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The androgen-signaling pathway is important for the growth and progression of prostate cancer. The cell cycle signaling regulated by the mitogen activated protein/extracellular-signal-regulated kinase (MAPK/ERK) have been linked to tumorigenesis and tumor progression. The p21-activated kinases (PAKs) are members of a growing class of Rac/Cdc42-associated Ste20-like ser/thr protein kinases. Recent studies have shown that MAPK/ERK signaling can be mediated via Cdc42/Rac-stimulation of PAK activity. Recently, we demonstrated a specific interaction between a novel PAK protein, PAK6, and AR. This finding provided the first link between PAK-mediated signaling to the steroid hormone receptor pathway. In this study, we propose to assess the biological roles of PAK6 in prostate cancer cells, and to examine the expression of PAK6 in prostate tissues. We anticipate that by completing the objectives proposed in this grant, we will obtain fresh insight into the regulatory processes of AR and PAK6 that may contribute to the development of new targets for the treatment of prostate cancer.

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INTRODUCTION:

Like most other cancers, prostate carcinogenesis involves a multistep progression from precancerous cells to cells that proliferate locally in an unregulated fashion and then metastasize. During the metastatic phase of this illness, tumor cells often lose cell-cell contact and possess a more aggressive growth phenotype. Observations from androgen ablation treatment of prostate cancer have shown that the androgen-signaling pathway is important in the growth and progression of prostate cancer (6). The growth-promoting effects of androgen are mediated mostly through the androgen receptor (AR). It has also been shown that growth factor stimulated signal transduction pathways can modulate the activity of AR through modification of the protein such as phosphorylation. PAK6 was first identified as an AR-interacting protein able to inhibit AR-mediated transcription. A novel AR-interacting protein, PAK6, was identified in our group previously (8). PAK6 is a serine/threonine kinase belonging to the p21-activated kinase (PAK) (5). The goal of this study is to determine the biological roles of PAK6 in the tumorigenesis of prostate cancer. Two specific aims, including assessing biological roles of PAK6 in cell proliferation and detecting alterations in PAK6 expression in prostate cancer tissues, have been proposed and approved by both the scientific and programmatic reviewers. We have made a substantial progress in achieving our objectives within this funding year.

BODY:

Currently, six members of the p21-activated kinase (PAK) family of protein kinases have been identified and can be classified into two groups based on their sequence homology and regulatory properties: group I, including PAKs 1–3, and group II, including PAKs 4–6 (4). PAKs are serine/threonine kinases that contain a Cdc42/Rac-interactive binding (CRIB) domain and a Ste20-related kinase domain. The PAK family members have been implicated in the regulation of multiple cellular functions, including actin reorganization, cell motility, gene transcription, cell transformation, apoptotic signaling, and more recently, steroid hormone receptor signaling (2, 4). The interactions between the AR and PAK6 may be an important event both for the development and progression of prostate cancer. This year, we continued to investigate the biological roles of PAK6 and the significance of its interaction with AR. We focused on two specific objectives: 1) To characterize the biological roles of PAK6 in prostate cancer cells, and 2) To detect expression of PAK6 protein in prostate cancer samples by immunohistochemistry.

Objective 1: To characterize the biological roles of PAK6 in prostate cancer cells.

Using yeast two-hybrid screening, we identified PAK6 as an AR-interacting protein (8). Our previous works have showed that PAK6 interacts with the ligand binding domain of the AR and translocates to the nucleus along with the AR, where PAK6 inhibits AR-mediated transcription. The results of Northern blot analysis showed that PAK6 is mainly expressed in brain, testis, prostate, and breast tissues (7, 8). In the previous year, we demonstrated that PAK6 was capable of phosphorylating histone H3/H4 in the presence of both Cdc42-GDP and Cdc42-GTPS, and that the activity was not enhanced by the presence of the active GTPase. In addition, we generated several PAK6 mutants and used them in studies of the phosphorylation of PAK6 and the repression of AR mediated transcription.

The Rho family GTPase Cdc42 has been shown to bind to PAK proteins, presumably via its CRIB domain (4). Our preliminary results showed that this interaction does not affect PAK6 kinase activity. The binding of Cdc42 to PAK4 and PAK5 also does not increase their kinase activity but rather modulates their subcellular localization (1). To determine whether the interaction of PAK6 with GTPase is important in enabling PAK6 to inhibit AR-mediated transcription, we generated a series of PAK6 constructs in which the CRIB domain was mutated in a conserved pair of histidine residues known to be important for GTPase binding, which include PAK6 (H20L/H23L), PAK6 (H20L/H23L/K436A), and PAK6 (H20L/H23L/S531N). The binding of Cdc42 to these PAK6 constructs was analyzed by immunoprecipitation of EGFP-tagged PAK6 mutants in the presence of Cdc42 loaded with either GDP or GTPS. PAK6wt was able to pull down Cdc42 loaded with GTPS, whereas the H20L/H23L mutation abrogated binding of Cdc42 to PAK6. We also demonstrated that the mutation H20L/H23L did not modify the kinase activity of PAK6, but significantly modified the inhibitory effect of AR activity by wt and kinase-active (S531N) forms of PAK6. These results demonstrate that PAK6-induced inhibition of AR-mediated transcription is independent of Cdc42 binding.

Our group, along with others, have shown that AR transcriptional activity can be enhanced by the presence of AR coactivators, including β-catenin, SRC1, ARA55, p300, and Tip60 (3). We sought to determine whether PAK6wt and PAK6 mutants were able to inhibit to the same extent AR-mediated transcription in the presence of these differentially binding AR coactivators. Using transient transfection approach, we showed that β-catenin, SRC1, and ARA55 enhanced AR-mediated transcription in CV1 cells. In the presence of the highly active PAK6(S531N/S560E) mutant, these coactivators were not able to significantly increase the transcriptional activity of AR. In contrast, in the presence of PAK6wt or K436A, these coactivators all increased AR transcriptional activity. Our results suggest that the inhibitory effect of PAK6 on AR-mediated transcription is dominant over the presence of the AR activating cofactors β-catenin, SRC1, ARA55, and Tip60.

To better understand the potential biological role(s) of PAK6, we extended our previous study by examining the expression of PAK6 protein in different cancer cell lines. The antiphospho-PAK6 (Ser(P)-560) antibody was used to analyze PAK6 in prostate cancer cell lines, DU145, PC-3, ARCaP, LNCaP, and LAPC4, breast cancer cell line MCF7, and the uterine cancer cell line HeLa. The western-blot showed that phospho-PAK6 was detected most abundantly in the LAPC4 cell line. High expression/activity levels were also detected in the DU145 and PC-3. MCF7 and ARCaP expressed only a small amount of phospho-PAK6, and no expression was detected in HeLa and LNCaP. These results are consistent with our Northern blot results (8) and indicate that the expression and/or activity level of PAK6 in prostate cell lines is highly variable, suggesting that PAK6 could be involved in differential regulation of AR signaling in these cells.

To further study the biological role of PAK6 in the tumorgenesis of prostate cancer, we made several sublines of LNCaP cells that were stably transfected with wt and mut of PAK6 constructs. We confirmed the expression of PAK6 in these cells and will further assess these lines by examining cell proliferation, colony formation, and cell cycle progression. We are also in the process of making adenoviral siRNA construct of PAK6, which will be used in future

studies. With these tools, we will be able to determine the biological role of PAK6 and the interaction between AR in the tumorigenesis of prostate cancer.

Objective 2: **To detect expression of PAK6 protein in prostate cancer samples by immunohistochemistry.**

In our previous studies, we examined the expression of PAK6 protein using a rabbit polyclonal antibody against human PAK6 that was generated in our lab. This antibody has been successfully used in Western blot and immunohistochemistry in detecting the transfected protein. However, we have not been able to use it to detect the endogenous PAK6 protein. Therefore, we produced two new antibodies against the peptides that were chosen from the N- or C-terminal region of human PAK6 based on their predicted immunogenicity and sequence specificity. The rabbit antisera were collected and purified by the proteinA affinity column. However, the preliminary results have shown that the antisera and the purified antibodies were unable to detect the endogenous PAK6 proteins in prostate cancer tissues by immunohitochemistry. Currently, we are in the process of purifying the antibodies using the specific peptides in order to increase the sensitivity and specificity of the antibodies. We hope that the additional purification process will help us to overcome the technical problem. In addition, we are planning to make one or two new antibodies by using GST-PAK6 fusion proteins. In this case, the fragments containing both the N- and C-terminal regions of PAK6 will be fused to GST and used for raising the antibodies. The GST fusion protein approach is generally very successful. If the antibodies cross-react with other PAK proteins, the antibodies will be absorbed with other GST-PAK proteins to deplete the non-specific reactivity.

In the unlikely event that a specific antibody to PAK6 protein is not available and cannot be generated by the above or related methods, in *situ* hybridization will be used to assess message expression. There has been significant experience with this method in the Pathology Department at Stanford Medical Center and it will be done collaboratively. Accordingly, we started to make the construct that will be used to make either the sense or antisense RNA probe of PAK6. Our efforts will be adjusted based on our progresses.

KEY RESEARCH ACCOMPLISHNETS:

1) Demonstrate that PAK6-induced inhibition of AR-mediated transcription is independent of Cdc42 binding.
2) Construct PAK6 SiRNA adenoviral vectors and adenoviral vectors of PAK6.
3) Establish LNCaP sublines that were stably transfected with wild type and mutants of PAK6.
4) Examine the expression of the PAK6 protein in prostate cancer cell lines.

REPORTABLE OUTCOMES:

Publications:

1. Sharma M, Li X, Wang Y-Z, Zarnegar M, Huang C-Y, Palvimo JJ, Lim B, **Sun ZJ** (2003). HZimp10 is an Androgen Receptor Coactivator and Forms a Complex with SUMO-1 at Replication Foci. *The EMBO Journal*, 22:6101-6114.

2. Schrantz N, Fowler B, Ge Q, **Sun ZJ**, and Bokoch GM. (2003). Mechanism of p21-activated kinase 6 (PAK6) - mediated inhibition of androgen receptor signaling. *J. Biol. Chem.*, 279:1922-31.

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   Beta-catenin and androgen signaling in prostate cancer

3. 2003-2006 The Dept. of Defense/PC020763, Principal Investigator: Zijie Sun
   PTEN regulates beta-catenin in androgen signaling: Implication in prostate cancer progression

CONCLUSIONS:

The precise mechanism for the development and progression of prostate cancer has remained obscure. Previously, we demonstrated that PAK6 interacts with AR and represses AR-mediated transcription. The goal of this study is to characterize the interaction between the PAK6 and AR proteins and to assess the expression of PAK6 in prostate cancer tissue samples. In this funding year, we continued to investigate biological roles of PAK6 in prostate cancer cells. We demonstrated that PAK6-induced inhibition of AR-mediated transcription is independent of Cdc42 binding and dominant over the presence of the AR coactivator. The expression of PAK6 protein in different cancer cell lines was also assessed. In addition, we are in the process of characterizing the two new antibodies, which were made recently in the lab. We hope that through these efforts we can gain more information regarding a central role of PAK6 in the regulation of androgen signaling and prostate cancer growth.

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Appendix Cover Sheet

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

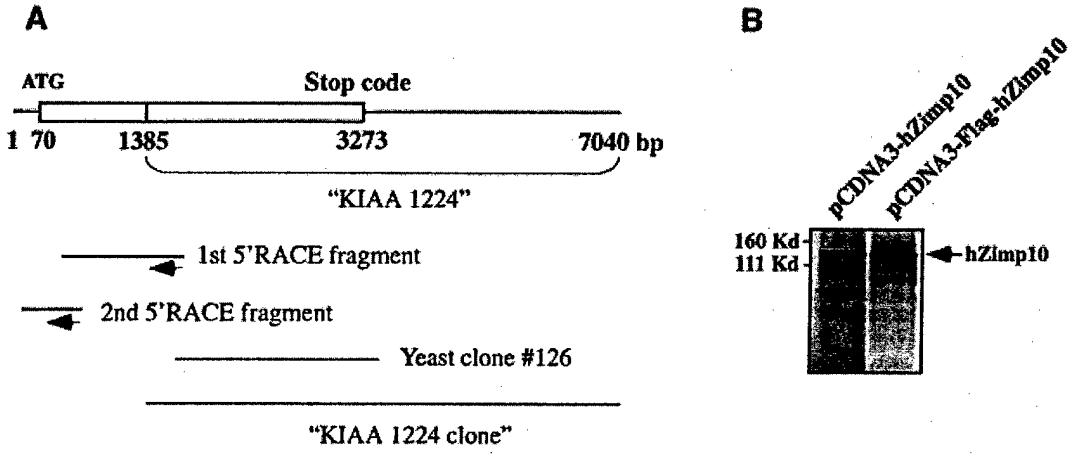
Introduction

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



D

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hZimp10          VSLKCPITFRRIQLPARGHDCKHVQCFDLESYLQLNCERGTWRCPVCNKTALL
ZimpB            VSLNCPLGKMKMLLPCRASTCSHLQCFDASLYLQMNERKPTWNCPVCDKPAIY
PIASxβ           VSLMCPLGKMRLTIPCRAVTCTHLQCFDAALYLQMNEKKPTWICPVCDKKAAY
PIASxα/ARIP3     VSLMCPLGKMRLTIPCRAVTCTHLQCFDAALYLQMNEKKPTWICPVCDKKAAY
PIASy            VSLICPLVKMRLSVPCRAETCAHLQCFDAVFYLQMNEKKPTWMCPVCDKPAPY
                 *** ***   *  ** *   * ******   *** *  * ** **** * *
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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.

