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hZimp10 is an androgen receptor co-activator and forms a complex with SUMO-1 at replication foci

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The androgen receptor (AR) plays a central role in male sexual development and in normal and malignant prostate cell growth and survival. It has been shown that transcriptional activation of AR is regulated through interaction with various co-factors. Here we identify a novel PIAS-like protein, hZimp10, as an AR-interacting protein. The transactivation domain (TAD) of AR and the central region of hZimp10 were found to be responsible for the interaction. A strong intrinsic transactivation domain was identified in the C-terminal, proline-rich region of hZimp10. Endogenous AR and hZimp10 proteins were co-stained in the nuclei of prostate epithelial cells from human tissue samples. In human prostate cancer cells, hZimp10 augmented the transcriptional activity of AR. Moreover, hZimp10 co-localized with AR and SUMO-1 at replication foci throughout S phase, and it was capable of enhancing sumoylation of AR *in vivo*. Studies using sumoylation deficient AR mutants suggested that the augmentation of AR activity by hZimp10 is dependent on the sumoylation of the receptor. Taken together, these data demonstrate that hZimp10 is a novel AR co-regulator.

Keywords: androgen receptor/nuclear hormone receptors/PIAS/prostate cancer/sumoylation

Introduction

The effects of androgens are mediated by the androgen receptor (AR), which plays a critical role in male sexual development and in prostate cell growth and survival (Jenster, 1999). AR belongs to the nuclear receptor superfamily and contains four functional domains: a transactivation domain (TAD), a DNA-binding domain (DBD), a hinge region and a ligand-binding domain (LBD) (Zhou *et al*., 1994). The unbound AR forms a complex with heat-shock proteins (HSPs) (Sanchez *et al*., 1990). Upon binding to ligand, the AR dissociates from the HSPs and translocates into the nucleus, where it binds to the androgen response element (ARE) and stimulates ligand-dependent transcription (Zhou *et al*., 1994). Like other receptors, AR can bind to different co-factors through its distinct functional domains (Heinlein and Chang, 2002). Through such interactions, the co-factors can modulate AR activity. One of the mechanisms by which co-factors regulate AR activity is by modification of the AR protein. It has been documented that the AR can be modified by phosphorylation (Nazareth and Weigel, 1996), acetylation (Fu *et al*., 2000) and sumoylation (Poukka *et al*., 2000) in cells.

Members of the PIAS (protein inhibitor of activated STAT) family were originally identified as transcriptional co-regulators of the JAK–STAT pathway (Chung *et al*., 1997). PIAS1 and PIAS3 have been shown to block DNA binding of STAT1 and STAT3, respectively, and to inhibit their action (Chung *et al*., 1997; Liu *et al*., 1998). Crosstalk between PIAS proteins and other signaling pathways has also been demonstrated to be involved in various cellular processes (see review by Jackson, 2001). PIASxα was first isolated as an AR-interacting protein (ARIP3), which binds to AR and modulates AR-mediated transcription (Moilanen *et al*., 1999). Other members of the PIAS family have also been shown to play a role in regulating the activity of AR and other nuclear hormone receptors (Kotaja *et al*., 2000; Tan *et al*., 2002).

PIAS and PIAS-like proteins share a zinc finger domain, termed Miz, in the central region (Wu *et al*., 1997). The Miz domain (Msx-interacting zinc finger) was shown to mediate the interaction between Msx2 and PIASxβ. An increasing number of proteins from invertebrates have been found to contain the Miz domain, suggesting a conserved and biologically important role of PIAS proteins throughout evolution.

The SUMO (small ubiquitin-related modifier) conjugation system has been extensively studied and it shares similarity with the ubiquitin-conjugation system (Melchior, 2000). Modification by SUMO-1 (sumoylation) is a three-step process, involving the E1 enzyme Aos1/Uba2, the E2 enzyme Ubc9 and the recently identified E3-like ligases, such as the nucleoporin RanBP2 (Melchior, 2000; Jackson, 2001; Pichler *et al*., 2002). While modification of proteins by SUMO-1 is a covalent process, it is reversible through the activity of a number of specific isopeptidase enzymes (Nishida *et al*., 2001). Although the specific mechanism by which SUMO-1 modification modulates cellular functions remains unclear, it is believed that unlike ubiquitination, sumoylation does not promote protein degradation but rather is involved in mediating protein–protein interactions, subcellular compartmentalization and protein stability. Recent data suggest that sumoylation is important in the regulation of transcription (Verger *et al*., 2003). Sumoylation of both steroid

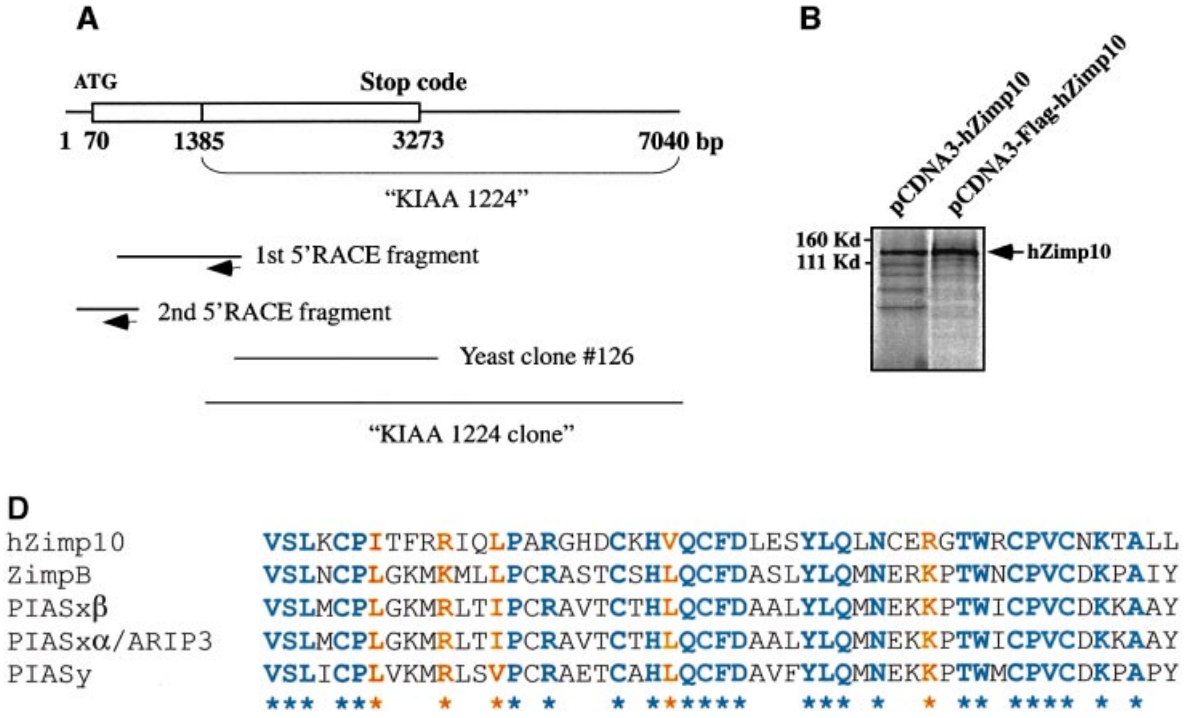


Fig. 1. Nucleotide and predicted amino acid sequence of hZimp10. (**A**) Diagram of the hZimp10 transcript. Both 5′ and 3′ untranslated regions are shown as a solid line and the coding region is displayed as an open box. The translation start site (ATG) and stop codon are labeled with nucleotide numbers. (**B**) The pcDNA3 constructs containing full-length hZimp10 cDNA, either with or without Flag-epitope tag, were transcribed and translated *in vitro*. Proteins were labeled and separated on an SDS–PAGE. (**C**) The full-length cDNA and predicted amino acid sequences of hZimp10 are shown (DDBJ/EMBL/GenBank accession No. AY235683). The putative functional domains are colored: a nuclear localization signal (green), a Miz domain (red) and two proline-rich regions (pink). (**D**) Alignment of the Miz domain of hZimp10 with those of other PIAS proteins is shown. Identical or similar amino acids are shaded with either blue or orange.

