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

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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)† 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 Mx1, 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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‡ 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 PIAS1 (mPIAS1) were recovered from digestions of pFLAG-PIASxα, -xβ, -xγ, and mPIAS1 and cloned into pBS to create pBSG-PIASxα, -xβ, -xγ, and mPIAS1 pFLAG-PIAS vectors were provided by Ke Shuai, UCLA (36). BamHI-Klenow filled in fragments of PIASαx and -xβ derived from pSG-PIASαx and -xβ were cloned into the Smal site of pBDGALCAM to create yeast Gal4 DNA binding domain vectors, pBDGalCAM-PIASαx and -xβ. The same fragments were cloned into the filled in XhoI site of pGADGH to create yeast Gal4 activation domain vectors pGADGH-PIASαx and -xβ. A specific probe for PIAS1 was generated by PCR of pGADGH-PIAS1 (37) using 5′ primer (GGTCTAGAATGCAACACAGGACACGACAAGGCTGCA) and 3′ primer (CACAATCAGTAATGCAGTG) using pSG5-PIASyα and -xβ as templates. A probe specific for PIASy was generated by PCR with 5′ primer (GCTCTAGAAGGAGCGCAGCTGCA) and 3′ primer (CA- 

GAATTCCAGAATGCAGTG) with pSG5-PIASxα as template. PCR products were expressed in XbaI-EcoRI and cloned into the same sites of pBKCMV (Stratagene).

Probes specific for PIASαx and -xβ mRNAs were generated by PCR with 5′ primer (CACTCTGAGTCCAAACCGGGCCCCTCTGC) and 3′ primer (GGTCTAGAATGCAACACAGGACACGACAAGGCTGCA) and 3′ primer (CACAATCAGTAATGCAGTG) using pSG5-PIASxα and -xβ as templates. A probe specific for PIASy was generated by PCR with 5′ primer (GCTCTAGAAGGAGCGCAGCTGCA) and 3′ primer (CA- 

GAATTCCAGAATGCAGTG) with pSG5-PIASxα as template. PCR products were digested with XbaI-BglII and cloned into pBKCMV XbaI-BamHI site. To create the vector pGTEST-P1A51 amino acids 7–651 of PIAS1 were excised with SmaI-XhoI and cloned into pGCT8: pGEX-PIAS1 (1–135) and pGEX-PIAS1delISAP (1–166 del 11–45) were constructed by PCR of templates pSGS-PIAS1 and pSGS-PIAS1delISAP with 5′ primer CTCTTAGCTCACAAGCGGCTCCTGC and 3′ primer TCTC- 

GAAGCCGCTAGCTTGTCTAGTG. For cell-free binding assays GST-P1A51 (amino acids 7–651) was created by digesting pGADGH- PIAS1 (37) with SpeI-XhoI and cloning the purified PIAS1 into the XhoI-EcoRI GST-2BamHI site. After induction 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 [35S]methionine as described above were incubated with GST-P1A51 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** [35S]PIAS Proteins—pSGS-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).

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**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). PIAS proteins are modulated by interactions with other members of the PIAS family and a number of other eukaryotic proteins.

**PIAS Are SAF-like Proteins and Interacting AR Coregulators**—Probes specific for PIASαx and -xβ mRNAs were generated by PCR with 5′ primer (CACTCTGAGTCCAAACCGGGCCCCTCTGC) and 3′ primer (GGTCTAGAATGCAACACAGGACACGACAAGGCTGCA) and 3′ primer (CACAATCAGTAATGCAGTG) using pSG5-PIASyα and -xβ as templates. A probe specific for PIASy was generated by PCR with 5′ primer (GCTCTAGAAGGAGCGCAGCTGCA) and 3′ primer (CA- 

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**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, PIASβ, and PIASγ as indicated above. Ribosomal RNA was stained with methylene blue to compare the amounts of sample loaded in each lane. Sample loading was checked by hybridization of 185 RNA. The DNA for 18S RNA was obtained from Ambion (Austin, TX) and labeled with [32P]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-AB, Acius, PIAS (SAP) domain (49). Secondary structure modeling predicts the sequence forms two amphipathic helices (Fig. 1) with homology to helices 1 and 2 of 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 (pSGS-hAR) 0.1 μg, and various amounts of pSGS expression vectors containing PIAS cDNAs. To control for possible DNA effects, CV1 cells were transfected with or without 80 ng pSGS vector, pSGS-PIAS1 antisense vector, or pSGS-BTG1 that expresses the B cell translocation gene 1 (44). Cells were grown in 6-cm culture dishes and transfected by the CaPO4 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 μM dithyroxysterone (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 measured as an invariant glycine (49). In contrast to homeodomains that contain aromatic and polar amino acids separated by a region that contains several times in 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, protein containing 102 C-terminal amino acids (549–560) provided by Ke Shuai, UCLA (36). 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.
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 32P-labeled A/T-rich oligonucleotide using a batch method. Either unlated A-T-rich oligonucleotide or *E. coli* DNA was used in competition with the 32P-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-PIAS1aa 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 reporters. 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.