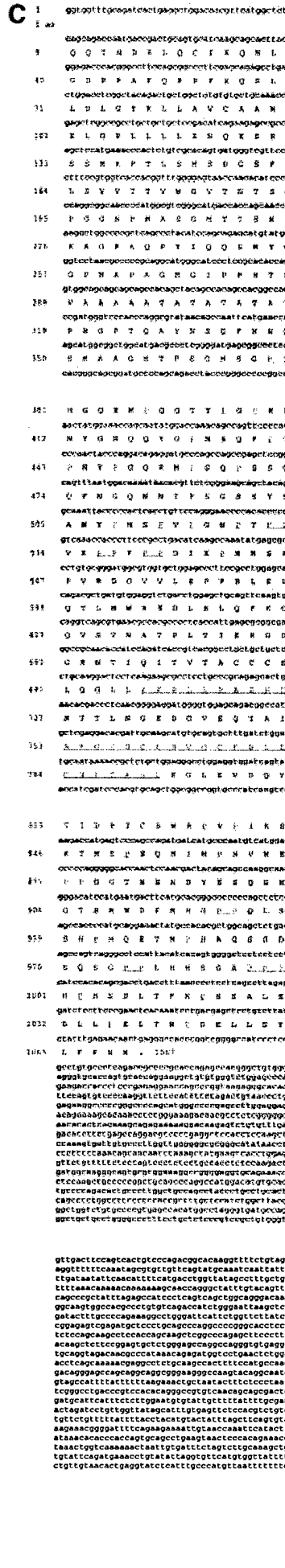
receptors and their co-factors have been demonstrated (Poukka *et al.*, 2000; Kotaja *et al.*, 2002).

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



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.

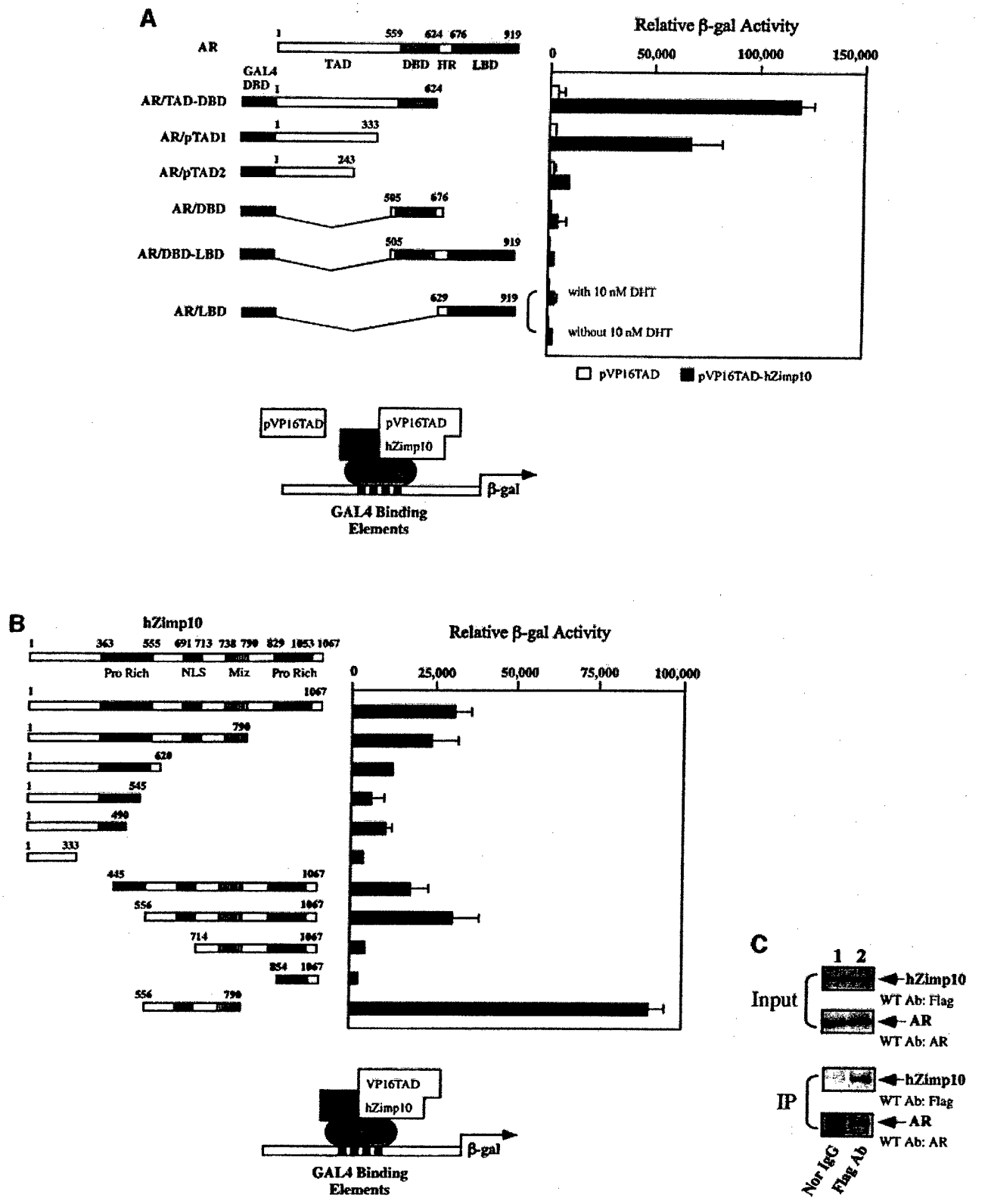


Fig. 2. Specific interaction between hZimp10 and AR. **(A)** cDNA fragments containing different portions of the human AR were fused to GAL4DBD in the pGBT9 vector. Numbers correspond to amino acid residues. The AR vectors were co-transformed with the pVP16 vector alone or the pVP16-Zimp10 construct. 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).

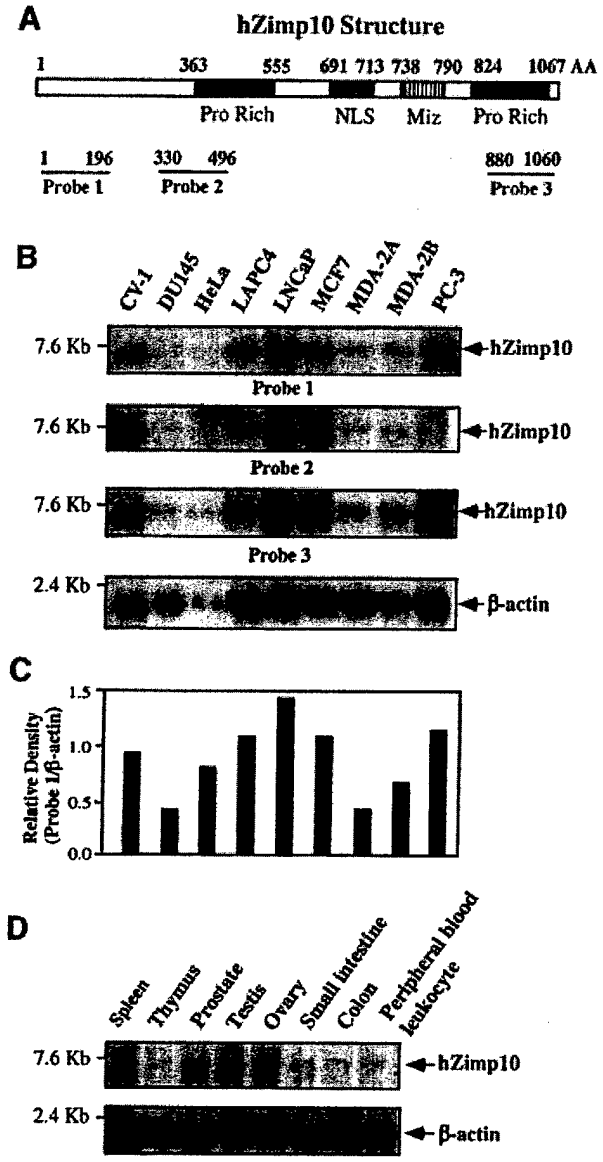


Fig. 3. Expression of hZimp10 in cell lines and human tissues. (**A**) A schematic representation of the hZimp10 protein is shown with the locations of three probes indicated. The numbers correspond to the amino acid sequence of the protein. (**B**) Northern blots containing poly(A)$^+$ RNA samples from 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

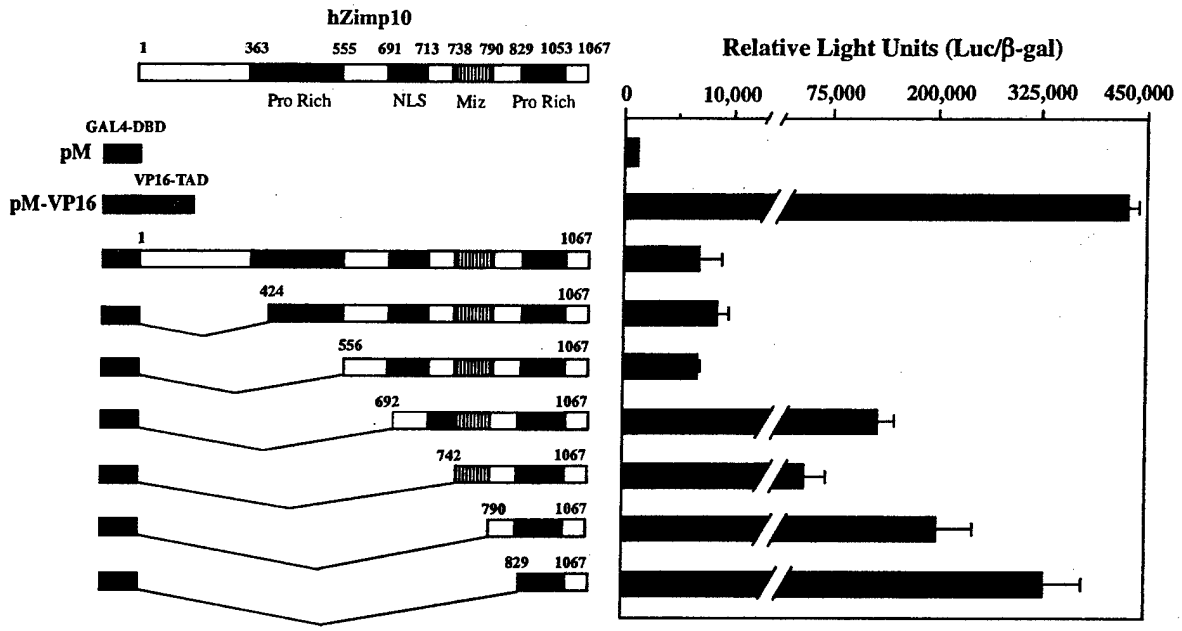


Fig. 4. Detection of intrinsic transcriptional activity of hZimp10. Full-length or truncated fragments of hZimp10 were fused to GAL4 DBD in the pM vector. Numbers correspond to amino acid residues. The pM constructs were co-transfected with luciferase reporter constructs containing the chicken myelomonocytic 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