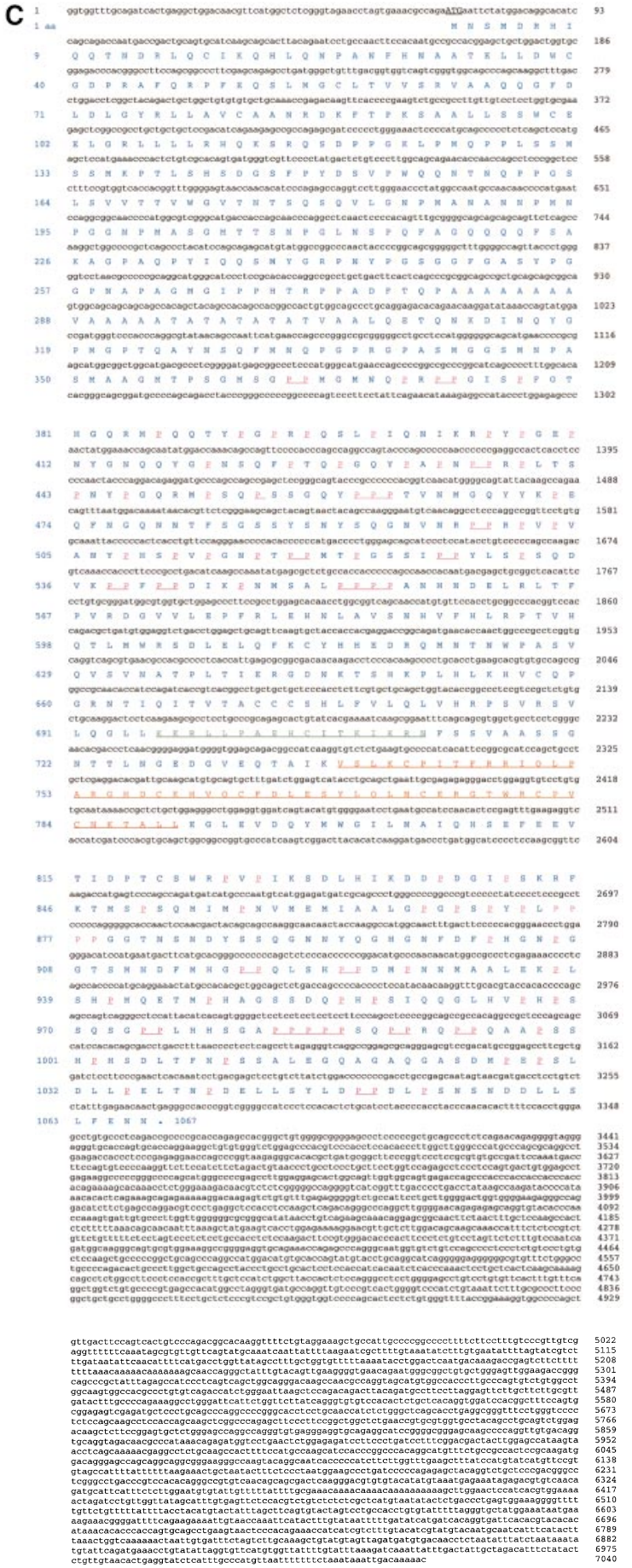
receptors and their co-factors have been demonstrated (Poukka *et al*., 2000; Kotaja *et al*., 2002).

Recently, PIASxα, xβ, 1 and 3 have been found to interact with SUMO-1 and Ubc9 and mediate sumoylation of steroid receptors and other transcription factors (Kahyo *et al*., 2001; Kotaja *et al*., 2002; Nishida and Yasuda, 2002). PIAS proteins are also modified by sumoylation and are involved in the process of sumoylation of other binding partners (Jackson, 2001; Kotaja *et al*., 2002). Here we identify a novel AR-interacting protein that augments AR-mediated, ligand-dependent transactivation in prostate cells. Sequence analysis of the protein showed that it contains a conserved Miz domain and is a new PIAS-like protein. Based on these features, we named the protein as hZimp10 (human zinc finger-containing, Miz1, PIAS-like protein on chromosome 10). Specific interaction between AR and hZimp10 was demonstrated both *in vitro* and *in vivo*. Importantly, endogenous AR and hZimp10 proteins were co-stained in the nuclei of prostate epithelial cells in human tissue samples. Fusion of hZimp10 to a heterologous DNA-binding domain revealed a strong transactivation domain in the C-terminal region of hZimp10. In addition, we have demonstrated that hZimp10 co-localizes with AR and SUMO-1 in the nucleus and forms a protein complex at replication foci. Moreover, we showed that hZimp10 is able to enhance the sumoylation of AR in intact cells. Using AR with mutations in the two major sumoylation sites, we demonstrated that augmentation of AR activity by hZimp10 is dependent on the sumoylation of the receptor. Thus, these data demonstrate that hZimp10 acts as an AR co-regulator and modifies AR activity in transcription and DNA replication.

Results

Isolation of a novel PIAS-like protein, hZimp10

To understand the mechanism of AR-mediated transactivation of genes, we employed a modified yeast two-hybrid system using a bait construct containing a partial TAD sequence [amino acids (aa) 1–333] of the AR (James *et al*., 1996). Of 7.4×10^6 transformants, 13 grew under selective conditions and showed increased β-galactosidase (β-gal) production. Rescue of the plasmids and sequencing of the inserts revealed several different cDNAs. Among them, two cDNAs (clone numbers P126 and P233) encoded a novel sequence, and analysis of these mouse cDNA clones with the NCBI DDBJ/EMBL/GenBank database suggested that the human KIAA1224 cDNA clone was the homolog. Since KIAA1224 is a truncated fragment, we performed the rapid amplification of 5′ cDNA ends (5′-RACE) to isolate the full-length cDNA (Figure 1A). Sequence analysis of the full-length clone created by combining the 5′-RACE fragments and the KIAA1224 clone revealed a methionine initiation codon at nucleotide 70, followed by an open reading frame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encoding a 1067 aa protein with a predicted molecular weight of 123 kDa (DDBJ/EMBL/GenBank accession No. AY235683) (Figure 1C). Using *in vitro* transcription and translation, an ~130 kDa protein was generated by the full-length clone (Figure 1B), confirming the identity of the predicted initiation codon. The clone with an N-terminal Flag epitope-tag encoded a protein with a molecular weight similar to the clone with the natural initiation codon.

A BLAST search of the human genome database showed that this full-length sequence is located on human chromosome 10 at 10q22.1–22.3 and is comprised of 21 putative exons. Analysis of the protein sequence showed that the clone contains several functional domains, including a Miz domain, a nuclear localization signal sequence and two proline-rich regions (Figure 1C). PIAS and PIAS-like proteins share a highly conserved Miz domain, and a high degree of sequence similarity was observed when our clone was aligned with the zinc fingers of other PIAS proteins (Figure 1D). Based on these features, we named this protein as hZimp10 (human zinc finger-containing, Miz1, PIAS-like protein on chromosome 10).

hZimp10 interacts with the AR

We initially identified Zimp10 through a yeast two-hybrid screen with a 'bait' containing the partial TAD of AR. To confirm the interaction and identify the specific region of AR responsible for the interaction, we co-transformed either the clone of Zimp10 that was isolated from the initial screen or the constructs containing either GAL4 DBD alone or various fusions of different fragments of AR, respectively (Figure 2A). A liquid β-gal assay was performed to quantify the interactions. The AR/TAD-DBD and AR/pTAD1 constructs showed an ~23- or 16-fold induction with pVP16-Zimp10 compared with pVP16 alone. However, the clone with deletion of the region between aa 243–333 (AR/pTAD2) showed virtually no interaction with Zimp10, suggesting that the region between aa 243–333 is critical for the interaction. No significant production of β-gal was observed in the samples co-transformed with Zimp10 and other AR constructs containing the DBD, DBD-LBD and LBD.

To map the interaction region of Zimp10, we generated several truncated mutants of hZimp10 and assessed their abilities to interact with AR. As shown in Figure 2B, the full-length fragment and the C-terminal deletion mutant containing aa 1–790 possess strong interaction activity. Further deleting the C-terminal sequence of hZimp10 gradually reduces β-gal activity. The fragment containing aa 1–333 showed only minimal activity. The mutant containing aa 556–1067 showed the highest levels of β-gal activity among the N-terminal truncation mutants. These results suggest that the region between aa 556 and 790 contributes to the interaction with the AR. An additional mutant that contains only the central region of the protein (aa 556–790) was generated and used to map the precise interaction region. As shown in Figure 2B, this mutant showed the highest β-gal activity, indicating that the central region between aa 556 and 790 is the primary binding region for AR.

To confirm that AR and hZimp10 are physically associated in intact cells, co-immunoprecipitation assays were carried out to detect a possible protein complex. We expressed the Flag-tagged hZimp10 together with AR in CV-1 cells. Whole-cell lysates containing equal amounts of proteins were immunoprecipitated with normal mouse IgG or an anti-Flag monoclonal antibody. As shown in Figure 2C, Flag-hZimp10 proteins were detected in the immunoprecipitates with anti-Flag antibody, but not in the ones with normal IgG. These data demonstrate that an interaction between AR and hZimp10 occurs in mammalian cells.

hZimp10 is selectively expressed in human ovary, testis and prostate

Northern blot analysis was carried out to examine the expression of hZimp10. Three probes isolated from the N-terminal (aa 1–196), central (aa 330–496) and C-terminal (aa 880–1060) regions of the hZimp10 cDNA were used to ensure that the full-length sequence of hZimp10 constructed by combining three cDNA fragments together corresponded to a natural transcript (Figure 3A). A 7.2 kb hZimp10 transcript was detected by all three probes in various tumor cell lines (Figure 3B), which is consistent with the cDNA size of hZimp10 (Figure 1C). LAPC4, LNCaP, MCF7 and PC3 showed relatively high signals (Figure 3B and C). Using probe 1, we also examined the transcript profile of hZimp10 in human tissues. As shown in Figure 3D, a 7.2 kb transcript of hZimp10 was detected most abundantly in ovary, and at lower levels in prostate, spleen and testis. There was little or no detectable signal in thymus, small intestine, colon and peripheral blood leukocytes.

