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Table of Contents

Cover.....

SF 298.....

Table of Contents.....

Introduction.....1

Body.....1

Key Research Accomplishments.....3

Reportable Outcomes.....3

Conclusions.....3

References.....3

Appendices.....3

Introduction

It has been well established that androgen receptor (AR) plays an important role in prostate cancer. Previous studies have suggested that the PIAS (protein inhibitor of activated STAT) family may be involved in the regulation of AR-mediated gene activation. The overall goal of this proposal is to study the role of PIAS proteins in androgen signaling and prostate tumor progression.

Body

Aim 1. - To examine the role of PIAS SUMO ligase activity on androgen signaling (month 16-36).

This aim has been accomplished and the results have been published in our recent paper (Gross et al., 2004, 23:3059).

1.1 To examine the specificity of PIAS proteins on the sumoylation of AR

PIAS proteins do not display specificity on promoting protein sumoylation when assayed under over-expression conditions. As described in Aim 1.2 below, our data do not support a role of PIAS SUMO ligase activity in the regulation of AR.

1.2 To examine the involvement of PIAS SUMO ligase activity on AR transcription

Using PIASy as an example, we showed that the SUMO ligase activity of PIASy is not involved in AR regulation. Instead, we showed that the ability of PIASy to repress the transcriptional activity of AR is largely dependent on histone deacetylase (HDAC) activity. Our results suggest that PIASy represses transcription by recruiting HDACs. These results have now been published (Gross et al., 2004, 23:3059).

Aim 2. - To study the role of PIAS proteins in AR signaling in prostate cancer cell lines (months 1-12)

As described in my last year's progress report, we have accomplished the goals of this Aim.

Aim 3. To study the role of PIAS proteins in prostate tumor progression (months 6-36)

We have not been able to complete this aim on time due to the unexpected difficulties we have had on the generation of certain PIAS knockdown prostate cancer cell lines. Thus, we have requested a one-year no-cost extension on this grant. This request has been approved by the agency. Despite the delay, we have been continuously making progress. There are four members in the PIAS family: PIAS1, PIAS3, PIASy, and PIASx. In order to systematically analyze the role of PIAS proteins in AR signaling and prostate cancer,

we wish to knockdown each PIAS protein in prostate cancer cells. As indicated in the last year's report, we had been successful in generating PIAS3 knockdown prostate cell line using the RANi strategy. During the past year, we have successfully obtained RNAi constructs that can knockdown PIASy or PIASx expression (Fig. 1). Experiments are now underway to generate prostate cancer cells with PIASy or PIASx knockdown. Finally, we are in the process of generating PIAS1 knockdown constructs using the same strategy. Once we have obtained knockdown prostate cancer cell lines for each PIAS protein, we will inject these cells into SCID mice to carry out studies on the potential role of PIAS proteins in prostate tumor progression.

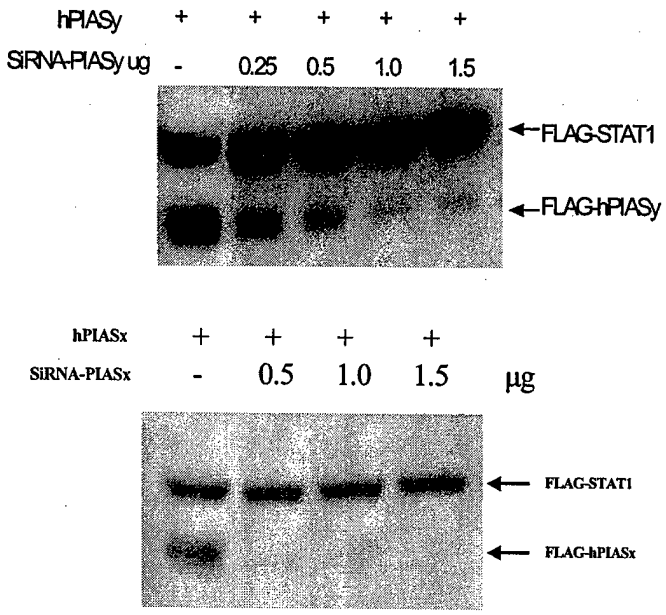

Figure 1. Generation of siRNA constructs for PIASy and PIASx knockdown. *The upper panel*: 293 cells were transfected with FLAG-PIASy together with either an empty vector or various amounts of the siRNA-PIASy construct as indicated. FLAG-STAT1 was included in the transfection assays as a control. Protein extracts from transfected cells were analyzed by immuno blot with anti-FLAG. *The lower panel*: same as the upper panel except FLAG-PIASx and siRNA-PIASx constructs were used.

Key research accomplishments

- We have demonstrated that PIASy represses the transcriptional activity of AR through the recruitment of HDAC.
- We have successfully obtained siRNA constructs for the knockdown of PIASx and PIASy.

Reportable outcomes

A manuscript describing these findings has been published.

1. Gross, M., Yang, R., Top, I., Gasper, C., Shuai, K. (2004) PIASy-mediated repression of the androgen receptor is independent of sumoylation. *Oncogene*, 23:3059-3066.

Conclusions

A controversial issue in the field of AR transcriptional regulation is whether the SUMO ligase activity of PIAS is involved. We have now provided evidence to demonstrate that PIASy represses the transcriptional activity of AR by recruiting histone deacetylases (HDAC1 and HDAC2), independent of its SUMO ligase activity. These studies provide important information for the understanding of the role of PIAS proteins in androgen signaling in prostate cancer cells.

Reference

Gross, M., Liu, B., Tan, J., French, F. S., Carey, M., and Shuai, K. (2001). Distinct effects of PIAS proteins on androgen-mediated gene activation in prostate cancer cells. Oncogene *20*, 3880-3887.

Appendices

The paper by Gross et al. in PDF format.