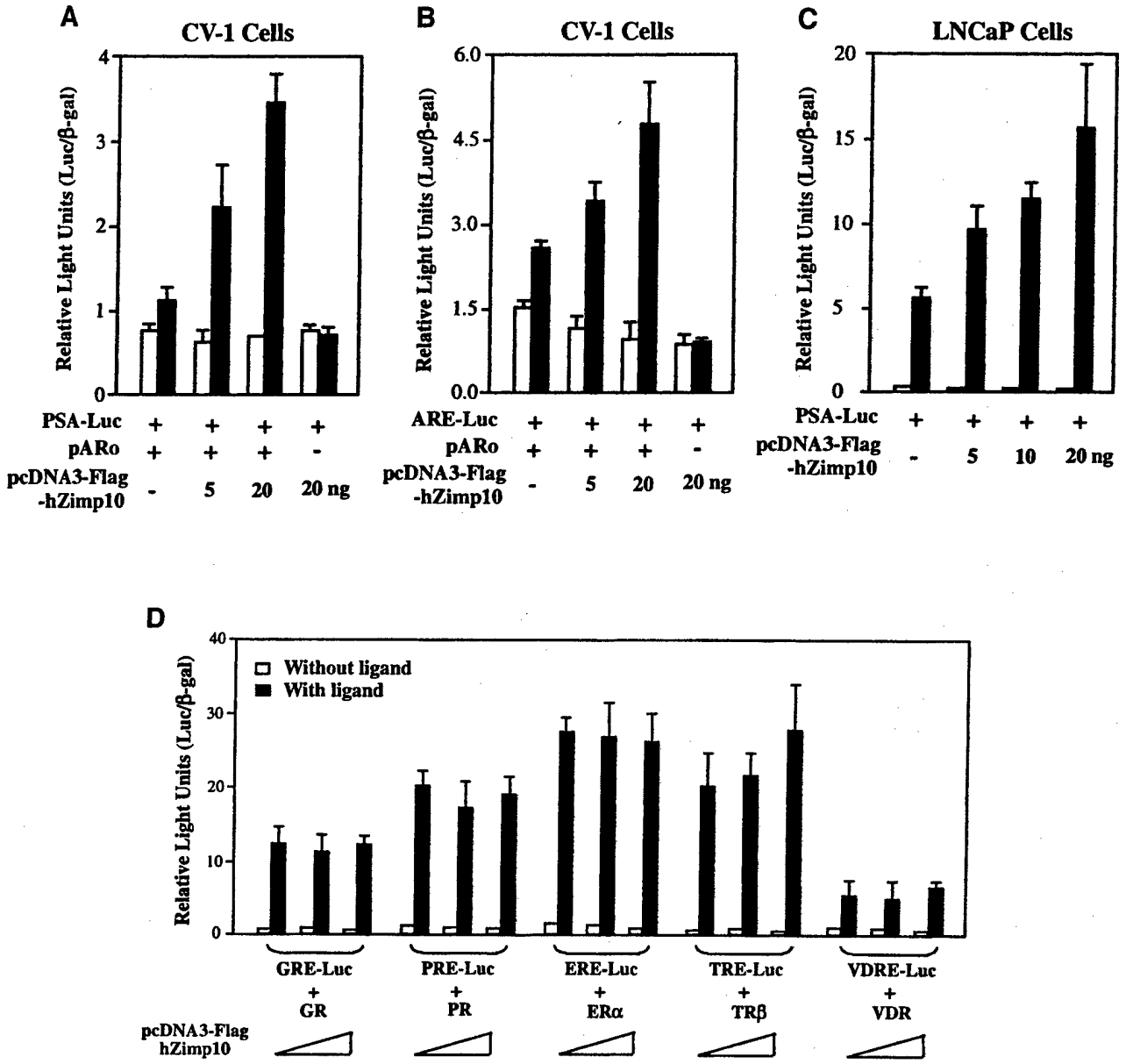


Fig. 5. hZimp10 selectively enhances AR-mediated transcription. (**A**) CV-1 cells were transfected with a luciferase reporter driven by the 7 kb PSA promoter (100 ng), pcDNA3-β-gal (25 ng), pSV-hARo (5 ng) and different amounts of the pcDNA3-Flag-hZimp10 as indicated. Twenty-four hours after transfection, 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

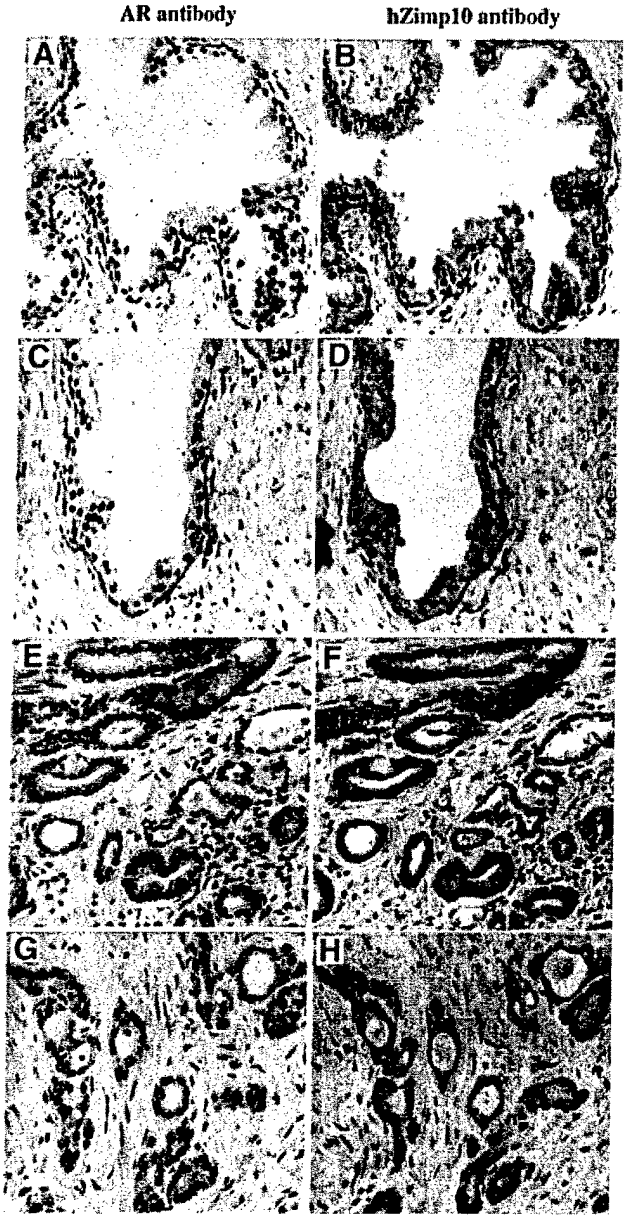


Fig. 6. The AR and hZimp10 co-localize in prostate epithelial cells. Four pairs of human prostate tissue samples (**A–H**) were stained either with anti-AR (left panel) or anti-hZimp10 (right panel) antibody. Color was developed with DAB in PBS. All sections used for immunohistochemistry were lightly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

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.

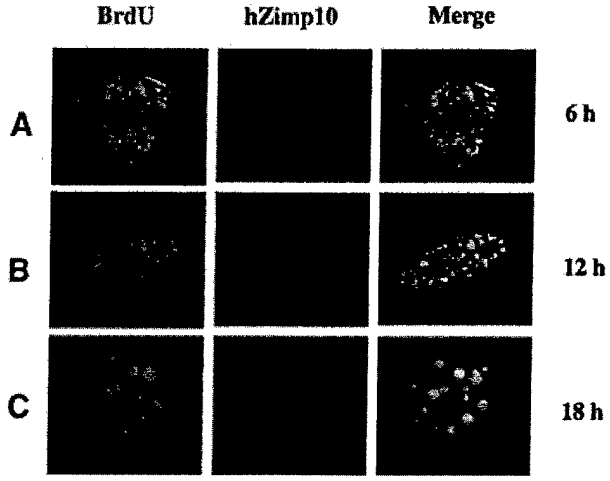


Fig. 7. Immunofluorescent localization of hZimp10 at replication foci. The pcDNA3-Flag-hZimp10 construct was transfected into CV-1 cells. Cells were synchronized with 0.5 mM mimosine (see Materials and methods). Representative confocal laser scanning microscopy images of nuclei from cells expressing 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).

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

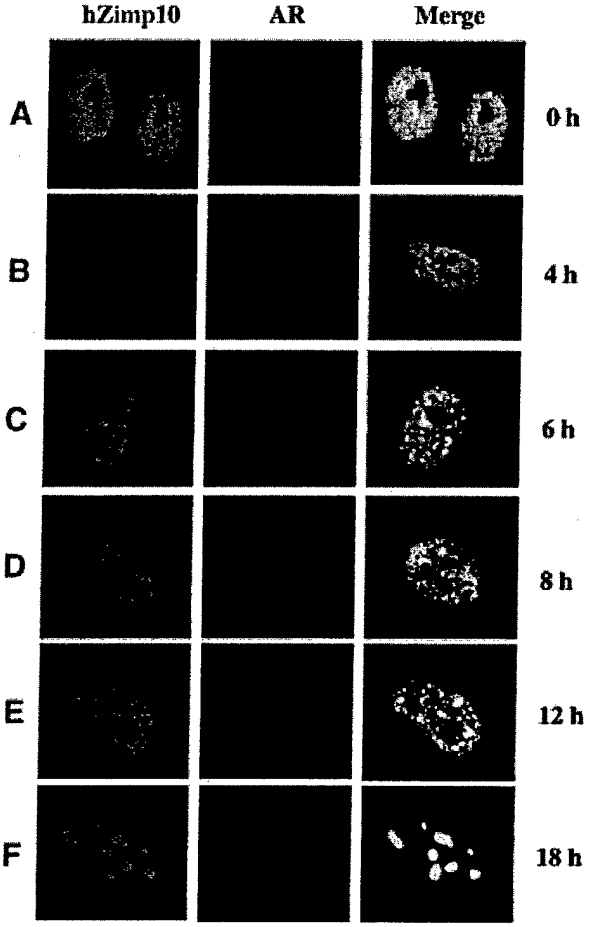


Fig. 8. Immunofluorescent co-localization of hZimp10 and AR. CV-1 cells co-transfected with pcDNA3-Flag-hZimp10 and pSVARo were synchronized with mimosine. Double immunostaining was conducted with anti-Flag and anti-AR antibodies followed by the secondary antibodies conjugated with FITC (green) and rhodamine (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**).

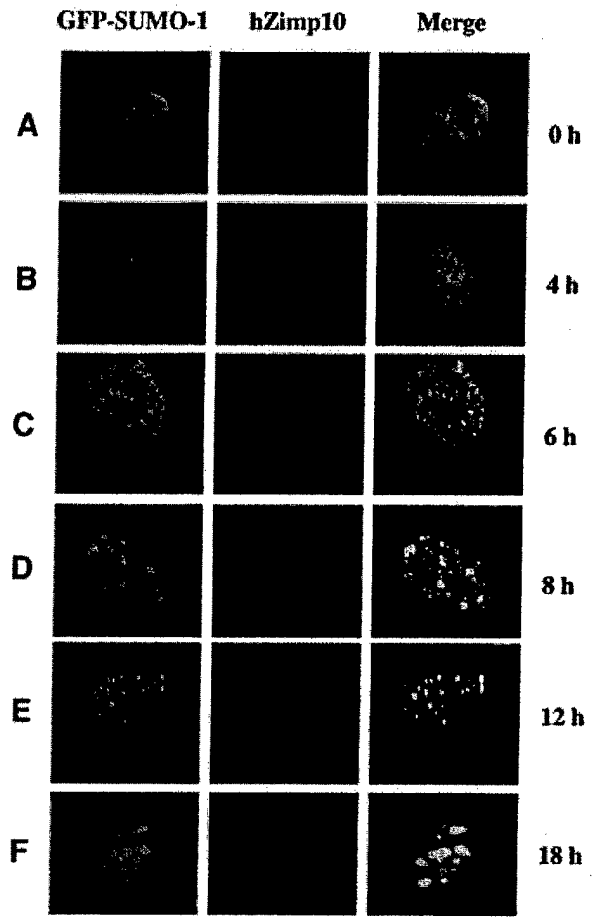


Fig. 9. Immunofluorescent co-localization of GFP–SUMO-1 and hZimp10 at DNA replication foci throughout S phase. CV-1 cells co-transfected with pcDNA3-Flag-hZimp10 and pGFP-SUMO-1 were synchronized with mimosine. Immunostaining was conducted with anti-Flag monoclonal antibody followed by secondary antibodies 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.

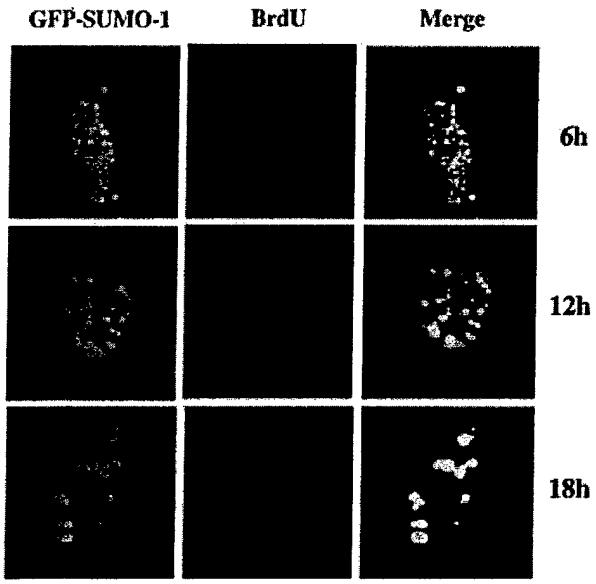


Fig. 10. Immunofluorescent localization of SUMO-1 at replication foci. CV-1 cells were transfected with GFP–SUMO-1 plasmid, and then synchronized as described in Materials and methods. Representative confocal laser scanning microscopic images of nuclei from cells expressing GFP–SUMO-1 proteins and pulsed 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).