hZimp10 contains a very strong transactivation domain

Next, we investigated a possible role for hZimp10 in transcription. Fragments containing the full-length or N-terminal truncation mutants of hZimp10 were targeted to DNA by fusion with the GAL4 DBD. These constructs were then tested for their abilities to modulate transcription from a minimal promoter, derived from the chicken myelomonocytic growth factor gene (Sterneck *et al.*, 1992). Fusion of the GAL4 DBD to the full-length fragment of hZimp10 showed an ~7-fold induction compared with the GAL4 DBD alone (Figure 4). Deletions of the N-terminal region between aa 1 and 556 did not significantly affect transcription. However, removal of the region between aa 556 and 692 produced a dramatically elevated transcriptional activity. The mutant containing the C-terminal proline-rich region (aa 829–1067) showed 60- to 80-fold more transcriptional activity than that of the full-length hZimp10 construct. It should be noted that the transcriptional activity mediated by the C-terminal proline-rich region of hZimp10 is much stronger than that mediated by the TADs of other transcriptional factors that we have examined, including p53, Smad3, PU1, GATA2, AR and estrogen receptor α (ERα) (data not shown), and is comparable to the activity of the TAD of VP16 (Figure 4). Identification of the strong transcriptional activation domain confirms a functional role for hZimp10 in transcriptional regulation, providing the first line of evidence showing that a PIAS-like protein can regulate transcription through an intrinsic TAD.

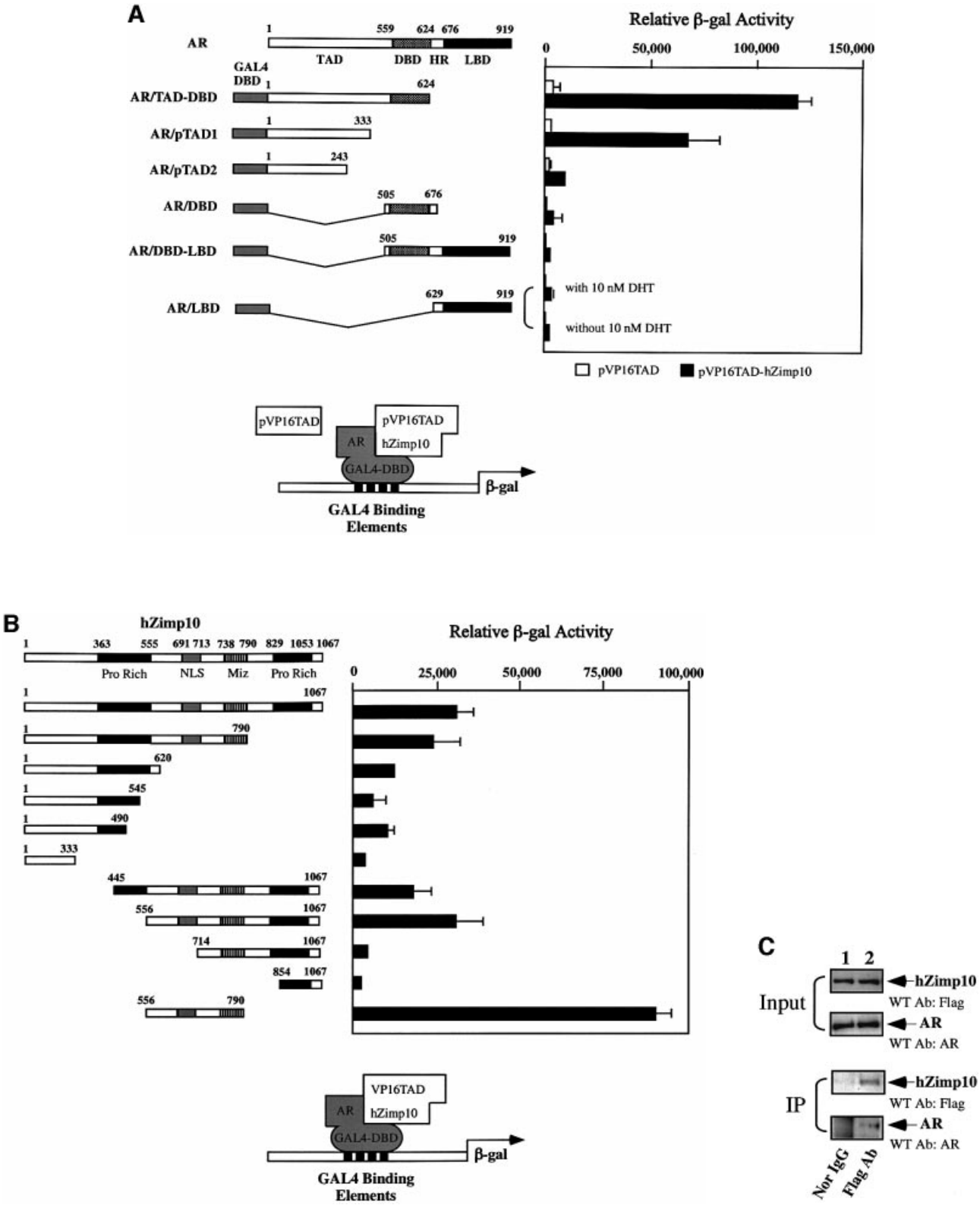

Fig. 2. Specific interaction between hZimp10 and AR. (**A**) cDNA fragments containing different portions of the human AR were fused to GAL4DBD in the pGBT9 vector. Numbers correspond to amino acid residues. The AR vectors were co-transformed with the pVP16 vector alone or the pVP16-Zimp10 construct. Three independent colonies were inoculated from each transformation experiment for a liquid β-gal assay. The data are shown as the mean ± SD. (**B**) Different truncation mutants of hZimp10 were generated by fusing fragments of hZimp10 to the TAD of VP16, and co-transformed with the pGBT9-AR/pTAD1. Transformants were selected and analyzed. (**C**) CV-1 cells were transiently co-transfected with AR and Flag-tagged hZimp10. Equal amounts of whole-cell lysates were blotted with AR or Flag antibodies to detect expression of the two proteins (input) and subjected to immunoprecipitation with normal mouse IgG or anti-Flag monoclonal antibody. The precipitated fractions were then resolved by SDS–PAGE and analyzed by western blotting using anti-Flag antibody or anti-AR antibody (IP).

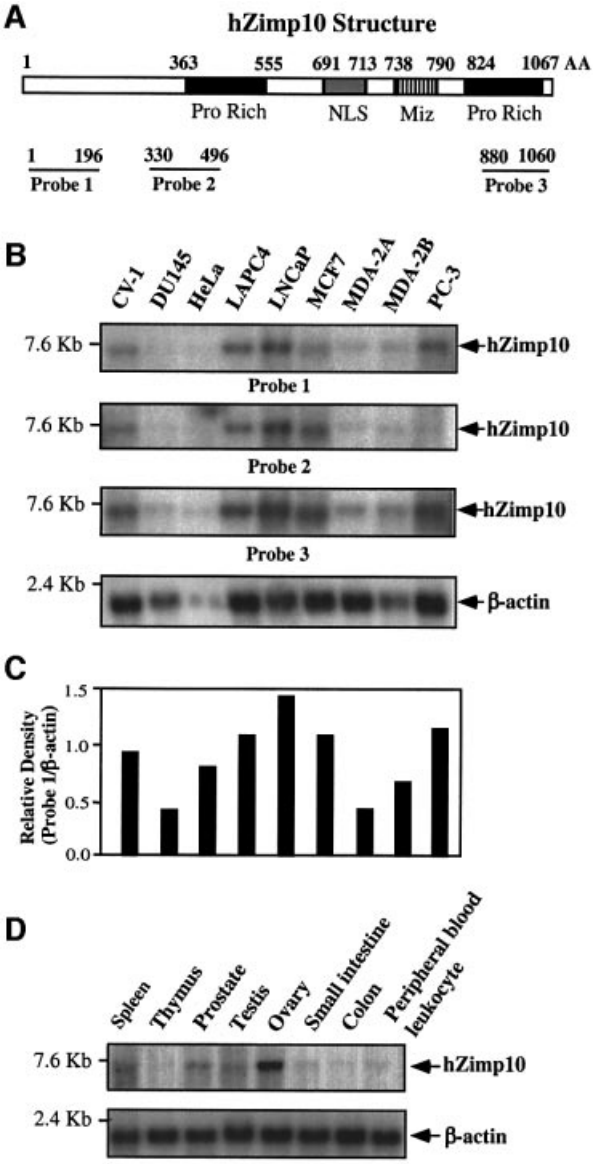

Fig. 3. Expression of hZimp10 in cell lines and human tissues. (**A**) A schematic representation of the hZimp10 protein is shown with the locations of three probes indicated. The numbers correspond to the amino acid sequence of the protein. (**B**) Northern blots containing poly(A)$^+$ RNA samples from various cell lines were hybridized with the three hZimp10 probes and a probe derived from human β-actin cDNA. (**C**) Relative densities (signals of probe 1 divided by those of β-actin) were used to measure the expression levels. (**D**) The blot with multiple human tissues was probed with probe 1 and the fragment of β-actin.

hZimp10 selectively augments AR-mediated transcription

Given the identification of an intrinsic activation domain within hZimp10 and a protein–protein interaction between AR and hZimp10, we further examined the effects of hZimp10 on AR-mediated transcription. Transient transfection experiments were carried out with plasmids expressing AR, hZimp10, and a luciferase reporter driven by the 7 kb promoter of the prostate-specific antigen (PSA) gene (Pang *et al.*, 1997). A basic ligand-dependent transactivation was observed in the CV-1 cells transfected with AR plasmid alone (Figure 5A). Co-transfection of hZimp10 construct augmented AR activity. With 20 ng of hZimp10 plasmid, AR-dependent transcription was increased 4-fold. To ensure that augmentation of the PSA promoter by hZimp10 was mediated through the AR rather than through other transcription factors, we examined the effect of hZimp10 on the transcription from a luciferase reporter driven by a minimal promoter with two AREs. A similar ligand-dependent enhancement of AR-mediated transcription by hZimp10 plasmid was observed from the ARE-luciferase reporter (Figure 5B). These results indicate that hZimp10 functions as a co-activator of AR and is able to enhance AR-mediated transcription.