Oncogene (2004) 23, 3059–3066

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PIASy-mediated repression of the androgen receptor is independent of sumoylation

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PIASy, a member of the protein inhibitor of activated STAT (PIAS) family, represses the transcriptional activity of the androgen receptor (AR). In this report, we investigate the mechanism of PIASy-mediated repression of AR. We show that AR binds to the RING-finger like domain of PIASy. PIASy contains two transcriptional repression domains, RD1 and RD2. RD1, but not RD2, is required for PIASy-mediated repression of AR. We show that the RD1 domain binds HDAC1 and HDAC2 and that HDAC activity is required for PIASy-mediated AR repression. PIAS proteins possess small ubiquitin-related modifier (SUMO) E3 ligase activity. Conjugation of SUMO-1 to AR has been implicated in the regulation of AR activity. We examine if the SUMO ligase activity of PIASy is required for PIASy to repress AR. We show that a mutant PIASy, defective in promoting sumoylation, retains the ability to repress AR transcription. In addition, mutation of all the known sumoylation acceptor sites of AR does not affect the transrepression activity of PIASy on AR. Our results suggest that PIASy may repress AR by recruiting histone deacetylases, independent of its SUMO ligase activity.
Oncogene (2004) **23**, 3059–3066. doi:10.1038/sj.onc.1207443
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Keywords: protein inhibitor of activated STAT; androgen receptor; sumoylation

Introduction

The androgen receptor (AR) plays an important role in many normal and abnormal physiologic processes. Aberrant activation of the androgen-signaling pathway is central to prostate cancer development and progression. The molecular details responsible for the tissue- and ligand-specific control of androgen signaling remain largely unknown. However, a complex network of interactions between AR and accessory proteins are thought to explain the tight physiologic control of androgen signaling.

The protein inhibitor of activated STAT (PIAS) family was originally identified in a screen for proteins that interact with signal transducer and activator of transcription (STAT) (Shuai and Liu, 2003). PIAS1 and PIASy inhibit Stat1 through distinct mechanisms (Liu *et al.*, 1998; Liu *et al.*, 2001). PIAS3 specifically inhibits Stat3 (Chung *et al.*, 1997). Our laboratory and others have studied the interaction between AR and PIAS family members in the regulation of AR signaling. The PIAS family is unique among AR-interacting proteins in that related proteins exhibit strikingly different effects on AR-mediated gene transcription. PIAS1 and ARIP3/PIASxα have been shown to function as activators of androgen signaling (Moilanen *et al.*, 1999; Tan *et al.*, 2000). In contrast, PIASy is a potent inhibitor of AR (Gross *et al.*, 2001). PIASy interacts with the AR DNA-binding domain (DBD), but does not inhibit the ability of AR to bind to DNA. PIAS proteins are highly expressed in androgen-dependent tissues, including Sertoli cells and spermatagonia in the testes (Moilanen *et al.*, 1999; Tan *et al.*, 2000). A role for PIAS family members in the normal physiology of AR is further supported by the observation that the temporal pattern of expression corresponds with AR expression and function during rat testes development (Tan *et al.*, 2002).

The conjugation of small ubiquitin-related modifier (SUMO) to a variety of target proteins has recently been implicated in a variety of cellular processes, such as nuclear transport, signal transduction, apoptosis, autophagy, cell cycle control, and protein degradation (reviewed in Melchior, 2000). Recent studies point towards a potential role of PIAS family members as E3-like ligases that control the conjugation of SUMO (sumoylation) to critical target proteins, such as p53, c-Jun, and LEF1 (Kahyo *et al.*, 2001; Sachdev *et al.*, 2001; Kotaja *et al.*, 2002; Schmidt and Muller, 2002). PIAS proteins share a highly homologous central RING-finger like domain (RFD) that is required for their SUMO ligase activity (Aravind and Koonin, 2000; Johnson and Gupta, 2001). AR has recently been shown to be a target of sumoylation (Poukka *et al.*, 2000; Nishida and Yasuda, 2002). Consensus SUMO acceptor

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sites were identified in the amino-terminal region of AR that overlap with transcriptional synergy control motifs (Iniguez-Lluhi and Pearce, 2000). Sumoylation is promoted by the addition of androgenic ligands and mutations which abolish sumoylation result in a slightly more transcriptionally active AR mutant, suggesting that sumoylation maybe involved in the downregulation of AR transcription (Poukka *et al.*, 2000; Nishida and Yasuda, 2002).

This report describes our efforts to understand the molecular mechanism of PIASy-mediated AR repression. We show that PIASy directly interacts with AR and demonstrate that the RFD mediates the PIASy-AR interaction. We identify two distinct repression domains in PIASy, named RD1 and RD2. RD1, but not RD2, is required for PIASy-mediated AR repression. We show that PIASy physically interacts with HDAC1 and HDAC2 and that histone deacetylase activity (HDAC) is required for PIASy-mediated AR repression. We examine the involvement of the PIASy-associated SUMO ligase activity in the regulation of AR transcription. We show that mutations that abolish the SUMO ligase activity of PIASy and sumoylation acceptor sites in AR do not affect the ability of PIASy to inhibit AR signaling. Our data suggest that sumoylation is not involved in the transrepression of AR transcription by PIASy.

Results

PIASy directly binds to AR via the RFD

It has been shown that PIAS proteins can interact with an isolated portion of AR, namely the DNA-binding domain (Moilanen *et al.*, 1999; Tan *et al.*, 2000; Gross *et al.*, 2001). To examine if PIASy interacts with full-length AR in mammalian cells and to identify the AR interaction domain of PIASy, a GST-affinity binding assay was performed. A vector was designed to express full-length PIASy fused to GST (GST-PIASy). Cells were transfected with expression vectors for Flag-AR, GST, and GST-PIASy as indicated (Figure 1a). Lysates were incubated with glutathione-sepharose beads, thoroughly washed, and the association of AR with GST-PIASy was analysed by immunoblotting with anti-Flag antibody. The results show that full-length AR strongly interacts with GST-PIASy, but not GST alone.