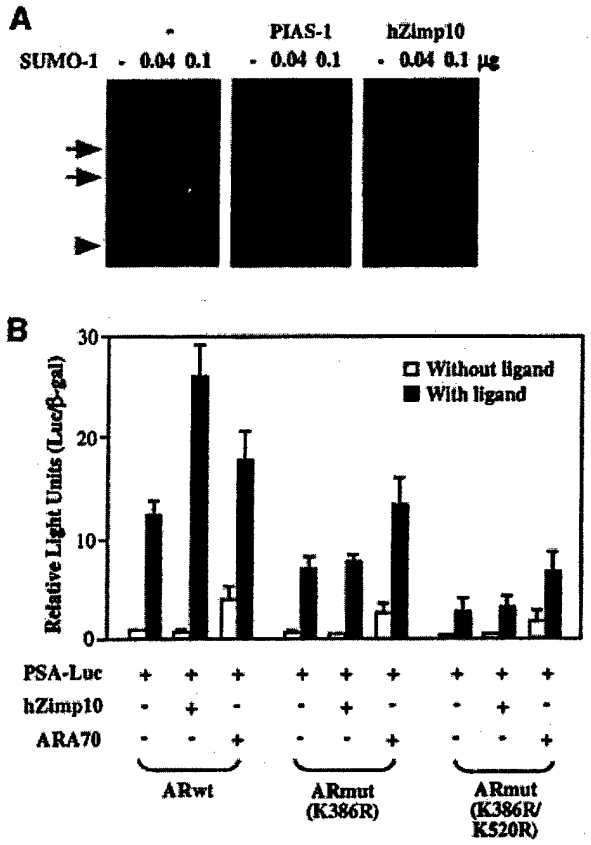


Fig. 11. hZimp10 enhances the sumoylation of AR and augments AR-mediated transcription. **(A)** COS-1 cells were co-transfected with 200 ng of pSG5-AR in the presence and absence of 200 ng of pFlag-PIAS1 or 360 ng of pcDNA3-Flag-hZimp10 along with 40 or 100 ng of pSG5-His-SUMO-1 as indicated. The cells 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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Mechanism of p21-activated Kinase 6-mediated Inhibition of Androgen Receptor Signaling*

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PAK6 was first identified as an androgen receptor (AR)-interacting protein able to inhibit AR-mediated transcriptional responses. PAK6 is a serine/threonine kinase belonging to the p21-activated kinase (PAK) family implicated in actin reorganization and cell motility, gene transcription, apoptosis, and cell transformation. We investigated the biochemical basis for inhibition of AR signaling by PAK6. We compared the kinase activity of PAK6 with two other well characterized members of the PAK family, PAK1 and PAK4. Like PAK4, PAK6 possesses a constitutive basal kinase activity that, unlike PAK1, is not modulated by the binding of active Rac or Cdc42 GTPases. In order to test the involvement of PAK6 kinase activity in suppression of AR-mediated transcription, we generated kinase-dead (K436A) and kinase-active (S531N) mutants of PAK6. We show that PAK6 kinase activity is required for effective PAK6-induced repression of AR signaling. Suppression does not depend upon GTPase binding to PAK6 and is not mimicked by the closely related PAK1 and PAK4 isoforms. Kinase-dependent inhibition by PAK6 extended to the enhanced AR-mediated transcription seen in the presence of coactivating molecules and to the action of AR coinhibitors. Active PAK6 inhibited nuclear translocation of the stimulated AR, suggesting a possible mechanism for inhibition of AR responsiveness. Finally, we observe that autophosphorylated, active PAK6 protein is differently expressed among prostate cancer cell lines. Modulation of PAK6 activity may be responsible for regulation of AR signaling in various forms of prostate cancer.

Currently, six members of the p21-activated kinase (PAK)[1] family of protein kinases have been identified and can be classified into two groups based on their sequence homology and regulatory properties: group I, including PAKs 1–3, and group II, including PAKs 4–6 (1–3). PAKs are serine/threonine kinases that contain a Cdc42/Rac-interactive binding (CRIB) domain and a Ste20-related kinase domain. The PAK family members have been implicated in the regulation of multiple cellular functions, including actin reorganization, cell motility, gene transcription, cell transformation, apoptotic signaling, and more recently, steroid hormone receptor signaling (see below).

The binding of activated GTP-bound Cdc42 or Rac to group I PAKs dramatically stimulates their ability to phosphorylate exogenous substrates. In contrast, the group II PAKs, PAK4 and PAK5, possess a substantial "basal" kinase activity that is not further stimulated by binding of activated GTPase (4, 5). GTPase binding does mediate kinase relocalization: after binding Cdc42, PAK4 is relocalized to the Golgi (4), and PAK5 shuttles from the microtubule network to actin-rich structures (6). The mechanisms by which PAK6 activity is regulated and the role of PAK6 kinase activity in its biological functions have not yet been studied.

PAK6 was identified by yeast two-hybrid screening as an androgen receptor-interacting protein (7). After androgen stimulation, PAK6 was reported to interact with the ligand binding domain of the androgen receptor (AR) and to translocate to the nucleus along with the AR, where PAK6 inhibits AR-mediated transcription. Northern blot analysis shows that PAK6 is mainly expressed in brain, testis, prostate, and breast tissues (7, 8). PAK6 has also been shown to bind the estrogen receptor (ER) and to inhibit ER-mediated gene transcription (8). Interestingly, the inhibitory effect of PAK6 on AR- and ER-mediated gene transcription is opposite to the transactivation of the ER induced by PAK1-mediated receptor phosphorylation (9).

The AR and ER are hormone-activated transcription factors that belong to the nuclear receptor superfamily (10, 11). The AR has a fundamental role in the development and differentiation of androgen-sensitive tissues and also plays an important role in the pathogenesis of prostate cancer (12). Structurally, the AR is composed of three important functional domains, an N-terminal transactivation domain (TAD), a DNA binding domain (DBD), and a ligand binding domain (LBD). In the absence of androgen, the AR is localized in the cytoplasm in association with heat shock proteins (13, 14). Upon stimulation by dihydrotestosterone (DHT) or DHT analogues, including R1881, heat shock proteins are released, and homodimerization and translocation of the AR to the nucleus occur (15, 16).

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[1] The abbreviations used are: PAK, p21-activated kinase; AR, androgen receptor; ARA, androgen receptor-associated; β-gal, β-galactosidase; CRIB, Cdc42/Rac-interactive binding; DBD, DNA binding domain; DHT, dihydrotestosterone; LBD, ligand binding domain; Luc, luciferase; GST, glutathione *S*-transferase; TAD, transcription activation domain; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; wt, wild type; DTT, dithiothreitol; PBS, phosphate-buffered saline; EGFP, enhanced green fluorescent protein; ER, estrogen receptor; MMTV-LTR, murine mammary tumor virus-long term repeat.

The ligand-bound nuclear AR is capable of mediating transactivation and/or transcriptional repression of target genes. The transcriptional activity of AR is modulated by interaction with cofactors, including coactivators that enhance AR activity and corepressors that inhibit AR activity (17). SRC1, p300, Tip60α, β-catenin, and ARA55 are among these coactivating molecules that have been shown to increase AR-mediated transcription (18–23). The transcriptional activity of the AR can also be regulated either by direct phosphorylation of the receptor and/or by phosphorylation of cofactors (24–28).

In this paper, we investigated the mechanism(s) by which PAK6 inhibits AR-mediated transactivation. By using both kinase-inactive and constitutively kinase-active mutants of PAK6, we showed that inhibition of AR transcriptional activity by PAK6 is dependent on its kinase activity. The binding of Cdc42 GTPase to PAK6 was not required for transcriptional inhibition. The inhibitory effect of PAK6 on AR-mediated transcription was dominant to AR coactivator functions and synergized with corepressors. PAK6 phosphorylated the DNA binding domain of the AR in *in vitro* kinase assays. Finally, we observed that phosphorylated, activated PAK6 protein is expressed differently among the different prostate cancer cell lines. Our results suggest that modulation of PAK6 expression and/or kinase activity may be an important component in the regulation of AR signaling in various forms of prostate cancer.

EXPERIMENTAL PROCEDURES

Materials—Cell culture media, fetal bovine serum, and supplements were from Invitrogen. [γ-^{32}P]ATP (specific activity 4500 mCi/mmol) was from ICN, Costa Mesa, CA. Plasmids for transfection were purified using the Qiafilter purification system of Qiagen, Chatsworth, CA. Thrombin was purchased from Sigma; GDP and GTPγS were from PerkinElmer Life Sciences. For PCR, the Expand High Fidelity PCR system from Roche Applied Science was used.

Cell Cultures—Human cancer cell lines PC-3, PC3MM2 (41), Du145, and HeLa were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, penicillin (25 units/ml), and streptomycin (25 μg/ml). MCF-7, ARCaP (42), and LNCaP were maintained in RPMI 1640 with 10% fetal calf serum, penicillin (25 units/ml), and streptomycin (25 μg/ml). PC3zj is a subclone of the human prostate carcinoma cell line PC3.

Plasmid Construction—PAK1, PAK4, and PAK6 were subcloned into pcDNA3-EGFP. PAK6 was inserted into the Myc tag vector pCMV6M. To produce K436A, S531N, and S560E single mutations in PAK6, overlapping PCR was performed using outer boundary primers (PAK6-EcoRI-5′ and PAK6-XhoI-3′) and overlapping primer pairs to introduce the desired mutations (for K436A, forward primer 5′-CGC CAG GTG GCC GTC gca ATG ATG GAC CTC AGG-3′; reverse primer 5′-CCT GAG GTC CAT CAT tgc GAC GGC CAC CTG GCG-3′; for S531E, forward primer 5′-GAC ATC AAG AGT GAC aac ATC CTG CTG ACC-3′; reverse primer 5′-GGT CAG CAG GAT gtt GTC ACT CTT GAT GTC-3′; for S560N, forward primer 5′-GTC CCT AAG AGG AAG gag CTG GTG GGA ACC CCC-3′; reverse primer 5′-GGG GGT TCC CAC CAG ctc CTT CCT CTT AGG GAC-3′; and wt PAK6 as a template (lowercase letters indicate the introduced base mutation)). The different fragments were inserted into EcoRI/XhoI-cut pcDNA3-EGFP. To produce the S531N,S560E double mutations in PAK6, overlapping PCR was performed using outer boundary primers (PAK6-EcoRI-5′ and PAK6-XhoI-3′), the overlapping primer pairs S560N, and using PAK6 S531N as a template. The fragment was inserted into EcoRI/XhoI-cut pcDNA3-EGFP. The mutations H20L,H23L were introduced by site-directed point mutation using Quickchange kit (Stratagene, La Jolla, CA) according to the manufacturers instruction. Primer sequences are as follows: PAK6(H20L,H23L) 5′-CCA CAG AAC TTC CAG CTC CGT CTC CTC ACC TCC TTC-3′ and its complementary sequence. The reporter plasmid MMTVpA3-Luc, containing the luciferase gene under the control of the steroid hormone-response elements in the MMTV-LTR, was provided by Dr. Richard Pestell (Albert Einstein Medical College, New York). The human AR cDNA, cloned into SV40 promoter-driven expression vector, pSV-hAR, was provided by Dr. Albert Brinkmann (Erasmus University, Rotterdam, The Netherlands). An SV40-driven β-galactosidase reporter plasmid (pSV-β-GAL) (Promega, Madison, WI) was used in this study as an internal control. The mouse SMRTa cDNA, cloned into pCMX, was provided by Dr. Ronald M. Evans (The Howard Hughes Medical Institute, and the Salk Institute for Biological Studies, La Jolla, CA).

Purification of Recombinant Proteins—GST-AR (TAD, DBD, and LBD) and Cdc42 proteins in vector pGEX-2TK were expressed in BL21 cells and purified according to the protocol of Amersham Biosciences. Buffers for the GST-Cdc42 purification contained 1 μM GDP starting from the lysis step, excluding the dialysis buffer. The GST moiety was cleaved off Cdc42 with thrombin at a final concentration of 10 units/ml glutathione beads. Thrombin was removed by incubation with *p*-aminobenzamidine beads (Sigma), and the protein was dialyzed four times against 25 mM Tris/HCl, pH 7.5, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride. After dialysis, 1 μM GDP was added again, and the purified protein was concentrated by ultrafiltration.

Immunoprecipitation—HeLa cells were seeded on 10-cm cell culture dishes at 50–70% confluency and transfected using LipofectAMINE (Invitrogen). 2 μg of plasmid DNA and 30 μl of LipofectAMINE were used per dish, and the transfection protocol was essentially followed according to the manufacturer's guidelines (Invitrogen). After 30 h the cells were washed with PBS, lysed in 0.5 ml of Lysis Buffer (25 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT, 10% glycerol, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride), and centrifuged for 5 min at 4 °C at 15,000 × *g*, and the supernatant was collected. Protein expression in the lysates was determined by immunoblotting. For precipitations of EGFP, Myc, or FLAG-tagged PAK6 proteins, lysate containing equal amounts of the proteins was incubated with equilibrated protein G beads and anti-EGFP (3E6; Molecular Probes, Eugene, OR), anti-Myc (9E10; Santa Cruz Biotechnology, Santa Cruz, CA), or anti-FLAG (M2; Molecular Probes) antibodies for at least 3 h or overnight at 4 °C. The bead fraction was washed four times with lysis buffer, twice in kinase buffer, and used for kinase assays.

Loading of Cdc42—Cdc42 was loaded with GTPγS or GDP under the following conditions. 5–20 μg of Cdc42 was incubated in 25 mM Hepes/NaOH, pH 7.5, containing 20 mM EDTA and 1 mM GTPγS or GDP for 10 min at 30 °C in a total volume of 25–100 μl. The reaction was stopped by addition of $MgCl_2$ at 100 mM final concentration. GTPγS or GDP-loaded Cdc42 was directly used in kinase or pull-down assay.

In Vitro Kinase Assay—Kinase reactions with immunoprecipitated PAK were performed in kinase buffer (50 mM Hepes/NaOH, pH 7.5, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 0.2 mM DTT) in a volume of 60 μl with 250 μM ATP. Radiolabeled ATP was used at 10 μCi/reaction. The reactions were incubated for 30 min at 30 °C and stopped by addition of sample buffer. Histones H3/H4 or AR proteins were used as a substrate at 1 μg/reaction.