To evaluate the enhancement by hZimp10 in a physiologically more relevant cellular context, an AR-positive prostate cancer line, LNCaP, was transfected with an hZimp10 construct along with the luciferase reporter driven by the PSA promoter. As shown in Figure 5C, overexpressed hZimp10 enhanced endogenous AR-mediated transcription from the PSA promoter. These data demonstrate the capability of hZimp10 to augment endogenous AR activity in prostate cancer cells.

The specificity of hZimp10-mediated augmentation was further investigated with other nuclear receptors, including the glucocorticoid receptor (GR), progesterone receptor (PR), ERα, thyroid receptor β (TRβ), and vitamin D receptor (VDR), As shown in Figure 5D, there was no significant effect of hZimp10 on GR, PR, ERα and VDR-mediated transcription. hZimp10 showed a slight enhancement of TRβ-mediated transcription. These results are consistent with our previous observation that there is no significant interaction between the hZimp10 protein and other nuclear receptors in yeast (data not shown).

hZimp10 protein is specifically expressed in prostate epithelial cells and co-stained with the AR protein

Our results demonstrate that hZimp10 is a novel AR-interacting protein that augments AR-mediated transcription. To further explore the potential biological role of hZimp10, we examined the expression of hZimp10 in human prostate tissues. Two adjacent sections from each tissue sample were stained with either anti-AR or anti-hZimp10 antibody. As reported previously, AR was found exclusively in the nucleus of prostate epithelial cells (Figure 6A, C, E and G). hZimp10 proteins showed a strong nuclear and cytoplasmic staining in prostate epithelial cells (Figure 6B, D, F and H). There was no, or very weak, staining in the stromal elements with either antibody in all samples examined. Comparing the staining patterns from the two adjacent sections, a clear co-staining of AR and hZimp10 proteins was found in the nucleus of prostate epithelial cells. These data provide the first line of evidence that hZimp10 can interact with AR in a biologically relevant manner and indicate that hZimp10 may play a role in modulating androgen signaling.

hZimp10 is found at cell cycle-regulated DNA replication foci throughout S phase

A number of PIAS proteins have been shown to localize to the nucleus and to display speckled patterns of nuclear distribution (Kotaja *et al.*, 2002). Transfection with expression plasmids encoding Flag-tagged hZimp10 in CV-1 cells showed a similar pattern of nuclear distribution

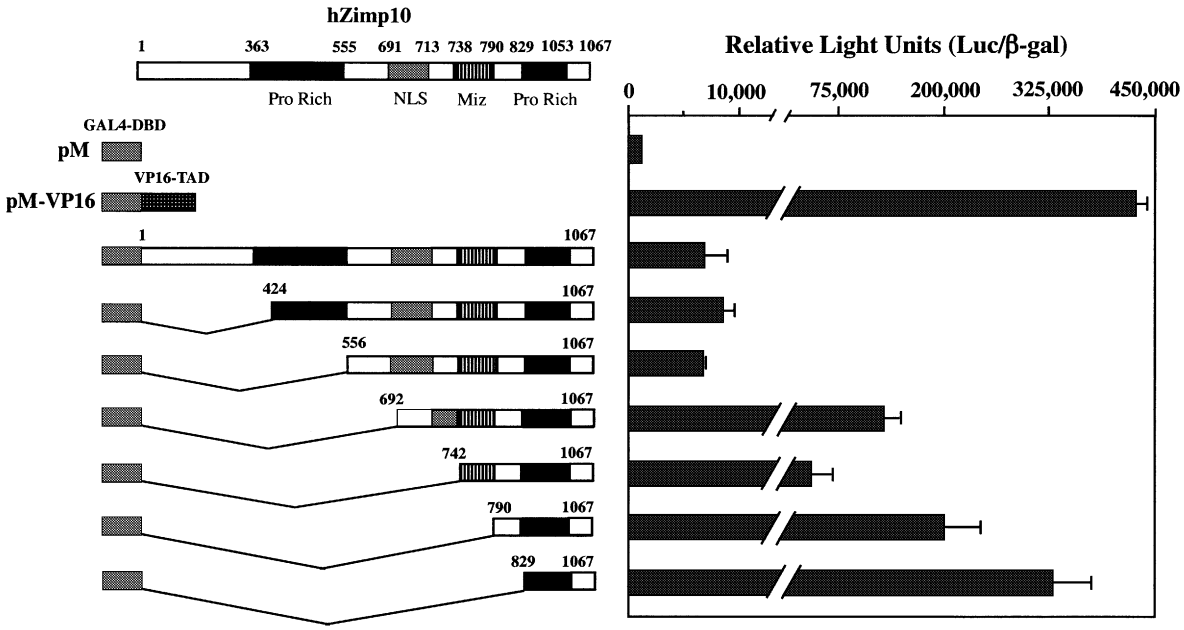

Fig. 4. Detection of intrinsic transcriptional activity of hZimp10. Full-length or truncated fragments of hZimp10 were fused to GAL4 DBD in the pM vector. Numbers correspond to amino acid residues. The pM constructs were co-transfected with luciferase reporter constructs containing the chicken myelomonocytic growth factor gene minimal promoter (–41 to +61) into CV-1 cells. Data are presented in relative light units (RLU), which were obtained by normalizing the activities of luciferase to those of β-gal. The results are reported as the mean ± SD from representative experiments.

as with the Flag-ARIP3 plasmid (data not shown), suggesting that hZimp10 may be involved in DNA replication structures in interphase nuclei. Distinct patterns of DNA replication have been identified during S phase in mammalian cells, and the size, shape and number of replication foci change during cell cycle progression (O'Keefe *et al*., 1992). Using immunofluorescence imaging, we systematically probed a potential role of hZimp10 in DNA replication. Cells were synchronized in late G_1 phase with mimosine and then allowed to enter S phase (Krude, 1999). Newly synthesized DNA was identified by 5-bromodeoxyuridine (BrdU) labeling and staining. An identical pattern of replication foci in synchronized cells was observed during S phase progression (Figure 7, left panel). Replication foci changed from numerous small, punctate structures in early S phase cells to large, toroidal structures in late S phase cells. Intriguingly, hZimp10 labeled by the Flag monoclonal antibody and newly synthesized DNA labeled by BrdU displayed a similar pattern of nuclear distribution (Figure 7, left and middle panels), and were overlaid throughout S phase (right panel). Our results demonstrate that hZimp10 can be found at sites of DNA synthesis throughout all phases of DNA replication, suggesting that hZimp10 may have a role in DNA synthesis.

hZimp10 co-localizes with AR and SUMO-1 during cell cycle progression

Next, we examined whether hZimp10 co-localizes with AR during cell cycle progression. To more systematically characterize localization of AR and hZimp10 throughout S phase, we synchronized cells in late G_1 phase, and then allowed the cells to progress through S phase. In cells synchronized in late G_1 phase, both AR and Flag-hZimp10 showed diffuse nuclear staining (Figure 8A). When merged, these staining patterns showed a considerable amount of overlaying (yellow) throughout the nucleus. When the cells were allowed to progress into S phase, Flag-hZimp10 became associated with the distinctive small punctate structures of early S phase replication foci, whereas a portion of AR tended to retain a diffuse nuclear staining pattern (Figure 8B). When the cells progress further into S phase, Flag-hZimp10 demonstrated the slightly larger punctate staining indicative of mid-S phase (Figure 8C–E), and then the large, toroidal replication foci characteristic of late S phase (Figure 8F). A significant amount of overlaying between Flag-hZimp10 and AR appeared throughout S phase, although a portion of AR remained diffusely localized throughout the nuclei. Based on these observations, we conclude that hZimp10 can co-localize with AR in replication foci throughout S phase and that in addition to its role in transcription, AR may play a role in DNA replication.