To identify the region of PIASy responsible for the interaction with AR, GST fusion proteins were constructed which express various portions of PIASy joined in-frame to GST. Similar GST-pull down analysis was performed to analyse the binding of PIASy domains to AR. The results show that a 70-amino-acid region encompassing the RFD of PIASy was sufficient to mediate the interaction with AR (Figure 1b). GST-PIASy fusion proteins with deletions of the RFD domain show no significant binding to AR. We conclude that the RFD domain of PIASy mediates the PIASy-AR interaction.

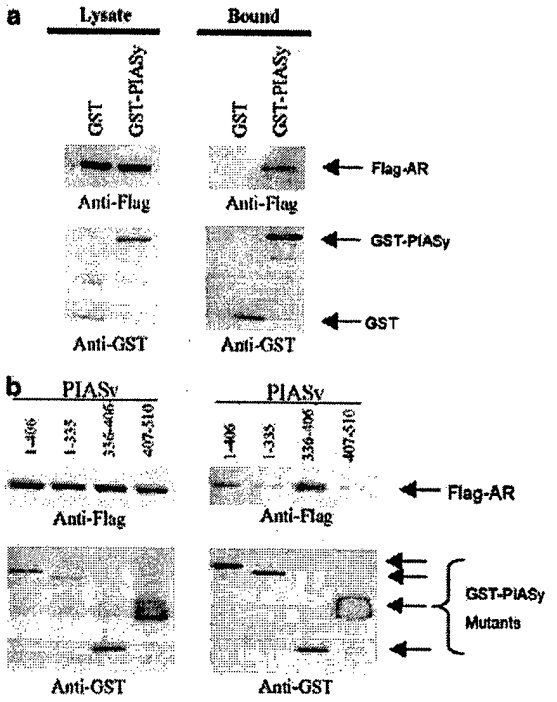

Figure 1 Full-length AR binds to the RING-finger domain of PIASy. (**a**) 293T cells were transfected with expression vectors for Flag-AR, GST, and GST-PIASy. Lysates were incubated with glutathione-sepharose beads, thoroughly washed, and bound proteins were analysed by immunoblotting with Flag antibody (right panel). The filter was stripped and probed with GST antibody to demonstrate protein loading. The input level of expression of Flag-AR and GST proteins was analysed by loading 2% of each lysate for immunoblotting before addition of GST beads (left panel). (**b**) Binding of Flag-AR to portions of PIASy fused to GST was analysed as in (**a**). Right panel demonstrates proteins bound to glutathione sepharose beads. Left panel demonstrates input level of protein expression in lysates used for binding reactions

PIASy RFD is required for the PIASy-mediated AR repression

If the RFD of PIASy is responsible for interacting with AR, then removing the RFD should abolish the ability of PIASy to repress AR. Therefore, we generated a PIASy mutant that contains an internal deletion of the 70 amino-acid region required for the AR interaction (PIASyΔRFD). The effect of this mutant on AR transcription was examined by luciferase reporter assays in 293T cells. Consistent with our earlier findings, wild-type PIASy is a potent repressor of AR (Gross *et al.*, 2001). However, PIASyΔRFD failed to inhibit AR-mediated transcription (Figure 2a). Western blot analysis confirmed comparable expression of wild-type and mutant PIASy proteins (Figure 2b). These results are consistent with the finding that the RFD of PIASy directly interacts with AR.

PIASy possesses distinct transcriptional repression domains

PIASy has been found to repress diverse classes of transcription factors including AR, Stat1, LEF1, and

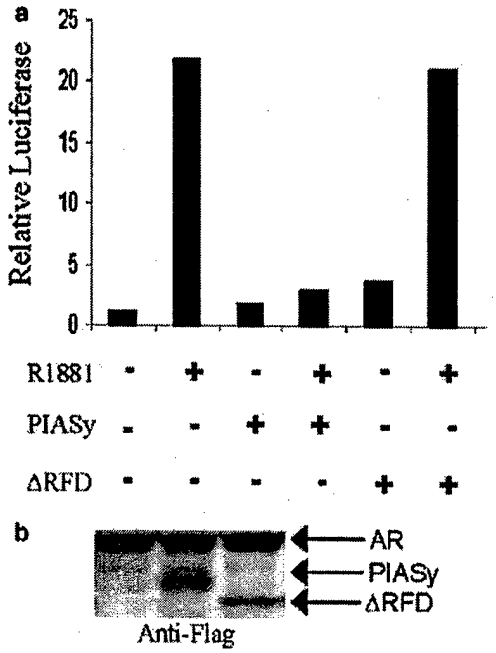

Figure 2 The RFD is required for PIASy-mediated AR repression. (**a**) 293T cells were transiently transfected with empty vector, PIASy, or PIASyΔRFD expression vectors. Human Flag-AR, PSA-E Luc and β-galactosidase plasmids were included in each transfection. Results are expressed as luciferase activity relative to β-galactosidase activity. (**b**) Expression of Flag-AR, PIASy, and PIASyΔRFD in the lysates shown in (**a**) was analysed by immunoblotting for Flag expression