Western Blot—Cells were lysed and supernatants collected as described above. β-Mercaptoethanol and bromphenol blue were added, and cell lysate proteins were resolved by SDS-PAGE. Proteins were then electroblotted onto nitrocellulose filters, and filters were blocked by incubation for 1 h with 5% bovine serum albumin in Tris-buffered saline, 0.1% Tween 20 and then incubated overnight at 4 °C with anti-EGFP antibody (rabbit antibody, Molecular Probes, Eugene, OR) or anti-PAK6 phospho-antibody (anti-PAK4(Ser-474) anti-PAK5(Ser-602), and anti-PAK6(Ser-560) phospho-antibody 3241; Cell Signaling Technology, Inc. Beverly, MA). Blots were washed three times for 10 min in Tris-buffered saline plus 0.1% Tween 20 and incubated for 1 h with peroxidase-labeled anti-rabbit immunoglobulins. Blots were developed with the use of the enhanced chemiluminescence detection system (Pierce).

Pull-down Assay—Cells were transfected and lysed, and supernatants were collected as described above. Clarified lysate was incubated with either 1 μg of GTPγS or GDP-loaded Cdc42 for 2 h at 4 °C. Anti-EGFP antibody and protein G beads were then added and incubated at 4 °C for another hour. Beads were washed three times in lysis buffer, boiled in SDS sample buffer for 5 min, separated by SDS-PAGE, and subjected to a Western blotting with either an EGFP antibody (rabbit antibody, Molecular Probes) or a Cdc42 antibody (SC-87, Santa Cruz Biotechnology).

AR Transcriptional Activity—The monkey kidney cell line CV-1 containing no detectable levels of endogenous steroid hormone receptor activity was maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum (Hyclone, Denver, CO). Transient transfections were carried out with LipofectAMINE (Invitrogen). Cells were plated on 12-well plates at 50–70% confluency 16 h before transfection, and 400 ng of total plasmid DNA per well was used in the transfection. Approximately 16 h after transfection, the cells were washed and fed with medium containing 5% charcoal-stripped (steroid

hormone-free) fetal calf serum (Hyclone) in the presence or absence of R1881. Cells were incubated for another 24 h, lysates prepared, and luciferase and galactosidase activities measured with the Luciferase Assay kit (Promega) and Galactolight kit (Tropix, Bedford, MA), respectively.

Immunofluorescence—HeLa cells were plated onto 2-well chamber slides. After 24 h, cells were transiently transfected with wt or mutated EGFP-tagged pcDNA3-PAK6 and pSV-hAR with LipofectAMINE PLUS reagent (Invitrogen) according to the manufacturer's instructions. 24 h post-transfection, cells were left untreated or treated with 10 nM R1881 for another 24 h. Cells were fixed for 5 min with 4% paraformaldehyde in PBS and placed in methanol for 1 min, followed by sequential washes with PBS and 1% fetal bovine serum in PBS. Cells were incubated with polyclonal anti-AR antibody (Santa Cruz N20816) for 1 h at room temperature. Cells were washed with PBS and incubated with Texas Red-conjugated anti-rabbit secondary antibody (Molecular Probes). Cells were dried and mounted with fluor mounting medium (Vector Laboratories). Pictures were taken using confocal microscopy.

RESULTS

Analysis of PAK6 Kinase Activity—In order to evaluate the kinase activity of PAK6 and compare it to different members of the PAK family, HeLa cells were transfected with vectors containing wild type (wt) PAK6, PAK1, or PAK4. Equal amounts of EGFP-PAK1wt, EGFP-PAK6wt, and EGFP-PAK4wt were immunoprecipitated with an anti-EGFP antibody, and an *in vitro* kinase assay was performed using histone H3/H4 as an exogenous substrate, in the presence of either GDP-loaded (inactive) or GTPγS-loaded (active) Cdc42. As expected, significant phosphorylation of H3/H4 by PAK1wt was stimulated only in the presence of Cdc42-GTPγS. In contrast, substantial phosphorylation of H3/H4 by PAK6wt and PAK4wt was observed in the presence of both Cdc42-GDP and Cdc42-GTPγS, and the activity was not enhanced by the presence of the active GTPase (Fig. 1*A*). The relative kinase activity of Cdc42-activated PAK1wt was stronger than with PAK6wt or PAK4wt at comparable protein expression levels. An additional slower migrating band was observed with PAK6wt, suggesting that, as with PAK1 and PAK4wt, PAK6wt became autophosphorylated. However, unlike the autophosphorylation of PAK1wt, which is activation (Cdc42-GTPγS)-dependent, autophosphorylation of PAK6wt and PAK4wt was Cdc42-independent.

In order to verify that the presence of the relatively large EGFP tag did not alter the ability of Cdc42 to activate PAK6, HeLa cells were transfected with vectors containing Myc-, FLAG-, or EGFP-tagged versions of PAK6wt, and then protein was immunoprecipitated with the relevant epitope tag antibody. An *in vitro* kinase assay was performed in the presence of Cdc42 loaded with either GDP or GTPγS using histone H3/H4 as an exogenous substrate. As shown in Fig. 1*B*, phosphorylation of H3/H4 mediated by each FLAG-, Myc-, and EGFP-tagged PAK6wt was not modulated by the presence of Cdc42 loaded with either GDP or GTPγS. These results show that PAK6 possesses a substantial basal kinase activity that is not further stimulated by Cdc42 (or Rac; data not shown).

Characterization of PAK6 Mutants—Several PAK6 mutants were generated in an attempt to obtain kinase-inactive and highly kinase-active forms of PAK6. PAK6 (K436A) introduced a lysine-to-alanine mutation at amino acid 436 in the activation loop within the kinase domain (Fig. 2*A*). Mutation of this residue is predicted to inhibit the binding of ATP and abolish kinase activity. PAK6(S560E) converted serine in a predicted autophosphorylation site (based on homology to PAK1) to glutamic acid. PAK6(S531N) introduced a serine-to-asparagine mutation at amino acid 531 in the catalytic loop. Mutation of this residue is predicted to stabilize the catalytic loop, as is the double mutant PAK6(S531N,S560E) (Fig. 2*A*). The EGFP-tagged version of each construct was transiently transfected into HeLa cells; equal amounts of PAK6 protein were immunopurified from cell lysates using EGFP antibody, and an *in vitro* kinase assay was performed using histone H3/H4 as an exogenous substrate. As shown in Fig. 2*B*, PAK6(K436A) exhibited no autophosphorylation nor exogenous kinase activity. Mutation S560E alone did not change the exogenous kinase or autophosphorylation activity of PAK6 from that of PAK6wt. In contrast, the mutation S531N strongly enhanced both exogenous kinase and autophosphorylation activity of PAK6. A similarly enhanced PAK6 activity was observed with the PAK6(S531N,S560E) double mutant. These results suggest that the S531N mutation stabilized the catalytic loop within the kinase domain of PAK6 to increase the kinase activity. The kinase activity of these mutants was not modulated by the presence of Cdc42 loaded with either GDP or GTPγS (Fig. 2*C*).

The ability of an anti-phospho-PAK6 antibody directed against the Ser-560 predicted autophosphorylation site to detect active PAK6 was evaluated. Phosphorylation of the corresponding serine 423 residue in PAK1 was observed after activation by Rac/Cdc42 and reflected PAK1 kinase activity (29–31). HeLa cells were transfected with PAK6wt, K436A, S531N, and S560E, and cell lysates were prepared and analyzed by Western blot using anti-phospho-PAK6 antibody (Fig. 2*D, upper panel*). An anti-EGFP antibody was used to confirm similar protein expression levels (Fig. 2*D, lower panel*). As expected, PAK6(K436A) kinase-inactive and PAK6(S560E)-modified serine were not detected by the anti-phospho-PAK6 antibody. In

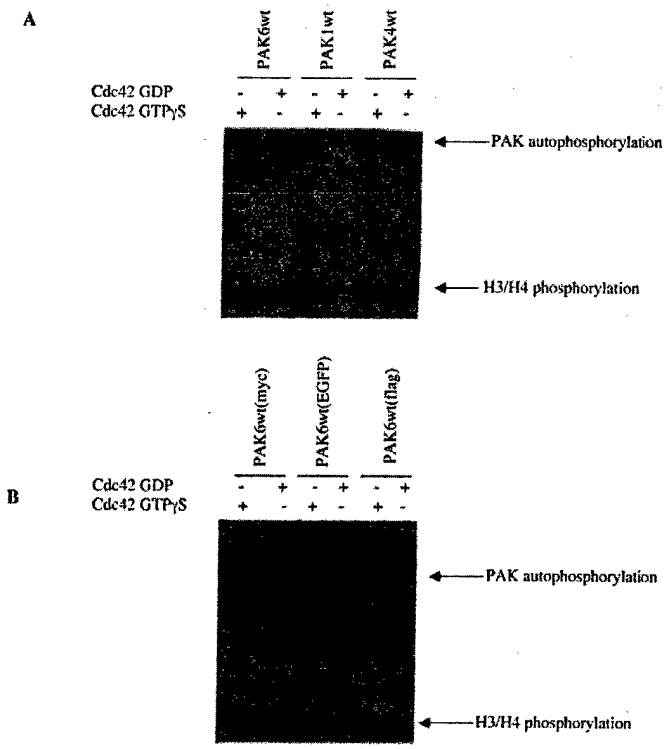

FIG. 1. **Analysis of PAK6 kinase activity.** *A*, HeLa cells were transiently transfected with pcDNA3-EGFP expression vector containing wild type PAK1, PAK4, or PAK6. After cell lysis, the amount of each PAK in the lysate was evaluated by Western blot and densitometry of the common EGFP fusion. Equal amounts of each PAK were incubated with Cdc42 loaded with either GDP or GTPγS, and an *in vitro* kinase assay was performed using histone H3/H4 as substrate. Phosphorylation was detected by autoradiography. *B*, HeLa cells were transfected with pcMV-myc, pcDNA3-EGFP, or pcDNA3-FLAG expression vectors containing PAK6wt. PAK6wt Myc-tagged, EGFP-tagged, or FLAG-tagged proteins were immunoprecipitated with an anti-Myc, anti-EGFP, or anti-FLAG antibody, respectively. Immunoprecipitates were incubated with Cdc42 loaded with GDP or GTPγS, and kinase assays were performed using histone H3/H4 as substrate. Phosphorylations were detected by autoradiography. Results shown are indicative of 3–4 similar experiments.