Previous studies have shown that PIAS proteins can interact with SUMO-1 and function as SUMO-1 ligases (Kotaja *et al*., 2002). To further examine whether hZimp10 forms a protein complex with SUMO-1, we repeated the above experiments with cells co-transfected with both green fluorescent protein (GFP)–SUMO-1 and Flag-hZimp10. CV-1 cells were synchronized in late G_1 phase. As shown in Figure 9A, both GFP–SUMO-1 and Flag-hZimp10 displayed a diffuse pattern of nuclear staining in late G_1. When cells progressed into S phase, both of the proteins were found in the small, punctate structures of replication foci in early S phase (Figure 9B and C) and in the large, toroidal structures in late S phase (Figure 9D–F). To confirm that SUMO-1 localizes at replication foci, we labeled newly synthesized DNA with BrdU in the cells transfected with the GFP–SUMO-1 plasmid. As shown in Figure 10, the staining of SUMO-1

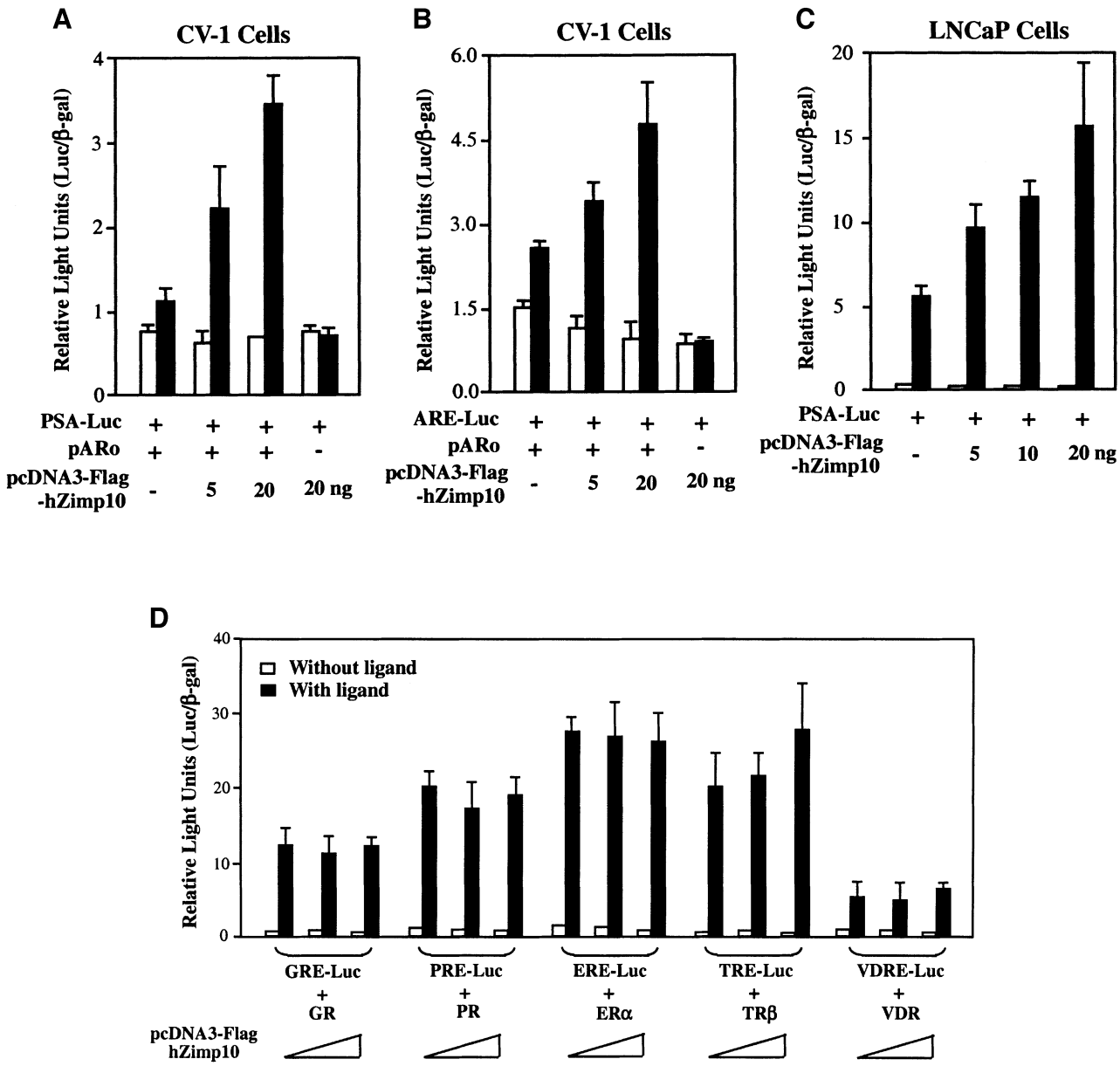

Fig. 5. hZimp10 selectively enhances AR-mediated transcription. (**A**) CV-1 cells were transfected with a luciferase reporter driven by the 7 kb PSA promoter (100 ng), pcDNA3-β-gal (25 ng), pSV-hARo (5 ng) and different amounts of the pcDNA3-Flag-hZimp10 as indicated. Twenty-four hours after transfection, cells were incubated with or without 10 nM DHT for 24 h. Cell lysates were prepared for assessment of luciferase and β-gal activities. (**B**) Similar to (A), except that a 2×ARE-luc reporter (100 ng) was used. (**C**) LNCaP cells were transfected with the PSA7kb-luc reporter (100 ng), pcDNA3-β-gal (25 ng) and the pcDNA3-Flag-hZimp10 as indicated. Twenty-four hours after transfection, cells were treated with or without 10 nM DHT for 24 h. Cell lysates were measured for luciferase and β-gal activities as described above. (**D**) One hundred nanograms of luciferase reporters driven by different response elements, as labeled in the figure, were co-transfected with 50 ng of pSV40-β-gal and 10 ng of different receptors into CV-1 cells. Either 0, 5 or 20 ng of pcDNA3-Flag-hZimp10 was co-transfected. Cells were cultured either in the presence or absence of the specific ligands to each receptor, including 10 nM dexamethasone (DEX), 10 nM progesterone, 100 nM β-estradiol, 10 nM tri-iodothyronine and 10 nM 1α, 25-dihydroxyvitamin D3.

was mainly overlaid with the BrdU staining throughout S phase, particularly in the late time point. The results demonstrate that SUMO-1 can localize at DNA replication foci, suggesting a role of SUMO-1 in DNA synthesis. In addition, we also confirmed the above observations through the co-localization of endogenous hZimp10, SUMO and AR with BrdU during S phase (see Supplementary data, available at *The EMBO Journal* Online).

hZimp10 is able to enhance the sumoylation of AR and the augmentation of hZimp10 is linked to the AR sumoylation sites

It has been shown that members of PIAS family can function as SUMO ligases (Kotaja *et al.*, 2002). To determine whether hZimp10 has a role in the sumoylation of AR, we co-transfected hZimp10, AR and SUMO-1 into COS-1 cells. As shown in Figure 11A, the expression of hZimp10 with AR in the presence of different amounts of

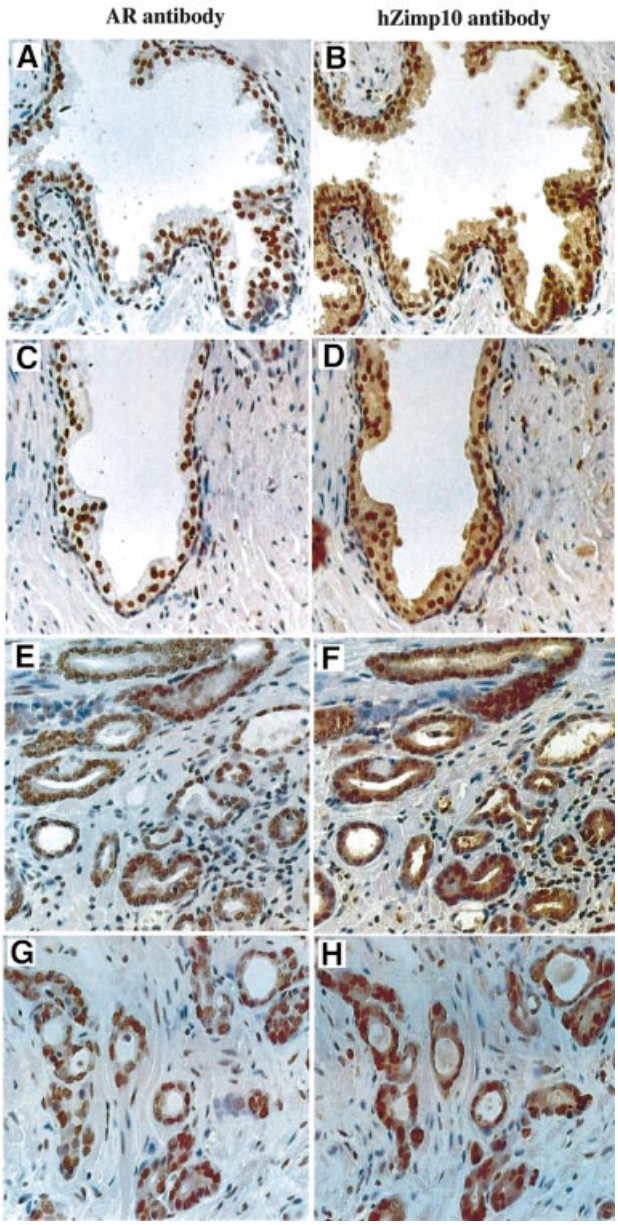

Fig. 6. The AR and hZimp10 co-localize in prostate epithelial cells. Four pairs of human prostate tissue samples (**A**–**H**) were stained either with anti-AR (left panel) or anti-hZimp10 (right panel) antibody. Color was developed with DAB in PBS. All sections used for immunohistochemistry were lightly counterstained with 5% (w/v) Harris hematoxylin.