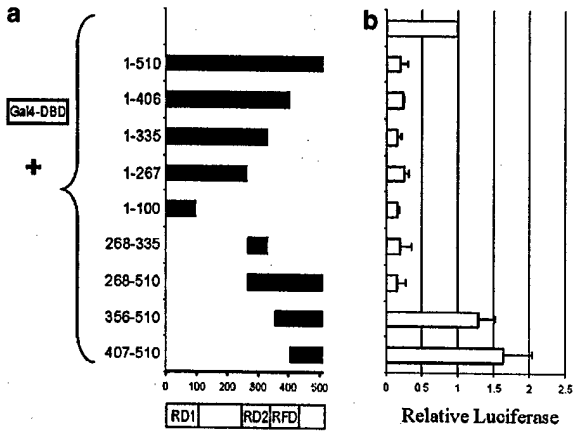

Figure 3 One-hybrid system identifies repression domains in PIASy. (**a**) Schema demonstrating expression vectors designed to express the Gal4-DNA-binding domain (Gal4-DBD) alone, or chimeric proteins with Gal4-DBD fused to various regions of PIASy. (**b**) Equal amounts of the Gal4-DBD vectors were transfected into 293T cells along with Gal4(5x)-Luc reporter construct. β-Galactisidase reporter was included to control for transfection efficiency. Data are expressed as the mean of at least three independent experiments (±s.d.) normalized to Gal-DBD alone

p53 (Gross *et al*., 2001; Nelson *et al*., 2001; Sachdev *et al*., 2001). We examined if PIASy contains intrinsic repressor domains. A strategy to identify transcriptional repression domains is to construct fusion proteins which contain a heterologous DNA binding domain, such as the GAL4-DBD, fused to putative repression domains from a protein of interest (Zamir *et al*., 1997; Ordentlich *et al*., 1999; Kotaja *et al*., 2000). The effect of these fusion proteins on the transcriptional activity of a reporter containing five copies of the GAL4 response element was examined. 293T cells were transiently transfected with expression vectors for GAL4-DBD, GAL4-DBD-PIASy, and GAL4-DBD fused to various portions of PIASy (Figure 3a). As expected, GAL4-DBD-PIASy strongly repressed transcription (Figure 3b). The removal of amino-acid residues 1–355 completely abolished the trans-repression activity of PIASy. Further deletional analysis uncovered two transcriptional repression domains within the amino-terminal region of PIASy. The first repression domain (RD1) spans residues 1–100 and includes an alpha-helical LXXLL motif that we have previously shown to be essential in AR- and Stat1-mediated repression (Gross *et al*., 2001; Liu *et al*., 2001). In addition, a second repression domain (RD2) is identified spanning residues 268–336 in the region immediately before the RFD. The GAL4-DBD-PIASy fusion protein can repress transcription in the presence of either the RD1 or RD2 domain (Figure 3b). Therefore, RD1 and RD2 are distinct transcriptional repression domains present in PIASy.

We next examined the roles of RD1 and RD2 domains in the repression of AR transcription by PIASy. Expression constructs encoding PIASy mutants lacking either RD1 (PIASy(268–510)) or RD2 (PIASyΔRD2) were generated. The ability of these PIASy mutants to influence AR-mediated gene activation was examined by transient reporter assays (Figure 4). Interestingly, while PIASy(268–510) failed to repress AR-mediated gene activation, the deletion of the RD2 domain had no effect on the ability of PIASy to repress AR. These results suggest that RD1, but not RD2, is required for PIASy to repress AR transcription.

To confirm that removal of the RD1 domain does not affect the ability of PIASy to interact with AR, we examined the interaction of PIASy(268–510) with AR using a GST-affinity matrix assay. PIASy(268–510) bound to GST-ARDBD equally as well as full-length PIASy (Figure 5a). We reasoned that if PIASy(268–510) is able to bind to AR but cannot repress AR transcription, it might display a dominant-negative effect on PIASy-mediated repression of AR. To test this hypothesis, PIASy(268–510) was transfected into 293T cells alone or together with wild-type PIASy. Coexpression of PIASy(268–510) with PIASy dramatically reduced the repressive effect of wild-type PIASy on AR-mediated luciferase activity (Figure 5b). Western blot analysis of the same samples confirmed the proper expression of wild-type and mutant PIAS proteins (Figure 5c). This is consistent with the conclusion that the RD1 domain of PIASy is a transcriptional repression domain that is required for PIASy to repress AR transcription.

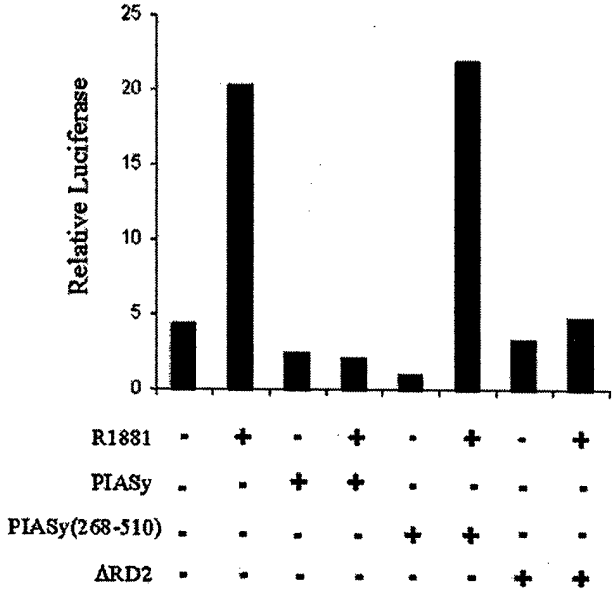

Figure 4 RD1, but not RD2, is required to repress AR transcription. AR signaling was reconstituted in 293T cells by transient transfection with Flag-AR and PSA E-Luc. The activity of empty vector, PIASy, PIASy(268–510), and PIASyΔRD2 on R1881-stimulated transcription was analysed as indicated