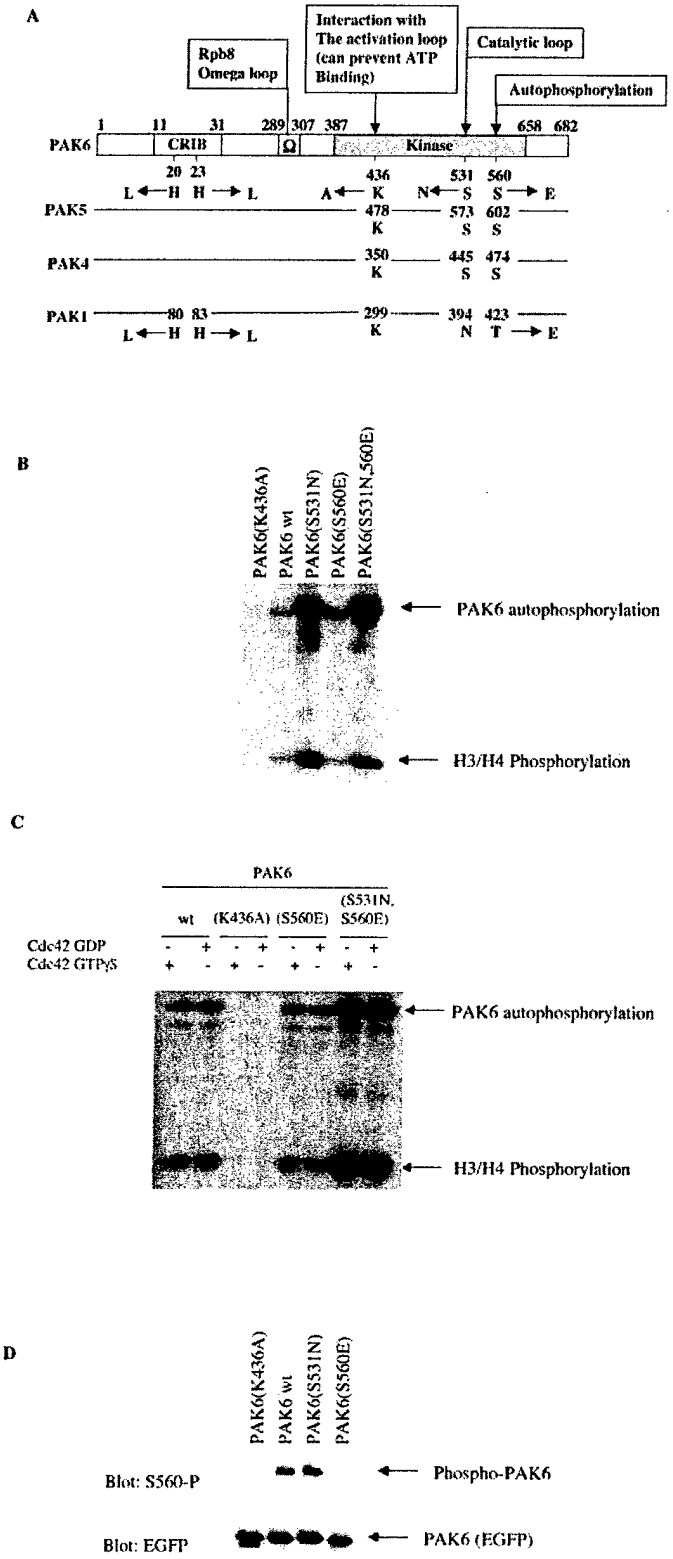

FIG. 2. **Kinase activity of PAK6 mutants.** *A*, schematic representation of PAK6 showing functional domains and point mutations used in this study. An alignment of the kinase domain and the CRIB domain of PAK1, PAK4, PAK5, and PAK6 shows the position of conserved amino acids. Mutation PAK6(K436A) corresponds to the kinase-inactivating mutation of the ATP-binding site K299A in PAK1. Mutation PAK6(S531N) corresponds to the activating mutation in the catalytic loop S445N in PAK4. The mutation S560E in PAK6 corresponds to the activating mutation of the autophosphorylation site T423E in PAK1. The mutations H20L,H23L in PAK6 correspond to mutations H83L,H86L in the CRIB domain of PAK1, which has been shown to abolish the binding of Rac or Cdc42 to PAK1 (3, 33). The Rbp8 Ω loop domain of PAK6, homologous to RNA polymerase subunit 8 Omega loop, is also shown. *B*, HeLa cells were transfected with pcDNA3-EGFP expression vector containing PAK6wt or PAK6 mutants K436A, S531N, S560E, and S531N,S560E. An *in vitro* kinase assay using histone H3/H4 as substrate was performed on the immunoprecipitated PAK6 proteins and then analyzed by autoradiography. *C*, HeLa cells were transfected with pcDNA3-EGFP expression vector containing PAK6wt or PAK6 mutants as indicated. PAK6 proteins were immunoprecipitated and incubated with Cdc42 loaded with GDP or GTPγS, and a kinase assay was performed. Phosphorylations were detected by autoradiography. *D*, HeLa cells were transfected with pcDNA3-EGFP expression vector containing PAK6wt or PAK6 mutants K436A and S531N,S560E. An aliquot of each lysate was analyzed by Western blot using an anti-EGFP antibody (*top panel*) or an antibody recognizing PAK6 protein phosphorylated on serine 560 (*bottom panel*). Results shown in *B–D* are representative of three similar experiments.

contrast, this antibody was able to detect PAK6wt and PAK6(S531N) mutants. These results are consistent with phosphorylation at serine 560 as an indicator reflecting PAK6 kinase activity.

Repression of AR-mediated Transcription by PAK6 Is Dependent on PAK6 Kinase Activity—It has been shown previously (7, 8) that overexpression of PAK6 specifically repressed AR-mediated transcription. To assess whether the kinase activity of PAK6 was required to inhibit AR-mediated transcription, CV1 cells were cotransfected with AR (pSVAR) and PAK6 plasmids, along with a luciferase gene reporter under the control of the steroid hormone-responsive elements in the MMTV-LTR (MMTVpA3-Luc). As shown in Fig. 3*A*, PAK6wt was able to repress in a dose-dependent manner AR transcriptional activity induced by different concentrations of R1881 ranging from 0.1 to 100 nM. wt PAK6 and mutant (S560E), which both have the same relative level of kinase activity, repressed to a similar extent AR transcriptional activity induced by R1881 (Fig. 3*B*). Significantly, PAK6(S531N) and PAK6(S531N,S560E) mutants that exhibited strong kinase activities inhibited ligand-dependent AR transcriptional activity more effectively (*i.e.* by more than 85%) at similar levels of transfected DNA. In marked contrast, kinase-inactive mutant PAK6(K436A) inhibited less than 10–20% of AR-dependent transcriptional activity (Fig. 3*B*). There is thus a correlation between relative kinase activity of PAK6 proteins and their ability to suppress AR-mediated transcription.

We next tested the specificity of the inhibition of AR transcriptional activity by transfecting CV1 cells with wt or dominant active versions of PAK1, PAK4, and PAK6. Fig. 3*C* shows that PAK4 only weakly repressed AR transcriptional activity, whereas PAK1 modestly stimulated AR transcriptional activity. PAK6(S531N) dominant active was used as control. These results provide evidence that the inhibition of AR-mediated transcription is specific to the testis/prostate-enriched PAK6 isoform.

PAK6 Inhibition of AR-mediated Signaling Is Independent of GTPase Binding—The Rho family GTPase Cdc42 has been shown to bind to PAK6, presumably via its CRIB domain (9, 32), but we have demonstrated that this interaction does not affect PAK6 kinase activity. The binding of Cdc42 to PAK4 and PAK5 also does not increase their kinase activity but rather modulates their subcellular localization (4, 6). We wanted to determine whether the interaction of PAK6 with GTPase was important in enabling PAK6 to inhibit AR-mediated transcription. We generated a series of PAK6 constructs in which the CRIB domain was mutated in a conserved pair of histidine residues known to be important for GTPase binding (PAK6(H20L,H23L), PAK6(H20L,H23L,K436A), and PAK6(H20L,H23L,S531N)) (33). The binding of Cdc42 to these PAK6 constructs was analyzed by immunoprecipitation of EGFP-tagged PAK6 mutants in the presence of Cdc42 loaded

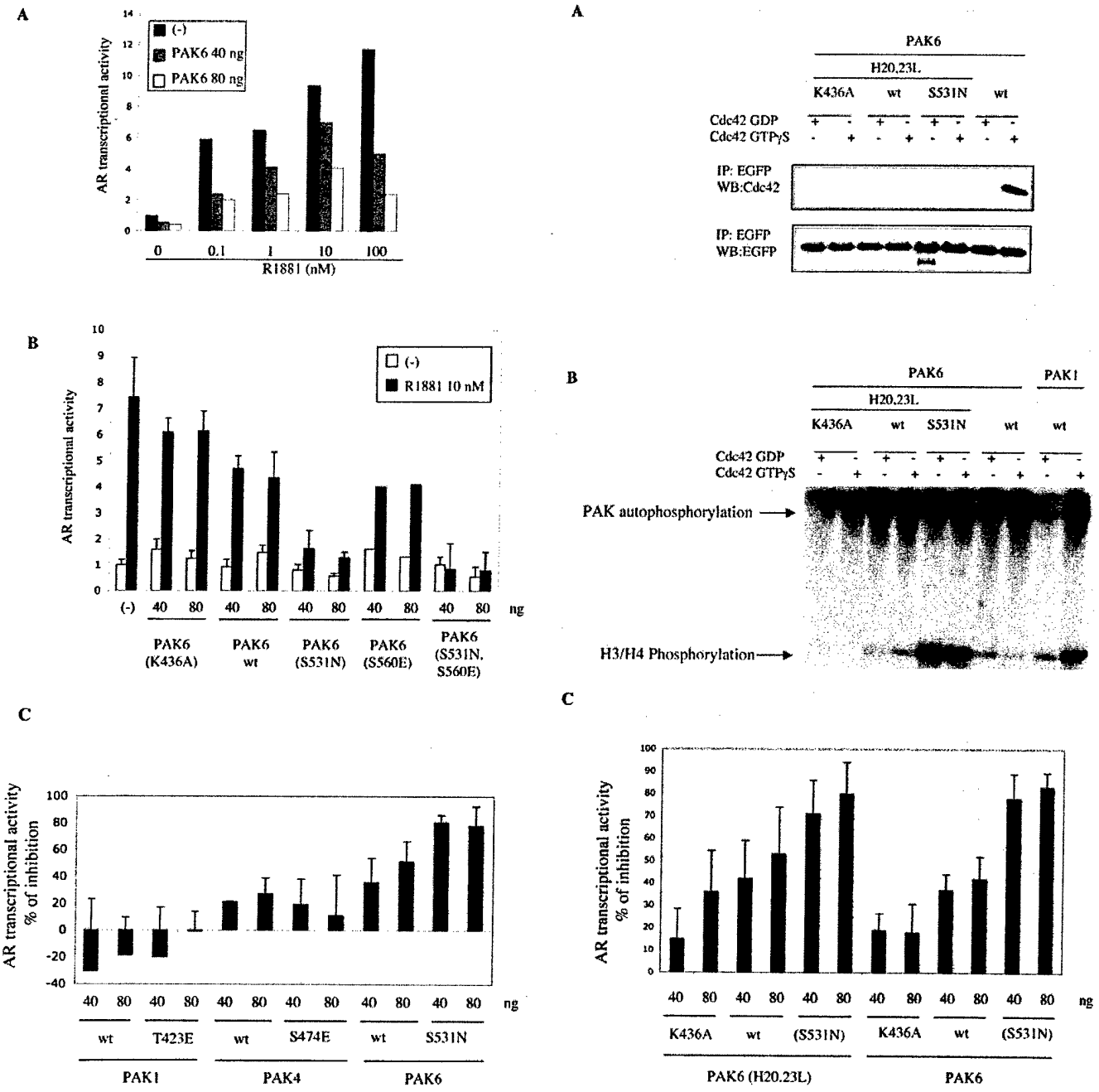

FIG. 3. **Repression of AR-mediated transcription by PAK6 is dependent on PAK6 kinase activity and is specific to PAK6.** *A*, CV-1 cells were transiently transfected in 12-well plates with 400 ng of pMMTV-Luc, 100 ng of pSV40-β-gal, 40 ng of pSV-hAR, and 40 or 80 ng of pcDNA3-EGFP-PAK6 constructs. The total amount of DNA was normalized with pcDNA3. 18 h after transfection, cells were treated with the indicated concentrations of R1881 (0.1–100 nM) and cell extracts prepared 24 h after stimulation, and luciferase activity was monitored. Luciferase activity is reported as relative light units normalized to β-galactosidase activity. *B*, CV-1 cells were transiently transfected in 12-well plates with 400 ng of pMMTV-Luc, 100 ng of pSV40-β-gal, 40 ng of pSV-hAR, and 40 or 80 ng of pcDNA3-EGFP expression vector containing PAK6 mutants K436A, wt, S531N, S560E, and S531N,S560E cDNAs. 18 h after transfection cells were treated with 10 nM R1881 (*gray bars*) or left untreated (*white bars*), and luciferase activity was determined (reported as relative light units). *C*, CV-1 cells were transiently transfected in 12-well plates with 400 ng of pMMTV-Luc, 100 ng of pSV40-β-gal, 40 ng of pSV-hAR, and 40 or 80 ng of expression vector containing wild type (wt) PAK1, -4, -6 or active kinase mutant PAK1(T423E), PAK4(S474E), and PAK6(S531N). Total amount of DNA was normalized with pcDNA3. 18 h after transfection, the cells were stimulated with 10 nM R1881 for another 24 h and cell extracts prepared, and luciferase activity was determined. The *bar graphs* shown in *B* and *C* represent the mean ± S.E. % inhibition of AR-mediated transcription observed in three separate experiments.

FIG. 4. **PAK6 inhibition of AR-mediated transcription is independent of Cdc42 binding.** *A*, binding of GTPγS loaded Cdc42 to PAK6. HeLa cells were transfected with pcDNA3-EGFP expression vector containing either PAK6wt or PAK6 mutants H20L,H23L,K436A, H20L,H23L, and H20L,H23L,S531N cDNAs. Equal amounts of cell lysate were incubated with Cdc42 loaded with either GDP or GTPγS and immunoprecipitated with an anti-EGFP antibody. The precipitated fractions were then resolved by SDS-PAGE and analyzed by Western blot using an anti-Cdc42 antibody or an anti-EGFP antibody. *B*, kinase activity of different PAK6 mutants. HeLa cells were transfected with pcDNA3-EGFP expression vector containing 40 or 80 ng of either PAK6wt, PAK1wt, or PAK6 (H20L,H23L,K436A; H20L,H23L; and H20L,H23L,S531N) cDNAs. PAK proteins were immunoprecipitated and incubated with Cdc42 loaded with either GDP or GTPγS; a kinase assay was performed in the presence of histone H3/H4, and the result was analyzed by autoradiography. *C*, CV-1 cells were transiently transfected in 12-well plates with 400 ng of pMMTV-Luc, 100 ng of pSV40-β-gal, 40 ng of pSV-hAR, and 40 or 80 ng of pcDNA3-EGFP-PAK6 constructs. Total amount of DNA was normalized with pcDNA3. 18 h after transfection cells were stimulated with 10 nM R1881 for another 24 h and cell extracts prepared, and luciferase activity was monitored. The *bar graph* shown represents the mean ± S.E. % inhibition of AR-mediated transcription by different PAK6 mutants observed in three independent experiments.