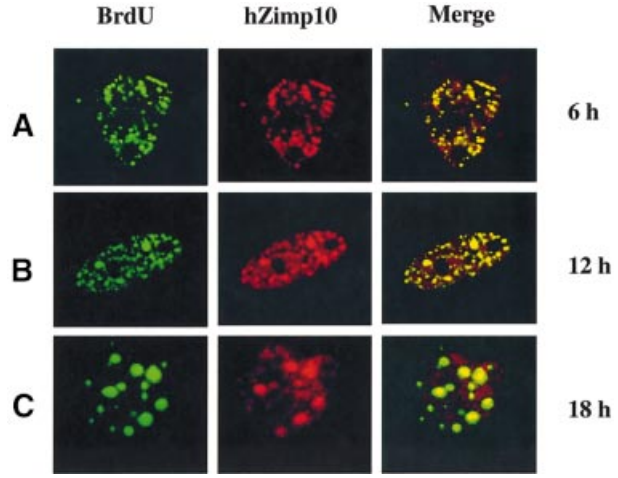

Fig. 7. Immunofluorescent localization of hZimp10 at replication foci. The pcDNA3-Flag-hZimp10 construct was transfected into CV-1 cells. Cells were synchronized with 0.5 mM mimosine (see Materials and methods). Representative confocal laser scanning microscopy images of nuclei from cells expressing Flag-tagged hZimp10 proteins and pulsed with BrdU are shown. Color was developed with either FITC-conjugated monoclonal anti-BrdU antibody (green) or the anti-Flag primary antibody followed by secondary antibodies conjugated with rhodamine (red). Merge (right panel) of left and middle panels indicates areas of co-localization (yellow).

SUMO-1 enhanced the intensity of the two slowly migrating AR immunoreactive bands. Although the effect of hZimp10 was relatively modest in comparison with that of PIAS-1, there was an ~40% increase in the intensity of the sumoylated AR forms in the presence of 0.04 or 0.1 μg of SUMO-1. Our results indicate that hZimp10 is capable of enhancing the sumoylation of AR.

The two major sumoylation sites (K386 and K520) have been identified within the AR (Poukka *et al*., 2000). Mutation of these sites significantly reduces the sumoylation of AR. Using constructs containing either single or double mutation at the above amino acids, we studied a link between the sumoylation and transactivation of AR. Transfection of the wild-type or mutant AR with a luciferase reporter driven by the 7 kb PSA promoter showed variable ligand-dependent activity (Figure 11B). Mutation at one sumoylation site (K386R) reduced the activity of AR by ~50%, and mutation of both sumoylation sites (K386R/K520R) further decreased the residual activity by another 50%. These results suggest that sumoylation of AR is essential for the activity of AR. The link between sumoylation and transactivation of AR by hZimp10 was further tested by co-transfection of hZimp10 with the wild-type and mutants of AR. As we observed previously, co-transfected hZimp10 enhanced the transcriptional activity of the wild-type AR (Figure 11B). Interestingly, hZimp10 was not able to modulate the activity of the mutant ARs. In contrast, a well-characterized AR co-activator ARA70 (Yeh and Chang, 1996) augmented both the wild-type and the mutants of AR, albeit to a different degree. Taken together, the results suggest that sumoylation of AR is involved in hZimp10-mediated augmentation of AR activity. Thus, given the fact that hZimp10 is able to enhance the sumoylation of AR, we have provided a link between hZimp10-mediated enhancement of AR sumoylation and modulation of AR-mediated transcription.

Discussion

Like other nuclear hormone receptors, transcriptional activity of the AR can be modulated through interactions with various co-regulators (Rosenfeld and Glass, 2001). Aberrations in the expression or function of these co-factors may lead to enhancement of AR activity and provide an adaptive advantage for tumor cell growth. Changes in the transcriptional programs of the AR are important but poorly understood events in prostate cancer

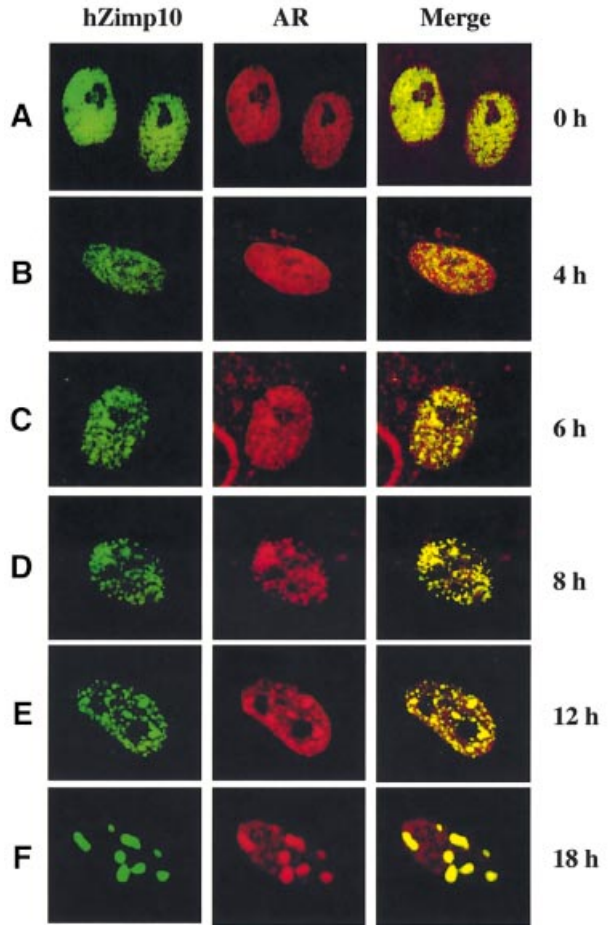

Fig. 8. Immunofluorescent co-localization of hZimp10 and AR. CV-1 cells co-transfected with pcDNA3-Flag-hZimp10 and pSVARo were synchronized with mimosine. Double immunostaining was conducted with anti-Flag and anti-AR antibodies followed by the secondary antibodies conjugated with FITC (green) and rhodamine (red), respectively. Representative confocal laser scanning microscopy images of nuclei from cells expressing Flag-tagged hZimp10 and AR proteins are shown. Merged images demonstrating co-localization of proteins are shown on the right panels (yellow). Staining was performed at different time points, including late G_1 phase before entering S phase (0 h, **A**), early S phase (4 and 6 h, **B** and **C**), mid-S phase (8 and 12 h, **D** and **E**) and late stages of S phase (18 h, **F**).

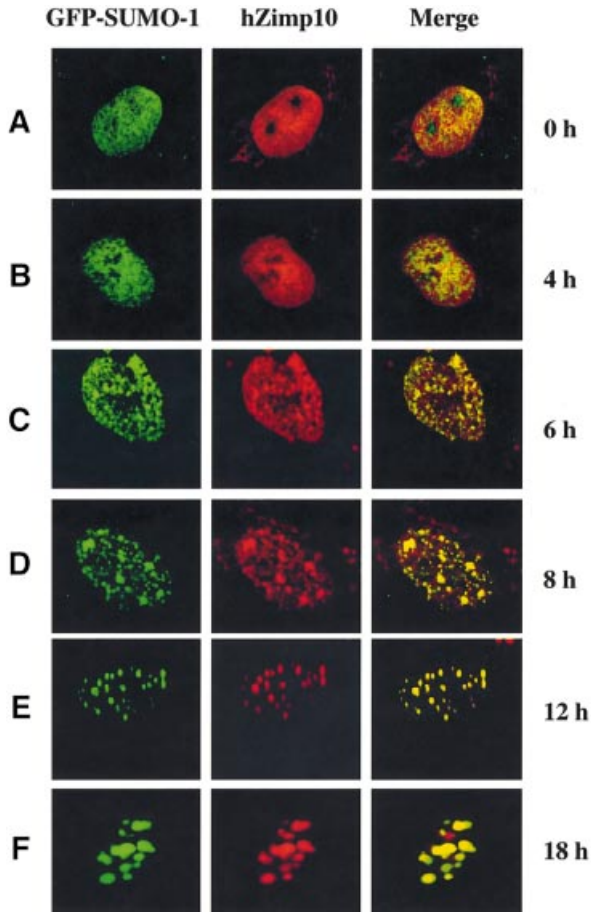

Fig. 9. Immunofluorescent co-localization of GFP–SUMO-1 and hZimp10 at DNA replication foci throughout S phase. CV-1 cells co-transfected with pcDNA3-Flag-hZimp10 and pGFP-SUMO-1 were synchronized with mimosine. Immunostaining was conducted with anti-Flag monoclonal antibody followed by secondary antibodies conjugated with rhodamine (red) to contrast with the GFP proteins. Merged images demonstrating co-localization of proteins are shown in the right panels (yellow). Staining was conducted at different time points as described in Figure 8.

development and progression. In our search for AR co-regulators that contribute to prostate cancer tumorigenesis, we identified a novel PIAS-like protein, hZimp10, which interacts with AR and augments AR-mediated transcription. Importantly, endogenous AR and hZimp10 are co-stained in the nuclei of prostate epithelial cells in human prostate tissues. This intriguing evidence, combined with other evidence, strongly suggests that the interaction between hZimp10 and AR is biologically relevant and may play a role in prostate cells.