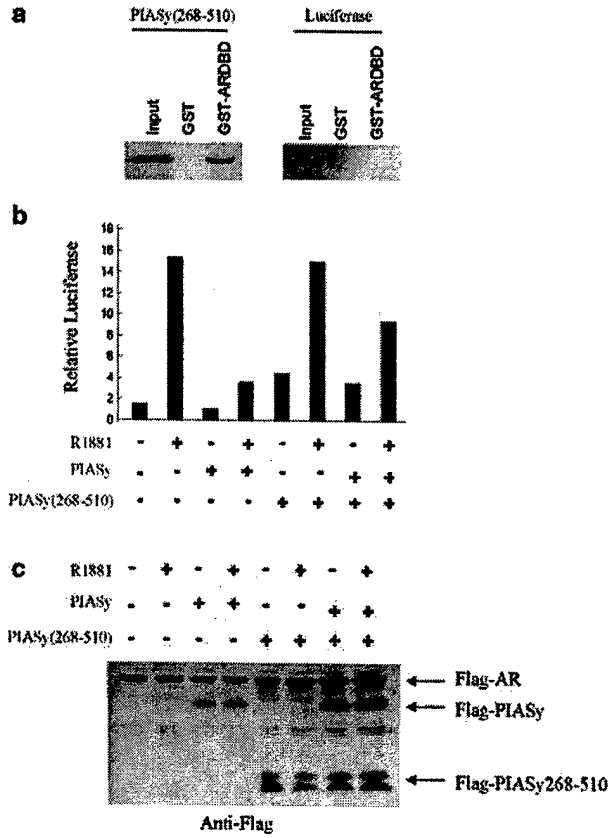

Figure 5 RD1 is not required for AR binding and functions as a dominant-negative mutant of PIASy. (**a**) Glutathione-agarose beads coated with approximately 100 μg of GST or GST-ARDBD were incubated with ^{35}S-labeled PIASy (268–510) or luciferase, as a control. The samples were washed and analysed by SDS–PAGE and autoradiography. Input represents 10% of the ^{35}S-labeled proteins in each binding reaction. (**b**) 293T cells were transiently transfected with empty vector, PIASy (25 or 80 ng), and PIASy(268–510) (400 or 500 ng) along with equal amounts of Flag-AR and reporter vectors. Expression vectors were adjusted to equalize protein expression levels. (**c**) Immunoblot demonstrating expression of Flag-tagged PIASy, PIASy(268–510), and AR in lysates shown in (**b**)

PIASy interacts with HDAC1 and HDAC2

HDACs are found in many repressor complexes and are part of a general mechanism of transcriptional repression in a wide variety of transcriptional contexts (Xu *et al*., 1999). We hypothesized that PIASy-mediated AR repression may involve the recruitment of HDACs. 293T cells were transfected with expression vectors for GST, GST-PIASy, and GST-PIASy(1–90), GST-PIASy(407–510) with or without Flag-HDAC1 (Figure 6a). Lysates were incubated with glutathione-sepharose beads. The beads were thoroughly washed and retained proteins analysed by immunoblotting. The results show that full-length PIASy physically interacts with HDAC1. There is no interaction with GST alone or GST-PIASy(407–510). Similar results were obtained when we examined the interaction of GST-PIASy and GST-PIASy domains with Flag-HDAC2 (Figure 6b). We conclude that the amino-terminal portion of PIASy (residues 1–90 containing the majority of the RD1 domain) is sufficient to mediate the interaction between PIASy and HDAC1/HDAC2.

If PIASy directly interacts with HDACs, then inhibition of HDAC activity should be able to overcome PIASy-mediated AR repression. The transient transfection assay was used to test this hypothesis (Figure 7). AR and ARE4xLuc constructs were transfected into 293T cells with an empty vector or a PIASy expression vector. Transfected cells were stimulated with 1 nM R1881 in the presence or absence of TSA, as indicated. Stimulation with 1 nM R1881 was associated with an increase in luciferase activity that is not appreciably changed by the presence of 100 nM TSA. As expected, PIASy inhibited the R1881-induced ARE reporter activity. However, simultaneous treatment with TSA overcomed the PIASy-mediated repression of AR signaling. Thus, TSA is able to reverse the PIASy-mediated AR repression. Taken together, these results suggest that PIASy directly interacts with HDAC1/2 through the amino-terminal RD1 domain, and that this interaction and HDAC-activity are required for PIASy-mediated AR repression.

Sumoylation is not required for PIASy-mediated AR repression

Protein sumoylation plays a role in the function and stability of many target proteins (Melchior, 2000). PIAS proteins have been suggested to promote the SUMO conjugation of several transcription factors, including p53, c-Jun, and LEF1 (Kahyo *et al*., 2001; Sachdev *et al*., 2001; Kotaja *et al*., 2002; Schmidt and Muller, 2002).

