeared to be independent of LEF1 sumoylation because it was unaffected by a mutation in the LEF1 SUMO conjugation site (95). Neither did this mutation interfere with PIASy inhibition of LEF1 transactivation. Thus the mechanism of PIASy inhibition remains to be established. One possibility is that PIASy promoted the sumoylation of other coregulators that repressed LEF1 transactivation and subnuclear sequestration (95). Another possibility would relate these effects more to the self-assembly properties (61) and scaffold functions of RING domain proteins.

The binding of PIAS family members to A-T-rich DNA in regions of active transcription, the presence of RING domains with E3 ligase activity, and the potential to form nuclear scaffolds for attachment of regulatory proteins provide a basis for understanding the multifunctional nature of PIAS. Expression of PIAS in androgen-regulated peritubular myoid cells, Sertoli cells, and in spermatogenic cells throughout the germinal epithelium indicates this family of interactive coregulators is involved in controlling multiple stages of germ cell development.

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