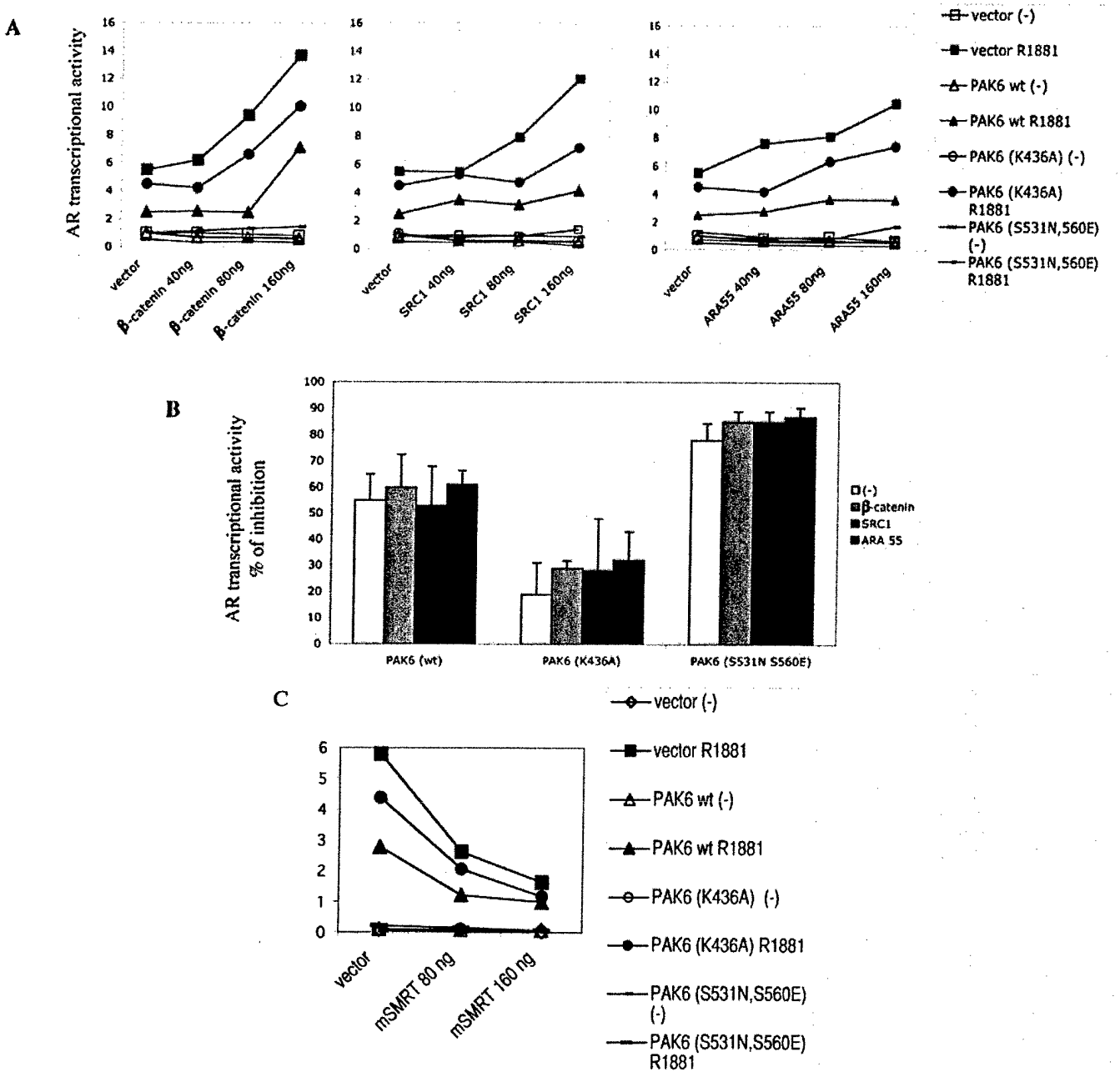

FIG. 5. **PAK6 inhibits AR-mediated transcription in the presence of AR coactivators.** *A*, CV-1 cells were transiently transfected in 12-well plates with 400 ng of pMMTV-Luc, 100 ng of pSV40-β-gal, 40 ng of pSV-hAR, 40 ng of pcDNA3-EGFP-PAK6 construct, and increasing amounts of pcDNA3-β-catenin, pcDNA3-SRC1, or pSG5-ARA55 constructs (40, 80, and 160 ng). The amount of DNA was normalized with pcDNA3. 18 h after transfection, cells were stimulated with 10 nM of R1881 (*black symbols*) or left untreated (*open symbols*) for another 24 h. Luciferase activity was determined, reported as relative light units. Results shown are representative of three similar experiments. *B*, the *bar graph* shown represents the mean ± S.E. % inhibition of AR-mediated transcription by PAK6 in presence of AR coactivators. *C*, CV-1 cells were transiently transfected in 12-well plates with 400 ng of pMMTV-Luc, 100 ng of pSV40-β-gal, 40 ng of pSV-hAR, 40 ng of pcDNA3-EGFP-PAK6 construct, and increasing amounts of pCMX-mSMRTa construct (80 and 160 ng). The amount of DNA was normalized with pcDNA3. 18 h after transfection, cells were stimulated with 10 nM R1881 (*black symbols*) or left untreated (*open symbols*) for another 24 h. Luciferase activity was determined, reported as relative light units. Results shown are representative of four similar experiments.

with either GDP or GTPγS (Fig. 4*A*). PAK6wt was able to pull down Cdc42 loaded with GTPγS, whereas the H20L,H23L mutation abrogated binding of Cdc42 to PAK6. In order to verify that the mutation H20L,H23L did not modify the kinase activity of PAK6, PAK6wt and CRIB mutants were transiently expressed in HeLa cells, and equal amounts of PAK6 were immunopurified from cell lysates using EGFP antibody. An *in vitro* kinase assay using histone H3/H4 was performed in the presence of Cdc42 loaded with either GDP or GTPγS. As shown in Fig. 4*B*, PAK6(H20L,H23L) exhibited the same relative level of exogenous kinase and autophosphorylation activity as did PAK6wt, whereas PAK6(H20L,H23L,K436A) exhibited no kinase activity at all. The mutant H20L,H23L,S531N displayed a strong exogenous kinase and autophosphorylation activity.

We next analyzed the ability of the non-Cdc42-binding H20L,H23L PAK6 mutants to suppress AR-mediated transcriptional activity. CV1 cells were transfected with pSVAR, MMTVpA3-Luc, PAK6wt, and PAK6 mutants. Mutation of H20L,H23L did not significantly modify the inhibitory effect of wt and kinase-active (S531N) forms of PAK6 (Fig. 4*C*). These

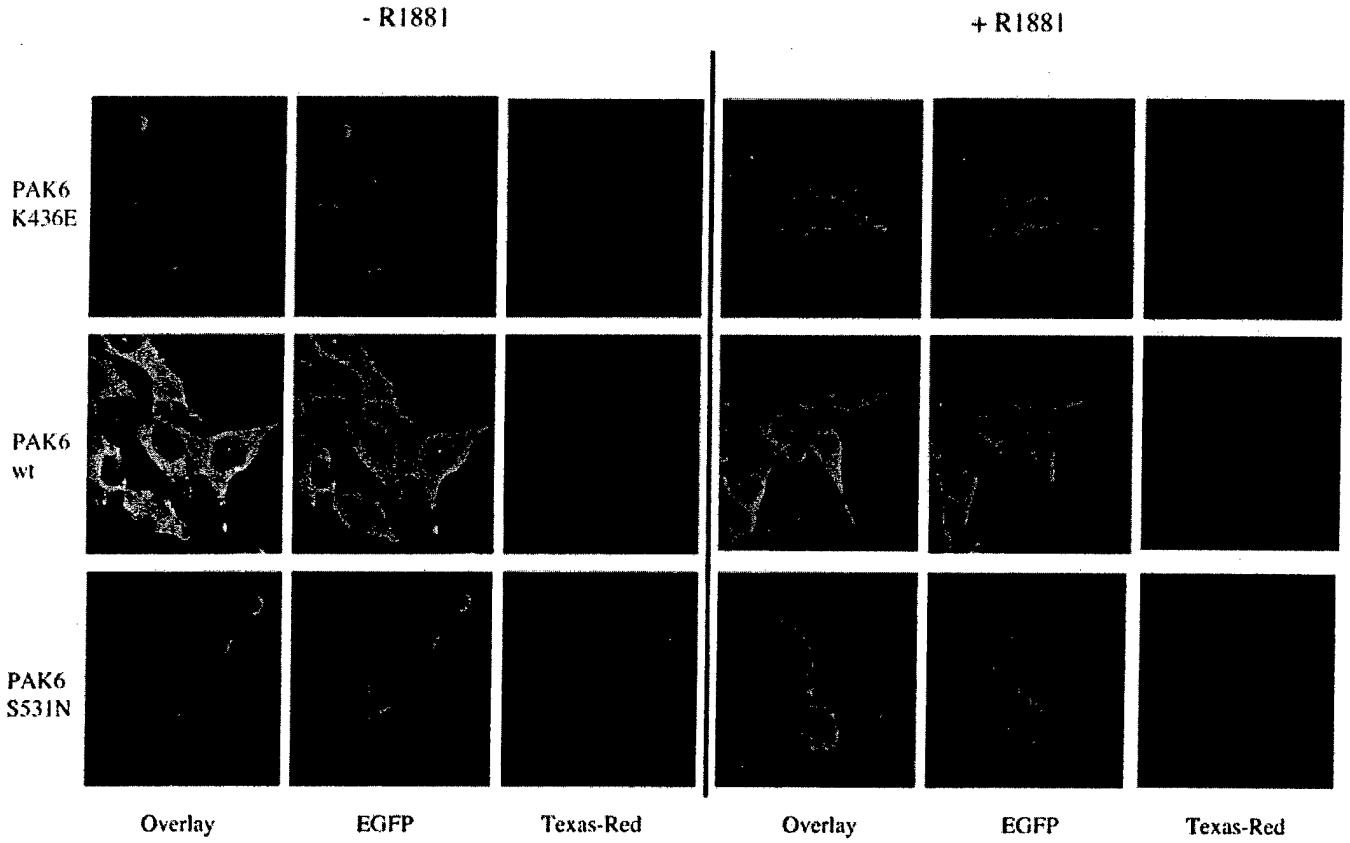

FIG. 6. **PAK6 inhibits AR nuclear translocation.** HeLa cells were transfected with wt or mutated EGFP-PAK6 and AR expression constructs and were cultured in the presence or absence of R1881 (10 nM). AR protein was detected with a polyclonal anti-AR antibody and revealed by Texas Red-conjugated secondary antibody. Pictures of cells were taken with confocal microscopy.

results demonstrate that PAK6-induced inhibition of AR-mediated transcription is independent of Cdc42 binding.

PAK6 Inhibits AR-mediated Transcription Enhanced by AR Coactivators and Inhibited with AR Corepressors—AR transcriptional activity can be enhanced by the presence of AR coactivators, including β-catenin, SRC1, ARA55, p300, and Tip60α, which have been shown to bind to distinct regions of the AR and increase AR-mediated transcription. We sought to determine whether PAK6wt and PAK6 mutants were able to inhibit to the same extent AR-mediated transcription in the presence of these differentially binding AR coactivators. CV1 cells were transfected with pSVAR, MMTVpA3-Luc, PAK6wt, and PAK6 mutants and serial concentrations of either β-catenin, SRC1, or ARA55. The coactivators β-catenin, SRC1, and ARA55 all increased AR-mediated transcription in a dose-dependent manner (Fig. 5*A*). At a concentration of 160 ng, β-catenin, SRC1, and ARA increased AR-mediated transcription by 2.3-, 2-, and 1.6-fold, respectively. In the presence of the highly active PAK6(S531N,S560E) mutant, these coactivators were not able to increase significantly the transcriptional activity of AR. In contrast, in the presence of PAK6wt or K436A, these coactivators all increased AR transcriptional activity. As shown in Fig. 5*B*, the inhibitory effect of PAK6wt on AR-mediated transcription was 55% in the absence of the coactivator and 60, 53, and 61% in the presence of β-catenin, SRC1, or ARA55, respectively. The inhibitory effect of PAK6(K436A) on AR-mediated transcription was 20% in the absence of the coactivator and 29, 28, and 32% in the presence of β-catenin, SRC1, or ARA55, respectively. The inhibitory effect of the dominant active mutant PAK6(S531N,S560E) on AR-mediated transcription was 80% in the absence of the coactivator and 85, 85, and 87% in the presence of β-catenin, SRC1, or ARA55, respectively. PAK6 also inhibited AR-mediated transcription in the presence of the coactivators p300 and Tip60α (data not shown). In addition, as shown in Fig. 5*C*, PAK6 increased the inhibition of AR signaling seen with the AR corepressor SMRT. These results show that the inhibitory effect of PAK6 on AR-mediated transcription is dominant over the presence of the AR activating cofactors β-catenin, SRC1, ARA55, p300, and Tip60α and are observed in the presence of a corepressor, SMRT, suggesting that PAK6 may act to directly modulate AR function.

Nuclear Translocation of the AR in Response to Androgen Is Inhibited by Kinase-active Mutants of PAK6—To assess the biological function of PAK6 kinase activity, we examined the subcellular localization of the AR after stimulation with androgen in the presence of PAK6 and PAK6 mutants (K436A and S531N) by immunofluorescence. As shown in Fig. 6, in the absence of R1881 the AR localized to the cytoplasm and the nucleus. In control (GFP-transfected) cells, R1881 induced a total nuclear accumulation of the AR, as reported previously (not shown). Similarly, in the presence of the kinase-inactive PAK6(K436A), treatment with R1881 resulted in the total accumulation of the AR in the nucleus. In contrast, upon coexpression of the AR with PAK6wt or highly active PAK6(K531N), R1881-induced translocation of the AR was partially inhibited, with a good deal of receptor remaining in the cytoplasm. These results suggest that PAK6 kinase activity negatively modulates steroid-induced AR translocation into the nucleus.