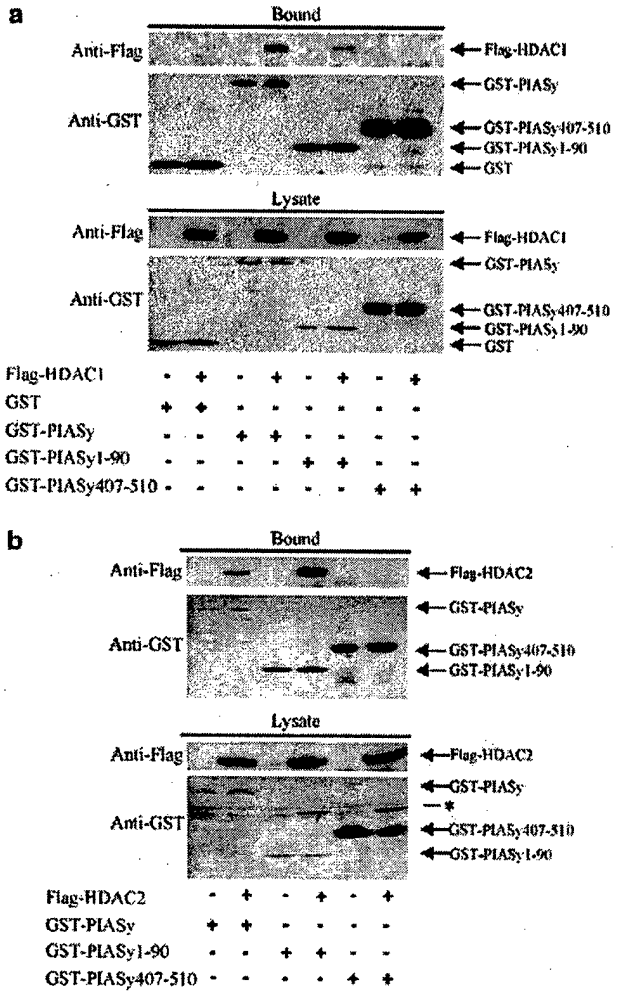

Figure 6 PIASy interacts with HDAC1 and HDAC2. (**a**) 293T cells were transfected with expression vectors for GST and GST-PIASy fusion proteins with or without Flag-HDAC1 as indicated (lower panel). Lysates were incubated with glutathione-beads, washed, and bound proteins analysed by immunobloting (upper panel). (**b**) Same as (**a**) except Flag-HDAC2 was used in the assays *: retained signal from Flag-HDAC2 immunoblot (upper panel)

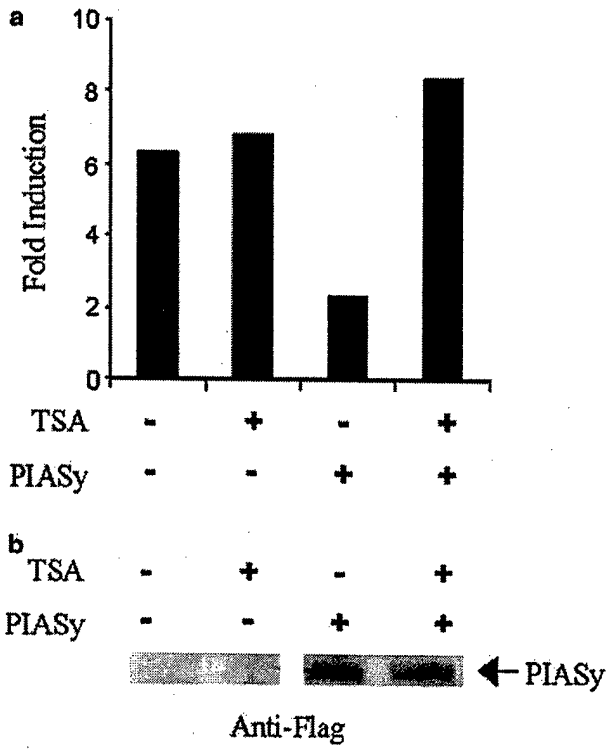

Figure 7 HDAC activity is required for PIASy-mediated AR repression. (**a**) 293T cells were transfected with AR and PIASy expression vectors (0.3 μg/well) along with ARE4xLuc and β-galactisidase reporter vectors. Cells were stimulated with 1 nM R1881 with or without 100 nM TSA. Fold induction is calculated as the ratio of relative luciferase units for stimulated versus unstimulated wells. (**b**) Immunoblot of R1881-stimulated lysates from (**a**)

A tryptophan residue in the RFD of PIAS family members is required for the SUMO activity of PIASxα/ARIP3 (Kotaja *et al*., 2002). Site directed mutagenesis was used to generate the corresponding PIASyW363A mutant. Cotransfection with Myc-tagged SUMO-1 in 293T cells demonstrated that PIASy potentiated the transfer of SUMO to many target proteins, and that this activity was defective in the PIASyW363A mutant (Figure 8a). We next investigated if the PIASy SUMO ligase activity was required for AR repression. PIASy and PIASy W363A were expressed at comparable levels in 293T cells and the effect on R1881-stiumulated AR transcriptional activity was examined (Figure 8b). The results show that both PIASy and PIASyW363A were equally effective at inhibiting AR-dependent transcription. We conclude that the PIASy-associated SUMO-ligase activity is not required for AR repression.

To examine the possibility that sumoylation was indirectly linked to AR repression, we generated an AR mutant unable to be a target of sumoylation (Poukka *et al*., 2000). A combination of *in vitro* and *in vivo* assays identified two lysine residues within (I/L/V)KXE sumoylation acceptor sites (K386 and K520) located in the amino-terminal region of AR (Johnson and Blobel, 1999; Sternsdorf *et al*., 1999; Poukka *et al*., 2000). A mutant AR in which K386 and K520 were replaced with arginine (AR-RR) was generated. The ability of the AR-RR mutant to activate transcription in the presence or absence of PIASy was examined by reporter assays. Consistent with earlier observations, AR-RR was able to activate transcription (Figure 9a). However, we did not observe a significant difference in the transcriptional activity between wild-type AR and the AR-RR mutant (data not shown). Coexpression of PIASy with AR-RR significantly repressed the ability of AR-RR to activate transcription. The magnitude of repression by PIASy on AR-RR was comparable with that on wild-type AR. Western blot analysis of the same samples showed proper expression of PIASy and AR proteins (Figure 9b). These results suggest that sumoylation of AR is not involved in PIASy-mediated repression.