In this study, we tested the functional consequences of the interaction between AR and hZimp10. We demonstrated that hZimp10 augments the ligand-dependent activity of AR on both the natural AR-dependent enhancer and promoter from the PSA gene and on a mini-promoter containing only two AREs. In addition, hZimp10 enhances endogenous AR-dependent activity in prostate cancer cells. These results provide the first line of evidence as to the functional consequence of the interaction between AR and hZimp10.

Sequence analysis suggests that hZimp10 is related to the PIAS proteins. To determine the molecular basis by which hZimp10 functions as a transcriptional factor, we tested the intrinsic transcriptional activity of the protein. We showed that the C-terminal proline-rich domain possesses significant intrinsic transcriptional activity. Intriguingly, the latter activity is much higher than the TADs of other eukaryotic transcription factors that we have tested, including p53, Smad3, PU1 and AR, and is even comparable to transactivation by the TAD of VP16. These data, combined with the results from our transient transfection experiments, demonstrate that hZimp10 can enhance transcription both *in trans* and *in cis*, and are consistent with the notion that hZimp10 is a transcriptional regulator. Sequence analysis showed that the C-terminal proline-rich region is not found in other PIAS or PIAS-like proteins, suggesting that the Miz domain family may consist of a group of proteins that contain unique structures and play distinct roles in regulating transcription and other cellular processes.

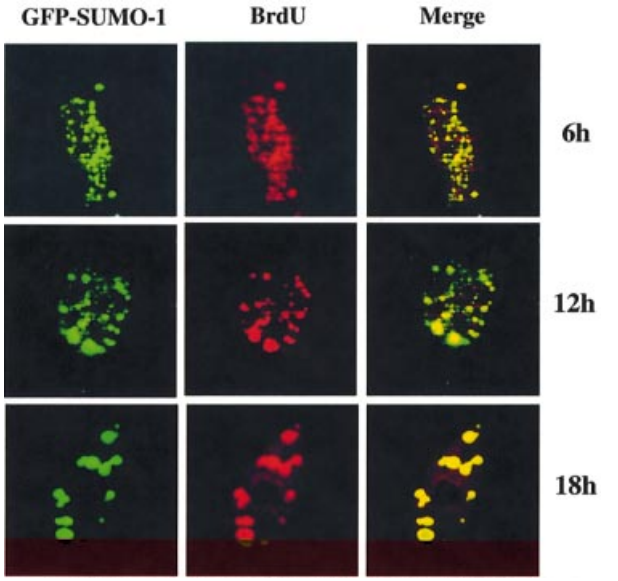

Fig. 10. Immunofluorescent localization of SUMO-1 at replication foci. CV-1 cells were transfected with GFP–SUMO-1 plasmid, and then synchronized as described in Materials and methods. Representative confocal laser scanning microscopic images of nuclei from cells expressing GFP–SUMO-1 proteins and pulsed with BrdU are shown. Color was developed by either FITC-conjugated monoclonal anti-BrdU antibody (red) or fluorescent images of GFP proteins (green). Merge (right panel) of left and middle panels indicates areas of co-localization (yellow).

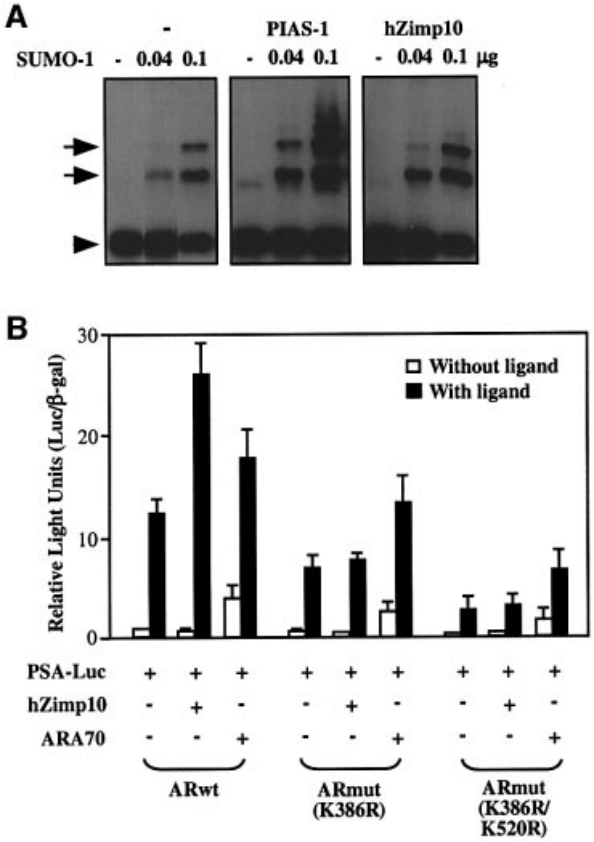

Fig. 11. hZimp10 enhances the sumoylation of AR and augments AR-mediated transcription. (**A**) COS-1 cells were co-transfected with 200 ng of pSG5-AR in the presence and absence of 200 ng of pFlag-PIAS1 or 360 ng of pcDNA3-Flag-hZimp10 along with 40 or 100 ng of pSG5-His-SUMO-1 as indicated. The cells were collected 48 h after transfection and the lysates were immunoblotted with the AR antibody. The slower migrating AR bands (sumoylated) are labeled with solid arrows. (**B**) CV-1 cells were transfected with a luciferase reporter driven by the 7 kb PSA promoter (100 ng), pcDNA3-β-gal (25 ng), pcDNA-hARwt or mut (5 ng) and pcDNA3-Flag-hZimp10 (10 ng). Cell lysates were prepared for assessment of luciferase and β-gal activities.

Interestingly, the full-length hZimp10, when fused to the DBD of GAL4, displays a very limited activity compared with the truncated mutants containing the C-terminal proline-rich domain. We determined that the N terminus of hZimp10 (aa 1–691) significantly inhibits the activity of the C-terminal proline-rich region. The auto-inhibition of the transcriptional activity of hZimp10 indicates that a complicated regulatory mechanism may be involved in the enhancement of transcriptional activation by hZimp10. Therefore, identification of regulatory mechanisms that can release the inhibition and switch the protein from an inactive form into an active form will be extremely important.

Like other PIAS-like proteins, hZimp10 contains a conserved Miz domain. Another member of the PIAS family, ARIP3, was originally identified as an AR-interacting protein (Moilanen *et al*., 1999). The zinc finger region of AR was shown to be involved in the interaction with ARIP3/PIASxα (Moilanen *et al*., 1999). Other members of the PIAS protein family have also been shown to be capable of interacting with AR and other steroid receptors and to regulate their activity (Kotaja *et al*., 2002; Tan *et al*., 2002). Our data showing that the Miz region of hZimp10 is involved in the interaction with AR are consistent with previous reports, and suggest a biological role of hZimp10 in androgen signaling.

Using immunofluorescence imaging, we confirmed the interaction between hZimp10 and AR. In synchronized cells, both hZimp10 and AR were detected at replication foci and a significant amount of overlay between these two proteins was observed throughout S phase. In addition, we also demonstrated a co-localization of hZimp10 and SUMO-1. These findings suggest a potential role for hZimp10 in both chromatin assembly and maintenance of chromatin. hZimp10 may act as a transcriptional regulator to initiate and mediate formation of an active transcriptional complex. It may also be involved in the modification of chromatin and participate in other steps during DNA synthesis. Interestingly, a novel *Drosophila* gene, termed the *tonalli* (*tna*), was identified recently (Gutierrez *et al*., 2003), and shares some sequence similarity to hZimp10. Intriguingly, *tna* was shown to genetically interact with SWI2/SNF2 and the Mediator complex. These data are consistent with our discoveries, suggesting that hZimp10 may play a critical role in chromatin modification.