PAK6 Phosphorylates the AR in the DBD Domain—In order to further investigate the biochemical basis of PAK6-induced inhibition of AR-mediated signaling, we sought to determine whether PAK6 could phosphorylate the AR. To that end, we assessed the ability of PAK6 to phosphorylate a series of recombinant AR proteins (Fig. 7*A*). Highly active PAK6(S531N) was immunoprecipitated from HeLa cell lysates and incubated

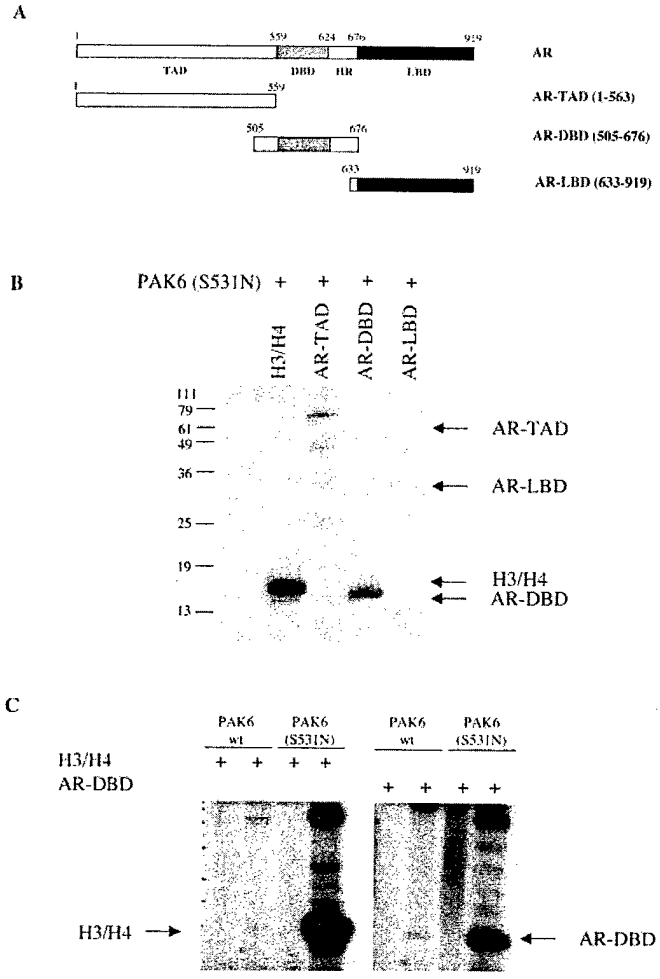

FIG. 7. **PAK6 phosphorylates the AR-DBD domain.** *A*, schematic representation of AR proteins. AR-TAD, AR-DBD, and AR-LBD were expressed in *Escherichia coli* and used as substrates in kinase assays. TAD, DBD, LBD, and hinge region (*HR*). *B*, immunoprecipitated PAK6(S531N) was used in a kinase assay with 1 μg of H3/H4 or 1 μg of the different domains of the AR. Phosphorylation of H3/H4 and the AR fragments was detected by autoradiography. *C*, PAK6wt or PAK6(S531N) was immunoprecipitated from HeLa cells and incubated with 1 μg of H3/H4 or DBD-AR purified from *E. coli*, and a kinase assay was performed. Phosphorylation of H3/H4 and the DBD fragments were detected by autoradiography.

with recombinant AR proteins in an *in vitro* kinase assay. PAK6(S531N) strongly phosphorylated a construct containing the DBD of the AR (Fig. 7*B*). In contrast, no phosphorylation of the AR TAD or the AR LBD could be detected. PAK6wt also phosphorylated the DBD-containing fragment, although phosphorylation was weaker than with PAK6(S531N) (Fig. 7*C*). These results suggest that PAK6 could modify AR activity through the direct phosphorylation of the DNA-binding element, thereby potentially preventing transcriptional regulation from taking place.

PAK6 Protein Expression in Prostate Cancer Cell Lines—To better understand the potential biological role(s) of PAK6, we analyzed the expression of PAK6 protein in different cancer cell lines. The antiphospho-PAK6 (Ser(P)-560) antibody was used to analyze PAK6 in prostate cancer cell lines DU145, PC-3zj, PC-3, ARCaP, PC3MM2, LNCaP, and LAPC4, breast cancer cell line MCF7, and the uterine cancer cell line HeLa (Fig. 8). As shown in Fig. 2*D*, this antibody was able to detect active versions (PAK6wt and PAK6(S531N)) but did not recognize the inactive PAK6(K436A) mutant. Phospho-PAK6 was detected most abundantly in the LAPC4 cell line. High expression/activity levels were also detected in the DU145, PC-3, and PC-3zj lines. MCF7 and ARCaP expressed only a small amount of phospho-PAK6, and no expression was detected in HeLa, LNCaP, and PC3-mm2 cells. These results indicate that the expression and/or activity level of PAK6 in prostate cell lines is highly variable and suggest that PAK6 could be involved in differential regulation of AR signaling in these cells.

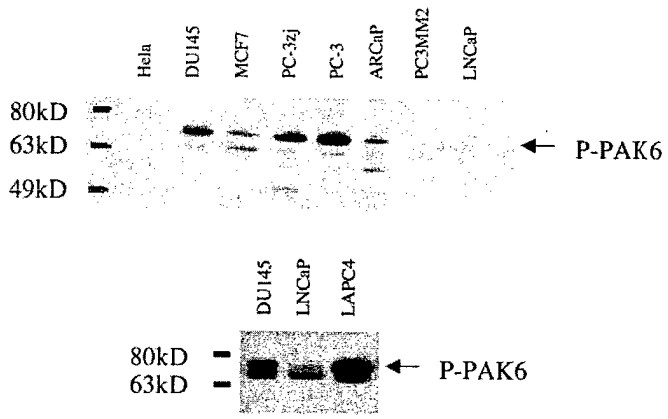

FIG. 8. **PAK6 protein expression in human cancer cell lines.** A Western blot was performed on equal protein amounts of lysates from prostate cancer cell lines (DU145, PC3zj, PC3, Arcap, PC3MM2, Lncap, and LapC4), breast cancer cell lines (MCF7), and uterus cancer cells line (HeLa) by using antibody recognizing PAK6 protein phosphorylated on serine 560.

DISCUSSION

In this study, we have investigated the mechanism by which the protein kinase PAK6 inhibits AR-mediated transcription. In order to better characterize the regulation of this serine/threonine kinase, we compared the kinase activity of PAK6 to PAK1 and PAK4, two well characterized members of the group I and group II PAK protein family, respectively. It is well established that the binding of activated Rac and Cdc42 GTPases to PAK1 markedly stimulates its kinase activity, both autophosphorylation and activity toward exogenously supplied substrates (3, 33, 34). In contrast, Rac or Cdc42 GTPase binding to PAK4 does not stimulate the already rather high basal kinase activity (4). We found that, like PAK4, PAK6 exhibits a constitutive kinase activity that is not increased by active Cdc42 (or Rac, not shown) GTPase (Figs. 1*A* and 2*D*). These findings are in accordance to BLAST comparisons showing that, like PAK4 and PAK5, PAK6 lacks the autoinhibitory domain adjacent to the CRIB domain in the group I PAKs whose release by GTPase binding accounts for GTPase-dependent kinase activation.

Whereas two prior studies (7, 8) have described the ability of PAK6 to suppress transcriptional signaling downstream of the androgen and estrogen nuclear receptors, the mechanism(s) underlying this effect have not been determined. We showed that mutation of PAK6 (PAK6(S531N)) to increase its kinase activity relative to the wild type protein dramatically increased its inhibitory effect on AR-mediated transcription (Fig. 3). Conversely, abrogating PAK6 kinase activity (PAK6(K436A)) considerably decreased its inhibitory effect on AR transcriptional activity. These results demonstrate that PAK6 kinase activity is involved in inhibition of AR-mediated transcription. It is also interesting to notice that the kinase-dead mutant of PAK6 (K436A) is still able to inhibit ~20% of AR transcriptional activity (Figs. 3 and 4). This result suggests that PAK6 is also able to suppress the transcriptional activity of the AR by a phosphorylation-independent mechanism. Neither PAK1 nor PAK4 was effective at inhibiting AR signaling, suggesting a mechanism specific to PAK6 is operative. Both prior studies

have indicated a physical interaction of PAK6 with the AR, including a region between the hinge and LBD. By BLAST comparison we have found that PAK6 possesses a unique region localized between amino acids 289 and 307. This domain is homologous to the Ω loop of Rbp8, one of the subunits of RNA polymerase (35, 36). We speculate that this domain could also be important for the PAK6 and AR interaction.

Because the regulation of transcription by the hormone-bound AR requires relocalization of the receptor to the nucleus, we considered the possibility that the binding of GTPase to PAK6 might play some role in this translocation process. We mutated conserved residues within the PAK6 CRIB domain known to be critical for GTPase binding (H20L,H23L). We established that this mutation impeded the binding of Cdc42 to PAK6 without affecting the inhibitory action of PAK6 on AR transcriptional activity (Fig. 4). These results demonstrate that Cdc42 binding is not required for PAK6-mediated inhibition of AR transcriptional activity, and also suggest that PAK6 activity is not modulated by GTPase-dependent relocalization within the cell. It is possible that the interaction of PAK6 with Cdc42 modulates an as yet unknown aspect of PAK6 function that is independent of the observed regulation of AR transcriptional activity.

The inhibition of AR signaling by PAK6 might be the result of a direct effect on the AR or an indirect effect on other AR-associated proteins. To address this issue, we studied the effect of PAK6 on AR signaling in the presence of different AR-associated coactivator and corepressor molecules, β-catenin, SRC1, ARA55, p300, Tip60α, and SMRT, all of which have been reported to increase or decrease AR transcriptional activity (Fig. 5). The presence of these coregulators did not modify the relative inhibitory effect of PAK6 and PAK6 mutants (K436A and S531N) on AR transcriptional activity. These results indicate that the mechanism for AR inhibition by PAK6 does not involve an indirect effect on the AR coactivators, β-catenin, SRC1, and ARA55, and that inhibition occurs through a common component utilized by each coactivator or corepressor (*i.e.* the receptor itself).

Given the fact that PAK6 inhibition of AR-mediated transcription is dependent on its kinase activity and is independent of AR coactivators, we examined the effects of PAK6 on AR nuclear translocation (Fig. 6). Kinase-active versions of PAK6 partially blocked steroid-induced nuclear translocation of the receptor. The lack of nuclear AR would tend to inhibit the transcriptional activity of the receptor. Interestingly, we did not observe steroid-induced nuclear translocation of PAK6, as had been originally reported by Yang *et al.* (7). We cannot rule out that this difference is because of cell type differences or to the use of GFP-tagged PAK6 in the current studies.

We also tested whether PAK6 was able to directly phosphorylate the AR (Fig. 7). We found that wild type and the highly active PAK6 mutant (S531N) were able to phosphorylate a fragment of the AR including the DBD and the hinge domains *in vitro*. It has been reported that PAK6 binds to a region between the hinge region and LBD of the AR. This suggests a scenario in which the *in vivo* binding of PAK6 to this region positions PAK6 to phosphorylate the AR and regulate activity of the nearby DBD domain. Because this region is important for DNA binding by the AR, phosphorylation in this domain by PAK6 could inhibit the binding of AR to DNA and, by this mechanism, suppress its transcriptional activity. There are several potential phosphorylation sites for PAK6 in the AR DBD domain. Of particular interest, Gioeli *et al.* (37) have reported that Ser-650 in the DBD is phosphorylated in response to epidermal growth factor receptor stimulation, which is known to recruit PAK (38). A Ser-650 → Ala mutation has been suggested previously by Zhou *et al.* (39) to regulate AR transactivation of the mouse mammary tumor virus promoter when suboptimal levels of steroid were used. This phosphorylation site in the hinge region is conserved in many of the steroid receptors (40). The identification of the relevant PAK6 phosphorylation sites in the AR and the investigation of this potential regulatory mechanism will be a subject of future studies.

We have shown by using PAK6 mutant proteins of varying activity that serine 560 phosphorylation reflects the kinase activity of PAK6. We used a phosphoserine 560 PAK6 antibody to examine in prostate cancer cell line the expression of active PAK6 (Fig. 7). We found that expression of phosphorylated PAK6 in these cell lines is quite variable. Interestingly, we did not detect phospho-PAK6 expression in the androgen-sensitive cell line LNCaP, but we observed a strong expression of phospho-PAK6 in the androgen-insensitive cell lines DU145 and PC3. These results suggest that PAK6 up-regulation might account for the development and/or maintenance of androgen independence, which is known to be associated with more aggressive tumors. In this regard, the activity common to the PAK family members of modulating cell motility and dynamics may come into play for PAK6 as well. It will be of interest in future studies using a larger sampling of prostate cancer cell lines and tumor tissues to assess the relationship of PAK6 activity to AR phosphorylation state, responsiveness to androgens, and the growth rates and metastatic capabilities of these cancer cells.

In conclusion, this study demonstrates that PAK6 inhibition of AR-mediated transcription is dependent on the kinase activity of PAK6 and is specific to this PAK isoform. PAK6 may act by multiple mechanisms to antagonize transcriptional activity of the AR. Most importantly, we present evidence that expression of active PAK6 in cancer prostate cells is variable but may correlate with androgen sensitivity of the lines. These data suggest that modulation of PAK6 activity may be an important mechanism contributing to the regulation of AR signaling in various forms of prostate cancer.

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