**PIAS Proteins Interact Directly with AR in Vitro**—In affinity matrix assays full-length 35S-PIAS proteins bound glutathione...
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**FIG. 4. Coregulatory effects of PIAS proteins on AR transactivation.** The AR expression vector pSG5AR (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 mM dihydrotestosterone. The PIAS expression vectors were pSG5-PIAS1, pSG5PIAS1delF (full-length PIAS1 with deletion of amino acids 341–537) (37), pSG5PIA5Sxα, 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 [35S]metabolic-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 [35S]-PIAS protein (10%); lane 2, GST control; lane 3, [35S]-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 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 staining of PIASx 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...
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 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, PIASxα (Fig. 7, bottom panel), or PIASxβ 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 PIASxβ mRNA was abundant and PIASy was a weaker signal (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 coinexpressed 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
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PIAS were cloned into the Gal DNA binding domain vector, pGBT8. PIAS\(\alpha\) and PIAS\(\beta\) were cloned into the Gal activation domain vector, pGADGh. Y190 yeast were transformed with the individual vectors and with combinations of PIAS1 + PIAS\(\alpha\) and PIAS1 + PIAS\(\beta\). The yeast liquid \(\beta\)-galactosidase (\(\beta\)-gal) assay was performed as described under “Experimental Procedures.”

PIAS\(\gamma\) 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 \(\mu\)g) was offset by cotransfection of an equal amount (0.5 \(\mu\)g) of PIAS\(\alpha\), PIAS\(\beta\), or PIAS\(\gamma\). Luciferase levels obtained with each of these family members in combination with PIAS1 approached the levels obtained with PIAS\(\alpha\), PIAS\(\beta\), or PIAS\(\gamma\) alone. In contrast, when 0.5 \(\mu\)g PIAS1 was cotransfected with an equal amount of the same vector, PIAS1 (0.5 \(\mu\)g), luciferase activity was unchanged (Fig. 9, middle panel).

The dose-response effect of PIAS1 on DHT-dependent AR transactivation was quite different from PIAS\(\alpha\), PIAS\(\beta\), or PIAS\(\gamma\) in this system. With the PIAS1 there was inhibition of AR transactivation at the lower doses of expression vector (0.025–0.1 \(\mu\)g) and a steep transition to stimulation of transactivation between 0.1 and 0.25 \(\mu\)g. On the other hand PIAS\(\alpha\), PIAS\(\beta\), and PIAS\(\gamma\) 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 PIAS\(\alpha\) and PIAS\(\beta\) 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 PIAS\(\alpha\), PIAS\(\beta\), or PIAS\(\gamma\). Luciferase levels obtained with each of the individual vectors and with combinations of PIAS1 + PIAS\(\alpha\) and PIAS1 + PIAS\(\beta\) were 4–6-fold higher than with either vector alone. Similarly the activity with PIAS1 + PIAS\(\alpha\) was 3–4-fold higher than either PIAS1 or PIAS\(\alpha\) alone indicating that PIAS1 interacts with PIAS\(\beta\) and PIAS\(\alpha\).

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 \textit{in vitro} (Fig. 11). In these assays, \(^{35}\)S-PIAS1, PIAS\(\alpha\), PIAS\(\beta\) and PIAS\(\gamma\) each bound to GST-PIAS1, whereas binding to GST-glutathione-Sepharose was negligible. The results indicated that PIAS1 can...
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 PIASx3 are influenced by cell type and the reporter gene enhancer/promoter in transient cotransfection assays. By using a simple ARE, 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 cellular 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 LXXLL motif (amino acids 19–23) (36, 37). LXXLL motifs of p160 coactivators interact with activation function 2 domains (63–65) in the C-terminal region of nuclear receptors. 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 FXXLF motif in the AR N terminus (41, 66). The AR N-terminal region binds p160 coactivators independent of LXXLL motifs (67, 68). At present we have no evidence that LXXLL motifs of PIAS family members are involved in PIAS binding to the AR.

Gross et al. (69) reported that the LXXLL 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 LXXLL 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 LXXLL motif enables PIASy to function as an adaptor protein to link STAT1 to a transcriptional corepressor. In our experiments with PIAS1, mutating the LXXLL motif to LXXAA 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-

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**Fig. 11.** Binding of PIAS1 to itself and to other PIAS family members in an affinity matrix assay. [35S]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.
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 PIASxβ. 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-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 PIASy promoting the sumoylation of other coregulators that 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 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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