PIAS proteins can function as SUMO-1 E3 ligases to facilitate sumoylation of steroid receptors and other transcription factors (Kotaja *et al*., 2002). It has been shown that the sumoylation of AR can be enhanced by PIAS proteins (Kotaja *et al*., 2002) and two major sumoylation sites have been identified in the TAD of AR (Poukka *et al*., 2000). Co-localization of hZimp10 with AR and SUMO-1 in the same subnuclear structures suggests that the sumoylation may play a role in the

interaction between the proteins. In this study, we further demonstrated that hZimp10 is able to enhance the sumoylation of AR. While the effect mediated by hZimp10 was relatively modest in comparison with PIAS1, it may reflect the fact that the role of hZimp10 in sumoylation is distinct from that of the PIAS proteins. Based on the experimental design, we were not able to conclude whether hZimp10 is acting directly as an E3 ligase. The mechamism by which hZimp10 enhances the sumoylation of AR needs to be investigated further. Trancription assays showed that in contrast to the wild-type AR, the activity of the sumoylation deficient AR mutant was not augmented by hZimp10. The above results not only provide evidence demonstrating that hZimp10 enhances the sumoylation of AR, but also demonstrate a link between the sumoylation and augmentation of AR activity by hZimp10. Although the precise mechanisms by which hZimp10-mediated sumoylation affects the transcriptional activity of AR remain unclear, our findings suggest several possibilities, one of which is that the sumoylation of AR may enhance the interaction between AR and hZimp10, and/or their localization to replication foci.

Recently, the PIAS and SUMO proteins have been found in a PML-related nuclear body (Schmidt and Muller, 2002). Our observation that hZimp10 and SUMO-1 co-localize to replication foci is novel and interesting. Given the recent result showing that PCNA is a target of SUMO-1 (Hoege *et al*., 2002), sumoylation may be involved in both transcriptional regulation and DNA synthesis (Hoege *et al*., 2002; Schmidt and Muller, 2002). In both cases, our discovery of hZimp10 and its link to the SUMO pathway provide a new line of evidence, suggesting that sumoylation, combined with other modification pathways such as methylation, phosphorylation and acetylation, is involved in regulating transcriptional activity of AR by modifying chromatin formation during the early and late S phases of the cell cycle.

Materials and methods

Yeast two-hybrid system

Yeast two-hybrid experiments were basically performed as described previously (Sharma *et al*., 2000). The DNA fragment containing the partial TAD of human AR (aa 1–333) was fused in-frame to the GAL4 DBD in the pGBT9 vector (Clontech, Palo Alto, CA). The construct was transformed into a yeast strain PJ69-4A (James *et al*., 1996). A cDNA library from the mouse EML cell line was used for this screening (Lioubin *et al*., 1996). β-gal activities were measured using the Galacto-light Plus kit (Tropix Inc., Bedford, MA).

Plasmid construction

The C-terminal region of Zimp10 was isolated in the initial yeast two-hybrid screening. A BLAST search found KIAA1224 and several human EST cDNA clones that contained sequences similar to the mouse fragment. To isolate the full-length Zimp10, 5′-RACE was used with human brain and prostate Marathon Ready cDNAs (Clontech). The specific reverse primers spanning amino acid residues 464–472 (5′-CTGGCTTGTAATACTGCCCCATGTTG) and 176–184 (5′-TTCCCAAGGACCTGGCTCTGGGATG) were used in first and second rounds of 5′-RACE, respectively. A full-length hZimp10 cDNA was created by combining the cDNA fragments coding for the N-terminal region isolated by 5′-RACE and the KIAA1224 cDNA fragment in the pcDNA3 vector. Subsequently, truncated mutants of hZimp10 were generated from the full-length clone.

The human AR plasmid, pSV-hAR, was provided by Dr Albert Brinkmann (Erasmus University, Rotterdam, The Netherlands). A β-gal reporter was purchased from Promega (Madison, WI). The human ERα plasmid and pERE-luc plasmid were supplied by Dr Myles Brown (Dana-Farber Cancer Institute, Boston, MA). A human PR construct (hPR1) and the PRE-luc reporter were provided by Dr Kathryn B.Horwitz (University of Colorado, Denver, CO). The expression constructs of human GR and VDR, and the pVDRE-luc reporter were gifts from Dr David Feldman (Stanford University, Stanford, CA). The pARE-luc reporter was a gift from Dr Chawnshang Chang (Yeh and Chang, 1996). The pPSA7kb-luc was obtained from Dr Jan Trapman (Cleutjens *et al*., 1996). The SUMO-1 and ARIP3 vectors were generated as reported previously (Poukka *et al*., 2000; Kotaja *et al*., 2002). Luciferase reporters containing the chicken myelomonocytic growth factor gene minimal promoter were provided by Dr Donald Ayer (University of Utah, Salt Lake City, UT).

Cell culture and transfections

The monkey kidney cell line, CV-1, and other human cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum (HyClone, Denver, CO). An AR-positive prostate cancer cell line, LNCaP, was maintained in T-medium (Invitrogen, Carlsbad, CA) with 5% fetal calf serum. Transient transfections were carried out using LipofectAMINE for CV-1 cells, and LipofectAMINE 2000 for LNCaP cells (Invitrogen). The RLU from individual transfections were normalized using β-gal activity in the same samples. Individual transfection experiments were done in triplicate and the results are reported as mean RLU/β-gal (±SD).

Immunoprecipitation, western blotting and antibody production

pSV-hAR alone or with the Flag-tagged hZimp10 and GFP–SUMO-1, was transfected into CV-1 cells. Co-immunoprecipitation assays and western blotting were carried out as described previously (Sharma *et al*., 2000). The membranes were then probed with a polyclonal antibody against the N-terminus of AR (Santa Cruz Biotech, Santa Cruz, CA), or a monoclonal Flag tag antibody (Sigma, St Louis, MO). Proteins were detected using the ECL kit (Amersham, Arlington Heights, IL).

The C-terminus of hZimp10 (aa 917–1067) was inserted into the pGEX-4T1 vector (Amersham). The GST fusion protein was prepared as described previously (Smith and Johnson, 1988). The GST–hZimp10 fusion protein was used as a source of antigen for antibody production. The antibody was produced and purified by BioChain Institute Inc. (Hayward, CA).

Northern blot analysis

A blot with RNA from multiple human tissues was obtained from Clontech Inc. Poly(A)$^+$ RNAs were isolated by Oligo-dT (Invitrogen) chromatography from prostate cancer cell lines (LNCaP, DU145, PC-3, ARCaP and LAPC4) and non-prostate cancer cell lines (MCF7 and HeLa). Northern blots were performed as described previously (Sharma *et al*., 2000).

Cell synchronization, BrdU labeling and immunofluorescence

CV-1 cells were seeded onto gelatin-coated (0.2%) coverslips 24 h before transfection. Transfected cells were fed with the fresh medium after 6 h and then incubated for an additional 12 h before synchronization (Krude, 1999). For detection of DNA replication, cells were pulsed with 10 μM 5-BrdU and 1 μM fluorodeoxyuridine (Sigma) to inhibit thymidylate synthetase. Cells were then washed twice with cold PBS and fixed with 3% formaldehyde for 30 min. After fixation, cells were washed again. To visualize the newly synthesized DNA labeled with BrdU, the rinsed permeabilized cells were treated with 4 N hydrochloric acid to denature the DNA, rinsed several times in TBS-T, and incubated at 37°C for 1 h with FITC-conjugated monoclonal anti-BrdU antibody (PharMingen, San Diego, CA).

At various time points after synchronization, cells grown on coverslips were fixed with 3% formaldehyde for 30 min at 22°C, permeabilized with 0.1% Nonidet P-40 in PBS, and blocked with 5% milk in 1× TBS-T for 30 min at room temperature. The cells were then incubated with monoclonal anti-Flag or polyclonal anti-AR antibodies alone or together at a 1:1000 dilution in 1% BSA/PBS for 1 h at 22°C. Cells were washed three times followed by incubation with fluorescein isothiocyanate-conjugated or rhodamine-conjugated anti-mouse, or rhodamine-conjugated anti-rabbit secondary antibody, respectively (Molecular Probes, Eugene, OR).

Immunohistochemistry

Human prostate tissue samples were fixed in 10% neutral buffered formalin, and processed to paraffin. Sections were cut at 5 μm intervals, de-waxed in Histoclear (National Diagnostic, Atlanta, GA), and then hydrated in graded alcoholic solutions and distilled water. Two adjacent sections from each tissue sample were used for immunohistochemistry. Endogenous peroxidase activity was blocked with 0.5% hydrogen peroxide in methanol (ImmunoVision Technology, Spingdale, AR). The sections were then incubated with either the rabbit polyclonal anti-AR antibody (Affinity BioReagents, Golden, CO) at a 1:100 dilution, or the chicken anti-hZimp10 antibody at a 1:150 dilution overnight at 4°C. Sections were washed and incubated with a 1:200 dilution of either a biotinylated anti-rabbit (Zymed Corp., South San Francisco, CA) or anti-chicken antibody (Amersham), and then washed and developed with DAB in PBS and 0.03% H_2O_2. All sections used for immunohistochemistry were lightly counterstained with 5% (w/v) Harris hematoxylin.

SUMO-1 conjugation

The experiment was performed essentially as described previously (Kotaja *et al*., 2002). COS-1 cells seeded onto 6-well plates were transfected with 200 ng of pSG5-AR in the presence and absence of 200 ng of pFlag-PIAS1 or 360 ng of pcDNA3-Flag-hZimp10 along with 40 ng or 100 ng of pSG5-His-SUMO-1 as indicated. The cells were supplied with 100 nM testosterone 24 h after transfection, collected 48 h after transfection and lysed in RIPA-1 buffer containing 10 mM *N*-ethylmaleimide. The lysates were immunoblotted and developed with the anti-AR antibody.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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