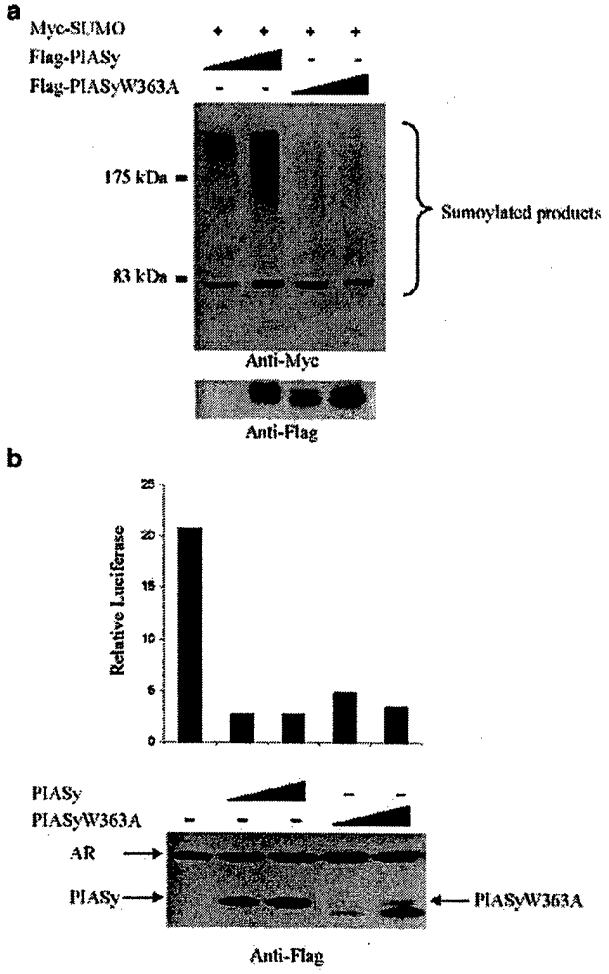

Figure 8 AR repression is independent of PIASy SUMO ligase activity. (**a**) 293T cells were transfected with expression vectors for Myc-SUMO along with Flag-PIASy (20 and 50 ng) or Flag-PIASyW363A (200 and 300 ng). Equivalent amounts of each cell lysates were probed with anti-Myc antibody (upper panel) and anti-Flag antibody (lower panel). (**b**) 293T cells were transfected with empty vector, Flag-PIASy, and Flag-PIASyW363A. Relative luciferase activity was measured as in Figure 3. The relative expression levels of PIASy and PIASyW363A were examined by Western blot analysis with anti-Flag antibody (lower panel)

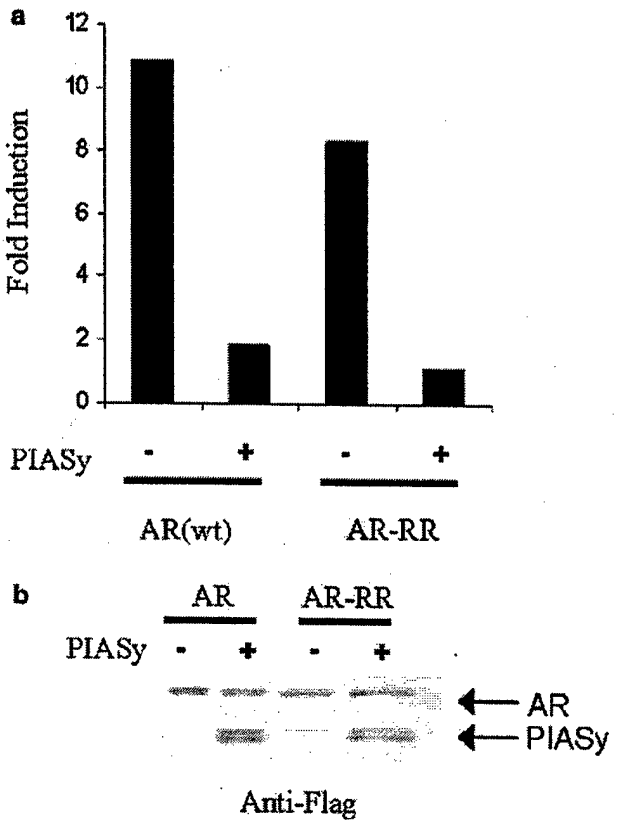

Figure 9 PIASy mediated AR repression is independent of AR sumoylation. (**a**) 293T cells were transfected with Flag-AR, Flag-AR-RR, and Flag-PIASy or empty vectors to equalize total amount of DNA. PSA-E Luc and β-galactosidase vectors were included in each transfection. Cells were grown in the presence or absence of 10 nM R1881. Fold induction is defined as the relative luciferase activity from paired samples grown in the presence or absence of 10 nM R1881. (**b**) Expression of AR, AR-RR and PIASy in lysates from (**a**)

Discussion

This paper reports the characterization of the molecular mechanism of PIASy-mediated repression on AR transcription. Our studies reveal several significant findings: (1) the RFD mediates the interaction of PIASy with full-length AR; (2) PIASy contains two transcriptional repression domains, but only the most amino-terminal of these is required for AR repression; (3) PIASy interacts with HDAC1/2 through the RD1 domain; (4) HDAC-activity is required for PIASy-mediated AR repression; and (5) PIASy-mediated repression of AR is independent of sumoylation.

The central portion of the PIAS family, including the RFD region, is the most highly conversed region among PIAS family members (Liu *et al.*, 1998). Multiple studies have shown that the RFD region is a particularly important domain in mediating the binding of PIAS family members to other proteins. The RFD is required for the interaction of both ARIP3/PIASxα and PIAS1 with Ubc9, the E2 SUMO conjugating enzyme (Kotaja *et al.*, 2002; Nishida and Yasuda, 2002). The RFD also mediates the interaction of ARIP3/PIASxα with the nuclear receptor coactivator, GRIP-1 (Kotaja *et al.*, 2002). The finding that the RFD also mediates the interaction of PIASy with AR highlights the need to further explore how sequences within the RFD may determine the specificity of the interaction of PIAS family members with different protein targets.

We have identified two transcriptional repression domains of PIASy. We show that the most amino-terminal of these is required for PIASy to repress AR. This is consistent with our previous observation that an LXXLL motif contained within the RD1 domain is required for repression of AR and Stat1 (Liu *et al.*, 2001; Gross *et al.*, 2001). The RD1 domain also contains a motif that has been suggested to interact with the

nuclear matrix (Sachdev *et al.*, 2001; Tan *et al.*, 2002). It is not known if the nuclear matrix attachment activity of PIASy may contribute to its repression on AR.

In contrast to PIASy, PIAS1 and PIAS3 enhance the transcriptional activity of AR (Gross *et al.*, 2001). Interestingly, an RD1-related domain is also present in PIAS1 and PIAS3. It remains to be determined if theRD1-related domain is involved in the enhancement of AR-mediated transcription by PIAS1 and PIAS3.

We show that PIASy can directly interact with HDAC1 and HDAC2. HDACs are found in many repressor complexes and are part of a general mechanism of transcriptional repression in a wide variety of transcriptional contexts (Xu *et al.*, 1999). PIASy has been found to be a potent repressor for diverse classes of transcription factors including AR, Stat1, LEF1, and p53 (Gross *et al.*, 2001; Nelson *et al.*, 2001; Sachdev *et al.*, 2001). Our results suggest that PIASy may recruit HDACs or HDAC-containing corepressor complexes as a mechanism of AR repression.

It has been suggested that the SUMO ligase activity of PIAS proteins may be involved in the regulation of several transcription factors. We have directly tested if the SUMO ligase activity of PIASy is involved in AR repression. We show that a mutant PIASy that is defective in SUMO ligase activity retains the ability to repress AR transcription. In addition, mutation of all known sumoylation acceptor sites does not affect the transrepression activity of PIASy on AR. Our results suggest that sumoylation is not required for PIASy to repress AR transcription.

Materials and methods

Plasmids

The mammalian expression vector pEBG was produced by inserting the coding sequence of glutathione-S-transferase (GST) upstream of the EF-1α promoter in pEBB (Mizushima and Nagata, 1990). The mammalian expression vector pCDNA3-Gal4 was produced by subcloning the coding sequences corresponding to residues 1–147 of Gal4 (Emami and Carey, 1992) into the *Bam*HI/*Eco*RI sites of pCDNA3 (Invitrogen). Plasmids designed to express GST-PIASy or Gal4-PIASy chimeric proteins were produced by subcloning the desired region of PIASy into the *Bam*HI/*Not*I sites of pEBG and pCDNA3-Gal4, respectively.

A PCR-based strategy was used to generate PIASyΔRFD, PIASyW363A, PIASy(268–510), and PIASyΔRD2 based on the Flag-PIASy vector (Gross *et al.*, 2001). Flag-AR was produced by cloning the coding sequence from pBS-fAR (Huang *et al.*, 1999) into the *Xba*I site of pCDNA3 (Invitrogen). A PCR-based mutagenesis strategy was used to generate Flag-AR-RR. Individual primer sets were designed to introduce AAG to AGG mutation corresponding to the Lys-386 to Arg and AGG to AGA corresponding to the Lys-520 to Arg mutations described by Poukka *et al.* (2000). All mutations were confirmed by standard sequencing reactions. Flag-HDAC1 was produced by cloning HDAC1 (a gift from Dr Eisenman (Laherty *et al.*, 1997)) into the *Bam*HI/*Sal*I sites of pCF. Flag-HDAC2 has been previously described (Laherty *et al.*, 1997). The PSA E-Luc, ARE(4x) E-Luc, and GAL4(5x) E-Luc reporter vectors have also been described previously (Gross *et al.*, 2001; Wu *et al.*, 2001; Zhang *et al.*, 2002).

Cell culture

293T cells were maintained in DMEM. Media was supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen/Life Technologies). Trichostatin A (TSA) was purchased from Sigma-Aldrich (St Louis, MO, USA).

Gst-affinity matrix binding assays

293T cells were transiently transfected with Flag-AR or Flag-HDAC1 and equal amounts of pEBG, pEBG-PIASy, or pEBG-PIASy deletion constructs. Cells were lysed in buffer containing 150 mM NaCl, 50 mM Tris pH 8.0, 0.5% NP-40, 10% glycerol, 1 mM EDTA with proteinase inhibitors (1 mg/ml aprotonin, 1 mg/ml leupeptin, 1 mM PMSF). For AR interaction assays, an equal portion of each lysate (50 μl) was added to 450 μl of binding buffer (100 mM NaCl, 25 mM Tris pH 7.5, 1 mM EDTA, 1% Triton X-100, 10 mM NaF, 0.5 mM DTT) with proteinase inhibitors. A measure of 30 μl of glutathione-sepharose 4B beads (Amersham Biosciences) were added to each binding mixture and the samples were rotated at 4°C for 1–2 h. For HDAC1 interaction assays, 50 μl of glutathione-sepharose beads were added directly to lysates. The beads were washed four to five times with binding buffer followed by one wash with 100 mM NaCl/25 mM Tris pH 7.5 or PBS. SDS-sample buffer was added and the samples were boiled. Bound proteins were analysed by immunoblotting. *In vitro* GST-pull down assays with ^{35}S-labeled proteins were performed as previously described (Gross *et al.*, 2001).

Transfection and reporter assays

293T cells were transiently transfected with equal amounts of pCDNA3-Gal4, Gal4-PIASy, or Gal4-PIASy mutants along with Gal4(5x)-Luc. An expression vector for β-galactisidase was included in each well to control for transfection efficiency. Cells were harvested 28–36 h after transfection and luciferase activity was determined according to the manufacture's instruction (Promega Corp.) The activity of each Gal4-PIASy construct determined relative to the pCDNA3-Gal4 control was determined for each experiment and the values were averaged over at least three independent experiments. Androgen signaling was monitored in 293T cells transfected with Flag-AR and PSA-E Luc or ARE (4x) Luc as previously described (Gross *et al.*, 2001). Cells were transfected via the calcium phosphate method and then grown in DMEM supplemented with 5% charcoal-dextran treated FBS (Omega Scientfic) with or without 1 nM R1881 for an additional 24 h. Luciferase activity was determined relative to β-galactosidase activity. Fold induction is defined as the relative luciferase activity of stimulated versus unstimulated paired samples. Each experiment was repeated at least three times, and one representative experiment is shown.

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